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"SULFITING AGENTS"

Appendix A Part 2

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ON THE EFFECTS OF PROLONGED EXPOSURE TO SULPHUR DIOXIDE*

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PURPOSES OF STUDY

IT is highly desirable to have definite information as to whether or not any residual or permanent damage to health is sustained by persons who are employed in trades in which there is continual or frequent exposure to sulphur dioxide. Much of the hearsay information in these trades is to the effect that no injury results from ordinary exposure, and that a marked tolerance is quickly developed. Indeed, it is not uncommon to find workmen who believe their occupational exposure to be beneficial to their health. A body of opinion arising in this way often has some foundation in fact but requires critical investigation before it is accepted.

Furthermore, the common use of sulphur dioxide as a refrigerant, with its consequent introduction into the household, brings about a more than ordinary interest in the effects which may result from its inhalation and absorption. Although it is generally regarded as an irrespirable gas, its presence in the air makes itself known in low concentration and provides a warning which is not likely to be disregarded by an unobserving or even a sleeping person. No accurate information is available, however, as to the limits of concentration borne without immediate or delayed injury, and as to the effects, if any, of prolonged exposure upon life, health, and the incidence of acute or chronic diseased states. It has seemed of some importance, therefore, to make careful studies both under laboratory conditions and under practical conditions of human exposure. The following paragraphs relate to observations of the latter type.

Since the study of sulphur dioxide

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was supported by one of the large manufacturers of electric refrigerators,[1] the fullest co-operation in obtaining suitable subjects for observation was assured. A brief survey of the occupations in which exposure to sulphur dioxide is incurred demonstrated that the workrooms of the company's plants presented as nearly ideal conditions for observation as could be found, in that a large number of subjects were available who had had long exposure to a known basic concentration, as well as exposures to high concentrations, which were brief but in many cases frequent. There were also a large number of men of the same type in completely separated departments of the plant, whose ages and general conditions of work and life were similar to those of the subjects under study in every respect save that of sulphur dioxide exposure.

Selection of Subjects

From the workmen who were engaged in handling sulphur dioxide, 100 subjects were selected on the basis of severity and duration of exposure. Among these were forty-seven employees who had had from four to twelve years of exposure. The sulphur dioxide storage and distributing department, the refrigerating unit charging department, and the repair department furnished most of the subjects.

One hundred control subjects were chosen from the assembling departments, tool room, punch press room, carpentry shop, and shipping department, in which there occurred no exposure to sulphur dioxide or to other noxious materials such as paints, lacquers, and metallic dusts. Selection was made on the basis of age groups comparable to those of the exposed subjects.

Severity of Exposure

The charging department of the plant provided a large proportion of the exposed subjects. Here, the units of refrigerating apparatus receive their charge of sulphur dioxide. The liquid sulphur dioxide is delivered to a large number of operating panels through a series of pipes leading from the distributing and storage center. These panels are close together and a large number of operators work in the same room. Thus careless or unskilled breaking of a connection or an accidental leak in a line affects many workmen. In 1927 an effective ventilating system was established in this department, and a system of air analysis was instituted which gave full information as to the concentration of sulphur dioxide present under operating conditions. Figure 1 shows the results of such analyses during the months of August, 1929 and February, 1930. Prior to 1927 the concentration was higher and subject to greater fluctuations, analyses at irregular intervals showing average concentrations of from 80 to 100 parts per million. Under present conditions the concentrations as shown in Figure 1 run somewhat higher during winter months when the windows are closed. During periods of rush production, and when inexperienced operators are being trained, the average concentration is also higher.

The storage and distributing department shows a generally higher concen-

[1] The Frigidaire Corporation of Dayton, Ohio.

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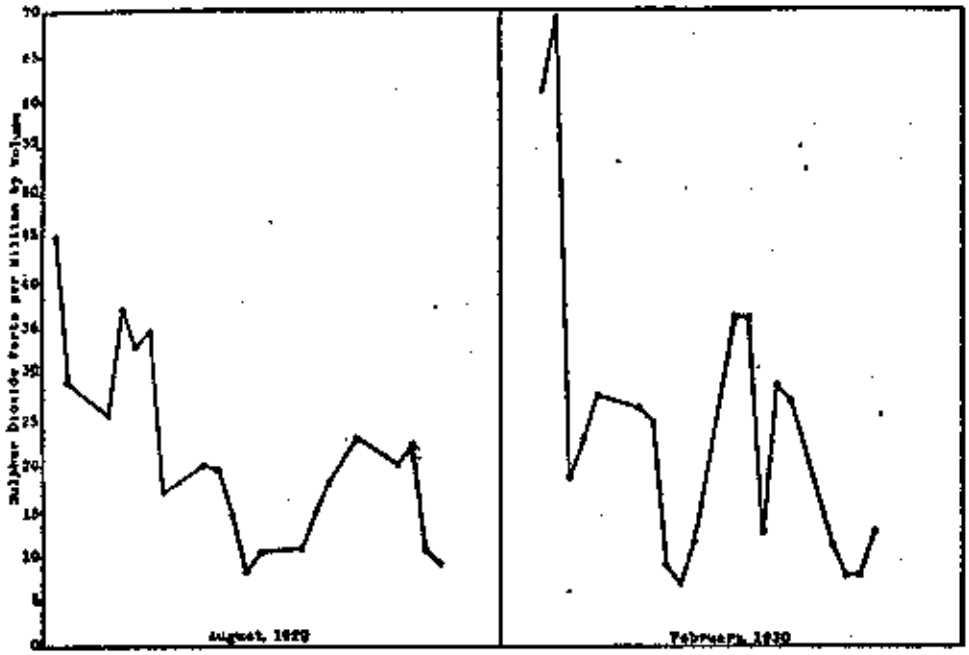

FIG. 1.—Sulphur dioxide content of air in charging room.

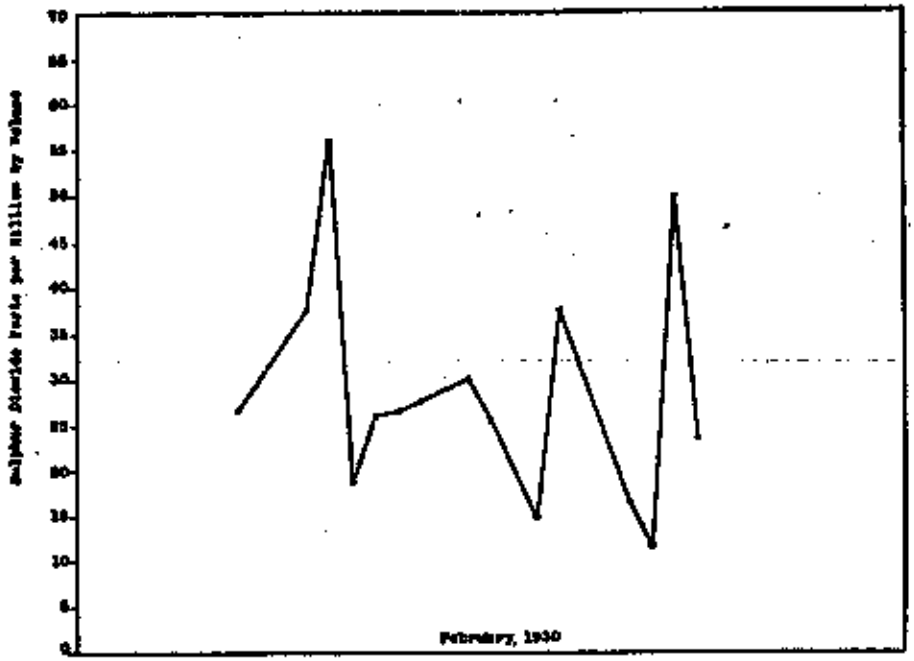

FIG. 2.—Sulphur dioxide content of air in distribution and storage room.

HISTORY SHEET

Number Examiner's Initials Date
Name Age Race Color Birthplace
Marital Ages Children Miscarriages Date Stage
Residence
Other Places Lived in, with Dates
Place of Employment Type of Work (exact description of nature and conditions)
Length of Present Employment and
Previous Employment at Same Work
Previous Occupations Exact Description of Work Done Dates
Gasoline Refining
Lead
Benzene
Lacquer Paints
Paper Manufacturing
Acids
Garage Work
Other Refrigerating Equipment
Previous Illnesses, with Dates:
Tbc. Malaria Rheumatism Lues Gc. Scarlet Diph.
Typhoid Convulsions Heart Disease
Significant Family History:
SO_2 History with Reference to Duration of Exposure, Acclimatization, Injury, Residual Effects, and Recovery:
Sleep Hours in Bed Dreams Restful Disturbed
Consumption per Day { Milk, Cream, Water } { Tea, Coffee, Alcohol }
Bowel Movements Frequency Hour
Tendency to Constipation Cathartics
Tendency to Frequent Stools
Teeth Brushing When Last Trip to Dentist
Usual Weight Best Weight Change of Weight { Seasonal, Occupational }
General Health
Rate of Tiring Recent Change
Headaches Time
Eye Trouble
Taste in Mouth Character Time
Pains in Joints Swelling of Joints
Muscular Strength Cramps in Muscles
Pains in Belly Character Frequency
Appetite Different Meals
Digestive Disturbances Nausea or Vomiting
Skin Infection or Eruption General Hands
Polyuria Nocturia Frequency Pain or Burning on Voiding
Nervousness General Weakness Numbness
Other Complaints
Respiratory History:
Nasal Irritation Hemoptysis
Frequent Colds Bronchitis
Cough Asthma
Sore Throat Shortness of Breath
Laryngitis

FIGURE 3

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PHYSICAL EXAMINATION SHEET

Number Examiner's Initials Date Height Weight
General Appearance Posture
Nutrition Musculature
Pulse Blood Pressure (seated)
Color of Skin (exact) Sclera
Condition of Skin Glands
Condition of Skin of Hands Tremors
Romberg
Glasses Vision R.15/ L.15/ Near-Sighted Far-Sighted
Eyes: Muscles Pupils Size Regularity
Cornea and Sclera Conjunctiva
Nose Ears: Audition
Abnormalities
Tongue Throat Tonsils
Teeth (exact condition)
Gums (cleanliness) Pyorrhea
Lead Line (exact appearance—location)
Heart: Apex Rate R.C.D. x
After 25 Hops R.S.D.
Two Minutes after
Lungs: K. I.—R. D.E.—R. L.L.B.—R.
L. L. L.
Chest Diagnosis
Abdomen
Liver Spleen Kidneys
Rectum Genitalia
Reflexes {Biceps, Triceps} Abdominal Tendon Jerks Sensations: Painful, Tactile, Thermal, Kinesthetic
Upper Extremities Lower Extremities
Special Remarks and General Diagnosis:

FIGURE 4

LABORATORY SHEET

Number Examiner's Initials Date
Urinalysis: Quantity
Sp. G.
Reaction (Methyl Red)
Albumin (Heller's) Heat and Acetic
Sugar (Fehling's)
Acetone (Nitroprusside)
Microscopic
Blood: White Count Hemoglobin (Dare)
Red Count
Differential (100 cells): Poly. Neutrophils
Poly. Eosinophils
Poly. Basophils
Lymphocytes
Large Mononuclear {Endothelial, Transitional}
Abnormal
Polychromasia
X-Ray Chest Report
Special Examinations:

FIGURE 5

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tration, averaging approximately 30 parts per million, as shown in Figure 2.

In both the charging and the discharging departments, and especially in the repair department, occasional or frequent escape of sulphur dioxide causes a brief but temporarily unendurable exposure from which the operator finds it necessary to escape.

Methods of Study

Each subject in the two groups was questioned in detail as to the length and nature of his exposure to sulphur dioxide, as well as to other occupational hazards. Care was taken to elicit information as to past and present symptoms and illnesses. The record blanks used as an outline for obtaining information are shown in Figures 3, 4, and 5. No attempt was made to limit the examiner to the subject matter or the details of the blank form, but in each case the findings relating to each item were recorded in a uniform manner.

Under sulphur dioxide history, the examiner recorded all information which could be obtained by inquiring into the following matters:

1. Duration of Exposure
2. Acclimatization
   a. Symptoms of initial period of exposure
   b. Date acclimatization was observed
3. Symptoms Arising from Basic Exposure since Acclimatization
   a. Symptoms from usual minimum amounts
   b. Influence of exposure on common colds (frequency, susceptibility, or prolongation)
4. Heavy (Intolerable) Periodic Exposure
   a. Frequency
   b. Symptoms
      1. Eye, nose, throat
      2. Chest pain and its duration
      3. Aggravation of usual symptoms
      4. Hemoptysis or epistaxis
      5. Hacking cough—night or morning
      6. Polydipsia
      7. Polyuria
      8. Weakness
      9. Appetite and digestion
      10. Headaches and nervous system symptoms
      11. Constipation or diarrhea
5. Residual Symptoms
   a. Altered susceptibility to other chemical or physical irritants
   b. Alteration in sense of taste or smell
   c. Permanent injury of any type

The physical examinations were carefully carried out in a uniform manner, and the laboratory tests of the blood and urine were all made by the same technician. X-ray examinations of the chest of each exposed and each control subject were made by an experienced roentgenologist who also classified the findings in the two groups.

A selected group of subjects representing the severest and longest exposures were studied as to their alveolar carbon dioxide content and their blood plasma carbon dioxide combining capacity. Since the latter tests yielded no information of value, they were not extended to the remainder of the subjects, and they will receive no further mention.

Analysis of Findings

The results of the examinations were subjected to a careful individual and general study, in an attempt to evaluate them properly from a clinical point of view; they were then tabulated and subjected to statistical

analysis by one of the authors (T. J. Lell.). The conclusions arrived at from the study of individual cases are included in those obtained by the application of biometric methods, with the exception of the X-ray findings, to which reference will be made subsequently.

The biometric characteristics of the exposed and control groups are shown in Table 1. Since all the subjects are males, the question of sex is eliminated. The number in each group is the same, *i.e.*, 100. From the table it is seen that the variability as indicated by the respective standard deviations is relatively low and as a result the probable errors of the respective means are low, thus showing that the mean values are stable and that the groups are large enough. The only significant difference exhibited by the table is in the case of years employed in industry, the control group having been employed a significantly longer time than the exposed. In fact, the very close agreement between the respective pairs of means in the case of age, height, weight, and number of years worked in this plant shows that the control group has been exceptionally well chosen and that it constitutes an adequate control for the purpose of this study.

In order to test the validity of the control group further, certain physical findings of a quantitative nature, arrived at by the examination of the clinicians, were analyzed. The resulting constants are listed in Table 2, which in general exhibits the same characteristics as Table 1. Standard deviations are low and the means are stable. Two differences are shown: The polymorphonuclear leukocyte count is *significantly higher in the control group*; the lymphocyte count, on the other hand, is higher in the exposed group. The fact that systolic and pulse pressures, erythrocyte and leukocyte counts, and percentage of hemoglobin as shown by the Dare hemoglobinometer are not significantly different for the two groups constitutes further evidence that the control group is well chosen.

TABLE 1.—SHOWING THE MEAN WITH ITS PROBABLE ERROR AND STANDARD DEVIATION FOR CERTAIN VARIABLES

	EXPOSED GROUP		CONTROL GROUP	
	Mean	Standard Deviation	Mean	Standard Deviation
Age in years	37.0 ±0.70	10.40	35.7 ±0.55	8.75
Height in inches	68.1 ±0.21	3.14	68.4 ±0.13	1.96
Weight in pounds	158.6 ±1.87	27.70	153.3 ±1.41	21.00
Years in plant	4.0 ±0.13	1.98	4.5 ±0.17	2.51
Years in industry	8.0 ±0.35	5.26	12.3 ±0.65	9.65

Here it may be noted that in the control group there was a subgroup of twenty-nine individuals who exhibited pallor. The average hemoglobinometer reading for this group was 76 per cent., which was lower than the

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mean of the total of either exposed or control groups.

Physical Findings

All the physical findings on the case records were tabulated, but by inspection a considerable number of them were seen to be of no importance —that is, there was an equal or nearly equal distribution of the findings between the exposed and control groups. This was true of the following rubrics:

Overnutrition, undernutrition, normal pulse, bradycardia, tachycardia, normal temperature, elevated or lowered temperature, adenopathy, tremor, chest findings, cardiac lesions, hypertension and hypotension, abdominal and pelvic lesions, sensory disturbances, skin lesions, impaired vision, lesions of the cornea and sclera, conjunctivitis, chronic nasal discharge, pyorrhea and dental caries.

The remaining findings were not so obvious and were further tabulated. The results are shown in Table 3. It will be noted that the four entries under each pair of rubrics are mutually exclusive and constitute a fourfold table. The Chi-square[7] test applied to the values in this table showed that the differences exhibited were significant. Column 3 lists the values of Chi-square and column 4 shows the value of P arising from the corresponding values of Chi-square, *i.e.*, the probability that the differences shown arose from the operation of chance. In each case this probability is so low that the validity of the difference is almost certainty and the findings have been listed in descending order of *statistical significance*. Emphasis is placed on this point because *clinical significance* may be quite different—that is, a finding might occur with much greater frequency in one group and be of no clinical significance.

TABLE 2.—SHOWING THE MEAN WITH ITS PROBABLE ERROR AND STANDARD DEVIATION, FOR CERTAIN PHYSICAL FINDINGS[1]

PHYSICAL FINDINGS	EXPOSED GROUP		CONTROL GROUP	
	Mean	Standard Deviation	Mean	Standard Deviation
Systolic pressure in mm. Hg	126.7 ±1.30	10.2	124.4 ±1.02	15.0
Pulse pressure	48.5 ±0.78	11.5	48.5 ±0.75	11.1
Erythrocytes	4,784,000 ±29,827	440,000	4,642,000 ±28,517	422,800
Leukocytes	8,170 ±133	1,070	7,840 ±196	2,010
Hemoglobin	80 ±0.59	8.5	81 ±0.45	6.6
Polymorphonuclear leukocytes	61 ±0.56	8.2	*65 ±0.45*	6.7
Lymphocytes	*36 ±0.49*	7.6	32 ±0.48	7.2

[1] Only those entries in italics are statistically significant.

Exposure and Acclimatization

Exposures resolved themselves into a fairly normal frequency which gave the following constants:

Mean length of exposure in years	3.82 ±0.12
Standard deviation	1.81
Modal group in years	2-3.9
Below the modal group	33%
At the modal group	26%
Above the modal group	41%

In addition it may be noted that 74 per cent. of the total exposures fell within the limits of 2 to 4.9 years so that the majority grouped themselves quite closely about the mean, with extremely short and extremely long exposures constituting the remaining 26 per cent. This is also indicated by the standard deviation.

Of this exposed group, 80 per cent. became acclimatized—that is, they acquired the ability to withstand the customary basic exposure without experiencing a notable intensity of the symptoms later described as initial symptoms. The mean length of time necessary for acclimatization was 2.84 ±0.15 months with a standard deviation of 2 months. The standard deviation is high, and is almost equal to the mean. This shows that the variability in time necessary for acclimatization is great, as might have been expected since acclimatization is a personal reaction and as a biologic phenomenon is entitled to relatively high variability. This variability was such that the mode was not defined. Some of this, of course, was due to the fact that acclimatization was not amenable to measurement and the times recorded were reported by each individual, such values necessarily involving not only physiologic variability but variability in intelligence, memory, attention, etc.

TABLE 3.—SIGNIFICANT PHYSICAL FINDINGS[1]

PHYSICAL FINDINGS	EXPOSED GROUP	CONTROL GROUP	CHI-SQUARE	P[2]
Pharyngitis, chronic and slight	70	31	338	0.000000
No pharyngitis	30	69		
Urine acid to methyl red	87	47	41	0.000000
Urine alkaline to methyl red	10	53		
Chronic pharyngitis	38	9	33	0.000000
No pharyngitis	30	69		
Tonsillitis, chronic and slight	34	12	14	0.000180
No tonsillitis	66	88		
Slight pharyngitis	32	22	11	0.000000
No pharyngitis	30	69		
Abnormal reflexes	31	15	7	0.007000
Normal reflexes	69	85		

[1] Arranged in descending order of statistical significance.
[2] P = probability that difference arose through operation of chance.

Symptoms

The symptoms associated with exposure were tabulated in order to determine the frequency of their

of the cold, which is extended in the exposed group to 2.3 times the average duration in the control group. This fact is also supported by the total man weeks of colds and the number having colds lasting all winter, the values for the exposed group being two to three times those of the control group.

The application of the Chi-square test failed to demonstrate any relationship between the prolongation of colds and the frequency of severe exposure.

Symptoms Associated with Severe Exposure.—The symptoms arising from the irregular exposure to high concentrations were chiefly an intensified form of those occasioned by the original minimum exposure. Coughing and sneezing, conjunctival irritation, and soreness of the throat and larynx were the most frequent results of such exposure, while thirst, weakness, transitory chest pains, loss of appetite for the succeeding meal, polyuria, and chest pain of a more or less prolonged type were sufficiently frequent to be taken into account. These facts merely demonstrate the incompleteness of the acclimatization.

General Symptomatology

A comparison of the general symptomatology of the two groups showed that many of the symptoms had no relationship to sulphur dioxide exposure. That is to say, they occurred with approximately equivalent or lower frequency in the exposed as compared with the unexposed group. This situation existed in the case of the following:

Skin eruptions during employment, weight gain in employment, weight loss in employment, poor general health, bad taste in mouth, joint pains, abdominal cramps, loss of appetite, dyspepsia or vaguely described digestive disturbances, constipation, diarrhea, nocturia, burning on urination, headache, disturbed sleep, nervousness, numbness or tingling of extremities, general weakness, frequency of colds, sore throat or laryngitis, bronchitis, asthmatic attacks, and eye troubles. (See Table 4 for comparative frequencies of certain items.)

A significant increase was found, however, in the frequency of occurrence in the exposed subjects of shortness of breath on exertion, increased fatigability during employment, altered sense of taste or smell, and increased sensitivity to other irritants. Table 5 shows the application of the Chi-square test to these items.

Only two of these complaints occurred with sufficient regularity in the exposed group to be subjected to further analysis, *i.e.*, an increased fatigability during employment and shortness of breath on exertion. The relationship of these to the frequency of severe exposure was studied by arranging fourfold tables as shown in Table 6. The average number of severe exposures per man per annum was 230. This was used, therefore, as the dividing line between high and low frequencies of severe exposure. The Chi-square test under these conditions showed that the occurrence of each symptom was positively correlated with the frequency of severe exposure.

X-Ray Findings

Each group of subjects (exposed and control) was classified by the roentgenologist into four subgroups made up according to the results of the chest examinations: (1) average

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TABLE 4.—FREQUENCY OF CERTAIN SYMPTOMS HAVING NO STATISTICAL SIGNIFICANCE

SYMPTOMS	EXPOSED GROUP	CONTROL GROUP
Weight gained	17	13
No weight gained	61	77
Weight lost	22	10
No weight lost	61	77
Alteration in health	5	2
No alteration in health	95	98
Tendency to constipation	33	42
No tendency to constipation	67	58
Complaint of headaches	16	12
No complaint of headaches	84	88
Headache and constipation	9	0
Headache and no constipation	7	3
Constipation and headache	9	9
Constipation without headache	24	33

TABLE 5.—FREQUENCY OF CERTAIN SYMPTOMS OF STATISTICAL SIGNIFICANCE[1]

SYMPTOMS	EXPOSED GROUP	CONTROL GROUP	CHI-SQUARE	P
Alteration in sense of smell	14	0	15	0.0001
No alteration in sense of smell	86	100		
Increase in sensitivity to other irritants	14	0	15	0.0001
No increase in sensitivity to other irritants	86	100		
Shortness of breath on exertion	42	19	12	0.0005
No shortness of breath on exertion	58	81		
Increase in fatigability in employment	27	9	11	0.0009
No increase in fatigability in employment	73	91		
Alteration in sense of taste	10	0	11	0.0000
No alteration in sense of taste	90	100		

[1] Arranged in descending order of statistical significance.

normal chests; (2) chests in which there may be slight reasons for suspecting the existence of pathologic development; (3) chests in which the

existence of disease, active or healed, is suspected; (4) chests in which there is definite lung pathology. In accordance with this classification the two groups are compared in Table 7.

There was nothing peculiar in the appearance of the roentgenograms of the exposed subjects, and nothing from an X-ray point of view to indicate the existence of injury to the lungs or bronchi as a result of the exposure. The four cases showing chest pathology in the exposed group consisted of three in which an abnormal amount of peribronchial infiltration with mottled areas in the lung tissue suggested the existence of active infection, and one in which thickening of the pleura and calcified areas in the lung tissue indicated the presence of either a chronic or a healed disease process. Upon checking these findings with the histories of previous illness and the physical findings, one case was seen to be a chronic bronchitis of long standing, two were instances of chronic nasopharyngitis with a degree of involvement of the respiratory passages characteristic of this condition, while the last-mentioned was a case of healed pleuritis, the acute illness having occurred over a period of four months in 1901.

The four cases in the control group were made up of three cases of peribronchial infiltration, with areas of calcification in the lung tissue, the former finding being associated with

TABLE 6.—RELATIONSHIP OF CERTAIN SYMPTOMS TO FREQUENCY OF SEVERE EXPOSURE

SYMPTOMS	EXPOSURES PER ANNUM		CHI-SQUARE	P
	Below 230	Above 230		
Shortness of breath on exertion	24	18	7	0.0082
No shortness of breath on exertion	49	9		
Increase in fatigability in employment	14	13	8	0.0047
No increase in fatigability in employment	59	14		

TABLE 7.—FINDINGS BY X-RAY EXAMINATION OF CHEST

GROUP	CLASSIFICATION	NO. OF SUBJECTS	
		Exposed to SO_2	Not Exposed to SO_2
1	Average normal chests	70	64
2	Slightly suspicious chests	14	12
3	Suspicious chests	7	9
4	Definite chest pathology	4	4
Total no. of persons observed		95	89

chronic rhinitis, and one case of healed, infected gunshot wounds of the chest.

Discussion

The interpretation of the main results of the foregoing analysis is essentially self-evident. Exposure to sulphur dioxide under the conditions observed produces two distinct sets of effects: (1) irritation of the upper respiratory tract, which is at first acute in character, and subsequently subacute or chronic; and (2) systemic symptoms of a mild type such as are associated with any type of increased acid absorption or production in the tissues. The high degree of correlation shown between the systemic symptoms and the frequency of severe exposures indicates that the human organism has a high degree of adaptability to a regular moderate exposure, and that it suffers no apparent injury from such an exposure. In the case of intense exposures, even though they occur frequently, there is no evidence of damage of a serious or a permanent type.

It might be expected that the effects of a persistent chronic rhinitis and pharyngitis would be more noticeable in the production of systemic symptoms and in the development of chronic disease of the lungs and bronchi. The absence of such results leads one to suspect that the irritation of the upper respiratory tract is not regularly associated with infectious processes. If this should be found to be true it would follow that the persistent "colds" described by many of the subjects are not colds in the ordinary sense, but rather a chronic coryza due to chemical irritation.

The variation in the relative numbers of the types of leukocytes in the two groups is not so readily interpreted. Lymphocytes occur with higher than normal frequency in both groups. This not uncommon finding is probably associated with the high incidence of low grade chronic infection of the nose, mouth, and throat in both groups. The difference in the two groups, while of statistical significance, is of doubtful clinical importance. A much greater difference would be required to establish any certainty in this regard.

The matter of abnormal reflexes requires some further consideration. Under this heading were listed sluggish and hyperactive reactions. In no case were the abnormalities of the type associated with localized lesions of the central nervous system. They signify, therefore, a degree of variation in general irritability. It would be presumptuous to ascribe any important clinical significance to such variation.

The roentgenograms show that no appreciable residual injury to the lungs and bronchi has been produced by the exposure to sulphur dioxide, either of a prolonged or of a severe type. It cannot be doubted that injury to the pulmonary epithelium occurs when high concentrations are inhaled. Nor can one doubt the significance of hemoptysis, constricting pain in the chest, and a deep productive cough after a period of intense exposure. It is clear, however, that under the conditions of exposure here considered, no permanent damage is done to the lungs.

Considering the number of subjects studied, the variety of their exposure, and the number who did not become acclimatized to exposure but who never-

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theless continued to be exposed, it would seem reasonably certain that the foregoing observations represent an adequate cross-sectional picture of the results of exposure to sulphur dioxide. On this basis it may be concluded that there is no appreciable danger to health in a frequent or more or less continuous exposure to endurable concentrations, and that the effects of exposure to unendurable concentrations are negligible under conditions which allow of quick escape.

Summary

1. A significantly higher incidence of nasopharyngitis, both chronic and slight, of alteration in sense of smell and sense of taste, and of increased sensitivity to other irritants was found in a group of workers exposed to sulphur dioxide. These may be thought of as conditions arising from local irritation.

2. A significantly higher incidence of abnormal urinary acidity, of tendency to increased fatigue, of shortness of breath on exertion, and of abnormal reflexes was found in the exposed group. These are in the nature of systemic symptoms.

3. There was no demonstrable association between frequency or severity of initial symptoms and frequency of heavy exposure, nor was there any relation between exposure and the frequency and severity of symptoms arising from the customary exposure.

4. A positive relation exists between frequency of heavy exposure and presence of certain symptoms indicative of acid accumulation, namely, increased fatigability during the period of employment, and shortness of breath on exertion.

5. The incidence of colds is not significantly different in the two groups. The duration, however, is extended in the exposed group.

6. In all other respects as indicated in the text, there was no significant difference between the two groups.

mittelmäßig bezeichneten Jahrgänge sowohl nach der einen, als nach der anderen Seite weniger deutlich als in meiner Studie erfolgte. Im Jahrhundert 1898–1907 sind ebenfalls keine öfteren oder längeren Fehljahrintervalle in der Qualität als 1908 bis 1957 zu verzeichnen.

Die Erforschung der Gründe für die außerordentlich großen jährlichen Schwankungen in den Mostgewichten und damit in der Güte der Rieslingweine ist nicht Aufgabe dieser Studie. Die Erklärung der Zusammenhänge zwischen Sonnenscheindauer und anderen klimatischen Auswirkungen auf die Ausreife der Trauben soll der Wissenschaft einschließlich der Meteorologie überlassen bleiben. Ihr ist auch die Frage zu stellen, ob und gegebenenfalls wie in Zukunft eine Beeinflussung der Mostgewichtsschwankungen möglich erscheint. Der Betriebswirtschaftler muß sich z. Zt. auf die Fortdauer der geschilderten außerordentlich großen jährlichen Qualitätsunterschiede einstellen.

(Wird fortgesetzt.)

Weinberg Keller 5 (1958)

Ist die schweflige Säure im Wein mit unvergorenem Zucker gesundheitlich weniger zuträglich als im durchgegorenen Wein?

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Die Ansicht, daß diese Frage bejahend zu beantworten ist und infolgedessen die Weine mit unvergorenem Zucker auch weniger bekömmlich sind, ist unter Weintrinkern heute weit verbreitet. Die Hauptursache dafür ist darin zu suchen, daß gerade diese Weine bisher häufig (aber erfreulicherweise jetzt immer mehr abnehmend) überschwefelt wurden. Sie stützt sich aber auch, und zwar auch für Weine mit einem nicht übermäßigen Gehalt an schwefliger Säure (im folgenden der Kürze halber, aber nicht ganz korrekt mit SO_2 bezeichnet), auf einen Vortrag und eine im Gefolge davon erschienene Publikation von Schandert (1). Darin wird die Ansicht vertreten, daß die in Weinen mit unvergorenem Zucker hauptsächlich an Glukose gebundene schweflige Säure besonders dann nicht unbedenklich ist, wenn diese Weine von Personen mit anaciden (d. i. salzsäurefreiem) Magensaft getrunken werden, da hierdurch im Magen aus Glukose-SO_2 besonders viel freie SO_2 gebildet wird.

Schandert geht dabei von der Beobachtung aus, daß der Atem mancher, aber nicht aller Weintrinker nach SO_2 riecht und schließt daraus, daß bei diesen Personen im Magen durch die spezifische Wirkung ihres Magensaftes besonders viel SO_2 freigesetzt werden muß. Auf Grund einer Angabe von von der Heide, wonach die Freisetzung der an Glukose gebundenen SO_2 in saurer Lösung im Vergleich zu einer weniger sauren Lösung stark verlangsamt wird, wird nun von Schandert hauptsächlich bei Dessertweinen (Samos, süßem Muskateller) untersucht, in welchem Umfange durch verschieden saure Modellmagensäfte bei Körpertemperatur (37° C) SO_2 freigesetzt wird. Er findet dabei

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nach 60—120 Minuten die größte freigesetzte SO_2-Menge beim Zusatz von anaciden (und auch subaciden) Magensaft zum Wein, erheblich mehr als bei Zusatz von normaciden und besonders hyperaciden, d. h. stark saurem Magensaft, und auch mehr als beim Kontrollversuch, bei dem der Wein ohne Zusatz von Magensaft nur der Körpertemperatur ausgesetzt wird. Da diese Aufspaltung der Glukose-SO_2 auch die Bildung von gasförmigem SO_2 mit den damit verbundenen gesundheitlich nachteiligen Wirkungen (Brennen im Hals, Magenschmerzen usw.) zur Folge hat, kommt SCHANDERL zu der Schlußfolgerung, daß solche Weine gerade für anaciide und subaciide Weintrinker schlecht bekömmlich sind.

Im hiesigen Institut sind in der letzten Zeit eingehende Untersuchungen über die Bindung von SO_2 an verschiedene Weinbestandteile, sowie über den Einfluß verschiedener Faktoren auf diese Bindung und auch Freisetzung durchgeführt und kürzlich in dieser Zeitschrift publiziert worden (2). Die dabei enthaltenen Befunde lassen sich mit den Schlußfolgerungen von SCHANDERL in mehrfacher Hinsicht nicht vereinbaren. Nachstehend soll deshalb untersucht werden, worauf diese sich widersprechenden Ergebnisse zurückzuführen sind. Eine Klärung dieses Problems erschien uns nicht nur aus wissenschaftlichen Gründen notwendig, sondern vor allem wegen der Konsequenzen, die sich aus den Befunden von SCHANDERL sowohl für die hygienische Beurteilung der Weine mit Restsüße wie auch in weingesetzlicher Hinsicht ergeben.

Bei unseren früheren Untersuchungen, die wir an Modellösungen ausführten, hatten wir uns besonders eingehend mit der Bindung von SO_2 an Glukose (Traubenzucker), mit ihrer Beeinflussung durch Temperaturerhöhung und durch Änderung des Säuregrades (pH-Wertes) befaßt, sowie auch mit der Änderung der Geschwindigkeit dieser Bindung und auch Freisetzung durch die genannten Faktoren. Auf Grund unserer Ergebnisse läßt sich u. a. berechnen, wieviel SO_2 jeweils bei einem bestimmten Gehalt der Weine an Glukose und an freier SO_2 an Glukose gebunden ist. Man kommt bei den Weinen mit normaler Restsüße, worunter wir (in diesem Zusammenhang) Weine mit etwa 25 g/l unvergorenem Zucker verstehen, und mit einem innerhalb der gesetzlichen Grenzen liegenden Gehalt an freier SO_2 zu einem Wert von höchstens 3—4 mg/l an Glukose gebundener SO_2. Dies ist vielweniger als allgemein angenommen wird und auch sehr wenig gegenüber dem Gehalt an freier SO_2 wie auch besonders an Aldehyd gebundener SO_2, welch' letzterer in der Regel 30—50fach größer ist.

In den von SCHANDERL als Versuchswein benutzten sehr süßen Dessertweinen mit einem vielfach höheren Zucker- und Glukose-Gehalt ist naturgemäß auch die Menge der an Glukose gebundenen SO_2 entsprechend größer. Aber auch dabei errechnen sich, wie nachstehend noch gezeigt wird, dafür Werte, die nur einen kleinen Bruchteil der anderweitig gebundenen SO_2 betragen. Man kann also auch bei diesen Weinen nicht davon sprechen, daß darin hauptsächlich an Glukose gebundene SO_2 enthalten ist. Es ergeben sich somit schon von vornherein erhebliche Zweifel hinsichtlich der Stichhaltigkeit der von SCHANDERL gezogenen Schlußfolgerungen über die hygienische Bedenklichkeit der Glukose-SO_2, speziell für unsere deutschen Tischweine mit Restsüße.

VERSUCHE

Infolge Fehlens der notwendigen analytischen Angaben war eine rechnerische Überprüfung der Befunde von SCHANDERL auf Grund unserer früheren Versuchsergebnisse an Modellösungen nicht möglich. Aus dem gleichen Grunde stießen wir auch bei der Nacharbeitung dieser Versuche auf Schwierigkeiten. Vor allem fehlen dabei zwei Angaben, die gerade im Hinblick auf die von SCHANDERL gezogenen Schlußfolgerungen von entscheidender Bedeutung sind, nämlich einmal die Angabe des Glukosegehaltes (oder doch des Zuckergehaltes) der Versuchsweine und zum anderen des Säuregrades, d. i. des pH-Wertes, welchen der Wein jeweils durch Vermischung mit anaciidem, subaciidem usw. Magensaft annimmt. Beide Angaben sind offensichtlich unentbehrlich, wenn man die Aufspaltung der Glukose-SO_2 in Abhängigkeit vom Säuregrad untersuchen will.

Modell-Magensäfte

Wir haben deshalb zunächst die pH-Werte der Modell-Magensäfte, die nach den Angaben von SCHANDERL hergestellt wurden, ermittelt und dabei für hyperaciiden Magensaft pH 1,2, für normaciiden 1,7, für subaciiden 10,8 und für anaciiden Saft 11,2 gefunden.

Nach klinischen Angaben (3) liegt der pH-Wert des Nüchternsekretes des Magens bei Hyperacidität unter pH 1,6, bei Subacidität und Anacidität bei pH 2,0—3,5. Demnach würden die beiden ersteren Modellsäfte den natürlichen Verhältnissen entsprechen, die beiden letzteren liegen offensichtlich in ihrem pH-Wert erheblich zu hoch.

Es hat sich nun weiter gezeigt, daß der Zusatz dieses anaciden und subaciden Modellsaftes zu Wein oder auch zu „Modellwein" (n/10 Weinsäure-Tartrat-Puffer pH 3,3 mit 10 Vol.% Alkohol) trotz ihres sehr viel höheren pH-Wertes fast keine Verschiebung des pH-Wertes des Weines bewirken. So wurde z. B. der pH-Wert eines Weines durch Zusatz der gleichen Menge subaciden bzw. anaciden Magensaftes von ursprünglich 3,4 nur auf 3,5 bzw. 3,6 erhöht, der pH-Wert des Modellweines von 3,3 auf 3,5 bzw. 3,6. Beide Modellsäfte sind also schlecht gepuffert, weit weniger als Wein. Normacider und hyperacider Modell-Magensaft ist dagegen viel stärker gepuffert und vermindert deshalb den pH-Wert des Weines erheblich, z. B. von 3,5 bzw. 1,8.

Daraus muß man, wie dies auch durch unsere nachstehenden Versuche noch bestätigt wird, folgern, daß bei den Versuchen von SCHANDERL — entgegen seiner Annahme — durch die Vermischung mit anaciden und subaciden Modell-Magensaft kaum eine Verminderung des Säuregrades der Versuchsweine bewirkt wurde. Demnach kann auch die gefundene Freisetzung von SO_2 nicht oder höchstens nur in sehr geringem Ausmaß durch den Zusatz dieser anaciden und subaciden Magensäfte bewirkt worden sein; es müssen dafür vielmehr andere Ursachen vorliegen.

VERSUCHSTECHNIK

Zu den Versuchen wurden unter Angleichung an die Angaben von SCHANDERL ein „süßer italienischer Muskateller" sowie zwei „vollsüße Insel-Samos" benutzt, ferner später noch ein weiterer Samoswein und schließlich auch noch ein „Modell-Samos", d. h. ein deutscher Wein, der durch Zusatz von Glukose, Alkohol und durch Abstumpfung seiner Säure den Samos-Weinen analytisch in etwa angeglichen worden war. Da alle diese Versuchsweine keine (oder kaum) freie SO_2 enthielten, wurden sie zuerst durch Zusatz von SO_2 auf einen normalen, d. h. unseren deutschen Weinen entsprechenden SO_2-Spiegel gebracht, da ja Bindung (und Freisetzung) von SO_2 auch von der vorhandenen freien SO_2-Menge abhängig ist. Mit dem Versuchsbeginn mußte dann jeweils bis zur vollständigen Einstellung des Bindungsgleichgewichtes gewartet werden (mindestens 3 Tage). Je 200 ml Wein wurden mit der gleichen Menge Modell-Magensaft versetzt und dann sofort auf ihren Gehalt an freiem SO_2 untersucht. Die Hälfte dieser Ansätze verblieb bei Zimmertemperatur (ca. 20° C), die andere Hälfte wurde im Wasserbad innerhalb ½ Std. auf 37° C gebracht und sodann im Brutschrank auf dieser Temperatur gehalten. In bestimmten Zeitabständen wurde auf freie SO_2 untersucht.

Die Bestimmung der freien SO_2 erfolgte jodometrisch in der üblichen Weise. Die Bestimmung durch Destillation (4) war meistens zeitlich unmöglich; auch bestanden dagegen Bedenken wegen der bei 37° C schnellen Verschiebung des Gleichgewichtes. Der der jodometrischen Bestimmung anhaftende, vor allem durch andere jodreduzierende oder jodbindende Substanzen verursachte Fehler (von uns als Restreduktionswert bezeichnet), konnte bei diesen Versuchen in Kauf genommen werden, da es hierbei weniger auf genaue absolute Werte als auf relative Unterschiede ankam.

Neben den Ansätzen mit Magensaftzusatz wurde jeweils auch eine Kontrolle angesetzt, wobei der Versuchswein mit der gleichen Menge dest. H_2O vermischt wurde, zum Unterschied von den Versuchen von SCHANDERL, welcher den unverdünnten Wein als Kontrolle benutzte. Dieser Unterschied erwies sich, wie sich nachstehend noch ergeben wird, als bedeutungsvoll.

ERGEBNISSE

Für zwei unserer Versuchsweine (Samos und Muskateller) ist die Freisetzung von SO_2 in Abhängigkeit von der Zeit sowohl bei 20° C wie auch bei 37° C in der Tabelle 1 im Einzelnen wiedergegeben. In Tabelle 2 findet sich eine Zusammenstellung der bei allen Versuchsweinen gefundenen Maximalwerte an freigesetzter SO_2, in Tabelle 3 die analytischen Daten der Versuchsweine.

Aus Tabelle 1 läßt sich zunächst erkennen, daß in Übereinstimmung mit SCHANDERL eine mehr oder minder starke Zunahme der freien SO_2 gegenüber dem Anfangsgehalt (berechnet durch Halbierung des ursprünglichen Gehaltes an freier SO_2) stattfindet und weiterhin, daß bei 37° C die Höchstwerte (kursive Zahlen) durch anaciden Magensaft und im Kontrollansatz nach ½ bis 1 Stunde, im hyperaciden Ansatz nach 1 bis 1½ Std.,

Tabelle 1

Freie SO_2 mg/l nach Zusatz von hyperacidem bzw. anacidem Magensaft bzw. Wasser im Verhältnis 1:1

nach Stunden	Samos II (41 mg/l freie SO_2)						Muskateller (44 mg/l freie SO_2)		
	20° C			37° C			37° C		
	hyperacid	anacid	Kontrolle	hyperacid	anacid	Kontrolle	hyperacid	anacid	Kontrolle
pH	1,7	3,7	3,4	1,7	3,7	3,4	1,9	4,0	3,7
Anfangsgehalt (ber.)	20,5	20,5	20,5	20,5	20,5	20,5	22	22	22
sofort	22	24	23	21	23	23	22	28	28
0,5	—	—	—	—	32	29	41	44	44
1	—	—	—	27	33	32	44	42	41
1,5	—	—	—	28	33	31	44	41	41
2	22	27	25	28	33	31	—	—	—
4	23	27	26	—	—	—	—	—	—
6	22	27	26	—	—	—	—	—	—
Maximale Freisetzung	3,5	6,5	5,5	7,5	12,5	11,5	22	22	22

also nur wenig später, erreicht sind. Bei weiterer Versuchsdauer ist dann bei 37° C wieder eine Verminderung des SO_2-Spiegels infolge Verflüchtigung und auch Oxydation festzustellen.

Daraus ergibt sich (auch in Übereinstimmung mit unseren früheren Modellversuchen), daß die schon von KASE beobachtete und von VON DER HEIDE erwähnte in stark saurer Lösung verlangsamte Spaltung speziell von Glukose-SO_2 bei 37° C (anders als bei 20° C) nur wenig in Erscheinung tritt. Für die Beurteilung der Freisetzung von SO_2 im Magen ist deshalb in erster Linie die Lage des Bindungsgleichgewichtes maßgebend und nicht die Geschwindigkeit, mit der dasselbe erreicht wird. Auch die von SCHANDERL am Ende seiner Versuchszeit angegebenen Werte an freier SO_2 müssen nach diesen unseren Befunden Maximalwerte sein, wenn auch aus der Darstellung (als Menge-Zeit-Kurve) leicht der Eindruck entsteht, daß die Freisetzung von SO_2 danach noch weiter geht.

Tabelle 2

Maximale Zunahme der freien SO_2 mg/l

Anfangswert (ber.)	Samos II 20,5			Muskateller 22		Samos I 18			Modell-Samos 22		
	1 pH	2 20° C	3 37° C	4 pH	5 37° C	6 pH	7 20° C	8 37° C	9 pH	10 20° C	11 37° C
1 hyperac. Magensaft	1,7	3,5	7,5	1,9	22	1,6	1	7	—	—	—
2 normac. Magensaft	—	—	—	—	—	2,5	0	7	—	—	—
3 subac. Magensaft	—	—	—	—	—	3,7	5	10	3,5	3	11
4 anac. Magensaft	3,7	6,5	12,5	4,0	22	3,9	5	9	3,5	3	11
5 Kontrolle	3,4	5,5	11,5	3,7	22	3,5	3	9	3,5	3	11

SCHANDERL findet eine maximale Freisetzung (aus den Kurven entnommen) beim Samos-Wein von 45 mg/l, beim Muskateller von 63 mg/l. Demgegenüber ist bei unseren Versuchen die Zunahme an freier SO_2 erheblich niedriger (nach Tab. 2, Querspalte 4, unter Verdoppelung dieser Werte), nämlich bei Samos-Wein 18 und 25 mg/l, bei Muskateller 44 mg/l. Ebenso konnten wir im Gegensatz zu den Befunden von SCHANDERL keine erhöhte Freisetzung von SO_2 durch anaeiden Magensaft gegenüber dem Kontrollansatz beobachten; durch Vergleich der Querspalten 4 und 5 der Tabelle 2 ergibt sich, daß der Unterschied kaum außerhalb der analytischen Fehlergrenze liegt. Demgegenüber ist dieser Unterschied bei SCHANDERL mit 22 und 39 mg/l sehr bedeutend und führte ihn auch zu seinen einleitend erwähnten Schlußfolgerungen.

SO_2-Freisetzung durch Verdünnung

Die Erklärung dieser letzteren unterschiedlichen Ergebnisse liegt nun darin, daß SCHANDERL als Kontrollansatz unverdünnten Wein benutzt, während unser Kontrollansatz mit H_2O ebenso verdünnt war wie die Versuchsansätze mit Magensaft, so daß hierbei die gleiche Konzentrationsminderung der Weinbestandteile stattfindet.

Tabelle 3

Analytische Daten der Versuchsweine

	Zucker (Invertzucker) g/l	Glukose g/l	Arabinose gesäuert g/l	pH 20° C	freie SO_2 mg/l	ges. SO_2 mg/l	geb. SO_2 mg/l	Aldehyd mg/l	an Aldehyd geb. SO_2 mg/l	an Glukose geb. SO_2 mg/l	an Arabinose geb. SO_2 mg/l	Rest-SO_2 mg/l
Samos II	162	80	1	3,4	42	184	142	48	70	27	4	41
Muskateller	133	70	1	3,7	44	242	198	43	62	25	4	107
Samos I	180	87	1	3,5	36	136	100	34	50	25	4	21
Modellsamos	115	107	1	3,5	44	277	233	—	—	37	4	—
Samos III	150	88	1	3,5	42	160	118	37	54	30	4	30

Nach den bei den früheren Modellversuchen mit Glukose-SO_2 erhaltenen Befunden ist nun die gebundene SO_2 sowohl der SO_2-Konzentration wie auch der Glukose-Konzentration proportional. Wenn beide Komponenten verändert werden, wie hier bei der Verdünnung mit Wasser oder Magensaft, muß also die gebundene SO_2 um mehr als die Hälfte abnehmen und damit auch die freie SO_2 entsprechend stärker zunehmen. Es läßt sich, worauf hier nicht im einzelnen eingegangen werden kann, unter Benutzung des von uns früher ermittelten Proportionalitätsfaktors von 0,80 errechnen, daß z. B. bei dem Samos I mit 25 mg/l an Glukose gebundener SO_2 (gemäß Tab. 3) nach der Verdünnung im Verhältnis 1:1 statt 12,5 mg/l nur noch 8 mg/l SO_2 an Glukose gebunden sind; daß also durch diese Verdünnung 4,5 mg/l oder, bezogen auf den unverdünnten Wein, 9 mg/l freigesetzt wurden.

Dieser Verdünnungseffekt, d. h. die Freisetzung von SO_2 allein schon durch den Wasserzusatz, ist von SCHANDERL übersehen und die dadurch verursachte Freisetzung von SO_2 irrtümlich der Wirkung des Magensaftes zugeschrieben worden. Die Größe des Verdünnungseffektes bei unseren Versuchsweinen läßt sich aus den Werten der Querspalte 5 und der Längsspalten 2, 7 und 10 der Tabelle 2 erkennen (beim Muskateller zunächst zu berechnen, da hierbei die Werte bei 20° C fehlen). Diese Werte geben die Zunahme an freier SO_2 gegenüber dem Anfangswert bei gleicher Temperatur und bei gleichem pH-Wert an. Sie liegen zwischen 3 und 3,5 mg/l oder, auf unverdünnten Wein bezogen, zwischen 6 und 11 mg. Sie entsprechen also etwa dem vorgenannten für Glukose-SO_2 errechneten Wert.

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früher von uns gezeigt, daß die Lage des Bindungsgleichgewichtes von Glukose-SO_2 bei einer pH-Verschiebung um 1—1,5 Einheiten in den weniger sauren Bereich sich nur unwesentlich ändert.

Durch Zusatz von normacidem und besonders hyperacidem Magensaft wird dagegen, wie auch schon erwähnt, der pH-Wert der Versuchsweine beträchtlich vermindert, nach Querspalte 1, 2 und 3 der Tabelle bis zu 1,9 Einheiten. Die maximale Freisetzung ist dabei aber bei allen Versuchsweinen gegenüber dem Kontrollansatz nicht nur nicht erhöht, sondern in der Regel sogar deutlich vermindert und zwar sowohl bei 20° C wie auch bei 37° C.

SCHANDERL hat auch hierbei eine Zunahme der freien SO_2 gegenüber der Kontrolle gefunden, allerdings wesentlich weniger als bei Zusatz von anacidem Magensaft. Wenn man aber dabei die Wirkung der Verdünnung berücksichtigt, dann kommt man auch bei den Weinen von SCHANDERL zu einem den unsrigen entsprechenden Ergebnis, d. h. es ist hierbei ebenfalls keine Zunahme an freier SO_2 festzustellen.

Dies läßt sich nun nicht vereinbaren mit unseren früheren Befunden über die Lage des Bindungsgleichgewichtes von Glukose-SO_2 bei niedrigem pH-Wert. Danach liegt das Maximum der Bindung bei etwa pH 3,5. Es vermindert sich bereits bei einer pH-Verschiebung um eine Einheit nach dem sauren Bereich erheblich, nämlich auf etwa ⅔ des maximalen Wertes. Man müßte also demnach gerade hierbei, d. h. bei Zusatz von normal saurem und übersaurem Magensaft (im Gegensatz zu der Ansicht von SCHANDERL) eine merkliche Freisetzung von SO_2 erwarten.

Bei näherer Prüfung dieser sich widersprechenden Befunde hat sich nun gezeigt, daß hierbei zwei Faktoren nicht berücksichtigt sind, welche die analytisch feststellbare Menge an freier SO_2 beeinflussen, nämlich einmal die verlangsamte Gleichgewichtseinstellung bei niedrigem pH-Wert und zum anderen die sehr erhöhte Flüchtigkeit von SO_2-Gas aus stark sauren Lösungen. Daraus resultiert, daß bei Abschluß der Versuche (nach längstens 6 Stunden gemäß Tab. 1) das Bindungsgleichgewicht bei 20° C in der hyperaciden Lösung (pH 1,7 bezw. 1,9) noch nicht erreicht ist, d. h. daß auch danach immer noch SO_2 freigesetzt wird. So fanden wir in einem Modellversuch mit Glukose-SO_2, daß bei diesem pH-Wert und bei 20° C das Gleichgewicht erst nach 4 Tagen vollständig eingestellt ist. Andererseits verflüchtigt sich während dieser Zeit zunehmend SO_2-Gas und entzieht sich dadurch der analytischen Bestimmung.

Bei 37° C ist zwar auch bei pH 1,7 die Gleichgewichtseinstellung gegenüber 22° C vielfach beschleunigt und dieselbe gemäß Tabelle 1 dabei auch schon nach 1,5—2 Std. erreicht. Andererseits sind aber hierbei verständlicherweise die Flüchtigkeitsverluste noch erheblich größer.

Offenbar gehen diese Flüchtigkeitsverluste bei dem Zusatz von hyperacidem Magensaft hauptsächlich zu Lasten der freien SO_2 und die tatsächlich freigesetzte SO_2-Menge liegt infolgedessen wesentlich höher als die analytisch gefundene Menge.

Dies fanden wir auch durch die Bestimmung der gesamten SO_2 bei dem Muskateller-Wein bestätigt. Hierbei wurden — mittels der Destillationsmethode (4) — 3 Std. nach Versuchsbeginn (37° C) bei dem Kontrollansatz (pH 3,7) 120 mg/l, mit anacidem Magensaft (pH 4,0) 119 mg/l, mit hyperacidem Magensaft (pH 1,9) 104 mg/l ges. SO_2 gefunden, also beträchtlich weniger als bei den Ansätzen mit höherem pH-Wert.

Durch einen weiteren Versuch (mit einem Samos-Wein III, Tab. 4) wurden die vorstehenden Überlegungen und Befunde über die Wirkung von hyperacidem Magensaft auf die Freisetzung von SO_2 bestätigt. Es läßt sich daraus erkennen, daß bei 20° C durch hyperaciden Magensaft im Vergleich zum Kontrollansatz die Gleichgewichtseinstellung und dadurch die Freisetzung von SO_2 merklich verzögert wird, daß aber dieser Vorgang durch die zunehmenden Flüchtigkeitsverluste an SO_2, wie sie durch die Abnahme der gesamten SO_2 zu erkennen sind, überlagert wird. Bei 37° C spielt sich der gleiche Vorgang ab, aber vielfach schneller, wodurch die Gleichgewichtseinstellung zeitlich kaum verschieden ist von der des Kontrollansatzes.

Insgesamt läßt sich hieraus erkennen, daß tatsächlich, wie nach den vorerwähnten Befunden über die Gleichgewichtsverschiebung bei Glukose-SO_2 zu erwarten ist, im hyper-

acidem Milieu (pH 1,7) die Freisetzung von SO_2 größer ist als bei pH 3,5, da man die Mehrabnahme der ges. SO_2 (4–5 mg/l) – infolge Verflüchtigung – zu der analytisch gefundenen freien SO_2 hinzuaddieren muß.

Tabelle 4

Freie und gesamte SO_2 (mg/l) im Samoswein III nach Zusatz von hyperacidem Magensaft bzw. Wasser im Verhältnis 1:1

	20° C				37° C			
	hyperacid pH 1,7		Kontrolle pH 3,5		hyperacid pH 1,7		Kontrolle pH 3,5	
	freie SO_2	ges. SO_2	freie SO_2	ges. SO_2	freie SO_2	ges. SO_2	freie SO_2	ges. SO_2
Anfangswert (ber.)	21	80	21	80	21	80	21	80
sofort	21	78	22	78	20	79	23	80
0,5 Std.	—	—	—	—	25	74	30	78
2,0 „	—	—	—	—	27	72	31	77
3,5 „	22	74	26	77	—	—	—	—
4,0 „	—	—	—	—	28	70	31	75
21 „	25	74	26	75	—	—	—	—
45 „	24	71	24	75	—	—	—	—

Tab. 5

Anteil der undissoziierten SO_2 an der vorhandenen SO_2 (bei 25° C und 64 mg/l SO_2)

pH	4,0	3,5	3,3	3,0	2,8	2,2	1,8	1,0
% undissoz. SO_2	0,86	1,3	3	5	8	37	48	86

Tabelle 6

Berechnete und gefundene SO_2-Freisetzung im Vergleich mit der Rest-SO_2 mg/l

	1 Rest-SO_2	2 gef. Freisetzung	3 ber. Freisetzung	4 ber. Freisetzung aus Glukose-SO_2
Samos I	21	18	16	13
Samos II	41	23	17	14
Samos III	30	20	18	15
Muskateller	107	44	16	13
Modellsaures	—	22	20	17

Daß bei niedrigem pH-Wert aus einer SO_2-Lösung erheblich mehr gasförmiges SO_2 entweicht als bei hohem pH-Wert, ist leicht durch Ansäuern einer SO_2-Lösung geruchlich zu erkennen und läßt sich auch bei verschieden sauren Weinen feststellen. Es hängt dies damit zusammen, daß (bei gleicher Temperatur) umso mehr SO_2-Gas aus der Lösung entweicht, je mehr sie undissoziierte, d. h. nicht in Ionen gespaltene H_2SO_3 enthält; denn es besteht das Gleichgewicht

$H_2SO_3 \rightleftarrows H_2O + SO_2$. Der Gehalt einer Lösung an undissoziierter H_2SO_3 ist aber abhängig vom pH-Wert, indem bei einem höheren pH-Wert mehr H_2SO_3 dissoziiert ist, als bei niedrigem pH-Wert. Die Zahlen der Tabelle 5 lassen erkennen, wie stark der pH-Wert die Dissoziation von H_2SO_3 und damit auch die Flüchtigkeit von SO_2-Gas beeinflußt. Daraus ergibt sich, daß z. B. bei einer Verschiebung des pH-Wertes des Weines von 3,5 auf 1,3, wie sie durch Zusatz von hyperaciden Magensaft stattfindet, die Konzentration an undissoziierter H_2SO_3 von 1,8 auf 48%, d. h. etwa auf das 25fache erhöht wird und damit auch die Flüchtigkeit des SO_2-Gases.

Daraus ergibt sich, daß im stark sauren Magensaft SO_2 viel stärker flüchtig sein muß als im weniger sauren Magensaft und daß ein SO_2-Geruch des Atems deshalb auf Hyperacidität oder allenfalls auch normale Acidität des Magensaftes hinweist, keinesfalls aber auf Anacidität oder Subacidität. Es ist also gerade das Gegenteil von dem richtig, was SCHANDERL annimmt.

Von den drei Faktoren, welche auf die Freisetzung von SO_2 im Wein im Magen einen Einfluß ausüben können (Verdünnungseffekt, Temperatureffekt und pH-Effekt) haben sich nach Vorstehendem die Verdünnung, die bei der Vermischung des Weines mit Magensaft eintritt und die dadurch bewirkte Temperaturerhöhung auf Körpertemperatur als wirksam erwiesen, keinesfalls jedoch die Säureveränderung im Wein, wie sie durch den anaciden und subaciden Magensaft verursacht werden kann.

Der Einfluß der Rest-SO_2 auf die Freisetzung von SO_2

Es verbleibt nun noch näher zu untersuchen, warum in den Versuchsweinen die berechnete Freisetzung von SO_2 geringer ist, als die gefundene freigesetzte SO_2-Menge, insbesondere als die von SCHANDERL gefundenen Werte.

Dazu wurde zunächst die SO_2-Menge, welche an die einzelnen bekannten Komplex-Bildner gebunden ist, auf Grund unserer früheren Befunde berechnet. Wenn man die Summe dieser bekannten SO_2-Bindungen vom Gesamtwert an gebundener SO_2 abzieht, erhält man offensichtlich die an unbekannte Substanzen gebundene SO_2-Menge. Wir bezeichnen sie als Rest-SO_2. Diese Werte finden sich zusammengestellt in Tabelle 3. Es ergibt sich daraus, daß die Menge der bekannten SO_2-Komplexe (Aldehyd-SO_2, Glukose-SO_2, Arabinose-SO_2) bei den einzelnen Versuchsweinen nicht sehr verschieden ist. An Aldehyd-SO_2 ist bei diesen Mustern, die nur eine geringe Gärung erfahren haben, erwartungsgemäß weniger enthalten als bei Tiefweinen.

Demgegenüber zeigen die Rest-SO_2-Werte (wozu hier auch die an Acetoin gebundene SO_2 gerechnet wird) erhebliche Unterschiede; sie sind durchweg höher als bei einigen von uns früher untersuchten deutschen Weinen mit Restsüße (10—20 mg/l).

In Tabelle 6 sind nun diese Rest-SO_2-Werte gegenübergestellt einmal die bei den einzelnen Versuchsweinen bei 37° C gefundenen Maximal-Werte der Freisetzung (gemäß Tab. 2, Querspalte 3, Längsspalten 3, 5, 8, 11, nach Verdoppelung derselben), sodann die Summe der aus den einzelnen bekannten Komponenten (nach Tab. 3) unter den gleichen Bedingungen (37° C, Verdünnung 1:1) freigesetzten SO_2-Mengen, wie sie sich mittels der früher gefundenen Proportionalitäts- und Temperaturfaktoren berechnen lassen, und schließlich auch die SO_2-Mengen, die lediglich aus Glukose-SO_2 freigesetzt werden.

Man findet nun, daß die berechnete Freisetzung von SO_2 umso mehr von der gefundenen Freisetzung abweicht, je höher der Rest-SO_2-Wert ist. Besonders hoch ist derselbe bei dem Muskateller-Wein mit 107 mg/l. Hierbei werden auch 28 mg/l SO_2 mehr freigesetzt, als sich aus den einzelnen bekannten Komponenten der gebundenen SO_2 berechnet. Dieser Mehrwert muß demnach aus noch unbekannten SO_2-Komplexen stammen, was auch verständlich ist, da ja an diese unbekannten Substanzen 107 mg/l SO_2 gebunden sind.

Wie aus der letzten Spalte der Tabelle 6 zu ersehen ist, ist die aus Glukose-SO_2 freigesetzte SO_2-Menge bei den einzelnen Versuchsweinen nur wenig verschieden; sie ist in jedem Falle niedriger als die gefundene Freisetzung der 2. Spalte, bei dem Muskateller machte sie sogar noch nicht ein Drittel von der insgesamt freigesetzten SO_2-Menge aus, während mehr als zwei Drittel derselben anderer, im wesentlichen unbekannter Herkunft sind.

Diese Befunde bestätigen die vorstehend wiederholt geäußerte Vermutung, daß die von SCHANDERL gefundene besonders große Freisetzung von SO_2 vor allem auf in seinen Versuchsweinen reichlich enthaltene SO_2-Komplexe unbekannter Natur zurückzuführen sein müssen. Zum anderen ergibt sich daraus aber auch – und hierauf kommt es in diesem Zusammenhang besonders an – daß für diese starke Freisetzung von SO_2 keinesfalls, wie SCHANDERL annimmt, hauptsächlich die Glukose-SO_2 verantwortlich zu machen ist. Es läßt sich vielmehr, wenn man etwa den gleichen Zuckergehalt (und Glukose-Gehalt) wie bei unseren (gleichartigen) Versuchsweinen zugrunde legt, berechnen, daß bei dem angegebenen Gehalt an freier SO_2 bei diesen Weinen nur etwa der dritte bis fünfte Teil der insgesamt freigesetzten SO_2 (von 45 bis 63 mg/l) auf die Spaltung der an Glukose gebundenen SO_2 zurückgeführt werden kann.

Als Substanzen, die für die Bindung der Rest-SO_2 verantwortlich sind, kommen vor allem solche in Betracht, deren SO_2-Komplexe, ebenso wie das schon oben erwähnte Acetoin, auf Temperaturerhöhung und auch auf Konzentrationsänderungen durch starke Spaltung reagieren. Es ist dabei vor allem an Oxymethylfurfurol zu denken, welches gerade in diesen süßen Dessertweinen auf Grund ihrer Herstellungsart enthalten sein kann und welches als Aldehyd vermutlich SO_2 zu binden vermag, was aber noch zu untersuchen ist.

ZUSAMMENFASSUNG UND SCHLUSSFOLGERUNGEN

Zu unseren Versuchen, die zur Nachprüfung der Befunde von SCHANDERL über die beträchtliche Freisetzung von SO_2 in Weinen mit unvergorenem Zucker vorgenommen wurden, verwendeten wir ebenfalls süße Dessert-Weine (Samos, süßer italienischer Muskateller). Es wurde dabei unter den von SCHANDERL gewählten Bedingungen (Vermischung von Modell-Magensaft mit Wein im Verhältnis 1:1 bei 37° C) zwar ebenfalls eine Freisetzung von SO_2 gefunden, sie ist aber wertmäßig wesentlich geringer, nämlich 18–38 mg/l anstatt 45 und 63 mg/l.

Diese Freisetzung von SO_2 ist aber nicht, wie SCHANDERL annimmt, überwiegend auf die Spaltung von Glukose-SO_2 zurückzuführen, sondern aus dieser kann auch bei diesen süßen Weinen mit hohem Glukosegehalt nur etwa 13–15 mg/l an freier SO_2 gebildet werden, d. h. nur etwa der dritte bis fünfte Teil der nach SCHANDERL freigesetzten SO_2. Die Hauptmenge der hierbei gebildeten freien SO_2 stammt vielmehr aus unbekannten SO_2-Komplexen, die in diesen Dessertweinen in zum Teil vielfach größerer Menge als in unseren (deutschen) Tischweinen mit Restsüße enthalten sind. Dies läßt auch der hohe Gehalt der ersteren an Rest-SO_2, d. h. an unbekannte Substanzen gebundener SO_2 erkennen, der nach unseren Untersuchungen bis 107 mg/l betragen kann, gegenüber 16–30 mg/l bei unseren Tischweinen.

Die Freisetzung von SO_2 im menschlichen Magen kann durch die Temperaturerhöhung des Weines auf 37° C, durch seine Verdünnung mit Magensaft, sowie auch durch die Veränderung des Säuregrades (pH-Wertes) dabei verursacht werden. SCHANDERL schreibt die größte Wirkung der Säureänderung durch den Magensaft und zwar durch den anaciden, d. h. salzsäurefreien Magensaft zu. Wir stellten demgegenüber fest, daß dadurch kaum eine Freisetzung von SO_2 stattfinden kann und daß die von SCHANDERL der Säurevermindernng des Weines durch diesen Magensaft zugeschriebene Wirkung auf der Konzentrationsänderung im Wein infolge Verdünnung beruht.

Wir konnten dagegen eine (geringe) Freisetzung von SO_2 durch hyperaciden, d. h. übersauren Modell-Magensaft nachweisen, wobei jedoch die freigesetzte SO_2 wegen der etwa 25fachen größeren Flüchtigkeit des SO_2-Gases in dieser sehr sauren Lösung sich teilweise der analytischen Erfassung entzieht. Dadurch kann der Atem der hyperaciden und allenfalls auch normaciden Weintrinker nach SO_2 riechen, keinesfalls aber, wie SCHANDERL annimmt, der Atem von anaciden Personen.

Wenn schon die Freisetzung von SO_2 aus Glukose-SO_2 bei diesen süßen Dessertweinen ganz erheblich geringer ist, als SCHANDERL annimmt, dann stellt sich die Frage, wie sich diese Aufspaltung der Glukose-SO_2 bei unseren deutschen Tischweinen mit unvergorenem Zucker auswirkt, auf die ja in erster Linie diese Untersuchungen abgestellt sind. Da diese Tischweine bei einer „normalen" Restsüße von etwa 20 g/l unvergorenem Zucker nur etwa den 8. bis 10. Teil der Glukose dieser Dessertweine enthalten, beträge (bei gleichem Gehalt an freier SO_2) auch die an Glukose gebundene SO_2 nur den 8. bis 10. Teil der ersteren und ebensoviel auch die daraus mögliche Freisetzung an SO_2, d. h. diese kann bei Körpertemperatur im Magen höchstens 1,5–2 mg/l ausmachen. Diese Menge liegt kaum außerhalb der analytischen Fehlergrenze und ist absolut und im Vergleich mit der schon in Wein normalerweise vorhandenen freien SO_2 (30–40 mg/l) so gering, daß sich jede Diskussion über die gesundheitliche Unbedenklichkeit einer solchen Zunahme an freier SO_2 im Magen erübrigt. Das gleiche gilt auch für Weine mit „erhöhter" Restsüße, mit 30–40 g/l unvergorenem Zucker, die in der Praxis bei Weinen, die in größerer Menge getrunken werden, wohl kaum überschritten werden.

Hierbei ist noch weiter zu berücksichtigen, daß die den Versuchen von SCHANDERL zugrunde liegende und für unsere Versuche übernommene Verdünnung des Weines mit Modell-Magensaft im Verhältnis 1:1 (woraus ja in erster Linie die Freisetzung von SO_2 resultiert) in vivo, d. h. im menschlichen Magen kaum realisiert ist. Normalerweise wird bei vollem Magen und nicht zu schnellem Trinken eine wesentlich stärkere Verdünnung des Weines als 1:1 stattfinden und dadurch auch eine noch stärkere Freisetzung von SO_2. Diese wird aber mehrfach überkompensiert durch die Konzentrationsverminderung, welche die bereits im Wein vorhandene freie SO_2 hierdurch erfährt, ferner auch durch die Bindung oder Adsorption durch den Mageninhalt, worauf schon SCHANDERL hinweist. Bei reichlichem und schnellem Weintrinken in den leeren Magen findet dagegen eine geringere Verdünnung des Weines statt und infolgedessen auch eine geringere Freisetzung aus Glukose-SO_2 als bei den Modellversuchen gefunden wurde. Wenn man noch dazunimmt, daß sich im gefüllten Magen auch der pH-Wert weitgehend ändern kann, und das Magensekret selbst bei der gleichen Person einen verschiedenen pH-Wert aufweisen kann (vgl. Anm. 3), erscheint die Übertragung solcher Modellversuche auf die Vorgänge im menschlichen Magen überhaupt sehr problematisch.

Darüber hinaus ergeben sich aus diesen (und früheren) Feststellungen aber auch Folgerungen für die hygienische Beurteilung der insgesamt im Wein gebundenen SO_2, speziell in den hier zur Diskussion stehenden deutschen Tischweinen mit Restsüße.

In diesen besteht die gebundene SO_2 auch bei Weinen mit unvergorenem Zucker weit überwiegend (bis zu mehr als 90%) aus Aldehyd-SO_2. Aldehyd-SO_2 ist aber so stabil, daß sie bei einer Verdünnung keine und bei Erwärmung auf 37° C nur eine kaum nachweisbare Spaltung erfährt, also nicht an der Zunahme der freien SO_2 im Wein beteiligt ist. Eine Freisetzung von SO_2 und damit eine hygienisch nachteilige Wirkung kann deshalb nur von den übrigen SO_2-Komplexen im Wein ausgehen. Für die Glukose-SO_2 ist sie aber nach Vorstehendem bei derartigen Weinen belanglos. Das gleiche gilt auch für die ebenso geringe oder noch geringere Menge an Arabinose-SO_2 und auch für die bei diesen Weinen nach unseren bisherigen Feststellungen ebenfalls geringe Menge an Rest-SO_2.

Viel gebundene SO_2 bei derartigen Weinen ist stets durch einen hohen Gehalt an Aldehyd-SO_2 bedingt, während die übrigen SO_2-Komplexe mengenmäßig weitgehend konstant sind, und auch die Glukose-SO_2, selbst bei größerem Unterschied im Zuckergehalt, nur wenig zur Erhöhung der gebundenen SO_2 beiträgt. Daraus muß man schließen, daß selbst bei einem hohen Gehalt an gebundener SO_2 bei derartigen Weinen keine nachteiligen gesundheitlichen Wirkungen infolge Freisetzung von SO_2 im menschlichen Magen zu erwarten sind.

Anders liegen die Verhältnisse jedoch bei süßem Dessertwein mit hohem Gehalt an wärmelabilen Rest-SO_2-Komplexen. Gerade aus diesen SO_2-Verbindungen finden, wie besonders die Ergebnisse von SCHANDERL zeigen, eine beträchtliche hygienisch nicht unbedenkliche Freisetzung von SO_2 statt.

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Bei unseren Tischweinen kann nach Vorstehendem ein Niedrighalten des Gehaltes an gebundener SO_2 im wesentlichen nur zu Lasten der Aldehyd-SO_2 stattfinden. Dies bedeutet, daß entweder der Aldehydgehalt des Weines niedrig gehalten werden muß, oder aber daß man freien, nicht an SO_2 gebundenen Aldehyd im Wein in Kauf nehmen muß. Wie das erstere, d. h. die Entstehung von möglichst wenig Aldehyd im Wein zu erreichen ist, darüber können noch keine sicheren Angaben gemacht werden; wahrscheinlich wirkt sich der Zusatz von SO_2 vor und während der Gärung in einer erhöhten Aldehydbildung aus (5), indem die Hefe dadurch die für sie giftige SO_2 unschädlich zu machen versucht. Daraus würde folgen, daß zur Niedrighaltung des Aldehydgehaltes die bisherige Art unserer Weinbereitung, bei der die S[illegible]efelung des Gärgutes eine große Rolle spielt, geändert werden müßte. Anderers[illegible] würde freier Aldehyd im Wein das heutige Geschmacksbild unserer Weine grundlegen[illegible] und für die meisten Weintrinker nachteilig verändern und kann deshalb ohne zwingende Gründe nicht in Frage kommen. Es ergibt sich daraus, daß die Festlegung eines Höchstgehaltes an gebundener SO_2 und damit auch an gesamter SO_2 im Wein sorgfältiger Überlegung bedarf, um der Weinwirtschaft keinen nachhaltigen Schaden zuzufügen.

Abschließend kann die in der Überschrift gestellte Frage klar und zweifelsfrei verneinend beantwortet werden: Die schweflige Säure ist (bei Vermeidung einer Überschwefelung, d. h. eines zu hohen Gehaltes an freier schwefliger Säure) in den Weinen mit unvergorenem Zucker gesundheitlich genau so unbedenklich wie in den durchgegorenen Weinen und zwar auch (und sogar erst recht) für Weintrinker mit anacidem Magen.

Meinen Mitarbeitern Herrn Dr. Ing. G. Würdig und Frl. M. Frere danke ich bestens für ihre Unterstützung bei den Untersuchungen und ihrer Auswertung.

LITERATURHINWEISE UND BEMERKUNGEN

(1) Schanderl, H.: Die schweflige Säure des Weines in hygienischer Sicht. Zeitschr. für Lebensmittel-Unters. Forsch., *103*, 379 (1953)

(2) Kielhöfer, E.: Die Bindung von schwefliger Säure an Weinbestandteile. Weinberg und Keller, *3*, 461 (1956)

(3) Wir beziehen uns dabei sowohl auf die Auskunft eines Spezialarztes für Magen- und Darmkrankheiten wie auch auf die Angaben in L. Hallmann, Klinische Chemie u. Mikroskopie, Leipzig 1952. Nach S. Ehrbacher u. F. Leuthardt, Lehrbuch der physiologischen Chemie, Berlin 1954, liegt der pH-Wert des normalen Magensekretes bei pH 1.5. Es wird auch angegeben, daß bei der gleichen Person der pH-Wert des Magensekretes sich, beeinflußt durch die aufgenommenen Speisen, innerhalb weiter Grenzen ändern kann.

(4) Kielhöfer, E. u. H. Aumann: Die Bestimmung der gesamten und freien schwefligen Säure im Wein, auch in Gegenwart von Ascorbinsäure. Mitt. Klosterneuburg, *A 7*, 287 (1957).

(5) Vgl. dazu die Befunde von F. Paul, Über den Acetaldehyd im Wein. Mitt. Klosterneuburg, Serie *A 8*, 123 (1958).

Berichtigung zu der Mitteilung des gleichen Autors in Heft 9/1958, Seite 464:

In der Abbildung 1 ist die Bezeichnung der beiden Kurven I und II versehentlich vertauscht worden. Es muß richtig heißen: I in % der freien SO_2 und II in % der ges. SO_2.

Auf derselben Seite muß es daher in der 10. Zeile von unten richtig heißen: ... aus der Kurve I ... anstatt aus der Kurve II.

DIE WEIN-WISSENSCHAFT
BEILAGE ZUR FACHZEITSCHRIFT
„SÜDDEUTSCHE WEINZEITUNG"

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Der Einfluß von Wein, Alkohol und schwefliger Säure auf die bakterizide Kraft des Blutes

Von H. Kliewe und G. Gillissen
Aus dem Hygienischen Institut der Universität Köln
Vorstand: Prof. Dr. H. D. Gerstenbergk

In Tierversuchen mit Kaninchen suchten wir die Frage zu beantworten, ob und in welchem Ausmaß orale Gaben von Wein, Alkohol und schwefliger Säure die bakterizide Kraft des Blutes verändern. Ferner untersuchten wir das Verhalten der Leukozyten (Granulozyten — und Lymphozyten-Zahl), die Diapedesisfähigkeit der weißen Blutzellen und die Veränderung des Komplementtiters, die vielleicht einen Anhaltspunkt für die gestaigerte oder verminderte Abwehrkraft des Organismus geben können.

Unsere Fragestellung ergibt sich im Rahmen eines größeren Arbeitsprogramms, das die vielseitigen biologischen Effekte von Wein und anderen alkoholischen Getränken klären soll, um zu einer gesicherten Beurteilung von Indikation und Gegenindikation dieser Getränke zu gelangen (Übersichten s. Kliewe 1955, Roller 1957, Lacis 1956 und 1956, Stary 1955, Reach 1957, Zieve und Lieberman 1955). In diesem Zusammenhang ist die Frage nach der antibakteriologischen Bedeutung von schwefliger Säure (SO_2) (Kliewe und Gillissen) heute von besonderem Interesse, da gerade diese Substanz für manche nach Weingenuß auftretenden Beschwerden verantwortlich gemacht wird. Dabei ist offen, ob SO_2 wirklich und in welchem Umfang die Ursache darstellt, andererseits in welche biologischen Reaktionen sie eingreift. Die aufgeworfene Frage wurde bisher trotz häufiger Diskussionen nur verhältnismäßig wenig sachlich bearbeitet. Eine der älteren und gleichzeitig auch umfänglichsten Untersuchungen in dieser Richtung ist die von Beuch an einer größeren Anzahl von Probanden unter ziemlich definierten Versuchsbedingungen. Die Versuche ergaben sehr differente Reaktionen der einzelnen Versuchspersonen. Während bei einem Teil der Probanden ein Kratzen im Hals und Kopfschmerzen schon nach Einnahme von 45 mg freier SO_2 bzw. 240 mg gebundener SO_2 bereits in 0,3 l Wein beobachtet wurden (bei 80 mg freier SO_2 und 370 mg gebundener SO_2 starke Kopfschmerzen und Diarrhoen), betrugen andere innerhalb von 25 Tagen in acht Dosen von 45 bis 85 mg zusammen 500 mg freie SO_2 bzw. innerhalb von drei Tagen in drei Dosen von 150 bis 350 mg zusammen 1400 mg gebundene SO_2 ohne Symptome. Beuch

empfiehlt nach seinen Untersuchungen eine vollständige Nachsthonung mit von 100 mg gebundener SO_2 und 20 mg, allenfalls auch bis 40 mg freier SO_2 für jeden Wein. Diese Konzentrationen sollen also ausreichen, um den Wein vor oxydativen Veränderungen zu schützen und die Bildung der gesundheitsschädlichen sog. Reduktone zu verhindern. Zu endlichen Resultaten führen, wie Reichel, sowie frühere Untersuchungen des Reichsgesundheitsamtes.

Die Toxizität von SO_2 wurde unter definierten Bedingungen von Rentschler und Franz im Tierversuch mit Kaninchen untersucht. Wenn damit auch noch kein direkter Schluß auf das Verhalten des Menschen gegenüber der SO_2 gezogen werden kann, so wird zumindest sicher, daß SO_2 nicht nur als Gas, sondern auch als schweflige Säure u.U. eine deutliche Giftwirkung hat. Nach den Untersuchungen waren für Kaninchen 0,25 g SO_2/kg noch tödlich, 0,20 g/kg nicht mehr über 0,20 g/kg. Der Tod der Tiere erfolgte durch Atemlähmung mit Beteiligung des Gefäßsystems. Kleinere Dosen bei Tieren wurden dagegen über Jahre symptomlos vertragen. In Gegensatz zu diesen vielseitigen Feststellungen vertritt das radikalste Ausgangsbuch von Bordeaux (1910) (zit. Schander) die Auffassung einer völligen Unschädlichkeit der SO_2 für den Menschen auch in hohen Dosen.

Im einzelnen wurde festgestellt, daß beim Menschen ab der Körpertemperatur von 37° C im Falle eines sauren oder neutralen Magensaftes freie SO_2 aus gebundener SO_2 schnell und in ausgeprägtem Maße entbunden wird (Schander). Hiermit wird die Unbedenklichkeit reichlich geschwefelter Weine für diesen Paragraphen abgeleitet.

Eine Untersuchung des Einflusses von SO_2 auf den Blutzuckerspiegel (Versuche mit Kaninchen) ergab, daß Wein, äquivalente Mengen von Aethanol und z.T. sehr hohe Dosen von SO_2 keine beweisende Aenderung des Blutzuckerspiegels ergaben. Andererseits scheint aber SO_2 die nach Glukosebelastung oder nach Injektion von Suprarenin auftretende Hyperglykämie zu hemmen (Kliewe und Gillissen). Diese Resultate lassen den Schluß auf eine Beeinflussung der A-Zellen des Pankreas durch SO_2 als Reduktionsmittel zu, da die Vitamin-C-reichen A-Zellen durch reduzierende Stoffe beeinflußt werden können. Dieser Feststellung dürfte bei den für Wein gesetzlich zugelassenen Mengen von SO_2 bei normalen Körperverhältnissen keine Bedeutung zukommen. Die Versuche geben aber doch den beachtenswerten Hinweis, daß größere SO_2-Mengen im Wein abgelehnt werden müssen. Ferner konnte gezeigt werden (Kliewe und Gillissen), daß auch die Funktion der Nebennierenrinde durch SO_2 beeinflußt wird. Oral verabreichte Mengen führten zu einer deutlichen Stimulierung, zu einer Streß-Situation, die durch gleichzeitige Gaben von Aethanol gebremst wird. Darüber hinaus ist die antibakterielle Wirkung der SO_2 von Interesse. Es kann mit Sicherheit gesagt werden, daß der SO_2 keine hohe antibakterielle Wirkung zukommt. Trotzdem ist die noch immer verbreitete Meinung, man könne damit den Wein konservieren, falsch (Schander). Zur Diskussion steht aber noch die Frage, ob große Gaben von SO_2 Wein oder Aethanol beim Tier oder beim Menschen die normale antibakterielle Kraft des Blutes zu beeinflussen vermögen. Es wäre denkbar, daß dabei die verschiedenen chemischen Stoffe des Weines synergistisch oder antagonistisch wirken, wie vergleichsweise die antibakterielle Wirkung von Wein in vitro kein reiner Aethanol-Effekt ist (Kliewe und Gillissen), sondern durch die Kombination von Aethanol und Säure hervorgerufen wird (Drnczynski).

Cremer beobachtete bei Kaninchen nach täglichen Gaben von 5 mg SO_2 bei einer Versuchsdauer von 110 Tagen am Ende dieser Zeit eine Abnahme der antibakteriellen Kraft des Blutes, während bei den Kontrolltieren (keine Zuteilungsgaben) kaum eine Aenderung eingetreten sein soll. Da nach den Untersuchungen von Pfannenstiel, Holsen und Henrich Vitamin-C-Ge-

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ten einen Anstieg der Blutbakterizidie bewirken, SO_2 dieses Vitamin in Traubensäften aber reduziert (Büsing und Raabe), waren die Resultate von Pfannenstiel und Eckhardt über eine Abnahme der Blutbakterizidie durch geschwefelten Traubensaft im Verhältnis zu ungeschwefeltem verständlich. Allerdings ist durch diese Versuche nicht entschieden, ob die Unterschiede durch einen Vitamin-C-Verlust oder durch SO_2 selbst bedingt sind. Auch wurden die Resultate mit Tieren ermittelt, die unter einer Art Mangelkost standen, so daß ein Schluß auf die natürlichen Verhältnisse nicht gerechtfertigt ist.

Ausgehend von diesen Beobachtungen untersuchten wir bei Kaninchen die Veränderungen der bakteriziden Kraft des Blutes bei Normalkost nach Gaben von Wein, Aethanol und SO_2 unter Verwendung von Leitungswasser zur Kontrollreihe.

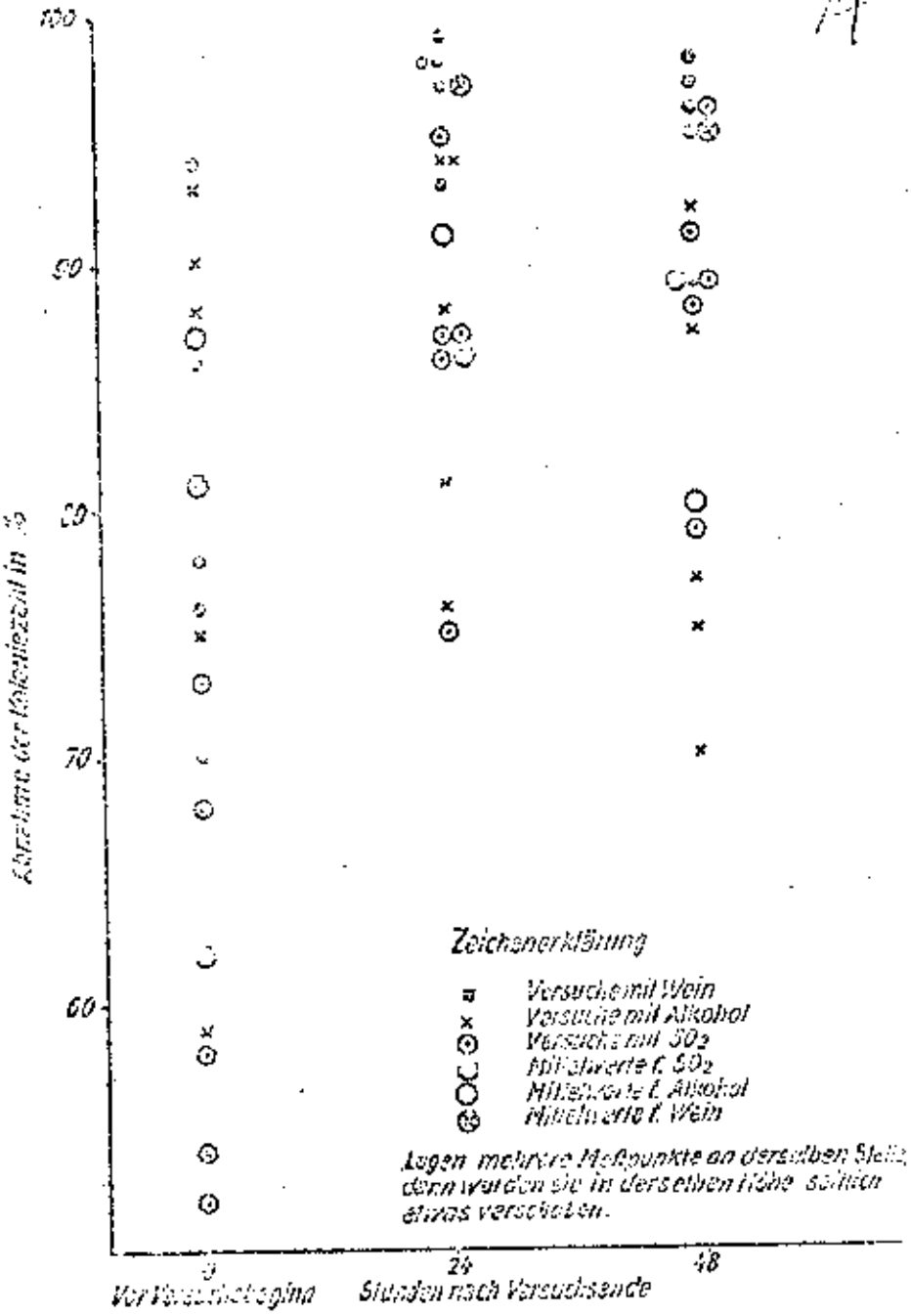
Material und Methoden:

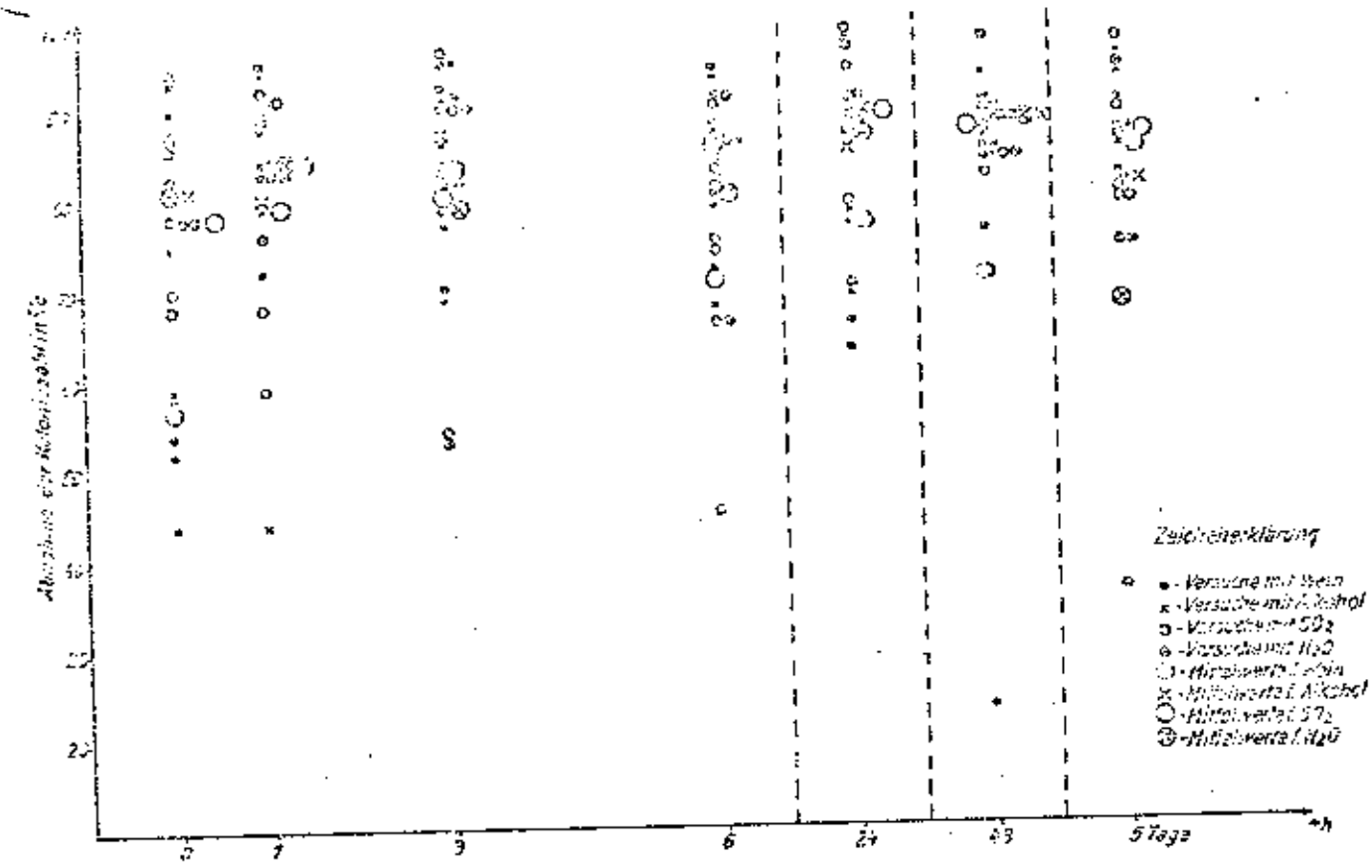
Als Versuchstiere dienten männliche Kaninchen mit einem Gewicht von 2200 bis 2600 g. Die Tiere wurden bei Normalkost stets in den gleichen Stallungen gehalten. Vom Verabend der Blutentnahme an erhielten die Tiere keine Nahrung mehr. Das Blut wurde aus der Ohrvene unmittelbar vor und in verschiedenen Intervallen nach einmaligen oder mehrmaligen Gaben der Testlösungen entnommen (1 ml Blut + 0,25 ml 3,8prozentige sterile Na-Citrat-Lösung). Zu jedem Versuchsansatz wurden 5 Tiere genommen. Beim Einzelversuch erhielten die Tiere 1 % und beim Dauerversuch 5 % täglich 30 ml der Versuchslösungen.

Die Bestimmung der Blutbakterizidie erfolgte nach den Angaben von Kriegel und Spode. Dabei wurde demnach die prozentuale Abnahme der Zell (Kolonie-)-Konzentration von Staphylokokken nach 4 Std. langer Inkubation des Blut-Staphylokokken-Gemisches bei 37° C. Zu diesem Zweck versetzten wir jede Blutprobe zu gleichen Teilen mit einer auf 10^{-4} mit steriler phys. NaCl-Lösung verdünnten 18 Std. alten Bouillonkultur von M. pyogenes var. aureus Sp. 511. Aliquote Anteile wurden unmittelbar danach sowie nach 4 Std. langer Inkubation im Wasserbad bei 37° C nach entsprechender Verdünnung mit Aqua dest. sterilis. (zur Erzielung einer gut auszählbaren Koloniezahl) zu je 0,3 ml auf 3 Agarplatten ausgestrichen und die prozentuale Abnahme der Koloniezahl je Volumeneinheit als Maß der Blutbakterizidie berechnet.

Von jeder Blutprobe bestimmten wir ferner in üblicher Weise unter Verwendung einer 4prozentigen Suspension von 3 % mit phys. NaCl-Lösung gewaschenen und in einer täglich neu hergestellten Verdünnung (1 : 5) von Veronalpuffer (pH 7,4) aufgenommenen sensibilisierten Hammelerythrocyten den Komplementtiter des Serums. Darüber hinaus wurde eine Leukocyten- und Erythrocytenzählung durchgeführt, sowie ein Differentialblutbild (Schermer) angelegt. Wir untersuchten den Einfluß von Wein, SO_2 und von Wasser. Da Kaninchen nicht brechen können, kam das gesamte, mit der Schlundsonde verabreichte Volumen zur Auswirkung.

Als Wein wurde ein Weißwein verwandt (1957er Jugenheimer Angerborn nat., Gewürztraminer). Seine Analyse ergab: Spez. Gewicht (bei 20° C) 1,0037, spez. Gewicht des alkoholischen Destillats 0,9869 und in g/l (= 1000 g) Alkohol = 76,8 (= 9,70 Vol.%), Zucker = 11,5, Gesamtsäure (Weinsäure) = 9,9 und freie SO_2 = 22 mg/l. Reines Aethanol wurde in einer Konzentration von 12 Prozent und SO_2 in einer solchen von 300 mg freie schweflige Säure pro l benutzt. Wir wählten bei SO_2 eine weitaus höhere Konzentration als im


100
90
80
70
60
Zeichenerklärung
Versuche mit Wein
Versuche mit Alkohol
Versuche mit SO2
Mittelwerte f. SO2
Mittelwerte f. Alkohol
Mittelwerte f. Wein
Lagen mehrere Meßpunkte an derselben Stelle, dann wurden sie in derselben Höhe seitlich etwas verschoben.
0
24
48
Vor Versuchsbeginn
Stunden nach Versuchsende


Zeichenerklärung
Versuche mit Wein
Versuche mit Alkohol
Versuche mit SO_2
Versuche mit H_2O
Mittelwerte f. Wein
Mittelwerte f. Alkohol
Mittelwerte f. SO_2
Mittelwerte f. H_2O
0
1
3
6
24
48
5 Tage
h

Wenn, da in Vorversuchen mit kleinen Konzentrationen keine Beeinflussung der Blutbakterizidie erreicht wurde und eine [illegible] Aussage über eine Beeinflussungsmöglichkeit schließlich erwünscht war.

Ergebnisse:

1.) Die Untersuchungsergebnisse über eine Aenderung der Blutbakterizidie sind für einmalige Gaben in Abb. 1 mit Mittelwertbildung angegeben. Es ist daraus zu ersehen, daß zwischen den einzelnen Versuchsanordnungen kein nennenswerter Unterschied besteht. Auch die Mittelwerte liegen in einer vergleichbaren Ebene. Der mit Stauenbach-Test vorgenommene Vergleich der Mittelwerte unter Berücksichtigung der Streuung ergab keine Signifikanz. Demgegenüber ergaben die Dauerversuche (Abb. 2) mit Wein [illegible] Höchstes, und mit SO₂ meist die niedrigsten Werte. Das Ergebnis mit Aethanol nimmt eine Mittelstellung ein. Die statistische Berechnung ergab jedoch beim Vergleich der Mittelwerte aus dem Aethanol- und SO₂-Versuch mit denen der Weinversuche ebensowenig eine Signifikanz für die Differenzen wie die Vergleiche der einzelnen Zeitwerte unterei[illegible] bei der gleichen Versuchsanordnung.

2.) Die Ergebnisse der Komplementtitration s. Tab. 1

Tabelle 1

Ergebnis der Komplement-Titrationen — Versuche mit Einzelgaben
(Titerangabe der Mittelwerte durch den reziproken Wert)

Testlösungen

t n. Vers. Ende	Wein	Aethanol	SO_2	H-C
0 h	102	44	51	79
1	44	32	51	70
3	51	57	43	26
6	51	51	71	42
24	57	61	44	44
48	85	44	51	80
5 Tg.	64	51	51	48
Versuche mit Dauergaben				
24 h	57	35	76	—
48	89	44	76	—

Die Streuung der in Tab. 1 wiedergegebenen Werte war stets mindestens ± 50 Prozent, so daß eine Nichtsignifikanz der Differenzen zwischen den Mittelwerten verständlich ist.

3.) Die Ergebnisse der Auswertung des Blutbildes s. Tab. 2 (Nomenklatur und Differenzierung nach den Angaben von Schermer).

Tabelle 2

Auswertung der Blutbilder
(Angabe der Mittelwerte je Versuchsgruppe)

a) Versuche mit Einzelgaben der Testlösungen

Versuch	t n. Vers. Ende	Ery. × 10³	Leucos. × 10³	Differentialblutbild-Zellzahlen in % P	E	L	Mono.	Bas.
H_2O	0 h	4,7 (2,8–3,3)	8,3 (7,4–10,8)	26 (11–79)	4 (1–15)	55 (17–79)	1 (0–5)	4 (0–13)
	1	4,4 (3,7–5,5)	10,6 (5,4–18,8)	27	2	59	2	10
	3	4,5 (3,6–5,6)	10,5 (6,2–16,5)	27	1	63	3	6
	6	3,5 (2,7–4,9)	8,4 (5,2–13,4)	26	1	63	3	7
	24	3,4 (2,9–3,7)	6,0 (5,2–12,2)	28	2	63	4	3
	48	2,9 (1,5–4,2)	7,2 (3,6–9,8)	47	2	43	2	6
	5 Tg.	3,3 (2,5–4,6)	6,6 (5,2–9,9)	33	3	54	1	9
Wein	0 h	5,2	8,2	24	3	67	4	2
	1	4,6	6,2	35	4	56	3	2
	3	4,4	5,3	39	3	53	4	1
	6	4,3	8,0	22	1	71	2	1
	24	3,7	7,7	44	5	43	3	5
	48	3,9	8,3	44	4	46	3	3
	5 Tg.	3,4	7,4	45	1	47	4	3
Aethanol	0 h	4,3	7,7	47	4	44	3	2
	1	4,5	7,4	39	2	47	4	8
	3	4,6	7,9	42	2	46	2	8
	6	3,9	9,2	40	1	52	6	1
	24	4,3	8,6	39	2	49	3	7
	48	3,7	7,4	39	3	52	2	4
	5 Tg.	3,3	7,7	40	5	39	3	4
SO_2	0 h	4,6	7,0	46	3	44	3	4
	1	4,5	6,7	42	3	50	2	3
	3	4,3	10,2	45	1	51	1	2
	6	4,1	6,7	36	2	53	5	4
	24	3,5	9,8	40	2	54	2	2
	48	3,2	5,9	34	4	57	3	2
	5 Tg.	3,8	6,5	51	3	40	1	2

b) Dauerversuche

Versuch	n. Vers. Woche	Ery. x 10^6	Leuko. x 10^4	Differentialblutbild-Zellzahlen in % P	N	L	Mono.	Ba.
Wein	24	6,3	3,6	53	3	43	3	3
	48	5,2	6,5	31	4	56	4	5
Äthanol	24	5,4	9,8	47	2	42	4	5
	48	5,0	7,3	42	4	46	3	5
SO_2	24	4,7	9,3	38	2	51	2	9
	48	4,5	8,8	34	5	46	3	12

Zeichenerklärung:

( ) = Zeigende für Extremwerte als Maß der Streuung
P = Pseudoeosinophile, spezialgranulierte Leukozyten
N = Neutrophile
L = Lymphozyten
Mono. = Monocyten
Ba. = Basophile Leukozyten

Aus Tab. 2 ist zu entnehmen, daß bemerkenswerte Veränderungen des Blutbildes durch die verabreichten Versuchsmedien nicht eintraten. Dies ist nicht mehr auf die große, bei Kaninchen bekannte Streuung dieser Daten zurückzuführen (Uebersicht s. Scheunert). Nur größere Veränderungen könnten im Sinne einer verschiedenartigen Beeinflussung gedeutet werden, solche waren aber nicht festzustellen.

Diskussion:

Die vorstehenden Daten besagen, daß weder Wein, noch Äthanol oder SO_2 oral verabreicht unter den gewählten Versuchsbedingungen merkliche oder statistisch gesicherte Veränderungen der Antikörperzahl, des Komplementtiters oder des Blutbildes verursachen. Diese Feststellung hat insofern noch ein besonderes Interesse, als in der Literatur verschiedentlich anders lautende Angaben vorliegen. Verwiesen sei vornehmlich auf die bereits genannten Arbeiten von Pfennenstiel und Eckhardt und von Cremer. Letzterer stellte eine starke Schädigung der bakteriziden Kraft des Blutes durch SO_2-Gaben fest. Eine Beurteilung dieser Befunde ist aber schwierig, da nur die Daten der Versuchsreihe und nicht die der Kontrollreihe angegeben wurden. Es müßte noch berücksichtigt werden müssen, daß bei den einzelnen Tieren die Werte vor Versuchsbeginn mit den nach über 3 Monaten gemessenen Werten verglichen wurden, ein Zeitintervall, in dem zahlreiche andere Faktoren zur Auswirkung kommen konnten. Der angegebene Meßwert muß deswegen nicht unbedingt Ausdruck eines SO_2-Effektes sein.

Bei den Versuchen von Pfennenstiel und Eckhardt wurde die Wirkung von unverschwefelten mit der von geschwefelten Trockensaft, einschließlich einer Kontrolle mit Leitungswasser, verglichen. Diese Versuchsanordnung weicht aber von unserer Fragestellung insofern ab, als bei den genannten Autoren die Versuchstiere ein mehlhaltiges (1 Std. bei 120° C), sonst Mischfutter erhielten, während unsere Versuche unter natürlichen Ernährungsbedingungen abliefen. Um einen Ueberblick über die Streuung der von Pfen-

[illegible] und Eckhardt ungeeignete Versuchstiere zu erhalten, berechneten wir ihre Beweiskraft unter statistischen Gesichtspunkten. Zu berücksichtigen, der Ansicht dieser Autoren folgt nach, daß die Steigerung der Erythrozytenzahl nach Gaben von ungeschwefeltem Saft, ferner die Abnahme der Erythrozytenzahl nach Gaben von geschwefeltem Saft in einer Wahrscheinlichkeit von 99 Prozent beweisend bzw. ablehnend ist, daß auch die bei Verwendung von [illegible] unpasteurisierter beschriebene Abnahme der Blutbakterizidie (von 70 Prozent auf [illegible]) wirksam ist, wenn man als Kriterium für die Signifikanz eine Wahrscheinlichkeit von 5 Prozent für die Zufallsbedingtheit der Änderung annimmt. Die Differenz zwischen der Versuchsgruppe (geschwefelter Saft) und der Kontrollgruppe (ungeschwefelter Saft) ist statistisch signifikant. Ein Rückschluß auf unsere Resultate ist aber nicht nur wegen der verschiedenen Erkrankung der Tiere unzulässig, sondern auch deswegen, weil die bei unseren Versuchen bei der Gegenüberstellung von geschwefeltem und ungeschwefeltem Saft beobachteten Unterschiede (Vitamin C und SO2) gleichzeitig höher und bei ein in Vergleich mit Leitungswasser noch niedrigen, so daß sowohl an eine Wirkung unterschiedlicher Vitamin-C-Konzentrationen als auch an eine direkte Wirkung der SO2 auf den Organismus gedacht werden kann.

Unsere hier beschriebenen Versuchsergebnisse mit maximaligen Gaben der Testsubstanzen deuten — wenn auch nicht beweisend — an, daß SO2 die bakterizide Kraft des Blutes vielleicht doch etwas vermindert. Sollte dies zutreffen, dann könnte zweifellos mit einer geringfügig großen Tierzahl und der dadurch bedingten Verkleinerung der Streuung eine geringere Beeinflussung der Blutbakterizidie versuchsmäßig gesichert werden. Es dürfte aber nicht ratsam sein, vor einer Unterbewertung statistischer Schätzungen bei biologischen Versuchen dieser Art zu warnen, da eine kleine Änderung eines Mittelwertes, die statistisch nur mit großen Versuchszahlen gesichert werden kann, praktisch kaum bedeutungsvoll ist.

Zusammenfassung:

In Tierversuchen mit Kaninchen wurde der Einfluß von Wein, Äthylalkohol und schwefliger Säure auf die Blutbakterizidie, das rote und weiße Blutbild und auf das Komplementsystem untersucht. Während der ganzen Versuchszeit erhielten die Tiere Normalkost, um außergewöhnliche Lebensbedingungen zu vermeiden. Bezeichnende Veränderungen konnten bei einmaligen und wiederholten Gaben der Testsubstanzen nicht festgestellt werden.

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mit der Schlundsonde verabreicht. Die KSt.-Bestimmung erfolgte täglich bis zu 5 Tagen.

Tabelle I. Einfluss von schwefliger Säure auf die C-17-Ketosteroidausscheidung

Tage nach Versuchsbeginn	Zweimal 5 cm³ SO_2 (50 mg/l) am 1. Tag Abweichung der KSt.-Ausschüttung in Prozent vom normalen Mittelwert	
	1. Serie	2. Serie
1	– 17,4 N.	– 1,2 N.
2	+ 100,2	+ 94,7
3	– 0,2 N.	+ 4,0 N.
4	– 4,0 N.	+ 5,2 N.
5	– 1,0 N.	+ 6,5 N.

N. = Normbereich

Schweflige Säure bewirkt demnach an dem auf die Applikation folgenden Tag einen deutlichen Anstieg der KSt.-Ausscheidung.

Da Äthanol allein die KSt.-Ausschüttung nicht erhöht, nach den Untersuchungen von LAVES[1] aber einen Cortisoneffekt bremst, wurde den Versuchstieren am 1. Versuchstag zweimal 5 cm³ einer 12%igen Lösung von Äthanol in schwefliger Säure (50 mg SO_2/l) wie oben oral verabreicht und täglich die KSt.-Ausscheidung gemessen.

Tabelle II. C-17-Ketosteroidbestimmung nach Gaben von Äthanol und schwefliger Säure

Tage nach Versuchsbeginn	Abweichung der KSt.-Ausscheidung in Prozenten vom normalen Mittelwert	
	1. Serie	2. Serie
1	– 31,4	– 29,2
2	+ 23,0	+ 24,4
3	+ 8,7 N.	+ 7,5 N.
4	+ 4,0 N.	+ 4,5 N.
5	– 6,0 N.	+ 0,3 N.

N. = Normbereich

Die gleichzeitige Gabe von Äthanol und schwefliger Säure führt anfänglich zu einer geringen, den Normbereich unterschreitenden KSt.-Ausscheidung. Am 2. Versuchstag liegen die Werte dann wenig über dem Normbereich. Da nach den früheren Beobachtungen orale Weingaben zu einer deutlichen Mehrausschüttung führen – ein Ergebnis, das in keinem Verhältnis zu den oben wiedergegebenen Werten steht –, müssen noch andere Faktoren als schwefelige Säure für den Stresseffekt verantwortlich sein. Andererseits ist aus den oben beschriebenen Versuchen zu entnehmen, dass Äthanol den durch Gaben von schwefliger Säure hervorgerufenen Stresseffekt bremst. Dieses Resultat steht in Einklang mit demjenigen von LAVES[1] über den Antagonismus von Äthanol und Cortison.

H. KLEWE und G. GILLISSEN

Hygiene-Institut der Universität Mainz, den 7. Februar 1955.

Summary

Ethanol lessens the stress effect which is produced by application of sulphureous acid.

[1] W. LAVES, Arch. exp. Path. Pharmakol. 222, 182 (1954).

On the Vasopressor and Antidiuretic Activities of Synthetic Oxytocin

DU VIGNEAUD and his co-workers[1] identified the synthetic product with the natural oxytocic principle by different tests, such as: fowl blood-pressure, rat uterus test, induction of labour in humans, and finally milk ejecting action. The question of whether hypophysis posterior-lobe hormones are similar or identical agents and whether the differences existing between them are of qualitative or quantitative nature, has been widely discussed in the literature. As a result of newer investigations[2], the oxytocic and vasopressor peptides are found to differ from each other in their amino acid contents, lysine or arginine being characteristic for the vasopressor hormone. This statement led us to the question of idea whether interconnection may exist between the presence of the amino acids mentioned above and the vasopressor activity.

In order to clear up this question, we examined a sample of oxytocic octapeptide synthesized according to DU VIGNEAUD's method for its vasopressor and antidiuretic activities. (This preparation was not subject to Craig's counter-current purification process.) As standard was chosen the posterior-lobe extract standard powder of the State Institute for Public Health, Budapest, which is assayed by the guinea-pig uterus method. Determination of the vasopressor activity was carried out on decapitated cats (four point assay) that of the antidiuretic activity on rats (six point assay)[3]. The oxytocic activity of the preparation was measured by the fowl blood-pressure method.

The synthetic oxytocin examined proved to have an antidiuretic activity of $0{\cdot}067 \pm 0{\cdot}00825$ (S.E.) U. for each oxytocic unit (mean of 4 experiments) and a vasopressor activity of $0{\cdot}032 \pm 0{\cdot}0031$ (S.E.) U. for each oxytocic unit. (Mean of 6 experiments.) The difference between these two relative values is statistically significant ($p < 0{\cdot}01$).

We conclude, therefore, that the vasopressor and antidiuretic activities of the synthetic oxytocin which measured about 1/15 to 1/30 of the oxytocic activity concerned are independent of the arginine or lysine content of such polypeptides. Our results are not in accordance with those of LAWLER and DU VIGNEAUD obtained with a purified natural oxytocin; their preparation had an antidiuretic activity of less than 0·001 U./oxytocic unit and was entirely lacking in vasopressor activity[4]. On the other hand, purified arginine and lysine vasopressins show a 1/10–1/20 oxytocic activity according to the test employed.

On the grounds of these facts, we must assume that either the identity of the synthetic and natural oxytocin still remains questionable, or that the different quantitative and qualitative activity-relations between the posterior-lobe hormone are not yet clearly determined in spite of the possession of synthetic products.

The authors are greatly indebted to Drs. M. BONIKOWSKY, F. SZELKE, A. TOMORKENY, and E. WEISS who have prepared and supplied the synthetic oxytocin used in these experiments.

L. GYERMEK and GY. PÉKÁS

Department of Pharmacology, Pharmaceutical Industry Research Institute Budapest, January 30, 1955.

[1] V. DU VIGNEAUD, C. RESSLER, J. M. SWAN, C. W. ROBERTS, and J. KATSOYANNIS, J. Amer. Chem. Soc. 74, 4225 (1952).

[2] C. H. LAWLER, V. DU VIGNEAUD, and E. A. POPENOE, J. Amer. Chem. Soc. 75, 48, 80 (1953).

[3] J. H. BURN, *Biological Standardization* (Oxford, 1950).

[4] C. H. LAWLER and V. DU VIGNEAUD, Proc. Soc. Exp. Biol. Med. 84, 114 (1953).

Z. Ges. Exp. Med. 91 (1933)

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(Aus den seminaristischen Übungen für pathologische Physiologie an der Universität Berlin [Leiter: Prof. Dr. A. Biekl].)

Über den Einfluß von Sulfid-, Sulfit- und Sulfatverbindungen auf den Stoffwechsel.

Von

Dr. M. Kujima, Kioto.

Mit 7 Textabbildungen.

(Eingegangen am 15. Juni 1933.)

In neuerer Zeit wird dem Schwefel als Heilmittel wieder in verstärktem Maße Aufmerksamkeit geschenkt, nachdem er lange Jahre vernachlässigt worden war. Hand in Hand damit geht eine höhere Wertschätzung der Schwefelquellen, die zu Trink-, Inhalations-, Bade- und Irrigationskuren Verwendung finden. Die wachsende Bedeutung der Schwefelquellen, der sog. „fauler Wässer", bei der Behandlung von Frauenleiden war die spezielle Veranlassung für mich als Gynäkologen, mich mit der Pharmakologie des Schwefels intensiver zu befassen. Über einige meiner dabei durchgeführten Untersuchungen will ich im folgenden berichten.

Die Schwefelquellen sind wesentlich dadurch gekennzeichnet, daß Schwefel in ihnen in der Sulfidbindung enthalten ist. Natriumhydrosulfid (NaHS) und Schwefelwasserstoff (H_2S) sind die beiden in diesen Quellen immer wiederkehrenden Substanzen, die zugleich Träger der spezifischen Wirkung dieser Quellen sind. Ein gleichzeitiger Gehalt an Sulfaten ist für die Rubrizierung dieser Quellen unter die Gruppe der Schwefelquellen belanglos. Aus diesem Grunde setzt die Erklärung der therapeutischen Wirkung der Schwefelquellen eine genaue Kenntnis der biologischen Wirkung des Schwefelwasserstoffs und seiner Salze — der Sulfide — voraus. Der Schwefelwasserstoff ist bekanntlich das Reduktionsprodukt des Schwefels, das schon durch den Luftsauerstoff unter Abscheidung von amorphem Schwefel oxydiert wird. Von dem Schwefeldioxyd, dem ersten Oxydationsprodukt des Schwefels, leiten sich die schweflige Säure und ihre Salze — die Sulfite — und bei weiterer Oxydation die Sulfate her. Die Wirkung der Sulfide auf das Geschehen im Körper läßt sich besonders anschaulich darstellen, wenn wir sie gegen diejenige von Sulfiten und besonders von Sulfaten kontrastieren und immer äquimolekulare Mengen unseren Versuchen zugrunde legen, d. h. Mengen, die den gleichen Gehalt an Schwefelelement besitzen.

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Bei einer solchen Untersuchung muß die zu prüfende Verbindung dem Körper peroral, subcutan oder intravenös in der bestimmten Dosis zugeführt werden. Schließlich kann ja auch ein Schwefelbad bei spezifischer Wirkung nur dann wirken, wenn seine wesentlichen Stoffe in das innere Milieu des Körpers vordringen.

Vom Schwefelwasserstoff ist bekannt, daß seine geringsten wirksamen Konzentrationen in der Luft reizend und entzündungserregend auf die Conjunctiven und die Schleimhäute der Atmungswege wirken. Eintretende Entzündungen sind sehr hartnäckig. Die Konzentration der Reizschwelle liegt bei 0,15—0,20 mg H_2S im Liter Luft. Bei 1 mg im Liter Luft treten bei Einatmung des Gemisches hervorzentral-nervöse Störungen auf: Starke Steigerung der Atmung, die von den Lungen her ausgelöst wird und nach Vagusdurchschneidung verschwindet, dann Atmungslähmung. Krämpfe, Salivation, Bradykardie begleiten die Vergiftung. Am Darm verursacht der Schwefelwasserstoff heftige Kontraktionen; noch in Verdünnungen von 1 : 10 Millionen erregt er den isolierten Meerschweinchendarm. Intramuskuläre Zufuhr von H_2S wirkt beim Menschen auf die Peristaltik anregend *.

Über die Pharmakologie der Salze der Säuren, Schwefelwasserstoff und schweflige Säure ist wenig bekannt. Zu diesen Salzen gehört auch das in den Schwefelquellen vorkommende Natriumhydrosulfid (NaHS).

Besonders durch die Untersuchungen von *Loedel* über die Einwirkungen der Kur in Neundorf, einem Schwefelbade, auf den Stoffwechsel wird als „Schwefelwirkung" in Anspruch genommen: Anstieg des Körpergewichtes, Grundumsatzsenkung, Hypoglykämie. Als allgemeine Schwefelwirkung waren diese Erscheinungen bereits früher von anderen Autoren festgestellt worden. Man hatte auch als Schwefelwirkung Glykogenanreicherung in der Leber gefunden **. Einen weiteren Beitrag zur Analyse der biologischen Wirkung der einzelnen Schwefelkomponenten in den Schwefelquellen, wie der hauptsächlichen Repräsentanten der anorganischen Schwefelverbindungen, soll die vorliegende Studie bringen.

Versuchsanordnung.

Ich arbeitete mit H_2S, $NaHSO_3$ und Na_2SO_4. H_2S löst sich in Wasser bei 15° C zu 3,2 Vol. auf 1 Vol. Wasser = etwa 5,14 mg pro Kubikzentimeter. Dieses H_2S-Wasser reagiert gegen Lackmus sauer, die Säurestärke ist geringer als die der Kohlensäure, weshalb auch diese den H_2S teilweise verdrängt. Es war also nötig, bei den Versuchen für jede H_2S-Zufuhr frisches H_2S-Wasser herzustellen. Hinsichtlich des S-Gehaltes der Schwefelwasserstoffmenge in 1 ccm H_2S-Wasser sind ihr Lösungen gleichzusetzen, die 13,9 mg $NaHSO_3$ und 19,0 mg Na_2SO_4 in 1 ccm enthalten. Bei ungefähr gleichschweren weißen Ratten wurden, auf den S-Gehalt berechnet, äquimolekulare Mengen von H_2S, $NaHSO_3$ und Na_2SO_4 in wässeriger Lösung peroral und subcutan während längerer Zeit verabfolgt, und es wurde der Ablauf des organischen intermediären Stoffwechsels durch Harnanalyse untersucht. Während der ganzen Versuchsdauer bekamen die Tiere qualitativ die gleiche Nahrung, von der sie nach Belieben fressen konnten. Die Nahrungszusammensetzung war, für ein Tier berechnet, folgende:

Kuhmilch	25 ccm	Casein	1 g
Trockenes Semmelmehl	4 g	Frische Butter	2 g
Kartoffelstärke	1 g	Leitungswasser	10 ccm

* Zit. nach *Heubner*[1], Z. Bäderkde 1928, H. 1, 16. Hier findet sich auch die einschlägige Literatur.

** Literatur siehe bei *E. Loedel*[2], Beitrag zur Einwirkung der Schwefelbäder auf den Stoffwechsel, dargestellt am Beispiel des Preußischen Staatsbades „Bad Neundorf". Veröff. Zentralstelle für Balneol., N. F., 1932, H. 20.

Die Milch wurde mit dem Wasser gekocht, dann erst wurden die übrigen Nahrungsbestandteile eingetragen. Es wurde täglich jedem Tier dieses Nahrungsgemisch in der genannten Menge verabfolgt, nach 24 Stunden wurde durch Wägung festgestellt, wieviel von dem Gemisch verzehrt worden war. Die diesbezüglichen Angaben erscheinen in den Tabellen als „Futtermenge %".

Im Harn wurden der Kohlenstoff nach *Osuka*, der Vakat-O nach der Halbmikromethode *Kunitz*, der N nach der Halbmikromethode von *Kjeldahl* bestimmt.

Es wurden in einzelnen Versuchsabschnitten die Mittelwerte für C, Vakat-O, N, Harnmenge und Körpergewicht berechnet und durch Division der entsprechenden Mittelwerte die Lage der Harnquotienten C : N, Vakat-O : N und Vakat-O : C festgestellt. Die Harne wurden immer auf Eiweiß- und Zuckerfreiheit kontrolliert. Die ermittelten Zahlen sind in den Tabellen 1—4 zusammengestellt.

Versuchsergebnis.

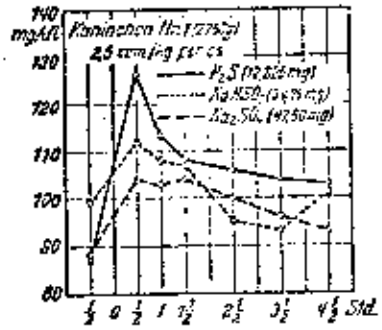
Die stärksten Veränderungen, die in diesen Versuchen an den Quotientlagen zur Beobachtung kamen, wurden bei den H_2S-Versuchen, also bei der Gabe des schwachsauren Schwefelwasserstoffs festgestellt. Dann folgten die Versuche mit $NaHSO_3$, also der Sulfitverbindung, an letzter Stelle standen die Versuche mit der Sulfatverbindung Na_2SO_4, die vielfach überhaupt keine Änderung in den Quotientlagen erkennen ließen.

Bei der Zufuhr größerer Mengen von H_2S (1,0 ccm = 5,14 mg pro Tag und Tier) stieg der Quotient C : N deutlich an; bei der Gabe von 0,5 ccm = 2,57 mg trat keine deutliche Änderung auf. Der Quotient Vakat-O : N sank im allgemeinen bei kleineren Dosen und erholte sich weitgehend bei den größeren. Vakat-O : C zeigte eine Neigung zur Senkung.

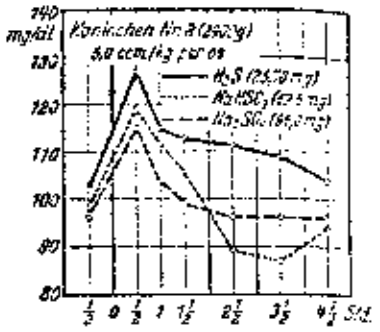
Der Anstieg von C : N bei Senkung von Vakat-O : N bei einem und demselben Tier in den gleichen Perioden bedeutet eine relative Vermehrung des C bei relativer Verminderung des Vakat-O. Im intermediären Stoffwechsel wird also C verschlechtert oxydiert (Steigerung C : N), trotzdem ist die Qualität der Gesamtoxydation im intermediären Stoffwechsel verbessert, offenbar durch eine die Verschlechterung der C-Oxydation überkompensierende qualitative Verbesserung der Oxydation an der übrigen oxydabelen Substanz. Dadurch kommt eine Änderung in der Zusammensetzung der harnfähigen Substanz in dem Sinne zustande, daß die harnfähige Substanz besser durchoxydiert ist, dabei kann auch eine Verschiebung der N-haltigen Harnsubstanz in der Richtung zum Harnstoff eintreten (Vakat-O : C sinkt). An der Oxydation des Kohlenstoffs liegt wohl auch der Grund, warum *Lendel* als Gesamtwirkung der Schwefelquellenbehandlung eine Granulumsatzsenkung, wenn auch manchmal nach vorübergehender Erhöhung, konstatierte, soweit hier nicht eine quantitative Einschränkung des Materialverbrauches vorliegt. Diese Erschwerung der C-Oxydation trägt auch vielleicht zu den Körpergewichtssteigerungen bei, die *Lendel* bei der bahntherapeutischen Schwefelbehandlung, und ich bei meinen Ratten im Versuch sah.

Bei der Gabe der Sulfitverbindung $NaHSO_3$ trat im Harnquotienten C : N überhaupt keine Veränderung auf; der Harnquotient Vakat-O : N

ging unter starker Schwankung nach oben, ähnlich verhielt sich der Quotient Vakat-O:C. Es wird also bei der Sulfitwirkung die

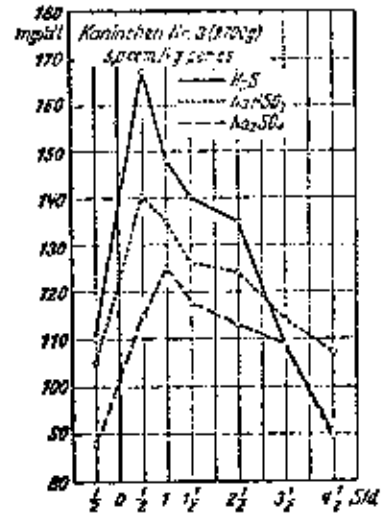


Kurve 1.

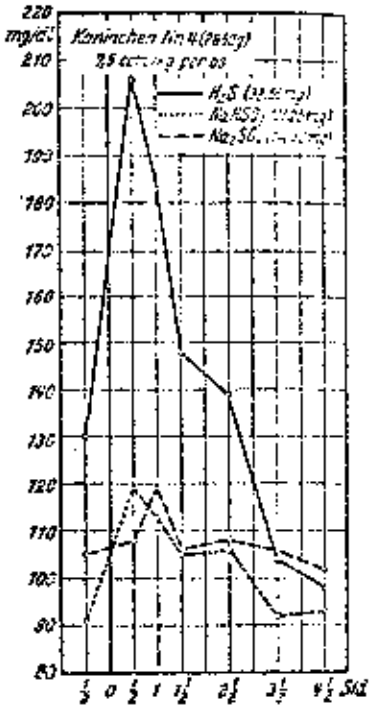


Kurve 2.

Oxydationshemmung am Kohlenstoff vermißt. Der Grad der Oxydation an den übrigen Molekülbestandteilen ist starken Schwankungen unterworfen, aber im allgemeinen nicht mehr im Sinne einer qualitativen Verbesserung der Oxydation. Das Sulfat (Na_2SO_4) änderte den Quotienten C:N nicht, Vakat-O:N sank entweder kontinuierlich oder



Kurve 3.

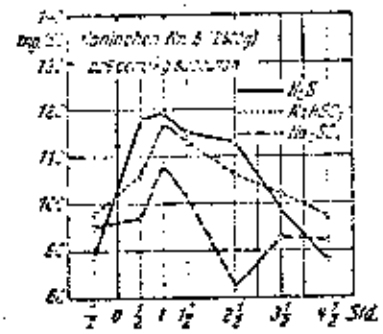


Kurve 4.

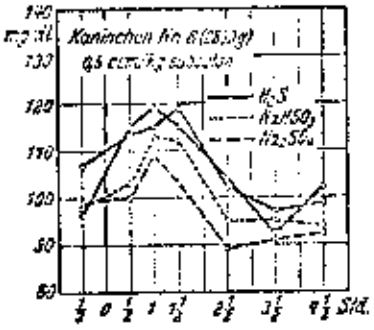
unter starken Schwankungen unerheblich. Vakat-O:C verhielt sich ähnlich. Die Wirkung der Sulfate steht also der Sulfitwirkung näher als der Sulfidwirkung. Zwischen Sulfat- und Sulfitwirkung scheint

nur ein kleiner Unterschied zu bestehen, vielleicht nur in dem Sinne, daß die Änderungen bei der Sulfitgabe etwas regelmäßiger auftraten als bei der Sulfatgabe, ein Umstand, der auch durch die wahrscheinlich gleiche chemische Struktur des sechswertigen Schwefels erklärlich ist.

Im Anschluß an diese Versuche habe ich dann noch den Einfluß der genannten Substanzen nach ihrer peroralen oder subcutanen Zufuhr

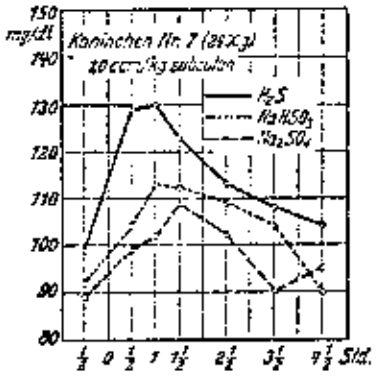


Kurve 5.



Kurve 6.

beim Kaninchen auf den Blutzucker mit der Methode nach *Hagedorn-Jensen* studiert. Auf das Versuchsergebnis ist der Weg der Zufuhr ohne wesentlichen Einfluß. Bei jedem Kaninchen wurden die 3 Substanzen, H_2S, $NaHSO_3$, Na_2SO_4 an verschiedenen Tagen in äquimolekularen S-Mengen in wässeriger Lösung gegeben.



Kurve 7.

In allen Versuchen trat eine deutliche initiale Blutzuckersteigerung bei den 24 Stunden nüchternen Kaninchen nach der Gabe aller Schwefelverbindungen auf, die langsam im Verlauf von 3—4¼ Stunden abklang und dabei manchmal die Norm etwas unterschritt.

Es ist bemerkenswert, daß die Blutzuckerveränderung bei der peroralen Gabe stärker war als bei der subcutanen Gabe. Ferner war die initiale Blutzuckererhöhung am stärksten bei H_2S, dann folgte $NaHSO_3$, an letzter Stelle stand Na_2SO_4. In manchen Versuchen war an der Kurvenlage die überragende Wirkung der Sulfidverbindung (H_2S) sehr ausgesprochen.

Diese Blutzuckerbefunde sind so eindeutig, daß an ihrer Richtigkeit nicht gezweifelt werden kann. Wie sich damit die angeblich blutzuckersenkende und den Diabetes bessernde Wirkung des Schwefels vereinbaren läßt, kann ohne weitere Untersuchungen in dieser Frage nicht gesagt werden (Literatur siehe bei *Lendel*).

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Die beigegebenen Kurven illustrieren die Schwefelwirkung auf den Blutzucker. Bei der Dosierung liegt den Versuchen folgende Berechnung zugrunde: 1,0 ccm H_2S-Lösung = 5,14 mg H_2S = 13,9 mg $NaHSO_3$ = 19 mg Na_2SO_4.

Tabelle 1. Ratte Nr. 1 ♂ (H_2S).

Datum	Körpergewicht in g	Futtermenge in g	Kotmenge in g	Harn-C in g	Harn-N in g	Harn-Vakat-O in g	C : N	Vakat-O : N	Vakat-O : C	Bemerkungen
8. 2.	158	90	12,0	0,1020	0,1408	0,1896	0,724	1,283	1,770	Physiologische Schwankungen der Vorperiode: C : N = 0,015 O : N = 0,123 O : C = 0,111
9. 2.	163	90	10,5	0,1092	0,1400	0,1710	0,780	1,221	1,566	
10. 2.	162	90	12,0	0,1267	0,1618	0,1890	0,783	1,168	1,491	
		Mittelwerte:		0,1126	0,1474	0,1502	0,763	1,222	1,600	
11. 2.	161	90	13,6	0,1253	0,1538	0,2304	0,816	1,500	1,838	
13. 2.	162	100	13,0	0,1375	0,1789	0,2118	0,769	1,510	1,184	
14. 2.	163	100	13,0	0,1320	0,1747	0,1894	0,756	1,086	1,437	
		Mittelwerte:		0,1316	0,1600	0,2239	0,781	1,375	1,486	
15. 2.	164	100	11,5	0,1337	0,1669	0,1754	0,787	1,032	1,302	Am 15. 2. 0,5 ccm H_2S-Lösung (per os)
16. 2.	164	80	13,0	0,1331	0,1707	0,1978	0,780	1,147	1,471	
17. 2.	161	95	13,0	0,0972	0,1365	0,1452	0,708	1,064	1,501	
		Mittelwerte:		0,1213	0,1590	0,1721	0,763	1,081	1,418	
18. 2.	163	95	12,0	0,1167	0,1525	0,1764	0,862	1,157	1,511	Am 18. 2. 0,5 ccm H_2S-Lösung (per os)
21. 2.	165	98	13,0	0,1188	0,1525	0,1855	0,779	1,216	1,561	
22. 2.	165	95	12,0	0,1174	0,2134	0,1907	0,550	0,894	1,240	
		Mittelwerte:		0,1278	0,1728	0,1842	0,740	1,065	1,441	
23. 2.	165	95	11,0	0,1116	0,1163	0,1582	1,002	1,360	1,357	Am 23. 2. 1,0 ccm H_2S-Lösung (per os)
24. 2.	166	90	10,0	0,1037	0,1322	0,1493	0,784	1,129	1,439	
25. 2.	165	95	10,0	0,1295	0,1225	0,1469	1,057	1,199	1,134	
		Mittelwerte:		0,1149	0,1237	0,1515	0,929	1,225	1,319	
26. 2.	163	100	9,5	0,1101	0,1215	0,1385	0,906	1,140	1,258	Am 26. 2. 1,0 ccm H_2S-Lösung (per os)
28. 2.	162	100	11,0	0,1233	0,1562	0,1677	0,789	1,074	1,360	
1. 3.	160	75	9,5	0,1428	0,1730	0,2256	0,825	1,304	1,580	
		Mittelwerte:		0,1254	0,1502	0,1773	0,835	1,180	1,414	
2. 3.	147	60	6,0	0,0074	0,0019	0,0077	0,733	1,063	1,447	Zwischenperioden
3. 3.	150	70	4,5	0,1032	0,1373	0,1169	0,555	0,708	1,074	
4. 3.	155	80	4,5	0,1132	0,1228	0,1178	0,922	0,959	1,042	
		Mittelwerte:		0,0946	0,1241	0,1088	0,762	0,877	1,150	
6. 3.	150	100	10,0	0,1615	0,2193	0,2598	0,751	1,184	1,579	
7. 3.	153	95	9,0	0,1289	0,1881	0,1509	0,685	0,802	1,172	
8. 3.	154	100	14,5	0,1511	0,2018	0,2874	0,749	1,424	1,902	
		Mittelwerte:		0,1182	0,2031	0,2327	0,730	1,145	1,601	
9. 3.	150	100	23,0	0,1739	0,2272	0,3712	0,768	1,648	2,146	Täglich 0,5 ccm H_2S-Lösung (subcutane Injektion)
10. 3.	146	100	18,0	0,1496	0,1947	0,2113	0,768	1,085	1,412	
11. 3.	150	100	15,0	0,1383	0,1825	0,2019	0,758	1,107	1,467	
		Mittelwerte:		0,1536	0,2008	0,2615	0,765	1,302	1,702	gestorben

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Tabelle 2. Ratte Nr. 2 ♂ (H_2S).

Datum	Körpergewicht in g	Futterverbrauch in g	Harnmenge in ccm	Harn-C in g	Harn-N in g	Harn-Vakat-O in g	C : N	Vakat-O : N	Vakat-O : C	Bemerkungen
										Physiologische Schwankungen der Vorperioden C : N = 0,025 O : N = 0,075 O : C = 0,101
8. 2.	156	90	10,0	0,0877	0,1011	0,1396	0,867	1,381	1,592	
9. 2.	159	80	8,5	0,0880	0,1116	0,1632	0,788	1,462	1,855	
10. 2.	158	90	9,0	0,0977	0,1225	0,1345	0,797	1,621	1,377	
		Mittelwerte:		0,0911	0,1117	0,1458	0,815	1,188	1,608	Vorperiode
11. 2.	168	95	16,5	0,1172	0,1450	0,2362	0,808	1,587	1,964	
13. 2.	170	90	14,0	0,1184	0,1452	0,1883	0,815	1,599	1,296	
14. 2.	170	100	16,0	0,1194	0,1595	0,2116	0,811	1,512	1,867	
		Mittelwerte:		0,1183	0,1499	0,2200	0,790	1,563	1,799	
15. 2.	172	100	14,0	0,1228	0,1564	0,2016	0,816	1,340	1,642	
16. 2.	—	100	14,0	0,1091	0,1316	0,1503	0,762	1,145	1,501	
17. 2.	174	100	14,0	0,1014	0,1378	0,1621	0,764	1,145	1,603	
		Mittelwerte:		0,1080	0,1419	0,1713	0,761	1,210	1,586	Täglich 0,5 ccm H_2S-Lösung (per os)
19. 2.	173	95	14,0	0,1021	0,1382	0,1681	0,739	1,422	1,216	
21. 2.	174	100	14,0	0,1145	0,1534	0,2061	0,746	1,343	1,800	
22. 2.	—	99	10,0	0,0763	0,0956	0,1282	0,798	1,341	1,679	
		Mittelwerte:		0,0976	0,1291	0,1675	0,756	1,297	1,716	
24. 2.	177	100	11,0	0,0920	0,1178	0,1600	0,781	1,358	1,739	
26. 2.	—	100	13,0	0,1481	0,1895	0,1881	0,900	1,258	1,270	
27. 2.	177	100	10,5	0,1030	0,1338	0,1552	0,770	1,114	1,507	
		Mittelwerte:		0,1144	0,1555	0,1678	0,841	1,238	1,467	Täglich 1,0 ccm H_2S-Lösung (per os)
28. 2.	175	100	13,0	0,1180	0,1284	0,1873	0,919	0,993	1,587	
28. 2.	172	65	9,0	0,0897	0,1040	0,1260	0,862	1,211	1,421	
1. 3.	172	30	6,0	0,0477	0,0608	0,0795	0,784	1,307	1,667	
		Mittelwerte:		0,0851	0,0977	0,1309	0,871	1,340	1,538	
2. 3.	170	50	10,0	0,1268	0,1850	0,1620	0,683	0,873	1,278	
3. 3.	168	90	11,0	0,1430	0,1900	0,1705	0,723	0,857	1,184	
4. 3.	170	90	8,5	0,0859	0,1165	0,1279	0,737	1,098	1,489	
		Mittelwerte:		0,1189	0,1608	0,1535	0,713	0,920	1,291	Zwischenperiode
5. 3.	171	85	13,0	0,1259	0,1765	0,1933	0,713	1,059	1,535	
7. 3.	—	95	13,0	0,1020	0,1440	0,1525	0,708	1,050	1,495	
8. 3.	176	100	15,0	0,1351	0,1653	0,1956	0,818	1,190	1,455	
		Mittelwerte:		0,1210	0,1619	0,1808	0,747	1,117	1,491	
9. 3.	174	100	16,0	0,112	0,1544	0,2175	0,720	1,141	1,955	Täglich 0,3 ccm H_2S-Lösung (subcutan)
10. 3.	—	90	17,0	0,1409	0,2235	0,2390	0,671	1,069	1,594	
11. 3.	175	90	15,0	0,1209	0,1791	0,1816	0,705	1,031	1,464	
		Mittelwerte:		0,1291	0,1869	0,2137	0,694	1,149	1,653	
12. 3.	174	90	18,0	0,1427	0,1834	0,2623	0,789	1,430	1,838	Täglich 0,5 ccm H_2S-Lösung (subcutan)
14. 3.	172	95	10,0	0,1156	0,1646	0,1589	0,703	0,966	1,375	
15. 3.	171	20	8,0	0,0677	0,0954	0,0801	0,731	0,840	1,183	
		Mittelwerte:		0,1087	0,1478	0,1671	0,735	1,431	1,537	gestorben

Tabelle 3. Ratte Nr. 3 ♂ (Na_2SO_4, $NaHSO_3$).

Datum	Körpergewicht in g	Futtermenge in g	Harnmenge in cc	Harn-C in g	Harn-N in g	Harn-Vakat. O in g	C : N	Vakat. C : N	Vakat. O : C	Bemerkungen
8. 2.	169	90	10,0	0,0975	0,1184	0,1141	0,824	1,222	1,484	Physiologische Schwankungen der Vorperiode C : N = [illegible] O : N = [illegible] O : C = [illegible] Vorperiode
9. 2.	180	90	10,0	0,1143	0,1400	0,1624	0,816	1,100	1,420	
10. 2.	180	90	12,0	0,1381	0,1844	0,1986	0,749	1,077	1,438	
		Mittelwerte:		0,1166	0,1476	0,1684	0,790	1,153	1,448	
11. 2.	185	100	17,0	0,1306	0,1620	0,2439	0,862	1,503	1,747	
13. 2.	185	100	18,5	0,1506	0,2148	0,2883	0,841	1,602	1,345	
14. 2.	—	100	16,0	0,1291	0,1758	0,2586	0,734	1,414	1,927	
		Mittelwerte:		0,1197	0,1842	0,2606	0,812	1,507	1,673	
15. 2.	187	100	17,0	0,1724	0,2040	0,2068	0,845	1,013	1,200	Täglich 0,5 ccm Na_2SO_4-Lösung (per os)
16. 2.	—	100	14,0	0,1365	0,1705	0,1953	0,801	1,145	1,430	
17. 2.	191	100	13,0	0,1529	0,1616	0,1972	0,822	1,221	1,484	
		Mittelwerte:		0,1173	0,1587	0,1938	0,823	1,126	1,571	
18. 2.	191	100	16,0	0,1126	0,1674	0,2204	0,683	1,421	1,611	
21. 2.	—	100	17,0	0,1326	0,1625	0,2509	0,859	1,503	1,555	
22. 2.	189	100	12,0	0,1117	0,1416	0,1812	0,789	1,300	1,640	
		Mittelwerte:		0,1313	0,1572	0,2242	0,855	1,426	1,708	
23. 2.	190	100	14,0	0,1348	0,1774	0,1969	0,816	1,105	1,354	Täglich 1 ccm Na_2SO_4-Lösung (per os)
24. 2.	195	100	16,0	0,1135	0,1910	0,1932	0,751	1,061	1,011	
25. 2.	195	100	11,0	0,1221	0,1487	0,1654	0,823	1,119	1,359	
		Mittelwerte:		0,1369	0,1724	0,1852	0,791	1,074	1,353	
26. 2.	196	100	10,5	0,0975	0,1212	0,1429	0,799	1,172	1,466	
1. 3.	198	95	13,0	0,1322	0,1738	0,1962	0,761	1,484	1,484	
2. 3.	190	80	12,0	0,1301	0,1759	0,1844	0,774	1,195	1,355	
		Mittelwerte:		0,1219	0,1570	0,1715	0,776	1,111	1,431	
3. 3.	188	90	11,0	0,1343	0,1813	0,1720	0,740	0,949	1,281	Zwischenperiode
4. 3.	190	95	9,0	0,1099	0,1341	0,1439	0,819	1,073	1,309	
		Mittelwerte:		0,1221	0,1577	0,1579	0,774	1,001	1,293	
5. 3.	193	100	10,0	0,1102	0,1488	0,1506	0,740	1,012	1,367	Täglich 0,5 ccm $NaHSO_3$-Lösung (per os)
6. 3.	195	100	15,0	0,1571	0,2050	0,2128	0,768	1,038	1,352	
		Mittelwerte:		0,1335	0,1769	0,1817	0,756	1,027	1,358	
8. 3.	196	100	16,0	0,1412	0,1548	0,2331	0,930	1,538	1,625	
9. 3.	195	100	18,0	0,1479	0,1834	0,2509	0,806	1,368	1,700	
10. 3.	191	100	13,0	0,1123	0,1848	0,2018	0,608	1,092	1,793	
		Mittelwerte:		0,1338	0,1733	0,2287	0,772	1,320	1,709	
11. 3.	192	95	12,0	0,1281	0,1611	0,1825	0,781	1,112	1,425	Täglich 1 ccm $NaHSO_3$-Lösung (per os)
12. 3.	192	90	13,0	0,1473	0,1716	0,1937	0,858	1,129	1,315	
13. 3.	193	90	13,0	0,1266	0,1951	0,2011	0,649	1,030	1,588	
		Mittelwerte:		0,1340	0,1769	0,1924	0,757	1,088	1,436	

Tabelle 3 (Fortsetzung).

Datum	Körpergewicht in g	Futterverbrauch in g	Harnmenge in ccm	Harn-C in g	Harn-N in g	Harn-Vakat-O in g	C : N	Vakat-O : N	Vakat-O : C	Bemerkungen
14. 3.	193	90	11.0	0,1396	0,1641	0,1722	0,721	0,890	1,234	Nachperiode
15. 3.	195	50	15.0	0,1528	0,1716	0,2251	0,712	1,049	1,473	
16. 3.	195	60	10,5	0,1385	0,1876	0,1726	0,739	0,692	1,246	
		Mittelwerte:		0,1436	0,1985	0,1900	0,723	0,957	1,323	
17. 3.	198	95	11,0	0,1494	0,2116	0,1850	0,706	0,870	1,244	
18. 3.	198	100	14.0	0,1721	0,2286	0,2560	0,753	1,032	1,320	
20. 3.	198	100	15.0	0,1871	0,2200	0,2460	0,848	1,117	1,371	
		Mittelwerte:		0,1695	0,2204	0,2220	0,769	1,011	1,315	

Tabelle 4. Ratte Nr. 4 ♀ (Na_2SO_4, $NaHSO_4$).

Datum	Körpergewicht in g	Futterverbrauch in g	Harnmenge in ccm	Harn-C in g	Harn-N in g	Harn-Vakat-O in g	C : N	Vakat-O : N	Vakat-O : C	Bemerkungen
										Physiologische Schwankungen der Vorperioden C : N = 0,020 O : N = 0,075 O : C = 0,135
8. 2.	164	100	9.0	0,0964	0,1221	0,1441	0,790	1,180	1,565	Vorperiode
9. 2.	172	100	8,0	0,0789	0,1014	0,1242	0,778	1,323	1,574	
10. 2.	172	100	9,0	0,1081	0,1323	0,1581	0,817	1,122	1,373	
		Mittelwerte:		0,0945	0,1186	0,1389	0,787	1,171	1,470	
11. 2.	170	100	13,0	0,1005	0,1505	0,1815	0,707	1,206	1,704	
13. 2.	170	100	14,0	0,1233	0,1544	0,1992	0,799	1,615	1,290	
14. 2.	170	100	9,0	0,0902	0,1071	0,1326	0,844	1,238	1,470	
		Mittelwerte:		0,1067	0,1373	0,1711	0,777	1,246	1,604	
15. 2.	172	100	0,5	0,0836	0,1015	0,1266	0,824	1,247	1,514	Täglich 0,5 ccm Na_2SO_4-Lösung (per os)
16. 2.	175	100	8,0	0,0851	0,1129	0,1179	0,759	1,053	1,385	
17. 2.	177	100	8,5	0,0813	0,1084	0,1111	0,750	1,025	1,333	
		Mittelwerte:		0,0843	0,1073	0,1185	0,776	1,108	1,411	
18. 2.	178	100	10,0	0,1004	0,1320	0,1468	0,758	1,112	1,466	
21. 2.	183	100	11,0	0,1085	0,1336	0,1595	0,810	1,194	1,470	
22. 2.	185	100	10,0	0,1062	0,1330	0,1521	0,813	1,136	1,406	
		Mittelwerte:		0,1056	0,1329	0,1528	0,794	1,150	1,447	
23. 2.	185	100	10,0	0,1023	0,1288	0,1465	0,795	1,137	1,432	Täglich 1,0 ccm Na_2SO_4-Lösung (per os)
24. 2.	187	100	10,0	0,1147	0,1331	0,1599	0,823	1,141	1,186	
25. 2.	187	100	10,0	0,1130	0,1428	0,1178	0,800	1,035	1,298	
		Mittelwerte:		0,1103	0,1370	0,1511	0,805	1,103	1,370	
26. 2.	188	100	10,0	0,0740	0,1333	0,1424	0,555	1,069	1,030	
1. 3.	188	100	9,5	0,1026	0,1266	0,1457	0,810	1,151	1,420	
2. 3.	188	100	8,3	0,1009	0,1243	0,1203	0,811	1,040	1,381	
		Mittelwerte:		0,0925	0,1281	0,1321	0,735	1,086	1,504	
3. 3.	189	100	8,0	0,1019	0,1422	0,1591	0,738	1,431	1,281	Zwischenperiode
4. 3.	189	100	9,0	0,1036	0,1345	0,1285	0,770	1,050	1,336	
		Mittelwerte:		0,1013	0,1384	0,1443	0,754	1,050	1,384	

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Tabelle 4 (Fortsetzung).

Datum	Körpergewicht in g	Futtermenge in %	Harnmenge in ccm	Harn-C in g	Harn-N in g	Harn-Vakat-O in g	C : N	Vakat-O : N	Vakat-O : C	Bemerkungen
5. 3.	189	90	7,5	0,0864	0,1115	0,1110	0,748	0,961	1,285	
7. 3.	190	100	10,5	0,0968	0,1222	0,1412	0,792	1,157	1,459	
		Mittelwerte:		0,0916	0,1169	0,1261	0,783	1,079	1,376	Täglich 0,5 ccm $NaHSO_3$ Lösung per ...
8. 3.	190	100	12,0	0,1010	0,1214	0,1599	0,832	1,317	1,583	
9. 3.	191	100	14,5	0,1204	0,1544	0,2079	0,780	1,368	1,727	
10. 3.	191	100	10,0	0,0870	0,1438	0,1558	0,605	1,083	1,791	
		Mittelwerte:		0,1028	0,1399	0,1745	0,735	1,247	1,697	
11. 3.	190	100	15,0	0,1142	0,1440	0,1632	0,793	1,133	1,240	Täglich 1,0 ccm $NaHSO_3$ Lösung per ...
12. 3.	189	100	12,5	0,1344	0,1809	0,1901	0,760	1,051	1,383	
13. 3.	192	100	12,5	0,1241	0,1710	0,1930	0,726	1,129	1,555	
		Mittelwerte:		0,1242	0,1653	0,1821	0,751	1,102	1,466	
14. 3.	197	100	10,5	0,1355	0,1792	0,1668	0,756	0,931	1,231	
15. 3.	197	90	13,0	0,1067	0,1512	0,1747	0,706	1,155	1,637	
16. 2.	198	100	12,5	0,1513	0,1985	0,2106	0,762	1,061	1,392	
		Mittelwerte:		0,1312	0,1763	0,1840	0,745	1,044	1,402	Nachperiode
17. 3.	200	100	14,0	0,1586	0,2082	0,2286	0,762	1,098	1,441	
18. 3.	200	100	13,0	0,1460	0,1901	0,2203	0,768	1,156	1,509	
20. 3.	200	100	11,0	0,1265	0,1613	0,1862	0,784	1,154	1,472	
		Mittelwerte:		0,1437	0,1865	0,2117	0,771	1,135	1,473	

Zusammenfassung.

1. Der Schwefel wirkt als zweiwertiger Schwefel im Schwefelwasserstoff stärker und eindeutiger auf die Oxydationslage des Körpers ein als in seinen Sulfit- und Sulfatverbindungen, in denen er sechswertig ist.

2. Sulfid-, Sulfit- und Sulfatverbindungen bewirken bei Kaninchen eine vorübergehende Hyperglykämie, die am stärksten bei Schwefelwasserstoff, weniger stark bei der Sulfit- und am schwächsten bei der Sulfatverbindung ist.

3. Da in den Schwefelquellen der wirksame Schwefel zweiwertig als H_2S oder als Salz des Schwefelwasserstoffs vorliegt, erklärt sich daraus die starke biologische Wirkung der Schwefelquellen.

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I 緒言

低濃度SO_2の人及び動物に対する影響に就いてIsidor Greenwald[1]は詳細な概説を掲げて居る。久保田[2]，桂[3]等は動物に於ける系統的検索から諸臓器に病変を指摘し，殊に桂は硫化鉱作業員の長管骨骨端部の変異に着目しこれをOsteoporoseと解して居る。

SO_2の系統的な刺激に依つてOsteoporoseが招来されるならば，それに先行する血中鉱物質代謝の異常が期待される。然るに骨組成代謝の生化学的機転に就いては未だ完全には知らされていないが，凡そ次の様に考えられて居る[4][5]。

1） Vitamin Cの助けを籍りて蛋白質性の繊維物質による骨組みが出来る。

2） 骨芽細胞でフォスファーターゼ分泌されMgにより3,000倍に賦活され，解糖過程中の分解産物，例えば六炭糖燐酸エステル等が基質となつてPO_4^{---}が遊離される。

3） 正常な血清及び組織液に於ては$[Ca]_3\times[PO_4^{---}]_2$の乗積は一定恒数でイオンの過飽和平衡に達して居るから，その何れか一方が増加すると忽ち$CaHPO_4$を生じ，次いで$Ca_3(PO_4)_2$が形成され骨沈着を招く。

逆に考えればCa・P両イオンの減少を来す原因は悉く化骨障碍の可能性がある。即ち食飼中のCa・P・紫外線・V. Dの缺乏・副甲状腺機能亢進・随伴の酸血症等は骨脱灰の原因となる。就中吾々が注目するのは，体液pHの態度である。何となれば$3Ca(PO_4)_2$ $CaCO_3$ $Mg(PO_4)_2$の人骨分析の主役であるCa・Pと体液pHは密接な関係に在るからである。

例えば血清中約10mg%を占めるCaの易はイオン状で，他の易は酸性反応で容易にイオン化する無機結合体であり，過剰の酸排除に利用される。反之，血中3 mg%を占める酸溶性無機燐酸はアルカリ反応でイオン化すると言われる。のみならずフォスファターゼには多種あり，その内燐酸モノエステラーゼにも4種あり，至適pH 9～10のものが骨・腎・腸・乳腺・肺・血清中に在つて骨化生に重要であるとされて居る。随つて若し血清が酸性に傾くほど此の酵素の動きは低下し燐酸エステルよりのPのイオン化は期待出来難くなる。尤も赤血球中の豊富なP源である有機燐酸エステルにopt. pH 6のフォスファターゼが如何に作動するか等のエネルギー代謝全般に亘る機転にはふれないで，血清アルカリフォスファターゼを通じてPの消長とCa代謝を考え骨化成との結びつきにふれるのが本検査の目的である。

II 検査期日・対象・方法

1） 検査期日：自昭和9年8月至29年9月

2） 検査対象：某化学工業$SO_2$2曝露作員405名を実勤続年数（～6年・～12年・13年～）3階級に層化後任意抽出の標本45名を選出した。別にSO_2非曝露健康男子数名を対象群とし我国検査を行つた。

3） 検査方法：検査試料は始業前安静時に解脈血を乾熱滅菌した注射器で遠かに採取し，脱繊維素後遠心分離氷冷の血清である。

Ca定量法は楊沢法[7]による光電比色法を用いた。

無機燐酸定量法はFiske & Subbarow法[14]に準拠した。

アルカリフォスファターゼ活性値はBodansky の

第1表 検査精度

月日	測定項目	測定値 mg%	添加量 mg%	総量 mg%	回収率 %
7月16日	血清 Ca	12.1	2.0	14.0	99.2
7月21日	〃	9.6	2.0	12.01	103.4
7月5日	無機 P	4.7	1.0	5.25	92.1
7月27日	〃	4.5	0.2	4.8	102.0

第2表 対照値（健康男子血清）

項目	例数	平均値	不偏分散	平均値の信頼限界（確率95%）
Ca (mg%)	5	10.38	1.351	10.38±1.68(12.06—8.7)
無機燐酸 (mg%)	13	4.28	0.741	4.28±0.45(4.73—3.83)
活性値 Bodansky 単位	8	1.28	0.92	1.28±0.75(0.53—2.03)

アルカリ基質 pH8.6 を用い，37°C 1 時間に分解されたP量より Bodansky Unt で計算した。

III 成績

第3表に各項目測定値を表括した。

第3表に就き SO_2 曝露群を所謂正常値に照合させると，Ca では84% が生理的範囲に含まれ増減の16% についても著しい逸脱のない事は就労可能の対象群として当然である。

無機燐酸も略々同様であるが，血清アルカリフォスファターゼの比較的低値であることは一応注目される。然し乍らこれを対照と比較すると対照群の少数である事の故障に災いされ乍らも尚，無機Pが比較的低値で，却つてアルカリフォスファターゼの活性値は遅延の著しくない事が第4表の勤続年数別平均値の検定とも相俟つて理解される。元来血清アルカリフォスファターゼの活性値の表現はその測定術式に依つて所謂正常値が異なるから，吾等の行つた Bodansky 単位，即ち，1mg のPも単位時間に遊離させる酵素量に就いては所謂正常値よりも同一手法の対照群との比較に於て評価さるべきで，此の意味からは寧ろ SO_2 曝露群の血清無機Pの低値が SO_2 の曝露の影響の1つとして注目さるべきであると思われる。

IV 考按

Phosphatase は燐酸結合を氷解する酵素群で殆どすべての組織・体液・細胞に分布し，特その他の重要物質の吸収排泄中間代謝に演ずる役割は大きい。その作用に随つて phosphomonoesterase・diesterase・Pyrophosphatase・Adenosin—Triphosphatase・Nucleofidase etc. があるが，その内 monoeterase は核酸・糖燐酸から燐酸を放つ。その至適 pH に依つて4つに大別され，opt. pH8.6—9.4 のものは腎・骨・腸・乳腺・肺・血漿中に在るとされ，体内代謝に重大な関与を持つて居て，その活性値は身体違和の際著明に動揺する。例えば[8] 骨組織代謝の旺盛な Paget 氏病では正常値の50倍も，Rachitis では20倍以上，副甲状腺機能亢進時は10倍餘も，その他の骨疾患・肝炎等にも多少増加し，生理的にも発育期の小児は成人より高い。山賀[9] は鉛・Benzen 等の工業従業員の該酵素活性値は中毒の早期又は軽症時に増加し，末期又は重症時には減少すると云う事から，矢ケ崎[10] は赤血球内フォスファターゼの出現率の増加により，何れも工業毒たる Stress に依つて惹起されるアドレナリン——脳下垂体前葉——副腎皮質系の反応，即ち Selye の適応症候群中警告期・抵抗期・疲憊期・不応期の段階に随つて phosphatase 活性値の正常規準は血清アルカリフォスファターゼの測定術式，例えば反応時間・温度・試薬，就中アルカリ基質に依つて異なり，例えば発色比色の原理を Bodensky 法に就いて前記山賀は泉高松法で 37°C 5時間乃至18時間作用後の燐分解能を，小西[11] は Fructose phosphorsaures Calcium の基質で38°C 1時間値の燐分解態を夫々正常値として 3.5—16.7 或は 13.5—33.5 及び 0—0.4 として居るが，吾々の場合 β-グリセロ燐酸ナトリウムを基質にした健康男子対照群よりの平均値の信頼限界は 1.28±0.75 を示し，所謂正常値よりは低いが，これを以て被検群の測定値を評価する方が妥当であろう。即ち被検群は所謂正常

第 3 表　動続年数別測定値及び対照群，正常値比較表

符号	動続年 ＼ 項目	Ca (mg%)			inorg. P (mg%)			alkali phosphatase (Bodensky単位)		
J_1	0 ～ 6	10.8	10.8	10.0	3.36	3.00	3.06	1.08	2.64	0.91
		10.8	10.0	9.9	3.12	3.10	3.66	0.84	1.70	3.18
		8.6	11.6	10.9	2.40	2.80	3.60	2.04	2.00	1.80
		10.1	10.8	10.2	2.80	3.10	2.40	1.62	1.06	2.34
		12.3	12.3	10.6	4.30	4.80	3.72	1.08	—	1.46
J_2	7 ～ 12	11.5	8.9	—	2.25	2.70	3.00	2.01	2.28	1.44
		10.2	10.7	—	3.84	4.44	3.66	3.12	—	2.49
		9.8	10.9	9.0	3.06	4.20	2.64	1.38	0.96	2.70
		10.6	—	10.2	3.36	3.18	2.34	1.56	3.42	2.64
		11.8	9.5	10.0	3.72	3.84	2.46	2.04	1.20	1.26
J_3	13 ～	12.5	11.5	11.8	3.36	2.60	2.80	1.26	1.78	1.82
		10.7	11.1	9.9	4.80	4.20	2.52	—	0.96	1.44
		9.9	10.7	10.7	2.10	3.96	1.68	2.52	2.76	3.36
		9.8	—	11.5	3.06	—	3.54	1.44	—	1.08
		10.6	8.2	9.7	3.42	2.64	2.64	1.44	2.16	1.32
J_4	歯牙酸蝕症群	9.9	9.9	11.6	2.70	2.94	3.00	1.74	1.26	1.62
		11.2	9.1	13.0	3.36	3.84	3.06	1.44	2.16	0.84
			11.5	9.8		3.36	4.20		1.02	1.05
				10.0			2.70			0.42
							3.12			2.88
J_5	対照群平均値	8.7～11.5 (10.38)			3.83～4.73 (4.28)			0.53～2.03 (1.28)		
所謂正常値		9～11.5 (10.0)			2.5～4.5 (3.7)			1.5～4.0 (2.7)		
所謂正常値よりの増減	比較	正常	増加	減少	正常	増加	減少	正常	増加	減少
	SO_2 群 例数	41	6	2	45	2	7	27	0	24
	SO_2 群 %	83.7	2.2	4.1	83.3	3.7	13.0	52.9	0	47.1
	対照群 例数	3	1	1	8	5	0	4	0	4
	対照群 %	60	20	20	61.5	38.5	0	50	0	50

第 4 表　動続年数別 (Jn) 平均値の検定表 (n＝1～5)

区分	要因	変動	自由度	不偏分散	分散比	有意性
Ca	J.	513.25	4	128.81	1.158	$\alpha>0.05$
	R. J	5429.18	49	110.30		
	J. R	5942.43	53			
無機 P	J.	1243.29	4	310.82	6.395	$\alpha<0.01$
	R. J	3013.42	62	48.60		
	J. R	4256.71	66			
アルカリフォスファターゼ活性値	J	232.98	4	58.245	1.102	$\alpha>0.05$
	R. J	2855.12	54	52.873		
	J. R	3088.10	58			

値に比べ一見減少者が多いように見えるが，対照群と有意の差はなく，むしろ若干上廻つて居るとさえ見える。即ち骨疾患時に見られると云う何十倍もの増加も示さない代りに所謂 Stress の著しい場合の減少とも見えない。

これに反して血清無機Pは所謂正常値にほぼ抱括されて居るに拘らず，尚対照群に比して有意差を以て低値である。回収精度より見てもその測定誤差は餘り大きくないから，対照群の方に高値に傾き易い原因が在つたのではなかろうか検討する必要がある。然し歩行或は軽微な動作以外に負荷のない対照群は時刻その他の条件も被検者群と同一に規定されて居るし，小池，小沢[12]は積ミの労作前後の血清無機燐の測定結果よりその倒れの少いことを報告して居る。即ち負荷の強度の場合，成績の動揺は時間と共に著しく，静的動作の場合は影響が少く，定常状態の成立するときは変化がないと云う。これによれば吾ミの被験者は正常出勤後始業前の安静時に採血を行つて居るから，作業の影響は一応除外出来る。尤も朝食・通勤時の労作・採血時の刺戟等の細かな個体差は此の限りでないので，これ等を考慮の外に置くと原因は詳かでないけれど，所謂正常値をすら下廻る無機燐減少者率の13%は注目すべき数字と云つてよい。

血清 Ca は略ミ生理的動揺範囲に留り，対照群とも甲乙が細い。斯の如き所見はフォスファターゼの著明な増加こそ伴わないが，一見，VitaminD 缺乏に因る佝僂病の場合に似ている。即ち甲状腺機能亢進時の脱灰では血中 Ca・P が正常であるに反し，副甲状腺のそれの場合は血中 Ca は正常だが，Pは減少[13]すると云う事を併せ考えると興味深い。一方アルカリフォスファターゼの活性値は至適 pH では対照群のそれに優るとも劣らないから，この酵素の in vivo での作動条件に缺陥があるか，Pの供給源に乏しいのか，或はPの排泄が著しいのか何れかであろう。此の内フォスファターゼは骨からの燐酸塩の排泄を制禦すると云われる一面，副甲状腺の Parathormon はその排泄を促進し，機能亢進燐酸カルシウムの排泄を高めると云う[14]。此のPの吸収排泄は暫く措いて，酵素の作動条件に就いては下のように考えられる。即ち SO_2 が直接に酸血症を来すとすれば勿論，或は脳下垂体前葉・副腎皮質の反応系に異常刺戟が加われば副甲状腺機能の亢進を招き得るから，血清アルカリフォスファスターゼの増加[11]を示し，他方Hイオンの増加に伴つて該酵素（opt. pH8.6—9.4）の作働を低下させる。

又該酵素活性値増加の所見は直ちに血漿内無機燐酸の増加を意味するものではない。蓋し適当な pH・温度・時間・基質を与えた活性値と生体内制約は異なるからである。例えばエステラーゼの動き得る基質に障碍があれば酵素活性値の増加に拘らず無機燐は不足するし，反応そのものもSH化合物等では阻害される。阻害物質に就いては Robinson は Ca^{++} PO_4^{---} の適宜濃度を含む媒体内でフォスファターゼの阻害とか，骨無機質に影響を来さない程度の弗化物・沃度酢酸・HCN等が軟骨化骨を障碍する事から，フォスファターゼの外に別の酵素的機序を想定して居る。随つて無機燐産生に直接関与するかに見える血清アルカリフォスファターゼの活性値増加も，条件が具備しなければ必ずしも血漿無機燐の増加を期待し得ない。むしろ斯る条件悪化を克服し，無機燐産生への努力が活性値の増加となつて現われる防禦反応の一部とさえ見られる。況して燐酸分解酵素はモノエステラーゼにも数種あり，又幾つかのフォスファターゼ，フォスファリラーゼ等の直接間接に作働して燐酸エステル，次いで燐分解へ進む鎖鎖な反応であるから，酵素活性値と無機Pの値の背馳はあやしむに足りない。

茲に於て該酵素活性値の如何に拘らず，血清無機質の比較的減少が骨変転に対し独立の意味を持つて来るようである。即ち Herery[4] によれば大腿骨・脛骨の如き大長管骨でさえその無機質の30%は2カ月を出ずに交替するから相当に弱しい代謝であるとされ，第一次大戦後の Hungerosteopathie[15] に於て尿中 Ca・P の排泄量に変化の少い事から，此等の所要無機質は骨中より動員されると考えられ，又片瀬は Alimentare Osteopathie として偏食動物の骨障碍は血液アチドーシスに基づくと考えた。Hans Hellner[5] は副甲状腺機能亢進の際摂取 Ca の確保が妨碍されるのか，単に Ca を洗い出す事に動くか判らないが，Ca 缺乏を来し，又過剰に体内に生産された酸を排除するために Ca イオンが消費されると記している。此の考えに立てば，吾ミの測定した血清 Ca の正常量は Ca に不足がないと云う保証でなくて，SO_2 に基づく直接・間接のHイオンの増加等を援護するための Ca の消費及び排泄の増加，次いでこれを補償するための骨中よりの動員等が輻輳現象として存在する事を推測出来る。

此のような場合は血清の無機燐の低値は必然的に生理的飽和であるべき血漿燐酸カルシウム（即ち $(Ca)_6 \times (PO_4^{---})_2$ のイオン乗積が過少となり $Ca_3(PO_4)_2$ の沈着を防碍するのみか，却つて骨中よりの脱灰作用が促されることも想定される。

7 総括並びに結語

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者トは第工場技術課員 465 名に於いて骨相場に対する SO_2 の影響をみるために勤続年数3階級区分の群化で得た任意抽出標本45名の血清無機燐・同アルカリフォスファターゼ活性値・血清カルシウムを測定して次の結果を得た。

1） SO_2 曝露作業員の各勤続年数区分の間には各測定値に有意差はない。但し何れも最短数ヵ年の実験経歴を有し，SO_2 非曝露群は被検者には含まれていない。

2） 血清 Ca は正常値或は対照非曝露群との間に有意差はない。

3） 血清アルカリフォスファターゼ活性値は対照群より僅かに増加の傾向を認め，SO_2 の刺激とすれば軽度の Stress として考えられるが，検定上有意ではない。

4） 血清無機燐は対照群より有意差を以て低値であり，且つ被検者中所謂正常生理値の下限にすら及ばないものが13%も占めて居る。

上の成績から SO_2 の作用機構は明らかではないが，工業毒としての Stress が Selye の所謂警告反応としてアルカリフォスファターゼ活性値の比較的増加を来すのか否かは措いても，SO_2 の直接乃至間接の結果副甲状腺機能亢進，或は血液Hイオンの増加，或はこれを防禦するための血液 Ca の減少，骨組織よりの排泄，血清無機燐の減少等の因果関係を想定する事は $Ca_3(PO_4)_2$ の沈着障害・骨軟化等の機構の理解を容易にするものと期待される。

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Enzymatische und Säure-Basen-Gleichgewichtsstörungen nach experimenteller SO_2-Vergiftung sowie Schutzwirkung von Ammoniakdämpfen

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Disturbance of Acid-Base and Enzyme Equilibrium in Experimental Intoxication with SO_2 and Protective Action of Ammonia

Summary. In connection with a new method for neutralizing SO_2 in combustion gases by means of ammonia, we have examined the changes induced in the organism by SO_2, compared with the effect of the products of the reaction of SO_2 with ammonia.

We made our experiments on one hundred guinea pigs, twenty of which were controls. The first group of twenty guinea pigs were exposed to SO_2 at a concentration of 2 g/Nm³ in a special toxicological chamber; the second group og twenty were similarly to the products resulting from the reaction of SO_2 with ammonia. The third group of twenty guinea pigs were exposed SO_2 at a concentration of 50 mg/Nm³ for eight hours a day for four month, and the fourth group of twenty to the reaction products of SO_2+NH_3 for a period of four months.

The blood of all the animals was examined for: level of hemoglobin number of erythrocytes and leukocytes, the coloured indicator and a percentage composition of leukocytes. We investigated the disturbances of acid-base equilibrium, and determined the pH of blood taken from the left ventricle, the partial pressure of CO_2 and the total pressure of CO_2 in the plasma. We also determined the total protein level as the activity of aldolase, lactic acid dehydrogenase and asparagine and alanine aminotransferase.

In the urine determined pH contents of sugar, protein and morphotic components. Samples of liver and brain were taken after the animals had been to death. These samples were then homogenized in the usual way, and the activity of their enzymes was determined as well as those of the blood.

The results show that the toxic activity of SO_2 is multiple and leads to the impairment of the function of several organs.

The toxic action of SO_2 is due to the disturbances of the acid-base equilibrium brought about by the inhalation of acid products and by the disturbances of ventilation resulting from changes in the respiratory system.

The decreased activity of aldolase and lactic-acid dehydrogenase in the blood and tissues in cases of SO_2 intoxication may interfere with the carbohydrate metabolism, thus accounting for hyperglycemia and disturbances of glycogen synthesis in the tissues, as described by many authors.

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Animals exposed to the reaction products of SO_2 with ammonia had a statistically lower mortality rate.

The chemical reaction of SO_2 with ammonia reduces the disturbances of the acid-base equilibrium observed in SO_2 intoxication and eliminates the enzymatic disturbances in the blood and tissues noted in SO_2 intoxication.

Zusammenfassung. Im Zusammenhang mit der Bearbeitung einer neuen Neutralisierungsmethode des SO_2 in Verbrennungsgasen mit Hilfe von Ammoniak wurden in toxikologischer Hinsicht unter dem Einfluß von SO_2 entstandene Strukturveränderungen untersucht. Außerdem wurde der durch die SO_2-Verbindung mit Ammoniak entstandene Einfluß der Reaktionsprodukte verfolgt. Diese Untersuchungen wurden an 100 Meerschweinchen durchgeführt. Die Kontrollgruppe bestand aus 20 Meerschweinchen. Die nächsten 20 Meerschweinchen wurden in einer speziellen toxikologischen Kammer durch SO_2 in einer Konzentration von 2 g/Nm³ vergiftet. Die weiteren 20 Tiere exponierte man den infolge der Verbindung von SO_2 mit Ammoniak entstandenen Reaktionsprodukten. Die 4. Gruppe von Tieren (20 Meerschweinchen) wurde langdauernd, und zwar 8 Std täglich innerhalb von 4 Monaten, SO_2-exponiert (50 mg/Nm³). Die 5. Gruppe von Tieren ist ebenfalls 4 Monate lang den Reaktionsprodukten $SO_2 + NH_3$ ausgesetzt worden. Bei allen untersuchten Tieren wurde im Blut Hämoglobinbestimmung, Erythrocyten- und Leukocytenzahl und prozentuelle Zusammensetzung der Leukocyten aufgezeichnet. Es wurden Störungen im Säure-Basen-Gleichgewicht verfolgt. Verzeichnet wurden in dem aus der linken Herzkammer entnommenem Blut pH-Wert, CO_2-Partialdruck sowie vollständiges Plasma-CO_2. Außerdem bestimmte man im Blut das vollständige Eiweißniveau und ebenso die Aktivität der Aldolase, Milchsäure-Dehydrogenase, der Alanin- und Asparagin-Aminotransferase. Im Urin der untersuchten Tiere wurde pH-Wert, Zucker- und Eiweißgehalt sowie morphologische Bestandteile bestimmt.

Aus den durch Ausblutung getöteten Tieren wurden Ausschnitte aus Leber und Hirn entnommen. Die entnommenen Ausschnitte wurden auf übliche Weise homogenisiert und, ähnlich wie beim Blut, die Aktivität der Enzyme bestimmt. Die erhaltenen Ergebnisse haben erwiesen, daß die toxische Wirkung des SO_2 sehr vielseitig ist und zur Funktionsschädigung einer Reihe von Organen führt. Grundlegend für die toxische SO_2-Wirkung sind Störungen im Säure-Basen-Gleichgewicht, hervorgerufen durch die Resorption saurer Produkte, als auch Ventilationsstörungen, die das Ergebnis von Veränderungen im Atmungssystem sind.

Der bei SO_2-Vergiftung beobachtete Funktionsabstieg der Aldolase und Milchsäure-Dehydrogenase im Blut und den Geweben kann auf Störungen des Kohlenhydratstoffwechsels hinweisen, wodurch man die von vielen Autoren beschriebene Hyperglykämie und Störungen in der Synthese von Glykogen der Gewebe deuten kann. Die Exposition der Tiere auf das Ergebnis der SO_2-Bindung durch Ammoniak entstandene Reaktionsprodukte veranlaßte eine statistisch signifikante Verringerung der Sterblichkeitszahl bei Tieren und verlängerte bedeutend ihre Lebensdauer. Das durch Ammoniak gebundene SO_2 läßt Störungsbeseitigungen im Säure-Basen-Gleichgewicht erkennen. Solche Störungen wurden dagegen bei Vergiftungen mit reinem SO_2 beobachtet. Die bei SO_2-Vergiftungen ermittelten Enzymstörungen im Blut und den Geweben werden behoben, wenn Ammoniak in gebührenden stöchiometrischen Mengen eingeführt wird. Die erhaltenen Ergebnisse zeigen, daß das durch Ammoniak gebundene SO_2 Stoffwechselstörungen in den Geweben evident verringert. Im toxikologischen Aspekt sprechen die Ergebnisse für die Wirksamkeit der Methode der Bindung von SO_2 durch Ammoniak.

Fortschreitende Industrialisierung, Ausbau der Städte und weitgehende Entwicklung der Motorisierung sind Ursachen für ständig ansteigende atmosphärische Luftverunreinigungen (Grzybowski, 1966). Die atmosphärische Luft kann durch Staub oder Gase verunreinigt werden. Gegenwärtig bestehen grundsätzlich keine technischen Schwierigkeiten, den in den industriellen Abgasen enthaltenen Staubgehalt vor der Emission in die Atmosphäre bis zu den zulässigen Grenzen zu verringern. Anders jedoch verhält sich das Problem der durch gasförmige Substanzen, besonders durch Schwefeldioxid, verunreinigten Atmosphäre. Dieses Problem wartet einer Lösung in weltweitem Ausmaß. Als Quellen für die Emission von Schwefeldioxid in die Atmosphäre werden hauptsächlich industrielle Abgase angesehen, welche beim Verbrennen von festen, flüssigen oder gasförmigen Brennstoffen entstehen und Schwefel in verschiedenen Verbindungen enthalten. Schwefelsäurefabriken, Röstungsabteilungen für Pyrit und Zinkblende sowie Cellulose-industrie liefern der Atmosphäre außerdem SO_2. Man berechnet gegenwärtig, daß in Polen alljährlich 2,7 Mill. Tonnen Schwefeldioxid in die Atmosphäre entflieht. Das in die Atmosphäre abgegebene SO_2 verbindet sich mit Wasser zu schwefliger Säure und zu Schwefelsäure nach der Oxydierung. Diese Verbindungen üben ihre schädlichen Einflüsse auf Pflanzen aus, führen schließlich zur Zerstörung des Holzbestandes und beschleunigen dadurch Geländeerosionen. Sogar auf geringe Konzentration des SO_2 sind eigentlich alle Formen der Vegetation empfindlich (Amdur u. Underhill, 1968). Die Zerstörungsstufe hängt bei Pflanzen von dem in der Luft konzentrierten SO_2, sowie von der Zeit der Gefährdung ab (Brandt, 1962).

Seit langem schon ist die schädliche SO_2-Wirkung auf den menschlichen wie den tierischen Organismus bekannt, obwohl sie bis jetzt noch nicht genügend ermittelt ist. Ständig anwachsende Luftverunreinigung durch Schwefeldioxid ergibt die Notwendigkeit, neue Bemühungen zu machen, um die Emission von SO_2 in die Atmosphäre zu beschränken. Möglich ist es z. B., wenn man Schwefel aus Brennstoffen vor deren Verbrennung entfernt oder entstandene Oxide des Schwefels vor dem Abflug der Abgase in die Atmosphäre neutralisiert. Als Beispiel für die Verringerung der Schwefelmenge in gasförmigen Brennstoffen dürfte Koksgas gelten, das während des Produktionsprozesses von Koks entsteht. Dieses wird einer Entschwefelung vor der Verbrennung unterzogen und aus diesem Grunde wird die Atmosphäre durch Oxide des Schwefels nicht verunreinigt. Gleichzeitig werden Versuche unternommen, den Schwefelgehalt in der Kohle auf dem Wege der mechanischen Aufbereitung zu verringern (Zarubin, 1963). Aus technologischen Gründen kann man gegenwärtig Schwefel aus Brennstoffen nicht genügend entfernen. Es bleibt daher der Weg der Neutralisierung von

Oxiden des Schwefels innerhalb der Feuerungen. Überall in der Welt werden solche Versuche unternommen. Eine der Methoden, Oxide des Schwefels in verbrennbarem Abgas zu neutralisieren, ist die SO_2-Bindung mit Hilfe von gasförmigem Ammoniak. Es wäre zu erörtern, was den durch SO_2 hervorgerufenen Störungen des Metabolismus zugrunde liegt, ob Ammoniak in gasförmiger Gestalt und in entsprechenden, stöchiometrisch berechneten, dem Schwefeldioxid zugeführten Mengen die SO_2-Toxicität verringert und die entstandenen Reaktionsprodukte geringere Toxicität als SO_2 aufweisen. Um eine Antwort auf die Fragen zu geben, wurden mehrere Experimente an Tieren durchgeführt.

Material und Methodik

Untersuchungen wurden an 100 Meerschweinchen (Männchen) mit einem Einzelgewicht von je 300—400 g vorgenommen. Diese Tiere, Eigenzucht der Zentralen Experimentellen Tieranstalt der Schlesischen Medizinischen Akademie, wurden einer Diät, bestehend aus Grünfutter, Hafer und Heu, unterzogen. Die Experimente wurden an mehreren Gruppen von Tieren durchgeführt, die vorerst einer akuten und langdauernden SO_2-Vergiftung unterzogen worden sind. Gleichzeitig wurde der Einfluß der SO_2-Bindung durch gasförmiges Ammoniak auf das Entstehen von enzymatischen Störungen und solchen im Säure-Basen-Gleichgewicht kontrolliert.

Die Tiere wurden in 5 Gruppen aufgeteilt:

1. Die Kontrollgruppe bestand aus 20 Meerschweinchen, welche sich in den gleichen Verhältnissen wie die der vergifteten Tiere befanden, mit dem Unterschied, daß die Atmosphäre, in welcher sich die Tiere befanden, kein SO_2 und $SO_2 + NH_3$ enthielt.

2. 20 Tiere der 2. Gruppe wurden in einer Kammer von 1 m³ Rauminhalt einer akuten Vergiftung durch SO_2 in einer Konzentration von 2 g/Nm³ unterzogen. Die Tiere verbrachten täglich 8 Std innerhalb von 3 Tagen in der Kammer.

3. Um den Wert der SO_2-Bindungs-Methode mit Hilfe gasförmigen Ammoniaks in toxikologischer Hinsicht abzuschätzen, wurde eine zweite Serie von Experimenten vorgenommen. In einen Mischer wurde entsprechend angefeuchtete Luft eingeführt und mit Schwefeldioxid so gemischt, daß das Gasgemisch SO_2 in einer Konzentration von 2 g/Nm³ wie im vorangegangenen Versuch enthielt. Nach der Vermischung wurden die Gase in die Reaktionskammer eingeführt, welche aus einem Quarzglasrohr von 1,2 m Länge, 3 cm Durchschnitt bestand und auf 150° C angewärmt wurde. Nach der Standardbestimmung der Durchflüsse wurde in die Reaktionskammer ein Strahl von Ammoniak in einer etwas größeren Menge, als sie sich aus den stöchiometrischen Verhältnissen ergab, eingeführt. Die entstandenen Reaktionsprodukte wurden in die Kammer abgeleitet, in der sich die Tiere befanden. In dem aus Quarzglas bestehenden Rohr kam es zur Reaktion zwischen SO_2 und NH_3. Die Reaktionsprodukte wurden mit Preßluft aus einer zusätzlichen Pumpe in die Kammer befördert. Um ständig die gleiche Konzentration zu gewährleisten, wurde der Luftdurchsatz mit einem Drosselmeßgerät und Barostat kontrolliert und reguliert. Die ausgeschiedenen Reaktionsprodukte gingen in die Kammer über. (Auf Grund einer 3fach durchgeführten Analyse wurde folgende Zusammensetzung festgestellt: 95,5% Ammoniumsulfat, 4,5% neutrales Sulfat. Es wurde jedoch keine Spur von Hydrogensulfat und Sulfit gefunden.) Besonders genaue Bestimmungen

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der SO_2-Konzentration in den Gasen vor, hinter der Reaktionskammer und in der toxikologischen Kammer wurden im Verlauf des Experiments ausgeführt. Die Konzentration des SO_2 wurde mit Hilfe des Jod-Stärke-Reagens, Ammoniak mit Hilfe des Nessler-Reagens bestimmt. Diese Versuche wurden an 20 Tieren vorgenommen.

4. 20 Meerschweinchen der 4. Gruppe wurden einer langdauernden Vergiftung durch SO_2 in Konzentration von 50 mg/Nm³ unterzogen. Innerhalb von 4 Monaten verbrachten die Tiere in einer toxikologischen Kammer täglich 8 Std.

5. Die 5. Gruppe von Tieren, die aus 20 Meerschweinchen bestand, wurde Reaktionsprodukten exponiert, welche durch gasförmiges Ammoniak mit SO_2 entstanden sind. In die Reaktionskammer wurde Luft mit einem SO_2-Gehalt von 50 g/Nm³ eingeführt. Danach wurde in die Reaktionskammer Ammoniak in solchen Mengen zugeführt, wie sie sich aus den stöchiometrischen Verhältnissen ergaben. Die entstandenen Reaktionsprodukte wurden in die toxikologische Kammer abgeleitet, in welcher die Tiere untergebracht waren.

Bei allen Tieren wurden Bestimmungen von Hämoglobin, Erythrocyten- und Leukocytenzahl, Färbeindex und prozentuale Zusammensetzung der Leukocyten durchgeführt. Es wurden Störungen des Säure-Basen-Gleichgewichts im Blut der Tiere verfolgt. Gasometrische Untersuchungen des Blutes wurden mit Hilfe eines Mikro-Astrup-Apparats vorgenommen. Aus dem der linken Herzkammer entnommenen Blut bestimmte man: pH-Wert, Partialdruck des Kohlendioxids in mm Hg sowie vollständiges CO_2 des Plasmas in Vol.-%. Außerdem wurde das gesamte Eiweißniveau im Parnas-Wagner-Apparat bestimmt (Bogdanikowa, 1968). Bei allen Tieren wurde gleichfalls die Aktivität der Aldolase nach der Methode von Sibley und Lehminger, modifiziert nach Brunns (1964), die Aktivität der Milchsäure-Dehydrogenase (LDH) nach der Methode von Cabaud und Wróblewski (1958), die Aktivität der Asparagin- und Alanin-Aminotranspherase nach Reitman und Frankel (1957) bestimmt. Im Tagesurin der Tiere wurden pH-Wert, Zuckergehalt und morphologische Bestandteile ermittelt.

Vier der durch SO_2 vergifteten Tiere gingen innerhalb von 5 Tagen ein, die übrigen 16 Tiere wurden nach 5 Tagen der Vergiftung durch Ausblutung getötet. Aus der Gruppe, welche einem 20 Tage dauernden Experiment (akute SO_2-Vergiftung bei gleichzeitiger Zufuhr von gasförmigem Ammoniak in die Reaktionskammer) unterzogen wurde, ging keines der Tiere ein. Sowohl die kontrollierten Tiere, als auch die durch SO_2 mit Zufuhr von gasförmigem Ammoniak vergifteten, wurden nach 20 Tagen getötet, und es wurden dieselben Untersuchungen angestellt, wie bei der durch bloßes SO_2 vergifteten Gruppe von Tieren. Bei langdauernder Vergiftung, sowohl nach der Exposition auf SO_2 als auch $SO_2 + NH_3$, ging keines der Tiere ein. Nach täglicher, 4 Monate dauernder Vergiftung wurden die Tiere ebenfalls durch Ausblutung getötet. In dem heparinisierten Blut führte man die oben angeführten Feststellungen aus. Danach wurden die Tiere seziert und Ausschnitte aus Leber und Hirn entnommen. Diese Ausschnitte wurden in üblicher Weise homogenisiert, und in ihnen wurde die Aktivität der Aldolase, Milchsäure-Dehydrogenase sowie Asparagin- und Alanin-Aminotranspherase bestimmt, die auf 1 g frischen Gewebes nach oben angegebener Methode umgerechnet wurden. Mit Hilfe des Students-Test „t" wurden die Er-

gebnisse bei den durch $SO_2 + NH_3$ vergifteten Tiere einer statistische Analyse unterzogen.

Untersuchungsergebnisse

In den ersten 2 Std des Experiments waren die durch 2 g/Nm³ Schwefeldioxid vergifteten Tiere unruhig, später wurden sie apathisch. Nach 2tägiger Vergiftungsdauer wurde serumhaltiger Ausfluß aus Nasenlöchern, Rötungen und Blutwallungen der Bindehaut sowie starker Tränenfluß beobachtet. Ebenso wurde ein Nachlassen der Atmungshäufigkeit bemerkt. Wegen Nasenverschluß atmeten die Tiere durch die Mundhöhle. Im Bereich des Kreislaufs wurde Bradykardie (durchschnittlich bis zu ungefähr 60 in der Minute) festgestellt. Bis zum 5. Tage des Experiments gingen 4 Tiere dieser Gruppe mit Erscheinungen von Darmlähmungen und Lungenentzündung ein. Die akute Vergiftung durch Schwefeldioxid führte außer Reizungen der Bindehaut, der oberen Atemwege und Kreislaufstörungen zu Körpergewichtsabnahme, durchschnittlich um ca. 81 g (20—158 g).

Die Tiere der 3. Gruppe (akute SO_2-Vergiftung, mit gleichzeitige Zufuhr von gasförmigem Ammoniak) wiesen nur unbedeutende Reizung der Bindehaut und der oberen Atemwege auf. Ein Nachlassen der Herzaktion wie in der vorigen Gruppe wurde nicht beobachtet, obwohl die Zeit der Exposition bedeutend länger war. Innerhalb von 20 Tagen ständiger Vergiftung war die Körpergewichtsabnahme geringer als in der 2. Gruppe und betrug durchschnittlich ungefähr 20 g (5—40 g).

Bei chronischer Vergiftung durch 50 mg/Nm³ Schwefeldioxid wurde anfänglich nur geringer Serumausfluß aus den Nüstern sowie leichte Blutwallung der Bindehaut beobachtet. Diese Erscheinungen traten nach einigen Tagen auf. In der Gruppe von Tieren dagegen, die der Wirkung der Reaktionsprodukte $SO_2 + NH_3$ unterzogen wurden, bemerkte man von seiten der Atemwege und des Kreislaufs keine klinischen Erscheinungen. Innerhalb von 4 Monaten beobachtete man bei den durch SO_2 chronisch vergifteten Tieren eine Gewichtszunahme in Höhe von ca. 254 g, bei den Reaktionsprodukten ausgesetzten durchschnittlich um 274 g.

Bei akuter SO_2-Vergiftung wurde eine statistisch nicht kennzeichnete Abnahme des Eiweißniveaus im Blutserum beobachtet. Ähnlich wie in der vorigen Gruppe wurde bei den mit $SO_2 + NH_3$ Exponierten ein Sinken des Eiweißniveaus im Blutserum bemerkt (Tabelle 1).

Verglichen mit der Kontrolle, wurden bei langdauernder Vergiftung, so durch SO_2 als auch $SO_2 + NH_3$, keine wesentlichen Abweichungen im Bereich des Eiweißniveaus beobachtet (Tabelle 2).

Bei akuter SO_2-Vergiftung wurde ein Anstieg des Hämoglobingehalts und der Erythrocytenzahl sowie des Färbeindex statistisch signifikant

Tabelle 1. *Das Gesamteiweißniveau im Blutserum und das periphere Blutbild bei gesunden und mit SO_2 sowie $SO_2 + NH_3$ vergifteten Tieren*

	Gesunde Tiere (20) Arithmetischer Mittelwert m_1 Abweichungen von — bis	Mit SO_2 vergiftete Tiere (10) Arithmetischer Mittelwert m_2 Abweichungen von — bis	Mit $SO_2 + NH_3$ vergiftete Tiere (20) Arithmetischer Mittelwert m_3 Abweichungen von — bis	Unterschied zwischen den Mittelwerten $m_2 - m_3$	Signifikanztest der Unterschiede (t)	Statistische Signifikanz (α)
Gesamteiweiß (g-%)	5,13 4,87—5,70	5,11 4,93—5,45	5,107 4,86—5,63	0,003	nicht kennzeichnend	
Hb (g-%)	9,53 8,19—11,7	10,01 8,97—10,92	10,14 7,93—11,31	0,13	1,8	nicht signifikant
Erythrocyten	3660000 3240000—4120000	3810000 3670000—4120000	3966000 3300000—4300000	156000	6,4	$\alpha < 0,01$
Hämoglobinwert	0,99 0,95—1,03	1,0 0,97—1,05	0,99	0,01	nicht signifikant	
Leukocyten	8330 3600—10500	6600 2000—10000	9420 6000—19300	2820	2,8	$\alpha < 0,01$
Neutrophile (%)	21 4—38	20,5 7—44	16,6 2—38	3,9	nicht signifikant	
Eosinophile (%)	1,0 0—4	0,5 0—2	0,7 0—2	0,2	nicht signifikant	
Monocyten (%)	0,7 0—2	0,5 0—2	0,7 0—2	0,3	nicht signifikant	
Lymphocyten (%)	77,3 62—90	78,5 59—93	82,5 62—98	4,0	nicht signifikant	

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Tabelle 2. *Das Gesamteiweißniveau im Blutserum und das periphere Blutbild der auf dem Inhalationsweg mit den Reaktionsprodukten $SO_2 + NH_3$ chronisch vergifteten Meerschweinchen*

	Gesunde Tiere (20) Arithmetischer Mittelwert m_1 Abweichungen von — bis	Mit SO_2 vergiftete Tiere (20) Arithmetischer Mittelwert m_2 Abweichungen von — bis	Mit $SO_2 + NH_3$ vergiftete Tiere (20) Arithmetischer Mittelwert m_3 Abweichungen von — bis	Unterschied zwischen den Mittelwerten $m_1 - m_3$	Signifikanztest der Unterschiede (t)	Statistische Signifikanz (α)
Gesamteiweiß (g-%)	5,13 4,87—5,70	5,11 5,0—5,11	5,03 4,95—5,08	0,1	1,4	nicht signifikant
Hb (g-%)	9,53 8,19—11,7	9,21 8,16—10,14	10,82 9,8—11,34	1,29	0,8	signifikant
Erythrocyten	3660000 3210000—4120000	3160000 2950000—3340000	3719200 3000000—4300000	502000	0,77	signifikant
Hämoglobinwert	0,99 0,95—1,03	1,05 0,99—1,08	1,06 0,95—1,04	0,01	0,2	nicht signifikant
Hämatokrit (%)	36,0 33,0—40,0	29,5 28,0—31,0	38,6 32,0—40,0	2,6	2,4	$\alpha < 0,05$
Leukocyten	8330 3500—10500	6180 5100—12000	9070 4000—10200	740	0,66	nicht signifikant
Neutrophile (%)	21 4—38	19 5—40	21,7 12—31	0,7	0,2	nicht signifikant
Eosinophile (%)	1,0 0—4	1,0 0—3	1,25 0—4	0,25	0,8	nicht signifikant
Monocyten (%)	0,7 0—2	1,0 0—5	0,95 0—3	0,25	1,0	nicht signifikant
Lymphocyten (%)	77,3 62—90	79 65—92	76 66—84	1,3	0,56	nicht signifikant

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beobachtet. Verglichen mit der vorigen Gruppe, hat die Zufuhr von gasförmigem Ammoniak einen Hämoglobin- und Erythrocytenanstieg hervorgerufen. Nach der SO_2-Vergiftung kam es zur Senkung der Leukocytenzahl, die Zufuhr von Ammoniak dagegen führte zur Leukocytose. Bei akuter SO_2-Vergiftung wurden im Blutausstrich eine statistisch nicht signifikante Abnahme von Monocyten, Neutrophilen und Eosinophilen, ein Anstieg dagegen von Lymphocyten beobachtet.

Ähnliche Veränderungen bemerkte man im Blutbild der durch $SO_2 + NH_3$ vergifteten Tiere.

Bei langdauernder Vergiftung durch die Reaktionsprodukte $SO_2 + NH_3$ beobachtete man einen Anstieg der Erythrocytenzahl, des Hämoglobins, des Färbeindex und des Hämatokrits. Im Blutausstrich wurden dagegen keine wesentlichen Abweichungen bemerkt.

Die akute Vergiftung der Tiere durch SO_2 hat Acidifikation des Organismus hervorgerufen. Ausdruck dessen war eine Senkung der pH-Werte, Anstieg des CO_2-Partialdrucks (ausgedrückt in mm Hg), sowie Anstieg des CO_2-Gesamtgehalts im Blutplasma (ausgedrückt in Vol.-%).

Veränderungen dieser Art waren statistisch signifikant (Tabelle 3).

Zu SO_2 zugeführtes Ammoniak beseitigte die durch SO_2 hervorgerufenen Störungen im Säure-Basen-Gleichgewicht.

Bei langdauernder SO_2-Vergiftung wurde Blut-pH-Herabsetzung festgestellt sowie eine geringere Abnahme des CO_2-Partialdruckes und des Totalgehalts von CO_2 im Blutplasma. Diese Angaben haben darauf hingewiesen, daß langdauernde Vergiftung durch SO_2 leichte, metabolische Acidose hervorruft. Nach langdauernder Exposition der Tiere auf Reaktionsprodukte von SO_2 und NH_3, wurde dagegen ein unbedeutender Anstieg (des Blut-pH) pCO_2-, sowie eine bedeutende CO_2-Steigerung festgestellt (Tabelle 4).

Langdauernde Exposition der Tiere auf die Reaktionsprodukte der SO_2-Verbindung mit Ammoniak führt also zur metabolischen Alkalose. Statistisch kennzeichnend stieg die Aktivität der Aldolase und Milchsäure-Dehydrogenase im Blutserum und in den Gewebehomogenaten der Tiere, die einer akuten SO_2-Vergiftung unterzogen worden sind, an (mit Ausnahme der Milchsäure-Dehydrogenase im Hirn). Eine Exposition der Tiere auf SO_2 mit NH_3-Zufuhr beseitigte die Störungen der Enzymaktivität, welche bei der Vergiftung durch SO_2 allein beobachtet wurde, auf statistisch kennzeichnende Art (Tabellen 5 und 6).

Bei langdauernder Vergiftung bei den Gruppen stieg die Wirkung der Aldolase und Milchsäure-Dehydrogenase im Serum und den Geweben an. In der Gruppe der Tiere, die durch Reaktionsprodukte gefährdet wurden, war der Anstieg jedoch kennzeichnend geringer.

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Tabelle 3. *Störungen des Säure-Basen-Gleichgewichts im Blut gesunder und der einer akuten SO_2- sowie SO_2+NH_3-Vergiftung unterworfenen Tiere*

	Gesunde Tiere (20) Arithmetischer Mittelwert m_1 Abweichungen von — bis	Mit SO_2 vergiftete Tiere (10) Arithmetischer Mittelwert m_2 Abweichungen von — bis	Mit SO_2+NH_3 vergiftete Tiere (20) Arithmetischer Mittelwert m_3 Abweichungen von — bis	Unterschied zwischen den Mittelwerten m_2-m_3	Signifikanztest der Unterschiede (t)	Statistische Signifikanz (α)
Blut (pH)	7,25 7,16—7,36	7,070 6,96—7,195	7,170 7,09—7,22	0,100	3,8	$\alpha < 0{,}01$
pCO_2 (mm Hg)	49,2 35,5—59,5	91,14 66,5—104,0	58,1 46,5—72,5	33,04	16,8	$\alpha < 0{,}01$
CCO_2 (Vol.-%)	45,9 29,5—57,0	65,03 51,0—86,5	46,1 31,5—54,5	18,93	4,9	$\alpha < 0{,}01$

Tabelle 4. *Störungen des Säure-Basen-Gleichgewichts im Blut der auf dem Inhalationsweg mit den Reaktionsprodukten $SO_2 + NH_3$ chronisch vergifteten Meerschweinchen*

	Gesunde Tiere (20) Arithmetischer Mittelwert m_1 Abweichungen von — bis	Mit SO_2 vergiftete Tiere (20) Arithmetischer Mittelwert m_2 Abweichungen von — bis	Mit $SO_2 + NH_3$ vergiftete Tiere (20) Arithmetischer Mittelwert m_3 Abweichungen von — bis	Unterschied zwischen den Mittelwerten m_1—m_3	Signifikanztest der Unterschiede (t)	Statistische Signifikanz (α)
Blut (pH)	7,25 7,10—7,36	7,21 7,20—7,30	7,29 7,22—7,38	0,04	1,43	nicht signifikant
pCO_2 (mm Hg)	49,2 35,5—59,5	48,75 47,5—50,0	50,0 28,0—71,5	0,8	0,21	nicht signifikant
CCO_2 (Vol.-%)	45,9 29,5—57,0	43,5 41,0—45,0	56,2 41,0—72	10,3	3,1	$\alpha < 0,01$

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Tabelle 5. *Die Aktivität der Aldolase im Blutserum und in den Homogenaten gesunder, mit SO_2 vergifteter sowie mit $SO_2 + NH_3$ vergifteter Tiere in Einheiten nach Sibley und Lehninger, modifiziert nach Brunns auf 1 g frischen Gewebes umgerechnet*

	Gesunde Tiere (20) Arithmetischer Mittelwert m_1 Abweichungen von — bis	Mit SO_2 vergiftete Tiere (16) Arithmetischer Mittelwert m_2 Abweichungen von — bis	Mit $SO_2 + NH_3$ vergiftete Tiere (20) Arithmetischer Mittelwert m_3 Abweichungen von — bis	Unterschied zwischen den Mittelwerten $m_1 - m_3$	Signifikanztest der Unterschiede (t)	Statistische Signifikanz (α)
			Akute Vergiftung			
Blutserum (E)	86 58—102	124 98—151	84 67—107	40	8,7	$\alpha < 0{,}01$
Leber (E)	11058 8750—13750	16247 10250—22500	12251,8 10240—14650	3905	4,02	$\alpha < 0{,}01$
Hirn (E)	2827 2750—3200	3057 8830—3320	2859 2690—3020	207,5	3,3	$\alpha < 0{,}01$
			Chronische Vergiftung	$m_1 - m_3$		
Blutserum (E)	86 58—102	120,5 95—132,0	97,0 78,5—106,5	11,6	3,4	$\alpha < 0{,}01$
Leber (E)	11058 8750—13750	16059,7 13070—10665	13683 11450—15082	2525	4,8	$\alpha < 0{,}01$
Hirn (E)	2827 2750—3200	3051,8 2781—3272	3017,6 2708—3320	190,6	2,9	$\alpha < 0{,}01$

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Tabelle 6. *Die Aktivität der Milchsäure-Dehydrogenase im Blutserum und in den Homogenaten gesunder, mit SO_2 vergifteter, sowie mit SO_2+NH_3 vergifteter Tiere in Einheiten nach Cabaud und Wróblewski auf 1 g frischen Gewebes umgerechnet*

	Gesunde Tiere (20) Arithmetischer Mittelwert m_1 Abweichungen von — bis	Mit SO_2 vergiftete Tiere (16) Arithmetischer Mittelwert m_2 Abweichungen von — bis	Mit SO_2+NH_3 vergiftete Tiere (20) Arithmetischer Mittelwert m_3 Abweichungen von — bis	Unterschied zwischen den Mittelwerten m_2-m_3	Signifikanztest der Unterschiede (t)	Statistische Signifikanz (α)
			Akute Vergiftung			
Blutserum (E)	566 360—710	790 750—850	625 475—725	164	3,6	$\alpha < 0{,}01$
Leber (E)	1345 1205—1510	1704 1490—1880	1445,0 1275—1640	259	5,7	$\alpha < 0{,}01$
Hirn (E)	490 430—580	509 450—560	486 425—535	23	2,0	$\alpha < 0{,}05$
			Chronische Vergiftung	m_1-m_2		
Blutserum (E)	566 360—710	769,5 720—780	726,3 669—815	160,3	5,5	$\alpha < 0{,}01$
Leber (E)	1345 1205—1510	1570,0 1427—1719	1469,3 1289—1636	124,3	2,9	$\alpha < 0{,}01$
Hirn (E)	490 430—580	509,5 483—520	493,1 466—594	3,1	0,23	nicht signifikant

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Tabelle 7. *Die Aktivität der Asparagin-Aminotransferase im Blutserum sowie in den Homogenaten gesunder, mit SO_2 vergifteter, sowie mit $SO_2 + NH_3$ vergifteter Tiere, umgerechnet auf 1 g frischen Gewebes in Einheiten nach Reitmann und Frankel ausgedrückt*

	Gesunde Tiere (20) Arithmetischer Mittelwert m_1 Abweichungen von — bis	Mit SO_2 vergiftete Tiere (16) Arithmetischer Mittelwert m_2 Abweichungen von — bis	Mit $SO_2 + NH_3$ vergiftete Tiere (20) Arithmetischer Mittelwert m_3 Abweichungen von — bis	Unterschied zwischen den Mittelwerten $m_2 - m_3$	Signifikanztest der Unterschiede (t)	Statistische Signifikanz (α)
			Akute Vergiftung			
Blutserum (E)	49,9 28,5—67,5	32,2 23,0—42,0	25,5 17,0—39,0	6,7	2,8	$\alpha < 0{,}01$
Leber (E)	77641 69325—85000	65438 56000—78000	72102 59830—81345	6664	3,5	$\alpha < 0{,}01$
Hirn (E)	7131 6000—8450	6793 5830—7480	6732 5430—7315	61	0,31	nicht signifikant
			Chronische Vergiftung	$m_1 - m_2$		
Blutserum (E)	42,9 28,5—67,5	30,86 20,0—36,1	29,8 26,5—32,5	13,1	5,2	$\alpha < 0{,}01$
Leber (E)	77641 69325—85000	69235 56040—73606	70412 67853—72814	7229	10,7	$\alpha < 0{,}01$
Hirn (E)	7131 6000—8450	6368,0 5670—7017	6524,4 5795—7311	606,6	3,5	$\alpha < 0{,}01$

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Tabelle 9. *Die Aktivität der Alanin-Aminotranspherase im Blutserum sowie in den Homogenaten gesunder, mit SO_2 vergifteter, sowie mit $SO_2 + NH_3$ vergifteter Tiere, in Einheiten nach Reitman und Frankel auf 1 g frischen Gewebes umgerechnet*

	Gesunde Tiere (20) Arithmetischer Mittelwert m_1 Abweichungen von — bis	Mit SO_2 vergiftete Tiere (16) Arithmetischer Mittelwert m_2 Abweichungen von — bis	Mit $SO_2 + NH_3$ vergiftete Tiere (20) Arithmetischer Mittelwert m_3 Abweichungen von — bis	Unterschied zwischen den Mittelwerten $m_2 - m_3$	Signifikanztest der Unterschiede (t)	Statistische Signifikanz (α)
			Akute Vergiftung			
Blutserum (E)	36,8 29,5—43,5	25,6 10,5—31,0	17,62 14,5—23,0	8,0	7,02	$\alpha < 0{,}01$
Leber (E)	4645,7 3940—5500	3772 3000—4500	4378 3080—5390	606	2,8	$\alpha < 0{,}01$
Hirn (E)	344,0 280—440	298,0 270—300	303 260—360	5	0,66	nicht signifikant
			Chronische Vergiftung	$m_1 - m_3$		
Blutserum (E)	36,8 29,5—43,5	24,7 21,0—28,2	22,2 17,0—27,0	14,6	10,5	$\alpha < 0{,}01$
Leber (E)	4645,7 3940—5500	3666 3240—4115	3944,2 3687—4142,5	701,5	4,8	$\alpha < 0{,}01$
Hirn (E)	344,0 280—440	300,1 276—321	308,7 287—330,5	35,3	2,09	nicht signifikant

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Als interessantes Ereignis, beobachtet an Tieren, die durch SO_2 akut vergiftet wurden, kann die statistisch signifikante Abnahme der Aktivität von asparaginer und alaniner Aminotranspherase im Blutserum und den Geweben angesehen werden (mit Ausnahme von Alat in der Leber).

Die Gruppe von Tieren jedoch, die SO_2 mit einer Ammoniakzufuhr exponiert wurde, ließ eine Normalisierung der Enzymwirkung in den Geweben feststellen; im Serum dagegen beobachtete man eine Abnahme der Aktivität dieser Enzyme (Tabellen 7 und 8). Ebenso wurde im langdauernden Experiment in beiden untersuchten Gruppen eine Aktivitätsabnahme der asparaginen und alaninen Aminotranspherasen im Blutserum und den Geweben beobachtet (Tabellen 7 und 8).

Verglichen mit der Kontrollgruppe, bei welcher im Urin ein pH von 7,6 (7,2—9,1) mit gleichzeitig erwiesener Eiweißspur festgestellt wurde, beobachtete man im Urin der einer akuten SO_2-Vergiftung unterzogenen Tiere eine Ansäuerung durchschnittlich bis zu 6,6 pH (6,2—7,3). Die Anwesenheit von Zucker wurde im Urin nicht festgestellt. Einzelne Leukocyten und Erythrocyten, Kristalle des Calciumphosphats und Tyrosins wurden im Urinsediment dieser Gruppe von Tieren beobachtet. Im Urin der durch SO_2+NH_3 vergifteten Tiere beobachtete man einen pH-Wert von durchschnittlich 7,4 (7,1—8,0); die Anwesenheit von Eiweiß und Zucker wurde nicht erwiesen. Bei manchen Tieren wurden im Urinsediment einzelne Leukocyten und Kristalle des Calciumphosphats beobachtet. Im Urin der einer langdauernden SO_2-Vergiftung unterzogenen Tiere beobachtete man einen pH-Wert von durchschnittlich bis zu 7,07 (7,0—10,0), bei Vergiftung durch SO_2+NH_3 durchschnittlich bis zu 8,59 (7,8—10,0). In keiner der beiden untersuchten Gruppen konnten pathologische Veränderungen im Urin erwiesen werden.

Diskussion

Die erlangten Resultate haben ergeben, daß akute SO_2-Vergiftungen bei Tieren verschiedenartige Störungen im Organismus hervorrufen. Reizungen der Atemwege und der Bindehaut, die Ausgangspunkt zur Bindehautentzündung, Bronchial- und Lungenentzündung sind, stellten sich an die Spitze des klinischen Bildes. Die innerhalb des Atmungssystems entstandenen Veränderungen sind unmittelbares Ereignis der reizbaren Wirkung von Säureprodukten, welche infolge der Verbindung von Wasser mit SO_2 zu schwefliger Säure entstanden sind. Wie bekannt ist, findet dieser Prozeß in Anwesenheit von Wasserdampf und NaCl schneller statt (Amdur u. Underhill, 1968). An den entzündlichen Veränderungen des Bronchialbaums nehmen Bronchial-Krampfzustände teil, die schließlich zur Verminderung der Vital- und Atmungskapazität führen. Das Zusammenziehen der Bronchien wird reflektorisch und durch

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unmittelbare Säurewirkung der Produkte auf die Schleimhaut hervorgerufen (Frank et al., 1962; Nadel et al., 1965). Veränderungen im Atmungssystem werden von Kreislaufschädigungen in Gestalt von Bradykardie und Herzmuskelschwäche begleitet.

Ähnliche Ergebnisse erzielten auch andere Autoren mit akuter SO_2-Vergiftung an Hunden (Salem u. Aviado, 1961). Vergiftung der Tiere durch SO_2 in einer Konzentration von 2 g/Nm³ hat eine beträchtliche Gewichtsabnahme infolge der Entwässerung hervorgerufen. Ausdruck dessen war Hämoglobinzunahme und Erythrocytenanstieg. Innerhalb des in 5 Tagen durchgeführten Experiments gingen 4 Tiere mit Erscheinungen von Darmlähmung und Lungenentzündung ein. SO_2-Verbindungen mit Hilfe gasförmigen Ammoniaks verringerte evident die Toxicität des ersten. Die Gefährdung der Tiere durch dieselbe Konzentration von SO_2, doch mit gleichzeitiger Zufuhr von NH_3, das sich zu saurem und neutralem Sulfit sowie Ammoniumsulfat verbunden hat, hat nur Reizung des Bronchialbaums, mit übermäßiger Schleimabsonderung hervorgerufen. Kreislaufstörungen wurden dagegen nicht beobachtet. Innerhalb von 20 Tagen der Exposition der Tiere mit 2 g/Nm³ $SO_2 + NH_3$ (in gebührenden stöchiometrischen Mengen), ging keines der Tiere ein. Ähnliche Wahrnehmungen von schutzwirkendem Einfluß des Ammonium-Kations bei toxischer SO_2-Wirkung auf die Meerschweinchen machte Pattle u. Mitarb. [11]. Diese Autoren haben erwiesen, daß eine Zufuhr von Ammoniumcarbonat in die toxikologischen Kammer die Sterblichkeit der SO_2-exponierten Tiere um 50% verringert.

SO_2-Vergiftung führt zum Entstehen von schwefliger Säure und Schwefelsäure, auf den Schleimhäuten der Atmungsorgane, welche ins Blut eindringen und in alle Gewebe, einschließlich des Hirns, verbreitet werden und sich, wie isotopische Untersuchungen mit $S^{35}O_2$ [2, 6] erwiesen haben, kumulieren können. Schwefeldioxidvergiftung führt zum Konzentrationsanstieg von sauren Produkten im Blut und in den Geweben, infolgedessen kommt es zu Störungen im Säure-Basen-Gleichgewicht. Bei akuten Vergiftungen durch dieses Gas beobachteten wir pH-Abnahme, Anstieg des CO_2-Partialdrucks und des vollständigen CO_2-Plasmagehaltes. Störungen im Säure-Basen-Gleichgewicht hatten also gemischten Charakter; sie wurden durch Anstieg von sauren Produkten im Blut hervorgerufen sowie durch Ventilationsstörungen als Folge von Veränderungen im Atmungssystem. Ebenso wurde, als Ergebnis größerer Ausscheidungen von Sulfaten, eine Acidifikation des Urins bei den vergifteten Tieren beobachtet. Störungen im Säure-Basen-Gleichgewicht werden auf kennzeichnende Weise beseitigt, indem eine Ammoniakzufuhr zu SO_2 erfolgt. Bei langdauernder SO_2-Vergiftung wird eine leichte metabolische Acidose beobachtet. Verglichen mit einer akuten Vergiftung, war die Acidifikation bei langdauernder Vergiftung

geringer. Nach langdauernder Exposition der Tiere mit Reaktionsprodukten wurde keine Acidifikation beobachtet, es wurde jedoch umgekehrt eine metabolische Alkalose festgestellt.

Es scheint, daß der durch SO_2-Vergiftung entstandenen Acidose metabolische Veränderungen zugrunde liegen. Es wurden im Blut wie auch in den Geweben Funktionsstörungen einer Reihe von Enzymen erwiesen. Eine Funktionssteigerung der Aldolase und Milchsäure-Dehydrogenase kann auf Störungen des Kohlenhydratstoffwechsels als Folge einer SO_2-Vergiftung hinweisen. Bindung von Schwefeldioxid mit Ammoniak führt zur Funktionsherabsetzung dieser Enzyme. Bei SO_2-Vergiftung beobachtete man keine Funktionssteigerung der Indicatoren-Enzyme auf nekrotische Prozesse der Zellen, entgegengesetzt jedoch ermittelte man eine Aktivitätsherabsetzung der Aminotranspherasen im Blut und den Geweben. Möglich ist, daß diese Wahrnehmung das Ergebnis einer unmittelbaren Wirkung der sauren Produkte auf diese Enzyme ist. Die SO_2-Bindung durch NH_3 vermehrt tatsächlich wesentlich die Aktivität dieser Enzyme in den Geweben (im Blutserum dagegen nicht).

Zusammenfassend sei zu unterstreichen, daß die grundsätzliche Ursache der bei SO_2-Vergiftung beobachteten Störungen wahrscheinlich in Veränderungen des Säure-Basen-Gleichgewichts liegt, welche zu Veränderungen der Eiweißgewebe und insbesondere der Enzyme führen. Im Endergebnis kommt es zu Störungen vieler Glieder des Stoffwechsels und folglich zu morphotischen Veränderungen. Die durchgeführten Untersuchungen haben zweifellos ergeben, daß die aus der Bindung von SO_2 durch NH_3 entstandenen Reaktionsprodukte im tierischen Organismus unvergleichlich geringere Störungen hervorrufen als SO_2 allein in derselben Konzentration. Im toxikologischen Aspekt dürfte das für die Wirksamkeit der Methode, der Bindung von Ammoniak und SO_2, sprechen.

Schlußfolgerungen

1. Der toxischen Wirkung des SO_2 liegen Störungen im Säure-Basen-Gleichgewicht zugrunde, die folglich zum Funktionswechsel einer Reihe von Enzymen führen, welche die Ursache von Stoffwechselstörungen sind.

2. SO_2-Bindung mit gasförmigem Ammoniak läßt die im Säure-Basen-Gleichgewicht entstandenen Störungen beseitigen, welche bei der Vergiftung durch reines SO_2 beobachtet wurden.

3. Die Sterblichkeit bei Tieren ist bedeutend geringer nach der Exposition auf die Bindungsprodukte des SO_2 mit Ammoniak als nach der Gefährdung durch reines SO_2 in derselben Konzentration.

4. Der Aktivitätswechsel der Aldolase und Milchsäure-Dehydrogenase, im Blut als auch in den Geweben, dürfte auf Störungen des Kohlenhydratstoffwechsels hinweisen.

5. Die bei SO_2-Vergiftung ermittelten enzymatischen Störungen im Blut und in den Geweben werden wesentlich verringert, wenn Ammoniak zugeführt wird.

6. Für die im toxikologischen Aspekt gesehene Wirksamkeit der Methode einer Bindung von Ammoniak und SO_2 sprechen die erhaltenen Ergebnisse.

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MITOTIC RATES, GOBLET CELL INCREASE AND HISTOCHEMICAL CHANGES IN MUCUS IN RAT BRONCHIAL EPITHELIUM DURING EXPOSURE TO SULPHUR DIOXIDE

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PLATES XXI AND XXII

THE stigmata of human chronic bronchitis are hypertrophy of the mucous glands deep in the bronchial wall and a marked increase in the number of epithelial goblet cells, particularly in the peripheral small airways, where they are usually sparse. An increase in bronchial goblet cells beyond the normal has been produced without infection by allowing rats to inhale sulphur dioxide (Reid, 1963). In rats there is a difference in the histochemical nature of the intracellular mucus at different levels in the tracheo-bronchial tree. In the trachea the mucus is sulphated, in the proximal airways a mixture of sulphomucin and sialomucin is produced, whilst more peripherally all goblet cells produce sialomucin alone (McCarthy and Reid, 1964*a*).

The purpose of the experiments here reported was to discover whether epithelial destruction precedes the development of goblet cells and whether there is any increase in mitotic count; to measure gland hypertrophy, and to ascertain whether in the newly appearing goblet cells the distribution of acidic mucins is similar to the normal. A further objective was to find out whether the new goblet cells persisted, and whether there were further histochemical changes after the irritant was removed.

MATERIALS AND METHODS

Forty-eight rats bred from pathogen-free rats (Anticimex Stockholm) were used for this investigation. The rats were divided into three groups, one of sixteen, one of fourteen and one of eighteen. Each group was used for a different experiment and exposed for the periods shown in table I.

In the first two experiments the timing of exposure was so arranged that in each experiment all animals were killed at the same time, roughly 20 hr after their last exposure to sulphur dioxide. For mitotic counts three animals were used from each exposure time in the first experiment and one from each exposure time in the second experiment. The remaining animals were used for studying the uptake of radioactive sulphate. In the third experiment all were used to investigate the persistence of the changes. Three were kept as controls and the remaining 15 were exposed to SO_2 for 3 wk only, after which groups of three animals were killed 1 day and 4, 7, 14 and 35 days after exposure.

The irritant, sulphur dioxide, was applied as previously described (Reid, 1963) at a concentration of about 400 p.p.m. for periods of 3 hr a day, 5 days a week.

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The concentration was determined by withdrawing samples from the chamber through fine polythene cannulae distributed throughout the chamber and kept permanently in position. The air withdrawn from the chamber was absorbed into H_2O_2 solution and titrated against 0·02N borax solution; concentrations varied between 380 and 450 p.p.m.

Between exposure periods the exposed and control animals were kept in adjacent cages under identical conditions of feeding, supply of drinking water, cage cleaning and ventilation. All animals were weighed daily. The animals were killed by intraperitoneal injection of 1–2 ml. Nembutal solution (Pentobarbitone Sodium, B.P., 60 mg. per ml., Abbott Laboratories Ltd). For mitotic counts

TABLE I

Plan of experiments

Expt no.	Number of control unexposed animals	Number of animals exposed to sulphur dioxide for				Total animals
		2 days	4 days	3 wk	6 wk	
1	4	0	4	4	4	16
2	3	2	3	3	3	14
3	3	0	0	15	0	18

animals were given an injection of 0·1 mg. colchicine per 100 g. body weight 4 hr before they were killed; animals for histochemical study were given, 4 hr before they were killed, an intraperitoneal injection of 5 mc of radioactive sulphate ($^{35}SO_4$) in aqueous solution, carrier free and sterile, at *p*H 6·5–7·5, per kg. body weight; the $^{35}SO_4$ was supplied by the Radiochemical Centre, Amersham.

Preparation of lungs. The lungs and trachea were removed intact, the trachea being ligated just below the larynx before the chest was opened in order to prevent contamination with blood during removal. The lungs and trachea were fixed to pieces of card with thread, so as to maintain the normal laryngo-hilar distance. About 2 ml. of fixative were then injected into the trachea and the specimen was immersed in fixative. Heidenhain's Susa solution was used to fix lungs for mitotic counts and buffered formol-saline for lungs studied histochemically. After fixation overnight the carina and attached left lung were embedded in one block. From the upper third of the trachea 3 blocks were cut transversely, and the remainder of the trachea was embedded to be cut in longitudinal section. The blocks were embedded in paraffin and 4μ sections cut. For mitotic counts and general histological examination sections were lightly counterstained with eosin after Weigert's haematoxylin, as this accentuates the nuclei in mitosis. For study of the mucus, the periodic acid-Schiff (PAS) method both by itself and with alcian blue (AB/PAS) was used. Materials for both these methods were supplied by George T. Gurr Ltd, the alcian blue batch number being 08247.

Mitotic counts. In counting cells in mitosis only those in prophase or metaphase were included, later stages of mitosis being ignored to prevent confusion with pyknotic nuclei, lymphocytes migrating across epithelium and goblet cell nuclei. The number of nuclei in mitosis was expressed as a percentage of the total nuclei counted. For each bronchial region studied (see below), at least 4000 nuclei were counted, representing for each region all nuclei from several sections.

In the growing animal the mitotic count represents the increase in size of the organ as well as the replacement of cells lost from normal wear and tear (Bertalanffy and Lau, 1962). Bertalanffy and Lau suggest that the percentage of mitoses

representing growth can be assessed from the increase in body weight, reckoning the percentage increase in size of an organ over a given time as the same as the percentage increase in total body weight. In these experiments the weight gain of each rat over a 4-hr period was deduced from the weight gained over the previous week. This 4-hr gain was then expressed as a percentage of the weight at the beginning of the week and subtracted from the gross mitotic percentage for the 4-hr period. If no weight was gained, then the gross mitotic percentage was used without adjustment.

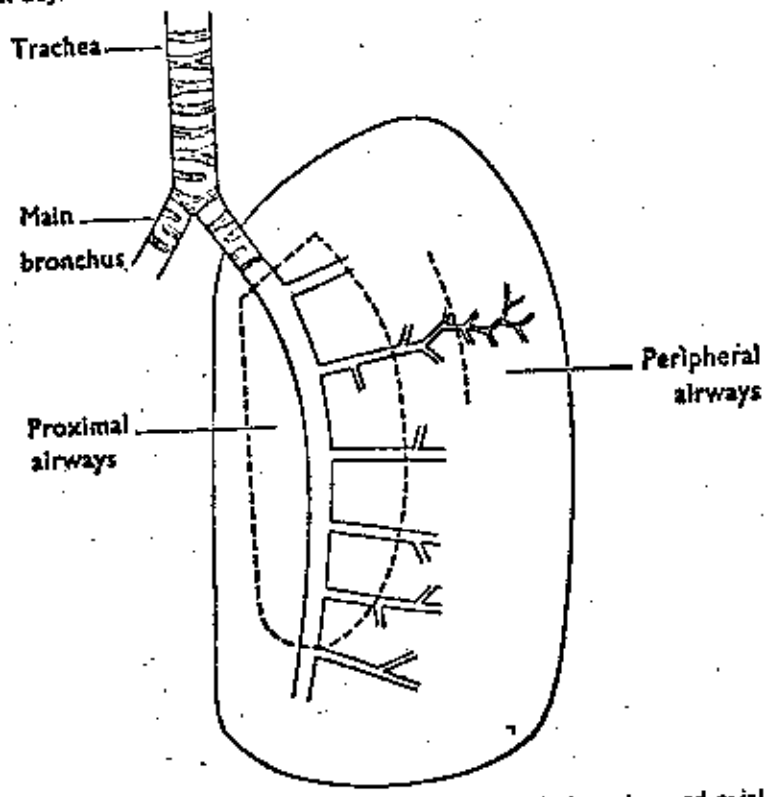

FIG. 1.—Diagrammatic representation of the trachea, main bronchus and axial pathway in the left lung of the rat, showing the four regions in which histological features have been assessed.

Mitotic counts were made separately in each of the following regions (fig. 1).

1. Upper trachea.
2. Main bronchus—below carina and proximal to the first branch, and having cartilage in its wall.
3. "Proximal airways"—lying distal to bronchi with cartilage in the wall and proximal to region 4.
4. "Peripheral airways"—if possible, the last 1·5 mm. of an airway seen in ideal longitudinal section before it opens into a respiratory bronchiole; if not, cross-sections of airways less than 0·3 mm. in diameter.

In addition, in the tracheal glands, mitoses were counted separately for particular structures.

5. Ducts—including all that part of the gland that has an obvious lumen and stains dark blue with AB/PAS.
6. Acini—groups of cells staining pink with AB/PAS and having canaliculi between the cells but no proper lumen.

Tracheal gland size. The tracheal glands are patchy in their distribution, lying mainly between the cartilage rings. To assess the increase in their size, we measured their length and thickness, as seen in cross-section of the trachea (see fig. 2). From each of the three blocks of the upper third of a trachea we examined sections at 7 levels 40 μ apart, i.e., 21 sections in all. From each section, we determined the maximum length and the maximum thickness of a single gland (see fig. 2). The mean of the findings for the twenty-one sections as well as the maximum for each measurement taken from each section were used for comparison between control and exposed animals.

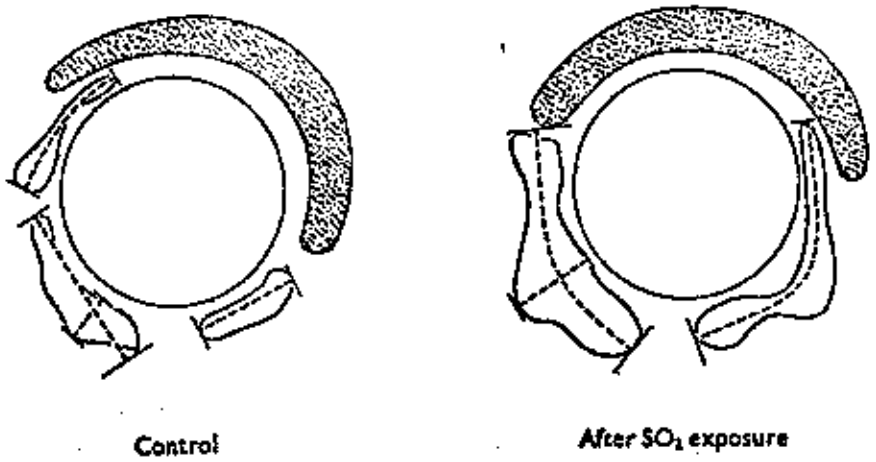

FIG. 2.—Diagrammatic representation of the submucosal glands in the rat trachea. The dotted lines show the sites at which measurements were made. After SO_2 exposure gland depth and length are both increased.

Goblet cell density. Goblet cell density is expressed by number per high-power microscopic field, i.e., per 0·3 mm. of wall. Goblet cells were counted in consecutive fields, and as far as possible, each value given is the mean of the findings of twenty fields.

Histochemistry of goblet cells. Sialomucins were localised by staining adjacent sections, one of which had been pre-treated at 37°C for 18 hr with sialidase (Spicer and Warren, 1960), with AB/PAS (Mowry, 1956). The sialidase was receptor-destroying enzyme (RDE) from *Vibrio cholerae* (Burroughs Wellcome and Co., London). If the cells contain sialic acid susceptible to sialidase, the blue staining of goblet cells with AB/PAS stain, indicative of acid glycoprotein, is lost after sialidase pre-treatment, the dark blue giving way to shades of red or red-purple. Acid glycoprotein resistant to the enzyme may contain, as acidic groupings, sialic acid not susceptible to sialidase, or sulphate radicles.

The assessment of these changes was made for the same anatomical regions as for the mitotic counts. Because of the findings a more detailed analysis of the proximal airways was made.

Autoradiography and sulphate uptake. Sulphomucins were localised by autoradiography after injection of radioactive sulphate into the animals. For autoradiography Kodak AR 10 stripping film was mounted over PAS-stained 4μ sections and exposed at 4°C in the dark for 4 wk. Development was carried out at 20°C for 5 min. with Kodak D-19b developer, and fixation for 15 min. in FIX-SOL (Johnson) diluted 1 in 5. After one hour's washing in tap water, sections were dried and mounted in Canada balsam. The silver grain density was measured on the autoradiograph by comparison with standard density charts ranging from 10 to 500 grains per unit area.

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Results

Histological changes in airways

Normal airways. The rat trachea (fig. 3) and main bronchi are lined by pseudostratified ciliated columnar epithelium, which becomes progressively thinner peripherally until, in the distal two-thirds of the airways, the epithelium consists of a single layer of cells, at first low columnar, more peripherally cuboidal. Goblet cells are present in the trachea and more proximal airways, whereas in the distal one-third or so of an airway of the normal rat no goblet cells are found. In the wall of the trachea there are mixed sero-mucous glands, opening by ducts on to its surface and lying mainly between the rings of cartilage. These glands are larger and more numerous in the proximal part of the trachea, and are not found in the main bronchi or beyond.

Airways after exposure. After 2 days' exposure to sulphur dioxide cilia are lost throughout the trachea and main bronchi and patchily in proximal airways. The normal epithelium in these regions is replaced by one or two layers of small rather flat cells with an irregular but mainly horizontal alignment. The presence of pyknotic nuclei and the separation of cells one from another suggest cell death. The most peripheral airways show no histological evidence of damage.

After 4 days' exposure these epithelial changes are even more severe in proximal airways, there being fewer cells. This is the time when mitoses are very obvious (fig. 4). The peripheral airways are still normal in appearance.

By 3 wk the epithelium in the more affected regions appears rather thicker than normal, having several layers of cells. Areas of flat regenerating epithelium are still present. Cilia, shorter than are normally seen, are irregularly distributed in main bronchi. There is marked variation in appearance, airways showing patches of flat regenerating epithelium giving rise to side branches showing no damage. By 6 wk cilia have returned to parts of the trachea, but they are still stunted and the epithelium is still thicker than normal (fig. 5). At no time is the epithelium completely denuded nor is there evidence in the submucosa of severe inflammation. After 4 days' exposure, however, an occasional polymorphonuclear leucocyte can be seen migrating through the epithelium. The submucous glands of the trachea show no evidence of damage, the ciliated cells of even the main ducts appearing normal throughout.

Mitotic counts. In the animals exposed to sulphur dioxide the number of mitoses is significantly increased in the trachea, main bronchi, proximal and distal airways, the counts for the distal airways being lower than for the other 3 regions (fig. 8). By 4 days a large number of mitoses is present in the trachea, main bronchi and proximal airways; the difference in mean values between these sites is not statistically significant. In peripheral airways the number of mitoses

seen is significantly less than in more proximal airways and is not significantly increased over levels in control animals.

By 3 wk the mitotic counts in trachea, main bronchi and proximal airways have fallen from the high values seen after 4 days' exposure,

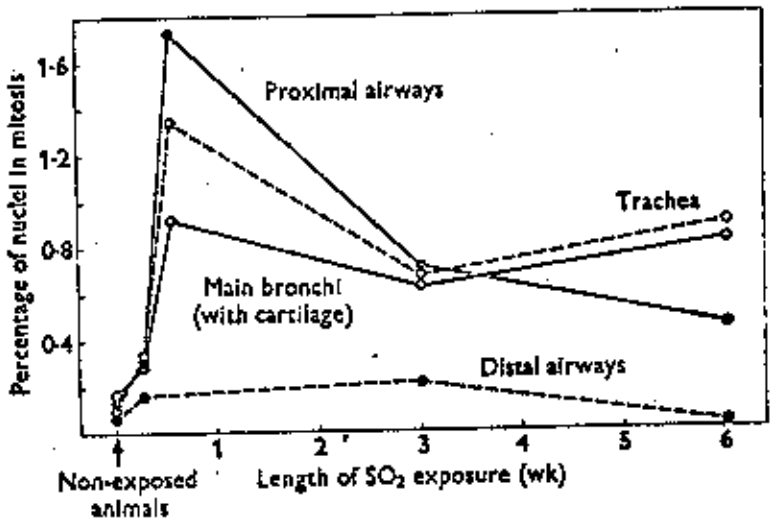

FIG. 8.—The mitotic count (4-hr period) after SO_2 exposure for periods up to 6 wk. The mitoses are represented as a percentage of the total nuclei counted. In the non-exposed animals the counts are low at all levels in the bronchial tree. At 1 wk after acute damage, there is a great increase in mitotic counts in the trachea and large airways: by 3 wk even here the number has fallen. In the distal airways the increase is scarcely significant.

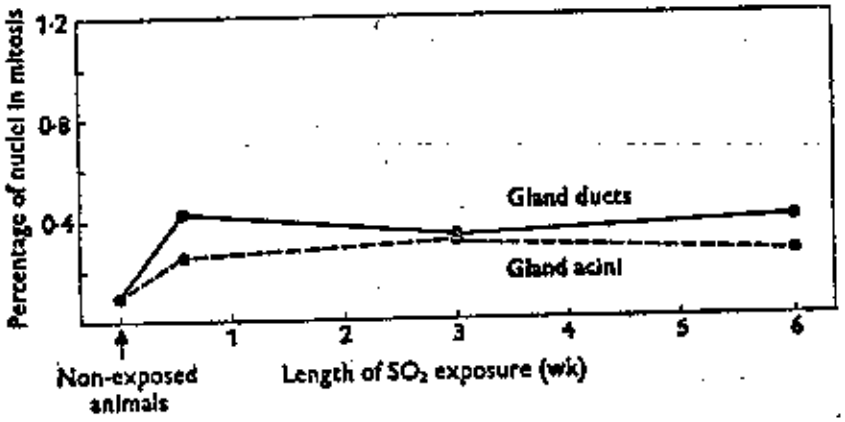

FIG. 9.—The mitotic count (as in fig. 8) for the gland ducts and acini. The increase is slight but significant and persists.

but they are still six times the control levels, and still significantly higher than the counts in distal airways, in which the figure is the same as at 4 days. After 6 weeks' exposure the mitotic counts in trachea and main bronchi are the same as after 3 wk: in proximal airways, however, the counts have fallen slightly. At 6 wk the count

in distal airways has fallen below the 3-wk value and to a level below that of the controls. All figures for distal airways are so near zero that analysis of their significance is unsatisfactory.

In the ducts and acini of the tracheal submucosal glands, despite lack of histological evidence of damage, the mitotic counts are in-

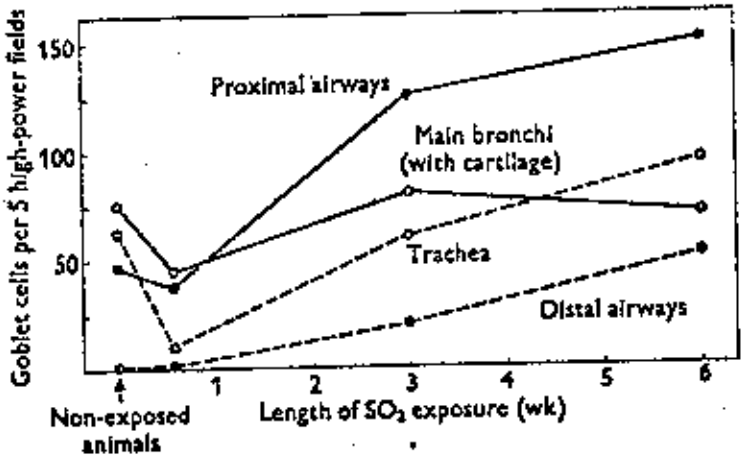

FIG. 10.—Goblet cell density expressed as the number of goblet cells per 5 high-power microscopic fields. In large airways there is a fall during the 1st wk associated with the acute damage and then a steady rise, seen also in distal airways.

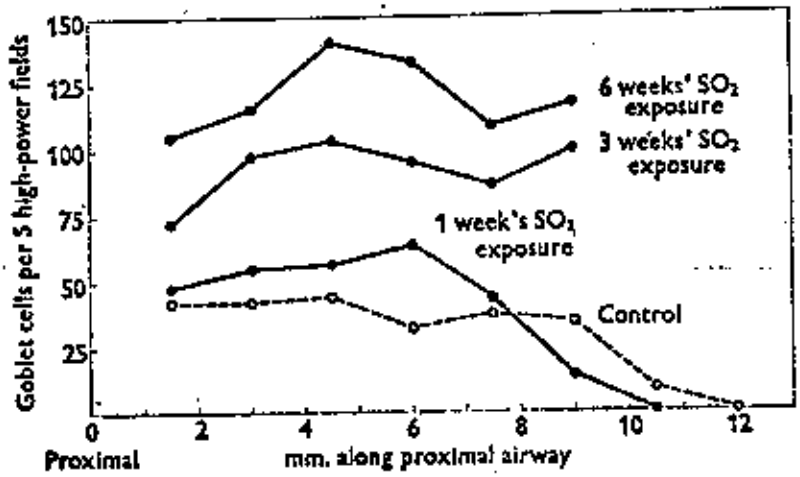

FIG. 11.—Increase in goblet cells along proximal airways.

creased (fig. 9). After 4 days' exposure, the values are significantly higher than in control animals and these levels are maintained at 3 and 6 wk.

Goblet cell counts. In the first days of exposure to sulphur dioxide there is a reduction in the number of goblet cells in the trachea and main bronchi, particularly in the trachea (see fig. 10). After 3 wk the

number of goblet cells in the regions where they are normally present has increased and, in addition, they have appeared in the distal airways where they are not normally found. By 6 wk they have increased still more in the trachea and proximal and distal airways but not in the main bronchi (figs. 6 and 7). By 3 wk there is a marked increase in size of the individual goblet cells.

A more detailed representation of the increase in goblet cells along the proximal airways is shown in fig. 11, which gives the total for each successive 1·5 mm. length. This shows that the marked increase in goblet cells is found evenly throughout this proximal region.

TABLE II

Increase in tracheal gland size after exposure to sulphur dioxide

Length of exposure (wk)	Tracheal gland			
	length (mm.)		thickness (mm.)	
	Mean	Maximum	Mean	Maximum
0	11·8	19·8	1·1	1·8
1	13	22	1·3	1·75
3	15·6	24·5	1·4	2·0
6	21	32	1·7	2·2

Seven levels on 3 rings of trachea measured for each case.

Increase in tracheal gland size. The mean and maximum values for the length and thickness of the tracheal glands are shown in table II for each exposure period. The gland size increases proportionately to the time of exposure to sulphur dioxide. The mean values give statistically rather better correlations than the maximum values. After 3 weeks' exposure to SO_2 the mean increase in both length and thickness is significant ($P<0·01$).

Histochemical properties of intracellular mucus

In the normal rat there is a characteristic distribution of goblet cells with different types of intracellular mucus (McCarthy and Reid, 1964*a*). In the trachea goblet cells containing acid glycoprotein are resistant to sialidase; in bronchi and upper proximal airways they are partly susceptible and in more distal parts of proximal airways they are completely sensitive to sialidase. These findings were confirmed by the results in the present investigation (see results for the non-exposed animals in fig. 12).

After only 4 days' exposure to sulphur dioxide there is marked increase in the proportion of goblet cells producing acid glycoprotein.

SULPHUR DIOXIDE BRONCHITIS IN RATS

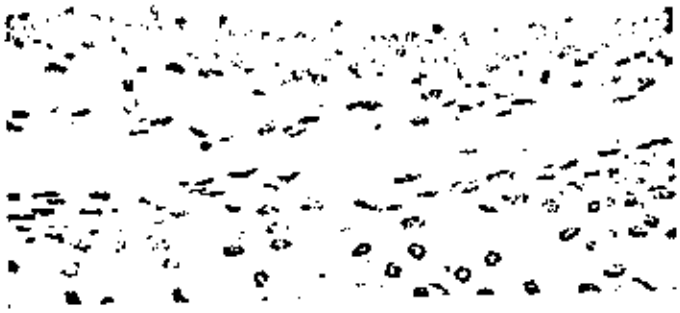

FIG. 3.—Tracheal epithelium from control rat. Susa, Weigert's haematoxylin-eosin (WHE). ×330.

FIG. 4.—Bronchial epithelium (main bronchus) from rat after 4 days' exposure to sulphur dioxide. Two layers of flattened epithelium with no cilia or goblet cells and several mitoses. Susa, WHE. ×330.

FIG. 5.—Bronchial epithelium (main bronchus) from rat after 6 weeks' exposure to sulphur dioxide. Tall epithelium with goblet cells and a cell in mitosis; a layer of mucus is apparent on the surface. Susa, WHE. ×330.

SULPHUR DIOXIDE BRONCHITIS IN RATS

FIG. 6.—Epithelium from proximal airway of control animal. Only an occasional goblet cell (black). Buffered formol-saline, periodic acid-Schiff. ×475.

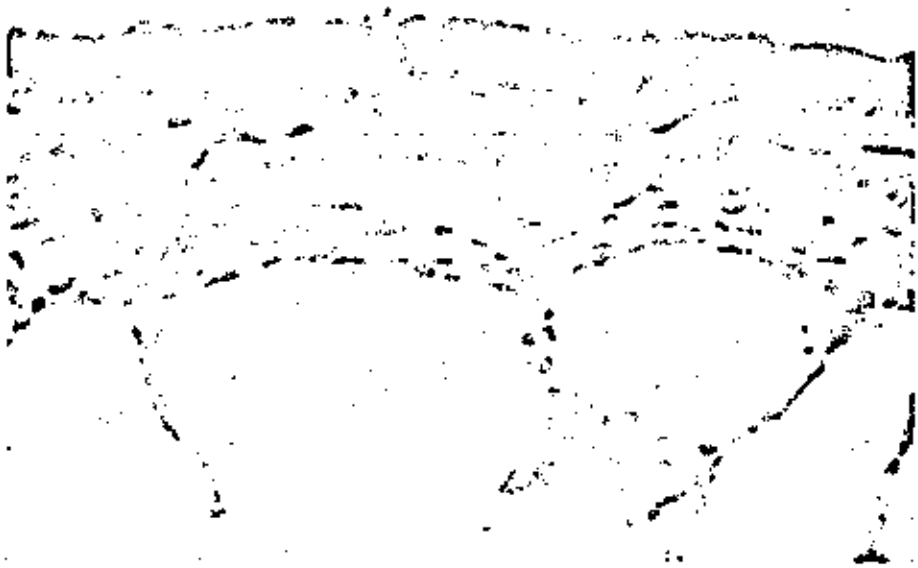

FIG. 7.—Bronchial epithelium from rat exposed to sulphur dioxide for 6 wk. Numerous goblet cells (black), each larger than in the normal. Buffered formol-saline, PAS. ×475.

There is also a marked change in the susceptibility of the acid glycoprotein to sialidase (see fig. 13). In regions where the goblet cells are normally sensitive to sialidase, as in proximal airways, a marked increase in resistance can be detected. In fig. 13 a more detailed

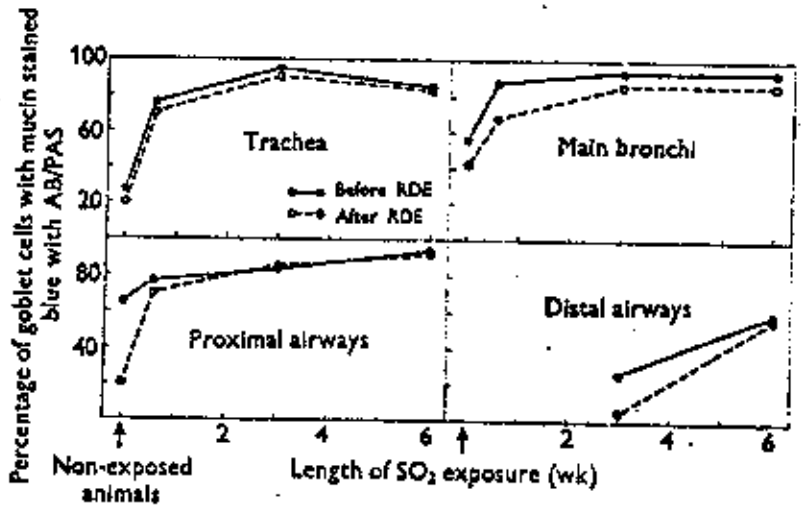

FIG. 12.—Effect of SO_2 exposure on neuraminidase (RDE) susceptibility of rat goblet cells. The ordinate shows the percentage of cells containing acid mucopolysaccharide (blue with AB/PAS stain) resistant to sialidase. At all sites this increases. In distal airways the cells on their first appearance are sensitive.

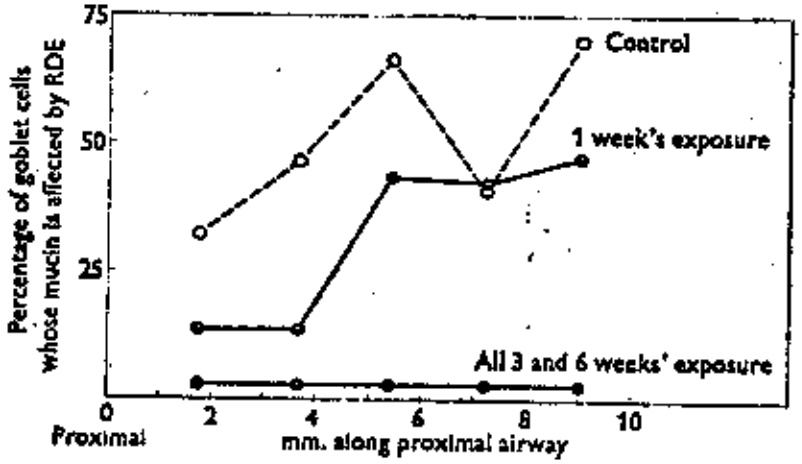

FIG. 13.—Increase in resistance to neuraminidase after SO_2 exposure. In control animals most of the goblet cells over the 10 mm. of the proximal airways studied are susceptible; as exposure increases the number susceptible falls so that by 3 wk almost all cells are resistant to the enzyme.

representation of the effect of sialidase on goblet cells is given. After 4 days the reduction in sensitivity is most marked proximally, but by 3 wk all goblet cells in this region are resistant to sialidase.

The findings in the distal airways are of particular interest. In this region there are normally no goblet cells, but as they appear all

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those containing acid glycoprotein are sensitive to sialidase. Three weeks after, all such cells are resistant to sialidase (see fig. 12).

Uptake of radioactive sulphate

In the autoradiograph of the rat lung the grain density over goblet cells in the trachea and proximal airways is low—10–20 grains per unit

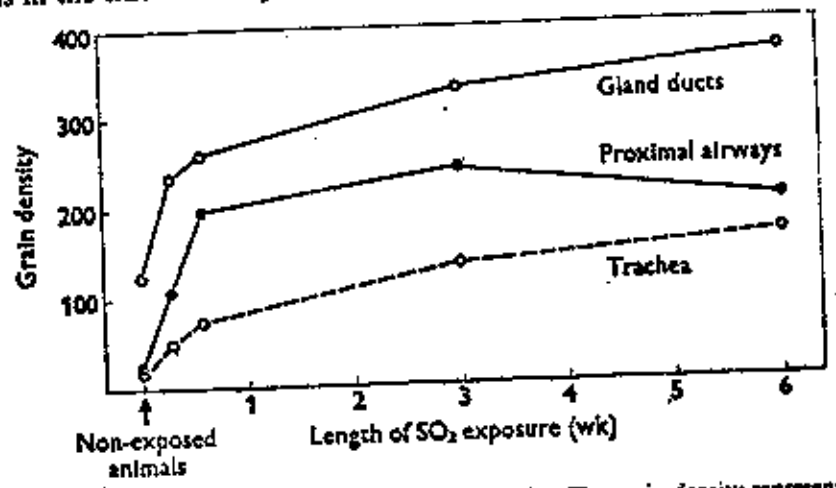

FIG. 14.—Uptake of radioactive sulphate by goblet cells. The grain density representing uptake of sulphate increases with increasing exposure.

TABLE III

Number of goblet cells and resistance of their mucins to neuraminidase up to 35 days after exposure to sulphur dioxide has ceased

Measurement	Number or percentage in proximal airways in					
	control non-exposed animals	exposed animals when interval between exposure and death was				
		1 day	4 days	7 days	14 days	35 days
Number of goblet cells per high-power field	4·8	?	14·5	12·8	12·6	10·3
Percentage of goblet cells affected by neuraminidase	65	?	10	3·5	6	11

area—but after sulphur dioxide exposure for only 2 days the grain density in the trachea is increased nearly 3 times and in proximal airways more than 4 times (see fig. 14). Further increase in radioactive sulphate uptake occurs with increased length of exposure; by 6 wk the uptake by tracheal goblet cells is 10 times that at the control level. In the normal animals the mucous cells of the tracheal glands have

well marked radioactive sulphate uptake and this also increases significantly on exposure, until after 6 weeks' exposure the level is 3 times that in the control animals. This suggests increased activity of individual cells.

Persistence of histological changes

In the third group of animals killed at intervals after removal from sulphur dioxide the proximal airways still show an increase in goblet cell number, which falls only slightly after removal from the irritant (table III). Over the proximal airways 65 per cent. of cells are susceptible to sialidase in the controls; this falls to 10 per cent. after 3 weeks' exposure and does not revert to normal over 35 days.

Discussion

Decrease in severity of damage peripherally

The acute damage is much more severe in the trachea than in more peripheral airways. This is unlikely to reflect any difference in the resistance of the epithelium to the sulphur dioxide, but is probably due to a reduction in the concentration of sulphur dioxide in the peripheral airways because of its high solubility. The study of Dalhamn and Strandberg (1961) suggests that sulphur dioxide dissolves so quickly in the tissues of the trachea that its concentration has fallen significantly by the time the main carina is reached.

Recovery

In view of the severe damage produced by the initial days of exposure to the sulphur dioxide it is strange that the epithelium heals and differentiates even with continuing exposure, so that by 6 wk the epithelium is somewhat thicker than normal and the cilia reappear. It may be that the basal cells are more resistant to the sulphur dioxide than is the superficial layer of differentiated cells. During the restoration of the central epithelium there is already an excess of mucus in the bronchial lumen from the glands and from peripheral goblet cells and it may be that this " protects " the epithelium if only by absorbing the sulphur dioxide.

In the normal rat or human lung, there are very few goblet cells at the periphery and no evidence of mucus on the tips of the cilia. The cilia themselves beat in a fluid layer (Lucas and Douglas, 1934; Hilding, 1943), which is either transudate or secretion from cells other than mucous cells. After several weeks' exposure a layer of mucus is often detected in the lung sections.

Mitotic counts

The mitotic counts give an estimate of cell turnover, and are a more sensitive criterion of the damage produced by SO_2 exposure

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than the histological appearances, since the mitotic counts in the distal airways are increased at 1 and 3 wk of exposure, although there is no histological evidence of damage. At 6 wk the fall of the mitotic count in proximal and distal airways supports the histological evidence of a decrease in the damage by sulphur dioxide.

In proximal sites the large number of mitoses seen after one week's exposure reflects the effect of regeneration following early cell damage. By 3 wk the count stabilises to a level that is maintained at 6 wk; although the histological appearances are still abnormal there is an intact epithelium of several cell layers, suggesting that regeneration exceeds any destruction. It is not possible to say from the techniques used here whether the persistently raised mitotic count arises from a small proportion of cells that bear the brunt of the damage, die and are replaced, or from shortening of the life-span of all cells.

Tracheal mucous glands

The rise in the mitotic count in the tracheal glands is not associated with any histological evidence of cell damage. It may be that as in distal airways the damage is too slight to be detected with the light microscope, or possibly the count reflects the hypertrophy of the gland which occurs during exposure as shown by the measurements of gland size.

The mechanism that produces gland hypertrophy is not known. Florey, Carleton and Wells (1932) have shown that in the cat and dog the tracheobronchial glands secrete in response to irritation of the surface epithelium through a reflex mediated through the vagus. Irritation of the bronchial surface may lead through hypersecretion to gland hypertrophy. Studies with the salivary glands of the rat suggest that irritation of the teeth leads to salivary gland hypertrophy (Wells, 1963).

Goblet cell increase

An increase in goblet cells can be produced by other irritants—formalin (Florey *et al.*), nitrous oxide fumes (Freeman and Haydon, 1964)—as well as by sulphur dioxide as used here. What is not known is the relation between the severity and duration of damage and the production of new goblet cells. Goblet cells, like other epithelial cells, seem to be susceptible to the acute effects of SO_2 exposure, for their numbers fall in the first days of the exposure. They reappear, and in larger numbers than normal, in all areas affected by the early fall, during continued exposure. The increase is least in the trachea where damage is most severe, either because it is not directly related to intensity of damage or because persisting exposure to the irritant does not permit of further differentiation to goblet cells; or the continuing loss of cells may be counterbalanced by new production.

At the periphery of the lung where there are normally no goblet

cells, they appear by 3 wk and continue to increase up to 6 wk, by which time they are found further out in the peripheral airways. In this region any early damage is minimal, but the increase continues even when evidence of this is no longer seen. New goblet cells seem to appear and increase in number progressively to the more peripheral regions; but not simultaneously throughout the bronchial tree.

Insofar as there is some increase in mitosis and goblet cell numbers throughout the bronchial tree it may be said that the two are related, but the goblet cell increase is not proportional to the mitotic count. At the periphery, where the goblet cell numbers continue to rise steadily through the 6 wk of exposure, the mitotic counts were only slightly raised, and to significant levels only in the first few days.

Change in intracellular mucus

Along airways of the rat the type of intracellular mucus varies; in the tracheal airways the acid mucopolysaccharide is resistant to sialidase and incorporates sulphate, whereas in the more distal airways the acid mucopolysaccharide is less and is susceptible to sialidase (McCarthy and Reid, 1964*a*). This has been confirmed in the present study in a different strain of rat.

During the experiments here described this distribution changed with an extension of acid mucopolysaccharide, resistant to sialidase, to the very smallest airways, those in which goblet cells newly appeared. This change in distribution of acid mucopolysaccharide could be detected in 4 days and, save for the most distal goblet cells, was complete in 3 wk.

Is this a new type of cell or is it a change in the type of mucus being produced by a cell? The fact that, at the periphery, when the cells first appear they contain a sialomucin that develops into sulphomucin suggests that the intracellular pattern of secretion can change. Because nearly all cells come to resemble each other it seems certain that these changes happen within the same cell.

Exposure to the irritant increases the rate of secretion of individual cells as shown by an increase in uptake of radioactive sulphate and an increase in its discharge. The former is measured by the increase in density of sulphate uptake and is not therefore simply a question of total cell size, although cell size also increases. It might be that the switch to a sulphomucin reflects an increased rate of synthesis in the cell. Most cells, even those that predominantly produce a sialomucin, take up some sulphate, and under conditions of irritation there may be a shift from the sialo- to the sulpho-mucin. However, this argument, based as it is on the rate of secretion, does not explain the presence of sulphomucins in the trachea when all cells are of low activity.

This change in the type of mucus is seen also with other irritants such as tobacco smoke (Lamb, Passey and Reid, unpublished).

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Reversibility of these changes

These changes, in particular the increase in goblet cells and the change in the type of intracellular mucus, do not revert to normal patterns even 5 wk after exposure ceases. Although speedily produced the changes persist in the absence of the external stimulus.

Model of human chronic bronchitis

The increase in size of the tracheal submucous glands and the extension of goblet cells to peripheral airways are typical of the changes in human chronic bronchitis. In human bronchial glands there are also both sulpho- and sialo-mucins, some cells within an acinus containing one, others both; and in chronic bronchitis the sialidase-resistant acid mucopolysaccharide is increased (McCarthy and Reid, 1964*b*; de Haller and Reid, 1965), including the sulphomucins (Lamb, unpublished).

Exposure to irritants causes hypertrophy of the bronchial mucous glands and extension of the goblet cells to the periphery, two key features of the lung in human chronic bronchitis (Reid, 1954, 1967); addition of the change in the type of intracellular mucus in rat lung after irritation makes sulphur dioxide bronchitis an even closer model of the human disease.

SUMMARY

Rats exposed to sulphur dioxide by inhalation developed a disease closely resembling human chronic bronchitis; an important feature of it was the relatively slight damage to the peripheral as compared with the central airways.

The mitotic count increased particularly in the first 4 days and in the central airways. In the ducts and gland cells it was also increased. Goblet cells increased at the periphery and centrally, the increase continuing even when the mitotic count had dropped back to nearly normal values. With exposure, cells producing acid glycoprotein increased and those with sulphomucin appeared in much more peripheral parts of the airways. New goblet cells seemed first to go through the stage of producing sialomucin; the activity of individual cells as measured by radioactive sulphate uptake increased. The gland size increased, showing that measurable hypertrophy had developed within days.

The presence of the new goblet cells and the change in the histochemical nature of the intracellular mucus persisted at least up to 5 wk after exposure.

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DÉTERMINATION ET COMPARAISON DES DL 50 DU MÉTABISULFITE DE POTASSIUM DE L'ÉTHANAL ET DE LEUR COMBINAISON (HYDROXY-ÉTHANE-SULFONATE DE POTASSIUM) PAR VOIE GRALE SUR LE RAT DE SOUCHE WISTAR

par

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RÉSUMÉ

Cette note concerne une série d'essais dont le but essentiel est de permettre la comparaison de la toxicité aiguë de l'anhydride sulfureux libre avec celle de sa combinaison avec l'aldéhyde éthylique que l'on rencontre dans le vin. Les auteurs ont opéré sur le rat Wistar mâle, en administrant les toxiques en solution aqueuse par sonde gastrique.

La considération des doses létales 50 montre que l'anhydride sulfureux engagé sous forme d'hydroxy-éthane-sulfonate de potassium est un peu moins toxique que celui engagé sous forme de métabisulfite. La toxicité de l'éthanal est comparable à celle du métabisulfite de potassium. Alors que cette dernière augmente avec la concentration, la toxicité de l'éthanal et celle de l'hydroxy-éthane-sulfonate de potassium semblent indépendantes de ce facteur. Le tableau clinique de l'intoxication par la combinaison SO_2-aldéhyde se rapproche beaucoup plus de celui de l'intoxication par l'éthanal que celui de l'intoxication par le métabisulfite.

I. Introduction.

Nous avons démontré dans un précédent travail [1] l'innocuité pour le rat Wistar de l'ingestion quotidienne d'une quantité d'anhydride sulfureux correspondant, toutes proportions gardées, à celle qu'absorberait un individu de 70 kg dont l'alimentation journalière comporterait 2 litres d'eau ou de vin renfermant 450 mg d'anhydride sulfureux par litre (dose limite autorisée pour le vin par le décret du 18 août 1921, modifié par le décret du 9 septembre 1931 et ramenée à 350 mg par le décret du 31 août 1964).

Il nous a été très justement objecté que la marge de sécurité offerte par cette expérience pour l'extrapolation à l'homme est assez faible si l'on considère la dose journalière susceptible d'être absorbée occasionnellement par ce dernier. Elle apparaît cependant importante si l'on tient

compte du fait que les animaux ont été traités quotidiennement sur trois générations successives. Il faut observer par ailleurs que dans les vins rouges de consommation courante, qui renferment une quantité moyenne voisine de 100 mg d'anhydride sulfureux par litre, les 2/3 environ de ce dernier sont combinés à l'aldéhyde éthylique sous forme d'acide hydroxy-éthane-sulfonique, plus communément désigné sous le vocable d'acide éthanol-sulfonique. Ce dernier est relativement stable, et l'on peut penser qu'il présente, par rapport à l'acide sulfureux lui-même, une toxicité atténuée. Tel n'est cependant pas l'avis de tous les auteurs, dont certains vont jusqu'à affirmer que la combinaison SO_2-éthanal est plus toxique pour l'organisme que l'acide sulfureux lui-même. La question mérite une réponse nette, car la toxicité de l'anhydride sulfureux demeure à l'ordre du jour ; son action éventuelle sur la thiamine a notamment donné lieu à de nouveaux et récents travaux (2, 3, 4, 5, 6), mais les effets de l'acide hydroxy-éthane-sulfonique sur cette vitamine n'ont pas été étudiés (*). En tout état de cause, il nous a paru indispensable dans un premier temps de procéder, le plus classiquement possible, à des essais de toxicité aiguë, permettant une comparaison précise des doses létales 50 de l'acide sulfureux engagé soit sous forme de métabisulfite de potassium, soit sous forme d'acide hydroxy-éthane-sulfonique. Nous avons naturellement été amenés à déterminer dans le même temps la toxicité propre de l'éthanal, dont la combinaison équimoléculaire avec l'acide sulfureux aboutit à la formation d'acide hydroxy-éthane-sulfonique.

Bien que le procédé d'administration le meilleur pour déterminer la dose létale d'un toxique irritant soit l'injection lente par voie intraveineuse, nous avons choisi en toute connaissance de cause la voie d'administration orale, plus conforme à la réalité physiologique. Si elle ne conduit pas aux mêmes valeurs absolues que la voie intraveineuse ou intrapéritonéale, elle n'en permet pas moins la comparaison des DL 50 des toxiques expérimentés.

Si les données bibliographiques abondent en ce qui concerne la toxicité aiguë de l'anhydre sulfureux gazeux, elles demeurent assez rares pour l'acide sulfureux ou ses sels en solution aqueuse, surtout par voie d'administration orale. La plupart sont déjà anciennes, et nous les avons citées dans notre précédent travail [1]. Aucune n'apporte d'ailleurs d'indication très précise ; seul Hoppe [7] a déterminé les doses létales 50 du bisulfite de sodium sur quatre espèces animales (souris, rat, lapin, hamster), mais en administrant le toxique par voie intraveineuse. Rappelons cependant que l'un de nous [8] a pu évaluer à 3 g environ de métabisulfite de potassium par kilogramme de poids corporel la dose minimale létale pour la souris, par voie orale. L'acide sulfureux peut donc être classé, selon Hodge et Steiner [9] parmi les « substances légèrement toxiques ».

En ce qui concerne l'éthanal, mention doit être faite des travaux de Supniewski, [10] qui a déterminé les DL de ce toxique sur la grenouille

(*) Nous avons entrepris ce travail *in vitro* et *in vivo* ; nous en rendrons compte prochainement.

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et le lapin par voie sous-cutanée ou intraveineuse. Plus récemment, STORZ [11] a opéré sur le rat par voie intrapéritonéale et SKOG [12] sur le rat et la souris par voie sous-cutanée. Mais seuls SMITH et CARPENTER [13] ont donné la valeur de 1,93 *g*/kg pour la DL 50 sur le rat par voie orale.

Quant à la toxicité de la combinaison SO_2-éthanal, elle n'a fait, à notre connaissance, l'objet d'aucune détermination.

II. Mode opératoire général.

Nous avons utilisé des rats Wistar de souche CF, mâles, âgés de 2 mois, présentant un poids moyen de 250 g et parfaitement comparables sur le plan qualitatif. Les toxiques leur ont été administrés sous forme de solution aqueuse, par sonde gastrique, en début d'après-midi, pour éviter la réplétion stomacale consécutive à l'alimentation nocturne. Nous avons ainsi pu suivre durant plusieurs heures les effets des toxiques. Les animaux morts durant la nuit étaient dénombrés le lendemain matin. Nous n'avons jamais observé de mort tardive survenant le lendemain ou les jours suivants.

Après des essais préliminaires, qui nous ont renseignés sur l'ordre de grandeur des doses toxiques et nous ont rapidement démontré, dans le cas du métabisulfite de potassium, l'influence considérable de la concentration sur la toxicité, nous avons expérimenté d'abord à concentration fixe et doses variables, puis à doses fixes et concentrations croissantes. Dans le cas de chaque toxique, les concentrations fixes nous ont été naturellement imposées par la limitation du volume administrable en dose unique par intubation gastrique. Les doses fixes expérimentées à concentrations variables ont été choisies en fonction de la mortalité constatée à concentration constante ; comme concentrations, nous avons choisi des multiples entiers de doses légalement tolérées dans les denrées les plus communément traitées par l'anhydre sulfureux.

Nous n'avons opéré que sur des animaux neufs : les survivants n'ont jamais été réutilisés pour un second essai.

Pour exprimer graphiquement la mortalité en fonction de la dose, nous avons utilisé la notation proposée par BLISS : au lieu de porter en abcisses les doses et en ordonnées les pourcentages de mortalité pour obtenir la courbe sigmoïde de TREVAN, nous avons fait figurer en abcisses les logarithmes des doses et en ordonnées les pourcentages de mortalité exprimés en « unités probits », ce qui a pour effet de rendre la courbe rectiligne : dès lors la détermination de quelques points bien choisis suffit pour tracer cette dernière, ce qui simplifie beaucoup l'expérimentation. Pour un maximum de commodité, nous avons utilisé le papier logarithme-probabilité de BONET-MAURY, sur lequel nous avons directement porté en abcisses (échelle logarithmique) les doses, et en ordonnées (échelle « probits »), les pourcentages de mortalité.

III. Détermination des doses létales 50.

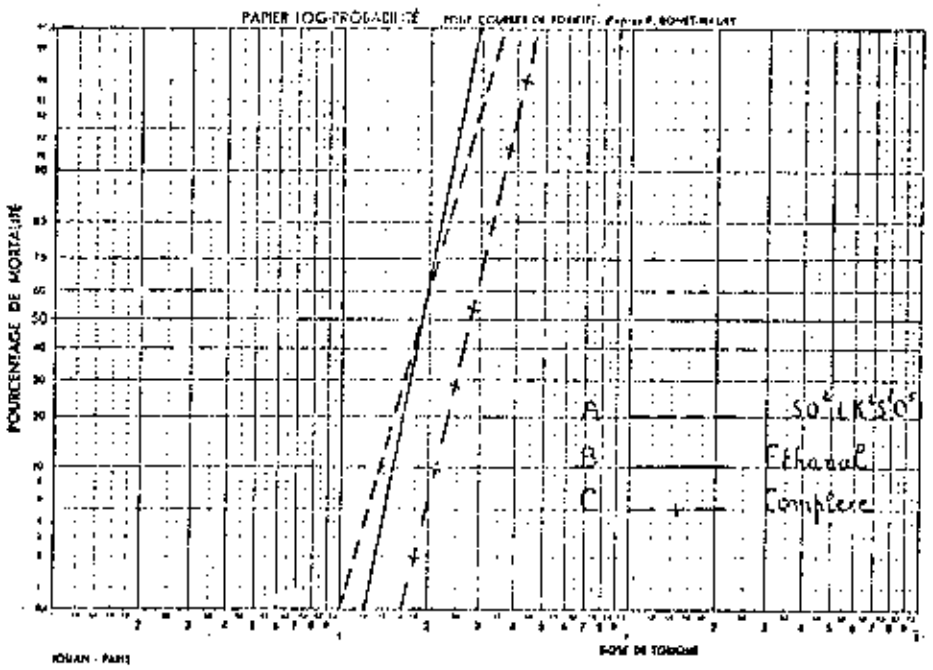
A. Métabisulfite de potassium.

L'acide sulfureux « libre » a été administré aux animaux sous forme de solution aqueuse de métabisulfite de potassium (anhydrosulfite de potassium ou bisulfite monopotassique $K_2S_2O_5$, qui résulte de la condensation de 2 molécules de sulfite avec élimination d'eau, et dont chaque molécule correspond à $2\ SO_2$).

1. *Concentration constante, doses variables.*

Après un certain nombre d'essais préalables, nous avons choisi la concentration de 125 g de métabisulfite de potassium par litre de solution aqueuse. Cette dernière a été préparée une heure avant l'emploi, car l'expérimentation préliminaire nous a montré que ce temps de « maturation » était susceptible d'intervenir sur le résultat, phénomène correspondant peut-être à la réaction $K_2S_2O_5 + H_2O \rightleftarrows HKSO_3$.

Nous avons opéré de façon très classique sur des groupes de 30 animaux, auxquels nous avons respectivement administré un certain nombre de doses, en répétant plusieurs fois (sur des groupes neufs) les essais correspondant aux trois doses ci-dessous, qui nous ont permis d'établir les points les plus sûrs de la courbe (voir fig., courbe A).



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Doses (g $K_2S_2O_5$/kg poids corporel ; concentration uniforme : 125 g/l)	Mortalité (pourcentage) (moyenne des essais effectués pour chaque dose)
1,5	28,6
2,0	56,7
2,5	85

L'exploitation de ces résultats selon la méthode de Miller et Tainter nous a conduits aux valeurs suivantes pour les doses létales :

DL (g/kg)	En $K_2S_2O_5$	En SO_2
DL 100	3,5	2,05
DL 50	1,8	1,01
DL 0	1,0	0,58

2. *Doses constantes, concentrations variables.*

Nous avons opéré avec les doses de 1,5 et 2,0 g par kilogramme, qui nous ont permis d'évaluer dans les meilleures conditions d'intubation la toxicité de solution de concentrations croissantes allant de 7 à 20 g de métabisulfite de potassium pour 100 ml. La dose de 1,5 g/kg nécessitait pour ce faire l'intubation de volumes allant de 0,75 à 2,15 ml pour 100 g de poids corporel ; la dose de 2,0 g/kg nécessitait l'intubation de volumes allant de 1,0 à 2,85 ml pour 100 g de poids corporel. Les premiers essais furent effectués avec la dose de 1,5 g/kg, mais la létalité ne dépassant pas 50 % pour la solution la plus concentrée (la plus toxique), ils furent repris avec la dose de 2,0 g/kg.

Voici les pourcentages de mortalité obtenus avec les concentrations extrêmes pour les deux « doses constantes » mentionnées :

Doses (g/kg) \ C° (g/l)	70	.....	200
1,5	10 %	.....	50 %
2,0	15 %	.....	85 %

Ces résultats mettent en évidence l'augmentation de la toxicité avec la concentration. Ce phénomène s'observe très fréquemment, mais semble dans le cas présent particulièrement marqué.

3. *Tableau clinique de l'intoxication.*

Les symptômes peuvent se décompter en deux phases qui se succèdent dans un laps de temps plus ou moins long, suivant la dose ingérée : d'une manière générale, de 5 à 20 minutes après l'administration du toxique apparaît une phase dépressive caractérisée par la prostration des sujets, qui semblent ne réagir à aucun stimulus extérieur ; puis dans une seconde phase, on observe des spasmes gastriques, des convulsions, puis des « pédalages », conduisant progressivement à une tétanisation et à la mort par arrêt cardiaque. Avec les doses les plus fortes, celle-ci survient une dizaine de minutes après l'ingestion du toxique, alors qu'avec les doses les plus faibles, les deux phases se succèdent en quelques heures. L'autopsie révèle une congestion généralisée de tous les organes, sans lésion spécifique, ce que confirme l'examen histologique (foie, reins, estomac, surrénales...).

B. Éthanal.

Nous avons suivi le même protocole en employant la même concentration pour la détermination des doses létales, et les mêmes doses et concentrations pour l'étude de l'influence de la concentration sur la toxicité.

1. *Concentration constante, doses variables.*

Les trois doses déjà essentiellement retenues pour l'établissement de la courbe de mortalité du métabisulfite de potassium nous ont conduits aux pourcentages de mortalité suivants :

Doses (g CH_3CHO/kg poids corporel ; concentration uniforme : 125 g/l	Mortalité (pourcentage) (moyenne des essais effectués pour chaque dose)
1,5	10
2,0	50
2,5	95

L'exploitation des résultats de l'ensemble des essais nous a permis le tracé de la courbe B (figure), qui correspond aux doses létales suivantes :

DL (g/kg)	CH_3CHO
DL 100	2,9
DL 50	1,9
DL 0	1,2

2. *Doses constantes, concentrations variables.*

En opérant avec les mêmes doses et les mêmes concentrations qu'avec le métabisulfite, nous avons obtenu des réponses bien situées en ce qui concerne les pourcentages de mortalité, mais ne mettant en évidence aucune variation nette de la toxicité avec la concentration.

3. *Tableau clinique de l'intoxication.*

On observe deux phases, comme avec le métabisulfite de potassium, mais leur ordre de succession est inversé : le plus généralement, de 5 à 15 minutes après l'administration du toxique apparaît une phase d'excitation comparable à celle de l'état d'ébriété, avec incoordination des mouvements, perte d'équilibre, puis parésie du train postérieur. Puis vient une période pendant laquelle l'animal paraît sombrer dans un coma analogue au coma éthylique. Le rat est en décubitus dorsal, l'œil en mydriase ; le rythme cardiaque est accéléré et le rythme respiratoire ralenti, entrecoupé de phases d'apnée plus ou moins longues. Quelques spasmes gastriques provoquent alors la régurgitation du contenu stomacal. Pour les plus fortes doses, la mort survient une en dizaine de minutes par collapsus cardio-respiratoire ; elle ne survient qu'après plusieurs heures pour les doses mortelles les plus faibles. L'étude microscopique n'apprend rien de plus qu'avec les animaux intoxiqués par le métabisulfite.

C. — Hydroxy-éthane-sulfonate de potassium.

Nos essais préliminaires nous ont montré que l'acide sulfureux combiné était moins toxique que l'acide sulfureux libre.

La combinaison SO_2-éthanal a été préparée en ajoutant un très léger excès d'éthanal à des solutions aqueuses de métabisulfite de concentrations doubles de celles utilisées plus haut. C'est ainsi que, dans une solution de métabisulfite de potassium à 250 g/l, nous avons ajouté 13 ml d'éthanal pur. Après mélange et attente à l'air libre, nous avons vérifié que la solution ne donnait plus les réactions caractéristiques de l'aldéhyde en excès, et qu'après déblocage de l'acide sulfureux en milieu boraté, on retrouvait par dosage iodométrique la totalité de ce dernier.

1. *Concentration constante, doses variables.*

Doses (g $K_2S_2O_5$ combiné à l'éthanal par kg de poids corporel ; concentration uniforme : 250 g/l)	Mortalité (pourcentage) (moyenne des essais effectués pour chaque dose)
2,0	10
3,0	65
4,1	90,6

Ces résultats correspondent à la courbe C (figure) et aux doses létales suivantes :

DL (g/kg)	$CH_3-CHOH-SO_3K$	
	exprimé en $K_2S_2O_5$	exprimé en SO_2
DL 100	4,6	2,65
DL 50	2,7	1,56
DL 0	1,55	0,89

2. *Doses constantes, concentrations variables.*

En opérant successivement avec les doses fixes de 2,0 et 3,0 g et des concentrations variant de 1,10 g/l à 100 g/l (en $K_2S_2O_5$), nous n'avons mis en évidence aucune variation de la toxicité en fonction de la concentration.

3. *Tableau clinique de l'intoxication.*

Les symptômes observés sont très analogues à ceux de l'intoxication par l'éthanal, dont l'action semble prédominante. L'examen anatomo-pathologique des organes essentiels ne révèle qu'une congestion généralisée, avec foyers hémorragiques microscopiques plus ou moins importants, sans lésion caractéristique.

IV. Comparaison des doses létales.

1. La considération de leurs doses létales respectives montre que la toxicité du métabisulfite de potassium n'est guère supérieure à celle de l'éthanal. Mais si l'on considère que $K_2S_2O_5$ conduit à 2 SO_2, et si l'on tient compte de leurs masses moléculaires respectives pour comparer

les toxicités des deux substances et pour permettre ultérieurement une comparaison avec la toxicité de la combinaison SO_2-éthanal, la DL 50 de la molécule SO_2 (64 g), engagée sous forme de $K_2S_2O_5$ correspond à 0,016 M, alors que celle de l'éthanal (44 g) correspond à 0,013 M. Bien que l'acide sulfureux ne puisse exister dans les aliments sous forme d'anhydride, il est commode de l'exprimer sous cette forme, puisque c'est ainsi que le font les textes officiels qui en limitent l'emploi.

2. L'anhydride sulfureux, engagé sous forme d'hydroxy-éthane-sulfonate de potassium, est un peu moins toxique que celui engagé sous forme de métabisulfite : la DL 50 de SO_2 engagé sous forme de CH_3—$CHOH$—SO_3K correspond en effet à 0,021 M. La détoxication de l'acide sulfureux, par sa combinaison avec l'aldéhyde, apparaît cependant plus évidente si l'on considère — en admettant que tel soit son mécanisme d'action physiologique — qu'en libérant une molécule de SO_2 (64 g), la molécule d'hydroxy-éthane-sulfonate de potassium libère aussi une molécule d'éthanal (44 g) ; donc, en libérant 1,56 g de SO_2, la DL 50 de la combinaison libère 44 × 1,56/64 = 1,07 g d'éthanal ; cette dose est inférieure à la dose maximale tolérée de l'éthanal libre, mais il n'est pas sûr que son introduction dans l'organisme soit sans conséquences. On ne peut pas dire en tout cas que les effets physiologiques de la combinaison aldéhyde-sulfureux sont dus au seul acide sulfureux, comme pourrait le donner à penser l'habitude de noter la toxicité de cette combinaison par la quantité d'anhydride sulfureux engagé ; ceci est d'autant plus vraisemblable que le tableau clinique de l'intoxication par la combinaison SO_2-aldéhyde se rapproche beaucoup plus de celui de l'intoxication par l'éthanol que celui de l'intoxication par le métabisulfite.

3. Quelle que soit celle des trois substances considérée, la marge observée entre la dose maximale tolérée (DL 0) et la dose minimale mortelle (DL 100) est très étroite ; la pente des courbes de létalité explique l'irrégularité fréquente des « réponses » enregistrées pour une même dose de toxique.

La marge la plus étroite est celle qui correspond à l'acétaldéhyde (DL 100/DL 0 = 2,1), alors que le même rapport est de 3,6 pour SO_2 engagé sous forme de métabisulfite de potassium.

En ce qui concerne SO_2 engagé sous forme d'hydroxy-éthane-sulfonate de potassium, la marge est intermédiaire (DL 100/DL 0 = 3,0).

4. La toxicité du métabisulfite de potassium augmente très nettement avec la concentration.

Par contre, les toxicités de l'éthanal et de l'hydroxy-éthane-sulfonate de potassium semblent indépendantes de la concentration (toute au moins dans l'intervalle dans lequel nous avons expérimenté).

Conclusions.

Si l'on exprime en molécules/grammes par kilogramme les DL 50 des trois substances considérées, on a :

pour SO_2 libre	0,016 M/K
pour l'éthanal libre	0,013 M/K
pour la combinaison	0,021 M/K

La toxicité de la combinaison aldéhyde-sulfureuse est donc intermédiaire entre celle de chacun de ses constituants, et inférieure à celle de l'anhydride sulfureux libre.

Cette conclusion n'est pas indifférente, du point de vue œnologique : au cours de la fermentation, l'acide sulfureux favorise en effet la formation de l'éthanal en bloquant une certaine quantité de ce dernier sous forme de combinaison stable, au fur et à mesure de son apparition. Or l'éthanal est beaucoup plus toxique (14-15) que l'éthanol correspondant ; il convient donc d'éviter cette combinaison.

On sait d'autre part, depuis longtemps, que l'acide sulfureux combiné à l'éthanal est pratiquement inactif, tant comme antiseptique que comme anti-oxygène. Le travail ci-dessus apporte un nouvel argument contre l'emploi de l'anhydride sulfureux au cours de la fermentation alcoolique ou dans la préparation et la stabilisation des vins moelleux. Par contre, l'introduction de petites quantités d'anhydride sulfureux (*) dans un vin sec, qui a achevé sa fermentation, en vue de le protéger contre la tourne ou l'acescence, ne présente pas le même inconvénient, car elle ne favorise pas l'augmentation de l'éthanal. L'acide sulfureux restant en partie libre demeure actif ; il disparaît d'ailleurs assez rapidement par oxydation en sulfate dès que le vin vient au contact de l'air au cours d'un soutirage ou d'un transvasement.

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(*) Moins de 50 mg par litre de vin, plus petite dose décelée à la dégustation par la majorité des consommateurs.

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THE REACTIVE REDUCING COMPONENTS OF SEMEN; THE PRESENCE OF SULFITE IN BOVINE SEMEN

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The metabolic pathway providing energy for the vigorous motility of spermatozoa in semen is considered by most authorities to be primarily glycolytic (1–4). Since the maintenance of buffered anaerobic conditions promotes fructolysis and maintains the life of the spermatozoa, reducing substances, apart from the monosaccharides present in semen, play a part in maintaining such conditions even in the presence of molecular oxygen. In addition, these reducing substances may enter into or be the product of direct physiological reactions.

By the use of suitable reagents and conditions, it is possible to distinguish between those reducing substances which are strongly reactive (such as ascorbic acid, various inorganic ions, and sulfhydryl-containing compounds) and the weaker reducing substances (such as the sugars). Several investigators have used such a reagent, acidified iodine, to evaluate the reducing power of semen. Using the method of Woodward and Fry (5) (for determining the glutathione content of blood by titrating iodine into a sulfosalicylic acid filtrate), Reid *et al.* (6, 7) found a considerable reducing titer and reported the probable presence of sulfhydryl compounds such as glutathione in bovine semen. Since ascorbic acid is also present in semen (and oxidized by iodine), its concentration has been evaluated usually by its power to reduce the dye 2,6-dichlorophenol indophenol (6, 7). In addition to these substances, apparently other materials are present in semen which reduce iodine, since the "sulfhydryl" content and the ascorbic acid present do not account for all of the reducing power (1–3, 6, 7). Recent work by Leone and Mann (8), for example, has shown that ergothioneine (the betaine of thiolhistidine) is present in the seminal vesicle secretion of boars.

Preliminary work in the present investigation indicated that reactive sulfhydryl compounds such as glutathione were not present in bovine seminal plasma. Since part of the iodine-reducing power could not be accounted for by substances known to be present in semen, the possibility that some other sulfur compound such as thiosulfate or sulfite might be present was considered. This was prompted by the recent reports of Lardy *et al.* (9, 10), who have reported the presence in ejaculated semen of a met-

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abolic regulator and have shown it to be elemental sulfur, the amount of which could be increased by alkaline hydrolysis. Fromageot (11) has discussed the presence of various oxidation forms of sulfur in biological systems, some forms of which will reduce iodine. With these considerations in mind, it appeared desirable to elucidate further the reactive reducing components of bovine semen and to determine in a quantitative manner their contribution to the total iodine-reducing power.

EXPERIMENTAL

A pooled sample of fresh semen was obtained by combining fourteen ejaculates from seven healthy bulls (of varying ages) on a morning in March. The semen (51 ml.) was cooled slowly to 5° and rushed to the laboratory. The sperm cells were removed by centrifugation and washed four times by redispersion with mixing and centrifugation in a volume of phosphate buffer, pH 6.9, ionic strength of 0.1, equal to that of the original semen sample. The seminal plasma was further centrifuged at high speed to remove completely all of the sperm cells and fractionated by dialyzing (in Visking sausage casing with a few drops of toluene) a portion for 24 hours with frequent agitation at 4° against an equal volume of phosphate buffer, pH 6.9, ionic strength of 0.1, to obtain the dialyzable constituents. A portion of the seminal plasma was also exhaustively dialyzed for 24 hours at 4° against frequent changes of the same strength phosphate buffer to obtain the seminal plasma proteins.

Ascorbic acid was determined by a modification of the method of Woessner *et al.* (12). In this procedure, a known amount of standardized 2,6-dichlorophenol indophenol dye is added to a matched spectrophotometer tube containing a metaphosphoric-oxalic acid-sodium chloride filtrate with known or unknown amounts of ascorbic acid. From readings made at 15 second intervals up to 1 minute, it is possible to extrapolate to a zero time reading so that other reducing materials such as sulfhydryl-containing compounds do not interfere in the analysis.

Iodine titrations were performed by the amperometric *o*-iodosobenzoate-iodine procedure of Larson and Jenness (13). This titration is essentially an acidified iodine titration, the *o*-iodosobenzoate being used to oxidize first to the disulfide any very reactive sulfhydryl groups which may be present so that the iodine will not overoxidize them subsequently in a nonspecific manner. Iodoacetamide was used also in some of the titrations. This reagent will combine with any free sulfhydryl groups present, which then will be removed from titration in the subsequent *o*-iodosobenzoate-iodine titration (14). Nitroprusside tests were conducted according to the procedure of Anson (15), and the guanidine hydrochloride was purified according to the procedure of Greenstein and Jeanette (16).

In order to obtain a concentrated seminal plasma dialysate for chromatographic analysis, the dialysate was dried from the frozen state under a vacuum and reconstituted to a concentration 10 times that of the original semen. Chromatograms were run with water-saturated phenol as the irrigating solvent and the dried papers were developed with an alcoholic ninhydrin spray (17, 18). To determine whether any unrecognizable ninhydrin color-producing substances were present, amino acid and glutathione solutions of different concentrations were also run with and without the dialysate. In this manner it was possible to determine in a quantitative manner the amount of such materials present as well as the presence or absence of reduced glutathione and oxidized glutathione (which possess an R_F of 0.12 and 0.50, respectively, in this solvent system).

Analyses for thiosulfate and sulfite were conducted on the 10 times concentrated seminal plasma dialysate by the specific seminiero qualitative procedures of Heisig (19). The procedure for sulfite, which involves precipitation as the strontium salt, washing, and identification as a filterable ferric-ferricyanide reducing substance when treated with HCl, was put on a quantitative basis by construction of a standard curve with graded amounts of sodium sulfite. It was found that, if some neutral phosphate buffer were added to the standard solutions, the supporting precipitate of strontium phosphate aided the retention of the fine strontium-sulfite precipitate. 1 drop of the ferric-ferricyanide-HCl-filtrate mixture was added to 10 ml. of distilled water and the absorption determined in a spectrophotometer set at 600 mμ. Fructose, glucose, ascorbic acid, thiosulfate, and other common reducing anions were found not to interfere in the analysis.

Results

The results of the iodometric titrations, expressed as the concentration present in the original, undiluted, pooled semen, are summarized in Table I. The whole semen contained a total of 0.515 m.eq. of reactive reducing titer per 100 ml. Of this total, 0.009 m.eq. per 100 ml. was accounted for as ascorbic acid. When iodoacetamide was added, the total value for the whole semen was reduced to 0.483 m.eq., which indicated that 0.002 m.eq. was due to reactive sulfhydryl groups. Similar analyses on the separated washed spermatozoa and seminal plasma indicated that the seminal plasma contained none of the titratable material considered as reactive sulfhydryl-containing compounds. The entire amount present in semen was found in the 4 times washed spermatozoa.

The data show that of the iodine titer about 50 per cent each was contributed by the spermatozoa and the seminal plasma. Also given in Table I are the data for the two major fractions of the seminal plasma, the seminal

proteins, and the seminal plasma dialysate. The seminal plasma proteins accounted roughly for 18 per cent of the total reducing titer and ascorbic acid for a similar amount. Thus, the reducing titer in the seminal plasma dialysate, except for the 0.033 m.eq. due to the ascorbic acid (which is lower because of oxidation during the 24 hour dialysis period), was due to a substance as yet unaccounted for. Since the titration with iodoacetamide indicated that sulfhydryl compounds such as glutathione were not present, further investigation was instigated to confirm the absence of glutathione and to attempt to identify the unknown reducing material.

TABLE I

Iodine-Reducing Components of Bovine Semen

The values are given in milliequivalents per 100 ml. of the original semen.

Fraction	Total	Ascorbic acid	Total less ascorbic acid
Whole semen	0.545	0.099*	0.446
" " + iodoacetamide†	0.483	0.099	0.384
Sperm cells	0.270	0.000	0.270
" " + iodoacetamide	0.204	0.000	0.204
Seminal fluid	0.280	0.096	0.184
" " + iodoacetamide	0.280	0.096	0.184
" proteins	0.103	0.000	0.103
" fluid dialysate	0.134	0.033	0.101‡

* Equal to 8.7 mg. per cent of ascorbic acid.

† Determined by adding 0.1 gm. of iodoacetamide to 1 ml. of semen or equivalent with 4 ml. of phosphate buffer, pH 6.9, μ of 0.1, and titrating after 2 hours by the o-iodosobenzoate-iodine procedure.

‡ Equal to 8.1 mg. per cent of sulfite (as $SO_3^{=}$).

A nitroprusside test conducted on the 10 times concentrated seminal plasma dialysate was negative, which again indicated that active sulfhydryl compounds were absent or at least not present in quantity.

Chromatographic analysis, the results for which are shown in Table II, indicated that neither the oxidized nor the reduced form of glutathione was present in the seminal plasma dialysate and that all of the obvious ninhydrin-reacting substances could be accounted for by known amino acids. This result certainly does not preclude the possibility that other ninhydrin color-producing substances might be present in small quantities.

Since the unknown reducing substance or substances could not be accounted for by glutathione or any other sulfhydryl compound, the possibility of some other oxidation form of sulfur being present was next considered. Analyses for thiosulfate and sulfite were conducted on the 10

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...e original semen.

Ascorbic acid	Total less ascorbic acid
0.099*	0.446
0.099	0.384
0.000	0.270
0.000	0.204
0.096	0.184
0.096	0.181
0	0.103
0.	0.101‡

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times concentrated seminal plasma dialysate and, while the test for thiosulfate was negative, that for sulfite proved positive.

The results of the quantitative analysis indicated that sulfite was present to the extent of around 0.10 to 0.12 m.eq. per 100 ml. of the original semen (calculated as $SO_3^{=}$).[1] Thus, it appears that sulfite accounts for all or the major portion of the unknown reducing power. Further confirmatory evidence was obtained to show that, when sodium sulfite was added to phosphate buffer or to semen, it did not interfere in the ascorbic acid determination when extrapolation was made to zero time. It did react slowly, as glutathione does, and the rate of reaction over a minute interval was similar to the rate found with the semen filtrates.

TABLE II

Free Amino Acids of Bovine Seminal Plasma

Amino acid	Concentration
	mg. per ml.
Alanine	0.25
Aspartic acid	0.09
Glutamic "	0.35
Glycine	0.09
Histidine	0.16
Phenylalanine	0.16
Serine	0.13
Arginine	Trace
Cystine	"
Proline	"
Tryptophan	"
Tyrosine	"
Threonine	"
Leucine	Possible trace
Lysine	" "
Methionine	" "
Valine	" "
Glutathione (GSH)	Absent
" (GSSG)	"
Other ninhydrin-reacting substances	Not present in quantity

DISCUSSION

It is probable that the total iodine-reducing components of bovine semen are the same as the non-fermentable reducing substances remaining after

[1] Not the same lot of semen as used previously. Titration of several pooled samples of semen indicated that the unknown reducing material was present in all at about the same concentration.

yeast or bacterial fermentative removal of the carbohydrate-reducing substances in semen (1–3) or semen diluters (4), as determined by the alkaline ferricyanide procedure.

The results are in general agreement with those reported by other investigators (6, 7) for the total iodine-reducing power and the ascorbic acid. It has been shown that, unless proper precautions are taken, the sulfite present in semen will interfere in the ascorbic acid analysis, giving a result too high. Reid *et al.* (6, 7) have cautioned against considering all the substances oxidized ultimately by 2,6-dichlorophenol indophenol dye as being ascorbic acid. However, while they believed the interfering substances to be sulfhydryl groups, the results of the present investigation show it primarily to be sulfite, no active sulfhydryl compounds being found in the seminal plasma.

As the reaction with iodoacetamide and the nitroprusside test of the dialyzed seminal plasma proteins was negative in both the presence and absence of 8 M guanidine hydrochloride, it would appear that neither reactive nor potentially reactive sulfhydryl groups were present. This is in sharp contrast to the condition found in many other proteins, such as β-lactoglobulin and egg albumin (14, 15), where reactive sulfhydryl groups are released upon denaturation by heat, guanidine, or other means. Whether this is due to a concentration effect is not known, since some proteins of very low sulfhydryl content do not demonstrate a clearly positive nitroprusside test. Electrophoretic and ultracentrifugal analyses of the bovine seminal proteins have indicated that a great number of protein entities are present, with three in preponderance.[5] It could be expected that one or more of these fractions might possess some sulfhydryl-reducing groups. The possibility should not be overlooked, however, that sulfite might be formed, released, or held by the seminal proteins. The data in Table I indicate that slightly more sulfite-reducing power (about 5 per cent) was present in the 24 hour-dialyzed seminal fluid than would be expected. Whether this is due to analytical error or indicates a progressive formation of sulfite from the seminal proteins is not known.

Also, the results would indicate that the ergothioneine found by Leone and Mann (8) in boar semen is either not present in bovine semen or at such low concentration as to be of no reducing importance in comparison with the other components.

It is not known whether there is any relationship between the observations of Lardy *et al.* (9, 10), who have shown that the metabolic regulator sulfur is released upon holding and alkaline hydrolysis of semen, and the sulfite found in the present investigation. Since sulfite is rather readily converted into elemental sulfur by certain reducing agents and the reverse

[5] Larson, B. L., and Salisbury, G. W., unpublished experiments.

reaction is also mediated by certain oxidizing agents and in addition by several metabolic procedures (11), it would appear that there is a strong possibility that some relationship might exist. Certainly both observations indicate an important rôle of inorganic sulfur in semen and possibly the presence of a high degree of sulfur metabolism.

It is further interesting to speculate on a possible physiological rôle of sulfite in semen. Since the pK of the equilibrium $HSO_3^- \rightleftharpoons H^+ + SO_3^-$ is about 7.2, at the pH of semen about 50 per cent of the material is in the form of bisulfite. The effect of bisulfite on aldehydes and ketones is well known (20), reversible addition complexes being formed with such compounds. Whether enough bisulfite is present to alter or mediate some metabolic process in which such compounds are intermediates is not known.

The importance of and the rôle of sulfite in semen are undergoing further investigation in this laboratory.

SUMMARY

The reducing power of the various components of bovine semen was evaluated by means of a specific iodine titration. The sperm cells and the seminal plasma each contribute about 50 per cent to the total titer. Active sulfhydryl compounds are present only in the sperm cells and constitute about 12 per cent of the total titer.

Of the seminal plasma components, the seminal proteins, ascorbic acid, and sulfite each contribute about 18 per cent to the total titer, the latter two materials being present at a concentration of about 8 to 10 mg. per 100 ml. of semen.

No evidence was found for the presence of glutathione in the seminal plasma. Although glutathione reacts with ninhydrin, chromatographic analysis indicated that all of the dialyzable ninhydrin color-producing substances could be accounted for by known amino acids.

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Biological Effects of SO_2 Exposures on Guinea Pigs

A Preliminary Report

S. D. LEE, PhD, AND R. M. DANNER, BS, CINCINNATI

ALTHOUGH considerable data concerning the toxicological and physiological effects of SO_2 exposure on humans and experimental animals are available in the literature,[1] little information concerning the biochemical effects of SO_2 exposure on experimental subjects is available. The current investigations were initiated to provide a basis for a more critical evaluation and interpretation of studies related to SO_2.

This preliminary communication concerns observations made in a series of experiments conducted to establish the minimum SO_2 exposure concentration producing consistent detectable alterations in the respiration and biochemistry of experimental animals. Hemoglobin and blood inorganic sulfur concentrations were determined to detect alterations occurring in the blood compartment, which serves as the vehicle for translocation of absorbed sulfur.

Methods

Guinea pigs weighing 552 gm to 1275 gm (the Table), about 1 to 1.5 years old, were exposed in a 10-liter, cylindrical, transparent plastic (Lucite), dynamic exposure chamber receiving a constant supply of the desired concentration of 1% SO_2 in N_2 diluted with ambient air. The sulfur dioxide atmosphere was monitored continuously with an automatic SO_2 analyzer calibrated by the method of West and Gaeke.[2] The sampling probe in the exposure chamber was located within an inch of the nose of the experimental animal. Tidal volumes, respiration rates, and minute volumes were measured every 15 minutes using a capacitance respirometer* in conjunction with a multichannel recording respiration pattern analyzer. Each guinea pig served as its own control while receiving charcoal-filtered clean air for 30 minutes during the pre-exposure period. Following this control period, the guinea pigs were exposed to specific concentrations of SO_2 for 60 minutes and then to clean air for a 60-minute recovery period. As an additional control experiment, six animals received only clean air for the entire experimental period.

Hemoglobin concentrations were determined immediately before and after the SO_2 exposure.[3]

Analyses for blood inorganic sulfur were conducted according to the modified Berglund and Sörbo method.[5,6] Control values for blood inorganic sulfur concentration were obtained at least ten days prior to the exposure experiment to allow the animals to regenerate the lost blood (5.0 ml) before the initiation of the actual exposure.

Blood regeneration time was calculated as follows:

$$\frac{C \times B}{A} = D$$

(A) Total blood volume (ml), approximately 5% of the body weight; (B) life-span of red blood cells (82 days); (C) blood lost (ml); (D) time required to replace lost blood.

The waiting period, according to the above calculation, for a 1,000 gm guinea pig was 8.2 days and a 500 gm animal was 17 days. Post-experimental concentrations were determined on blood samples obtained immediately following the recovery period.

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* In Fig 2, 4, and 5 each circle, large or small, represents one experiment. Larger circles were used where more than one point fell together.

Blood Inorganic Sulfur Concentrations Pre- and Post-Experiment

Date	Animal No.	Body Wt. Gm	SO_2 Exposure Concentration PPM	Blood Inorganic Sulfur Concentration Mg/100 Ml Before	After	% Change
2/3/65	34	737	0	2.9	2.7	− 7
2/4/65	31	691	0	4.0	3.3	−18
2/10/65	35	553	0	4.3	3.2	−26
2/11/65	14	995	0	3.7	3.3	−11
2/12/65	23	917	0	2.9	3.0	+ 3
2/17/65	26	1,033	0	3.3	2.5	−24
12/29/64	17	1,056	6.5	3.2	3.6	+13
12/29/64	18	912	6.8	4.0	3.3	−18
12/29/64	13	825	10.2	4.0	3.5	−13
12/28/64	14	815	10.9	4.0	3.3	−18
12/28/64	15	823	10.9	3.2	3.6	+13
12/22/64	3	1,175	11.5	4.3	4.2	− 2
12/22/64	4	1,025	11.5	3.9	4.9	+26
12/22/64	5	1,093	11.5	3.9	4.6	+18
12/16/64	36	852	13.3	3.4	3.3	− 3
12/15/64	30	557	17.3	4.0	3.4	−15
12/15/64	31	601	17.3	4.0	4.1	+ 3
12/15/64	32	622	17.3	4.7	4.1	−13
12/16/64	34	708	17.3	3.7	4.2	+14
12/17/64	37	743	19.0	3.1	4.0	+29
12/17/64	38	582	19.0	2.9	3.1	+ 7
12/16/64	35	804	19.3	3.8	4.3	+10
11/20/64	27	1,012	23.7	3.7	5.0	+35
11/30/64	28	1,012	23.7	3.4	3.9	+15
11/30/64	29	1,011	29.5	2.7	2.9	+ 7
11/30/64	24	1,033	29.5	2.0	3.0	+50
11/30/64	25	1,015	29.5	2.7	3.7	+37
11/30/64	26	1,013	29.5	2.8	3.0	+ 7
12/17/64	5	1,076	40.0	3.9	4.6	+18
12/17/64	4	1,057	41.0	3.8	4.9	+26
12/17/64	3	1,028	47.5	2.9	3.4	+17
1/2/65	3	1,275	72.0	3.5	3.5	0
11/10/65	2	656	75.0	2.6	4.1	+58
1/19/65	113	845	77.0	2.8	5.5	+96
1/12/65	111	1,057	100.0	2.7	4.2	+56
1/21/65	117	724	100.0	3.1	4.2	+35
1/27/65	120	886	102.0	3.1	3.8	+23
1/12/65	112	748	122.0	2.7	3.9	+44
1/26/65	119	725	123.8	3.1	3.7	+19
1/14/65	4	1,150	130.0	3.9	5.5	+41
1/25/65	118	748	132.6	2.7	4.3	+59
2/11/65	16	857	167.5	3.6	3.4	− 6
2/10/65	5	1,130	170.0	3.7	4.1	+11
2/8/65	30	737	170.0	3.5	4.0	+14
2/12/65	28	927	178.0	2.7	3.8	+41
2/18/65	25	1,035	270.0	3.5	4.0	+14
2/18/65	29	965	300.0	2.9	4.0	+38
2/11/65	24	1,032	310.0	2.0	3.8	+90

Results and Comment

Respiration Patterns.—The six control animals receiving clean air throughout the entire experimental period (150 minutes) maintained relatively constant tidal volumes, respiration rates, and minute volumes, as shown in Fig 1. Mean values for the control animals' tidal volumes, minute volumes, and respiration rates were 4.7 ml, 514 ml, and 109, respectively. Fluctuations were caused by various degrees of excitation and adaptation of the experimental animal. This type of variation was observed in many experiments.

Thirty-one of 36 guinea pigs receiving SO_2 concentrations ranging from 19 to 310 ppm showed a general increase in tidal volumes and decrease in respiratory rates with wide individual variations (Fig 2). Ten of 12 guinea pigs exposed to 6 to 17 ppm of SO_2 exhibited decreased tidal volumes, with poorly defined changes in respiration rates (Fig 2). Six of these animals showed minor changes (less than 5%); five others exhibited 11% to 38% decreases in

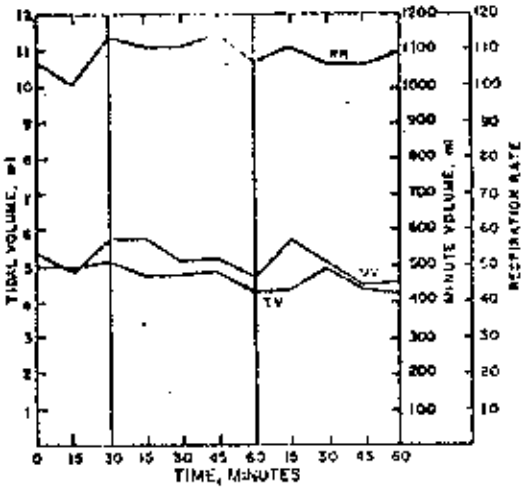

Fig 1.—The respiration patterns of control animals.

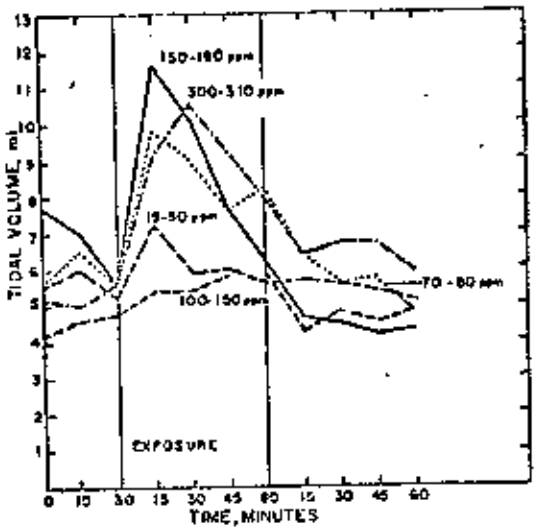

Fig 3.—Effect of SO_2 exposure on tidal volume of guinea pigs at various ranges of concentration.

tidal volume. One exception responded with a 44% increase in tidal volume. During the post-exposure period, the respiration patterns returned to control (pre-exposure) levels usually within 30 to 60 minutes if the animals had been exposed to SO_2 concentrations below 180 ppm. In the case of higher SO_2 concentrations (300 to 310 ppm), the recovery was not complete in 60 minutes (Fig 3). Maximum changes in tidal volumes occurred within the first 15 minutes of SO_2 exposure with the exception of the 300 to 310 ppm SO_2 exposures, in which tidal volumes continued to increase for about 30 minutes. The apparent partial recoveries occurring before termination of SO_2 exposure are difficult to interpret; however, they might be attributed to reflex adaptation (Fig 3).

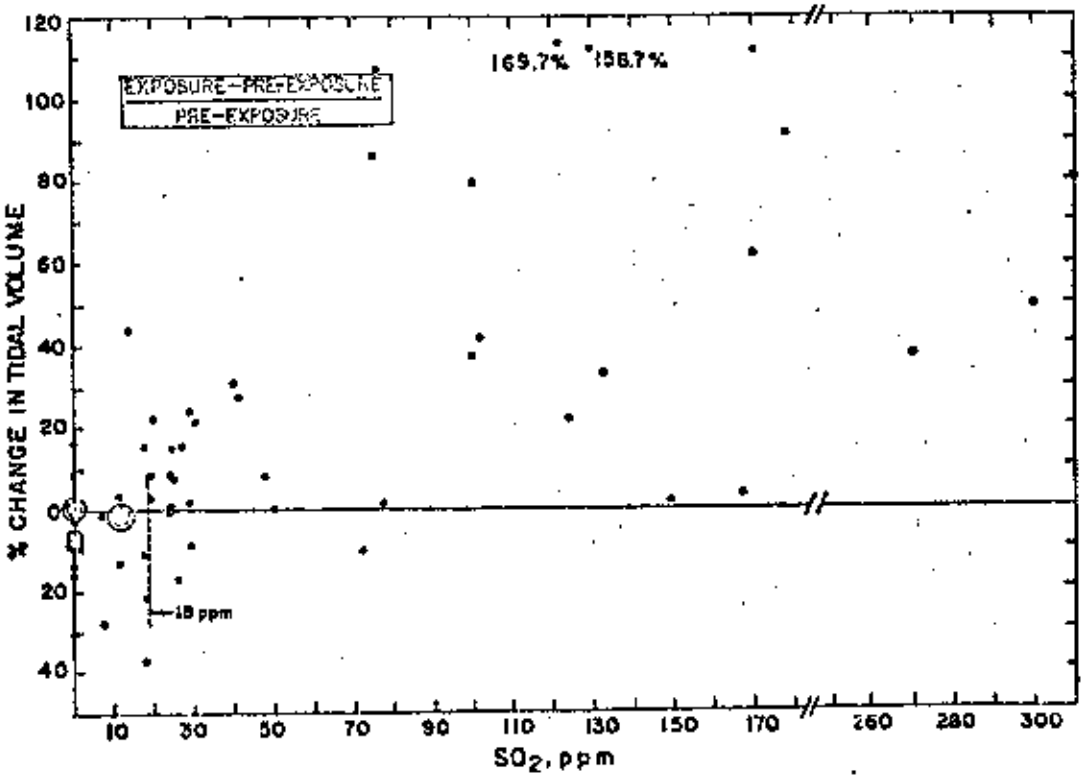

Fig 2.—Changes in tidal volume due to SO_2 exposure.

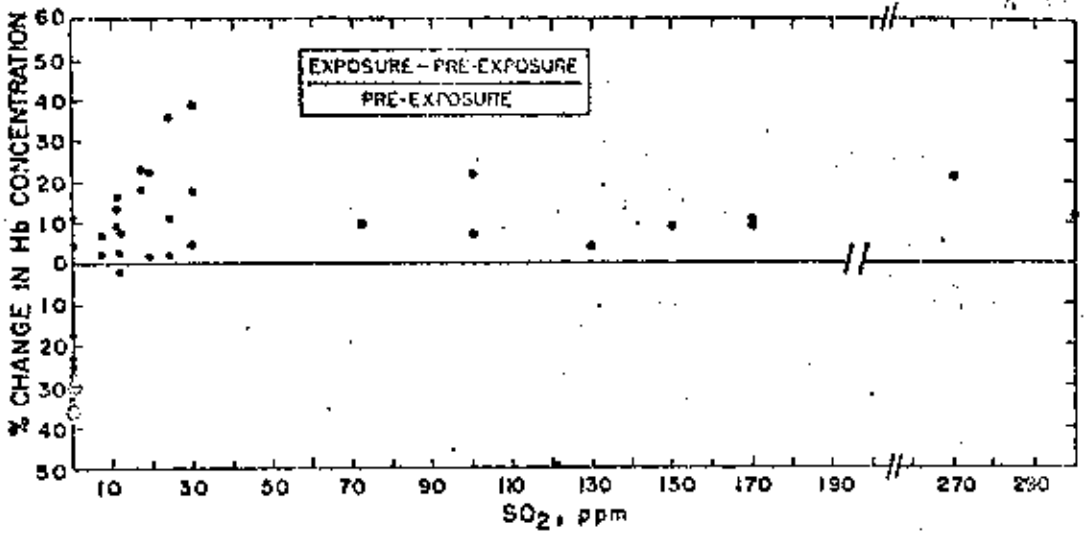

Fig 4.—The effect of SO_2 exposure on hemoglobin concentration.

These results are in general agreement with those reported for guinea pigs [8,9] and human subjects [10,11] receiving exposures of 15 to 30 ppm SO_2. Amdur and Mead [8] observed a reversal effect on tidal volume response of guinea pigs at approximately 25 ppm SO_2. Tidal volumes increased with decreased respiration rates at concentrations above 25 ppm SO_2 and decreased with increased respiration rates at concentrations below 25 ppm SO_2. During the experiments reported herein, this reversal effect occurred at approximately 18 ppm SO_2.

Hemoglobin.—Hemoglobin concentrations consistently increased during all exposures to SO_2 concentration ranging from 7 to 310 ppm with only one exception out of 30 experiments. The one exception exhibited a 2% decrease in hemoglobin concentration (Fig 4). The increase in hemoglobin concentration appeared linear with increasing concentrations of SO_2 between 6 and 20 ppm, but the linearity ceased at concentrations above 20 ppm SO_2. Of 11 controls, nine animals exhibited decreased blood hemoglobin concentrations. Two controls showed an

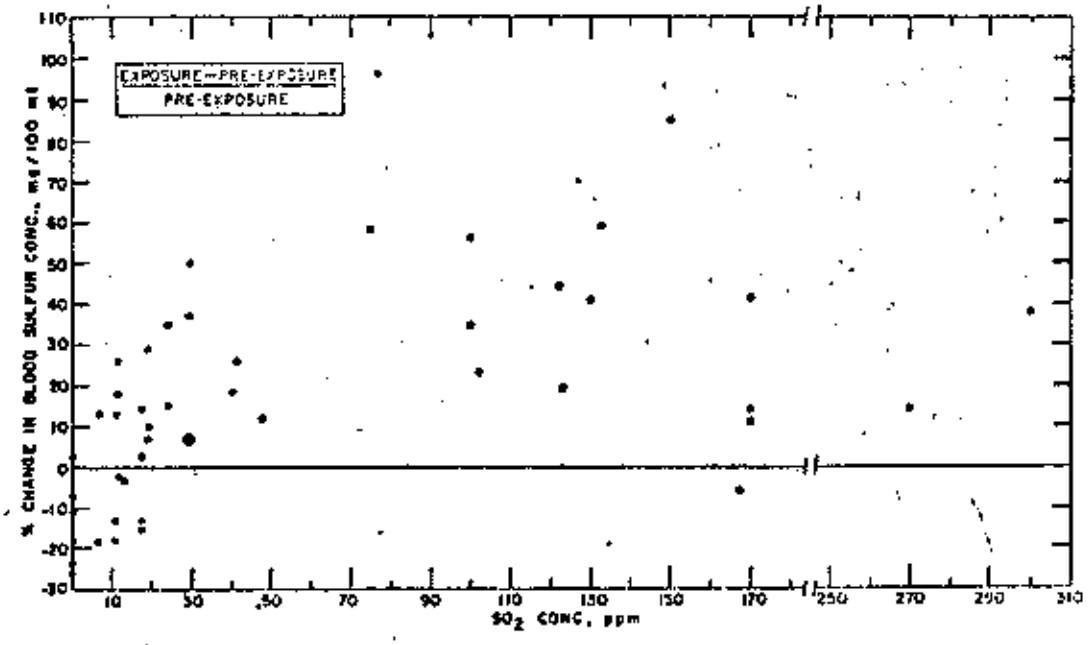

Fig 5.—The effect of SO_2 exposure on blood inorganic sulfur concentration.

increased hemoglobin concentration of 4% and 11%. Ball et al[12] observed increased hemoglobin concentrations in rats chronically exposed to 1 to 32 ppm SO_2.

Blood Inorganic Sulfur.—Blood inorganic sulfur concentrations consistently increased after exposures to SO_2 concentrations above 20 ppm. Twenty-two of 24 animals showed an increase (3% to 96%) in blood inorganic sulfur concentration after one hour of exposure to SO_2 levels ranging from 19 to 310 ppm, as shown in the Table. Blood inorganic sulfur concentrations varied widely in animals exposed to SO_2 concentrations ranging from 7 to 19 ppm. Interestingly, six control animals exposed to clean air during the entire experimental period exhibited a general decrease (+3% to −26%) in blood inorganic sulfur. This would indicate that the observed increases in the blood sulfur levels are true effects of SO_2 exposure.

Preliminary statistical analysis and inspection of the data showed a positive correlation between SO_2 exposure concentrations below 100 ppm and corresponding blood inorganic sulfur concentrations. For a given SO_2 exposure, animals with lower baseline blood sulfur concentrations showed greater percentage increases than animals with higher baseline concentrations. These observations will be subjected to more rigorous examination when more data become available.

Further investigations are being conducted to elucidate the mechanism responsible for the changes observed during these preliminary experiments.

Summary

Experiments were conducted to establish the minimum SO_2 exposure concentration that would consistently affect the respiration and biochemistry of guinea pigs.

SO_2 exposures at concentrations above 19 ppm for one hour caused an increase in tidal volume and a decrease in respiration rate, with irregular effects on minute volume. SO_2 exposures at concentrations ranging from 7 to 17 ppm caused a general decrease in tidal volume and an increase in respiration rate.

Hemoglobin concentrations increased after SO_2 exposure with all concentrations (7 to 310 ppm) tested. Control animals showed decreased hemoglobin concentrations following a comparable test period without SO_2 exposure.

Blood inorganic sulfur concentrations increased after exposures to SO_2 concentrations above 19 ppm and exhibited irregular responses below 17 ppm of SO_2. Control animals showed decreased blood inorganic sulfur concentrations.

Mr. K. Busch did the statistical analyses of the data and Mrs. Edith Joiner gave technical assistance.

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The Determination of Free Sulphur Dioxide in Soft Drinks by a Desorption and Trapping Method

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Experiments have shown that, under specified conditions, the desorption of free sulphur dioxide in soft drinks is much faster than the dissociation of combined sulphur dioxide. This principle has been used in developing a method for determining free sulphur dioxide as an alternative to methods involving direct titration of the sample with iodine. The proposed method does not suffer from drifting end-points or high blank values, which are often encountered in direct titration methods. Free sulphur dioxide is desorbed at room temperature and at pH 1 to 2 by a rapid stream of oxygen-free nitrogen. For 50 p.p.m. or more of free sulphur dioxide, it is trapped in in alkaline glycerol and the determination completed iodimetrically. Lower concentrations are trapped in sodium tetrachloromercurate reagent, and the determination is completed colorimetrically. With slight modification the method has been applied to samples having high and low concentrations of sulphur dioxide, samples having high and low contents of solids and to coloured and colourless samples. When the proposed method was applied to various soft drinks the results agreed satisfactorily with those obtained by a direct titration method.

THE classical method for determining free sulphur dioxide in fruit juices and soft drinks consists of direct titration of the sample with iodine, and then titration of a second portion with iodine in the presence of a carbonyl compound, the difference in titres being due to free sulphur dioxide. The chief disadvantages of this type of method are drifting end-points in one or both titrations, a relatively high second or blank titre compared with the titre difference (particularly if the ascorbic acid content of the sample is high) and difficulty in assessing the end-point with coloured samples. To overcome two of these disadvantages, Vas[1] showed that, at pH values of less than 2, the greater stability of glucose bisulphite leads to sharper end-points, and these can be further improved by an electrometric method described by Ingram,[2] which also permits determination in coloured samples.

Nevertheless, results from this type of method, which depends on the difference between the two iodine titrations, both of which are carried out in the presence of a large amount of reducing or potentially reducing organic material, are bound to depend to some extent on the personal judgment of the analyst. This could be avoided if it were possible to remove free sulphur dioxide from the sample in a similar way to the removal of total sulphur dioxide in the Monier-Williams method.[3]

Previous experience in desorbing dissolved oxygen from soft drinks had indicated that the desorption of sulphur dioxide from small volumes, though not as fast as that of oxygen, was still fairly rapid. Further, examination of the dissociation constants of glucose bisulphite showed that, at pH 1 to 2 and under conditions likely to be found in soft drinks, the extent of dissociation in 30 minutes would be extremely small; if this was also true for other sulphur dioxide complexes found in drinks, then it should be possible to complete the desorption of free sulphur dioxide before any effective dissociation of combined sulphur dioxide occurred.

EXPERIMENTAL

PRELIMINARY EXPERIMENTS—

The first experiment was carried out in a pear-shaped polarographic cell described by Lloyd and Parkinson,[4] which can be used to follow the rate of desorption of certain dissolved gases. It was found that approximately 40 p.p.m. of free sulphur dioxide could be desorbed at room temperature from a dextrose solution in acetate buffer, acidified to a pH value of 1 to 2, by a rapid stream of oxygen-free nitrogen at room temperature in about 5 minutes. Subsequent dissociation of combined sulphur dioxide was found to be negligible, and blank titres were extremely small.

These results were so promising, even when the near-ideal desorption conditions in the pear-shaped cell were taken into account, that an apparatus of the type shown in Fig. 1 was made. With this apparatus, a similar experiment to that in the pear-shaped polarographic cell was carried out, but the desorbed sulphur dioxide was trapped and determined. The choice of trapping reagent and subsequent method of determination was made at this stage. The use of neutral hydrogen peroxide in the traps and then titration with standard sodium hydroxide solution did not give sufficiently sharp end-points, even when screened methyl orange was used as indicator. By trapping in alkaline glycerol, however, the subsequent determination could satisfactorily be made by Aulenback and Bahnar's[5] polarographic method or alternatively by acidification and iodimetry. A colorimetric method, in which were used the reagents described by Beetch and Oetzel,[6] was found to be particularly suitable for small amounts of trapped sulphur dioxide. In our experiments, to avoid the use of a comparatively expensive polarograph, the iodimetric method was adopted for high concentrations and the colorimetric method for low concentrations of free sulphur dioxide.

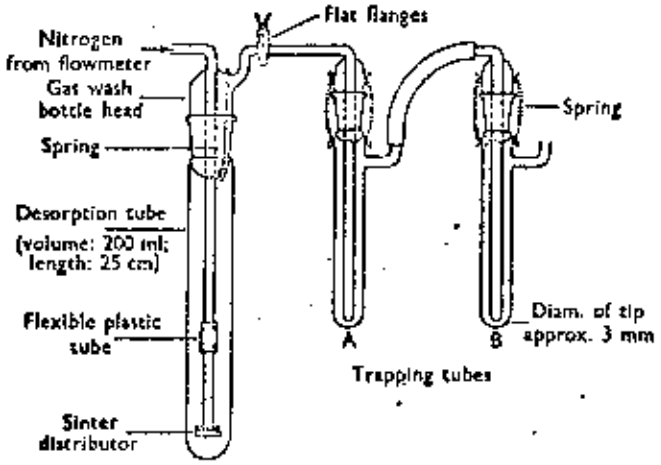

Fig. 1. Apparatus for determining free sulphur dioxide by a desorption and trapping method

Further preliminary experiments were carried out to study the application of the method to various sugar solutions and a sucrose - ascorbic acid solution, all of which had been sulphited and stored at pH 3 until equilibrium between free and combined sulphur dioxide was attained. Free sulphur dioxide was simultaneously determined by direct iodimetry of an acidified sample, formaldehyde being used as the binding agent in the blank determination as described by Ponting and Johnson[7]; for the sucrose - ascorbic acid mixture, this method gave poor end-points and Downer's method[8] was used. The results are shown in Table I.

TABLE I

COMPARISON OF RESULTS BY THE DESORPTION AND TRAPPING METHOD AND DIRECT IODIMETRY

Sugar solution	Total sulphur dioxide content, p.p.m.	Desorption and trapping method: Free sulphur dioxide found, p.p.m.	Desorption and trapping method: Desorption flow rate, ml per minute	Desorption and trapping method: Time required for desorption, minutes	Free sulphur dioxide found by direct iodimetry, p.p.m.
Dextrose, 10 per cent. w/v in acetate buffer	240	128	250	30	132
Liquid glucose, 10 per cent. w/v in acetate buffer	245	101	300	20	180
Fructose, 10 per cent. w/v in acetate buffer	258	254	300	20	245
Sucrose, 10 per cent. w/v in acetate buffer	251	258	300	20	245
Sucrose, 10 per cent. w/v in acetate buffer containing 100 mg of ascorbic acid per 100 ml ..	213	213	1500	15	224

During these experiments it was observed that a small amount of sulphur dioxide dissolved in droplets of moisture on the side of the desorption tube, but losses were avoided by washing back once during the desorption period. The size of desorption tube and the desorption flow-rate were also considerably increased at this stage. Recoveries of sulphur dioxide from standard solutions of sodium metabisulphite prepared in aqueous glycerol (to prevent oxidation) were carried out by the refined method. Results are shown in Tables II, III and IV.

TABLE II

PROPORTION OF TRAPPED SULPHUR DIOXIDE IN FIRST TRAPPING TUBE

Total sulphur dioxide trapped, mg ..	3·53	4·42	5·25	6·09	7·58	8·31
Percentage in first tube	100	100	100	99	99	99

TABLE III

RECOVERY OF SULPHUR DIOXIDE FROM STANDARD SOLUTIONS (IODIMETRIC METHOD)

Sulphur dioxide added, mg ..	0·47	0·94	1·40	1·88	2·81	3·75	4·69	5·63	7·50	8·44	9·38
Sulphur dioxide recovered, mg	0·49	0·93	1·38	1·88	2·77	3·73	4·72	5·61	7·41	8·47	9·38

Average recovery, 100 per cent. Range, 99 to 104 per cent.

TABLE IV

RECOVERY OF SULPHUR DIOXIDE FROM STANDARD SOLUTIONS (COLORIMETRIC METHOD)

Sulphur dioxide added, mg ..	0·31	0·62	0·94	1·25	1·56	1·87	2·18
Sulphur dioxide recovered, mg	0·32	0·61	1·02	1·20	1·56	1·86	2·13

Average recovery, 100 per cent. Range, 96 to 108 per cent.

In the colorimetric method only one tenth of the volume of liquid in the traps is normally taken for measurement, but for extremely low concentrations of sulphur dioxide a larger volume can be taken to make the method more sensitive.

METHOD

PREPARATION OF APPARATUS—

Set up the apparatus as shown in Fig. 1. More than one assembly may be used by one operator. The flow-rate required is approximately 1½ litres per minute of oxygen-free nitrogen for each assembly. This is conveniently controlled by use of a gas regulator with built-in flowmeter (British Oxygen Gases Ltd., Medical Division, Oxygen Regulator No. 330018).

Lightly grease the interchangeable joints of the apparatus. Into each of the two trapping tubes place 10 ml of 10 per cent. v/v glycerol in 0·1 N potassium hydroxide (alkaline glycerol reagent) if the iodimetric method is to be used or 10 ml of sodium tetrachloromercurate reagent[6] if the colorimetric method is to be used. The iodimetric method is preferred for 1 to 10 mg and the colorimetric method for smaller amounts up to 2 mg of trapped sulphur dioxide. Place 25 ml of water or 10 per cent. v/v glycerol in water (as specified for the type of sample to be examined) in the desorption tube. Pass a stream of air-free nitrogen through the assembly at 1½ litres per minute for approximately 5 minutes, and check that the joints are gas-tight.

PREPARATION OF SAMPLE—

Samples containing not more than 50 per cent. of solids or 400 p.p.m. of sulphur dioxide—Use distilled water in the desorption tube when preparing the apparatus. After passing nitrogen for 2 to 3 minutes, fairly quickly carry out the sequence of operations described below. Weigh 25 ± 0·02 g of sample into a second desorption tube. Add a few drops of water-soluble thymol blue and sufficient 5 N sulphuric acid to change the colour of the indicator from orange-yellow to red, and add 1 drop in excess. (If the colour of the sample interferes, determine the amount of 5 N sulphuric acid required on a separate equivalent portion with thymol blue as an external indicator or by using a pH meter, and then add the same amount of acid to the portion under test.) Turn off the flow of nitrogen. Replace the desorption tube containing water with the one containing the acidified sample, and ensure that the joint is gas-tight. If the samples show a marked tendency to froth, place a smear of Midland Silicones Ltd. anti-foam paste A on the side of the desorption tube.

Samples containing more than 50 per cent. of solids or 400 p.p.m. of sulphur dioxide—Use 10 per cent. glycerol in the desorption tube when preparing the apparatus and, after passing nitrogen for approximately 5 minutes, fairly quickly carry out the sequence of operations described below. Remove the desorption tube, but do not empty it. Weigh by difference a suitable portion of sample, and add it to the desorbed 10 per cent. glycerol in the desorption tube, ensuring that none of the sample adheres to the side of the tube. (For samples containing up to 400 p.p.m. of free sulphur dioxide, 25 g is a suitable amount of sample to use; decrease the amount of sample taken as the expected content of free sulphur dioxide increases.) Stir to dissolve, and acidify in exactly the same way as described in the previous paragraph. Replace the desorption tube containing the dilute acidified sample, and ensure that the joint is gas tight.

DESORPTION OF FREE SULPHUR DIOXIDE—

Turn on the supply of nitrogen, adjust the rate of flow to approximately 1½ litres per minute, and desorb at room temperature for 10 minutes. Turn off the nitrogen, disconnect the assembly between the desorption tube and the first trapping tube, and wash down the outlet tube of the wash bottle head and the walls of the desorption tube with a jet of distilled water. Reconnect the apparatus, and desorb for a further 5 minutes. Turn off the nitrogen flow, and combine the contents of the trapping tubes, washing out the tubes with distilled water. Recharge the trapping tubes, desorb for a further 5 minutes, and combine the contents separately. (For most samples it will be possible to omit the second desorption period if the conditions during the first period are rigidly observed; the full procedure, however, must be carried out for the first few determinations in each product to ascertain if this is possible.)

MEASUREMENT OF SULPHUR DIOXIDE IN THE TRAPPED LIQUIDS—

Iodimetric method—To the combined alkaline glycerol solutions in a 150-ml conical flask add a few drops of thymol blue and sufficient 5 N sulphuric acid just to give a red colour. Titrate with 0·02 N iodine to the first permanent blue colour, with starch or starch substitute as indicator. Simultaneously carry out a reagent blank determination by acidifying and titrating 20 ml of alkaline glycerol in the same way.

$$\text{Free sulphur dioxide content, p.p.m. w/w} = \frac{32{,}000\,(t - b)\,N}{w}$$

where t and b are the titres (in millilitres) for the sample and blank solutions, respectively, N is the exact normality of the sodium hydroxide solution and w is the weight of sample taken (in grams).

Colorimetric method—Combine the trapped sodium tetrachloromercurate solutions in a 50-ml calibrated flask, wash in quantitatively, dilute to the mark with distilled water, and mix. Complete the determination on a suitable portion (usually 5 ml) by using the colour developing procedure described by Beetch and Oetzel.[6]

RESULTS

The method was applied to some soft drinks and a concentrate. Results compared with those obtained by Downer's method of direct iodimetry (with electrometric titration when the colour of the sample interfered) are shown in Table V.

It is considered that there is satisfactory agreement between the results for free sulphur dioxide obtained by the two different methods. Difficulty was found in obtaining reliable results by direct iodimetry for the glucose beverage because of its low sulphur dioxide content and for the compounded blackcurrant juice because of its colour and complex nature. This probably accounts for the apparent disparity between the results for these samples.

In all the desorption and trapping determinations complete desorption occurred generally within 15 minutes and always within 20 minutes. No subsequent dissociation of free sulphur dioxide of any significance was observed. The method has since been applied to carbonated lemon drinks containing free sulphur dioxide in the range 3 to 40 p.p.m. and has given precise results after 15 minutes desorption; further desorption did not yield any more free sulphur dioxide.

TABLE V

COMPARISON OF RESULTS BY DIFFERENT METHODS

Sample	Total sulphur dioxide, p.p.m.	Free sulphur dioxide found by— desorption and trapping method, p.p.m.	Free sulphur dioxide found by— direct iodimetry, p.p.m.
Lemon squash	300	160, 161 (I)	167, 166
Orange squash	269	156, 156 (I)	152, 154
Lemon barley A	345	232, 240 (I)	232, 236
Lemon barley B	237	83, 83 (I)	62, 62
Lemon barley C	225	70, 73 (I)	66, 62
Blackcurrant cordial	311	178, 180 (I)	179, 177
Lemon juice	317	132, 135 (I)	132, 122
Liquid glucose beverage	30	11, 11 (C)	5
Lime juice cordial A	203	193, 191 (I)	196, 197
Lime juice cordial B	237	116, 116, 112 (I)	116, 114
Lime juice cordial B with added dehydroascorbic acid	225	48, 49 (I)	47, 43
Syrup of blackcurrant	301	99, 112 (I)	97, 120
Concentrated blackcurrant juice compound	1000 to 1200	100 (I)	203

I = Iodimetric method. C = Colorimetric method.

CONCLUSIONS

The work described has shown that, for a variety of soft drinks and raw materials, desorption and trapping provides a valid method of determining free sulphur dioxide.

The desorption and trapping method takes rather longer than do the iodimetric methods, but, by using two or more assemblies, one operator can complete at least four determinations in an hour. The apparatus required is relatively simple.

Advantages of the proposed method over direct iodimetric methods are: low or negligible blank values; elimination of drifting end-points and hence factors of personal judgement; application to samples of high and low solids content, high and low free sulphur dioxide content and to coloured and colourless samples.

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A STUDY OF THE TOXICITY OF SULPHITE. I.

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The effect of sodium metabisulphite, 750 p.p.m. as SO_2, in the drinking water, has been studied through three generations of rats in experiments lasting for nearly 3 years. The metabisulphite was without effect on growth, the intake of food and fluid and the output of faeces. It did not influence fertility, the weight of the newborn or lactation; neither did it increase the frequency with which tumours developed.

It is well known that sulphite reacts with aneurine[1] and that the sulphiting of foodstuffs reduces their aneurine activity[2,3]. This alone, however, cannot fully explain the effects observed by Fitzhugh, Knudsen and Nelson[5] in the course of the only published long-term investigation of the toxicity of a sulphited diet. These workers fed a partially synthetic diet to young rats for periods of 12 weeks or 1 year and observed the effect of mixing sodium bisulphite with the food on the growth and health of the animals. The addition of 0·1 per cent sodium bisulphite, 615 p.p.m. as SO_2, produced multiple histopathological changes, only some of which were reduced in incidence or severity by the bi-weekly injection of 100 µg. of aneurine per rat. Deficiency of aneurine was therefore not the full explanation of the findings. The present work is a first step in the search for additional causes of the long-term toxicity of sulphite. We have examined the toxicity of sodium metabisulphite itself, and have administered it in the drinking water to prevent prior interaction between the metabisulphite and the constituents of the diet. The experiments have extended over three generations of rats.

Methods

Eleven male and 39 female newly weaned rats, not differing in age by more than 5 days, and of a uniform strain bred for cancer research by the Chester Beatty Research Institute, London, received individual earmarks for identification and were divided at random into three groups. Each group contained 13 females: there were in addition 5 males in group I and 6 males in group II. The animals of different groups and the sexes were segregated. All were kept in standard cages of Boot's type, not more than 7 in a cage, in the same room under similar conditions of lighting and humidity, at a temperature maintained within the range of 21·5° to 24·5°.

Solid diet. All rats received cube diet 41 b of Stein in wire baskets. A typical sample of this diet was assayed for us by Vitamins Ltd. and was found to contain 3·1 µg. of aneurine per g.

Fluids. All rats drank freely from graduated bottles. Those of group I were provided with tap water, those of group II received tap water containing sodium metabisulphite equivalent to 750 p.p.m. SO_2, and those of group III tap water containing metabisulphite equivalent to 375 p.p.m. SO_2. Calculated weights of sodium metabisulphite assayed

for SO_2 equivalent were added to tap water daily to yield solutions equivalent to 750 p.p.m. SO_2. The solutions containing 375 p.p.m. SO_2 were initially prepared every other day. Only later was it found that the SO_2 content of these had dropped by nearly 20 per cent in 48 hours, so that the female rats of group III had received only 90 per cent of the SO_2 intended in the period before their first mating. Thereafter all solutions of metabisulphite were prepared daily, and were found to deteriorate in SO_2 content by approximately 10 per cent in the 24 hours before a fresh solution was provided.

Estimation of growth rate. Each rat was weighed twice weekly. Curves relating body weight in g. to age in days were found to be linear from the time of weaning to the 70th to 80th day of life for both males and females. Rates of growth were therefore expressed as the slopes of these lines. Standard statistical methods, based on the normal curve of distribution were used to compare the rates of growth of rats of the same sex which had received different drinking fluids since the males and females were found to constitute two different populations in respect of growth, each homogeneous within itself.

Estimation of food intake, fluid intake and faecal output. The daily food consumption of the rats in each cage was measured as the loss in weight of the food basket in 24 hours and was expressed in g. 100 g. weight of rat per day. The bottles containing the drinking fluid were graduated at 5 ml. intervals. Fluid consumption was therefore measured daily to the nearest 5 ml. and was expressed as ml. fluid drunk per 100 g. weight of rat per day.

The faeces produced in a cage were sifted from the sawdust, twice weekly, through a No. 6 sieve. The weight of faeces produced was expressed as a percentage of the weight of food eaten in the corresponding period.

Mean values for the consumption of food and fluids and for the output of faeces, and the standard errors of these means were obtained for the rats of each sex in each group, and the corresponding mean values for control and sulphite treated rats were compared by means of t tests.

Tests of ability to reproduce. The 50 rats with which the experiment began were designated Generation I. Male and female rats from group II, Generation I, which had been treated with sulphite (750 p.p.m. SO_2) for 11 weeks were paired and each pair was left to run in a separate cage for a week. Males and females from control group I, Generation I, were similarly paired and mated. If no litter resulted from the first mating period, a second period of 2 weeks was allowed. The litters resulting from these matings of Generation I constituted Generation II (i). Newborn rats were weighed individually and the six heaviest were retained; this was to reduce the burden of suckling for the mother. Weaning was effected 20 to 25 days after birth, when the newly weaned were segregated according to sex. Six to 8 weeks after the birth of Generation II (i), the same pairs of rats from Generation I were again mated, allowing not more than two periods each of 2 weeks duration: the resulting litters constituted Generation II (ii).

The members of Generation II (i) were paired and mated within their dose groups when they had attained their plateau weights. Not more than two periods of 2 weeks were allowed for this purpose. The litters so produced constituted Generation III. They were weaned 21 days after birth. All the young were retained for suckling tests in Generation II (ii) and Generation III.

Postmortem and histological examinations. Weighed rats were killed with ether. The heart, lungs, liver, spleen, adrenals, testes or ovaries, seminal vesicles or uterus, and the kidneys, were removed and weighed. Parts of these, the stomach, ileum, colon, a gastrocnemius muscle and a sciatic nerve were fixed in formol saline for not less than three days before they were blocked in paraffin. By routine, all sections were stained in Ehrlich's haematoxylin and eosin except for those of sciatic nerve for which a modified Weigert-Pal technique was used with 1 per cent neutral red as the counter stain.

Results

Sodium metabisulphite has been added to the drinking water of rats throughout an experiment lasting for 2½ years and extending over three generations of animals. The concentrations of metabisulphite used corresponded to 350 and 750 p.p.m. SO_2. Observation has been made of the effect of these concentrations of metabisulphite on growth, food consumption, fluid intake, faecal output, reproduction, lactation and the incidence of tumours. Water drinking rats of Generation II were derived from experimental matings between water drinking rats of Generation I. Sulphite treated rats of Generation II were the product of experimental matings between rats of Generation I which had received sulphite (750 p.p.m. as SO_2) in their drinking water from the start of the experiment, throughout mating, pregnancy and lactation, and until they were subjected to histological examination. Rats of generation III were similarly derived from Generation II.

The Effect of Metabisulphite in the Drinking Water on Growth

Curves relating body weight in g. to postnatal age in days were linear for this strain of rats from shortly after weaning until the 8th to 12th week of life; then, rather abruptly, as the animals matured sexually, the curves flattened to a plateau on which growth advanced only slowly. It was therefore possible to measure the growth rate of each animal as the slope of the steep part of this curve, and to compare the growth rates of water drinking and sulphite-treated animals in each generation by mean slopes (Table I, column 4). Males and females have been entered separately since the males grew the faster. There was no significant difference between the growth rates of the water drinking rats and those which received sulphite in their drinking water in any generation, but the growth rate of the stock as a whole accelerated from one generation to the next. This was more marked amongst the females, and was accompanied by shortening of the steep linear part of the curve relating body weight to time. Thus, whereas female rats of Generation I grew rapidly for the

TABLE I

THE EFFECT OF SULPHITE IN THE DRINKING WATER ON THE GROWTH, THE INTAKE OF FOOD AND FLUIDS AND THE FAECES OF RATS

Generation	Sex	Treatment	Slope of growth curve Mean ± SE	Values per 100 g rat per day (Mean ± SE)		Faeces weight as per cent of food intake Mean ± SE
				Food intake g	Fluid intake ml	
I	Male	Water	5·89 ± 0·35 (5)*	7·2 ± 0·5 (22)†	7·3 ± 0·5 (22)†	22·7 ± 0·46 (22)†
		SO_2 750 p.p.m.	5·01 ± 0·39 (6)	6·7 ± 0·4 (22)	9·8 ± 0·6 (22)	23·2 ± 0·42 (22)
	Female	Water	2·45 ± 0·13 (12)	6·9 ± 0·3 (22)	10·4 ± 0·4 (22)	21·4 ± 0·42 (22)
		SO_2 375 p.p.m.	2·55 ± 0·10 (13)	7·1 ± 0·3 (22)	11·3 ± 0·5 (22)	21·3 ± 0·33 (22)
		SO_2 750 p.p.m.	2·21 ± 0·12 (13)	7·1 ± 0·3 (22)	12·0 ± 0·5 (22)	22·0 ± 0·36 (22)
II (i)	Male	Water	6·19 ± 0·12 (12)	10·1 ± 0·8 (22)	13·1 ± 0·9 (22)	19·9 ± 0·63 (20)
		SO_2 750 p.p.m.	5·19 ± 0·24 (11)	9·7 ± 0·6 (22)	13·4 ± 1·1 (22)	20·9 ± 0·67 (21)
	Female	Water	3·55 ± 0·27 (6)	11·6 ± 1·4 (17)	16·3 ± 2·3 (17)	20·5 ± 0·46 (17)
		SO_2 750 p.p.m.	3·23 ± 0·38 (6)	8·9 ± 0·6 (21)	15·0 ± 0·5 (21)	19·3 ± 0·73 (17)
II (ii)	Male	SO_2 750 p.p.m.	6·41 ± 0·57 (7)	8·6 ± 0·7 (18)	15·4 ± 1·0 (18)	24·2 ± 0·70 (18)
	Female	SO_2 750 p.p.m.	4·12 ± 0·14 (13)	9·4 ± 0·5 (15)	15·6 ± 0·9 (15)	21·2 ± 0·66 (15)
III	Male	Water	6·94 ± 0·51 (10)	13·6 ± 1·3 (9)	17·7 ± 1·2 (9)	19·9 ± 0·33 (9)
		SO_2 750 p.p.m.	6·81 ± 0·36 (5)	13·5 ± 1·3 (9)	19·8 ± 1·6 (9)	20·5 ± 0·89 (9)
	Female	Water	5·34 ± 0·15 (9)	13·9 ± 1·3 (6)	19·3 ± 1·6 (6)	21·1 ± 0·20 (6)
		SO_2 750 p.p.m.	5·16 ± 0·12 (8)	14·7 ± 1·3 (7)	23·3 ± 2·0 (7)	23·6 ± 0·92 (6)

* Number of animals.
† Number of observations.

first 80 days of life, those of the third generation reached the end of this phase in 60 days. The plateau weights of the stock (Table II) did not alter significantly. There was no significant difference between the adult weights of water drinking and sulphite drinking male rats in any generation. By contrast, the female sulphite drinking rats of Generations II (ii) and III grew at a normal rate (Table I) but matured more rapidly than the water drinking controls, so that the plateau weights of the sulphite drinking females were significantly lower than those of the water drinking females in the third generation. (t calc. = 2·5, where n = 15; P = < 0·05).

TABLE II

COMPARISON OF THE WEIGHTS OF FULL GROWN RATS OF THE FIRST, SECOND AND THIRD GENERATION TREATED AND UNTREATED WITH METABISULPHITE IN THE DRINKING WATER

Generation	Treatment	Mean body weight in g. ± S.E.	
		Male	Female
I	Water	530·0 ± 26·2 (5)*	301·7 ± 9·4 (12)*
	SO_2 375 p.p.m.		313·9 ± 8·7 (13)
	SO_2 750 p.p.m.	471·7 ± 27·1 (6)	295·4 ± 9·2 (13)
II (i)	Water	498·2 ± 23·4 (11)	320·0 ± 7·6 (10)
	SO_2 750 p.p.m.	430·5 ± 24·0 (11)	310·0 ± 10·5 (9)
(ii)	Water		353·3 ± 9·3 (5)
	SO_2 750 p.p.m.	494·3 ± 21·3 (7)	295·8 ± 7·5 (13)
III	Water	442·5 ± 10·7 (10)	287·3 ± 5·7 (9)
	SO_2 750 p.p.m.	428·0 ± 17·3 (5)	269·4 ± 5·5 (8)

* Number of animals.

The Effect of Metabisulphite in the Drinking Water on the Consumption of Food and Fluid

The spontaneous intake of food was unaffected by the addition of metabisulphite (to 750 p.p.m. as SO_2) to the drinking water (Table I, column 5) throughout three generations.

The female rats of the first generation which received metabisulphite (750 p.p.m. as SO_2) in their drinking water had a significantly higher fluid intake than did those rats of the same generation which drank only water (t calc. = 2·3, n = 42, P = < 0·05), but this difference was not maintained amongst the females of later generations and was absent throughout amongst the males (Table I, column 6).

There was a progressive increase in the consumption of food and of fluid from generation to generation in the stock as a whole, so that rats of the third generation ate and drank twice as much during rapid growth as did those of the first generation. This difference can be correlated with the steady increase in the growth rate of the stock (see above).

The Effect of Metabisulphite in the Drinking Water on the Output of Faeces

The net weights of faeces excreted by each group of rats expressed as a percentage of the weight of food eaten during the period of faecal collection, followed by the standard errors of the means, are shown in Table I, column 7. There was no difference for the values for normal male and female rats, or between the generations. The only difference appearing between comparable groups of water drinking and sulphite-treated animals

was amongst the females of Generation III, where mean percentage found for the sulphite-drinking female significantly exceeded that for the group of water drinking controls (t calc. = 2·2, n = 11, P = < 0·05). This difference may have reflected a greater water content in the stools or a lesser degree of digestion or absorption of foodstuffs by the groups receiving sulphite (750 p.p.m. as SO_2) in the drinking water: in either case greater than normal speed of passage through the intestines is indicated.

The Effects of Metabisulphite in the Drinking Water on Fertility

The fertility of sulphite-treated animals was compared with that of control animals by comparing the number of periods paired animals had to be run together to produce pregnancies and the number of young per

TABLE III

THE EFFECT OF METABISULPHITE (750 P.P.M. AS SO_2) IN THE DRINKING WATER ON THE REPRODUCTION OF RATS

	Sulphite-treated rats	Control rats
Generation I		
First reproduction test		
Number of matings required/litter	1·2 ± 0·20 (5)	1·5 ± 0·07 (6)
Number of young/litter	12·8 ± 2·04 (5)	10·7 ± 1·66 (6)
Number surviving lactation/litter (six only returned to each mother)	3·6 ± 1·17 (5)	4·5 ± 1·12 (6)
Second reproduction test		
Number of matings required/litter	1·0 ± 0·00 (3)	1·0 ± 0·00 (2)
Number of young/litter	9·0 ± 0·58 (3)	10·5 ± 1·50 (2)
Number surviving lactation/litter (all the young were retained)	6·7 ± 1·50 (3)	2·5 ± 0·50 (2)
Generation II		
Single mating/litter test		
Number of matings required/litter	1·3 ± 0·33 (3)	1·3 ± 0·33 (3)
Number of young/litter	8·3 ± 2·35 (3)	14·6 ± 2·49 (3)
Number surviving lactation/litter	4·3 ± 3·75 (3)	6·3 ± 3·48 (3)

All results are expressed as means ± S.E. (number of pregnancies).

litter. Table III shows that there was no difference in the numbers for the sulphite-treated and control pairs of Generation I. The young of litters from the first mating of Generation I were weighed within 24 hours of birth, and only six young were returned to each mother. Six to 8 weeks after the birth of the first litter (Generation II. (i)) some of the same pairs of rats from Generation I were remated. All the young born remained with the mothers and were not handled for the first week of life (Generation II (ii)). Reduction in the handling of the young did not increase the proportion of those surviving lactation. The chief cause of loss was cannibalism by the mother. This was most marked amongst the water drinking animals.

A similar fertility test was made using rats of Generation II (i). Again there was no significant difference for rats of these two groups. All the young were weighed within 24 hours of birth, and were returned to their mothers. The proportion of the young surviving to the end of lactation did not differ significantly between control and sulphite-treated animals. The weights of the young are compared in Table IV. A significant difference appears (P = < 0·05) between the two groups of young at birth

after the first mating of Generation I, but this does not recur in the larger samples of young born to Generation II (i).

The growth of the young during the first ten days did not differ for sulphite-treated and control animals either in the first reproduction test on Generation I or in the reproduction test on Generation II (i). An apparent difference shown in the second test on Generation I should be attributed to a failure of lactation in the control water drinking animals (Table IV).

The Effect of Metabisulphite in the Drinking Water on the General Health of Rats

The general health of the control and sulphite-treated animals remained excellent throughout the first 9 months of the experiment. Occasional

TABLE IV

THE EFFECT OF METABISULPHITE IN THE DRINKING WATER SUPPLIED TO RATS ON THE YOUNG BORN TO THEM

	Treated rats 750 p.p.m. as SO_2	Control rats
Generation I		
First test		
Weight of newborn in g.	7·2 ± 0·23 (23)	5·8 ± 0·15 (19)
Weight of young at 10 days in g.	23·2 ± 1·97 (11)	19·6 ± 1·61 (6)
Second test		
Weight of young at 10 days in g.	20·2 ± 0·50 (24)	14·1 ± 1·45 (8)
Generation II (i)		
Weight of newborn in g.	6·7 ± 0·12 (25)	6·4 ± 0·11 (21)
Weight of young at 10 days in g.	19·8 ± 0·52 (15)	20·2 ± 1·27 (21)

All results are means ± S.E. (number of animals).

snuffles and sticky eyes developed, but these were as frequent amongst the controls as the experimental animals.

An epidemic respiratory infection broke out in the tenth month of the trial. This killed some of the rats of the first generation, but nearly all of those of the second and third generations recovered. Incidence of infection was spread evenly between the control and sulphite-treated animals. The latter could not, therefore, be claimed to show either increased or decreased susceptibility to the infection. There were as many deaths among the control as among the experimental animals.

Postmortem and histological findings. Four or five rats were selected at random from each group in Generation I for postmortem and histological examination of their organs a few days after the onset of the respiratory infection in the tenth month of the experiment. Metabisulphite treatment was maintained throughout the infection and thereafter. All survivors of the epidemic were kept until they had either reached 2 years of age or developed a well established tumour, then they were killed and examined.

There were no significant differences between the body weights of rats killed at 10 months and at 2 years of age. Therefore the weights of all animals subjected to postmortem examination are shown together in Table V combined according to the treatment they had been given. The

drinking of sodium metabisulphite solution (750 p.p.m. SO_2) throughout life affected neither the weights of the rats (Table V) nor the percentage of the weight contributed by the various organs (Table VI).

There was no evidence of clinical polyneuritis before death, nor were spectacle eyes, blanching of the incisor teeth, or browning of the uteri evident in animals which had received 750 p.p.m. SO_2 in their drinking water. These changes were described by Fitzhugh, Knudsen and Nelson[5] in rats fed solid diets containing sulphite.

TABLE V

POSTMORTEM WEIGHTS OF ADULT RATS IN G.

Sex	Drinking fluid supplied throughout life		
	Water	Solution of sodium metabisulphite	
		375 p.p.m. SO_2	750 p.p.m. SO_2
Females	409 ± 15·1 (13)*	399 ± 24·5 (10)	402 ± 10·0 (22)
Males	626 ± 36·1 (13)	—	599 ± 23·3 (18)

* Number of animals

TABLE VI

POSTMORTEM WEIGHTS OF THE ORGANS OF ADULT RATS EXPRESSED AS MEAN PERCENTAGE BODY WEIGHT ± S.E.

Organ	Drinking fluid supplied throughout life		
	Water	Solution of sodium metabisulphite	
		375 p.p.m. SO_2	750 p.p.m. SO_2
FEMALES			
Liver	4·1 ± 0·15 (13)*	3·8 ± 0·33 (10)*	3·8 ± 0·33 (22)*
Heart	0·47 ± 0·02 (13)	0·52 ± 0·03 (10)	0·43 ± 0·02 (22)
Spleen	0·45 ± 0·03 (20)	0·37 ± 0·04 (10)	0·32 ± 0·02 (22)
Kidneys	0·91 ± 0·04 (20)	0·99 ± 0·04 (10)	0·86 ± 0·03 (22)
Lungs	1·43 ± 0·08 (20)	2·08 ± 0·11 (10)	1·12 ± 0·19 (22)
Adrenals	0·03 ± 0·002 (20)	0·04 ± 0·01 (10)	0·03 ± 0·01 (22)
Uterus	0·34 ± 0·03 (20)	0·21 ± 0·15 (10)	0·18 ± 0·08 (22)
MALES			
Liver	3·49 ± 0·02 (13)	—	3·50 ± 0·09 (18)
Heart	0·70 ± 0·03 (13)	—	0·69 ± 0·04 (18)
Spleen	0·27 ± 0·01 (13)	—	0·23 ± 0·01 (18)
Kidneys	0·92 ± 0·30 (13)	—	0·84 ± 0·02 (18)
Lungs	0·89 ± 0·04 (13)	—	1·02 ± 0·01 (18)
Adrenals	0·02 ± 0·002 (13)	—	0·014 ± 0·001 (18)
Testes	0·73 ± 0·06 (13)	—	0·69 ± 0·05 (18)

* Number of animals.

Histological examination was made of the tissues of thirteen females and four males from each group, water drinking and sulphite drinking (750 p.p.m. as SO_2), 10 months after the beginning of treatment. No abnormalities were found in the spleens, adrenal glands, stomachs, ileums, colons, gastrocnemius muscles, sciatic nerves, uteri or testes or in the seminal vesicles of any of these animals. The nine cases of focal nephritis and two of cloudy swelling of the liver were almost evenly distributed between sulphite treated and control animals. These changes should most probably be ascribed to the infection prevalent at that time because

there was evidence of acute infection in the lungs of every one of the animals irrespective of their treatment group. None had developed tumours.

Thirty-seven per cent of the 54 animals kept for 2 years developed tumours. The incidence was higher among the females (44 per cent) than among the males (27 per cent), and was unaffected by the addition of sodium metabisulphite (750 p.p.m. as SO_2) to the drinking water. Of 16 females drinking water, one developed a lipoma, two had tumours of lymphoid type, and three tumours which had arisen from the duct of a sebaceous gland. Among the 16 females drinking sulphite in their water, three developed lymphoid tumours, four had tumours arising from sebaceous ducts, and one a fibroid. There were two lymphoid tumours, one duct tumour and one lipoma among nine untreated males; two lymphoid tumours arose in a group of 13 sulphite drinking males.

The full histological examination of the tissues of the 54 animals kept for 2 years under test revealed no abnormalities of the spleens, adrenal glands, ileums, colons, gastrocnemius muscles, sciatic nerves, ovaries or testes, or of the uteri or seminal vesicles, except when these were involved in a growth. Twenty-seven cases of excess fat, three of cloudy swelling and seven of infiltration of the portal tracts in liver were evenly distributed among the males and females of both control and treatment groups. So were the 28 cases of chronic and seven of acute infection of lungs. Renal scarring with old nephron damage was found in nineteen animals of either sex, spread evenly over control and treatment groups. Hyaline degeneration of heart muscle was confined to two treated and two untreated males. Two small acute gastric ulcers were found in water drinking males and one in a sulphite drinking male. Evidence of hyperplasia of the gastric mucosa, which occurred when rats were fed a solid diet containing 650 p.p.m. SO_2[4], was absent.

Discussion

The toxicity of sodium bisulphite (615 p.p.m. as SO_2) mixed in a solid diet was only in small part prevented by biweekly injection of 100 µg. aneurine to each rat[5]. The residual toxicity could have been due to the sulphite itself or to the products of its interaction with constituents of the solid food. The first of these two possibilities has now been excluded because sodium metabisulphite (750 p.p.m. as SO_2) in drinking water has proved non-toxic and the weight of fluid drunk has exceeded that of diet eaten by each generation of rats.

This work was undertaken during the tenure, by I. L. Natoff, of a studentship for training in research, presented by Carter and Co. Ltd., who also defrayed the expenses of the work.

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Sodium Metabisulfite and Sulfur Dioxide Gas (Silage Preservative) Poisoning in Cattle

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THE BENEFICIAL EFFECTS of sodium metabisulfite as a silage preservative have been reported.[1,2,3,4,5] The metabisulfite method of silage preservation has been extensively used, and there have been no confirmed cases of intoxication associated with the feeding of this silage. However, there have been occasional obscure cases of intoxication in which the feeding of metabisulfite silage was suggested as a possible cause.[6]

Studies on the toxicity of metabisulfite and sulfur dioxide gas for cattle and observations on the effects of feeding metabisulfite silage to cattle over a period of several years are reported in this paper.

MATERIALS AND METHODS

Sodium metabisulfite was given twice daily to fistulated dairy cattle immediately after it was prepared as a water solution. Only the measured amount of metabisulfite introduced into the rumen through the fistula was used in calculating intake. One half of the daily dose was given in the morning and the other half in the late afternoon. Small additional but variable amounts of metabisulfite were consumed when some of the animals were fed preserved silage, but no attempt was made to use these amounts in calculating the intake. A small cylinder of sulfur dioxide was used to introduce this gas constantly through a tube in the fistula. The flow was regulated by a needle valve and cylinder weights were recorded in order to determine the amount of gas introduced during each 24-hour period.

Information on experimental animals is given (table 1). All animals were clinically examined daily.

Blood was collected from the jugular vein of each animal and smears for differential leukocyte determinations were made within 15 minutes after collection. Sodium fluoride was used as the anticoagulant in samples collected for blood glucose determinations, and potassium oxalate was used in the samples collected for blood urea nitrogen determinations. Potassium and ammonium oxalated blood was used for all other samples.

The following procedures were used for hematological and biochemical determinations: Wright's stain for differential leukocyte counts,[7] Peter and Van Slyke's method for blood glucose,[7,8] and Van Slyke and Cullen's method for blood urea nitrogen.[9] Sections were taken at necropsy and stained with hematoxylin and eosin.

Baseline clinical, hematological, and biochemical data were collected for at least one week on animals B121, B130, B230, and B262 prior to giving the preservatives.

EXPERIMENTAL PROCEDURE AND CLINICAL RESULTS

Experiment 1.—Cows B164 and B145, on a ration of clover silage and concentrate, were given bisulfite at the rate of 80 Gm. per day. The rate was increased 40 Gm. every two days. On the sixth day, both cows quit eating and bisulfite feeding was discontinued. Appetites gradually returned and, six days after the experiment was stopped, both cows were on full feed. Three days after the cows returned to full feed, they were regarded as being normal. A new trial was then initiated at the level of 80 Gm. per day. Only the amounts of metabisulfite given in the second trial were used in calculating the total intake on this experiment.

Cow B164, which was in the third month of gestation at the start of the experiment, was given 80 Gm. per day for 180 days, at which time she calved normally. Neither the cow nor the calf showed any abnormalities at any time.

Cow B145 was given metabisulfite at the rate of 80 Gm. per day for the first week, 120 Gm. per day the second week, and 200 Gm. per day the third week. On the twentieth day of the experiment, she was unable to stand and showed occasional convulsions. By the twentieth day, there was complete anorexia and the cow was killed for necropsy.

Experiment 2.—Heifers B121 and B130 were used in experiment 2. At the beginning of the experiment, heifer B121 was

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fed preserved grass silage ad libitum. As the dosage of metabisulfite increased, the silage intake declined, and it was necessary to add hay and grain to the ration. Heifer B190 was given only hay and grain throughout the experiment.

Heifer B121 was started at a daily dosage of 80 Gm. of metabisulfite. The daily dosage was increased to 120 Gm. for the second week, 160 Gm. for the third week, 200 Gm. during the fourth and fifth weeks, and 240 Gm. per day for three days of the sixth week, when she died. The first signs of anorexia occurred during the second week, when the dosage of metabisulfite was 120 Gm. per day. Other signs of illness in the order of their appearance were: constipation, mild depression, dyspnea, vomiting, excessive salivation, diarrhea, severe dysentery, and terminal depression.

Heifer B190 was started on a daily dosage of 80 Gm. of metabisulfite, the dose was increased to 120 Gm. for the second week, and 160 Gm. per day was given for two days of the third week, at which time she died. This animal exhibited a marked decline in hay and grain consumption starting on the third day of the experiment and continuing until the twelfth day, when anorexia was complete. Other clinical signs observed were occasional coughing, constipation, and severe terminal dysentery.

Experiment 3.—Sulfur dioxide gas was introduced through rumen fistulas in steers B230 and B262.

In steer B230, the gas was released at a point well above the liquid contents of the rumen. The amount of gas introduced was 50 Gm. per day for the first week, 162 Gm. per day for the second week, and 255 Gm. per day for two days of the third week, when the steer died.

A temperature of 104.8 F. was observed on the fifth day and the first signs of anorexia occurred on the tenth day. A heavy mucopurulent nasal discharge, followed by diarrhea, constipation, and coughing, was observed on the ninth day and continued until the animal died.

Since considerable necrosis of the dorsal rumen wall of steer B230 occurred as a result of direct contact with sulfur dioxide gas, in steer B262 the gas was introduced into the liquid portion of the rumen contents. This steer was given 53 Gm. per day the first week; 73 Gm. per day the second week; 145 Gm. per day the third week; and 47 Gm. on the first day of the fourth week, when death occurred. Anorexia began on the fourteenth day. Other clinical signs were similar to those observed in steer B230, except for terminal nystagmus and severe depression.

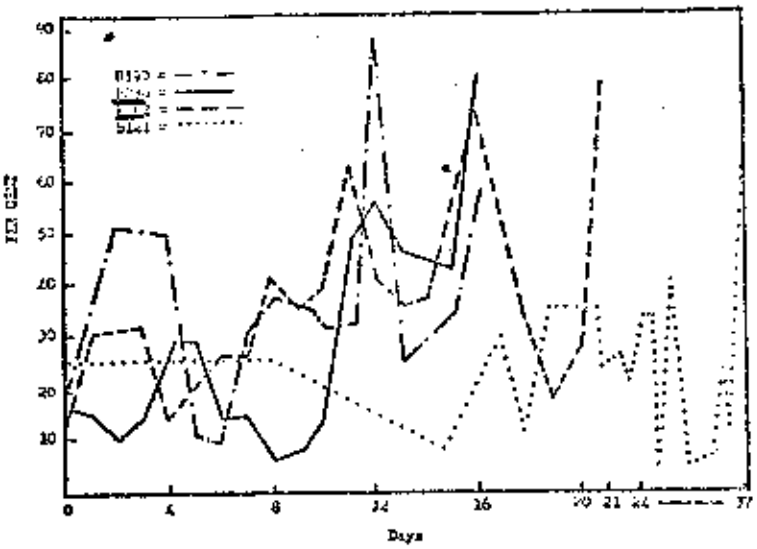
TABLE 1—Results from Feeding Sodium Metabisulfite and Sulfur Dioxide Gas to Fistulated Cattle

No. and breed	Sex and age	Estimated body weight (lb.)	Estimated body weight lost (lb.)	No. experiment days	Total bisulfite or SO_2 (Gm.)	Results
B164 Guernsey	Cow adult	1,200	None	180	14,400	Normal
B145 Holstein	Cow adult	1,100	———	20	2,360	Death
B121 Ayrshire	Heifer 16 months	850	250–300	37	6,040	Death
B190 Shorthorn	Heifer 16 months	600	200	16	1,720	Death
B230 Jersey	Steer 14 months	600	250	16	1,995 (SO_2)	Death
B262 Holstein	Steer 4 months	600	200	21	1,944 (SO_2)	Death

100 lb. of well preserved silage contains approximately 45 Gm. sulfite.

GENERAL RESULTS

The clinical course was 37, 16, 16, and 21 days for animals B121, B190, B230, and B262, respectively (table 1). The onset and severity of the signs observed were closely correlated with the amounts and rates of administration of the preservatives. Anorexia and severe body weight loss were the predominating signs of illness. Constipation, diarrhea, depression, and dysentery were common signs. Anorexia was observed at three and eight days, respectively, in the 2 animals given sodium metabisulfite, and at 10 and 14 days in the 2 steers given sulfur dioxide gas. Anorexia in heifer B190 was complete for six days.



Graph 1—Changes in neutrophil percentage during the course of sodium metabisulfite and sulfur dioxide gas intoxication.

Depression was more severe and constant in animals given metabisulfite, but discharge from the external nares was not observed in these animals. One of the animals given sulfur dioxide gas had considerable heavy mucopurulent discharge, and the other a thick mucous nasal discharge. Coughing varied from mild to moderate and was inconsistent and sporadic.

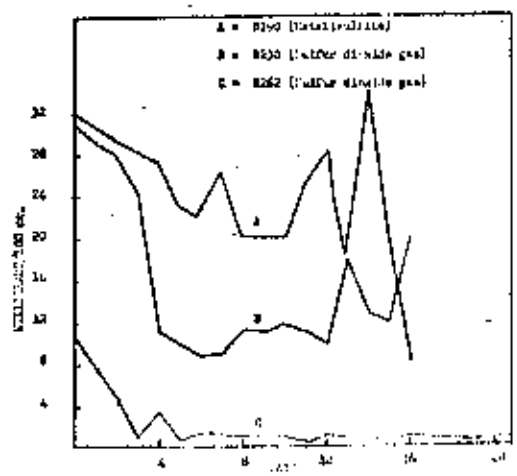
Hematological Observations

Coincident with the first signs of anorexia, there was a general but variable increase in hematocrit values which was related to the duration and degree of anorexia. Erythrocyte counts tended to parallel the hematocrit values. The mean corpuscular hemoglobin concentration gradually decreased, except for minor fluctuations in all 4 animals.

Leukocyte counts generally ranged from 9,500 to 15,000 cmm., but several counts of 21,000 to 35,000 were observed. The higher counts were one- or two-day peaks occurring in the latter half of the experimental period.

The usual range of icterus index was 100 to 150 during the last five to ten days of the experimental periods. In 1 animal, it reached 21.1.

A constant shifting of the differential leukocyte counts occurred when the use of preservative administration was at the higher levels. The more pronounced change occurred in the neutrophils with a corresponding shift of the large and small lymphocytes (graph 1). An occasional mild to moderate single-day increase occurred in the eosinophils, monocytes, and basophils but, in general, no significant change occurred.



Graph 2—Changes in blood urea nitrogen determinations during the course of sodium metabisulfite and sulfur dioxide gas intoxication.

Biochemical Observations

No significant change in blood glucose levels was observed. Blood urea nitrogen values decreased significantly in all animals (graph 2).

Residual sulfite of the urinary contents determined by the method of Nichols and Reed,[10] showed that added sulfite disappeared rapidly until the dosage level of

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Graph 1—Changes in neutrophil percentage during the course of sodium metabisulfite and sulfur dioxide gas intoxication.

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200 Gm. per day was reached (graph 3). At this point, the residual sulfite of the rumen contents was extremely high; during the two days preceding death, the level was so high that an accurate determination could not be made.

NECROPSY OBSERVATIONS

Rumen.—Rumen lesions varied with the preservative given. General thickening of the rumen wall and mild, subserous, suffusion hemorrhage occurred in the 2 animals given sulfur dioxide gas. In all 5 animals which died, there was a mild to moderate congestion of the mucosa. In 1 animal, the gas directed toward the dorsocaudal portion of the rumen produced a severely thickened rumen wall over an area of 10 square inches. Marked suffusion hemorrhage occurred in the serosa, while a 1/4-inch-thick membranous exfoliation resulted on the mucosal surface.

Microscopically, congested portions of the rumen showed severe congestion of the lamina propria as well as edema. There were a few focal areas of hemorrhage, erosions and leukocyte infiltrations of the tips of the papillae. In the more severely thickened portions of the rumen wall, there was complete structural disorganization. Most of the mucosa was eroded; considerable leukocyte infiltration and marked increase of connective tissue were observed.

Reticulum.—Moderate congestion occurred in 4 of the 5 cattle. Upon microscopic observation, there were variable erosions and leukocyte infiltrations of the tips of the papillae. In 1 animal, mild coagulation necrosis occurred under the cornified stratified squamous epithelium.

Omasum.—Gross and microscopic lesions similar to those in the reticulum were observed. In addition, in 1 animal the contents were fluid, while in another there was localized necrosis of the "leaves."

Abomasum.—One animal on each preservative developed acute hemorrhagic gastritis. The animal given sulfur dioxide gas had numerous bleeding points, and the abomasal contents were chocolate colored. The other 3 animals showed mild to moderate congestion of the mucosa.

Histologically, there was usually some erosion, leukocytic infiltration, congestion, and hemorrhage of the mucosa. In the animals with more severe cases, there was severe edema of the submucosa and tunica muscularis.

Small Intestines.—Gross pathological changes varied from none to acute hemorrhagic enteritis and corresponded with the degree of changes in the other organs. These varied with the type and total amount of preservative administered, and the duration of administration. Animals B145 and B121 showed no gross lesions. B190 and B230 showed a few petechial hemorrhages and marked catarrhal enteritis, and B262 had marked hemorrhagic enteritis of the duodenum that changed into marked catarrhal enteritis.

Histopathologically, there were varying degrees of hyperemia, edema, and cellular infiltration of the propria. The villi were swollen. In animals with more severe cases, the inflammatory reaction extended to the submucosa, with severe mucous degeneration in Lieberkühn's glands to the extent that few of the gland cells remained intact. The usual superficial effects of desquamation, erosion and, in a few cases, leukocytic infiltration were observed.

Large Intestines.—Grossly, acute catarrhal enteritis was observed in steer B262, whereas only a few sporadic petechial hemorrhages and hyperemia were observed in the other animals.

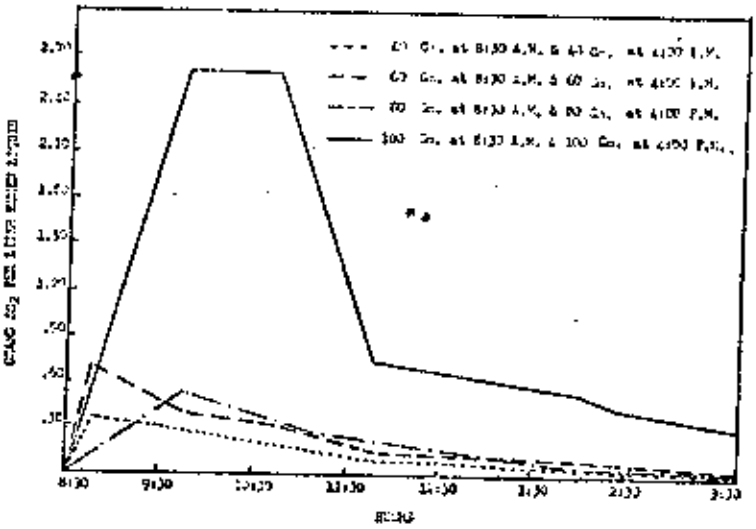
Microscopically, only the superficial effects of desquamation, erosion, hyperemia, and occasional petechial hemorrhages were found.

Liver and Gallbladder.—Grossly, the liver showed either mild congestion and a few localized areas of fatty degeneration or no pathological changes. The variation was associated with the severity and duration of illness and the total amount of preservative administered. The gallbladder of steer B262 showed several ecchymotic hemorrhages in the mucosa and thickened bile. The bile was also thickened in heifer B121.

Microscopically, the livers of animals B121 and B262 showed focal areas of coagulation necrosis, cloudy swelling, and fatty degeneration as compared with severe fatty degeneration, coagulation necrosis, and congestion in heifer B190.

Spleen and Lymph Nodes.—A few petechial hemorrhages were observed in the spleen of steer B262; in heifer B121, the spleen capsule was thickened and the mesenteric lymph nodes were enlarged and edematous. In cow B145, the parotid and submandibular lymph nodes were enlarged and edematous.

Kidney.—Mild to moderate swelling oc-



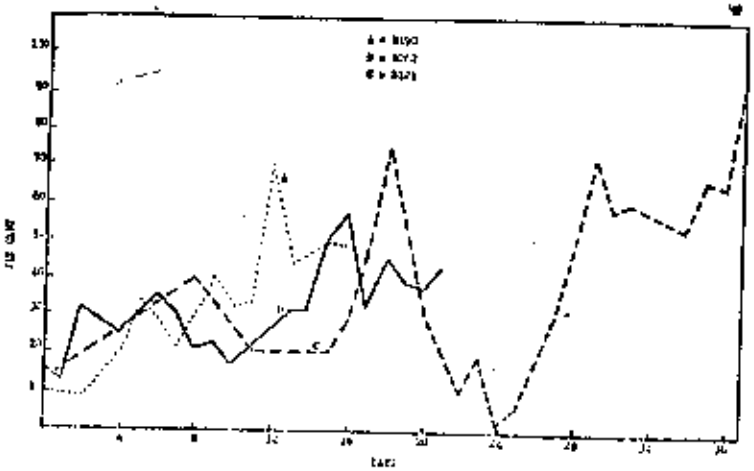
Graph 3—Rate of disappearance of sodium metabisulfite in rumen contents of heifer B121 when added through a fistula.

curred in the kidneys of 3 of the 5 animals, with mild to moderate congestion in 2 of the cattle. The kidneys appeared shrunken in 1 animal.

Microscopically, the kidneys of animals B121, B190, B230, and B262 showed moderate to severe nephrosis with some moderate cystic dilatation of the convoluted tubules. Occasionally leukocytic infiltrations were seen.

Respiratory System.—In 3 of the animals, the lungs were mildly to moderately emphysematous. In heifer B121, there was marked emphysema, numerous subpleural petechial hemorrhages, and a small area of acute bronchopneumonia in the dependent portion of the apical lobe of the right lung. In 2 animals, there was severe hemorrhage and edema of the larynx, while in the other 3 animals necropsied the larynx was apparently normal. Animals B121, B190, and B262 had severe petechial ecchymotic and suffusion hemorrhages along the ventral wall of the cranial portion of the trachea (fig. 1). In addition, there was moderate submucosal edema of the trachea. Only a mild to moderate congestion was observed in 1 of the remaining 2 animals.

Cardiovascular System.—Steer B262 had a moderate number of petechial, ecchymotic, and suffusion hemorrhages of the myocardium and endocardium. In the other 4 animals, there were only a few petechial and occasionally mild, diffuse suffusion-type hemorrhages. Microscopically, only hemorrhages were observed.



Graph 4—Changes of percentage of degenerating leukocytes in 3 cattle during the course of sodium metabisulfite and sulfur dioxide gas intoxication. Curve A is also typical for steer B230.

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Central Nervous System.—In animals B146 and B121, which showed clinical central nervous system disturbances, there was a moderate congestion of the leptomeninges, and apparently an increase in quantity of gelatinous, cerebrospinal fluid. In the other 3 animals, there was a mild congestion and edema of the leptomeninges, with some apparent increase in cerebrospinal fluid.

Microscopically, only mild to moderate inflammatory changes of the leptomeninges were observed.

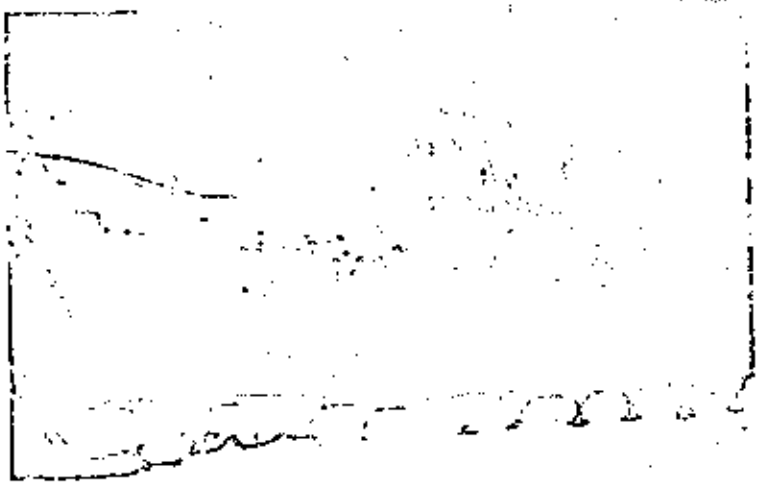

Fig. 1—Hemorrhages in the mucosa of the ventral wall of the cranial part of the trachea of steer B262 following the introduction of sulfur dioxide gas into the rumen contents.

Discussion

Severe intoxication in cattle from feeding silage preserved with these agents seems highly improbable because of the resultant anorexia. Furthermore, the animal's appetite for preserved silage was decreased prior to general anorexia. The dosage which produced anorexia was within the range of 80 to 160 Gm. of metabisulfite or sulfur dioxide gas per day.

Nearly all of the 1,000 to 1,500 tons of grass silage produced annually at the university is treated with metabisulfite. Each year, for the past five years, approximately 100 cattle have been fed preserved silage with no apparent harmful effects.

Alderman *et al.*[1] reported that 48 samples of well-preserved, metabisulfite-treated silage showed an average residual sulfite content of 0.295 per cent in the dry matter. This mean value was the equivalent of 810 p.p.m. in the fresh silage, and represented only 25 per cent of the sodium metabisulfite originally added. A well-preserved silage, made from forage treated with sodium metabisulfite at the recommended rate of 8 lb. per ton of fresh forage, would contain approximately 2 lb. of residual sulfite per ton. An animal eating 100 lb. of this silage per day would consume approximately 45 Gm. of sulfite per day, or about half as much as cow B164 was given each day for six months with no adverse effects. Since anorexia occurs when a greater concentration of the agent is given, it appears that an animal can obtain toxic or lethal amounts only by forced feeding.

Sodium metabisulfite and sulfur dioxide gas are inhibitors of bacteria and certain enzyme systems. It is logical to assume that these preservatives would have a depressing effect on the microorganisms of the rumen. However, cow B164 received 80 Gm. of metabisulfite per day for six months and remained apparently normal, indicating that this effect may not be significant in the cow. The evidence obtained in rumen residual bisulfite determinations (graph 3) shows that when as much as 160 Gm. of metabisulfite per day is given the disappearance from the rumen is fairly rapid. At a level of 200 Gm. per day there was a bisulfite saturation, but the cow did not die until 15 days later.

The consistent and immediate drop in blood urea nitrogen in animals B190, B230, and B262 suggests that this may be a constant feature of metabisulfite intoxication. Since urea is the most important nitrogenous waste product, a disturbance in the

nitrogen balance of the body probably occurs.

Blood glucose values were quite variable and erratic and, consequently, not significant.

The only fairly consistent lesion produced was the hemorrhagic trachea. Since this lesion occurred in 2 of 3 animals given metabisulfite, it was believed to be caused by inspired rumen gases (other than methane), possibly by sulfur dioxide gas. However, sulfur dioxide gas placed in the dorsal portion of the rumen of steer 13230 resulted in only a mild to moderate congestion of the trachea. Furthermore, when the gas was delivered into the liquid contents of the rumen of steer 13262, severe tracheal lesions were observed. If the tracheal lesions result from sulfur dioxide, they are not caused by the gas per se. The ventral location of the lesions may indicate that it was due to a condensation of inspired rumen gas into moisture droplets.

Summary

Cattle eating 100 lb. of silage treated with sodium metabisulfite at the recommended rate would consume approximately 45 Gm. of sulfite per day. One pregnant cow was given about twice this amount daily for 180 days with no deleterious effects. The 600 cattle maintained by the university have been fed metabisulfite-treated silage for more than five years with no deleterious effects.

When massive doses of sodium metabisulfite or sulphur dioxide gas were administered through rumen fistulas to 5 cattle, they died in 16 to 37 days. Anorexia was a consistently observed sign of the intoxication. The pathological changes varied considerably in each animal and with the preservative given. In general, the more severe tissue changes were confined to the first portion of the digestive tract. The most consistent lesions were in the larynx and in the mucosa of the ventral wall of the trachea.

There was no significant change in blood glucose, but blood urea nitrogen determinations in 3 animals revealed a definite drop in blood levels during the course of the experiment. Hematological data were highly variable.

A residual bisulfite determination on the rumen contents of 1 animal indicated that a strong homeostasis body function existed until the animal apparently became saturated with the preservative. Saturation occurred when the daily dose of metabisulfite reached 200 Gm. However, the animal did not die until 15 days later.

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DETERMINAZIONE SPETTROFOTOMETRICA DELL'ANIDRIDE SOLFOROSA TOTALE NEI VINI

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Laboratorio Chimico Provinciale di Pescara

Il dott. MICHELE LUISE si è laureato a Bologna in Chimica pura nel 1954; dopo una breve permanenza nel Laboratorio Chimico Provinciale di Pescara, è stato per alcuni anni in Inghilterra dove ha prestato servizio nel Laboratorio Ricerche di una grande industria. Dal 1959, vinto il pubblico concorso per titoli ed esami, copre il posto di assistente nel Laboratorio Chimico Provinciale di Pescara; dal 1963 è anche assistente alla Cattedra di Merceologia dell'Università di Pescara. È autore di numerosi lavori nei quali si è prevalentemente occupato di problemi colorimetrici e cromatografici.

Riassunto — *Viene proposto un metodo spettrofotometrico per la determinazione dell'anidride solforosa totale nei vini. Il metodo si basa sul fatto che la pararosanilina, decolorata con acidi, quando reagisce con formaldeide e ioni bisolfito produce una intensa colorazione violetta.*

Vengono anche riportati e paragonati i risultati di determinazioni di anidride solforosa totale eseguiti con i correnti metodi ufficiali e il metodo colorimetrico qui proposto.

La determinazione dell'anidride solforosa negli alimenti richiede, in generale, la distillazione del campione acidificato e la raccolta dell'anidride solforosa, così liberata, in adatte soluzioni nelle quali viene poi determinata. Il fatto che sono stati proposti tanti metodi e tante modifiche alle condizioni di distillazione e determinazione dell'anidride solforosa indica che finora non è stato trovato un procedimento generale abbastanza soddisfacente. La vasta bibliografia sulla determinazione dell'anidride solforosa è confusa ed alcune volte contradittoria forse a causa della valutazione degli errori inerenti ad un particolare metodo, specialmente se viene applicato ad un prodotto per il quale non è affatto idoneo.

Nei vari metodi già esistenti, le sorgenti di errore sono parecchie, alcune causano risultati alti, altre bassi, così che, apparentemente, si possono ottenere risultati corretti per fortuita combinazione di questi due effetti; conoscendo le diverse cause di errore, questo può essere eliminato, ridotto e quanto meno valutato; resta però il fatto che il completo recupero dell'anidride solforosa aggiunta ad un alimento non è una garanzia della precisione e riproducibilità di un metodo. Inoltre i diversi alimenti presentano differenti problemi a causa della presenza di varie sostanze interferenti.

Ogni metodo, per la determinazione accurata dell'anidride solforosa nelle bevande alcooliche, dovrebbe tenere conto della presenza:

1) di sostanze volatili oltre l'anidride solforosa e che possono dare valori del bianco abbastanza alti;

2) di sostanze riducenti specialmente quando si effettui la titolazione diretta con iodio;

3) di acetaldeide che può reagire con l'anidride solforosa prima che essa venga ossidata nel raccoglitore.

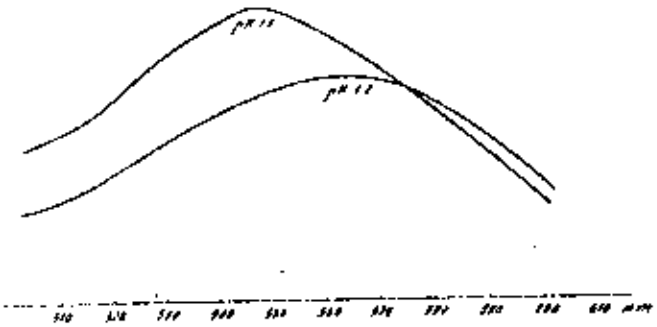
Quest'ultimo fattore è spesse volte trascurato perché l'errore che causa è generalmente piccolo (1 o 2%) ma se ne deve tenere conto in una accurata determinazione.

Il metodo colorimetrico che qui viene proposto è quello di West e Gaehr[1] che è stato adottato dalla American Society of Brewing Chemists e proposto come metodo ufficiale dell'AOAC[2] per la determinazione dell'anidride solforosa nella birra.

Sebbene questo metodo sia largamente adoperato per la determinazione dell'anidride solforosa nell'aria, inizialmente è stato criticato soprattutto per la sua mancanza di riproducibilità. Il metodo si basa sul fatto che la pararosanilina decolorata con acidi, quando reagisce con formaldeide e ioni bisolfito, produce una intensa colorazione violetta.

I fattori che influenzano questa reazione sono numerosi e sono stati studiati

ersi autori [1,4,5,6] ai lavori dei quali si rimanda per
ù completa conoscenza dell'argomento.
tavia qui vengono esposte in breve alcune norme che
ssario conoscere perché il metodo possa essere messo
o e dare risultati precisi e riproducibili. Innanzi tutto,
la concentrazione e la purezza della pararosanilina
erciale variano da partita a partita, occorre rifare la
di taratura ogni qualvolta si adoperi una nuova con-
e del colorante anche se della stessa marca, alcuni
consigliano invece la purificazione della pararosa-



1) Spettri d'assorbimento del complesso pararosanilina-anidride solforosa a due diversi valori di pH.

sviluppo del colore varia anche con il tempo e la tem-
ura, ma piccole differenze di temperature si possono
rare: a 25°C la variazione dell'intensità del colore con
mpo è molto simile a quella che si verifica alle diverse
erature ambiente del laboratorio, non controllate, ma
anti tra 18° e 28°, il che significa che normalmente
operare a temperatura ambiente. La massima intensità
lore viene raggiunta dopo tempi diversi a seconda della
eratura; per la normale temperatura ambiente questo
o è di 30 minuti. Il colore così sviluppato è stabile per
minuti se protetto dalla luce solare diretta.
pH della soluzione ha anche influenza sulla lunghezza
la del massimo assorbimento e sul valore dell'assorbi-
o stesso. La figura 1 mostra gli spettri di assorbimento
mplesso pararosanilina-SO_2 a due diversi valori di pH.
ti spettri sono simili a quelli di composti che mostrano
spostamento ipsocromico o batocromo con il variare
H. Il complesso appare quindi essere un composto sin-
con due forme isomere e può essere considerato come
pico indicatore di pH a due colori. Indipendentemente
H al quale la reazione viene eseguita, gli spettri pos-
trasformarsi l'un l'altro per aggiunta di acido o base.
to picco con un massimo di assorbimento a 548 milli-
n (colore rosa rosso) che si riscontra a pH 1,6, si sposta
millimicron (colore magenta) per un pH 1,2. Nella
intermedia di pH, lo spettro consiste di un effetto ad-
delle due forme dello stesso composto. Pertanto il
finale può essere letto a 548 millimicron se si opera
1,6 ± 0,1 o a 570 millimicron se il pH è 1,2 ± 0,1

Alla lunghezza d'onda di quest'ultimo pH si ottengono dei bianchi poco colorati ma il metodo è meno sensibile.

Logicamente è consigliabile ricontrollare questi massimi con la pararosanilina e lo spettrofotometro che si hanno a disposizione. Le concentrazioni di formaldeide aggiunte non sono critiche per lo sviluppo optimum del colore. Il metodo colorimetrico, poiché opera sul campione tal quale, presenta rispetto ai metodi ufficiali numerosi vantaggi, quali ad esempio la eliminazione della distillazione del campione e la successiva determinazione dell'acido solforico formatosi. Inoltre l'acido ascorbico spesse volte aggiunto ai vini, non ha alcuna influenza sul metodo colorimetrico.

PARTE SPERIMENTALE

Reattivi

1) *Soluzione di pararosanilina*

Si pesano 100 mg di pararosanilina cloridrato in un pallone tarato da 250 ml e si sciolgono con circa 200 ml di acqua distillata. Si aggiungono 40 ml di acido cloridrico diluito (1:1), si mescola e si porta a segno con acqua. La soluzione deve stare circa 15 minuti prima di essere usata. Si conserva in bottiglia scura ed in frigorifero.

2) *Soluzione di formaldeide*

Si diluiscono 5 ml di formaldeide al 40% a 1 litro con acqua. La soluzione deve essere preparata ogni giorno di fresco.

3) *Soluzione stabilizzante di cloromercurato di sodio*

27,2 g di cloruro mercurio e 11,7 g di cloruro sodico vengono disciolti in 1 litro d'acqua.

4) *Bisolfito di sodio purissimo*

Curva di taratura

Si adopera bisolfito di sodio purissimo di cui si stabilisce il titolo iodometricamente; con esso si prepara una soluzione contenente 1 mg di anidride solforosa per ml (sol. A).

Ml 10 della soluzione A vengono portati in pallone tarato da 1 litro contenente 100 ml di soluzione stabilizzante di cloromercurato di sodio e portati a segno con acqua distillata (sol. B).

Con una buretta di precisione si portano in palloni tarati da 50 ml quantità scalari da 0,5 a 5 ml della soluzione B. A ciascun pallone si aggiungono 5 ml della soluzione di pararosanilina e 5 ml della soluzione di formaldeide. Contemporaneamente si fa un bianco con i soli reattivi. Si mescola bene, si porta a segno con acqua e si tengono i palloni a temperatura ambiente ed al riparo della luce per 30 minuti. Poi si legge l'intensità del colore ad uno spettrofotometro a 570 millimicron rispetto al bianco. Si costruisce una curva riportando in ordinata i valori degli assorbimenti ed in ascissa le concentrazioni di anidride solforosa in gamma per ml.

La curva deve essere rifatta ogni volta che si cambia la pararosanilina.

PROCEDIMENTO

In un pallone tarato da 200 ml si versano 2 ml della soluzione stabilizzante di cloromercurato di sodio, 10 ml di vino e 20 ml di NaOH N/10. Trascorsi 10 minuti si aggiungono 20 ml di acido solforico N/10 e si porta a volume con acqua (soluzione 1).

A 5 ml della soluzione 1, posti in pallone tarato da 50 ml, si aggiungono 5 ml della soluzione di pararosanilina e 5 ml della soluzione di formaldeide. Si agita e si porta a volume con acqua. Contemporaneamente si prepara un bianco nel modo seguente: a 5 ml della soluzione 1 si aggiungono, in pallone tarato da 50 ml, 0,5 ml di salda d'amido all'1% e poi, goccia a goccia, una soluzione di jodio 0,05 N fino ad ottenere una colorazione bluastra permanente e poi una goccia in eccesso. Si agita e quando la colorazione bluastra scompare, si aggiungono 5 ml della soluzione di pararosanilina e 5 ml della soluzione di formaldeide. Si agita e si porta a segno. Si mettono i palloni al riparo della luce e, dopo 30 minuti, si legge l'intensità del colore a 570 millimicron rispetto al bianco. Con l'aiuto della curva di taratura si risale al contenuto di anidride solforosa espresso in gamma per ml. Moltiplicando questo valore per 200 si ottiene il contenuto di anidride solforosa espresso in milligrammi per litro di vino.

Tab. 1 — **Confronto di risultati per la determinazione dell'anidride solforosa totale nei vini con il metodo spettrofotometrico e quello ufficiale**

Vini bianchi		Vini rossi	
Metodo spettrofotometrico mg per litro	Metodo iodometrico mg per litro	Metodo spettrofotometrico mg per litro	Metodo iodometrico mg per litro
138	147	40	48
116	122,5	142	161
114,6	121,5	196	226
116	122,5	112	123,7
160	158	152	161
180	192	142	126
80	85	150	121,5
120	125	152	129,2
32	38	137	137
16	19	110	107,5
136	140	40	42,8
186	186,5	150	137,2
90	93	81,6	70,8
206	206	145	145,1
190	183	70	73,5
198	198,4	70	72
202	199	40	44
60	64	52	57,8
70	83	206	205
152	161	45	45
131	139	94	96,5
71	69,5	94	109
106	113,7	100	109,3
90	85,3	110	109,3
157	155	85	90
110	104,3	90	91
157	155	58	61
88	85,3	36	36
109	104	33,4	31
80	79	160	161,2
—	—	128	128
—	—	135	134
—	—	33,6	34
—	—	30	30
Medie 122,4	124,4	100,7	101,6

RISULTATI

Nella tabella 1 sono riportati e confrontati i risultati per la determinazione dell'anidride solforosa totale in n. 30 campioni di vino bianco e n. 34 campioni di vino rosso.

I risultati, anche se sono individualmente piuttosto discordanti, mediamente sono sufficientemente concordanti; è questa una conferma delle notevoli cause di errore che possono interferire nei metodi ufficiali e che, come abbiamo detto, possono compensarsi tra loro.

I valori medi ottenuti con il metodo spettrofotometrico sono leggermente più bassi di quelli ottenuti con i metodi ufficiali, ma la differenza è più sensibile (1,6%) nei vini bianchi che nei vini rossi (0,9%) ed anche questo è in relazione con il metodo ufficiale che è titrimetrico nei vini bianchi e ponderale nei vini rossi.

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STABILIMENTI
Viale IV Novembre 13
REGGIO EMILIA

ENOCIANINA FORNACIARI

Dott. Ing. Aldo Fornaciari fu Riccardo

SEDE
Corso Sempione N. 41
MILANO

COLORANT NATUREL EXTRAIT DU RAISIN ○ GRAPE COLOR EXTRACT ○ WEINTRAUBENFARBENEXTRAKT

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Levels of Free and Combined Sulfur Dioxide in Fruits during Sulfuring and Drying

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SUMMARY

A method for determining free SO_2 in cider has been adapted for use with sulfured apricots and peaches. This permitted a study which demonstrated, for the first time, the pattern of changes in the levels of free and combined SO_2 in these fruits during sulfuring and during drying. Between 10 and 20% of the total SO_2 absorbed was in the combined form at the end of sulfuring, and the rate of combination was linear throughout. In sulfuring, solution of SO_2 in the liquid phase and combination with components of the fruit are probably of greater importance than previously thought. The degree of combination had an important influence on the total amount of SO_2 retained during drying. Dried fruits contained 80-90% of their residual SO_2 in the bound form, most likely in combination with glucose.

INTRODUCTION

Sulfur dioxide is used in the production of dried fruits and vegetables mainly because of its effectiveness in reducing browning of these products while they are drying and during their subsequent storage. This inhibitory effect is due to the ability of SO_2 to modify the course of complex chemical reactions which are induced by drying and which involve amino compounds, sugars, and organic acids (Reynolds, 1965).

Maximum permissible limits of SO_2 are stipulated by most countries for dried foods, and for fruits these range from 2,000 to 3,000 ppm. Such limits are invariably specified in terms of total SO_2, which can be determined by well-established analytical methods. Joslyn and Braverman (1954), in reviewing extensive literature on the chemistry and technology of SO_2 utilization for treating fruits and vegetables, pointed out that in the treated products this preservative must exist in both free and combined forms, the relative proportions of these being determined by such factors as the composition of the food, its pH, and its temperature, all of which will play a part in determining the final state of equilibrium attained.

In the literature, though levels of free and combined SO_2 have often been quoted for beverages such as wines and ciders, analogous information relating to dried fruits is notably lacking. The absence of a reliable published method for determining free SO_2 in material which, like fruits, also contain appreciable amounts of other natural reducing compounds, seemed a likely reason for this deficiency, and suggested that the development of a suitable method was an essential preliminary to more extensive studies of the SO_2 distribution in sulfured fruits.

In the work now reported, a recently published method for determining free SO_2 in cider was appropriately and successfully modified for use with sul

fured fruit. Application of this method, in conjunction with the established procedure for the determination of total SO_2, permits changing levels of free and combined SO_2 to be followed in apricots and peaches being subjected to sulfuring and drying. Some results of this investigation are also presented.

MATERIALS AND METHODS

Fruit and its preparation. Apricots (var. Trevatt) and freestone peaches (var. Halehaven) were used as test material. The apricots were halved and pitted only, but the peaches were first steam-peeled. Most of the test fruit was grown without irrigation, but some apricots came from an irrigated region.

Sulfuring. Prepared fruit was sulfured in equipment described by McBean *et al.* (1964), which permitted air speed, temperature, and SO_2 concentration to be controlled simultaneously.

Drying. Sulfured fruit was dried under controlled conditions in the cross-flow dehydrator described by McBean *et al.* (1965).

Determination of SO_2. Total SO_2 was determined by the Monier-Williams method in all-glass apparatus, as recommended by Shipton (1954).

Free or uncombined SO_2 was determined by a method based on that developed by Burroughs and Sparks (1964) to estimate free SO_2 in ciders. Fruit was blended in orthophosphoric acid to lower its pH to between 1 and 2, thereby largely suppressing dissociation of combined SO_2 during displacement of the free SO_2 for subsequent estimation. This displacement was accomplished by drawing a stream of oxygen-free nitrogen through subsamples of the blended fruit at room temperature by applying suction. The SO_2 so displaced was absorbed from the emergent nitrogen stream by hydrogen peroxide and estimated by titrating the resulting sulfuric acid with standard sodium hydroxide solution.

The following minor modifications were made to the method of Burroughs and Sparks.

1) Apparatus used was as shown in Fig. 1. A fine-bore pressure relief tube with tap A was installed to prevent suck-back. The splash-bead Y prevented carry over of phosphoric acid from the sample flask B to the absorption tube C, and the fine bubblers, Z, assisted in ensuring efficient absorption of SO_2 from the emergent stream of N_2. Ball joints at X facilitated assembly, and a standard pressure of 50 mm Hg was used throughout.

2) The hydrogen peroxide was used at 3% strength (10-vol), because SO_2 levels found in sulfured fruit are higher than in ciders.

3) Bromophenol blue was used as indicator, as in the Monier-Williams method.

4) Caprylic alcohol (0.5 ml) was added to flask B to reduce frothing.

Burroughs and Sparks bubbled nitrogen through their samples for 15 min to displace free SO_2, then replaced absorption tube C by a fresh one before continuing the bubbling operation for a further 15 min. Any SO_2 absorbed by the second tube was presumed to have originated solely from dissociation of combined SO_2 during the second 15 min, and to provide an appropriate dissociation correction for the total amount of SO_2 displaced from the sample during the first 15-min bubbling period. It may be noted that the assumptions imply that all free SO_2 was eliminated in the first period and that the rate of dissociation of combined SO_2 was constant, i.e., the same amount of SO_2 was liberated in each of the two successive runs.

In the present work, preliminary tests on sulfured fruit, prepared and treated as described, invariably resulted in the collection of negligible quantities of SO_2 during the second of the two 15-min gas displacement runs. Moreover, when a series of aqueous solutions of different but known SO_2 content were examined similarly, it was found that a single 15-min period of displacement of SO_2 by nitrogen always sufficed to give recoveries of SO_2 close to 100% of theory.

From these findings it was concluded that a single 15-min period of gas bubbling was adequate for the displacement of free SO_2 from the sample under test, and that under the conditions used, no significant displacement of combined SO_2 occurred from sulfured fruit during this operation.

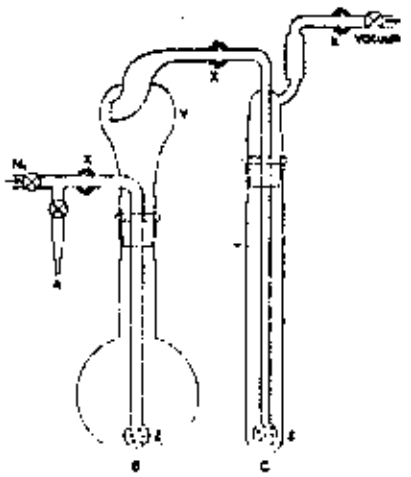

Fig. 1. Equipment for determining free SO_2 in sulfured fruit.

In view of the probability that in any case the principal SO_2-combining agent in fruits is glucose, and that very little dissociation of SO_2-glucose compounds occurs at pH 1.5 (Vas, 1949), blank corrections such as those used by Burroughs and Sparks were not used in the present work. It may be noted that a method similar in many respects to that employed here was used by Lloyd and Cowie (1963) on soft drinks in which glucose is the chief SO_2-binding agent, and these workers also found that blank corrections were unnecessary.

After displacing free SO_2 from experimental fruit samples in the manner described above, the SO_2 combined in the residue left in flask B was determined by the Monier-Williams method. Determinations of free and combined SO_2 were done on individual samples of fruit, and at the same time corresponding samples were taken for analysis for total SO_2 content.

It is impossible to prevent entirely some loss of SO_2 through volatilization or oxidation during the above analytical procedures, particularly in the determination of free SO_2 where fruit is blended in phosphoric acid (Ponting and Johnson, 1945). However, the use of carefully standardized sampling procedures (McBean *et al.*, 1964) and short blending times (15 sec for wet fruit and 30 sec for partly dried material) are believed to have restricted such losses to insignificant amounts. The view that only slight losses occurred in the analyses reported here is supported by the fact that the sum of free and combined SO_2 in any one sample generally lay within 5% of the total SO_2 content determined directly in the other. Total SO_2 values of fruit pieces sampled from a single tray are known to differ by up to 3% (McBean *et al.*, 1964).

Other analytical methods. Sampling procedures and methods for determining water content at all levels of dryness were those used by McBean *et al.* (1964, 1965).

RESULTS

Sulfuring. (Duration). Apricots were sulfured by exposure for 8 hr to SO_2 at 2% gaseous concentration (Fig. 2) and peaches by exposure to 1% SO_2 for about 6 hr (Fig. 3). These different sulfuring conditions, allied with inherently different characteristics of SO_2 absorption exhibited by the two kinds of fruit (McBean *et al.*,

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1961), account for differences in the observed levels of SO_2 in the apricots and peaches after equal periods of sulfuring. Yet comparison of corresponding curves in Figs. 2 and 3 show a general similarity in the pattern of

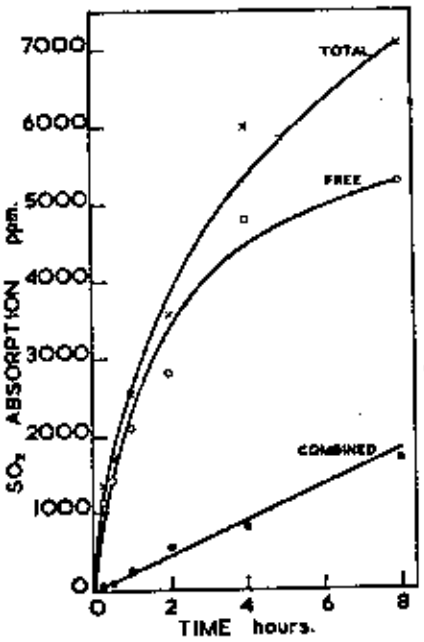

Fig. 2. Combined, free, and total SO_2 contents in apricots sulfured for various times at 2% gaseous SO_2 concentration.

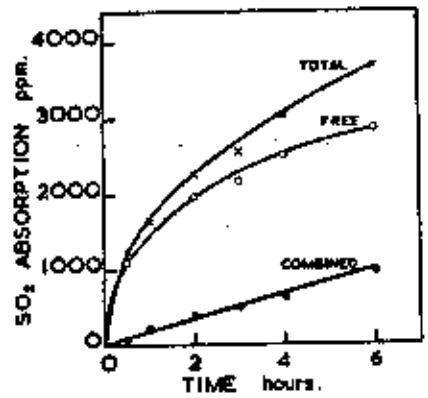

Fig. 3. Combined, free, and total SO_2 contents in freestone peaches sulfured for various times at 1% gaseous SO_2 concentration.

increases in free, combined, and total SO_2 for each kind of fruit. In both systems, the rate of increase of free SO_2 and of total SO_2 in the fruit was initially rapid and gradually diminished as sulfuring proceeded, whereas the rates of increase of combined SO_2 were constant (though different) over the whole period of sulfuring. Towards the conclusion of the sulfuring runs, these rates of increase of combined SO_2 were actually slightly greater than the corresponding rates of increase of free SO_2 in the fruits.

Table 1 shows that for apricots and peaches sulfured under the conditions specified above, longer periods of sulfuring increase the proportion of combined SO_2 relative to total SO_2 uptake. Though similar in magnitude in the present tests, the proportions of combined SO_2 relative to total SO_2 in apricots and peaches differed appreciably under different test conditions. For example, the combined SO_2 in a sample of apricots sulfured for 5.5 hr at 2% gas concentration approached 30% of the total SO_2 uptake, whereas some peaches sulfured at 1% SO_2 contained only 10% of their total SO_2 content in the combined form after 5 hr of sulfuring.

Irrigation. McBean *et al.* (1964) found that apricots grown under irrigation absorbed more SO_2 in sulfuring than did those grown without irrigation. This finding was continued in the present work by sulfuring irrigated and non-irrigated apricots together in an atmosphere containing 2% SO_2 gas. Fig. 4 shows that under these sulfuring conditions the apricots grown under irrigation contained more free SO_2 than non-irrigated fruit treated similarly. Also, in relation to the total SO_2 content of the fruit at any given stage of sulfuring, the proportion combined was consistently greater in irrigated apricots.

SO_2 concentration. In other similar tests the concentration of SO_2 continuously maintained around fruit samples during the course of sulfuring was varied over levels ranging from 0.5 to

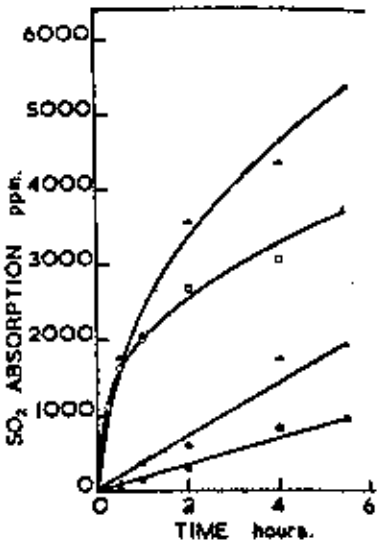

Fig. 4. Combined and free SO_2 in non-irrigated and irrigated apri sulfured for various times at 2% gas SO_2 concentration.

● combined SO_2, non-irrigated fr
▲ combined SO_2, irrigated fruit
○ free SO_2, non-irrigated fruit
△ free SO_2, irrigated fruit.

2.0% SO_2. Although increases in gas concentration enhanced total take of SO_2 by the fruit, there were consistent trends in the content either free or combined SO_2.

Loss of SO_2 before drying. Tabl shows levels of free and combined found in irrigated and non-irrig apricots held for 3.5 hr in still air 25°C immediately after they had b sulfured at 2% SO_2 concentration 2 hr. In both types, though there a fall of about 50% in the level free SO_2 in the fruit over the 3.5 holding period, a simultaneous occurred in the content of combi SO_2 in relation to total SO_2 in fruit. This is indicated at the bot of Table 2 by decreases in the lev of free SO_2 exceeding those for t SO_2 over the same period.

From the differences in Table 2 is clear that of the SO_2 initially pres

Table 1. Increasing percentage of combined SO_2 in apricot and peach tissue with increasing time of sulfuring.

Time of sulfuring (hr)	Combined SO_2 (% of total SO_2): Apricots sulfured at 2% concentration	Peaches sulfured at 1% concentration
0.25	5.5	
0.5	11.5	7.9
1	10.8	12.5
2	16.0	16.4
3		18.8
4	14.7	20.3
6		21.0
8	24.3	

Table 2. Changes in the levels of total, free, and combined SO_2 in apricots held 25°C for 3.5 hr after sulfuring for 2 hr at 2% SO_2 concentration.

Holding time at 25°C after sulfuring (hr)	SO_2 in irrigated apricots: Total (ppm)	Free (ppm)	Combined (% of total)	SO_2 in non-irrigated apricots: Total (ppm)	Free (ppm)	Combin (% of to
0	3650	3310	6.0	3140	2700	11.1
3.5	2500	1750	30.0	1890	1370	27
Changes during holding period (ppm, by difference)	1150	1560		1250	1330	

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a- free SO_2 in sulfured irrigated fruit, 400 ppm combined during the next 3.5 hr, while only 80 ppm combined in the non-irrigated apricots under similar holding conditions. In general, at the end of the 3.5-hr holding period after sulfuring, 30% of the total SO_2 in irrigated or non-irrigated apricots was in the combined form, whereas immediately after sulfuring, the relative proportion of SO_2 combined was only about 10% of the total present in each type.

Drying. (Duration). Fig. 5 indicates changes in the levels of free and combined SO_2 in irrigated and non-irrigated apricots as drying proceeded. Reckoned on a moisture-free basis (dwb), free SO_2 contents diminished throughout the whole drying period, whereas combined SO_2 increased during the first 2 hr or so before diminishing gradually to approximately constant levels roughly equal to those obtaining before commencing drying.

In irrigated apricots, combined SO_2 both at the start of drying and during the subsequent initial rise exceeded that present in the apricots grown without irrigation; hence at all times during drying the content of combined SO_2 in the irrigated fruit was greater than

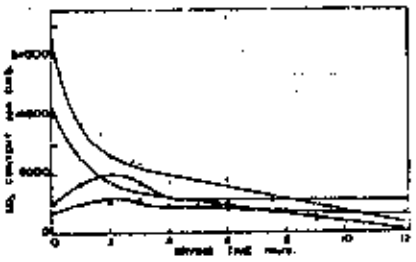

Fig. 5. Changes in combined and free SO_2 contents in non-irrigated and irrigated apricots during drying at 140°F.

● combined SO_2, non-irrigated fruit
▲ combined SO_2, irrigated fruit
○ free SO_2, non-irrigated fruit
△ free SO_2, irrigated fruit

Table 3. The increasing percentage of combined SO_2 in apricots during drying.

Drying time (hr)	SO_2 in combined form (% of total) Irrigated	Non-irrigated
0	12.0	14.0
1	31.0	28.0
2	43.4	34.4
3	.....	39.9
4	46.4	43.6
5	..	43.6
6	48.6	39.2
7.5	50.0	40.0
9	53.1	57.8
10.5	56.1	60.7
12	61.0	62.8

that in the non-irrigated fruit.

Table 3 shows the relative proportion of SO_2 in the combined form in these same apricots at various times during drying, expressed as a percentage of the total SO_2 present. This proportion rose from initial values of 12–14% to more than 80% for each type of fruit, sharp rises occurring during the first hour, and again towards the end of drying. However, these increases were mainly due to the marked falls in free SO_2 and the consequent decreases in total SO_2 contents that occurred at these stages of drying.

Influence of sulfuring concentration. Table 4 shows the free and combined SO_2 contents during the drying of peaches that had been sulfured for 1 hr at gaseous concentrations of 0.5, 1.0, or 2.0%. As with apricots, free SO_2 levels fall rapidly during the first hour and then more slowly during the remaining time. In contrast, combined SO_2 increased rapidly during the first 2 hr and again near the completion of drying.

At the end of drying (20% H_2O) all samples contained in the combined state more than 80% of the total residual SO_2. The percentages of SO_2 in the combined form at any particular time of drying were reasonably similar for the three concentrations of SO_2 used, despite differences in the total amounts absorbed at these levels.

Storage. A number of dried samples from these experiments were stored at 1.1°C for 4 months, and their free, combined, and total SO_2 contents were then determined again. The values obtained differed only slightly from the corresponding initial levels, and showed no consistent trend, the observed differences being reasonably accounted for by sampling variations alone.

DISCUSSION

Melean *et al.* (1964) adduced evidence that during sulfuring, gaseous diffusion in the tissue could be an important factor controlling the absorption of SO_2 by fruit before it was dried; but they recognized that solution of SO_2 in the liquid phase and chemical combination with components of the tissue may also provide alternative mechanisms.

Results presented in this paper show that during sulfuring, SO_2 combines to a slight-to-moderate degree with components in the fruit, the quantity combined at this stage depending mainly on the time of exposure; but it has also been shown that most combination occurs during subsequent drying of the fruit. As SO_2 is readily soluble in water, in which its concentration can range from 7% w/w at 25°C down to 5% w/w at 40°C, this gas can dissolve freely in the aqueous phase of raw fruit containing 82–87% water. Since the pH of apricot and peach tissues and juices usually lies between 3.5 and 4, it follows from the results of Vas and Ingram (1949) that about 95% of the ionic species originating from SO_2 in aqueous solution will be bisulfite ions (HSO_3^-), minor amounts of sulfite ion (SO_3^{--}) and undissociated sulfurous acid (H_2SO_3) also being present.

Ash and Reynolds (1955) showed that apricots and peaches contain the same sugars in roughly the same pro-

Table 4. The influence of gaseous SO_2 concentration during sulfuring on the levels of free and combined SO_2 in peaches as they were dried.

Drying time (hr)	Concentration of gaseous SO_2 during sulfuring (v/v) 0.5% Free SO_2[1]	Bound SO_2[1]	% of total bound	1.0% Free SO_2[1]	Bound SO_2[1]	% of total bound	2.0% Free SO_2[1]	Bound SO_2[1]	% of total bound
0	7140	420	5.6	14270	520	3.7	25650	470	2.9
1	2650	1210	31.0	4320	1360	23.3	6110	2170	26.2
2	2020	1380	39.0	2880	2090	31.0	3510	3280	34.1
3	960	1170	52.2	2300	1850	43.9	4010	3270	44.1
4	1260	1330	51.4	2120	2100	49.8	3270	3430	51.2
6	1430	1150	46.2	1660	2270	52.1	2600	3160	57.1
11	910	1250	57.9	1510	2250	59.1	2570	3650	60.1
14	360	2180	89.3	500	2870	85.2	920	4800	83.3

[1] Free and combined SO_2 contents of peaches are expressed in ppm, water-free basis.
After 14 hr, drying was stopped overnight for a period of 16 hr (water level in peaches about 40%) and then continued for a further 3 hr.

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portions. Sucrose predominates, at over 3% reckoned on the fresh weight of the fruit, while on the same basis the quantities of other sugars may be: glucose, between 1.5 and 3%; fructose, up to 1.5%; and xylose, below 0.2%.

Since Ingram and Vas (1950) found that unheated sucrose and fructose solutions did not combine with SO_2, and since apricots and peaches are sulfured at the relatively low temperatures between 25 and 50°C, the major reaction initially binding SO_2 in the fruit is likely to be that between glucose and SO_2.

Braverman (1953) prepared a glucose-bisulfite addition compound *in vitro*, and Ingles (1959) has prepared a series of aldose-bisulfite compounds by reacting potassium bisulfite with a range of aldose sugars that included glucose. In both instances, reaction temperatures were kept below 37°C.

Theoretically, in the reaction:

$$HSO_3^- + \text{glucose} \underset{k_1}{\overset{k_2}{\rightleftharpoons}} \text{glucose bisulfite}$$

one molecule of glucose reacts with one molecule of SO_2.

Working with pure solutions, Vas (1949) found that the equilibrium constant K (i.e. k_2/k_1), defining the steady state of this reversible reaction, depended decisively on pH, and was lowest and almost constant between pH 3.0 and 5.5, a range which generally includes the pH of both apricots and peaches. Vas also found that K was independent of reactant concentrations and that of the total SO_2 present in the tissue at pH 3.9, the proportion in the combined form was 64.1%.

Though it is uncertain whether such theoretical parameters could validly be applied to practical sulfuring, calculations on fruits containing 3% glucose (0.16*M*) would have a hypothetical maximum of 6,500 ppm of combined SO_2. In fact, in our work the highest levels observed towards the end of sulfuring were about 2,000 ppm, but levels were still rising linearly with time when the sulfuring runs were terminated.

Ingram and Vas (1950) found the amount of SO_2 combined in concentrated orange juice to be always higher than that theoretically calculable as the maximum that could be bound by the glucose present, and therefore concluded that other compounds, notably aldehydes and pectin, were also involved. However, Braverman (1953) showed that when a constant glucose level was maintained while the total soluble solids content was progressively raised by adding sucrose (a sugar unreactive to SO_2), the percentage of combined SO_2 increased proportionately. Subsequently Joslyn and Braverman (1954) reexamined the data of Ingram and Vas and concluded that the degree of combination of SO_2 depended on the total concentration of soluble solids in the food.

Since in the present work, non-irrigated fruits invariably contained more total soluble solids (13–15%) than those grown under irrigation (10–11%), it might be expected from Braverman's observations that the former would have bound more SO_2 during sulfuring. Nevertheless, we found that after a certain period of sulfuring, the level of combined SO_2 was higher in the irrigated apricots (Fig. 4) and, moreover, the ratio of combined to free SO_2 was always higher in such fruit. The obviously higher porosity of irrigated fruit probably permits SO_2 to penetrate the fruit pieces better, thereby reaching the available reaction sites more readily. If this were so, the cellular structure should prove to be a rate-limiting factor.

The view that cellular structure is likely to limit the uptake rate is supported by the steep SO_2 concentration gradients observed in sulfured fruit tissue by McBean *et al.* (1964). However, the precise reasons for differences shown by irrigated and non-irrigated fruits in their response to sulfuring have yet to be elucidated.

McBean *et al.* (1965) noted that SO_2 retention (SR) during drying was greater in irrigated than in unirrigated fruit and was increased by extending the time of sulfuring. An explanation of these observations, based on results given, is that concomitantly with a relatively slow loss of bound SO_2 during drying, a rapid loss of free SO_2 in the fruit occurred.

The changing concentration and distribution of SO_2 during sulfuring and drying and the complex systems within fruit tissue do not permit reliable estimation of the rate of combination from the present results. Nevertheless, in view of the experiments of Braverman (1953), which indicate that the tendency of fruit to combine with SO_2 depends largely on the total solids concentration of the fruit, it is not surprising to find that 80–90% of the total SO_2 present in fully dried fruits is in the combined state.

The greatly increased SO_2 retention caused by steam blanching sulfured fruit (McBean *et al.* 1965) cannot be due to changes in levels of combined SO_2, since the heating times of 10–20 sec are too short to influence this. A probable explanation is the establishment on the exterior of the fruit of an SO_2-denuded barrier that inhibits loss of SO_2 during drying.

While gaseous diffusion of SO_2 in fruit tissue must be the initial step in absorption, these experiments suggest that solution of the gas in the liquid phase and penetration due to concentration gradients, as well as combination with components of the fruit, are important mechanisms in the process of sulfuring. The degree of combination of SO_2 during sulfuring has a large influence on the retention of this preservative during drying.

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Чувствительность определения МФДА 0,5 *мкг* в анализируемом объеме или 0,6 *мкг* в объеме пробы. При отборе 40 *л* воздуха данная методика позволяет обнаружить 0,015 *мг* МФДА в 1 *м*³.

Метод неспецифичен в присутствии анилина и других ароматических аминов. Определению не мешают аммиак, гидроксиламин, этилендиамин, триэтаноламин и этанол.

Разработанный метод можно также применять для определения суммы ароматических аминов в газо-воздушной среде. В отличие от описанных в литературе колориметрических методов обнаружения ароматических аминов, базирующихся на реакциях с образованием индофенолов и индаминов, использованная нами реакция конденсации с ДМАБ является быстрой, достаточно чувствительной и вместе с тем не требует добавления большого числа реактивов.

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К ВОПРОСУ О ВРЕДНОМ ДЕЙСТВИИ ОКИСИ УГЛЕРОДА, СЕРНИСТОГО ГАЗА И ИХ КОМБИНАЦИИ НА ПЛОДОВИТОСТЬ САМОК КРЫС

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Известно, что окись углерода и сернистый газ являются практически постоянными загрязнителями атмосферного воздуха современных городов, в связи с чем вопрос о возможном гонадотоксическом действии этих соединений представляет несомненный интерес.

Белых крыс (по 10 в каждой серии) 72 дня ингаляционно затравляли окисью углерода в концентрациях 2 и 1 *мг/м*³ и сернистым газом в концентрациях 4 и 0,15 *мг/м*³, а также смесью этих веществ — 2 и 4 *мг/м*³ соответственно. Все указанные концентрации реально встречаются в атмосферном воздухе современных городов, концентрация окиси углерода 1 *мг/м*³ утверждена в качестве среднесуточной ПДК для атмосферного воздуха, концентрация сернистого газа 0,15 *мг/м*³ считалась среднесуточной ПДК в период, когда проводились наши исследования.

Плодовитость животных неразрывно связана с нормальным функционированием половых желез, поэтому эстральный цикл является одним из удобных показателей плодовитости самок. Однако наблюдения над эстральным циклом не заменяют необходимости прямого изучения плодовитости, ибо наличие нормального цикла еще не служит полной гарантией нормальной плодовитости.

При исследовании эстрального цикла мы использовали метод влагалищных мазков. Пробы брали ежедневно.

При изучении плодовитости к затравляемым самкам подсаживали «чистых» самцов, регистрировали первый день беременности, ее продолжительность, количество особей в помете, первоначальный вес крысят и дальнейшее его увеличение в динамике. Для определения отдаленных последствий действия веществ исследовали изменение эстрального цикла у потомства первого поколения подопытных самок, скрещенных также с «чистыми» самцами.

Сернистый газ в концентрации 0,15 *мг/м*³ не оказал действия на ход полового цикла. Более высокие концентрации сернистого газа (4 *мг/м*³), окиси углерода (1 и 2 *мг/м*³), а также смесь этих веществ (4 и 2 *мг/м*³ соответственно) с первого месяца затравки вызвали нарушения полового цикла, что выразилось в достоверном уменьшении стадии диэструс и метаэструс, в некотором увеличении стадий проэструс и в резком увеличении стадии эструс по сравнению с контрольной группой. На 3-м месяце эксперимента в группе животных, затравляемых смесью веществ, вдвое увеличился процент появления стадии эструс и в 4 $^1/_2$ раза уменьшился метаэструс. Следовательно, отмечается прямая зависимость тяжести изменения от концентрации веществ и времени затравки.

Для оценки нормального хода полового цикла мы учитывали также последовательность определенных стадий и продолжительность каждого цикла. Средняя продолжительность цикла на 2-м и 3-м месяцах затравки увеличилась во всех опытных группах (кроме группы, подвергавшейся действию 0,15 *мг/м*³ сернистого газа); более выраженным было действие смеси.

Исследования плодовитости проводили на самках, имевших нормальный цикл в фоновых исследованиях и затравленных указанными концентрациями окиси углерода, сернистого газа и их смеси.

Сернистый газ в концентрации 0,15 *мг/м*³ не оказал влияния на процент оплодотворенных самок в группе, средний срок беременности и прирост веса крысят в динамике.

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В других опытных группах отмечены существенные изменения этих показателей, особенно в группе, где на крыс воздействовали смесью изучаемых веществ. В той же группе имелись мертворожденные крысята, чего не наблюдалось в других группах.

В приросте веса крысят отмечено существенное отставание по сравнению с контролем с 7-го и 14-го дня после рождения во всех опытных группах, за исключением той, которая подвергалась воздействию наименьшей концентрации сернистого газа.

Общим для всех указанных групп является увеличение количества особей в помете (при подсчете родившихся крысят и эмбрионов на вскрытии самки на 17-й день беременности), что хорошо коррелирует с увеличением стадии эструс и может указывать на гормоностимулирующее действие изученных нами веществ.

Изучение эстрального цикла у потомства первого поколения показало, что эстральный цикл был достоверно изменен во всех группах, кроме той, которая находилась под воздействием сернистого газа в концентрации 0,15 *мг/м*³. Эти изменения аналогичны описанным изменениям материнского организма. Эти сдвиги также выражены в потомстве, полученном от самок, затравленных смесью окиси углерода и сернистого газа. Отмеченные нами изменения оказались стойкими. Восстановление нарушенных функций эстрального цикла в группах, подвергавшихся воздействию высоких концентраций сернистого газа и окиси углерода (4 и 2 *мг/м*³), произошло только на 190-й день после затравки; в группе же, затравка которой проводилась смесью, не достигнуто полного восстановления.

Гистологическое изучение органов самок крыс показало, что в группе животных, подвергшихся воздействию сернистого газа в концентрации 0,15 *мг/м*³, все органы функционировали нормально, патологических изменений не было. В группе, находящейся под воздействием смеси окиси углерода и сернистого газа в концентрациях 4 и 2 *мг/м*³ соответственно, отмечались воспалительные процессы в яичниках, уменьшение количества примордиальных фолликул, что в свою очередь привело к изменению плодовитости животных. У них же была понижена функция надпочечников и щитовидной железы и повышена функция гипофиза.

В группе животных, подвергавшихся воздействию окиси углерода в концентрации 1 *мг/м*³, структура яичника была сохранена, отмечались все стадии развития половой клетки. Имелась лишь тенденция к увеличению функции гипофиза.

Таким образом, сернистый газ и окись углерода вредно действуют на эстральный цикл и плодовитость животных, оказывают гормоностимулирующее влияние, что может находиться, очевидно, в зависимости от увеличения функции гипофиза.

Наибольшее действие отмечено при суммарном использовании сернистого газа и окиси углерода в повышенных концентрациях.

Утвержденная среднесуточная предельно допустимая концентрация окиси углерода в атмосферном воздухе (1 *мг/м*³) оказалась не безразличной для экспериментальных животных. Однако для ее пересмотра необходимы дальнейшие исследования.

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О КОМПЛЕКСНОМ ИЗУЧЕНИИ ЗАГРЯЗНЕНИЯ АТМОСФЕРНОГО ВОЗДУХА В ГОРОДАХ С РАЗВИТОЙ НЕФТЯНОЙ И НЕФТЕХИМИЧЕСКОЙ ПРОМЫШЛЕННОСТЬЮ

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Оценивая загрязнение атмосферного воздуха в селитебных районах, специалисты по коммунальной гигиене всегда дают рекомендации по оздоровлению воздушной среды, однако часто эти рекомендации носят общий характер из-за отсутствия конкретных данных по гигиенической характеристике изучаемого производства или комплекса производств в соответствующем плане. Поэтому необходимо комплексное изучение вопросов, связанных с влиянием выбросов предприятия на состояние воздушной среды как на его территории, так и в селитебных районах. Опыт, накопленный Уфимским научно-исследовательским институтом гигиены и профзаболеваний, показывает не только правомерность, но и преимущества подобной организации исследований.

В работах проводилось параллельное изучение состояния воздушной среды на площадках нефтеперерабатывающих и нефтехимических предприятий и в селитебных районах ряда городов РСФСР.

В ходе обследования площадок заводов определялись состав газовыделений, источники выбросов в атмосферу, оценивались мощность и значимость каждого источника в

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TOXICOLOGIC STUDIES IN THE FUNCTIONAL CONDITION OF THE PITUITARY AND ADRENAL BODY ENDOCRINE SYSTEM UNDER ASSOCIATED EFFECTS OF LOW-GRADE CARBON MONOXIDE AND SULFUR DIOXIDE CONCENTRATIONS

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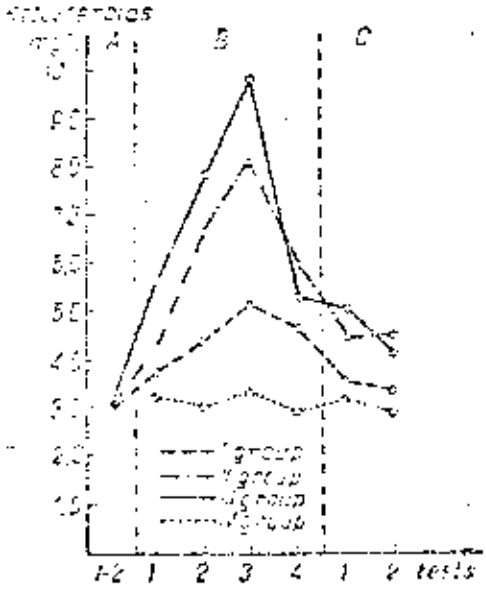
In recent years, the important role of the endocrine glands, particularly that of the pituitary and adrenal body system in defence and adaptive reactions of the organism against various chemical, physical, bacterial, allergic, and other noxious factors, has been emphasized time and again (2, 8, 13, 16, 17). In toxicologic studies, the high sensibility of the pituitary and adrenal body endocrine system is generally stressed, with insistence on the convenience and on the necessity of researching into such microfactors (4, 9, 12). Bibliographical data indicate that studies in the functional condition of that endocrine system are rather scarce and incomplete in chronic carbon monoxide and sulfur dioxide poisoning. Some research workers, at the end of their experimental investigations, tested the adrenal weight factors, ascorbic acid content, and histopathologic picture of the adrenal cortex in carbon monoxide and sulfur dioxide poisoning separately, moreover in high concentrations. As such experimental studies have been but accessory ones to their main points, they virtually imply incompleteness and discrepancy. Authors state now a hyperfunction, now a hypofunction of adrenal cortex, being at variance with each other. Such differences result from unequal concentrations applied, various phases of testing and duration of effect.

This paper deals with the results from studies in the functional condition of the pituitary and adrenal body endocrine system of experimental animals exposed to chronic carbon monoxide, and sulfur dioxide action, separately and in association, their concentrations having been chosen so as to approximate those found in atmospheric air over centres of industry in Bulgaria. The effect of these concentrations has not been studied as yet with regard to the adaptation and reactivity of the organism to them as well as to other associated harmful effects, say infections, intoxications, allergic diseases, etc.

To assess the functional condition of the anterior lobe of the pituitary gland and that of the adrenal cortex, the following test values have been put to use: 1) 17-ketosteroid excretion in twenty-four hour urine as determined after Norimbergski's method (5); 2) l-ascorbic acid content in the adrenals after Tillmanns's method, in its modification proposed by Birg,

Harris and Ney (7); 3) the weight of the adrenals; 4) eosinophil count in a blood-counting chamber after Dunger (11). Experimental animals were poisoned continuously 24 hours on 72 consecutive days. Prior to the beginning of poisoning, the 17-ketosteroid excretion and the count of eosinophiles were determined two times in order to ascertain basal level. Subsequent tests were carried out, at 18-day intervals, on the 12th, 36th, 54th, and 72nd day, respectively, from the beginning of poisoning. In the restoration period, after discontinuance of poisoning, two more tests were performed at 18-day intervals. Weight factors and ascorbic acid content of adrenals were determined on the 48th and 72nd day of poisoning, respectively, the experimental animals having been sacrificed by decapitation. All the data obtained were processed statistically as to their significance by means of the method of dispersion analysis (10).



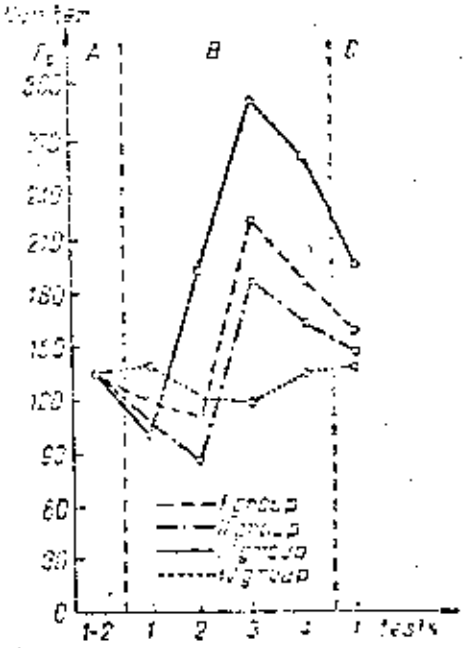
Diag. 1. Excretion of 17-ketosteroids in twenty-four hour urine: A — background; B — experimental period; C — period of restoration

Experimental animals — 40 male white rats, weighing on the average 110 g each — were divided into 4 groups: Group 1 was acted upon with carbon monoxide in concentrations of 6 mg±0.02 mg per cu. m of air; group 2 — with sulfur dioxide in concentrations of 0.06 mg per cu. m±0.0 mg; group 3 — with carbon monoxide and sulfur dioxide in association, their concentrations remaining the same; group 4 comprised control animals. These concentrations are six times as high as permissible limit concentrations in the free atmosphere and from twenty to thirty times lower than toxic ones with respect to working place environment. The animals were acted upon in a special dynamic chamber with secured microclimatic conditions in the zone of comfort, as expressed composite in the effective equivalent temperature of 15.80±2.11° and catadegree of 5.55±0.46 meal/sec.

Findings showed an early and intense increase of 17-ketosteroids already at the first testing from the beginning of the experiment, in all the groups exposed to poisonings, a fact indicative of a stress of adaptive mechanisms which is a potentiality to every change of the reactivity of the organism. Thus, for instance, in tracing the 17-ketosteroid excretion in group 1, we found a progressive increase from 2.00 mg% to 3.88 and 4.45 and 5.25 mg% on the 19th, 36th, and 54th day, respectively, followed by some diminution to 4.75 mg%, at the end of the experiment. In group 2, changes of 17-ketosteroids took a similar course; they proved more strongly marked, however, increasing from 2.06 mg% to 3.79, 6.57, 7.78 mg% on the 19th, 36th, and

54th day, respectively, then dropping down to 5.60 mg% at the end of the experiment. Carbon monoxide in association with sulfur dioxide called forth the most intense increase of 17-hetosteroids: from 2.97 mg% to 5.73 7.82/9.57 mg%, respectively, diminishing to 5.40 mg% at the end of the experiment. In the control animals, the excretion of 17-hetosteroids changed but slightly within the limits from 2.91 to 3.31 mg%. One can gather from the comparison of these data that the effects of carbon monoxide and sulfur dioxide on the function of the adrenal cortex associate additively.

Weight control of adrenals on the 40th day showed an increase by 16 per cent in group 1, by 9 per cent in group 2, and by 20 per cent in group 3, when compared to that of the adrenals of control animals. On the 72nd day, i. e. when poisoning was discontinued, the weight of adrenals had diminished by 20 per cent in group 1, by 17 per cent in groups 2 and 3, respectively, while in the control group it had increased by 7 per cent. According to bibliographical data, hypertrophy of adrenal cortex as a whole bears a direct proportional relationship to the intensity of hyperfunction of the anterior lobe of the pituitary gland. Therefore, under the conditions of our experiment, we can assert with good reason that the pituitary gland participates in the complex of physiological and pathological reactions, of the organism against the carbon monoxide and sulfur dioxide effects, respectively. In this index value too, their effects associate additively.



Diag. 2. Eosinophiles in the blood: *A* — background; *B* — experimental period; *C* — period of restoration

The content of l-ascorbic acid in the adrenals, when tested on the 40th day, had diminished by 21 per cent in group 1, by 29 per cent in group 2, and by 31 per cent in group 3, respectively, in comparison with that of the control animals. At the end of the experiment, l-ascorbic acid level in the adrenals rose in group 1 by 27%, in group 2 by 43%, and in group 3 by 28%, while in control animals by 4 per cent only. The decrease of l-ascorbic acid levels towards the 40th day, along with the other index values, is quite indicative of hyperfunction of the anterior lobe of the pituitary gland and of the adrenal cortex, respectively, under the influence of carbon monoxide and sulfur dioxide. The associated effect of the latter is not distinctly additive with regard to this index.

The absolute number of eosinophilous white blood cells, according to bibliographical data (13,20), is an easy and sensitive test to determine both

the functional condition and potentialities of the adrenal cortex. It has been stressed as well that, under the influence of an intense 17-oxykorticosteroid secretion, a permanent and characteristic diminution of the number of eosinophilous cells in the blood takes place. Bibliographical data stress that their numbers vary depending on the functional condition of the bone marrow, reticulo-endothelial system, thyroid gland, on the allergization of the organism, etc. When testing the absolute number of eosinophiles on the 18th and 36th day, we found in group 1 a diminution from 112 down to 102 and 95 cells, respectively, in group 2 from 154 to 118 and 96 cells, in group 3 from 97 to 74; towards the 36th day, however, there was already an increase to 152 cells. In the control group, variations remained within the limits of 147 down to 115 cells, i. e. they were insignificant. These changes are indicative of an intensified function of the adrenal cortex under the carbon monoxide and sulfur dioxide influence. Testing on the 54th day showed increased numbers of eosinophiles: in group 1 from 96 to 181, in group 2 from 95 to 208, and in group 3 form 74 to 210. As test findings (17-ketosteroid index values, weight factors of adrenals, l-ascorbic acid content) showed towards the 54th day, there was a hyperfunction of the pituitary and adrenal body endocrine system; the increase of eosinophiles can be explained by changes in the allergic reactivity of the experimental animals under the influence of carbon monoxide and sulfur dioxide. Testing of the histaminase activity level yields data which back the assumption of such changes of the allergic reactivity.

The 17-keto-steroid index values, adrenal weight factors, l-ascorbic acid-content of the adrenals, and the absolute number of eosinophiles in the blood, all of them indicate explicitely the active participation of the pituitary and adrenal body endocrine system in the complex of physiological and pathological reactions of the organism, even when carbon monoxide and sulfur dioxide are in very low concentrations, far below the toxic ones. The diminution of 17-keto-steroid excretion, adrenal weight factors and the increase of l-ascorbic acid as well as the normalization of the histologic picture towards the end of the experiment may suggest that the organism of the experimental animals, in this adaptive reaction against the effect of the toxic gases tested, does not stand any longer in need of the potentialities of the pituitary and adrenal body hormonal system, though they are not at all exhausted, and engages other adaptive mechanisms. The early, clear-cut functional changes traced by the indices of the pituitary and adrenal body system, non-specific as they are, proved to be an index by far more sensitive than a number of other tests we assayed, such as protein fractions, glutathion, coproporphyrine, phagocytic index, cholinesterase, neuromuscular irritability, etc. Test values of the functional condition of the pituitary and adrenal body system indicate explicitely that the carbon monoxide and sulfur dioxide effects associate additively. These indices can be used for the assessment of the summary effect on the organism of many homogeneous and even heterogeneous noxious factors after the uniform adaptive reaction of the organism (general adaptation syndrome). This is a matter of present-day practical importance, as in recent years the task of undertaking a hygienic assessment of harmful factors has been put into the foreground as a complex, and not only

separately. On account of the variety of such complexes of noxious factors and their intrinsic nature, it is often difficult, sometimes even impossible to study their ingredients separately. In working out such problems, the testing of the functional condition of the pituitary and adrenal body system as well as of the general adaptation syndrome can turn in information on the summary effect of complexes of harmful factors.

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ВЫСШИЙ МЕДИЦИНСКИЙ ИНСТИТУТ — СОФИЯ
КАФЕДРА ГИГИЕНЫ С КЛИНИКОЙ ПРОФЕССИОНАЛЬНЫХ ЗАБОЛЕВАНИЙ
Зав. проф. д-р Б. Сланков

ТОКСИКОЛОГИЧЕСКОЕ ИСПЫТАНИЕ ФУНКЦИОНАЛЬНОГО СОСТОЯНИЯ ЭНДОКРИННОЙ СИСТЕМЫ ГИПОФИЗА И НАДПОЧЕЧНИКА ПРИ КОМБИНИРОВАННОМ ВОЗДЕЙСТВИИ ОКИСИ УГЛЕРОДА И ДВУОКИСИ СЕРЫ В НЕЗНАЧИТЕЛЬНЫХ КОНЦЕНТРАЦИЯХ.

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РЕЗЮМЕ

Автором испытано комбинированное действие окиси углерода и двуокиси серы (в концентрациях 6 мг/м³ и 0,96 мг/м³) на функцию эндокринной системы гипофиза и коры надпочечника. Подопытные животные находились во время опыта в специальной динамической камере круглые сутки в течение 72 дней. Подвергались испытанию следующие показатели: выделение 17-кетостероидов в суточном количестве мочи, весовые коэффициенты, содержание витамина С в надпочечниках и абсолютное число эндоцифалов в крови. Установлено активное и раннее участие эндокринной системы гипофиза и коры надпочечника во физиологических и патологических ответных реакций организма. Автор указывает на высокую чувствительность исследуемых показателей для охарактеризирования данного состояния компенсаторных и адаптивных возможностей организма. Эффект комбинированного действия окиси углерода и двуокиси серы расценивается автором как аддитивный.

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The Effect of SO_2 upon the Sensitization with Albumin through the Airway in Rats

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There have been only a few publication on the effects of SO_2, a common pollutant in industrial and community atmosphere, from the immunological point of view. In 1908, Ronzani[1] reported that exposure to 500 ppm of SO_2 for a month lessened the production of antibodies and agglutinins against subcutaneously or intraperitoneally introduced typhus-toxin. Recently, Navrotzky[2] and Erban[3][4][5] described that chronic exposure to SO_2 of low concentrations reduced the antibody formation in rabbits and guinea pigs. Miyamoto[6], however, noted the enhancement of airway sensitization in guinea pigs by high concentration of SO_2.

The purpose of the present study was to know what effect SO_2 might have upon sensitization through the airways in rats.

Materials and Methods

Experimental Animals.—Forty-eight female, 4 weeks of age, Sprague-Dawley rats (SD-JCL*) were used for this study in a specific pathogen free (SPF) animal[7] room[8] to exclude the influence of common infectious diseases. To detect the latent infection in rats the provocation test by cortisone treatment**[9] was performed with random samples of rats before and after the experiment, but no signs of illness were observed. None of the searched organisms were detected in these samples by the microbiological examinations listed in Table 1. Using random numbers, rats were numbered and divided into two groups, one group to be exposed to SO_2 and the other group not to SO_2 (control). Two animals of the same number from both groups were always placed together in the same cage during the whole course of experiment except while being exposed to SO_2. Four rats in the SO_2 group were subjected to the pathological examinations. Two of them were killed by cutting the carotid artery immediately after the first exposure to SO_2, and the other two after 7th exposure.

Antigen.—The mixture of egg albumin (EA) (5 × crystalline: N.B.Co., Ohio) and bovine serum albumin (BSA) (Fraction V Powder: Armour Pharm. Co., Illinois) were used as sensitizing antigens, after solved in saline at a concentration of 1% and filtrated with Millipore Filter (HA type, poresize 0.45 $\mu \pm 0.2\ \mu$: Millipore Filter Corp., Massachusetts).

Exposure to SO_2 and Sensitization with the Antigens through the Airways.—One group of rats were exposed to SO_2 of 400 ppm for 30 minutes in a stainless steel exposure chamber, 60 × 90 × 20 cm in size (Fig. 1). The atmosphere in the chamber was changed once per 3 minutes. The room air was sucked through the chamber by a pump, and the controled dosage of dilute SO_2 (1%) joined the air stream before entering

A part of this study was reported at 17th Annual Meeting of the Japanese Society of Allergy in 1967, Kumamoto.

*The animals were considered as clean animals[10] or SPF animals: produced by Nihon CLEA Co., Tokyo.

**Each rat received intramuscularly 15 mg of cortisone acetate: "Curton", Nippon Merck-Banyu Co., Ltd.

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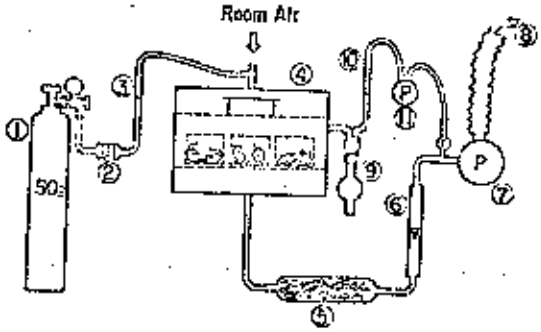
Table 1 Microbiological Examination of Rats*

Materials	Media	Incubating: at 37°C Aerobiosis Period	Objective organisms as specific pathogen
Heart blood Lung Spleen Liver Kidney	1. Blood agar plate (Trypto soya agar[b] containing 10% defibrinated horse blood) 2. Trypto soya agar plate	Aerobic 72 hrs.	*Corynebacterium kutscheri*, *Corynebacterium pyogenes*
Mesenteric lymph node	3. DHL agar[b] plate and Brilliant green agar[b] plate	Aerobic 24 hrs.	
Feces[a]	4.[c] Selenite broth[b] or Hajna's tetrathionate broth[b]	Aerobic 18 hrs.	Salmonella
Brain Middle ear Trachea Lung	5. Modified Edward's medium[11] Over-lay culture method Plate culture method	Aerobic 3—10 days 5%CO_2, 95%N_2 14 days	Mycoplasma

a) Feces were inoculated to the listed media 3 and 4, only.
b) Commercially dehydrated products by Nissui Seiyaku Co., Ltd., Tokyo.
c) After incubation, a loopfull was transfered to DHL agar and brilliant green agar plates.
* Performed by Dr. S. Yamazaki under the guidence of Dr. M. Murata, Department of Microbiology, the Institute of Public Health.

Fig. 1. The apparatus for the exposure and the sampling of SO_2-air mixture.



① SO_2 cylinder
② Glass filter
③④⑤ Flow meter
④ Exposure chamber
⑥ SO_2 absorber
⑦ Exhaust pump
⑧ Exhaust pipe on the ceiling
⑨ Impinger
⑩ Sampling pump

the chamber. After the completion of the exposure to SO_2 they were put together with the other group (control rats) in the chamber for the inhalation of antigen aerosols nebulized for 90 minutes by two nebulizers of Vaponeplrin type. Diameters of the most aerosols were considered smaller than 2 μ from a preliminary experiment. This treatment was repeated 7 times at intervals of a few days. Four weeks after the last inhalation of antigens all rats were bled and their sera were stored at −25°C until serological analysis. Times for the inhalation of antigens and the interval between the last inhalation and bleeding were determined by a preliminary experiment as described later in discussion.

Serological Analysis.—For the detection of antibodies against EA or BSA four techniques were used: a) double diffusion precipitation tests (Ouchterlony)[12], b) the passive cutaneous anaphylaxis (PCA)

on the skin of mice, c) the indirect hemagglutination (HA) tests with tannic acid and antigen-treated sheep red blood cells (Boyden)[13][14], d) and radioimmunoelectrophoresis using ^{131}I-labeled antigens. The iodination of antigens was carried out as described by Yagi et al[15]. Immunoelectrophoresis was performed by Scheidegger's method[16]. In each slide a well in one side was filled with a serum of SO_2 group and a well in the opposite side with that of a control rat of the same number. After completing the electrophoresis of rat sera, a channel was cut and filled with the mixture of rabbit anti-rat serum and the ^{131}I-labeled antigen. The precipitate arcs were allowed to develop for 40 hrs. in moist chambers. The slides were washed with several changes of buffered saline (35 hrs.), and dried at 37°C. Radioautographs were obtained by placing x-ray films (Sakura x-ray film, Konishiroku photo Ind. Co., Tokyo) in contact with the slides.

Results

General Observations on the Behavior of the Animals.—Immediately after the initiation of the exposure to SO_2, the rats became uneasy, struggled, and sneezed rubbing their eyes or noses with paws. These signs of irritation subsided in two or three minutes and the animals appeared to adapt themselves to the adverse conditions. No changes of behavior was observed by the inhalation of antigens.

Changes of Body Weight.—As shown in Fig. 2 there was no significant difference in body weight between the SO_2 group and the control group.

Serological Analysis.— a) In the double gel diffusion tests any arcs of precipitate appeared against neither EA nor BSA. b) No positive PCA reaction was found on the skin of the mice, even when undiluted serum was used. c) In HA test, among 17 rats exposed to SO_2 3 sera showed positive hemagglutination with EA and 4 sera, with BSA; whereas among 17 controls no sera made positive reaction with EA or with BSA

Fig. 2. Changes of mean body weight (± standard deviation) of each group. Seven arrows at the bottom show the exposure to SO_2.

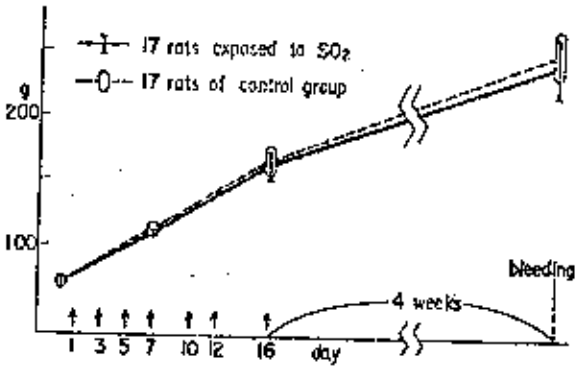


Table 2 Results of Indirect Hemagglutination Test

Antigen	Group	+*	−**	total
Egg Albumin	SO_2	3	14	17
	Control	0	17	17
Bovine Serum Albumin	SO_2	4	13	17
	Control	0	17	17

* Serum titer were between 1 : 40 and 1 : 320.
** Negative still at 1 : 20 dil.

(Table 2). The difference in these figures between the two groups is statistically significant at 11.3% level (EA) or at 5.1% (BSA) (by Fisher's exact probability test[17]). d) On the radioautographs (Fig. 3), vague radioactivities were seen on the arcs of albumin in all slides, which were considered of nonspecific binding between ^{131}I-antigen albumin and rat albumin. Different radioactivity on the long arc of 7s γ-globulin was found in some cases. It was considered to be specific binding between the ^{131}I-labeled antigen and an antibody as described later in discussion. As shown in Table 3, radioautography revealed that 12 of the 15 rats exposed to SO_2 and 7 of the 15 control rats had the antibodies against EA, and that 12 of the 15 exposed and 4 of the 15 control rats had the antibodies against BSA. The statistical difference

Fig. 3. An Example of Radioimmunoelectrophoresis. In the radioautograph a strong radioactivity is found on the long arc of γ-globulin in the SO_2 side but not in the control side although the patterns of arcs on the stained slide are practically the same. The weak radioactivity on the arcs of albumin in the both sides is considered of nonspecific binding.

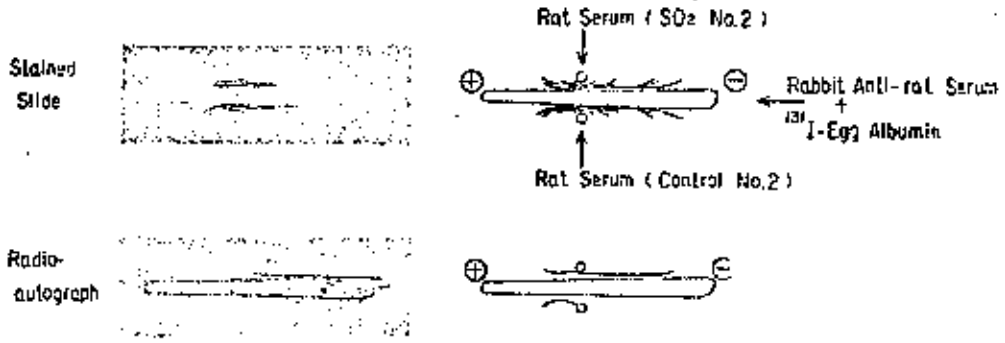


Table 3 Results of radioimmunoelectrophoresis

Antigen	Group	+*	−	total
Egg Albumin	SO_2	12	3	15
	Control	7	8	15
Bovine Serum Albumin	SO_2	12	3	15
	Control	4	11	15

* The radioactivity on the arc of γ-globulin was found on the radioautograph.

between the two groups is significant at 6.2% (EA) or 0.5% (BSA) level (by Fisher's exact probability test). On some radioautographs of ^{131}I-BSA there was found a weak arc beside the strong arc of 7s γ-globulin, but it has not been exactly determined in this study whether the arc was formed due to the reaction of γ-globulin of other class or the direct reaction between ^{131}I-BSA and precipitating antibody in rat serum.

Pathological Findings.—The lungs of 2 rats killed immediately after the first exposure to SO_2 showed no gross pathological lesions. In the histological examination of these lungs, no significant damage was found on the epithelial walls from alveoles to large bronchi. There were no signs of inflammation as infiltration or exudation, nor of emphysema. The lungs of 2 rats after 7th exposure to SO_2, however, showed pathological changes. A rat had many spots of hemorrhage on the surface of both lungs. In this animal the microscopic observation revealed an extensive hemorrhage filling alveolar spaces and lumina of small bronchi and bronchioles with partial lung edema and congestion. There were also slight changes on some epithelial walls with mild infiltrations in part. But the other animal after 7th exposure

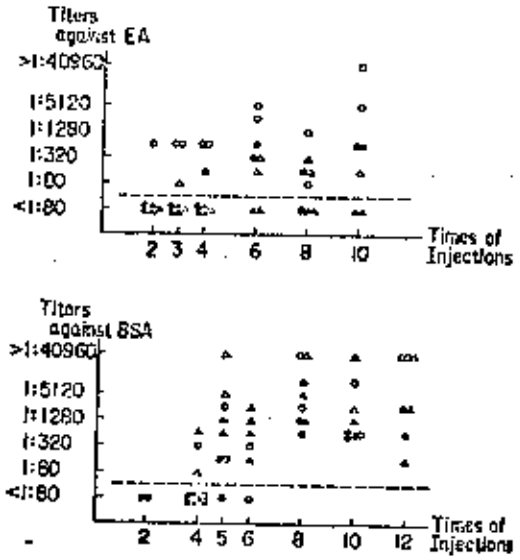
wed no significant pathological findings except only a minor congestion and exudate in alveoles in small parts.

Discussion

It is generally known that rats are difficult to be sensitized with soluble antigens as albumin without adjuvant[8]. A reason why rats were used in this study was because the exposure to SO_2, an adverse condition to the respiratory tracts and ciliary cleaning action, was expected to accelerate the sensitization of rats with albumin under such conditions that the healthy rats would not be sensitized. To find out such conditions a preliminary experiment was performed. Rats were injected intraperitoneally or subcutaneously with the antigens of EA and BSA three times a week. The serum titers were determined by HA test at two and four weeks after the last administration. As shown in Fig. 4 more than six times injections (2.5 mg antigen at a time) were needed to produce enough antibodies, and the titers were higher at four weeks than two weeks after the last administration. Based on those results *seven* times inhalations of antigen and *four* weeks interval between the last inhalation and bleeding were adopted.

In HA test, 3 out of 17 rats exposed to SO_2 showed positive reaction against EA, and 4 against BSA; whereas none of 17 controls reacted positive against EA or BSA. This showed the significant difference in antibody formation between the two groups. As the highest serum titer in HA test was 1 : 320, the serum antibody level of those rats were considered to be very low. It seems reasonable that no antibodies were detected by double gel diffusion tests or PCA on the skin of mice, for these methods were much less

Fig. 4. Sensitization of Sprague-Dawley rats with the injections of egg albumin (EA) and bovine serum albumin (BSA) given 3 times a week. The animals received 2.5mg antigen each time either intraperitoneally or subcutaneously. The number of times of injections is given on the x-axis. The rats were bled 2 and 4 weeks after the last administration. The serum titer by indirect hemagglutination test is given on the y-axis. (A preliminary experiment)



● : 2 weeks after intraperitoneal injections
▲ : 2 weeks after subcutaneous injections
○ : 4 weeks after intraperitoneal injections
△ : 4 weeks after subcutaneous injections

sensitive than HA test (Minimum detectable amount of antibody is about 1,000 times as large[19]). PCA test on the skin of guinea pigs is a very sensitive method as well as HA test when antisera of guinea pigs or rabbits are used, but no positive reactions occur when rat antisera are used[19]. PCA with rat antisera can be made on the skin of rats or mice, but they are not so sensitive.

Radioimmunoelectrophoresis was used as a much more sensitive and powerful method to detect antibodies in this study. It revealed antibodies in many sera that were negative in HA test. The radioactivity on γ-globulin arc was considered to have been developed by the specific antigen-antibody binding with the following reasons; a) the radioactivity on albumin arc was seen in all slides, b) the intenser the precipitate arc is, the more nonspecific binding is considered to occur, c) on the stained slide the intensity of albumin arc was always the highest and γ-globulin arc was very faint, while the radioactivity was much stronger on γ-globulin arc than on albumin arc in many cases, d) there were many other arcs which were intenser than that of γ-globulin but had no radioactivity, and e) the radioactivities on γ-globulin existed much more frequently in SO_2 side than control side, although no significant differences in the immunoelectrophoretic pattern or intensity were seen between the two sides of each slide. Thus it may be justified to compair statistically the difference between the SO_2 group and control group regarding the radioactivity on γ-globulin arc as the revelation of antibody. The radioimmunoelectrophoresis revealed that 12 of the 15 rats exposed to SO_2 and 7 of the 15 control rats had the antibodies against EA, and that 12 of the 15 exposed and 4 of the 15 control rats had the antibodies against BSA. The statistical difference in antibody formation between those two groups proved more significant than in HA test.

Based on these results it may be concluded that the pre-exposure to SO_2 enhanced the sensitization through the airway in rats, which agrees with the results of similar experiments in guinea pigs and rabbits[20], but its mechanisms have not been clarified by this study. Whether short-term exposure to SO_2 as done in this study stimulates the antibody production itself is unknown. SO_2 has been shown to damage the respiratory tracts or change the permeability of epithelial cells. Such histological or functional changes may facilitate antigens to enter the body. The inflammation caused by SO_2 might also enhance the sensitization like the case of Freund's adjuvant. Dalhamn[21] reported that 2 minutes' exposure to 25 ppm of SO_2 brought the ciliary beat in rat trachea to stop, and that the rate of mucous flow was still reduced a month after 18 days' exposure to 10 ppm of SO_2. Exposure to SO_2 of 400 ppm for 30 minutes may inhibit these cleaning action in the respiratory passage so that the introduced antigens could stay in the lung longer.

A direct comparison between the results of the author and that of Navrotzky or Erban is difficult due to the difference in the introduction route of antigen and the duration of exposure to SO_2. However, it is biologically common that noxious agent initially stimulates the cell functions but, as the exposure is prolonged, it acts to depress the functions. Effect on antibody formation may also be alternatively changed depending on the duration of exposure to SO_2. The problems of specificity or concentration of SO_2 must also be studied further.

Summary

The effect of SO_2 upon the sensitization through the airway was investigated in rats. Animals of one group were exposed to SO_2 at a concentration of 400 ppm for 30 minutes, then they were placed together with another group (control rats) in a chamber for the inhalation of antigen aerosols. Nebulized 1% solution of egg albumin (EA) and bovine serum albumin (BSA) was used as the sensitizing antigens. This treatment was repeated 7 times at intervals of a few days. All rats were bled four weeks after the last inhalation of antigens and their sera were stored at −25°C until serological analysis. Four techniques were used to detect antibodies against EA and BSA: double diffusion precipitin tests (Ouchterlony), passive cutaneous anaphylaxis on the skin of mice, hemagglutination tests with tannic acid and antigen-treated sheep red blood cells (Boyden), and radioimmunoelectrophoresis using ^{131}I-labeled antigen. No antibodies were detected by the first and the second methods. Among 17 rats exposed to SO_2, 3 sera showed positive hemagglutination with EA and 4 sera, with BSA; whereas no sera from 17 control rats showed

positive reactions with EA or BSA. Radioautography revealed that 12 of the 15 rats exposed to SO_2 and 7 of the 15 control rats had antibody against EA, and that 12 of the 15 exposed rats and 4 of the 15 control rats had antibody against BSA. It may be concluded from these results that the pre-exposure to SO_2 enhanced the sensitization through the airways in rats. The possible mechanisms are discussed briefly.

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亜硫酸ガス曝露の経気道感作に及ぼす影響

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（受付1月25日，1968）

はじめに

近年，大気汚染の呼吸器に及ぼす影響が社会問題として取り上げられているが，その多くは，慢性気管支炎，気管支喘息，肺線維，肺癌，等々の領域において論ぜられて来た．我々はさきに呼吸器に実質的な障害があると気道を介しての抗原感作がより容易になるのではなかろうかと思われる知見を報告し，さらに大気汚染が気道への障害を介在として免疫学的な面にも影響を及ぼし得るであろうと推定しこれを裏づけるための動物実験を行いその結果，大気汚染性化学物質として最も重視されて

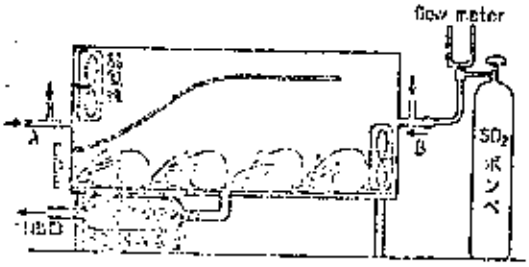
亜硫酸ガスの濃度変化に曝露させると経気道的な感作により容易になるという事実を報告したが，今回は異なつた2種の動物に異なる2種の抗原を用いてより広範に再検討したのでその結果を報告する．

対象及び方法

実験には，いずれも健康な400グラム前後のモルモットと3.0kg前後のウサギを用いた．用いた抗原はウシ血清アルブミンと卵白アルブミンで市販されているものである．

実験に先だち各々の動物より採血し，これ等の抗原に感作されていない事を確かめた．動物を図1.の如き密閉箱に入れ吸引装置で絶えず空気を引き乍らボンベから亜

図1．SO_2 及び抗原吸入装置．



（注）箱内は吸引装置に決まる，抗原の吸入は A及びBのカットから行なった．
C, D, E は SO_2 濃度測定のための採気口

硫酸ガスを流して，300ないし400ppm の亜硫酸ガスに約30分間曝露した．箱の中の亜硫酸ガス濃度はあらかじめの亜硫酸ガスの流量と吸引する流量から適当な濃度を選択し得る様にして置いたが，動物を中に入れると温度が高まり，亜硫酸ガスがそれに吸着又は溶解したり，動物の毛に吸着したりして予期した濃度が得られないので予備実験を繰り返えして，その時々に応じてかなり一定の濃度を保ち得る様にしたが，実験の最中に時々箱の中の数か所から気体を採取し，検知管（光明理化学工業株式会社の製作によるもの）により濃度を測定し，箱内での均一性をたしかめるとともに目的の濃度（300～400ppm）を保ち得る様に心掛けた．使用される亜硫酸ガスは，飽和した苛性ソーダー液に浸し，乾燥させたグラス，ファイバーを通過させる事により出来るだけ吸着させて大気への混入を防いだ．亜硫酸ガスに曝露した直後約同数の対照動物を一緒に同一の箱に入れ，1％前後の卵白アルブミンとウシ血清アルブミンの混合液をネブライザーを用いて約30分間吸入させた．動物は箱の中で自由に動き廻る事が出来るし，箱の両側で扇風機を廻わしてエロゾールが箱の中に均一に行きわたる様にしたので吸入に不均一性は殆んど考えられなかつた．亜硫酸ガスへの曝露と抗原の吸入は一日間隔で7回繰り返えし，最後の吸入から約2週間目に採血して血中の抗体を沈降反応，クンニン酸反応，血球凝集反応[2]，及びPCA (passive cutaneous anaphylaxis[3]) によりしらべた．PCAでは血清の倍々稀釈で同様の反応をしめした最高稀釈濃度を以つて抗体価とみなした．PCAに用いたモルモットは片側に亜硫酸ガスに曝露した動物の血清，対側に対照動物の血清を注射して同一モルモットで同時に比較出来るようにし，皮内反応の個体差にもとずく差を除く様に心掛けた．抗血清はいずれの検査にも倍々稀釈を用いた．そして同様での抗体価の指数（2^n の n）の平均値をt検定で比較し抗体価の有意差の有無をしらべてみた．

表1　両群での抗体価の比較

動物	抗原		動物の数	Boyden 法による血球凝集価 平均値±σ	t	p	PCA反応 平均値±σ	t	p
ウサギ	卵白アルブミン	SO_2曝露	8	$10\times 2^{8.25\pm.66}$ (1920～4800)	4.53	<0.01	$10\times 2^{5.5\pm2.45}$ (41.2～2490)	4.20	<0.01
		対象	7	$10\times 2^{6.14\pm1.11}$ (327～1910)			$10\times 2^{1.0\pm1.51}$ (7.0～57.9)		
	ウシ血清アルブミン	SO_2曝露	10	$10\times 2^{7.2\pm1.17}$ (653～3310)	3.60	<0.01	$10\times 2^{5.13\pm1.36}$ (136～893)	6.97	<0.01
		対象	9	$10\times 2^{3.67\pm2.79}$ (19.1～850)			$10\times 2^{0.57\pm1.14}$ (6.75～32.7)		
モルモット	卵白アルブミン	SO_2曝露	9	$10\times 2^{6.55\pm2.43}$ (174～5040)	3.27	<0.01	$10\times 3^{2.15\pm0.87}$ (41.3～279)	4.18	<0.01
		対象	11	$10\times 2^{5.18\pm1.97}$ (93.2～1420)			$10\times 3^{1.1\pm1.37}$ (7.46～151)		
	ウシ血清*アルブミン	SO_2曝露	9	--	—	—	—	—	—
		対象	11	—			—		

*：本文参照

成　績

沈降反応：いずれの血清もゲル内沈降反応では陽性の結果が得られなかつた．

血球凝集反応：表1にみられる様にウサギでも，モルモットでも，亜硫酸ガスをあらかじめ曝露させておいた群では，そうでない群に比べて血球凝集価に有意に高かつた．モルモットはウシ血清アルブミンに対して抗体を作りにくい事が知られているが，吸入操作に於いても注射と同様抗体価は低く，表にはしめさなかつたが亜硫酸ガスに曝露させなかつた群では1：10の血清稀釈で11匹中7匹が陰性をしめし，陽性をしめしたものでも最高価は80であつた．亜硫酸ガスにあらかじめ曝露させたモルモットでは9匹中5匹が陽性で，陽性の最高価は320であり，ウシ血清アルブミンに対しても亜硫酸ガスの曝露により抗体産生能がたかまるらしい事をしめした．

PCA：血球凝集価と同様PCAでも亜硫酸ガスにあらかじめ曝露させておいた実験動物群では対照群に比して明らかに抗体価が高かつた．ウシ血清アルブミンに対するモルモットのPCAの値は極めて低く表にはしめさなかつたが，亜硫酸ガスに曝露させなかつた群では，陰性は11匹中6匹にみられ陽性であつても最高価は8であつたが，亜硫酸ガスに曝露させた群では少くとも陽性群ではいずれも陽性をしめし，最高価は16であつた．

図2．同一のウサギでのウシ血清アルブミン（BSA）及び卵白アルブミン（EA）に対する血球凝集価（TRBC）とPCA価の比較．

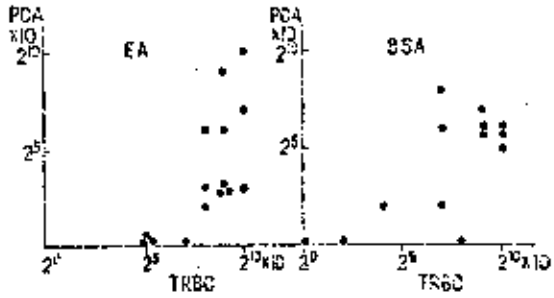
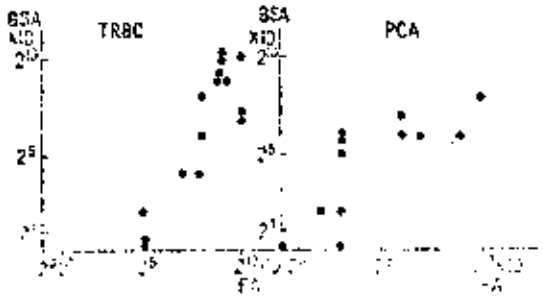


図3．同一のウサギでの血球凝集価（TRBC）及びPCA価のウシ血清アルブミン（BSA）と卵白アルブミン（EA）に対する方法の比較．



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血球凝集反応及びPCAによる抗体価相互間の関係：同一抗原に対する血球凝集価とPCA価とを比較してみると一方に高い価をしめした動物は他方に対しても概して高い価をしめした（図2.）．又同一動物群でウシ血清アルブミン及び卵白アルブミンに対する抗体価を凝集反応とPCAとで比較してみると，一方の抗原に対して高い抗体価を有したものは他方の抗原に対しても抗体産生能がたかいという事が明らかになつた（図3.）．

考　按

近代工業の急速な発展は社会福祉と共に，新らしい病因を人類にもたらして来た．即ち工場施設の拡大にともない空気の汚染が増加し，呼吸器疾病への悪影響が明らかになるにつれ空気汚染は重要な公害の一つとして人々の関心を集めたのである．

以前は空気汚染といえば，降下煤塵が対象になる事が多かつたが，近年，石炭工業から石油工業へと移向して来たため，降下煤塵量はむしろ低下して来た．併し，そのかわり空気汚染性の化学物質，特に亜硫酸ガス，窒素化合ガス，オゾン等が有害物質として重視されて来た．特に我が国においては硫黄化合物の含量の多い中近東の石油を主として使用しているため，多くの大気汚染性物質のうち亜硫酸ガスは特に重視されて来た．今迄，空気汚染を呼吸器系との関連に於いて論ずる場合には主として慢性気管支炎，気管支喘息，肺癌等が問題にされて，免疫学的な面はほとんど顧みられなかつた．我々は空気汚染性ガスの中，特に亜硫酸ガスに注目して，これに曝露する事により抗体産生がたかまり得るのではなかろうかと考えて，モルモットとウサギを亜硫酸ガスに曝露し，その直後に卵白アルブミン及びウシ血清アルブミンを噴霧吸入させて流血中の抗体価を血球凝集反応及びPCAによつてたしかめてみたところ，抗体価は対照に比べて明らかに高かつた．即ち亜硫酸ガスにあらかじめ曝露されると経気道的な抗原に対する抗体産生能がたかまる事が明らかにされたわけである．その理由としては種々の可能性が考え得られるが，その一つである亜硫酸ガスの吸入により生体内の抗体産生細胞の活性が増加させられるのではなかろうかと考えて亜硫酸ガスに吸入曝露した群と，そうでない群とに抗原を皮下注射で与えて，両者の抗体価を比較してみた我々の予備実験では，両者間に統計学的な有意差を認め得る事が出来なかつたので，上記の可能性は否定され得るのではなかろうかと考えられる．即ち亜硫酸ガス曝露の影響は局所作用にもとづくものと考えられ，局所作用としては，1)気道の抗原透過性を昂進させる，2)肺胞内喰食細胞（alveolar macrophage）の喰食能を増加させる，3)気道の線毛運動を抑制する等が抗体産生に影響を及ぼす可能性として考慮されるが，最後の線毛運動の抑制はすでに明らかにされていて，ウサギの気管支をTyrodo液にしたし，ケシの種を指標にして輸送活動（transport activity）を検討した実験では165ppmの亜硫酸ガス濃度に18秒曝露する事により線毛運動は約半分に抑制されるといわれる[1]．従つて線毛運動が抑制されれば吸入した抗原はより長く気道内に停滞し得るわけで，これも抗体産生に関与し得ると考えられる．

亜硫酸ガスに急性曝露した後，肺の組織像をとつてみると，白血球浸潤，急性壊死，鬱血，肺水腫，気管および気管支上皮の剥離等を主徴とする変化が目立ち[2][3]，これ等の変化から気道の障害により気道の透過性が増しているのではなかろうかと考えられるが若しそうであるならば同一の抗原が吸入された場合でも流血中の抗原は増加していて，抗体産生細胞をより刺激するという可能性が考えられる．又，亜硫酸ガスに曝露する事により肺胞内喰食細胞の数が増加したり，喰食能が増したりするならば，これも吸入した抗原を肺内に留めておく事になるので抗体産生機序に関与し得るわけであるが，これ等は現在のところ推測の域を脱しない．これ等両者の可能性についてはなお将来検索の予定である．

この実験に用いた亜硫酸ガスの濃度は，300～400ppmと極めて高濃度で，現在，社会的に大気汚染として問題になつているのは1ppm以下の濃度であるから，それよりはるかに高く，実社会に於いては特殊な場合を除いては遭遇し得ないであろう．併し乍ら気道に変化を来すと経気道的な抗原に対する抗体産生能がたかまるという事は注目すべき事であつて，人間においても肺に実質的な病変があると経気道的に入つて来る抗原に対する抗体価が高くなる傾向があるという事実[4]と相俟つて興味ある事である．ただ，ウシ血清アルブミンに対するモルモットや卵白アルブミンに対するマウスの如く，それ等に対して抗体を産生しにくいものは亜硫酸ガスに曝露しても抗体産生能は高まらないのであつて，人間の場合にもアトピー性の患者ないしは症状を現わしてはいないがレアギン抗体を作り得る者では，気道に障害があると経気道的な抗原に対するレアギン抗体の産生能が高まるであろうと考えられる．併しそういう素因の無いものではたとえ気道に障害があつても経気道的な抗原に対する抗体産生能には特に影響がないのではないかと推察され

る、この想定は，前に報告した喘息様症状を主体とする呼吸器異常患者でもアレルギー性素因のある者では潜在的な炎症状態と考えられる気管内壁に，より顕著されているが特に実質性の病変のあつたものでも，素因のないものでは正常対照群と比較して操作後に有意差を認めないという事実と合致して興味深い．

又，本実験の結果で明らかな如く，一つの抗原の吸入に対して抗体産生能が高いものは，他の抗原に対しても，抗体産生能が高く，抗体産生能の高低は単一抗原に限られるものではなく普遍的なものであると考えられる．

総括及び結論

隔日に7回，モルモット及びウサギを 300ないし 400 ppm の亜硫酸ガスに約30分間吸入し，その直後に卵白アルブミン及ゴウシ血清アルブミンを気管吸入させ，最後の吸入吸入から約2週間後に各々の動物から採血し，血中抗体を沈降反応，タンニン酸処理血球凝集反応及びPCAで検査し，その値を対照動物と比較してみたところ，亜硫酸ガスに暴露した群では対照群に比し血中抗体が明らかに高かつた．即ち気道に障害があると経気道的な抗原に対する抗体の産生能がたかまると考えられた．現在重視されている空気汚染の問題と考え合わせて興味深い．その原因として気道の抗原透過性の亢進，粘膜内吸収細胞の貪食能の上昇，及び補体価等の低下が考えられるが，後者についてはすでに明らかにされていて前二者の可能性については将来検討の予定である．

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LE ROLE DE L'ACIDE SULFUREUX DANS LE PROCESSUS D'OXYDATION PEROXYDASIQUE DE L'ACIDE L-ASCORBIQUE EN PRÉSENCE D'EAU OXYGÉNÉE

par Casimir MONIKOWSKI et Edvige CHMIELNICKA

Introduction.

L'acide sulfureux appartient au groupe des plus anciens agents de conservation des produits alimentaires d'origine végétale. D'un emploi très varié, il assure la stabilisation de produits crus tels que les sucs et les pulpes des végétaux. En général, l'acide sulfureux est doué de propriétés germicides, bactériostatiques. De plus, étant réducteur, il agit comme antioxydant sur les composés chimiques facilement oxydables en présence d'oxygène et notamment sur l'acide L-ascorbique (1).

On sait que les enzymes peroxydasiques contenus dans les tissus végétaux catalysent les oxydations non à partir de l'oxygène moléculaire, mais à partir d'un peroxyde. On distingue ainsi les hydroperoxydases agissant en présence d'eau oxygénée et les hydroxyalcoylperoxydases, qui utilisent comme donneur d'oxygène des hydroperoxydes d'alcoyle du type R-O-OH. La peroxydase du raifort appartient à la première catégorie.

Le mécanisme d'action des hydroperoxydases a été l'objet d'investigations de nombreux chercheurs (3, 4, 5, 6, 7) et a été élucidé par Heimann (8). Cet auteur s'est basé sur les observations cinétiques de Boll, qui montrent que la vitesse d'oxydation de l'acide L-ascorbique est en relation avec la concentration enzyme-substrat EAD qui elle-même dépend de la concentration des constituants E, A, D.

La vitesse V de la réaction enzymatique est donnée par la formule:

$$V = k.E.A.D$$

k est une constante,
E est l'enzyme (peroxydase du raifort),
A l'accepteur (H_2O_2),
D le donneur (acide L-ascorbique).

D'après Heimann le surplus de l'un quelconque des composants peut entraîner l'élimination de l'autre dans le complexe A.E.A.D. et par suite bloquer l'activité de l'enzyme.

Le travail ci-dessous a pour but d'élucider le rôle de l'acide sulfureux dans le mécanisme d'inactivation de la peroxydase du raifort et son influence sur l'oxydation de l'acide L-ascorbique en présence de peroxyde d'hydrogène.

Partie expérimentale :

Matériel et réactifs :

Les essais ont été effectués sur la peroxydase de raifort de « Boeringer », aimablement mise à notre disposition par M. le Dr Lomuller. Ce produit est de couleur brun clair, cristallisé, et contient environ 70% d'enzyme pur, selon les indications de la maison Boeringer.

Nous avons adopté la méthode de Diemair et Hänsser (10) pour évaluer l'activité de la peroxydase. L'acide L-ascorbique a été dosé par la méthode de Tillmans (11). La solution d'acide sulfureux a été préparée en saturant de l'eau bidistillée par l'anhydride sulfureux. La concentration en peroxyde d'hydrogène a été déterminée par la méthode manganimétrique (12). Pour les concentrations plus élevées en H_2O_2 on a procédé à des dilutions de perhydrol Merck à 30 %.

Les solutions tampons acéto-acétiques se sont montrées les plus avantageuses, car à pH 4,7, elles n'exercent pas d'influence sur l'activité de la peroxydase dans un délai de 24 heures, tandis que les tampons phosphato-citrique de pH 4,5 inactivent la peroxydase de raifort en 2 heures et à pH 5 en 7 heures.

Technique générale :

Afin d'étudier l'action inhibitrice de l'acide sulfureux sur la peroxydase dans le milieu complexe : enzyme-accepteur donneur-inhibiteur nous avons expérimenté 6 types de mélange, à diverses concentrations des composants : 1° inhibiteur-enzyme, 2° donneur-enzyme, 3° donneur-enzyme-inhibiteur, 4° accepteur-enzyme, 5° enzyme-accepteur-donneur, 6° enzyme-accepteur-donneur-inhibiteur.

Chacune de ces combinaisons avait pour but de résoudre partiellement le problème, du fait de l'élimination de composants définis, et rendant possible l'interprétation de l'action peroxydasique en l'absence de composants déterminés.

Réactifs :

1° Solution tamponnée aceto-acétique de pH 4,7.
2° Solution de peroxydase à 5 µg par 1 ml dans la solution tampon
3° Solution à 0,1 %, d'acide L-ascorbique dans la solution tampon.
4° Solution de peroxyde d'hydrogène à 0,5 %.
5° Solution d'acide sulfureux à 0,8 %.
6° Solution de 2-6-dichlorophenol-indophenol, 0,001 m
7° Solution d'acide sulfurique à 20 %.

Les solutions ont été préparées extemporanément, exception faite pour les solutions 1 et 7.

Pour nos expériences nous avons utilisé des flacons jaugés de 50 ml.

Après avoir introduit pour chacune des combinaisons, les réactifs indiqués ci-dessus, la solution est complétée à 50 ml avec la solution tampon acéto-acétique. La concentration des substances mises en réaction est comptée pour 1 ml. de solution.

Pour nos essais nous avons utilisé de 0,5 à 1 ml de solution et avons mesuré pendant un temps déterminé l'activité enzymatique de la peroxydase. Les résultats représentent la moyenne de 3 dosages et sont rapportés à 1 ml de solution mise en œuvre.

L'activité est exprimée en millilitres de la solution 0,001 m de 2-6-dichlorophenol-indophénol, selon la méthode de Diemair et Häusser (13). Cette méthode est basée sur la recoloration d'une solution aqueuse de 2-6-dichlorophénol-indophénol décolorée par l'acide L-ascorbique. La peroxydase mise en solution en présence de H_2O_2, oxyde le colorant, qui devient bleu et passe totalement dans l'éther éthylique. En utilisant toujours la même quantité d'éther : 50 ml, on peut mesurer l'activité peroxydasique par photocolorimétrie. Nous avons utilisé le photomètre de Pulfrich avec le filtre S.53.

L'extinction moyenne, après correction « en blanc » et multipliée par 8,76 indique la quantité en millilitres du colorant 2-6-dichlorophénol-indophenol 0,001 m dans 50 ml d'éther éthylique.

Le coefficient 8,76 est un nombre constant, il résulte du rapport entre l'extinction et la concentration du colorant 0 : $C_6H_2Cl_2$: NC_6H_4OH (14).

L'activité de la peroxydase peut être bloquée par l'acide acétique, c'est pourquoi l'éther éthylique est acidifié par l'acide acétique à la concentration de 0,1 ml d'acide acétique à 20 % pour 100 ml d'éther. On peut aussi bloquer l'action de l'enzyme par l'acide sulfurique. Nous avons utilisé ce procédé dans l'essai modèle 5.

Tous nos essais ont été effectués à la température de 20° C.

Comme pour les opérations qui précèdent, le dosage colorimétrique doit être exécuté le plus rapidement possible : nous avons fixé à 30 secondes le temps compris entre la prise d'essai et l'inactivation de la peroxydase par l'éther éthylique acidifié.

La couche éthérée séparée par décantation dans une ampoule à décantation est introduite dans le flacon jaugé de 50 ml et complétée jusqu'au trait de jauge par de l'éther.

La lecture des extinctions est effectuée immédiatement dans des cellules d'un centimètre d'épaisseur et en utilisant le filtre S.53.

Nous avons constaté que la méthode de D.H, décrite plus haut, entraine une déviation constante qui dépend de la concentration de la peroxydase, de la durée de la réaction de la peroxydase et de la solution décolorée du colorant 2-6-dichlorophenol-indophenol, en présence de peroxyde d'hydrogène. Pour une durée de 30 secondes et une concentration de la peroxydase de 0,375 à 0,525 µg/ml, la déviation est de 4,5 à 5,3 %.

Pour des concentrations plus fortes ou plus faibles l'erreur augmente et varie de 9,9 à 15,8 %. Si on prolonge la durée de la réaction, par exemple 1 minute, pour une concentration de l'enzyme de 0,525 µg l'erreur atteint 11,6 %. En conséquence, nous avons adopté une durée de réaction de 30 secondes et des concentrations de peroxydase de 0,375 à 0,525 µg/ml.

1° Réaction inhibiteur-enzyme.

Nous avons étudié le processus d'inhibition de la peroxydase du raifort (enzyme) par l'acide sulfureux (inhibiteur) en fonction du temps et de la concentration des deux substances mises en œuvre.

Les concentrations de peroxydedase utilisées sont de 0,3, 0,5 et 1 µg/ml et les concentrations d'acide sulfureux ajouté étaient 0,04, 0,08, 0,16, 0,32 et 0,48 mg/ml.

Les résultats de ces expériences sont indiqués dans le tableau I. Comme on le constate, l'inactivation de la peroxydase dépend de la concentration des composants et du temps de la réaction.

L'inactivation est d'autant plus rapide que la concentration en enzyme est plus faible et que la concentration en acide sulfureux s'accroît. Pour une même concentration en acide sulfureux : 48 mg SO_2, l'inactivation se produit après 2 heures pour 0,3 µg de peroxydase, après 5 heures pour 0,5 µg et après 10 heures pour 1 µg.

On peut donc régler l'inhibition de l'activité de la peroxydase du raifort par l'acide sulfureux en tenant compte du temps d'action et de la concentration en acide sulfureux.

TABLEAU I.

Influence de l'acide sulfureux sur le processus d'inhibition de l'activité de la peroxydase de raifort dans les solutions tamponnées acéto-acétiques à pH 4,7.

SO_2 - mg %	Activité initiale de peroxydase en ml selon D. H.	1/2	1	2	3	5	8	10	24 heures
		Modification de l'activité en fonction du temps							
4	1,6	1,6	1,6	1,6	1,6	1,6	1,6	1,1	0,7
8	»	1,6	1,6	1,6	1,6	1,6	1,46	0,08	0
16	»	1,6	1,6	1,6	1,4	0,87	0,43	0	0
32	»	1,6	1,13	0,97	0,87	0	0	0	0
48	»	1,6	0,08	0	0	0	0	0	0
4	2,8	2,8	2,8	2,8	2,8	2,8	2,8	2,8	2,6
8	»	2,8	2,8	2,8	2,8	2,6	2,6	2,0	0,87
16	»	2,8	2,8	2,6	2,5	2,0	0,96	0,96	0
32	»	2,8	2,8	2,0	1,2	0,43	0	0	0
48	»	2,6	2,01	1,6	1,13	0	0	0	0
4	5,69	5,69	5,69	5,69	5,69	5,69	5,69	5,25	5,25
8	»	5,69	5,69	5,69	5,69	5,69	5,25	4,6	2,29
16	»	5,69	5,69	5,69	5,69	5,69	2,89	0,7	0,43
32	»	5,69	5,69	5,69	4,65	3,76	2,01	0,08	0
48	»	5,69	5,25	4,64	3,76	2,0	1,13	0	0

L'activité 1,6 ml 2,6-dichlorophenol-indophenol 0,001 m correspond à 0,3 µg de la peroxyde de raifort.
» 2,8 » » » » » à 0,5 » » »
» 5,6 » » » » » à 1,0 » » »

2° *Réaction donneur-enzyme.*

Dans cette série d'essais, nous avons tenté de préciser le rôle stabilisateur de l'acide L-ascorbique sur l'enzyme.

Les concentrations en peroxydase de raifort étaient : 0,3, 0,5, 1 µg/ml et en acide ascorbique : 0,2 et 0,4 mg/ml. Les résultats obtenus sont indiqués dans le tableau II.

On constate que des quantités d'acide L-ascorbique proportionnellement très élevées par rapport à la quantité de peroxydase (acide L-ascorbique 20,000 peroxydase 0,3) n'exercent pas d'influence sur la peroxydase. D'autres acides organiques, p. ex. l'acide citrique peuvent peut-être inhiber cette activité (15). A pH 4,7, l'acide L-ascorbique est sans action. Mais en présence de peroxyde d'hydrogène, l'activité de la peroxydase est altérée.

TABLEAU II.

Influence de l'acide ascorbique sur l'activité de la peroxydase de raifort.

Acide l'ascorbique en mg %	Activité exprimée en ml d'après D.H. (10)	Les changements de l'activité peroxydasique sous l'influence du temps en heures				
		1	5	10	20	24 heures
20	1,6	1,58	1,58	1,55	1,55	1,55
20	2,8	2,8	2,8	2,8	2,8	2,8
20	5,69	5,69	5,7	5,69	5,69	5,68
40	1,6	1,6	1,58	1,58	1,57	1,57
40	2,8	2,8	2,8	2,8	2,8	2,8
40	5,69	5,65	5,7	5,68	5,69	5,69

L'activité 1,6 ml 2,6-dichlorophenol-indophenol 0,001 m correspond à 0,3 µg de la peroxyde de raifort.
» 2,8 » » » » » à 0,5 » » »
» 5,69 » » » » » à 1,0 » » »

3° *Réaction donneur-enzyme-inhibiteur.*

Dans cette série d'essais nous avons étudié l'influence des deux composants : acide sulfureux et acide L-ascorbique en mélange, sur l'activité enzymatique de la peroxydase.

Les concentrations en peroxydase étaient 0,3, 0,5 et 1 µg/ml, en acide sulfureux : 0,4, 0,8, 0,16, 0,32 et 0,48 mg/ml et en acide L-ascorbique : 0,2, 0,4 mg/ml. Le pH était de 4,7.

Dans ces conditions opératoires on constate que l'inactivation de la peroxydase est ralentie par rapport à ce que l'on observe dans le cas de la réaction inhibiteur-enzyme. Elle est complète en trois heures, tandis qu'en présence d'acide sulfureux, elle se produit en une heure.

En présence de concentrations croissantes d'acide L-ascorbique, l'inactivation de la peroxydase est ralentie.

TABLEAU III.

Influence de l'acide sulfureux (SO_2) sur l'activité de la peroxydase de raifort en présence d'acide ascorbique.

Acide ascorbique mg %	SO_2 mg %	Activité exprimée en ml d'après D.H. (10)	Changements d'activité sous l'influence du temps en heures				Pourcentage d'inactivation enzymatique
			1	3	7	24	
20	32	1,6	1,6	1,6	0,876	0,87	46
20	32	2,8	2,8	2,8	1,4	1,4	50
20	32	5,69	5,69	5,69	3,56	3,56	38,8
40	32	1,6	1,6	1,6	0,87	0,87	46
40	32	2,8	2,8	2,8	1,75	1,75	38,8
40	32	5,69	5,69	5,69	3,92	3,92	31

La correspondance d'activité exprimée en ml équivalant au poids de la peroxydase est indiquée dans le tableau II.

4° *Réaction accepteur-enzyme.*

Notre but a été de déterminer la relation qui existe entre l'activité de la peroxydase en présence de concentrations variables de peroxyde d'hydrogène dans le milieu à pH 4,7.

Les taux de peroxydase employés étaient : 0,3, 0,5 et 1 µg/ml et pour le peroxyde d'hydrogène : 0,01, 0,05, 0,1 mg/ml. Entre 1 à 3 heures l'enzyme, quelqu'en soient les concentrations, à perdu environ le tiers de son activité, et celle-ci est totalement inhibée entre 6 et 9 heures (tableau IV).

TABLEAU IV.

Influence du peroxyde d'hydrogène sur l'activité de la peroxydase.

Peroxyde d'hydrogène mg %	Activité exprimée en ml d'après D.H. (10)	Changements d'activité sous l'influence du temps, en heures				
		1	2	3	6	9
1	1,5	1,51	0,87	0,44	0	0
	2,8	1,92	1,51	0,87	0	0
	5,6	3,6		1,51	0,87	0
5	1,6	1,5	0,87	0,44	0	0
	2,8	1,92	1,51	0,87	0	0
	5,6	3,6		1,51	0,87	0
10	1,6	1,51	0,87	0,44	0	0
	2,8	1,92	1,51	0,87	0	0
	5,6	3,6		1,51	0,87	0

La correspondance d'activité exprimée en ml équivaut au poids de peroxydase est indiquée dan le tableau II.

5° Réaction Enzyme-Accepteur-Donneur.

Ici, l'activité de la peroxydase est confrontée avec la présence à la fois du peroxyde d'hydrogène et de l'acide L-ascorbique. Les concentrations en peroxyde d'hydrogène sont : 0,025, 0,05 et 0,1 mg/ml auxquelles on a ajouté 0,2 et 0,4 mg/ml d'acide L-ascorbique. Dans toute la série d'essais, la concentration en enzyme peroxydasique a été la même, 1 µg/ml, et le milieu acéto-acétique à pH 4,7.

Les quantités d'acide L-ascorbique oxydé par la peroxydase sont évaluées en fonction de la durée, en minutes, de la réaction. L'acide L-ascorbique oxydé par la peroxydase est dosé par différence.

L'activité enzymatique de la peroxydase est aussi déterminée en fonction du temps compté en heures.

L'acide L-ascorbique a été dosé par la méthode de Tillmans en utilisant la solution 0,001 M de 2-6-dichlorophenol-indophenol. L'activité enzymatique a été déterminée par la méthode D.H. Pour arrêter l'action enzymatique au moment du dosage de l'acide L-ascorbique restant on a ajouté de l'acide sulfurique à 20 % dans la proportion de 1 à 1.

Les résultats mentionnés dans le tableau V montrent qu'en 2 heures et à la concentration de 0,1 mg/ml de peroxyde d'hydrogène, l'acide L-ascorbique à 0,2 mg/ml est complètement oxydé. A la concentration de 0,05 mg/ml de H_2O_2 il reste 0,025 mg/ml d'acide ascorbique, soit donc une oxydation de 92,5 % ; et à la concentration de 0,025 mg/ml l'oxydation est de 62,5 %. En présence d'une concentration double en acide L-ascorbique pour les mêmes concentrations en H_2O_2 l'oxydation est moins importante. On observe, en ce qui concerne l'action enzymatique une chute d'activité qui dépend de la concentration « acide ascorbique-donneur ». Dans les essais 3 et 6 où la réaction d'oxydation est terminée, l'activité enzymatique est minimale, tandis qu'elle reste notable dans les essais 1, 2, 4, 5, où elle est protégée par l'acide L-ascorbique non oxydé (tableau V).

TABLEAU V.

Oxydation de l'acide ascorbique sous l'influence de peroxydase en présence de diverses concentrations de peroxyde d'hydrogène.

Concentration initiale d'acide L-ascorbique mg %	H_2O_2 mg %	Effet de l'oxydation exprimé en mg % d'acide L-ascorbique oxydé le temps en minutes								Activité peroxydasique initiale en ml D.H. (10)	Changements d'activité en heures		
		5	10	15	25	35	45	60	120		1	4	8
20	2,5	3,7	7,4	10,3	10,8	11,4	12,5	12,5	12,5	5,6	5,6	5,6	4,6
20	5,0	5,1	8,8	11,8	12,0	12,5	13,5	15,8	18,5	5,6	4,6	3,7	2,8
20	10,0	7,5	9,5	12,5	13,8	14,5	15,7	16,5	20,6	5,6	4,6	2,0	0,8
40	2,5	5,1	7,4	10,8	11,8	12,5	13,5	14,8	14,8	5,6	5,6	5,6	5,6
40	5,0	7,5	10,3	15,0	22,2	26,7	28,1	29,5	32,5	5,6	4,9	3,9	2,6
40	10,0	9,5	13,2	19,0	25,1	28,0	31,1	32,5	36,0	5,6	4,6	2,6	0,5

6° Réaction : Enzyme-Accepteur-Donneur-Inhibiteur.

Avant d'aborder l'étude de cette réaction, nous rappellerons :

a) Que dans la réaction Donneur (acide L-ascorbique)-Enzyme (peroxydase)-Accepteur (peroxyde d'hydrogène) l'oxydation totale de l'acide L-ascorbique se produit en 2 heures.

b) Qu'aux concentrations de 5, 10, 12,5 mg % d'acide sulfureux, celui-ci ne diminue pas en 2 heures d'activité enzymatique de la peroxydase (ce qui ressort des essais de la réaction 1 (Enzyme-Inhibiteur).

c) Que la réaction stéchiométrique SO_2 et H_2O_2 a lieu dans le rapport 64 : 34 soit 1,88 mg SO_2 pour 1 mg H_2O_2.

d) Que l'activité enzymatique de la peroxydase ne peut se manifester que par l'intermédiaire du complexe E.A.D. où A (accepteur), est représenté par H_2O_2 (voir réaction 5).

e) Enfin, il fallait aussi savoir si la vitesse de réaction $SO_2 + H_2O_2 \rightarrow SO_3 + H_2O$ est plus grande que celle de formation du complexe E.A.D. (réaction 6).

Si du mélange des composants du complexe E.A.D. le peroxyde d'hydrogène disparaît de la réaction par suite de la formation de SO_3 et H_2O la réaction qui se produira sera celle du Donneur-Enzyme (réaction 2).

Deux vitesses de réaction sont à considérer : celle de la réaction stéchiométrique SO_2 et H_2O_2 et celle de la formation du complexe E.A.D. Si la quantité

de SO_2 ajouté est insuffisante, il restera du peroxyde d'hydrogène et on revendra aux conditions de la réaction 5 (Enzyme-Accepteur-Donneur).

Ceci posé, nous avons opéré dans les conditions décrites précédemment : a) réaction en milieu acéto-acétique à pH 4,7 ; b) concentrations de 5 et 10 mg % de H_2O_2 ; concentrations de 5, 10 et 15,2 mg % de SO_2 ; concentrations en acide L-ascorbique de 20 et 40 mg %. La durée de la réaction est de 2 heures à la température de 22° C. L'activité de la peroxydase correspond à 5,6 ml de 2-6-dichlorophenol-indophenol (0,001 m = 1 µg/ml d'enzyme). Le volume du mélange était de 50 ml pour tous les essais.

En ajoutant 5, 10 et 15,2 mg % de SO_2 dans les essais 2, 4, 6, il reste encore à peu près 2,5, 5,0 et 2,5 mg % de peroxyde d'hydrogène. Les résultats doivent donc répondre à ceux des essais 1, 2 et 4 du tableau IV.

Les données du tableau VI montrent qu'il en est bien ainsi.

TABLEAU VI.

Oxydation de l'acide ascorbique sous l'influence de la peroxydase en présence de peroxyde d'hydrogène et d'acide sulfureux (SO_2) à diverses concentrations.

Concent. initiale d'acide ascorbique mg %	Activité peroxyd. en ml d'après D.H. (10)	H_2O_2 mg %	SO_2 mg %	Effet de l'oxydation exprimé en mg % d'acide L-ascorbique oxidé en fonction du temps en minutes							
				5	10	15	25	35	45	60	120
20	5,6	5	0	5,1	8,8	11,8	12,0	12,5	13,5	15,8	18,5
20	5,6	5	5	3,7	7,4	10,3	10,8	11,4	12,5	12,5	12,5
20	5,6	10	0	7,4	9,5	12,5	13,5	14,8	15,7	16,5	20,0
20	5,6	10	10	3,5	8,5	11,8	12,0	12,5	13,5	14,8	18,5
40	5,6	10	0	9,5	13,2	19,0	25,1	28,1	31,1	32,5	36,0
40	5,6	10	15,2	5,1	7,4	10,8	11,8	12,5	13,5	14,8	14,8

CONCLUSIONS.

De nos expériences, nous pouvons déduire les conclusions suivantes :

1° L'acide sulfureux est un inhibiteur de la peroxydase de raifort en provoquant la destruction du peroxyde d'hyrogène dans le complexe E.A.D.

2° La vitesse de réaction $SO_2 + H_2O_2$ est plus grande que la vitesse de formation du complexe E.A.D. (Enzyme-accepteur-donneur), de telle sorte que l'acide sulfureux, en provoquant le bloquage de l'activité de la peroxydase, maintient la stabilité de l'acide ascorbique présent.

3° L'acide sulfureux se comporte comme un inhibiteur de compétition puisqu'il diminue l'activité de la peroxydase d'autant plus vite, que la concentration en SO_2 est plus grande.

4° L'acide L-ascorbique seul, se conduit comme un corps indifférent. Son activité se déclenche en présence de peroxyde d'hydrogène.

***

Après nous être rendu compte du mécanisme de l'activité peroxydasique en présence de l'acide sulfureux, nous avons voulu confronter ces données dans les cas où l'acide sulfureux est ajouté aux produits alimentaires en vue d'éviter les pertes en acide L-ascorbique.

Nos essais ont porté sur des pulpes et des sucs sulfités : tomates, pommes, racine de raifort.

La composition des produits alimentaires rend le problème plus complexe. En effet, en plus des enzymes peroxydasiques propres, il y en a d'autres, comme la catalase qui est douée de la propriété d'être activée par le peroxyde d'hydrogène. Il y a aussi les ions métalliques activant ou inhibitant les activités enzymatiques. Néanmoins, si l'on se borne à observer les différences entre produits sulfités et non sulfités concernant l'activité peroxydasique et les pertes en acide L-ascorbique, on pourra déterminer le rôle spécifique de l'acide sulfureux.

Nos essais devaient s'effectuer à diverses températures étant donné que les pulpes et les sucs sulfités sont gardés en magasin à des températures variables. Les températures choisies étaient : 3 à 5° C, 10 à 12° C et 22° C, température du laboratoire. Les essais ont été maintenus à ces températures jusqu'à oxydation complète de l'acide L-ascorbique, soit environ 17 semaines. Nos expériences comportaient le dosage de l'acide L-ascorbique, la détermination de l'activité peroxydasique, du pH, de la matière sèche.

Méthodes utilisées :

1° L'activité enzymatique a été déterminée par la méthode Diemair-Hiusser (10). Les résultats sont rapportés à 1 gr de produit.

2° L'acide L-ascorbique a été dosé par la méthode iodométrique de Kielhöfer (15) à l'aldéhyde acétique, méthode adaptée à la détermination de l'acide L-ascorbique en présence de notables quantités d'acide sulfureux.

Des essais effectués par la méthode polarographique ont échoué à cause de la présence de matières pectiques.

3° Le pH a été déterminé au Ph-mètre.

4° La matière sèche a été dosée à 103° C sur le produit mélangé à du sable.

Mode opératoire :

Préparation des produits :

Les pulpes sulfitées ou non sulfitées sont lavées, puis égouttées et séchées extérieurement et ensuite émiettées au moyen d'une râpe de verre. La masse est ensuite passée au tamis de nylon. Les jus sont filtrés sur une toile épaisse de lin.

La matière non sulfitée est introduite dans des bocaux en verre formant à l'émeri. Les produits sulfités ont été préparés en introduisant une quantité déterminée de solution aqueuse d'acide sulfureux, préparée par saturation de l'eau par de l'anhydride gazeux. Après cette addition de SO_2, la masse est mélangée à nouveau. Les quantités de solution de SO_2 ne dépassaient pas 6 ml pour 100 ml.

Les produits ainsi préparés, placés dans des récipients en verre, bien fermés, sont maintenus aux températures choisies : 3 à 5° C, 10 à 12° C, 20° C.

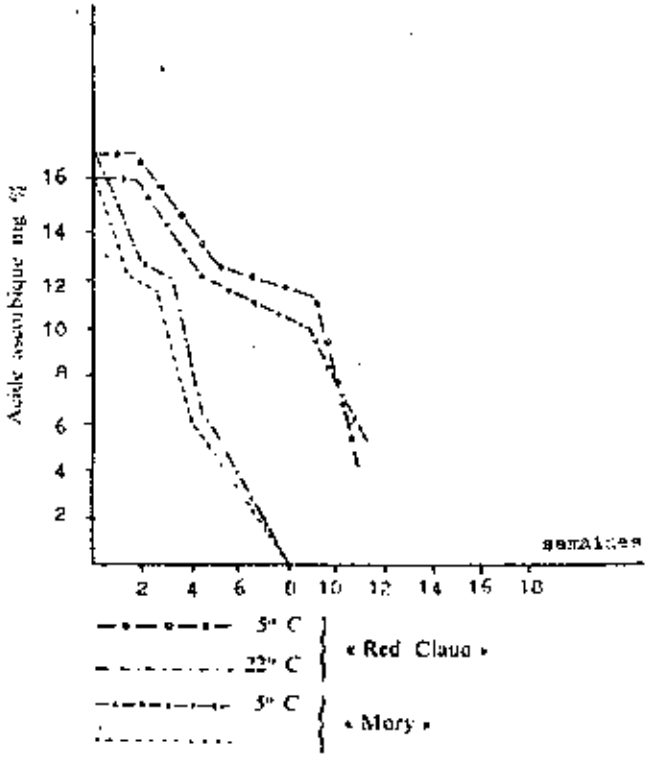
Détermination de l'activité peroxydasique et dosage de l'acide L-ascorbique restant.

Pour la détermination de l'activité peroxydasique, nous avons utilisé des prises de 5 g de produit. Celles-ci sont introduites dans des fioles jaugées de 100 ml. On complète jusqu'au trait de jauge par de l'eau distillée et laisse 30 minutes à la température du laboratoire. Le SO_2 est éliminé par barbotage de CO_2 pendant 10 minutes. L'activité a été déterminée sur 1 ml de la solution claire en observant strictement les conditions ci-dessous :

On mélange, dans une ampoule à décantation, 4 ml de la solution d'acide L-ascorbique à 0,05 % fraîchement préparée et 0,5 ml de la solution acéto-acétique à pH 4,7. On titre le mélange par le 2-6-dichlorophénol-indophénol jusqu'à obtention de la couleur grise. On ajoute alors 0,5 à 1 ml de la solution peroxydasique à déterminer, et immédiatement après, 50 ml d'éther éthylique acidifié (par l'acide acétique) et l'on secoue pendant 30 secondes. Après séparation de la couche éthérée (soit 5 à 7 secondes) on la décante et l'on introduit le liquide coloré dans une cellule du photomètre. La lecture doit être effectuée immédiatement.

FIGURE 1.

Pertes en acide ascorbique des pulpes de tomate sulfitée 180 mg % d'SO_2) aux températures de — 5° et — 22° C. Tomates « Red Claud » et « Mory ».

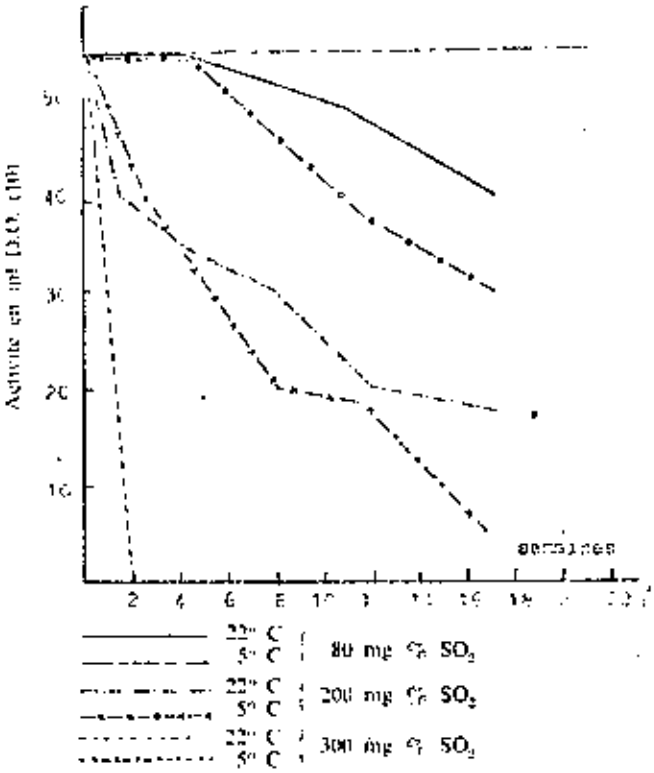


Pour le dosage de l'acide L-ascorbique, nous avons utilisé la méthode iodométrique de Kielhöfer (15) dans laquelle l'acide sulfureux, se combinant à l'aldéhyde acétique, ne réagit plus avec l'iode et ne fausse pas le dosage de l'acide L-ascorbique par la solution titrée d'iode. Nous avons constaté que dans une solution phospho-acétique de pH 3,5 à 4,5, l'aldéhyde acétique élimine complètement l'acide sulfureux de la réaction en présence d'iode, à condition d'introduire 3,38 mg d'aldéhyde par mg de SO_2. 10 gr de pulpes ou de sucs sont introduits dans une fiole jaugée de 100 ml que l'on remplit avec une solution d'acide oxalique à 3 %. Le tout est bien mélangé, puis centrifugé. A 10 ml de la solution claire on ajoute 3 ml de solution d'aldéhyde acétique à 5 % et après quelques minutes, on titre par la solution d'iode 0,02 N en présence d'empois d'amidon. Les résultats sont exprimés en mg d'acide L-ascorbique pour 100.

Le matériel utilisé pour nos essais nous a été fourni par l'Institut de l'art jardinier à Skierniewice. C'étaient des tomates « Red Claude » et « Mory » précoces, des pommes « Mac Intosch » et « C. Wilhem ». Nous avons aussi employé le suc du chou pommé et la racine de raifort.

Nos résultats sont mentionnés sur les figures 1 et 2.

FIGURE 2.
Changements d'activité de la peroxydase dans la pulpe de tomate « Red Chief ».



CONCLUSIONS.

Nos essais avaient pour but de déterminer: 1° les modifications de l'activité peroxydasique sous l'influence de l'acide surfureux et en fonction du temps et de la température ; 2° l'évolution des pertes d'acide L-ascorbique dans les produits sulfités.

Les tomates des 2 espèces possèdent une activité peroxydasique très semblable : 52-56. Cette activité a une tendance à diminuer sous l'influence du temps et de la température, et d'autant plus rapidement que la température est plus élevée et que la concentration en acide sulfureux est plus forte. Dans les pulpes non sulfitées, l'activité ne change pas.

L'influence de l'acide sulfureux sur la formation du complexe peroxydasique E.A.D. est incontestable. L'acide sulfureux étant un inhibiteur spécifique, l'activité enzymatique de la peroxydase est gravement perturbée, et l'oxydation de l'acide L-ascorbique est moindre.

L'activité peroxydasique des sucs est faible : 0,655-6,67, celle du chou est élevée et celle du raifort sauvage est très grande : 2628. Pour tous ces sucs on observe une chute marquée de l'activité peroxydasique pour des concentrations croissantes d'acide sulfureux ajouté. La perte totale de l'activité enzymatique (peroxydasique) peut s'observer déjà après 2 semaines pour les pulpes de tomates conservées à la température de 20° C (300 mg % SO_2) alors qu'elle est peu marquée à la température de 5° C.

Dans les sucs de pomme, l'activité cesse dans un délai de 3 heures, toutefois des traces d'activité subsistent pendant quelques semaines si la concentration en SO_2 ne dépasse pas 50 mg %.

Les pertes en acide L-ascorbique sont moindres dans les produits conservés par sulfitation, là où les concentrations en acide sulfureux sont plus grandes. On constate que les pertes les plus faibles apparaissent pour des concentrations de 80 mg de SO_2, ce sont vraisemblablement les doses les plus adéquates pour la conservation des tomates. L'addition de plus fortes concentrations en SO_2 provoque une chute marquée du pH, ce qui s'explique par la formation d'acide sulfurique.

L'étude des pertes en acide L-ascorbique dans les sucs de pomme conservés par sulfitation doit faire l'objet de nouvelles recherches.

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Determination of Sulfite in Biological Fluids: The Usefulness of Radioactive N-Ethylmaleimide

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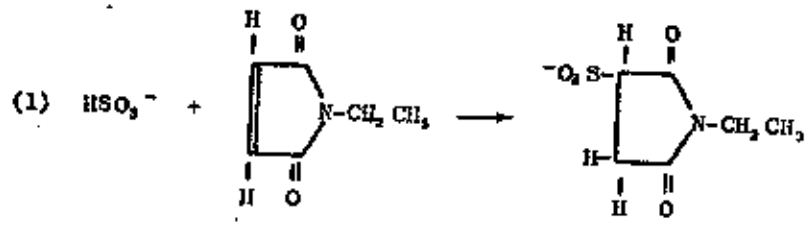
Sulfite is commonly accepted as a metabolic intermediate both in the oxidation of organic sulfur to sulfate by mammals and the reduction of sulfate to organic sulfur by autotrophic organisms (1). A number of methods are available for determination of sulfite. Precipitation of the strontium salt of sulfite (2) has been put on a quantitative basis and used to measure the rather high concentrations of sulfite present in bovine semen (3). Recently, most workers have used a method based upon the chromogenic reaction of sulfite with formaldehyde and a dye such as fuchsin (4, 5) or pararosaniline (6). During investigation of a human infant deficient in sulfite oxidase, it was necessary to identify and measure low concentrations of sulfite in urine and tissue extracts (7–9). Precipitation methods were too insensitive and development of the chromogenic material in the dye-formaldehyde method was inhibited by unidentified material present in urine.[1] A new method has now been developed and is described here. The method offers increased sensitivity and specificity. By its use, sulfite can be identified and quantitated at low concentrations in urine or crude tissue extracts. The method is based upon the reaction between sulfite and *N*-ethylmaleimide (NEM), a reaction first reported by Margolis and Mandl (10). Recently, Ellis emphasized the usefulness of NEM in the study of sulfur metabolism and employed this compound to demonstrate sulfite-S^{35} as a product of sulfate-S^{35} metabolism in *Escherichia coli* (11).

Sulfite and NEM combine quantitatively to form a stable adduct (equation 1):

[1] Irreverre, F., personal communication.



In the present work, radioactive NEM was used so that the adduct could conveniently be detected with high sensitivity.

EXPERIMENTAL METHOD AND MATERIALS

Radioactive NEM was used under conditions slightly modified from those of Ellis (11). The following were incubated in 0.12 ml at room temperature for 30 min in a nitrogen atmosphere: potassium phosphate, pH 7.4, 10 μmoles; NEM-1-C^{14} (Schwarz BioResearch, Inc.), 40 mμmole, 2.0–3.3 mc/mmole; urine, containing less than 40 mμmole of sulfite and other substances reacting with NEM. To minimize oxidation of sulfite, all solutions except the unknown and the NEM-1-C^{14} were gassed with N_2 just before use and the test tubes used for the incubation were flushed with N_2. After incubation, an aliquot of the reaction mixture was spotted for paper chromatography (Whatman No. 1, descending method). Usually a marker of authentic L-methionine was superimposed. After the chromatogram had been developed overnight, it was dried and sprayed with ninhydrin to locate the methionine. The chromatogram was cut into 1 cm strips which were each placed in a vial containing 5 ml of a solution of 4.0 gm/liter of 2,5-diphenyloxazole and 0.1 gm/liter of *p*-bis-2'-(5'-phenyloxazolyl)benzene in toluene. Radioactivity was then determined in a Packard Tri-Carb liquid scintillation spectrometer. Sodium sulfite-S^{35} was purchased from Volk Radiochemical Company.

RESULTS

Treatment of sulfite with NEM-1-C^{14} resulted in formation of a radioactive adduct. This adduct could readily be purified by paper chromatography. A typical chromatogram is shown in Figure 1. The migration of the compound formed as a result of treatment of sulfite-S^{35} with nonradioactive NEM is also shown in Figure 1. The two materials moved with identical mobilities and did not separate upon cochromatography. The authentic adduct formed from sulfite and NEM-1-C^{14} moved as a single spot in most solvents (Table 1). Other radioactive materials present in the reaction mixture were readily separated from the area to which the NEM-1-C^{14}-sulfite adduct moved. Paper electrophoresis served as an alternate means of separating the NEM-1-C^{14}-sulfite ad-

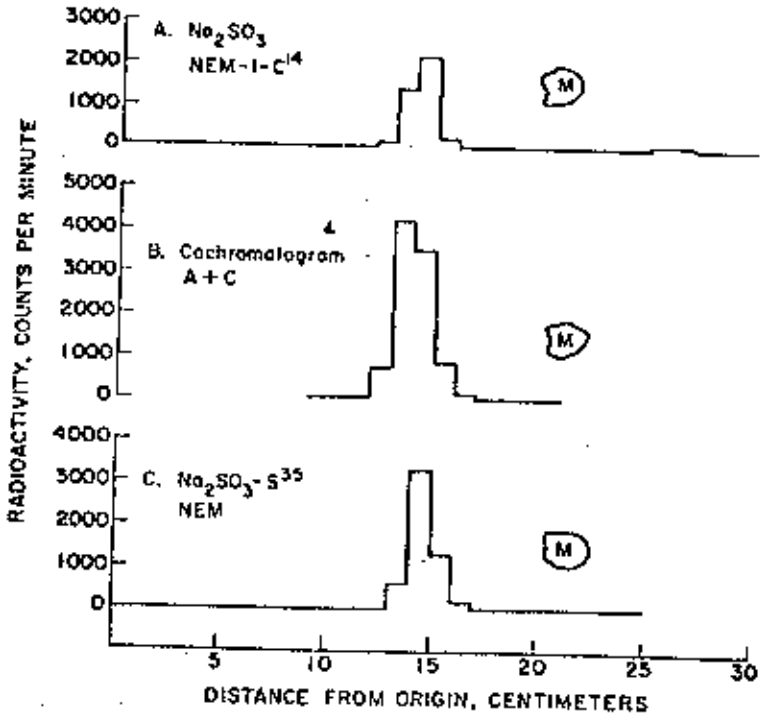


FIG. 1. Chromatography of radioactive adducts formed between sulfite and *N*-ethylmaleimide under conditions described in the text. Development was carried out overnight with solvent A (Table 1). The areas marked "M" are scale drawings of the ninhydrin-reacting material corresponding to authentic L-methionine.

duct from other components of the reaction mixture. The authentic compound moved as a single spot toward the anode with a mobility which changed little over the pH range examined (1.7–10.8).

Quantitation. The reaction between NEM-1-C^{14} and sulfite could be

TABLE 1
Mobility of the Adduct Formed between Sulfite and NEM-1-C^{14}

Solvent (v/v)	$R_{methionine}$*
A. *tert*-Butyl alcohol/88% formic acid/water, 70/15/15	0.70
B. Isopropyl alcohol/88% formic acid/water, 70/10/20	0.84
C. n-Butyl alcohol/glacial acetic acid/water, 60/15/25	0.45
D. Ethyl alcohol/21% NH_4OH, 95/5	1.50
E. n-Propyl alcohol/21% NH_4OH/water, 60/30/10	1.06, 0.93, 0.76 (trailing)
F. Ethyl alcohol/*tert*-butyl alcohol/21% NH_4OH/water, 300/100/4/75	1.47

* Mobility relative to that of L-methionine.

used for quantitative determination of sulfite as is shown in Figure 2. In this experiment a fixed amount of NEM-1-C^{14} was incubated under standard conditions with increasing amounts of sulfite. The radioactivity in the sulfite-NEM adduct area after chromatography with solvent A was then measured. Formation of adduct was linearly proportional to added sulfite until attainment of a plateau value corresponding to complete conversion of NEM-1-C^{14} to adduct. By suitable dilution of unknown samples this method can be used to determine as little as

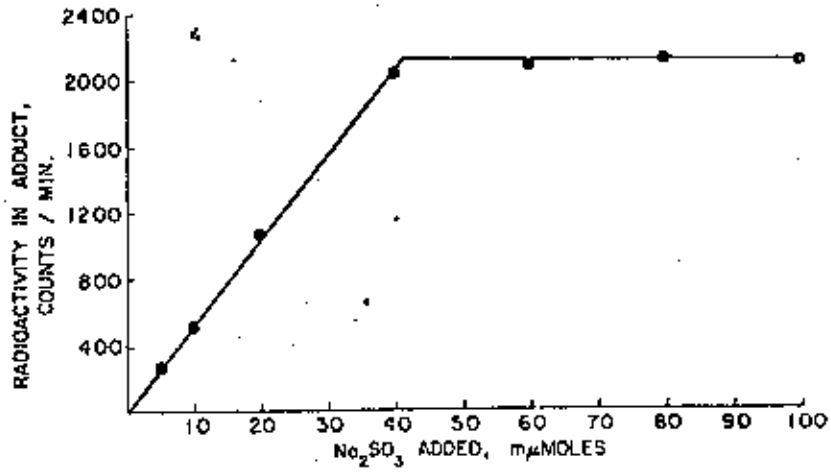


FIG. 2. Adduct formation as a function of amount of sulfite added. Reaction mixtures to which varying amounts of Na_2SO_3 and a constant amount of NEM-1-C^{14} had been added were incubated under standard conditions. After incubation, aliquots were subjected to paper chromatography in solvent A and the amount of radioactivity in the NEM-sulfite adduct area of the chromatogram was determined. The solid line is a theoretical curve calculated from the radioactivity added and the specific activity furnished by the manufacturer. The curve was drawn assuming product formation would be proportional to sulfite added until complete conversion of NEM-1-C^{14} and that complete conversion was attained with excess Na_2SO_3. The points represent experimental determinations.

5 mμmoles of sulfite with NEM-1-C^{14} of the specific activity of that now commercially available (2.9 and 3.3 mc/mmole in the two lots used). If other compounds which react with NEM are present in the unknown sample, care must be taken that an excess of NEM-1-C^{14} is used. If NEM-1-C^{14} is limiting, the ratio of adducts formed is not necessarily proportional to the ratio of reactive compounds initially present in the unknown.

Interfering Substances. Several experiments were carried out to determine the extent to which thiosulfate and other compounds likely to be present with sulfite in biological fluids would interfere. A reaction

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NEM-1-C^{14}

Reactivities*
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1.47

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mixture incubated with a several-fold excess of sodium thiosulfate did not contain a detectable peak of radioactive material in the area of the sulfite-NEM adduct after chromatography in solvent A (Table 1). By use of the spectrophotometric method of Ellis (11), it was shown that only a very slow reaction occurred between NEM and an 8-fold excess of thiosulfate. This amount of thiosulfate did not inhibit the reaction with sulfite. The NEM-sulfite adduct was readily separated by paper chromatography from the NEM-1-C^{14} adducts formed by several substances likely to be present in biological fluids. For example, the sulfide-NEM-C^{14} adduct moved with an $R_{methionine}$ of 1.37 in solvent A, whereas the sulfite-NEM adduct moved with an $R_{methionine}$ of 0.70. In solvent F, the adducts of NEM with reduced glutathione and cysteine moved with mobilities of 0.20 and 1.03, respectively, relative to methionine, whereas the sulfite-NEM adduct moved with a mobility of 1.47. In general, since the sulfite-NEM adduct retains a negative charge even at low pH's, it is separable from amino acid adducts by electrophoresis at, for example, pH 1.7 or by passage over a column of Dowex 50 (H^+).

Preliminary Purification Procedures. In some situations in which sulfite is present at low concentrations, it may be desirable to treat relatively large volumes of starting material with NEM-1-C^{14} and to purify and concentrate the radioactive adduct before paper chromatography or electrophoresis. In such cases, a large-scale reaction was carried out. The reaction mixture was then diluted, brought to pH 1–2 with HCl, and extracted twice with ethyl acetate to remove unreacted NEM-1-C^{14} and other radioactive materials. The two lots of NEM-1-C^{14} used were noted by the manufacturer to have 6% (lot 6602) and 30% (lot 6701) radioimpurities. About 2% (lot 6602) and 7% (lot 6701) of the radioactivity was not removed by extraction with ethyl acetate. During this extraction procedure the adduct formed between sulfide and NEM-1-C^{14} was removed from the aqueous phase whereas the adducts formed between NEM-1-C^{14} and sulfite, cysteine, or glutathione remained in the aqueous phase.[2] The sulfite adduct was freed of the amino acid adducts by passage of the diluted aqueous phase over a column of Dowex 50 (H^+). The sulfite-NEM-1-C^{14} adduct was recovered quantitatively in the combined effluent and water washings from the column. This solution was lyophilized to dryness without effecting the paper chromatographic mobility of the adduct. Caution should be used since

[2] If an unknown solution contains sulfite as the predominant component reacting with NEM-1-C^{14}, determination of the radioactivity in the aqueous phase at this point and comparison with a suitable blank can serve as a measure of the sulfite content of the unknown. Sensitivity and specificity are improved by further purification as described.

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there appeared to be some decomposition of the adduct during rigorous drying *in vacuo*.[2] The residue after lyophilization was dissolved in a small volume of water and used for paper chromatography.

DISCUSSION

The method described here is related to that of Ellis (11). Whereas Ellis detected radioactive sulfite by the use of nonradioactive NEM, the present methods detected nonradioactive sulfite by the use of radioactive NEM. This method is useful for situations in which it is impossible or inconvenient to work with radioactive sulfite. For example, the method has been satisfactory to allow proof of the presence of sulfite and determination of the concentration of this ion in urine from a human infant deficient in sulfite oxidase. The methods have also served for investigation of sulfite metabolism and excretion in other mammals. A sample of 0.1 ml containing 5 mμmoles of sulfite is sufficient for accurate quantitation and tentative identification. The method would appear to be at least equal in sensitivity to the commonly used dye-formaldehyde methods (4, 6), to be free from the interference with chromogen development encountered when the dye-formaldehyde methods were applied to human urine,[2] and to offer the potential of identification of the sulfite-NEM adduct by virtue of its paper chromatographic and electrophoretic properties. Other compounds which react with NEM may be determined simultaneously from the developed chromatogram. Thiosulfate and other reducing substances do not interfere with the determination of sulfite. The radioactive adduct formed is reasonably stable and may be subjected to further purification procedures as necessary. These methods may be applicable to a variety of problems in which it is desired to determine small amounts of sulfite with high specificity in crude biological materials. Extension of the method to determination of other compounds which react with NEM is clearly feasible.

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K+. In any case, with our results it is not possible to rule out a small participation of K+.

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27 February 1967

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Sulfite Oxidase Deficiency in Man: Demonstration of the Enzymatic Defect

Abstract. *An infant who died with neurological abnormalities, mental retardation, and dislocated ocular lenses excreted in his urine abnormally large amounts of S-sulfo-L-cysteine, sulfite, and thiosulfate and virtually no inorganic sulfate. The present report establishes the occurrence of an enzymatic defect in this infant. His liver, brain, and kidney specifically lacked sulfite oxidase activity. Deficiency of sulfite oxidase, which has not apparently been described in man, provides a reasonable explanation for the abnormalities in this infant.*

We have described (*1*) a male infant with a disorder of metabolism which we believe was previously unrecognized. At birth he had neurological abnormalities which progressed until he was virtually decerebrate. Dislocated ocular lenses were noted at age 1 year. He died at 32 months. Three of seven siblings were born with neurological abnormalities and died in early infancy for undefined reasons. The remaining siblings and the parents appear normal on clinical examination and have not yet been shown to have any disorders of their metabolism. The patient's urine contained greatly elevated concentrations of *S*-sulfo-L-cysteine, sulfite, and thiosulfate and virtually no inorganic sulfate (*2*). The abnormal chemical findings are readily reconciled by postulation of a deficiency of activity of sulfite oxidase, the enzyme that catalyzes the conversion of sulfite to inorganic sulfate. In the presence of such a deficiency, sulfate excretion would be abnormally low, and sulfite would accumulate. The raised concentration of sulfite in body fluids could lead secondarily, by way of known reactions, to the formation of abnormal amounts of *S*-sulfo-L-cysteine (*3*) and thiosulfate (*4*). We now report direct evidence that the patient was indeed markedly deficient in sulfite oxidase activity.

Sulfite oxidase [sulfite:oxygen oxidoreductase E.C. 1.8.3.1 (*5*)] of mammals has been studied by Fridovich and Handler and their associates (*6, 7*). Their methods, with minor modifications, were used for our study of tissues obtained from the patient and controls at postmortem examinations and stored at −50°C. To prepare an acetone powder, a tissue was minced in at least 15 times its weight of acetone at −50°C and thoroughly homogenized in a Waring Blendor. The suspension was filtered, and the residue was washed successively with acetone and dry, peroxide-free ethyl ether at −50°C. The resulting powder was dried at reduced pressure and stored at 2°C. For extraction, the powder was stirred at 0°C for 2 hours with 0.05*M* potassium phosphate buffer, *p*H 7.8, containing 0.005 percent Versene Fe-3 (*8*), 1 ml of buffer being used for each 100 mg of powder. Sulfite oxidase activity was assayed by determination of the sulfite- and enzyme-dependent reduction of cytochrome *c*. Absorbance changes at 550.5 mμ (A_{550}) were measured with a Beckman model DU spectrophotometer. The standard assay mixture, volume 3.1 ml, included: tris-HCl buffer, *p*H 7.6, 100 μmole; cytochrome *c* (horse heart, Type VI, Sigma Chemical Co.), 2.6 mg; Na_2SO_3, 10 μmole; Versene Fe-3, 5μg, and enzyme (20 to 50 units). The solution was contained in a cuvette with a 1.0-cm light path and maintained at room temperature (19° to 20°C). The reaction was started by addition of enzyme. The reference solution against which absorbance of the assay mixture was determined contained only tris-HCl buffer and cytochrome *c*. Reaction rate was determined during the period when extent of reaction varied linearly with time, generally between 2 and 12 minutes after enzyme addition. The observed reaction rate was corrected by subtraction of two blank values. One was determined by incubation of enzyme and cytochrome *c* in the absence of added sulfite; the other, determined by omission of enzyme, represented the slow nonenzymatic reduction of cytochrome *c* by sulfite (0.001 absorbance unit per minute). A unit of enzyme activity was considered that amount of enzyme required to catalyze a change of 0.001 absorbance unit per minute under the standard conditions. Protein was measured by the method of Lowry as described by Layne (*9*). All specific activities are expressed as activity per milligram of protein.

Sulfite oxidase activity was readily demonstrable in crude extracts made from acetone powders of human liver. With some extracts a slow rate of enzyme-dependent reduction of cytochrome *c* was observed in the absence of added sulfite. To minimize or prevent this reduction, extracts were routinely purified by passage over a column of Sephadex G-25, maintained at 2°C, equilibrated with tris-HCl buffer, 0.05*M*, *p*H 7.6, containing 0.005 percent Versene Fe-3. Sulfite oxidase was quantitatively recovered after elution with the same buffer without significant change in specific activity.

In tris buffer, the sulfite oxidase activity changed little from *p*H 7.6 to 9.1. The reaction rate at *p*H 6.9 was 60 percent of that at *p*H 7.6. The

Table 1. Sulfite oxidase and other enzyme activities in liver from the patient and control subjects. Sulfite oxidase was measured in Sephadex-treated extracts of acetone powders from livers obtained postmortem. Cystathionase (E.C. 4.2.1.15), methionine-activating enzyme (adenosine triphosphate: L-methionine adenosyltransferase, E.C. 2.5.1.6) and cystathionine synthase were assayed in extracts prepared in phosphate buffer. Standard assay conditions and control values for these enzymes in fresh tissues have been published (*14, 15*).

Source of liver	Sulfite oxidase (unit/mg)	Methionine-activating enzyme (mμmole/mg = 60 min)	Cystathionine synthase (mμmole/mg = 135 min)	Cystathionase (mμmole/mg = 30 min)
		Control subjects		
Postmortem				
No. of patients	10	7	8	
Range	22–87	0.3–5.5	17–237	
Mean	51	1.5	137	
Laparotomy or needle biopsy				
No. of patients		9	9	5
Range		4.4–9.2	133–610	6–18.5
Mean		6.4	252	13
		Patient T.A.		
Postmortem	Not detected (<2)	2.5	95	14.4*

* Assayed under modified standard conditions and converted by calculation to units previously defined (*14*).

standard *p*H of 7.6 was selected to minimize the nonsulfite-dependent rate. Replacement of tris buffer by potassium phosphate at *p*H 7.6 led to a 90 percent decrease in rate. Use of equimolar portions of tris and potassium phosphate led to a 73 percent decrease in activity. The rate of cytochrome *c* reduction was proportional to enzyme concentration. Decrease in the cytochrome *c* concentration to one-half the standard concentration led to a 17 percent decrease in rate. Cytochrome *c* at one-fourth the standard concentration yielded a 28 percent decrease in rate. The standard sulfite concentration could be reduced threefold without significant effect on rate. A tenfold decrease led to a barely detectable (about 10 percent) decrease in rate.

Figure 1 illustrates a typical experiment in which extracts from a control subject and from the patient were assayed. The reaction mixture to which control enzyme was added showed an immediate and linear increase in A_{550}. This continued until attainment of a plateau value corresponding to complete reduction of cytochrome *c*. The reaction mixture containing control enzyme, but without added sulfite, showed essentially no change. The reaction mixture to which extract from the patient's liver was added showed a very slow decrease in A_{550}. At 16 minutes, control enzyme was added to this reaction mixture. After a small immediate shift in A_{550} due to absorption by components of the enzyme solution, there was a progressive, linear rise at a rate equal to that attained with control enzyme alone. This experiment demonstrates that the extract of the acetone powder prepared from the patient's liver contained no detectable sulfite oxidase activity, and that there was no inhibitor or other factor present in this extract which interfered with assay of the control activity. Similar experiments were carried out with both crude and Sephadex-treated enzyme preparations from the patient, over the *p*H range between 6.9 and 9.1. In no case was significant sulfite oxidase activity detected nor was inhibition by the patient's extract observed.

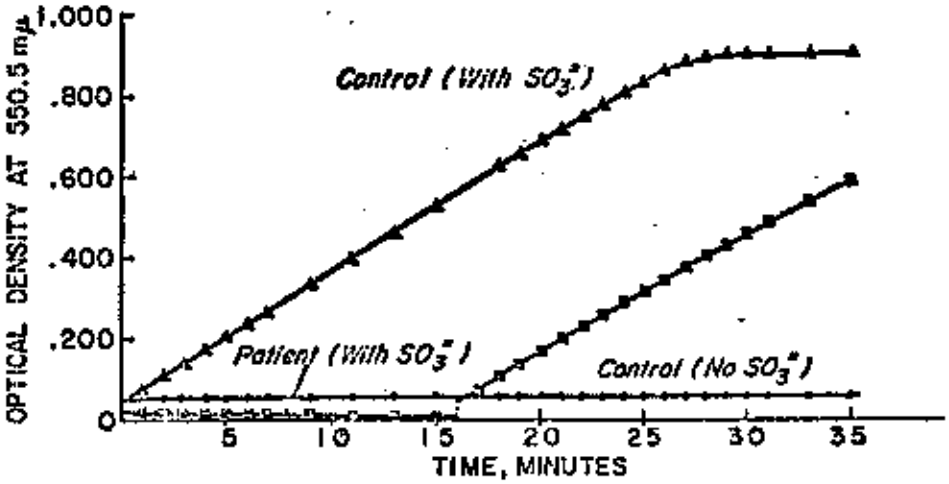

Fig. 1. Sulfite oxidase assays with extracts of acetone powders of liver specimens from the patient and from a control subject. Sodium sulfite was added as indicated. At zero time, extract from a control subject (0.07 ml containing 0.80 mg of protein) or the patient (0.07 ml containing 0.83 mg of protein) was added. At 16 minutes, extract from the control subject was added to the reaction mixture containing the patient's extract. Control experiments in which dithionite was used to reduce the cytochrome *c* demonstrated that the plateau attained with control enzyme at an absorbance of 0.905 was due to complete reduction of the cytochrome *c*.

The results of assays of liver specimens from control patients are summarized in Table 1. The patients had various diseases and ranged in age from 1½ months to 56 years. Postmortem examination was made as long as 14 hours after death. Each control liver had readily detectable sulfite oxidase activity. Activity would have been detected in the patient's liver extract had it been at least 2 unit/mg, a value equal to 4 percent of the mean control value or 10 percent of the lowest control value. The possibility that our failure to detect sulfite oxidase activity in the patient's liver is attributable to a general depression of enzymes in his tissue occurring either before or after death is excluded by several lines of evidence. (i) Tissues were taken from the patient no more than 8 hours after death. Sulfite oxidase activity did not disappear from control postmortem liver during a 14-hour period. There was no apparent tendency for the sulfite oxidase activity of control liver specimens to fall as the time of storage of the tissue at −50°C was increased. The specimens from the patient were stored for a shorter time before extraction than several of the control specimens were. (ii) Three additional enzymes were assayed in extracts prepared from the patient's liver (Table 1). The specific activities of methionine-activating enzyme and cystathionine synthase in the patient's liver were comparable to values determined for control postmortem specimens of human liver and were only slightly depressed even when compared to values for fresh liver (*10*). Cystathionase in the patient's liver was

Table 2. Sulfite oxidase and other enzyme activities in extracts of acetone powders of brain and kidney from the patient and control subjects. All tissues were obtained postmortem. Conditions of the assays were as in Table 1.

Subject	Sulfite oxidase (unit/mg)	Cystathionine synthase (mμmole/mg ≈ 135 min)	Cystathionase* (mμmole/mg ≈ 30 min)
	Brain		
Controls			
No. of patients	5	5	5
Range	10–13	21–38	0.03–0.22
Mean	11.5	27	0.13
Patient T.A.	Not detected (< 1.5)	26	0.13
	Kidney		
Controls			
No. of patients	4		3
Range	22–103		0–5.9
Mean	63		2.3
Patient T.A.	Not detected (< 1.0)		6.7

* Assayed under modified standard conditions and converted by calculation to units previously defined (*14*).

within the control range for fresh specimens.

These data prove that the patient's liver had a specific defect in its ability to oxidize sulfite with cytochrome *c* as electron acceptor. The physiological electron acceptor for sulfite oxidase is not known; it may be oxygen (*7*). For this reason, it was conceivable that normal activity of sulfite oxidase would have been detected in the patient's liver had oxygen been used in lieu of cytochrome *c*, and that the enzyme defect found with cytochrome *c* was fortuitous and without physiological significance. Direct evidence against this possibility was provided by two types of experiments. In one, crude extracts from acetone powders of liver were incubated aerobically with sulfite and without addition of cytochrome *c* or other electron acceptors. Sulfite disappearance was assayed by measurement of sulfite through use of *N*-ethylmaleimide-1-C^{14} (*11*). The patient's liver was less active in catalyzing aerobic sulfite disappearance than control specimens were. In the second experiment, sulfite-S^{35} served as substrate. After aerobic incubation with tissue extracts, reaction mixtures were acidified and gassed with nitrogen to remove unreacted sulfite-S^{35}. The radioactive material which did not volatilize under these conditions was taken as a measure of sulfate-S^{35} formation (*12*). With this assay, crude extract of acetone powder from the patient's liver was far less active than extracts made from control livers. Extracts of livers from ten control patients catalyzed the formation of from 0.31 to 1.33 mμmole of sulfate per milligram of protein per minute. The mean value was 0.66 mμmole. The extract from the patient's liver had an activity of 0.09 mμmole per milligram of protein per minute (*13*).

Of mammalian tissues reported to contain sulfite oxidase (*7*), we had specimens of liver, brain, and kidney from the patient (Table 2). For control subjects mean sulfite oxidase activity in brain was one-fifth that in liver. The patient's brain showed no detectable activity; specific activity equal to 13 percent of the mean control or 15 percent of the lowest control value would have been detected. Control values for kidney were comparable to those for liver. The patient's kidney showed no detectable activity. Sensitivity for detection was 2 percent of the mean control and 5 percent of the lowest control value. Recombination experiments similar to those performed with liver showed that the patient's brain and kidney extracts contained no inhibitors or other factors that interfered with determination of sulfite oxidase activity. The extracts of the patient's tissues had specific activities for cystathionine synthase and cystathionase within the range of values obtained with comparable extracts made from control tissues (Table 2). The controls included specimens obtained a longer time after death than the patient's tissues and the control subjects spanned a wide age range.

Our data demonstrate that the patient's liver, kidney, and brain were abnormally low, or entirely deficient, in sulfite oxidase activity. Although we studied only a single case, there can be little doubt that the enzyme defect demonstrated led to the observed chemical abnormalities. We also suggest a causal relation between these abnormalities and the clinical pathology (*2*). The question has been raised whether a compound so readily autoxidizable as sulfite requires an enzyme for its oxidation in the physiological situation (*7*). It has been suggested that this autoxidation, which is a metal-catalyzed, free-radical chain reaction, is ineffective in animal tissues where "chain-breakers" are plentiful and free unbound metal ions may be low in concentration (*7*). Our findings support this suggestion and indicate that enzymatic catalysis by sulfite oxidase is essential in vivo.

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10. As an additional control, cystathionine synthase was measured in the same extracts of acetone powders used for determination of sulfite oxidase activity. The extract from the patient's liver had the highest specific activity of cystathionine synthase of those tested.
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12. Tris-HCl buffer, pH 7.6, 7 μmole; Na_2SO_4-S^{35}, 9 mμmole containing approximately 20 × 10^6 count/min, and extract of an acetone powder of liver containing 0.3 to 0.5 mg of protein were incubated in a final volume of 0.15 ml in a test tube of 13 mm (inside diameter) at 26°C for 5 to 20 minutes (in air) with occasional shaking. To terminate the reaction, Na_2SO_3, 1 μmole in 1.85 ml of water, was added. The incubation was continued for 6 minutes. After addition of 0.5 ml of a solution containing 2.5 percent H_3PO_4, 1.2 percent mannitol, and 5 × 10^{-5} *M* EDTA, and a drop of caprylyl alcohol, the mixture was gassed with nitrogen for 1 hour. After dilution of the mixture to 3.0 ml, a portion (0.5 ml) was removed for determination of radioactivity in a Packard Tri-Carb liquid scintillation spectrometer.
13. The slight apparent activity with extract from the patient's liver may not correspond to true sulfate formation. During incubation with pa-

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The Mutagenic Specificity of Sodium Bisulfite

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Summary: Reversion studies with Escherichia coli mutants indicate that sodium bisulfite specifically induces mutations in only those mutants which are cytosine-guanine at the mutant site.

A base specific mutagen is of considerable value for the investigation of chemical mutagenesis. Chemical studies on the reaction of hydroxylamine with nucleic acid constituents (1), as well as biological studies with free single (2) and double (3) stranded bacteriophages have led to the inference that the major mutagenic effect of this mutagen is due to the alteration of cytosine (4, 5). While the mutational pattern for hydroxylamine has been in general agreement with the notion that cytosine-guanine to thymine-adenine base-pair transitions are chiefly induced by its reaction with cytosine, recent in vivo experiments with phage-infected host bacteria (6) and with bacterial mutants (7, 8) have indicated that hydroxylamine is also capable of producing thymine-adenine to cytosine-guanine transitions. Thus, the specificity of hydroxylamine for cytosine alone in mutagenicity studies remains to be established.

Recently, several authors (9-11) have reported on the chemical

reaction of sodium bisulfite with uracil and cytosine derivatives. They have shown that this chemical could deaminate cytosine to uracil without affecting adenine or guanine. One author (9) suggested that sodium bisulfite should be useful as a reference mutagen. The present report is concerned with mutational studies

Table 1 Characterization of E. coli mutants tested

Strain	Mutant Designation	Type	Assigned Change (wild type-mutant)
	A23 try⁻	Transition	CG - TA
	A46 try⁻	Transition	CG - TA
K12	A58 try⁻	Transition	CG - TA
	A78 try⁻	Transversion	GC - TA
	A38 arg⁻	Transition	TA - CG
	B23 cys⁻	Transition	TA - CG
	H-2 ilva⁻	Transition	TA - CG
	B14 his⁻	Transition	TA - CG
	S-3 leu⁻	Transition	TA - CG
	S-9 leu⁻	Transition	TA - CG
	A11 leu⁻	Transition	TA - CG
	S13 leu⁻	Transition	TA - CG
	A23 leu⁻	Transition	TA - CG
	A53 leu⁻	Transition	TA - CG
	A17 ser⁻	Transition	TA - CG
15	S25 ser⁻	Transition	TA - CG
	A12 try⁻	Transition	TA - CG
	S-1 met⁻	Transition	CG - TA
	S-8 pro⁻	Transition	CG - TA
	S-8 ser⁻	Transition	CG - TA
	S15 ser⁻	Transition	CG - TA
	S19 ser⁻	Transition	CG - TA
	S20 ser⁻	Transition	CG - TA
	B38 his⁻	Deletion	Deletion
	B-7 try⁻	Deletion	Deletion
	B16 try⁻	Deletion	Deletion

in E. coli which indicate that sodium bisulfite is a cytosine specific mutagen.

Materials and Methods: The strains of E. coli K12 and 15 used are listed in Table 1, together with relevant descriptive information. The type of genetic alteration assigned to the K12 mutants are from the amino acid replacement data of Yanofsky et al (12), while those assigned to the strain 15 mutants are deduced from the reversion experiments of Mukai and Troll (8).

Stationary growth phase cultures were used. Five ml of an overnight broth culture were centrifuged and washed once in saline. The washed pellet was resuspended in 5 ml of reaction mixture which was 1 M for sodium bisulfite in 0.2 M acetate buffer at pH 5.2. Control samples were resuspended in buffer alone at the same pH. At zero time and after 30 min incubation at 37°, sodium bisulfite and buffer were removed by centrifugation, and appropriate aliquots plated for titer and revertant colonies. The media and plating procedures have been described previously (8). Only large revertant colonies were counted after 36 hours incubation at 37° to exclude small suppressor type reversions.

Results and Discussion: Initial experiments to determine the optimal conditions for mutagenesis were performed with the CG mutant A38 arg⁻ and the results are shown in Table 2. There is no increase in the number of revertants after 30 min treatment at pH 5.2. Further, no killing of cells was observed under these conditions. Experiments using 1 M sodium bisulfite in 0.2 M phosphate buffer at pH 6, pH 7 and pH 8 showed that, only at pH 6 was there a significant increase in the number of revertants.

Table 2 Frequency of A38 arg$^-$ revertants induced by 1 M sodium bisulfite at pH 5.2

Agent	Frequency of revertants / 10^8 cells			
	0 min	30 min	60 min	120 min
Control buffer	22	18	23	22
Sodium bisulfite	20	125	94	100

However, at this pH, 20% of the cells were killed. Therefore, all subsequent work was done at pH 5.2 and 30 min exposure. These results are presented in Table 3.

Only those mutants identified as CG at the mutant site show an increase in reversion frequency. Since all of the TA and deletion mutants of strain 15 tested did not revert, the data for these mutants are omitted. In addition, reversion experiments with four UAG amber mutants of K12 were negative. We conclude from our observations that sodium bisulfite is specific for CG mutants alone.

While this manuscript was in preparation, Hayatsu and Miura (13) have reported that sodium bisulfite was mutagenic for the lambda phage and proposed that the mutagenicity may be due to CG to TA transitions. Studies on the chemical modification of phage DNA and yeast RNA have confirmed that bisulfite catalyzes the specific deamination of cytosine to uracil at the nucleic acid level (10, 13). The present data, in conjunction with the reported chemical data

Table 3 Frequency of reversions induced in E. coli mutants after 30 min exposure to 1 M sodium bisulfite at pH 5.2

Mutant	Assigned change at mutant site	Frequency of revertants / 10^8 cells	
		Control	Sodium bisulfite
A23 try⁻	TA	1.2	2.8
A46 try⁻	TA	5.2	4.2
A58 try⁻	TA	0.5	1.1
A78 try⁻	TA	11.6	11.6
A38 arg⁻	CG	18.0	125.0
B23 cys⁻	CG	2.6	80.1
H-2 ilva⁻	CG	12.9	37.9
B14 his⁻	CG	20.4	75.2
S-3 leu⁻	CG	10.7	74.3
S-9 leu⁻	CG	4.7	24.7
A11 leu⁻	CG	38.3	163.9
S13 leu⁻	CG	6.3	41.1
A23 leu⁻	CG	6.6	20.5
A53 leu⁻	CG	12.7	54.6
A17 ser⁻	CG	20.1	45.1
S25 ser⁻	CG	1.0	20.6
A12 try⁻	CG	1.9	26.7

strongly indicate that sodium bisulfite causes mutations solely by its modification of cytosine, and that it should be useful as a cytosine specific reference mutagen.

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Estudio sobre hidrólisis de proteínas

El uso del SO_2 en la protección de los aminoácidos, durante la hidrólisis de proteínas puras y en presencia de carbohidratos *

por

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Casi todos los métodos de valoración de aminoácidos exigen una hidrólisis previa de las proteínas. En los medios de hidrólisis corrientes, se observa frecuentemente destrucción de aminoácidos; ésta puede ser de dos tipos; por una parte destrucción de aminoácidos por razones inherentes a las condiciones de hidrólisis, tratándose de proteínas puras, es decir, que ciertos aminoácidos no resisten las condiciones de calentamiento, acidez y alcalinidad del medio; y un segundo tipo de destrucción que se observa cuando las proteínas son hidrolizadas en presencia de glúcidos.

En la hidrólisis de proteínas puras, el caso más conocido y estudiado es la destrucción masiva del triptófano en medio ácido y en este sentido numerosos trabajos se han realizado en este Instituto Español de Fisiología y Bioquímica por García-Asso (1, 2, 3) en los que estudia la importancia que la conservación de dicho aminoácido presenta en los hidrolizados ácidos que han de emplearse con fines terapéuticos y el empleo del SO_2 como protector, trabajos que concuerdan con los realizados por Moor (4), Pedersen y Backer (5, 6, 7).

Pero las cosas se complican cuando las proteínas están en

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presencia de carbohidratos; hecho sumamente interesante, ya que es bien conocido que los alimentos que contienen proteínas y glúcidos ven disminuido su valor nutritivo por las reacciones entre esos componentes, ya sea durante su estacionamiento, conservación, o durante los tratamientos térmicos a que son sometidos durante su elaboración (8, 9, 10, 11).

Esta interacción, proteína-carbohidratos, es importante, no sólo en los alimentos elaborados, sino también en el análisis de los alimentos que los contienen, como: cereales, leche, etc., los que son hidrolizados corrientemente con ácido ClH.

ROXAS (12), ha demostrado que durante la ebullición de proteínas y azúcares con ClH al 20 % se destruían los siguientes aminoácidos: tirosina 15 %, cistina 3 %, arginina 2,5 %, triptófano 71 % y lisina 17 %.

SENTHESHANMUGANATHAN y HOOVER (13), estudian la destrucción de lisina en presencia de carbohidratos en distintas condiciones de hidrólisis y establecen pérdidas de un 25 %.

Los medios de protección que se han ensayado para evitar este hecho han sido, por una parte, separar la proteína previamente a la hidólisis, ya sea con ácido fórmico (WILKINS [14]), o con ácido fórmico y alcohol (ALVANESE), o precipitándola en su punto esoeléctrico (MAHON y COMMON [15]), o incorporando al medio hidrolítico reductores como el cloruro estanoso (16) a una mezcla de ácido IH e hipofosforoso.

Evidentemente, en el primer caso no se recupera cuantitativamente la proteína, y la técnica tiene dificultades; y en el segundo, se incorporan elementos que pueden interferir en las determinaciones químicas. Es por esto que, basados en los trabajos realizados en este Instituto sobre la acción protectora del SO_2 en las hidrólisis ácidas de proteínas puras, pensamos estudiar su acción en las mezclas proteínas-carbohidratos con especial referencia a ciertos aminoácidos: lisina, triptófano, metionina y cistina.

Material y métodos

Caseína pura, fibrina Merck, pepsina Difco, glucosa anhidra pura Panreac, sacarosa pura Panreac y almidón soluble Merck.

Hidrólisis: Se llevó a cabo en todos los casos en tubos de vidrio Pyrex resistentes, cerrados a la llama, con 2 g de proteínas en 50 ml de líquido hidrolizante. Calentado en el caso de proteínas puras, en autoclave a 100° durante 12 y 24 horas (hidrólisis parciales) y en el caso de las mezclas proteína-carbohidratos, como la finalidad es ahora obtener la hidrolisis total

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Sulfitmetabolismus
Sulfit im Blut
Sulfit im Harn

Sulfat im Harn
Taurin im Harn
Thiaminmangel

Thiaminmangel und Unbedenklichkeit von Sulfit für den Menschen

3. Mitteilung

Untersuchungen zum Metabolismus von Sulfit bei Thiaminunterversorgung

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(Direktor: Prof. Dr. H.-D. Cremer)

Zusammenfassung: Männlichen und weiblichen Versuchspersonen wurden über einen Zeitraum von 3 Wochen bei Gabe einer thiaminarmen Kost täglich 400 mg SO_2 in Form von Natriumhydrogensulfit und «Natriumglucosesulfonat» in Wein und Saft oral verabreicht. Die Ausscheidung von Sulfat und Taurin im Urin war erhöht. Sulfit konnte in venösem Blut und Urin niemals festgestellt werden. Es konnte experimentell bei den eingehaltenen Bedingungen kein Hinweis dafür gefunden werden, dass die Oxidationsfähigkeit des Organismus für Sulfit bei Thiaminmangel beeinträchtigt ist.

Nach Angaben zahlreicher Autoren [1, 8, 13, 19, 20, 22, 26] wird oral oder parenteral aufgenommenes Sulfit im Säugetierorganismus praktisch vollständig oxidiert und mit Harn und Kot als Sulfat ausgeschieden. Die mit den recht unspezifischen Bestimmungsmethoden ermittelten Sulfitwerte im Harn waren stets sehr gering. Nach Untersuchungen von Pfleiderer [20] an der Ratte wird ein grosser Teil von oral aufgenommenem Sulfit bereits im Magen-Darmtrakt oxidiert, ein geringer Anteil dagegen soll immer in die Blutbahn gelangen und wird in der Leber und im Blut oxidiert. Ein sulfitoxidierendes Enzymsystem wurde von verschiedenen Autoren beschrieben und aus Säugetierlebern isoliert [9, 14, 15, 23]. Ein entsprechendes Enzym findet sich in Mikroorganismen [3, 12]. Sein natürliches Substrat ist intermediär gebildetes Sulfit, welches im Organismus regelmässig beim Abbau der schwefelhaltigen Aminosäuren entsteht [3, 4, 10,

17, 18, 24]. Cystein wird z. B. über Cysteinsulfinat und Sulfinylpyruvat zu Sulfit und Pyruvat umgesetzt. Die Umkehrung dieser Reaktion beschrieben Chapeville und Fromageot [6], die nach Inkubation von Präparaten aus Niere und Leber von Säugetieren mit Pyruvat, Glutamat und ^{35}S-Sulfit ^{35}S-Cysteinsulfinat nachweisen konnten.

Uns interessierte die Frage, ob die im Tierversuch bewiesene erhöhte Toxizität von Sulfit bei Thiaminmangel evtl. darauf beruht, dass die Oxidationsfähigkeit des Organismus für Sulfit beeinträchtigt ist, wodurch es möglicherweise zu einer erhöhten Ausscheidung von Sulfit im Harn, oder zu einem Auftreten von Sulfit im Blut kommen könnte. Da bei Thiaminunterversorgung der Pyruvatspiegel im Blut ansteigt [11], wäre auch an eine mögliche Reaktion von Sulfit mit Pyruvat zu Cysteinsulfinat zu denken, welches leicht durch Oxidation und Decarboxilierung in Taurin übergehen kann [21].

Wir bestimmten daher in den Perioden vor, während und nach der Sulfitapplikation Sulfit, Sulfat und Taurin im Urin, also in der Zeit, während der eine relativ einheitliche Kost verabreicht wurde, da sowohl die Sulfat- [5] als auch die Taurinausscheidung [27] im Harn vom Kalorien- und Eiweissgehalt der Kost abhängt. Auch das Serum von venösem Blut der Vpn wurde auf Anwesenheit von Sulfit geprüft.

Methoden

Sulfit im Serum: Bestimmung nach Dufaigne [6] in der Modifikation von Engelhardt [7].

Sulfit im Harn: Bestimmung nach Kiehnöfer und Aumann [16]. Die dabei verwendete Apparatur von Lieb und Zacherl wurde während der Destillation mit Stickstoffgas durchströmt, um eine vorzeitige Oxidation von Sulfit zu vermeiden. Es wurde in eine 1 %ige H_2O_2-Lösung destilliert und die Menge an gebildetem Sulfat durch Titration mit 0.1 n NaOH ermittelt.

Sulfat im Harn: Bestimmung gravimetrisch als Bariumsulfat, Berechnung als g Sulfat/24 h. Die tägliche Harnmenge wurde mit Hilfe des Kreatiningehalts errechnet.

Taurin im Harn: Bestimmung nach Sörbo [25]. Berechnung als mg Taurin/24 h.

Ergebnisse

Mit den von uns angewandten Analysenmethoden konnte während der gesamten Versuchsdauer weder im Blutserum noch im Harn Sulfit nachgewiesen werden. Die Sulfatausscheidung stieg in Periode III nicht nur bei der Testgruppe, sondern auch bei der Kontrollgruppe, offensichtlich wegen des Sulfatgehaltes des Weines und Traubensaftes, signifikant an. Die Sulfatausscheidung der Testpersonengruppe übertraf allerdings die Kontrollgruppe im Mittel um ca. 700 mg/Tag, die Unterschiede waren statistisch sicherbar. Bei völliger Oxidation der applizierten Sulfitmenge hätten 500 mg Sulfat entstehen müssen; der Überschuss von ca. 200 mg erklärt sich daraus, dass die mit Sulfit versetzten Ge-

Tab. 1: Sulfat im Harn (g/24 h)

		Testgruppe T							Kontrollgruppe K						
		♂			♀				♂			♀			
Periode	Datum	1	3	5	13	16	18	$\bar{x} \pm s$	6	7	8	11	14	15	$\bar{x} \pm s$
II	6. Nov.	1.84	1.12	1.26	2.81	2.69	2.17		1.76	2.45	1.85	1.87	2.77	–	
	8. Nov.	2.18	2.98	1.90	3.83	2.18	2.36		2.72	2.83	2.65	3.25	3.25	2.53	
	⌀	2.01	2.10	1.58	3.32	2.44	2.27	2.29 ± 0.59	2.24	2.63	2.25	2.56	3.01	2.53	2.54 ± 0.28
III	13. Nov.	3.30	4.51	4.78	5.51	3.90	6.27		2.84	3.65	3.16	3.40	3.96	2.83	
	15. Nov.	3.40	4.27	3.94	5.44	5.86	5.23		3.41	3.67	2.65	3.84	3.31	3.65	
	22. Nov.	3.86	2.75	1.99	3.21	4.50	4.83		2.16	2.21	2.98	3.44	3.36	4.15	
	27. Nov.	3.06	3.46	3.69	3.00	2.61	2.92		2.79	3.31	2.67	2.27	2.50	–	
	29. Nov.	4.17	4.96	3.43	3.61	4.11	5.35		2.86	–	2.51	3.30	2.98	2.96	
	4. Dez.	3.07	3.48	3.44	4.63	4.18	3.28		2.77	2.71	2.20	3.59	2.46	3.12	
	6. Dez.	4.12	5.77	3.43	4.18	4.28	4.43		–	3.41	2.93	4.35	3.14	3.50	
	⌀	3.57	3.80	3.52	4.23	4.21	4.54	3.98 ± 0.42	2.81	3.16	2.99	3.45	3.02	3.37	3.27 ± 0.36
IV	16. Dez.	3.60	3.83	2.76	3.51	3.15	3.34		3.60	3.40	2.92	2.93	2.84	3.68	
	17. Dez.	2.39	2.57	2.27	4.52	2.81	4.10		3.39	3.12	3.98	3.97	3.19	3.69	
	⌀	3.00	3.20	2.52	4.02	2.98	3.72	3.23 ± 0.41	3.49	3.26	3.45	3.48	3.02	3.69	3.46 ± 0.23

K_{II}: T_{III} $p < 0.001$ T_{III}: K_{IV} $p < 0.02$ K_{III}: T_{III} $p < 0.02$

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Tab. 2: Taurin im Harn (mg/24 h)

Periode	Datum	Testgruppe T ♂ 1	3	6	♀ 13	16	18	$\bar{x} \pm s$	Kontrollgruppe K ♂ 6	7	8	♀ 11	14	15	$\bar{x} \pm s$
II	4. Nov.	70.8	93.1	111.8	78.6	132.4	128.4		74.1	132.2	200.2	117.7	142.8	274.3	
	7. Nov.	81.8	102.7	89.2	113.2	119.4	34.4		69.6	54.9	104.1	165.6	104.9	154.4	
	11. Nov.	86.5	107.1	97.4	154.1	84.0	45.2		70.9	97.4	156.9	126.7	107.8	108.6	
	x	79.7	100.9	99.4	115.3	111.9	69.3	96.0 ± 18.0	71.5	94.8	155.7	156.6	118.5	179.1	126.0 ± 39.5
III	14. Nov.	79.1	108.5	113.2	123.9	163.3	37.8		70.2	91.7	121.5	80.0	76.2	175.1	
	18. Nov.	107.4	159.1	121.0	159.8	103.5	105.4		79.1	107.3	149.2	141.4	245.2	161.7	
	21. Nov.	142.7	169.6	144.1	149.9	90.5	84.7		137.3	76.8	163.4	171.3	186.3	155.6	
	25. Nov.	136.5	164.6	159.9	205.9	166.3	111.0		149.7	168.7	169.5	97.2	121.4	182.0	
	28. Nov.	125.6	128.9	136.3	242.3	144.2	117.6		96.9	148.6	120.8	95.6	58.5	210.3	
	2. Dez.	154.6	162.5	145.0	223.9	212.1	101.2		91.1	109.8	161.7	147.5	137.6	182.7	
	5. Dez.	119.7	181.5	130.8	103.3	122.9	114.6		137.8	173.9	189.3	106.1	76.9	163.3	
	x	123.6	153.5	135.7	172.7	143.2	96.0	137.4 ± 26.2	108.8	125.2	153.6	119.8	128.8	175.5	135.2 ± 24.6
IV	9. Dez.	100.0	114.7	125.4	101.5	93.3	81.5		98.4	113.1	132.4	111.7	92.8	114.2	
	12. Dez.	102.3	99.4	142.6	161.0	108.4	61.7		109.5	142.0	153.0	90.1	96.7	120.4	
	x	101.2	107.0	134.0	151.2	100.8	71.6	107.6 ± 22.9	105.9	127.5	142.7	100.9	94.7	117.3	114.5 ± 18.2

T_{II}: T_{III} $p > 0.05$

**	17. Dez.	2.39	2.57	2.27	4.52	2.81	4.10		3.39	3.12	3.98	3.97	3.19	3.69	
	x	3.00	3.20	2.52	4.02	2.98	3.72	3.28 ± 0.41	3.49	3.26	3.48	3.48	3.02	3.69	3.46 ± 0.23

K_{II}: T_{III} $p < 0.001$ T_{III}: K_{IV} $p < 0.02$ K_{III}: T_{III} $p < 0.02$

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tränke mehr Sulfat enthalten hatten, welches beim «Schwefeln» der Getränke mit Natriumhydrogensulfit und «Natriumglukosesulfonat» mit eingeschleppt worden war. Der Abfall der Sulfatausscheidung nach Absetzen der Sulfitgaben (Vgl. T_{III} mit K_{IV}) war statistisch sicherbar. Die Taurinausscheidung stieg in der Testgruppe um 43 %, in der Kontrollgruppe um 12 % bezogen auf den Ausgangswert. Die Mehrausscheidung nach Gabe von Sulfit war evident, liess sich aber statistisch dennoch nicht sichern, da die Ausgangswerte sehr stark differierten.

Diskussion

Die Frage, ob die im Tierversuch bewiesene erhöhte Toxizität von Sulfit bei Thiaminmangel evtl. darauf beruht, dass die Oxidationsfähigkeit des Organismus für Sulfit beeinträchtigt ist, lässt sich nach den Ergebnissen unsere Versuche verneinen: Es konnte unter den genannten Versuchsbedingungen weder Sulfit im Harn noch im venösen Blut nachgewiesen werden, während andererseits die Höhe der Sulfatausscheidung praktisch eine völlige Oxidation des Sulfits im Organismus vermuten lässt. Diese Ergebnisse widersprechen nicht unseren früheren Untersuchungen, in denen wir im Pfortaderblut von Ratten mit gleicher Bestimmungsmethode Sulfit nachweisen konnten, sondern zeigen, dass der Entgiftungsmechanismus der Leber auch bei Thiaminmangel voll wirksam ist. Die Frage, ob es im Thiaminmangel zu einer erhöhten Taurinbildung bei Gabe von Sulfit kommt, kann wegen der grossen Streubreite der Taurinwerte im Harn nicht sicher beantwortet werden. Dabei muss noch beachtet werden, dass intermediär gebildetes Taurin nur zum geringen Teil im Harn erscheint, während der allergrösste Teil an Gallensäuren gekoppelt und mit der Galle ausgeschieden wird [21].

***Summary:** Effect of thiamine deficiency on the metabolism of sulfite.*

For a period of 50 days male and female volunteers received a thiamine-deficient diet, the controls without and the test group during 25 days with 400 mg of SO_2/day given as sodiumhydrogensulfite and "sodium-glucose-sulfonate" dissolved in wine and grape-juice (experimental conditiones compare first communication).

No traces of sulfite could be detected either in the blood or in the urine. Compared to the preexperimental period the sulfate excretion in the urine increased in the experimental group as well as in the control group who received the same diet including wine and juice, but no added sulfite. The relatively high amount of naturally occurring sulfate in wine and grape juice actually contributed to the urine sulfate, but the increase was significantly higher in the experi-

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mental group on an average of 700 mg per person per day. The total oxidation of the applied sulfite should have amounted to 500 mg of sulfate. The surplus of 200 mg of urine sulfate can be explained by the fact that "glucose-sulfonate" always contains sulfate. At the end of the experiment, when no more sulfite was given to the volunteers, their sulfate excretion in the urine fell significantly.

Compared with the preexperiment levels the taurine-excretion increased by 43 % in the testgroup and by 12 % in the controlgroup. This increase was statistically not significant because of the extremly great variations in the taurine-excretion at the beginning of the experiment. There were not any experimental signs of thiaminedeficiency having an influence on the sulfite oxidizing ability of the organism under the conditions of the experiment.

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Microcolorimetric Estimation of Sulfur Dioxide in Fruits and Fruit Products

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A simple and rapid microcolorimetric method, which is based on the reduction of yellow colored peroxodisulfato titanic acid to colorless titanium oxysulfate, has been developed for the determination of sulfur dioxide in dried fruit, fruit sirups, beverages, and liquid concentrates. The reduction in intensity of the yellow color is directly proportional to the concentration of sulfur dioxide over the range of 0–30 μg/ml.

Detailed reviews on the chemistry of sulfur dioxide and analytical methods for its determination have been published by Joslyn and Braverman (1), Gehman and Osman (2), and Schuller and Veen (3). There are gravimetric (4), volumetric (4, 5), iodometric (1), and colorimetric methods (3) for the determination of sulfur dioxide in foods. The gravimetric and volumetric methods are time-consuming, although they are relatively accurate. The Monier-Williams method has been used as a standard method with which other methods are compared (6, 7). Most of the colorimetric methods are based on the well established technique of Steigman (8, 9), a modification of the well known reaction for the detection of aldehydes with colorless fuchsin-sulfurous acid. Other colorimetric methods depend on the bleaching of malachite green solution by sulfur dioxide (10). The present method involves the decolorization of yellow peroxodisulfato titanic acid by sulfur dioxide (11, 12). The reactions involved are given on p. 1147.

METHOD

Apparatus

(a) *Colorimeter.*—Klett-Summerson Photo-Electric (Klett Manufacturing Co., Inc., New York), or equivalent with No. 42 (blue) filter.

(b) *Distillation flask.*—100 ml round-bottom 3-neck flask with ₮ 14/23 ground glass joints.

(c) *Thistle funnel.*—With ₮ 14/23 ground glass joint.

(d) *Water-cooled condenser.*—With 22 cm long jacket and ₮ 14/23 ground glass joints. One end of condenser is tapered and bent as shown in Fig. 1. Bent tube almost touches bottom of receiving tube.

(e) *Receiving tube.*—100 ml, 19 cm long with ₮ 14/23 ground glass joint and side arm as gas vent.

(f) *Ice bath.*—0–4°C.

Reagents

(a) *Hydrogen peroxide.*—0.005*M* previously calibrated with 0.1*N* $KMnO_4$. Pipet 5.7 ml 3.0% H_2O_2 into 1 L volumetric flask and dilute to volume with water.

(b) *Peroxodisulfato titanic acid (PDT) stock solutions.*—Digest 1 g TiO_2 and 10 g K_2SO_4 in 150 ml concentrated H_2SO_4 by heating for 6–8 hr (after boiling begins, temperature >300°C) with flame under hood. (*1*) *Stock solution A.*—Cool, shake, and transfer digest to clean dry glass-stoppered bottle. (*2*) *Stock solution B.*—Transfer 30 ml stock solution A to 1 L volumetric flask. Add 70 ml concentrated H_2SO_4, shake thoroughly 2–3 min, carefully dilute to volume with distilled water, and filter.

(c) *Peroxodisulfato titanic acid (PDT) reagent.*—Thoroughly mix 200 ml 0.005*M* H_2O_2, 600 ml stock solution B, and 200 ml 1*N* H_2SO_4. Store in amber bottle. Reagent is stable at least 2 months.

(d) *Standard solutions of SO_2.*—(*1*) *Stock solution.*—Dissolve 1 g reagent grade potassium metabisulfite in 1 L volumetric flask and dilute to volume with water. Calibrate solution against standard solution of iodine. (*2*) *Working standard.*—Dilute calculated amount of stock solution to obtain working concentration of 100 μg SO_2/ml.

(e) *NaOH solution.*—Dissolve 4 g NaOH pellets in water and dilute to 1 L in volumetric flask.

Preparation of Standard Curve

Pipet 5 ml PDT reagent into 7 separate 10 ml glass-stoppered graduated cylinders. Add by pipet 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 ml working standard solution (100 μg SO_2/ml) to each cylinder. Dilute to volume with distilled water and mix thoroughly. Measure intensity of color in Klett-Summerson Photo-Electric Colorimeter, using No. 42 (blue) filter (absorption max 410 nm). Plot μg SO_2/ml vs. Klett units after subtraction from suitable controls (without SO_2) to obtain standard curve.

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$$TiO_2 + H_2SO_4 \xrightarrow[\text{(digestion)}]{K_2SO_4} TiOSO_4 + H_2O \quad (1)$$

$$TiOSO_4 + H_2O_2 + H_2SO_4 \longrightarrow \underset{\text{peroxodisulfate titanic acid}}{H_2(TiO_2(SO_4)_2)} + H_2O \quad (2)$$

$$SO_2 + \underset{\text{yellow}}{H_2(TiO_2(SO_4)_2)} + H_2O \longrightarrow \underset{\text{colorless}}{TiOSO_4} + 2H_2SO_4 \quad (3)$$

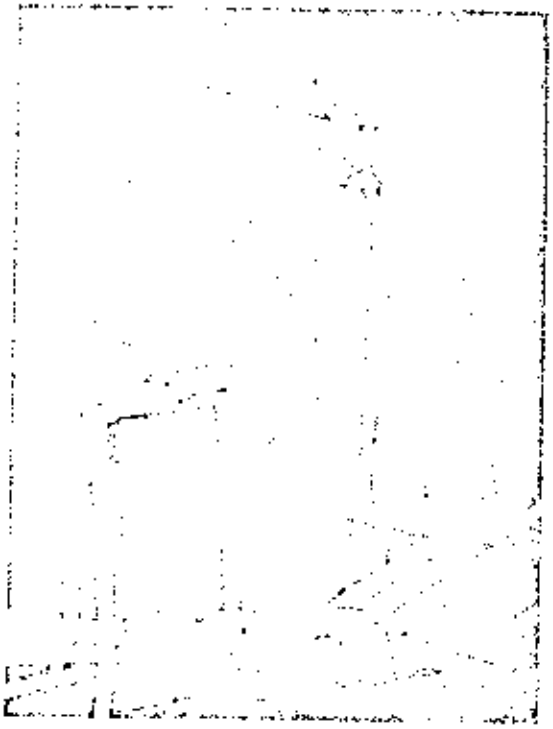

FIG. 1—Sulfur dioxide distillation assembly.

Estimation of SO_2 in Fruit Sirups and Liquid Concentrates

Set up apparatus as shown in Fig. 1. Circulate cold water through condenser. Pipet 5 ml 0.1N NaOH into receiving tube. Mix sirup or liquid concentrate thoroughly, weigh 10-12 g sample (containing 2-3 mg SO_2) in beaker, and wash into distillation flask through thistle funnel. Add 10 ml dilute HCl (ca 1N) to distillation flask, wash down thoroughly with 35-40 ml distilled water, and add 2 or 3 glass beads. Take care to see that all connections are air tight and tip of heat tube dips into dilute NaOH solution. Heat sample carefully over burner, distill SO_2 for 15 min after solution starts boiling, and collect ca 35-40 ml distillate. Transfer distillate to 100 ml volumetric flask. Rinse receiving tube with water and transfer rinsings to same volumetric flask. Dilute to volume with water.

Pipet aliquots of distillate (1-5 ml) into 10 ml glass-stoppered graduated cylinders each containing 5.0 ml PDT reagent and dilute to volume with water. Shake to mix and read color intensities as for standards. Obtain concentration of SO_2 in samples from standard curve.

Results and Discussion

The recoveries of sulfur dioxide from fruit sirups and liquid concentrates are shown in Tables 1 and 2. Interference likely to be encountered due to other volatile reducing substances is shown in Table 3.

The method is simple and rapid and does not require any elaborate assembly of equipment or complicated reagents. Pipetting errors are minimized, since only one reagent is required. The PDT reagent is easy to prepare and highly sensitive to traces of sulfur dioxide; it can be stored for 2-3 months. Values obtained for different concentrations of sulfur dioxide exhibit a linear relationship and are in close agreement with Beer's law for concentrations from 0 to 30 μg/ml. Results are reproducible.

The standard deviations between duplicate determinations for each method and sample are given in Table 1. The standard deviations obtained for the present method range from 0.05 to 0.10 and are in close agreement with the Monier-Williams method as modified by Shipton (5). Recoveries of sulfur dioxide range from 89.5 to 97.8%; these are also comparable to the results in ref. 5.

The applicability of this method to other fruit products such as dehydrated fruit and commercial liquid concentrates (orange, pineapple, and lemon concentrates) was also tested (Table 2). Dried fruits were prepared for analysis by blending 25 g chopped fruit with water (1:10) in a blender 3-4 min. The resulting slurry was used for analysis.

The values obtained by the present method (although consistently lower) are comparable to those obtained by the Monier-Williams method.

Table 1. Recovery of sulfur dioxide from sirup, fruit concentrate, and fruit beverage

Sample No.[a]	SO_2 Added, ppm	Shipton's Method: Found, ppm Mean±S.E.[b]	Shipton's Method: Rec., %	Shipton's Method: Std Dev.[c]	Present Method: Found, ppm Mean±S.E.[b]	Present Method: Rec., %	Present Method: Std Dev.[c]
			Pure Sucrose Sirup[d]				
1	Nil	—	—	—	—	—	—
2	50	44.18±2.22	88.4	1.05	44.82±0.10	89.6	0.35
3	75	71.67±0.53	95.6	0.64	70.76±0.28	94.3	0.71
4	100	93.21±0.52	93.2	0.61	92.35±2.88	93.2	0.05
5	200	181.3 ±0.45	90.7	2.21	178.9 ±0.73	89.5	0.56
6	250	242.2 ±2.28	96.9	3.35	235.2 ±0.27	94.1	2.68
7	300	292.4 ±3.55	97.5	7.36	286.4 ±1.80	95.5	1.02
8	350	327.7 ±1.33	93.6	2.06	319.8 ±1.00	91.4	2.34
9	400	377.2 ±0.40	94.3	2.40	369.9 ±4.70	92.5	4.65
			Mango Liquid Concentrate[e]				
10	Nil	—	—	—	—	—	—
11	200	185.6 ±0.37	92.8	1.01	184.9 ±1.70	92.5	3.11
12	250	238.6 ±3.55	95.4	2.31	238.5 ±0.80	95.4	0.51
13	300	296.5 ±2.23	98.8	3.64	286.8 ±0.15	95.6	0.98
14	350	332.3 ±1.45	94.9	5.17	323.8 ±1.13	91.8	6.10
15	400	372.5 ±3.83	93.1	2.10	369.0 ±2.08	92.3	0.21
			Mango Beverage[f]				
16	50	47.84±0.09	95.7	3.16	47.78±0.65	95.5	1.51
17	75	70.91±0.80	94.5	1.60	69.12±0.65	92.2	0.69
18	100	97.84±0.22	97.8	1.50	96.47±0.86	97.8	0.83

[a] Duplicate determinations were performed on each of 2 randomly selected samples.
[b] Standard error of the mean from duplicate readings of 2 samples.
[c] Standard deviation from duplicate readings pooled over both samples.
[d] Samples 1, 5, 6, 7, 8, and 9: 45° Brix, 1.2% citric acid; Samples 2, 3, and 4: 15° Brix, 0.3% citric acid.
[e] 45° Brix, 1.2% citric acid.
[f] 15° Brix, 0.3% citric acid.

Table 2. Sulfur dioxide in commercial fruit products of unknown sulfur dioxide content

Sample	Found, ppm[a]: Shipton's Method	Found, ppm[a]: Present Method
	Dehydrated Fruit	
Mango fruit bar	944.0	928.0
Banana fruit bar	714.8	717.2
	Liquid Concentrate	
Orange	184.05	178.85
Pineapple	310.05	300.68
Lemon	255.25	249.60

[a] Results are averages of duplicate readings per sample.

Table 3. Effect of some steam volatiles on results for sulfur dioxide determined by present method

Sample No.	Substances Added	Level, μg/ml	Klett Units (410 nm)	Variation from Control, %
1	Control[a]	—	45	—
2	Ethanol	50	45	0
3	Ethanol	100	45	0
4	Acetaldehyde	10	45	0
5	Acetaldehyde	20	45	0
6	Furfural	10	46	−2.2
7	Furfural	20	46	−2.2
8	Essential oil (lime)	10	45	0
9	Essential oil (lime)	20	46	−2.2

[a] 5 ml POT reagent and 200 μg sulfur dioxide in 10 ml total volume.

Other volatile constituents like ethanol, acetaldehyde, essential oils, and furfuraldehyde, likely to be present in the distillate from fruit juice, do not seem to interfere in the reaction between sulfur dioxide and POT reagent (Table 3). The reaction is, therefore, specific for sulfur dioxide.

Direct estimation of sulfur dioxide without prior distillation would be ideal for food products. Our efforts toward this objective, however, were not successful, because most of the beverages possess natural colors (yellow to red) which interfere with the color measurement. Nonetheless, the method can be of immense use to the food industry because of its simplicity, reliability, and reproducibility.

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REMINDER . . .

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SO_2 ガス濃度の気道排塵能力に及ぼす影響

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Effect of SO_2 Concentration in Atmopshere on Faculty of Dust Exhaustion in Respiratory Tract

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緒　言

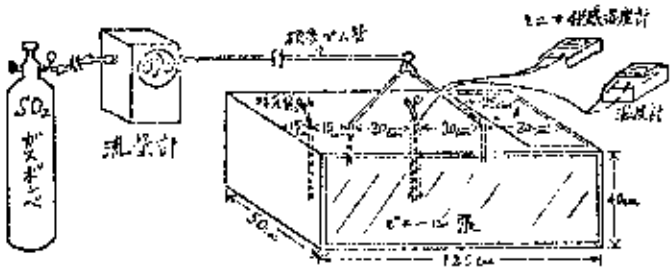
検近，大気汚染の生体への影響は一般の関心を惹起し，ことにこれが呼吸器系に及ぼす影響については大気汚染物質中の SO_2 が，その性質上，また量的にも重要な役割を演ずるは諸家[1)1)-5)]の等しく認めるところである．しかも昭和37年ばい煙規制法公布以来，名古屋，四日市などの指定地域の降下煤塵量はほぼ横ばいの状態にあるにかかわらず，SO_2 量は逐年的に増加を示しつつある[9)10)]現状は誠に憂慮にたえない．他方，気道の排塵作用は分泌物の排泄，異物の排除[11)]を司り，呼吸器疾病罹患と密接な関係を有するがゆえに排塵能力の良否は大気汚染の呼吸器におよぼす影響を論ずる上においてきわめて重要なものであるは容易に首肯しうるところであろう．しかるに SO_2 の呼吸器系におよぼす影響については多くの研究報告[12)11)-20)]があるも，SO_2 の気道排塵能力におよぼす影響を SO_2 濃度別に観たものについてはいまだにこれあるを知らない．

ここにおいて私は種々な SO_2 濃度の環境に家兎を毎日20分，連日5週にわたって暴露し，この間における気道排塵能力の変動，体重および血液性状の経過を比較観察して SO_2 濃度の排塵能力におよぼす影響を究明し，もって大気汚染の呼吸器系に及ぼす機序の一端を明らかにし，大気汚染防止対策の基礎的資料に資せんとして本研究を行なった．

第1章　実験方法

体重2.9 kg 内外の健康な同一系統雄性白色成熟家兎88羽を用い，4羽を対照群となし，84羽を21羽ずつ暴露 SO_2 濃度3 ppm, 5 ppm, 10 ppm および20 ppm 群の4群に分け，暴露箱内で毎日20分ずつ，連日5週間にわたり，それぞれ所定濃度の SO_2 ガスに暴露し，この間における気道排塵速度の変化を比較観察するとともに体重，赤血球数，血色素量，Ht 値および白血球数の経過を究明した．

SO_2 暴露には第1図に示すような木枠，ダンボール，ビニール布およびビニールテープで作製した長方形の暴露箱（50×120×40 cm）2個を用い，箱内の SO_2 濃度が所定の濃度になるように亜硫酸ガスボンベ（岩谷産業KK製）からの流量を加減したのち，暴露箱後方の蓋(A)を静かに開け，試獣を2羽ずつ素早く入



第1図　SO_2 暴露装置

れて密閉する．次で暴露開始後5分毎に2本の採気管より採気して箱内 SO_2 濃度を検知管法で測定し，その平均値が常に所定の濃度を保つように注意して流量を調節しつつ，20分間暴露を行なった．なお，本実験は秋季に行ない，暴露中の箱内の温，湿度の上昇は熱電的に測定したが，箱外気に比し，温，湿度の上昇は約2°C，6%内外にすぎなかった．しかして暴露は暴露条件を常に一定にするために暴露終了ごとに箱内を全換気した後，箱内 SO_2 濃度を所定濃度に調節して行なった．

気道排塵能力の測定は SO_2 暴露後に任意に選出した家兎4羽について逐週的に4週間行ない，暴露5週後には残りの5羽について行なった．すなわち SO_2 暴露終了後，血液検査に要する採血を行なった後，家兎を仰臥位に固定し，麻酔を用いずに頸胸部を剃毛した後，縦に切開し，可及的に出血を避けて気管を甲状軟骨上部および気管支分岐部直上において切断して摘出する．この際の気管の長さは約7 cmである．次いで直ちに水温37.5°Cの0.85%生理的食塩水で洗滌後，速かに気管後壁膜様部を縦に切開し，再び上記の生理的食塩水で軽く洗滌した後，気管を左右に開き喉頭部を上にして予め上記の生理的食塩水で湿したコルク板（8.5×14.0 cm）に留針で固定し，これを上記37.5°Cの生理的食塩水を満したシャーレ（直径9.5 cm）中に直立せしめて気管の下端が約0.6 cm浸漬するようにする．次いでこれを直ちに温度37.5°C，飽和湿度の電気恒温箱（50×50×48 cm）中の架台上に置き，速かに炭粉末（80 μ以下）を気管の浸漬境界線の直上に細い竹へらで薄く境界線に沿って1列に付着せしめた後，ガラス扉を閉じ，直ちに気道線毛運動による炭粉粒子の上昇速度をストップウオッチと上記コルク板の両側に固定した金尺とにより測定し，炭粉移動距離と排塵速度を算出して排塵能力を検した．なお，上記の操作は当教室設置の室温31°C，湿度60%の恒温室（2.0×2.6×2.5 m³）内で行ない，電気恒温箱内の架台下には39°Cの温水を満した琺瑯製バット（23×29×4.5 cm）を置いて箱内が常に飽和を保つように工夫し，恒温箱の観察用ガラス扉には石鹸水を塗布して曇りを防止した．

体重の測定は10 g目盛の自動台秤を用いて SO_2 暴露前の空腹時に行ない，血液検査は SO_2 暴露後の空腹時において耳様静脈を穿刺し，涌出する血液について行なった．すなわち赤血球数，血色素量および白血球数の測定には血液学会検定済のそれぞれのメランジュールを用い，計算法およびSahli—小宮山氏法[21]で行ない，Ht値の測定には毛細管ヘマトクリット法[22]を用いた．

第2章　実験成績

気道排塵能力と SO_2 濃度との関係については第1表（その1，2），第2表，第2，3図に示すごとく SO_2 濃度3 ppmでは暴露日数の長短にかかわらず，ほとんど変化を認めないが，5 ppm以上の濃度ではそれぞれ影響をうけ，排塵速度は SO_2 5 ppmでは暴露後2週間は亢進し，3週以後はもとに復するが，10 ppmおよび20 ppmでは暴露後3週までは低下し，以後は回復に向かうも後者は前者に比して低下が大きく，その回復は遅れる．すなわち180秒間の気道排塵平均速度は SO_2 3 ppm群では暴露

第1表（その1） SO_2 濃度別暴露日数別気道排除能力

暴露 SO_2 濃度別	暴露日数別（週）	家兎番号	体重（g）	経過時間（秒）→ / 排除能力↓	30″	60″	90″	120″	150″	180″
3 ppm 群	1 週間群	No. 11	2920	移動距離(mm)	8	17	25	33	38	46
				平均速度(mm/sec)	0.27	0.28	0.28	0.28	0.25	0.26
		No. 12	2860	移動距離(mm)	8	15	23	30	37	44
				平均速度(mm/sec)	0.27	0.25	0.26	0.25	0.25	0.24
		No. 13	2980	移動距離(mm)	9	16	27	33	40	47
				平均速度(mm/sec)	0.30	0.27	0.30	0.28	0.27	0.26
		No. 14	3020	移動距離(mm)	8	14	22	30	36	41
				平均速度(mm/sec)	0.27	0.23	0.24	0.25	0.24	0.23
		平　均	2945.0	移動距離(mm)	8.3	15.5	24.3	31.5	37.8	44.5
				平均速度(mm/sec)	0.28	0.26	0.27	0.27	0.25	0.25
	2 週間群	No. 21	2860	移動距離(mm)	9	15	24	32	40	45
				平均速度(mm/sec)	0.30	0.25	0.27	0.27	0.27	0.25
		No. 22	2960	移動距離(mm)	8	14	21	29	36	42
				平均速度(mm/sec)	0.27	0.23	0.23	0.24	0.24	0.23
		No. 23	3080	移動距離(mm)	8	16	24	31	37	44
				平均速度(mm/sec)	0.27	0.27	0.27	0.26	0.25	0.24
		No. 24	3020	移動距離(mm)	9	16	23	32	39	46
				平均速度(mm/sec)	0.30	0.27	0.26	0.27	0.26	0.26
		平　均	2980.0	移動距離(mm)	8.5	15.3	23.0	31.0	38.0	44.3
				平均速度(mm/sec)	0.29	0.26	0.26	0.26	0.26	0.25
	3 週間群	No. 31	3100	移動距離(mm)	8	16	25	32	36	42
				平均速度(mm/sec)	0.27	0.27	0.28	0.27	0.24	0.23
		No. 32	3020	移動距離(mm)	7	14	22	31	37	45
				平均速度(mm/sec)	0.23	0.23	0.24	0.26	0.25	0.25
		No. 33	2840	移動距離(mm)	9	16	26	34	41	46
				平均速度(mm/sec)	0.30	0.27	0.29	0.28	0.27	0.26
		No. 34	2820	移動距離(mm)	8	15	22	29	36	44
				平均速度(mm/sec)	0.27	0.25	0.24	0.24	0.24	0.24
		平　均	2945.0	移動距離(mm)	8.0	15.3	23.8	31.5	37.5	44.3
				平均速度(mm/sec)	0.27	0.26	0.26	0.26	0.25	0.25
	4 週間群	No. 41	3020	移動距離(mm)	8	15	23	34	41	47
				平均速度(mm/sec)	0.27	0.25	0.26	0.28	0.27	0.26
		No. 42	2860	移動距離(mm)	9	17	26	35	40	45
				平均速度(mm/sec)	0.30	0.28	0.29	0.29	0.27	0.25
		No. 43	2950	移動距離(mm)	8	14	22	29	35	42
				平均速度(mm/sec)	0.27	0.23	0.24	0.24	0.23	0.23
		No. 44	2900	移動距離(mm)	8	14	25	32	39	43
				平均速度(mm/sec)	0.27	0.23	0.28	0.27	0.26	0.24
		平　均	2932.5	移動距離(mm)	8.3	15.0	24.0	32.5	38.8	44.3
				平均速度(mm/sec)	0.28	0.25	0.27	0.27	0.26	0.24
	5	No. 51	2840	移動距離(mm)	8	14	21	30	37	44
				平均速度(mm/sec)	0.27	0.23	0.23	0.25	0.25	0.24
		No. 52	2800	移動距離(mm)	9	16	22	29	36	42
				平均速度(mm/sec)	0.30	0.27	0.24	0.24	0.24	0.23

第1表（その1）（続き）

最終SO_2濃度別	基礎日数別（週）	容器番号	体重（g）	経過時間（秒）→ 排煙能力↓	30″	60″	90″	120″	150″	180″
	週間群	No. 53	3000	移動距離(mm) 平均速度(mm/sec)	7 0.23	15 0.25	25 0.23	31 0.26	38 0.25	45 0.25
		No. 54	2880	移動距離(mm) 平均速度(mm/sec)	8 0.27	16 0.27	27 0.30	33 0.28	42 0.28	45 0.25
		No. 55	2960	移動距離(mm) 平均速度(mm/sec)	9 0.30	15 0.25	23 0.26	31 0.26	40 0.27	46 0.26
		平均	2896.0	移動距離(mm) 平均速度(mm/sec)	8.2 0.27	15.2 0.25	23.6 0.26	30.8 0.26	38.6 0.26	44.4 0.25
5 ppm 群	1週間群	No.111	3100	移動距離(mm) 平均速度(mm/sec)	8 0.27	17 0.28	26 0.29	34 0.28	40 0.27	47 0.26
		No.112	2960	移動距離(mm) 平均速度(mm/sec)	9 0.30	19 0.32	23 0.31	36 0.30	43 0.29	50 0.28
		No.113	2940	移動距離(mm) 平均速度(mm/sec)	8 0.27	16 0.27	24 0.27	33 0.28	40 0.27	46 0.26
		No.114	3000	移動距離(mm) 平均速度(mm/sec)	9 0.30	16 0.27	25 0.28	31 0.26	37 0.25	43 0.24
		平均	3000.0	移動距離(mm) 平均速度(mm/sec)	8.5 0.29	17.0 0.29	25.8 0.29	33.5 0.28	40.0 0.27	46.5 0.26
	2週間群	No.121	2860	移動距離(mm) 平均速度(mm/sec)	9 0.30	16 0.27	26 0.29	35 0.29	42 0.28	48 0.27
		No.122	2880	移動距離(mm) 平均速度(mm/sec)	9 0.30	20 0.33	29 0.32	38 0.32	46 0.31	52 0.29
		No.123	3010	移動距離(mm) 平均速度(mm/sec)	8 0.27	18 0.30	27 0.30	35 0.29	43 0.29	50 0.28
		No.124	2960	移動距離(mm) 平均速度(mm/sec)	10 0.33	21 0.35	30 0.33	39 0.33	48 0.32	55 0.31
		平均	2947.5	移動距離(mm) 平均速度(mm/sec)	9.0 0.30	18.8 0.31	28.0 0.31	36.8 0.31	44.8 0.30	51.3 0.29
	3週間群	No.131	3120	移動距離(mm) 平均速度(mm/sec)	8 0.27	15 0.25	24 0.27	30 0.25	36 0.24	42 0.23
		No.132	2820	移動距離(mm) 平均速度(mm/sec)	9 0.30	16 0.27	26 0.29	32 0.27	38 0.25	45 0.25
		No.133	2860	移動距離(mm) 平均速度(mm/sec)	8 0.27	14 0.23	23 0.26	31 0.26	40 0.27	47 0.26
		No.134	3030	移動距離(mm) 平均速度(mm/sec)	8 0.27	16 0.27	24 0.27	32 0.27	39 0.26	45 0.25
		平均	2970.0	移動距離(mm) 平均速度(mm/sec)	8.3 0.28	15.3 0.26	24.3 0.27	31.3 0.26	38.3 0.26	44.8 0.25
	4週間	No.141	3000	移動距離(mm) 平均速度(mm/sec)	8 0.27	14 0.23	20 0.22	29 0.24	38 0.25	45 0.25
		No.142	2900	移動距離(mm) 平均速度(mm/sec)	8 0.27	17 0.28	25 0.28	32 0.27	37 0.25	42 0.23
		No.143	2840	移動距離(mm) 平均速度(mm/sec)	9 0.30	15 0.25	22 0.24	30 0.25	39 0.26	47 0.26

第1表（その1）（続き）

暴露SO_2濃度別	暴露日数別（週）	家兎番号	体重（g）	経過時間（秒）→ 排痰能力↓	30″	60″	90″	120″	150″	180″
	群	No.144	2870	移動距離(mm) 平均速度(mm/sec)	8 0.27	16 0.27	24 0.27	32 0.27	38 0.25	44 0.24
		平　均	2902.5	移動距離(mm) 平均速度(mm/sec)	8.3 0.28	15.5 0.26	22.8 0.25	30.8 0.26	38.0 0.25	44.5 0.25
	5週間群	No.151	2900	移動距離(mm) 平均速度(mm/sec)	8 0.27	16 0.27	24 0.27	31 0.26	36 0.24	43 0.24
		No.152	3020	移動距離(mm) 平均速度(mm/sec)	7 0.23	15 0.25	23 0.26	30 0.25	38 0.25	44 0.24
		No.153	2840	移動距離(mm) 平均速度(mm/sec)	8 0.27	17 0.28	25 0.28	32 0.27	40 0.27	46 0.26
		No.154	2800	移動距離(mm) 平均速度(mm/sec)	8 0.27	15 0.25	21 0.23	29 0.24	37 0.25	42 0.23
		No.155	2950	移動距離(mm) 平均速度(mm/sec)	8 0.27	16 0.27	27 0.30	32 0.27	41 0.27	47 0.26
		平　均	2920.0	移動距離(mm) 平均速度(mm/sec)	7.8 0.26	15.8 0.26	24.0 0.27	30.8 0.26	38.4 0.26	44.4 0.25

第1表（その2）　SO_2 濃度別暴露日数別気管排痰能力

暴露SO_2濃度別	暴露日数別（週）	家兎番号	体重（g）	経過時間（秒）→ 排痰能力↓	30″	60″	90″	120″	150″	180″
	1週間群	No.211	2860	移動距離(mm) 平均速度(mm/sec)	8 0.27	15 0.25	23 0.26	29 0.24	33 0.22	39 0.22
		No.212	2920	移動距離(mm) 平均速度(mm/sec)	7 0.23	13 0.22	25 0.28	31 0.26	36 0.24	42 0.23
		No.213	2960	移動距離(mm) 平均速度(mm/sec)	8 0.27	14 0.23	21 0.23	29 0.24	37 0.25	41 0.23
		No.214	2820	移動距離(mm) 平均速度(mm/sec)	8 0.27	15 0.25	24 0.27	32 0.27	35 0.23	44 0.25
		平　均	2890.0	移動距離(mm) 平均速度(mm/sec)	7.8 0.26	14.3 0.24	23.3 0.26	30.3 0.25	35.3 0.24	41.5 0.23
	2週間群	No.221	2840	移動距離(mm) 平均速度(mm/sec)	7 0.23	14 0.23	22 0.24	28 0.23	34 0.23	41 0.23
		No.222	2980	移動距離(mm) 平均速度(mm/sec)	7 0.23	14 0.23	20 0.22	27 0.23	32 0.21	37 0.21
		No.223	3000	移動距離(mm) 平均速度(mm/sec)	8 0.27	15 0.25	24 0.27	30 0.25	35 0.23	40 0.22
		No.224	3020	移動距離(mm) 平均速度(mm/sec)	7 0.23	18 0.22	19 0.21	25 0.21	32 0.21	38 0.21
		平　均	2960.0	移動距離(mm) 平均速度(mm/sec)	7.3 0.24	14.0 0.23	21.3 0.24	27.5 0.23	33.3 0.22	39.0 0.22
10		No.231	3020	移動距離(mm) 平均速度(mm/sec)	6 0.20	12 0.20	18 0.20	25 0.21	30 0.20	34 0.13

第1表（その2）（続き）

基準SO_2濃度	冷蔵日数別（日）	家畜番号	体重（g）	経過時間（秒）→ 繁殖能力↓	30″	60″	90″	120″	150″	180″
ppm	3週間群	No.232	3040	移動距離(mm)	7	14	21	26	32	38
				平均速度(mm/sec)	0.23	0.23	0.23	0.22	0.21	0.21
		No.233	2800	移動距離(mm)	6	11	18	24	29	32
				平均速度(mm/sec)	0.20	0.18	0.20	0.20	0.19	0.18
群		No.234	2600	移動距離(mm)	7	12	17	24	30	35
				平均速度(mm/sec)	0.23	0.20	0.19	0.20	0.20	0.19
		平均	2865.0	移動距離(mm)	6.5	12.3	18.5	24.8	30.3	31.8
				平均速度(mm/sec)	0.22	0.20	0.21	0.21	0.20	0.16
	4週間群	No.241	2960	移動距離(mm)	7	14	20	26	33	39
				平均速度(mm/sec)	0.23	0.23	0.22	0.22	0.22	0.23
		No.242	2840	移動距離(mm)	6	13	18	24	30	36
				平均速度(mm/sec)	0.20	0.22	0.20	0.20	0.20	0.20
		No.243	2920	移動距離(mm)	8	15	20	27	31	41
				平均速度(mm/sec)	0.27	0.25	0.22	0.23	0.23	0.23
		No.244	2960	移動距離(mm)	7	14	22	28	32	37
				平均速度(mm/sec)	0.23	0.23	0.24	0.23	0.21	0.21
		平均	2925.0	移動距離(mm)	7.0	14.0	20.0	26.3	32.3	38.3
				平均速度(mm/sec)	0.23	0.23	0.22	0.22	0.22	0.22
	5週間群	No.251	2860	移動距離(mm)	8	15	23	29	36	42
				平均速度(mm/sec)	0.27	0.25	0.26	0.24	0.24	0.23
		No.252	3000	移動距離(mm)	8	16	25	32	39	46
				平均速度(mm/sec)	0.27	0.27	0.28	0.27	0.26	0.25
		No.253	2880	移動距離(mm)	7	14	21	29	33	42
				平均速度(mm/sec)	0.23	0.23	0.23	0.24	0.23	0.23
		No.254	2940	移動距離(mm)	8	15	24	30	37	43
				平均速度(mm/sec)	0.27	0.25	0.27	0.25	0.25	0.24
		No.255	2890	移動距離(mm)	7	16	25	33	40	45
				平均速度(mm/sec)	0.23	0.27	0.28	0.28	0.27	0.25
		平均	2914.0	移動距離(mm)	7.6	15.2	23.6	30.6	37.4	43.4
				平均速度(mm/sec)	0.25	0.25	0.25	0.26	0.25	0.24
	1週間群	No.311	2800	移動距離(mm)	8	14	23	28	33	38
				平均速度(mm/sec)	0.27	0.23	0.26	0.23	0.22	0.21
		No.312	2780	移動距離(mm)	7	13	24	29	35	41
				平均速度(mm/sec)	0.23	0.22	0.27	0.24	0.23	0.23
		No.313	2880	移動距離(mm)	7	14	21	27	34	40
				平均速度(mm/sec)	0.23	0.23	0.23	0.23	0.23	0.22
		No.314	3100	移動距離(mm)	8	15	22	29	34	39
				平均速度(mm/sec)	0.27	0.25	0.24	0.24	0.23	0.22
		平均	2890.0	移動距離(mm)	7.5	14.0	22.5	28.3	34.0	39.5
				平均速度(mm/sec)	0.25	0.23	0.25	0.24	0.23	0.22
	2	No.321	3000	移動距離(mm)	7	11	20	25	32	37
				平均速度(mm/sec)	0.23	0.23	0.22	0.21	0.21	0.21
		No.322	2880	移動距離(mm)	8	14	22	28	34	40
				平均速度(mm/sec)	0.27	0.23	0.24	0.23	0.23	0.22

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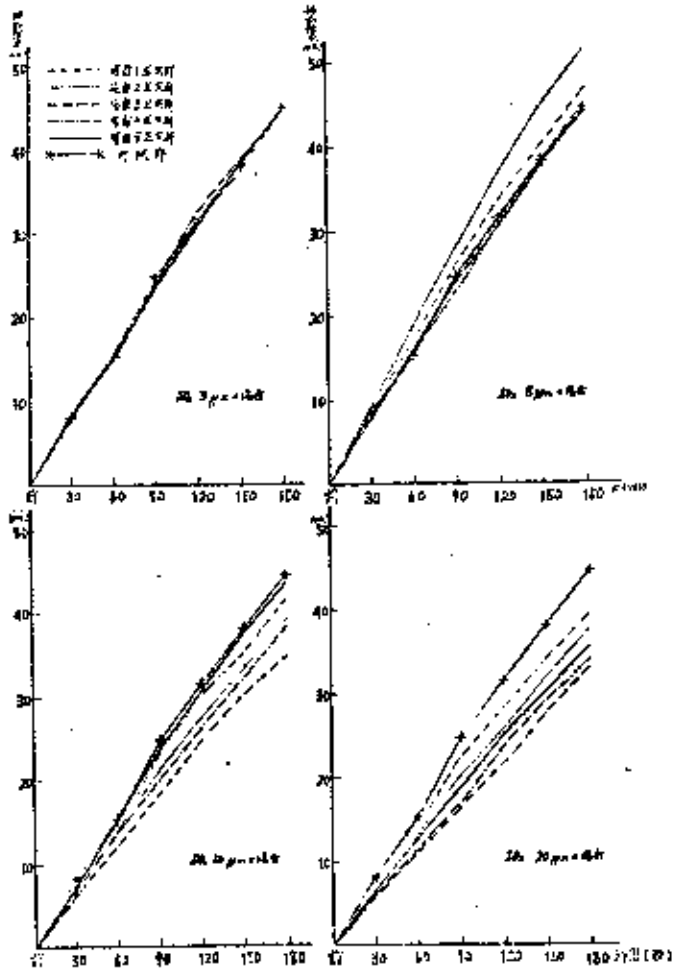
第1表（その2）（続き）

基礎 SO_2 濃度別	基礎日数別（週）	家兎番号	体重（g）	経過時間（秒）→ 接触能力↓	30"	60"	90"	120"	150"	180"
20 ppm 群	週間群	No.323	2920	移動距離(mm) 平均速度(mm/sec)	6 0.20	12 0.20	19 0.21	24 0.20	32 0.21	37 0.21
		No.324	2940	移動距離(mm) 平均速度(mm/sec)	7 0.23	13 0.22	20 0.22	26 0.22	30 0.20	36 0.20
		平均	2930.0	移動距離(mm) 平均速度(mm/sec)	7.0 0.23	13.3 0.22	20.3 0.22	25.8 0.22	32.0 0.21	37.5 0.21
	3週間群	No.331	2960	移動距離(mm) 平均速度(mm/sec)	5 0.17	10 0.17	15 0.17	20 0.17	28 0.19	32 0.12
		No.332	3100	移動距離(mm) 平均速度(mm/sec)	7 0.23	12 0.20	18 0.20	22 0.18	27 0.18	34 0.13
		No.333	2840	移動距離(mm) 平均速度(mm/sec)	6 0.20	12 0.20	17 0.19	23 0.19	30 0.20	35 0.19
		No.334	2820	移動距離(mm) 平均速度(mm/sec)	6 0.20	11 0.18	16 0.18	22 0.18	27 0.18	31 0.11
		平均	2930.0	移動距離(mm) 平均速度(mm/sec)	6.0 0.20	11.3 0.19	16.5 0.19	21.8 0.18	28.0 0.19	33.0 0.14
	4週間群	No.341	2940	移動距離(mm) 平均速度(mm/sec)	7 0.23	13 0.22	19 0.21	24 0.20	28 0.19	32 0.12
		No.342	2870	移動距離(mm) 平均速度(mm/sec)	5 0.17	10 0.17	15 0.17	22 0.18	29 0.19	35 0.19
		No.343	2900	移動距離(mm) 平均速度(mm/sec)	7 0.23	12 0.20	17 0.19	24 0.20	31 0.21	36 0.20
		No.344	2980	移動距離(mm) 平均速度(mm/sec)	6 0.20	11 0.18	17 0.19	25 0.21	29 0.19	33 0.13
		平均	2922.5	移動距離(mm) 平均速度(mm/sec)	6.3 0.21	11.5 0.19	17.0 0.19	23.8 0.20	29.3 0.20	34.0 0.16
	5週間群	No.351	2850	移動距離(mm) 平均速度(mm/sec)	8 0.27	14 0.23	20 0.22	26 0.22	31 0.21	35 0.19
		No.352	2900	移動距離(mm) 平均速度(mm/sec)	6 0.20	12 0.20	18 0.20	21 0.20	30 0.20	37 0.21
		No.353	3040	移動距離(mm) 平均速度(mm/sec)	7 0.23	13 0.22	20 0.22	27 0.23	32 0.21	39 0.22
		No.354	2920	移動距離(mm) 平均速度(mm/sec)	6 0.20	11 0.18	17 0.19	24 0.20	29 0.19	33 0.13
		No.355	2960	移動距離(mm) 平均速度(mm/sec)	7 0.23	12 0.20	19 0.21	25 0.21	30 0.20	34 0.13
		平均	2934.0	移動距離(mm) 平均速度(mm/sec)	6.8 0.23	12.4 0.21	18.8 0.26	25.2 0.21	30.4 0.20	35.6 0.18
対照群		No. 1	2980	移動距離(mm) 平均速度(mm/sec)	8 0.27	15 0.25	24 0.27	30 0.25	37 0.25	46 0.26
		No. 2	3060	移動距離(mm) 平均速度(mm/sec)	8 0.27	16 0.27	23 0.28	31 0.26	36 0.20	43 0.24
		No. 3	2850	移動距離(mm) 平均速度(mm/sec)	9 0.30	16 0.27	23 0.26	32 0.27	39 0.26	44 0.24
		No. 4	2890	移動距離(mm) 平均速度(mm/sec)	8 0.27	14 0.23	27 0.30	33 0.28	40 0.27	45 0.25
		平均	2945.0	移動距離(mm) 平均速度(mm/sec)	8.3 0.28	15.3 0.26	24.8 0.28	31.5 0.26	38.0 0.25	44.5 0.25

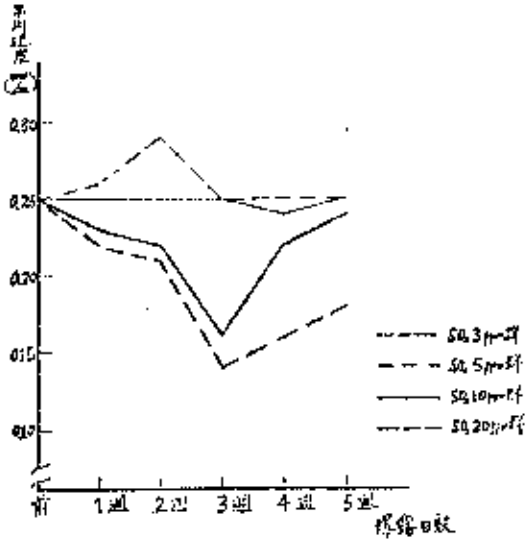
第2表　SO_2 濃度別平均排便速度経過

暴露日数（週）→ / SO_2 濃度別↓	暴露前 (mm/sec)	暴露 1週後	暴露 2週後	暴露 3週後	暴露 4週後	暴露 5週後	備考
3 ppm 群	0.25	0.25	0.25	0.25	0.24	0.25	平均排便速度は 180 秒平均を示す。（第1表参照）
5 ppm 群	0.25	0.26	0.29	0.25	0.25	0.25	
10 ppm 群	0.25	0.23	0.22	0.16	0.22	0.24	
20 ppm 群	0.25	0.22	0.21	0.14	0.16	0.16	



第2図　SO_2 濃度の炭粉移動距離の関係

後1～5週はいずれも 0.25～0.24 mm/sec を示して当初の値とほとんど変らないが，5 ppm 群では暴露後1週は 0.26 mm/sec，2週は 0.29 mm/sec と決定に亢進し，3週以後は 0.25 mm/sec となって当初の値に復する．10 ppm 群および 20 ppm 群ではそれぞれ暴露1週後は 0.23 mm/sec および 0.22 mm/sec，2週後は 0.22 mm/sec および 0.21 mm/sec，3週後は 0.16 mm/sec および 0.14 mm/sec と決定に低下し，以後は回復に向かい，4週後はそれぞれ 0.22



第3図　SO_2 濃度別平均沈降速度推移

mm/sec および 0.16 mm/sec，5週後は 0.24 mm/sec および 0.18 mm/sec となるも，後者は前者に比して沈降速度の低下が大きく，その回復も著しく遅れる．(第2表，第3図参照)

体重経過と SO_2 濃度との関係については第3表，第4図に示すごとく SO_2 濃度 3 ppm では日時の経過とともに体重は次第に増加し，対照群の増加率とほとんど変らないか，あるいはこれをやや上回る．これに対し SO_2 濃度 5 ppm 以上では体重は暴露後2～4週までは次第に減少し，以後は回復に向かうも，濃度の大なるほど減少が著しく，恢復の時期も遅れ，回復度もまた小さい．すなわち SO_2 5 ppm 群では暴露1週後は当初の値の平均 2.7% 減，2週後は平均 4.5% 減を示すも，以後は回復に向かい，3週後はほとんど当初の値に復し，4週後は平均 2.3% 増，5週後に平均 6.7% 増となる．SO_2 10 ppm および 20 ppm 群ではそれぞれ暴露1週後は当初の値の平均 2.6% 減および 5.4% 減，2週後は平均 5.5% 減および 6.7% 減，3週後は平均 7.9% 減および 8.8% 減を示して次第に減少し，前者では以後は回復に向かい，4週後は平均 4.6% 減，5週後は平均 1.6% 減となって当初の値に近づくも，後者では4週後は平均 9.0% 減を示して3週後とはとんど変らず，5週後において平均 6.2% 減となり，はじめて回復に向かうが，回復度は最も小さい．

赤血球数の経過と SO_2 濃度の関係については第4表，第5図に示すごとく SO_2 濃度 3 ppm および 5 ppm では対照群とほとんど差異を認めないが，5 ppm では暴露2週後まではやや初期増加の傾向を認める．SO_2 10 ppm および 20 ppm では暴露後3週までは次第に減少を示し，以後はいずれも回復に向かうも，後者は前者に比して減少が著しく，回復も極めて遅い．すなわち SO_2 5 ppm 群では暴露2週後は当初の値の平均 2.9% 増となり初期増加の傾向を示す．SO_2 10 ppm および 20 ppm 群ではそれぞれ暴露1週後は当初の値の平均 5.6% 減および 11.6% 減，2週後は平均 10.6% 減および 16.4% 減，3週後は平均 12.5% 減および 19.3% 減となって次第に減少し，以後はいずれも回復に向かい，4週後にはそれぞれ平均 6.6% 減および 18.6% 減，5週後には平均 1.9% 減および 16.8% 減となり，前者は後者に比して減少が少なく，回復もまた速かで，5週後には当初の値に近づくも，後者は減少が著しく，その回復度もまたきわめて遅い．

血色素量の経過と SO_2 濃度の関係については第5表，第6図に示すごとく，SO_2 濃度 3 ppm では対照群とほとんど変らなく，5 ppm では暴露1週後に初期増加を認めるも，以後は対照群とほとんど変らない．SO_2 濃度 10 ppm および 20 ppm ではいずれも暴露後3週までは次第に減少し，以後は回復に向かうも，後者は前者に比して減少が著しく，その恢復もまた遅い．すなわち 5 ppm 群では暴露1週後は当初の値の平均 2.4% 増を示し，2週以後はほとんどもとの値に復する．10 ppm および 20 ppm 群ではそれぞれ暴露1週後は当初の値の平均 4.5% 減および 7.6% 減，2週後は平均 8.3% 減および 13.1% 減，3週後は平均 9.6% 減および 15.7% 減となり次第に減少するも，以後は回復に向かい，4週後にはそれぞれ平均 4.6% 減および 10.7% 減，5週後には平均 3.4% 減および 11.4% 減となり，後者は前者に比して減少が大きく，回復は著しく遅れる．

Ht 値の経過と SO_2 濃度の関係については第

第3表 SO_2 濃度別体重経過

試験SO_2濃度別	試験番号	投与前 (g)	投与1週目 (g)	投与2週目 (g)	投与3週目 (g)	投与4週目 (g)	投与5週目 (g)
3 ppm 群	No. 41	3020 (100)	3040 (100.7)	3100 (102.6)	3140 (104.0)	3120 (103.3)	
	No. 42	2860 (100)	2920 (102.1)	2900 (101.5)	3100 (108.4)	3150 (110.1)	
	No. 43	2950 (100)	3050 (104.4)	3120 (105.8)	3190 (108.1)	3200 (108.5)	
	No. 44	2900 (100)	2960 (102.1)	3010 (104.8)	3070 (105.9)	3140 (108.3)	
	平均	2932.5 (100)	3000.0 (102.3)	3062.5 (104.4)	3125.0 (106.5)	3152.5 (107.5)	
	No. 51	2850 (100)	2940 (103.5)	2960 (104.2)	3080 (108.4)	3130 (110.0)	3160 (111.3)
	No. 52	2800 (100)	2900 (103.6)	2940 (105.0)	3040 (108.6)	3160 (112.9)	3180 (113.6)
	No. 53	3000 (100)	3180 (105.9)	3140 (104.7)	3250 (108.3)	3290 (109.7)	3270 (109.0)
	No. 54	2850 (100)	2830 (100.3)	2970 (103.1)	3100 (107.6)	3180 (110.4)	3230 (112.1)
	No. 55	2900 (100)	3030 (104.1)	3030 (104.1)	3160 (108.6)	3210 (110.4)	3210 (109.5)
	平均	2896.0 (100)	2998.0 (103.5)	3018.0 (104.2)	3126.0 (107.9)	3192.0 (110.2)	3210.0 (110.8)
	総平均	2912.6 (100)	2998.9 (102.9)	3038.2 (104.3)	3125.5 (107.3)	3174.0 (109.0)	3210.0 (110.2)
5 ppm 群	No. 141	3300 (100)	2920 (97.3)	2860 (95.3)	2910 (96.0)	3060 (102.0)	
	No. 142	2900 (100)	2810 (97.9)	2800 (96.5)	2880 (102.8)	2910 (104.8)	
	No. 143	2840 (100)	2720 (95.8)	2640 (93.0)	2790 (98.2)	2820 (99.3)	
	No. 144	2870 (100)	2760 (96.2)	2780 (96.9)	2860 (99.7)	2980 (103.8)	
	平均	2902.5 (100)	2810.0 (96.8)	2770.0 (95.4)	2892.5 (99.6)	2975.0 (102.5)	
	No. 151	2900 (100)	2810 (96.9)	2800 (96.5)	2890 (99.7)	2920 (100.7)	2990 (103.4)
	No. 152	3020 (100)	2940 (98.8)	2900 (96.0)	3040 (100.7)	3100 (102.6)	3120 (103.3)
	No. 153	2840 (100)	2800 (98.6)	2720 (95.8)	2860 (100.7)	2930 (103.5)	2990 (105.2)
	No. 154	2890 (100)	2830 (97.6)	2800 (96.9)	2870 (99.3)	2960 (102.4)	3150 (109.0)
	No. 155	2950 (100)	2860 (96.9)	2740 (92.9)	2900 (98.3)	2980 (101.0)	3170 (107.5)
	平均	2920.0 (100)	2854.0 (97.7)	2792.0 (95.6)	2912.0 (99.7)	2980.0 (102.1)	3108.0 (106.4)
	総平均	2912.0 (100)	2834.0 (97.3)	2782.0 (95.5)	2903.1 (99.7)	2977.7 (102.3)	3108.0 (106.7)
10 ppm 群	No. 241	2980 (100)	2900 (97.3)	2800 (94.0)	2690 (90.3)	2770 (93.0)	
	No. 242	2840 (100)	2800 (98.6)	2740 (96.5)	2720 (95.8)	2760 (97.2)	
	No. 243	2920 (100)	2850 (97.3)	2810 (96.2)	2740 (93.8)	2820 (96.6)	
	No. 244	2960 (100)	2850 (96.3)	2790 (94.3)	2760 (93.2)	2840 (95.9)	
	平均	2925.0 (100)	2847.5 (97.4)	2785.0 (95.2)	2727.5 (93.2)	2797.5 (95.6)	
	No. 251	2860 (100)	2790 (97.6)	2750 (96.2)	2700 (94.4)	2760 (96.5)	2800 (97.9)
	No. 252	3000 (100)	2940 (98.0)	2800 (93.3)	2670 (89.0)	2950 (98.3)	3100 (103.3)
	No. 253	2880 (100)	2800 (97.2)	2690 (93.4)	2520 (87.5)	2670 (92.7)	2780 (96.5)
	No. 254	2940 (100)	2820 (95.9)	2760 (92.9)	2760 (93.9)	2790 (94.9)	2850 (97.3)
	No. 255	2890 (100)	2740 (91.8)	2680 (92.7)	2630 (91.0)	2710 (93.8)	2820 (97.6)
	平均	2914.0 (100)	2938.0 (100.8)	2726.0 (93.9)	2656.0 (91.1)	2776.0 (95.3)	2872.0 (98.6)
	総平均	2919.0 (100)	2842.3 (97.4)	2758.3 (94.5)	2658.5 (92.1)	2783.8 (95.4)	2872.0 (98.4)
20 ppm 群	No. 341	2940 (100)	2880 (98.0)	2840 (96.6)	2680 (91.2)	2700 (91.8)	
	No. 342	2870 (100)	2500 (87.1)	2430 (85.7)	2120 (83.3)	2300 (83.6)	
	No. 343	2900 (100)	2780 (95.9)	2720 (93.8)	2700 (93.1)	2670 (92.1)	
	No. 344	2980 (100)	2800 (94.0)	2780 (93.3)	2750 (92.3)	2760 (92.6)	
	平均	2922.5 (100)	2740.0 (93.7)	2700.0 (92.4)	2637.5 (90.2)	2632.5 (90.1)	
	No. 351	2850 (100)	2760 (96.8)	2740 (96.1)	2680 (94.0)	2700 (94.7)	2740 (96.1)
	No. 352	2900 (100)	2790 (96.2)	2730 (94.8)	2700 (93.1)	2630 (90.7)	2720 (93.8)
	No. 353	3010 (100)	2870 (94.1)	2830 (93.1)	2810 (93.4)	2760 (90.8)	2800 (92.1)
	No. 354	2920 (100)	2730 (94.2)	2700 (92.5)	2630 (90.1)	2650 (90.8)	2690 (92.1)
	No. 355	2960 (100)	2820 (95.3)	2780 (93.9)	2670 (90.2)	2720 (91.9)	2780 (93.9)
	平均	2934.0 (100)	2798.0 (95.2)	2760.0 (93.9)	2698.0 (91.6)	2692.0 (91.6)	2740.0 (93.4)
	総平均	2928.8 (100)	2771.6 (94.6)	2732.7 (93.3)	2670.5 (91.2)	2665.0 (91.0)	2716.0 (93.8)

第3表（続き）

暴露 SO_2 濃度別	経過日数（週）→ 家兎番号↓	暴露前 (g)	暴露1週目 (g)	暴露2週目 (g)	暴露3週目 (g)	暴露4週目 (g)	暴露5週目 (g)
対照群	No. 1	2980 (100)	3100 (104.0)	3180 (106.7)	3200 (107.4)	3200 (109.4)	3280 (110.1)
	No. 2	3080 (100)	3080 (100.7)	3120 (102.0)	3150 (102.6)	3120 (102.0)	3170 (103.6)
	No. 3	2850 (100)	2890 (101.4)	2960 (103.9)	2990 (104.9)	3100 (108.8)	3150 (110.5)
	No. 4	2890 (100)	2910 (100.7)	2950 (102.1)	3080 (106.6)	3150 (109.0)	3200 (110.7)
	平均	2945.0 (100)	2995.0 (101.7)	3052.0 (103.6)	3102.0 (105.4)	3150.0 (107.2)	3200.0 (108.7)

註：括弧内は変動率を示す，以下これに準ずる．

第4表 SO_2 濃度別赤血球数経過

暴露 SO_2 濃度別	経過日数（週）→ 家兎番号↓	暴露前 (万)	暴露1週目 (万)	暴露2週目 (万)	暴露3週目 (万)	暴露4週目 (万)	暴露5週目 (万)
3 ppm 群	No. 41	606 (100)	626 (103.3)	632 (104.3)	614 (101.3)	603 (100.3)	
	No. 42	520 (100)	514 (98.8)	518 (99.6)	506 (97.3)	525 (101.0)	
	No. 43	574 (100)	590 (102.8)	565 (98.4)	583 (101.4)	570 (99.3)	
	No. 44	633 (100)	512 (95.2)	521 (97.4)	545 (101.3)	527 (98.0)	
	平均	559.5 (100)	560.5 (100.2)	559.8 (100.1)	561.8 (100.4)	557.5 (99.6)	
	No. 51	500 (100)	538 (96.1)	576 (102.9)	555 (99.1)	518 (97.9)	576 (102.9)
	No. 52	559 (100)	580 (98.5)	601 (102.0)	587 (99.7)	596 (101.2)	582 (98.5)
	No. 53	537 (100)	542 (100.9)	524 (97.6)	546 (101.7)	522 (97.2)	545 (100.9)
	No. 54	620 (100)	628 (101.3)	645 (104.0)	619 (99.8)	615 (99.2)	628 (101.3)
	No. 55	526 (100)	517 (98.3)	532 (101.1)	520 (98.9)	534 (101.5)	529 (100.6)
	平均	566.4 (100)	561.0 (99.0)	575.6 (101.6)	565.4 (99.8)	563.0 (99.4)	572.0 (101.0)
	総平均	563.3 (100)	560.8 (99.6)	568.4 (100.9)	563.7 (100.1)	560.5 (99.5)	572.0 (101.5)
5 ppm 群	No. 141	567 (100)	556 (98.1)	589 (103.9)	555 (97.9)	572 (100.9)	
	No. 142	514 (100)	519 (100.9)	572 (105.1)	552 (101.5)	538 (98.9)	
	No. 143	605 (100)	608 (100.5)	596 (98.5)	590 (97.5)	601 (99.8)	
	No. 144	524 (100)	531 (101.3)	539 (102.4)	521 (99.4)	528 (100.8)	
	平均	560.0 (100)	561.0 (100.2)	574.0 (102.5)	554.5 (99.0)	560.5 (100.1)	
	No. 151	601 (100)	617 (102.2)	620 (102.6)	588 (97.3)	612 (101.3)	603 (100.7)
	No. 152	583 (100)	585 (99.5)	602 (100.7)	541 (92.0)	551 (94.2)	573 (97.4)
	No. 153	545 (100)	528 (96.9)	573 (105.1)	549 (100.7)	536 (98.3)	542 (99.4)
	No. 154	532 (100)	546 (102.6)	558 (104.9)	525 (98.7)	542 (101.9)	540 (101.5)
	No. 155	616 (100)	621 (100.8)	634 (102.9)	607 (98.5)	621 (101.3)	618 (100.3)
	平均	577.0 (100)	579.4 (100.4)	595.4 (103.2)	562.0 (97.4)	573.6 (99.4)	576.2 (99.9)
	総平均	569.3 (100)	571.0 (100.3)	585.7 (102.9)	558.5 (98.1)	567.6 (99.7)	576.2 (101.2)
10 ppm 群	No. 241	581 (100)	532 (91.6)	519 (89.3)	528 (90.9)	516 (91.0)	
	No. 242	560 (100)	510 (89.6)	502 (88.2)	496 (87.2)	541 (95.1)	
	No. 243	577 (100)	551 (96.0)	524 (90.8)	512 (88.7)	550 (95.3)	
	No. 244	585 (100)	536 (91.6)	515 (88.0)	501 (86.2)	542 (92.6)	
	平均	578.0 (100)	546.0 (91.5)	515.0 (89.1)	510.0 (88.2)	544.8 (91.3)	
	No. 251	598 (100)	576 (96.3)	513 (85.8)	497 (83.1)	513 (85.8)	546 (91.3)
	No. 252	585 (100)	563 (96.2)	548 (93.7)	512 (87.5)	550 (93.6)	591 (101.0)
	No. 253	639 (100)	603 (91.7)	579 (90.6)	466 (88.6)	613 (95.8)	628 (98.3)
	No. 254	570 (100)	547 (95.7)	485 (80.8)	472 (87.4)	517 (95.7)	539 (90.8)
	No. 255	593 (100)	571 (96.3)	524 (88.4)	519 (87.5)	538 (90.7)	565 (95.3)
	平均	591.0 (100)	566.4 (95.8)	529.8 (89.6)	513.2 (86.8)	547.8 (96.7)	573.8 (97.1)
	総平均	585.1 (100)	552.4 (91.4)	523.1 (89.4)	511.7 (87.5)	546.4 (93.4)	573.8 (98.1)

第4表（続き）

暴露 SO_2 濃度別	経過日数（日）→ 家兎番号↓	暴露前（万）	暴露1週目（万）	暴露2週目（万）	暴露3週目（万）	暴露4週目（万）	暴露5週目（万）
20 ppm 群	No. 341	577 (100)	514 (94.3)	497 (86.1)	493 (85.4)	503 (87.2)	
	No. 342	604 (100)	452 (74.8)	425 (70.5)	420 (69.5)	438 (72.5)	
	No. 343	589 (100)	506 (85.9)	434 (82.2)	476 (80.8)	450 (76.4)	
	No. 344	526 (100)	498 (96.5)	462 (89.5)	447 (86.6)	466 (90.3)	
	平均	571.5 (100)	500.0 (87.5)	467.3 (81.8)	459.0 (80.3)	464.3 (81.2)	
	No. 351	521 (100)	470 (90.2)	428 (82.1)	416 (79.8)	449 (86.2)	457 (87.7)
	No. 352	614 (100)	518 (84.4)	491 (80.0)	486 (79.1)	470 (76.5)	483 (78.7)
	No. 353	527 (100)	472 (89.6)	456 (86.5)	430 (81.6)	438 (83.1)	446 (84.6)
	No. 354	611 (100)	556 (91.0)	531 (87.4)	519 (84.9)	502 (82.2)	527 (86.2)
	No. 355	553 (100)	501 (91.1)	498 (90.1)	438 (79.2)	446 (80.7)	450 (81.4)
	平均	565.2 (100)	504.0 (89.2)	481.4 (85.2)	457.8 (81.0)	461.0 (81.6)	472.6 (83.6)
	総平均	568.3 (100)	502.2 (88.4)	475.0 (83.6)	458.3 (80.7)	462.5 (81.4)	472.6 (83.2)
対照群	No. 1	538 (100)	526 (97.6)	558 (103.7)	527 (98.0)	554 (103.0)	512 (95.2)
	No. 2	515 (100)	537 (102.3)	504 (97.9)	518 (100.6)	559 (108.5)	515 (100.0)
	No. 3	580 (100)	582 (100.3)	539 (101.5)	574 (99.0)	560 (96.5)	582 (100.3)
	No. 4	604 (100)	611 (101.2)	617 (102.2)	602 (99.7)	596 (98.8)	608 (100.7)
	平均	557.4 (100)	559.0 (100.3)	567.0 (101.7)	555.2 (99.6)	567.3 (101.8)	562.0 (100.8)

第5表 SO_2 濃度別血色素経過

暴露 SO_2 濃度別	経過日数（日）→ 家兎番号↓	暴露前（%）	暴露1週目（%）	暴露2週目（%）	暴露3週目（%）	暴露4週目（%）	暴露5週目（%）
3 ppm 群	No. 41	88 (100)	85 (96.6)	90 (102.3)	86 (97.7)	85 (96.6)	
	No. 42	82 (100)	80 (97.6)	85 (103.7)	82 (100.0)	84 (102.4)	
	No. 43	90 (100)	92 (102.2)	92 (102.2)	88 (97.8)	90 (100.0)	
	No. 44	86 (100)	88 (102.3)	85 (98.8)	85 (98.8)	87 (101.2)	
	平均	86.5 (100)	86.3 (99.8)	88.0 (101.7)	85.3 (98.6)	86.5 (100.0)	
	No. 51	85 (100)	80 (94.1)	80 (94.1)	85 (100.0)	80 (94.1)	82 (96.5)
	No. 52	90 (100)	90 (100.0)	90 (100.0)	85 (94.4)	87 (96.7)	90 (100.0)
	No. 53	82 (100)	82 (100.0)	85 (103.7)	80 (100.0)	85 (103.7)	85 (103.7)
	No. 54	80 (100)	85 (106.3)	80 (100.0)	84 (105.0)	82 (102.5)	80 (100.0)
	No. 55	87 (100)	85 (97.7)	85 (97.7)	90 (103.4)	85 (97.7)	88 (101.1)
	平均	84.8 (100)	84.4 (99.5)	84.0 (99.1)	84.8 (100.0)	83.8 (98.8)	85.0 (100.2)
	総平均	85.6 (100)	85.2 (99.5)	85.8 (100.2)	85.0 (99.3)	85.0 (99.3)	85.0 (99.3)
5 ppm 群	No. 141	86 (100)	86 (100.0)	88 (102.3)	86 (100.0)	88 (102.3)	
	No. 142	84 (100)	88 (104.8)	86 (102.4)	84 (100.0)	86 (102.4)	
	No. 143	80 (100)	82 (102.5)	84 (105.0)	82 (102.5)	82 (102.5)	
	No. 144	87 (100)	90 (103.4)	86 (98.8)	84 (96.5)	87 (100.0)	
	平均	84.3 (100)	86.5 (102.6)	86.0 (102.0)	84.0 (99.6)	85.8 (101.8)	
	No. 151	85 (100)	85 (100.0)	82 (96.5)	80 (94.1)	85 (100.0)	87 (102.3)
	No. 152	82 (100)	85 (103.7)	85 (103.7)	80 (97.6)	82 (100.0)	80 (97.6)
	No. 153	84 (100)	88 (104.8)	85 (101.2)	82 (97.6)	82 (97.6)	84 (100.0)
	No. 154	90 (100)	90 (100.0)	87 (96.7)	80 (94.9)	90 (100.0)	88 (97.8)
	No. 155	83 (100)	85 (102.4)	80 (96.4)	80 (96.4)	85 (102.4)	82 (98.8)
	平均	84.8 (100)	86.6 (102.1)	83.8 (98.8)	82.2 (96.9)	84.8 (100.0)	84.2 (99.3)
	総平均	84.6 (100)	86.6 (102.4)	84.8 (100.2)	83.0 (98.1)	85.2 (100.7)	84.2 (99.5)

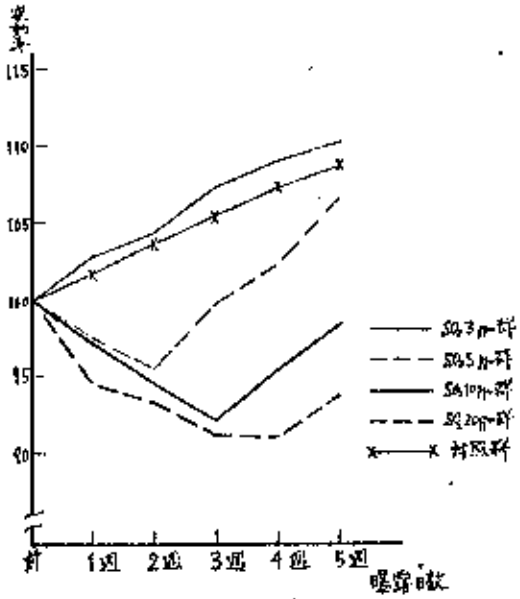
100

第5表（続き）

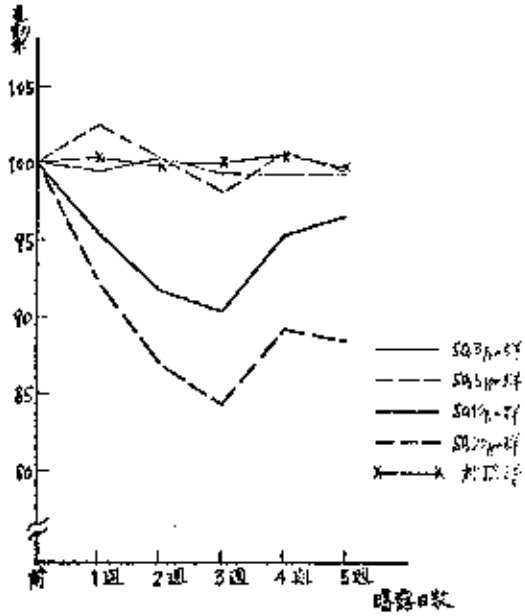
暴露SO_2濃度別	経過日数（%）→ 家兎番号↓	暴露前 (%)	暴露1週目 (%)	暴露2週目 (%)	暴露3週目 (%)	暴露4週目 (%)	暴露5週目 (%)
10 ppm 群	No. 241	87 (100)	80 (92.0)	75 (86.2)	75 (86.2)	81 (93.1)	
	No. 242	80 (100)	78 (97.5)	78 (97.5)	74 (92.5)	78 (97.5)	
	No. 243	85 (100)	82 (96.5)	80 (97.6)	78 (91.8)	82 (96.5)	
	No. 244	87 (100)	82 (94.3)	79 (90.8)	79 (90.8)	84 (96.5)	
	平均	84.8 (100)	80.5 (94.9)	78.0 (92.0)	76.5 (90.2)	81.3 (95.9)	
	No. 251	82 (100)	79 (96.3)	77 (93.9)	76 (92.7)	80 (97.6)	81 (98.8)
	No. 252	84 (100)	80 (95.2)	75 (89.3)	79 (94.0)	82 (97.6)	85 (101.2)
	No. 253	86 (100)	84 (97.7)	80 (93.0)	75 (87.2)	80 (93.0)	80 (93.0)
	No. 254	87 (100)	83 (95.4)	78 (89.7)	77 (88.5)	81 (93.1)	82 (94.3)
	No. 255	82 (100)	78 (95.1)	75 (91.5)	75 (91.5)	77 (93.9)	80 (97.6)
	平均	84.2 (100)	80.8 (96.0)	77.0 (91.4)	76.4 (90.7)	80.0 (95.0)	81.6 (96.9)
	総平均	84.5 (100)	80.7 (95.5)	77.5 (91.7)	76.4 (90.4)	80.6 (95.4)	81.6 (96.6)
20 ppm 群	No. 341	87 (100)	79 (90.8)	74 (85.1)	71 (81.6)	84 (96.5)	
	No. 342	88 (100)	79 (89.8)	76 (86.4)	75 (85.2)	80 (90.9)	
	No. 343	87 (100)	83 (95.4)	73 (83.9)	72 (82.8)	75 (86.2)	
	No. 344	85 (100)	78 (91.8)	76 (89.4)	72 (84.7)	76 (89.4)	
	平均	86.8 (100)	79.8 (91.9)	74.8 (86.2)	72.5 (83.5)	78.8 (90.8)	
	No. 351	84 (100)	82 (97.6)	77 (91.7)	76 (90.5)	80 (95.2)	79 (94.0)
	No. 352	90 (100)	83 (92.2)	80 (88.9)	75 (83.3)	82 (91.1)	80 (88.9)
	No. 353	88 (100)	78 (88.6)	74 (84.1)	72 (81.8)	69 (78.4)	72 (81.8)
	No. 354	89 (100)	83 (93.3)	78 (87.6)	76 (85.4)	79 (88.8)	77 (86.5)
	No. 355	86 (100)	80 (93.0)	73 (84.9)	72 (83.7)	75 (87.2)	78 (90.7)
	平均	87.4 (100)	81.2 (92.9)	76.4 (87.4)	74.2 (84.9)	77.0 (88.1)	77.2 (88.3)
	総平均	87.1 (100)	80.5 (92.4)	75.7 (86.9)	73.4 (84.3)	77.8 (89.3)	77.2 (88.6)
対照群	No. 1	83 (100)	85 (102.4)	82 (98.8)	84 (101.2)	84 (101.2)	82 (98.8)
	No. 2	88 (100)	86 (97.7)	90 (102.3)	87 (98.9)	85 (96.6)	90 (102.3)
	No. 3	86 (100)	84 (97.7)	85 (98.8)	89 (103.5)	87 (101.2)	85 (98.8)
	No. 4	89 (100)	92 (103.4)	88 (98.9)	86 (96.6)	92 (103.4)	88 (98.9)
	平均	86.5 (100)	86.8 (100.3)	86.3 (99.8)	86.5 (100.0)	87.0 (100.6)	86.3 (99.8)

6表，第7図に示すごとく，SO_2濃度3 ppmおよび5 ppmでは対照群の経過とほとんど変らず，初期増加も認め難い．SO_2 10 ppmでは暴露後3週間は次第に減少し，以後は回復して5週後には当初の値に近づくも，SO_2 20 ppmでは暴露後は日時の経過とともに減少し，かつ減少度も最も著しく，5週後においても回復の傾向を認めない．すなわち10 ppmおよび20 ppm群ではそれぞれ暴露1週後は当初の値の平均5.9%減および15.1%減，2週後は平均10.6%減および19.3%減，3週後は平均14.7%減および24.3%減となり，以後は前者では回復に向かい，4週後には平均8.1%減，5週後には平均4.2%減となって当初の値に近づくも，後者はさらに減少しつづけ，4週後には平均29.7%減，5週後には平均32.7%減となる．

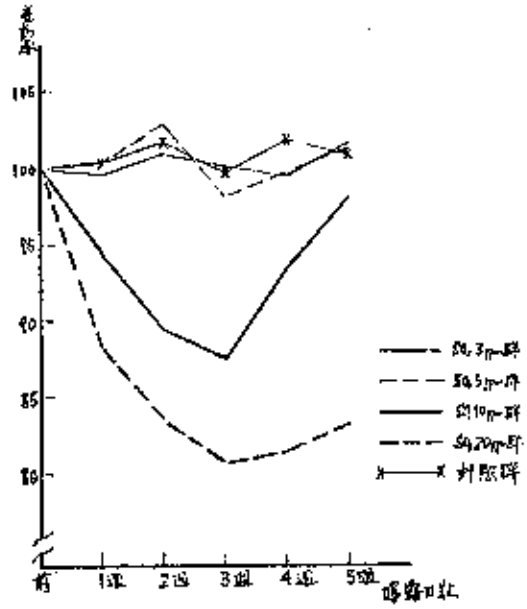
白血球数の経過とSO_2濃度の関係については第7表，第8図に示すごとく，SO_2濃度3 ppmでは対照群の経過とほとんど変らないが，5 ppm以上の濃度ではいずれも暴露1週後には白血球数は急増し，SO_2濃度5 ppmおよび10 ppmでは2週後，20 ppmでは3週後より減少し始め，5週後には5 ppm濃度ではほぼ当初の値に復するも，10および20 ppmではやや当初の値を下回る．しかしてSO_2濃度の



第4図　SO_2 濃度別体重経過



第6図　SO_2 濃度別血色素量経過



第5図　SO_2 濃度別赤血球数経過

高いほど初期増加が著しく，減少しはじめる時期が遅れる．すなわち SO_2 5 ppm，10 ppm および 20 ppm 群はそれぞれ曝露1週後には当初の値の平均 11.0% 増，17.4% 増および 18.3% 増を示し，2週後には 5 ppm および 10 ppm 群は減少し始め，それぞれ平均 6.2% 増および 11.9% 増となるも，20 ppm 群はさらに増加して平均 19.7% 増となり，3週後はいずれも減少を示して 5 ppm，10 ppm および 20 ppm 群はそれぞれ平均 1.6% 増，8.6% 増および 9.6% 増，5週後は平均 0.5% 減，2.5% 減および 1.5% 減となる．

気道排痰速度と血液性状との間の相関については第9，10，11，12図に示すごとく赤血球数，血色素量および Ht 値との間には高度の相関関係 ($r=+0.9$) を認めるも，白血球数との間には相関を認めない．(第1，4～7表参照)

以上要するに暴露 SO_2 濃度 3 ppm では気道排痰能力，血液性状および体重に影響を認めないが，5 ppm 以上の濃度では影響を認め，濃度の大なるほどその影響が著しい．すなわち SO_2 5 ppm では暴露初期には気道排痰速度は亢進を示し，赤血球数，血色素量および白血球数は増加し，体重は減少するが，遅くも3週後にはもとに復する．これに対し，SO_2 10 ppm 以上の濃度ではかかる排痰速度の初期亢進，赤血球数および血色素量の初期増加などは認められず，排痰速度，赤血球数，血色素量，Ht 値および体重はいずれも次第に減少し，白血球数

第6表 SO_2 濃度別 Hill 収穫量

SO_2 濃度別	処理区	対照区 (%)	処理1週目 (%)	処理2週目 (%)	処理3週目 (%)	処理4週目 (%)	処理5週目 (%)
3 ppm 区	No. 41	43 (100)	45 (104.6)	45 (104.6)	42 (97.7)	44 (102.3)	
	No. 42	38 (100)	38 (100.0)	35 (91.7)	40 (105.2)	40 (105.2)	
	No. 43	40 (100)	42 (105.0)	38 (95.0)	42 (105.0)	42 (105.0)	
	No. 44	45 (100)	45 (100.0)	42 (93.3)	42 (93.3)	44 (97.8)	
	平均	41.5 (100)	42.5 (102.4)	40.3 (97.1)	41.5 (100.0)	42.5 (102.4)	
	No. 51	42 (100)	40 (95.2)	40 (95.2)	42 (100.0)	40 (95.2)	40 (95.2)
	No. 52	39 (100)	39 (100.0)	39 (100.0)	37 (94.9)	40 (102.6)	39 (100.0)
	No. 53	45 (100)	45 (100.0)	45 (100.0)	42 (93.3)	42 (93.3)	44 (97.8)
	No. 54	40 (100)	40 (100.0)	42 (105.0)	40 (100.0)	38 (95.0)	40 (100.0)
	No. 55	42 (100)	44 (104.7)	42 (100.0)	43 (102.3)	44 (104.7)	42 (100.0)
	平均	41.6 (100)	41.6 (100.0)	41.6 (100.0)	40.8 (98.1)	40.8 (98.1)	41.0 (98.6)
	総平均	41.6 (100)	42.0 (101.0)	41.0 (98.6)	41.1 (98.8)	41.6 (100.0)	41.0 (98.6)
5 ppm 区	No. 141	38 (100)	40 (105.2)	38 (100.0)	38 (100.0)	40 (105.2)	
	No. 142	43 (100)	40 (93.0)	42 (97.7)	40 (93.0)	40 (93.0)	
	No. 143	38 (100)	36 (94.7)	35 (92.1)	35 (91.7)	38 (100.0)	
	No. 144	40 (100)	38 (95.0)	36 (90.0)	34 (85.0)	37 (92.5)	
	平均	39.8 (100)	38.5 (96.7)	37.8 (95.0)	37.0 (93.0)	38.8 (97.5)	
	No. 151	43 (100)	45 (104.6)	41 (95.3)	40 (93.0)	42 (97.7)	42 (97.7)
	No. 152	38 (100)	40 (105.2)	42 (106.3)	40 (105.2)	40 (105.2)	41 (103.7)
	No. 153	40 (100)	42 (105.0)	40 (100.0)	38 (95.0)	39 (97.5)	41 (102.5)
	No. 154	42 (100)	44 (104.7)	44 (104.7)	40 (100.0)	40 (100.0)	40 (100.0)
	No. 155	38 (100)	39 (102.6)	40 (105.2)	36 (94.7)	36 (94.7)	38 (100.0)
	平均	40.2 (100)	42.0 (104.5)	41.4 (103.0)	38.8 (96.5)	39.4 (98.0)	40.4 (100.5)
	総平均	40.0 (100)	40.4 (101.0)	39.7 (99.3)	38.0 (95.0)	39.1 (97.8)	40.4 (101.0)
10 ppm 区	No. 241	38 (100)	35 (92.1)	32 (84.2)	32 (84.2)	35 (92.1)	
	No. 242	40 (100)	39 (97.5)	37 (92.5)	35 (87.5)	38 (95.0)	
	No. 243	42 (100)	40 (95.2)	40 (95.2)	36 (85.7)	37 (88.1)	
	No. 244	39 (100)	37 (94.9)	36 (92.3)	34 (87.2)	37 (94.9)	
	平均	39.8 (100)	37.8 (95.0)	36.3 (91.2)	34.3 (86.2)	36.8 (92.5)	
	No. 251	41 (100)	39 (95.1)	39 (95.1)	35 (85.4)	38 (92.7)	39 (95.1)
	No. 252	42 (100)	38 (90.4)	35 (83.3)	32 (76.2)	37 (88.1)	39 (92.8)
	No. 253	45 (100)	42 (93.3)	38 (84.4)	38 (84.4)	40 (88.9)	40 (88.9)
	No. 254	39 (100)	37 (94.9)	35 (89.7)	35 (89.7)	38 (97.4)	39 (100.0)
	No. 255	40 (100)	38 (95.0)	36 (90.0)	35 (87.5)	37 (92.5)	38 (95.0)
	平均	41.4 (100)	38.8 (93.7)	36.6 (88.4)	35.0 (84.5)	38.0 (91.8)	39.0 (94.2)
	総平均	40.7 (100)	38.3 (94.1)	36.4 (89.4)	34.7 (85.3)	37.4 (91.9)	39.0 (95.8)
20 ppm 区	No. 341	40 (100)	37 (92.5)	32 (80.0)	30 (75.0)	28 (70.0)	
	No. 342	37 (100)	30 (81.1)	30 (81.1)	32 (86.5)	30 (81.1)	
	No. 343	45 (100)	39 (86.7)	36 (80.0)	36 (80.0)	34 (75.5)	
	No. 344	43 (100)	38 (88.4)	36 (83.7)	32 (74.4)	29 (67.4)	
	平均	41.3 (100)	36.0 (87.2)	33.5 (81.1)	32.5 (78.7)	30.3 (73.4)	
	No. 351	38 (100)	31 (81.6)	29 (76.3)	29 (76.3)	27 (71.0)	28 (73.7)
	No. 352	40 (100)	31 (77.5)	30 (75.0)	25 (62.5)	26 (65.0)	23 (57.5)
	No. 353	39 (100)	30 (76.9)	30 (76.9)	26 (66.7)	22 (56.4)	22 (56.4)
	No. 354	43 (100)	39 (90.7)	36 (83.7)	32 (74.4)	29 (67.4)	31 (72.1)
	No. 355	38 (100)	33 (86.8)	34 (89.5)	33 (86.8)	30 (78.9)	32 (81.2)
	平均	39.8 (100)	32.8 (82.4)	31.8 (79.9)	29.0 (72.9)	26.8 (67.3)	27.2 (68.3)
	総平均	40.4 (100)	31.3 (81.0)	32.0 (80.7)	30.6 (75.7)	28.4 (70.3)	27.2 (67.3)

第6表（続き）

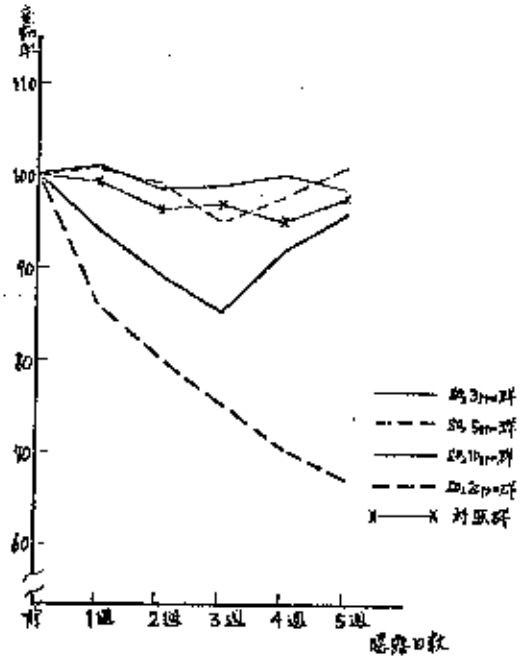
暴露 SO_2 濃度別	経過日数（月）→ 家兎番号	暴露前	暴露1週目	暴露2週目	暴露3週目	暴露4週目	暴露5週目
		(%)	(%)	(%)	(%)	(%)	(%)
対照群	No. 1	39 (100)	37 (94.9)	38 (97.4)	40 (102.6)	41 (105.1)	38 (97.1)
	No. 2	41 (100)	42 (102.4)	40 (97.6)	36 (87.8)	40 (97.6)	42 (102.4)
	No. 3	44 (100)	42 (95.4)	39 (88.6)	40 (90.9)	35 (81.8)	39 (88.6)
	No. 4	39 (100)	41 (105.1)	40 (102.6)	42 (107.7)	38 (97.4)	40 (102.6)
	平均	40.8 (100)	40.5 (99.3)	39.3 (96.3)	39.5 (96.8)	38.8 (95.1)	39.8 (97.5)

第7表 SO_2 濃度別白血球数経過

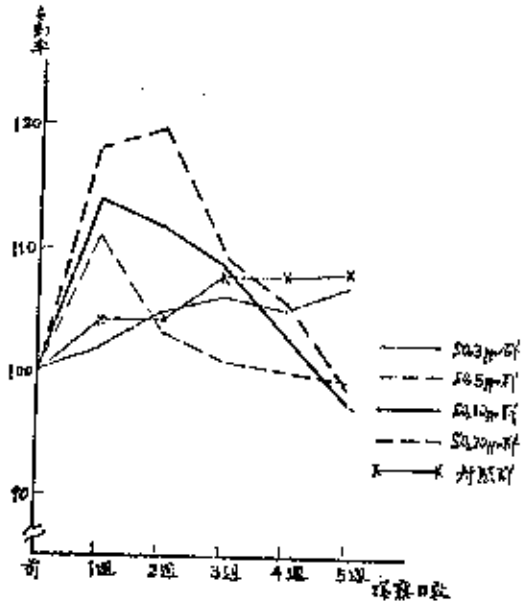
暴露 SO_2 濃度別	経過日数（月）→ 家兎番号	暴露前	暴露1週目	暴露2週目	暴露3週目	暴露4週目	暴露5週目
3 ppm 群	No. 41	7900 (100)	8200 (103.8)	8500 (108.9)	8000 (101.3)	8500 (107.6)	
	No. 42	8600 (100)	8800 (102.3)	9000 (104.6)	9500 (110.5)	9200 (107.0)	
	No. 43	8300 (100)	8200 (98.8)	8800 (106.0)	8500 (103.6)	8400 (101.2)	
	No. 44	7600 (100)	8000 (105.3)	7400 (97.4)	8200 (107.9)	8000 (105.3)	
	平均	8100.0 (100)	8300.0 (102.5)	8150.0 (101.3)	8575.0 (105.9)	8525.0 (105.2)	
	No. 51	8200 (100)	8600 (104.9)	8800 (107.3)	9000 (109.8)	8800 (107.3)	8600 (104.9)
	No. 52	7600 (100)	7400 (97.4)	8000 (105.3)	8400 (110.5)	8200 (107.9)	8400 (110.5)
	No. 53	8700 (100)	8500 (97.7)	9600 (110.3)	9200 (105.7)	8800 (101.1)	9200 (105.7)
	No. 54	7800 (100)	8200 (105.1)	8100 (103.8)	7800 (100.0)	8400 (107.7)	7900 (101.3)
	No. 55	8500 (100)	8800 (103.5)	8400 (98.8)	9100 (107.1)	8700 (102.3)	9400 (110.6)
	平均	8160.0 (100)	8200.0 (101.7)	8580.0 (105.1)	8700.0 (106.6)	8580.0 (105.1)	8700.0 (106.6)
	総平均	8133.0 (100)	8300.0 (102.0)	8521.0 (104.8)	8613.0 (106.3)	8555.0 (105.1)	8700.0 (107.0)
5 ppm 群	No. 141	8400 (100)	8800 (104.8)	8700 (103.6)	8000 (95.2)	8200 (97.6)	
	No. 142	7700 (100)	9200 (119.5)	8400 (109.1)	8600 (111.7)	7400 (96.1)	
	No. 143	8200 (100)	9400 (114.6)	9200 (112.2)	8800 (107.3)	8600 (104.9)	
	No. 144	8800 (100)	9700 (110.2)	9000 (102.3)	8300 (94.4)	8900 (101.1)	
	平均	8275.0 (100)	9275.0 (112.1)	8825.0 (106.6)	8450.0 (102.1)	8275.0 (100.0)	
	No. 151	8700 (100)	9300 (106.9)	9500 (111.8)	8800 (103.5)	8200 (96.5)	8700 (100.0)
	No. 152	8000 (100)	8500 (106.3)	7800 (97.5)	8200 (102.5)	8600 (107.5)	8000 (100.0)
	No. 153	7800 (100)	9000 (115.4)	8200 (105.1)	7000 (89.7)	7600 (97.4)	7400 (94.9)
	No. 154	8600 (100)	9100 (105.8)	9600 (111.6)	8000 (103.5)	8100 (97.7)	8200 (95.3)
	No. 155	7800 (100)	8600 (110.3)	8000 (102.6)	8300 (106.4)	8000 (102.6)	8500 (109.0)
	平均	8140.0 (100)	8960.0 (110.1)	8620.0 (105.9)	8240.0 (101.2)	8160.0 (100.2)	8160.0 (100.2)
	総平均	8201.0 (100)	9103.0 (111.0)	8713.0 (106.2)	8335.0 (101.6)	8212.0 (100.1)	8160.0 (99.5)
10 ppm 群	No. 241	8800 (100)	10000 (113.6)	10400 (118.2)	9200 (104.5)	9600 (109.1)	
	No. 242	7700 (100)	9300 (112.7)	7800 (101.0)	8300 (110.7)	7800 (101.0)	
	No. 243	8700 (100)	9600 (110.3)	9900 (113.8)	9000 (103.4)	8500 (97.7)	
	No. 244	7900 (100)	8800 (111.4)	8600 (108.9)	8100 (102.5)	7600 (96.2)	
	平均	8225.0 (100)	9400.0 (114.3)	9175.0 (111.5)	8725.0 (106.1)	8375.0 (101.8)	
	No. 251	8000 (100)	9200 (115.0)	9400 (117.5)	9000 (112.5)	7600 (95.0)	7800 (97.5)
	No. 252	7800 (100)	9800 (125.6)	8600 (110.3)	8800 (112.8)	8200 (105.1)	7600 (97.4)
	No. 253	8000 (100)	10000 (125.0)	9700 (112.8)	9200 (107.0)	9100 (104.3)	8000 (100.0)
	No. 254	8200 (100)	9600 (117.1)	9000 (109.8)	8700 (106.1)	8700 (106.1)	8000 (97.6)
	No. 255	7400 (100)	9000 (121.6)	8200 (110.8)	8000 (108.2)	8000 (108.1)	7200 (97.3)
	平均	8000.0 (100)	9600.0 (120.0)	8980.0 (112.3)	8860.0 (110.8)	8310.0 (104.3)	7900.0 (98.8)
	総平均	8102.0 (100)	9500.0 (117.4)	9060.0 (111.9)	8790.0 (108.6)	8356.0 (103.1)	7900.0 (97.5)

第7表（続き）

暴露 SO_2 濃度別	経過日数（週）→ 家兎番号	暴露前	暴露1週目	暴露2週目	暴露3週目	暴露4週目	暴露5週目
20 ppm 群	No. 341	8800 (100)	9700 (110.2)	10500 (119.3)	9000 (102.3)	9400 (106.8)	
	No. 342	7600 (100)	8200 (107.9)	8000 (105.3)	7400 (97.4)	7200 (94.7)	
	No. 343	8500 (100)	10400 (120.9)	8800 (102.3)	9200 (107.0)	8500 (98.8)	
	No. 344	8400 (100)	9800 (116.7)	10600 (126.2)	9800 (116.7)	8700 (103.6)	
	平均	8350.0 (100)	9525.0 (114.1)	9475.0 (113.5)	8850.0 (106.0)	8450.0 (101.2)	
	No. 351	8400 (100)	10200 (120.9)	11000 (127.9)	9400 (109.3)	8200 (95.3)	8000 (93.0)
	No. 352	7900 (100)	9400 (120.5)	9600 (123.1)	7800 (100.0)	8000 (102.6)	7600 (97.4)
	No. 353	8000 (100)	10600 (132.5)	10000 (125.0)	10200 (127.5)	10800 (135.0)	8400 (105.0)
	No. 354	8600 (100)	10300 (119.8)	10500 (122.1)	9300 (108.1)	8900 (103.5)	8800 (98.8)
	No. 355	7400 (100)	8500 (114.9)	9300 (124.3)	8600 (116.2)	8100 (109.5)	7800 (105.4)
	平均	8040.0 (100)	9800.0 (121.9)	10060.0 (125.1)	9060.0 (112.7)	8800.0 (109.4)	8060.0 (100.2)
	総平均	8161.0 (100)	9675.0 (118.3)	9794.0 (119.7)	8965.0 (109.6)	8641.0 (105.6)	8060.0 (98.5)
対照群	No. 1	8900 (100)	8200 (92.1)	9100 (102.2)	8000 (89.9)	9300 (104.5)	8800 (98.9)
	No. 2	8400 (100)	8800 (104.8)	8900 (105.9)	9500 (113.1)	8600 (102.4)	9200 (109.5)
	No. 3	7800 (100)	8500 (109.0)	8100 (103.8)	7800 (100.0)	8400 (107.7)	7900 (101.3)
	No. 4	8500 (100)	8700 (102.3)	8200 (96.5)	9300 (109.4)	9100 (107.1)	9600 (112.9)
	平均	8200.0 (100)	8550.0 (104.3)	8560.0 (104.4)	8850.0 (107.9)	8850.0 (107.9)	8875.0 (108.2)

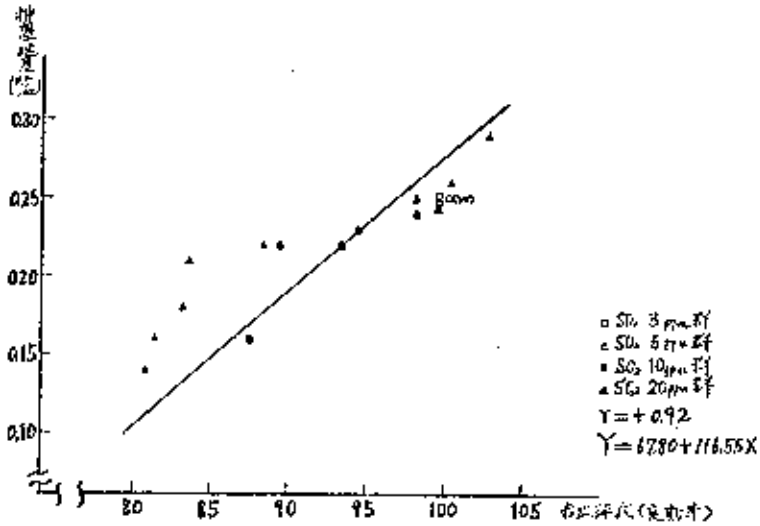


第7図 SO_2 濃度別 Ht 値経過

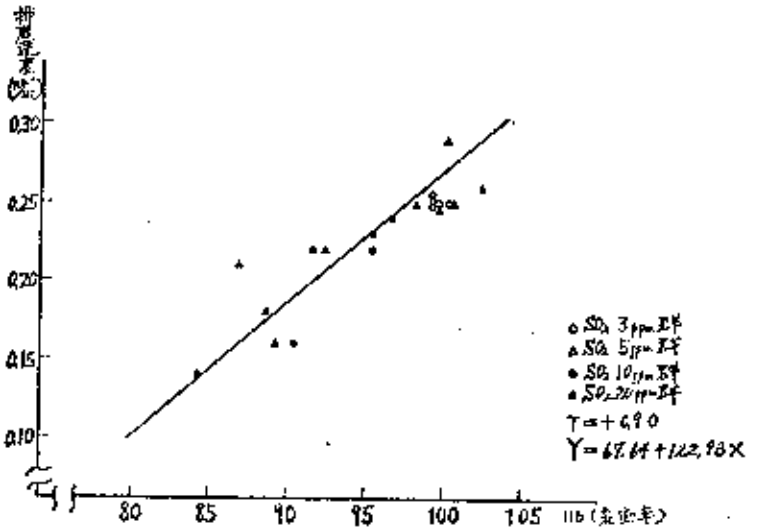


第8図 SO_2 濃度別白血球数経過

は急増し，SO_2 10 ppm ではこれらは遅くも4週後には回復に向かうが，SO_2 20 ppm ではその影響が大で，これらの減少あるいは増加が著しく，回復は一般に4週後より認められるも，回復度は遅く，体重は5週後に回復し始め，Ht 値は5週においてもなお回復の徴は認められない．



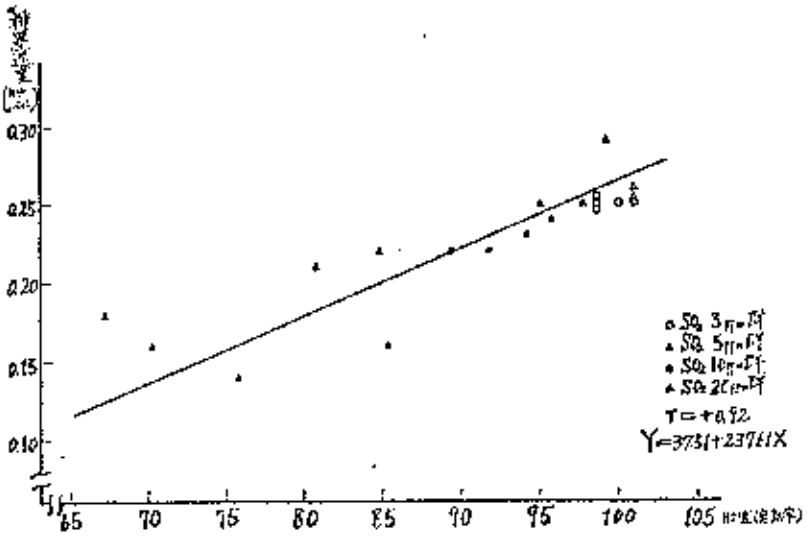
第9図　排膿速度と赤血球数の相関



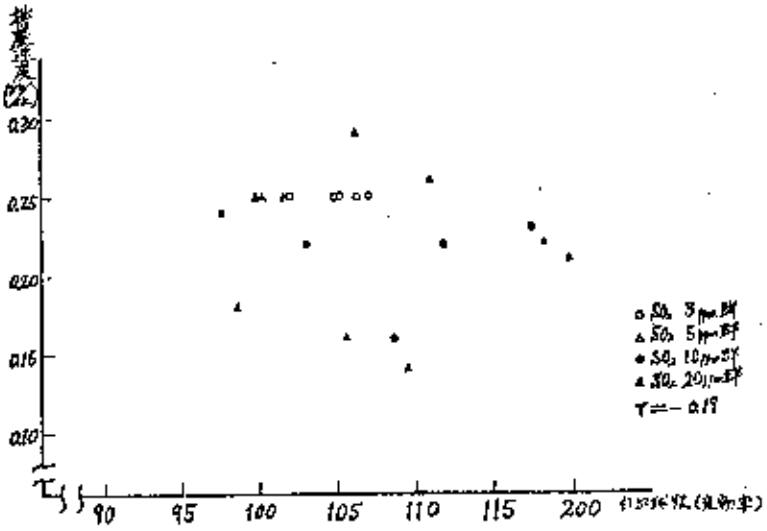
第10図　排膿速度と血色素量の相関

第3章　考　　察

以上の成績より按ずるに SO_2 ガスの気道排膿速度に及ぼす影響が SO_2 濃度の大なるほど著しいは薬物学的観点よりみても容易に想像しうるところであり，また Dalhamn[20]，Friberg[21] の報告よりしても明らかである．すなわち Dalhamn[20] はラッテおよび家兎の気管線毛運動を気管切開を施して in vitro および in vivo において顕微鏡下高速カメラで撮影し，ラッテでは線毛運動は SO_2 10 ppm 暴露では約5分で停止するが，家兎では吸気中の SO_2 濃度 100 ppm では運動停止を認めなく，200 および 300 ppm で 45 分以内に運動が停止すると報じ，Friberg[21] は家兎気管の線毛運動状態を電気装置（Dr. Rylander 法）で観察し，運動停止に必要な SO_2 濃度は気管内で約 10 ppm であり，これは吸入空気中の SO_2 濃度約 300 ppm に相当し，暴露終了後は数分で正常に復し，か



第11図　排痰速度と Ht 値の相関



第12図　排痰速度と白血球数の相関

かる影響は可逆的であると述べている．しかして両者の用いた SO_2 濃度は私の実験における濃度に比して高いが，私の実験においても 10 ppm 以上の濃度では気道線毛運動は低下して気道排痰速度は減少し，SO_2 濃度の大なるほどかかる低下は著しく，またその回復の遅れるを明らかに認め，その傾向を一にするを知る．他方，SO_2 濃度の低い場合は 3 ppm では気道線毛運動は何らの影響もうけず，5 ppm では初期亢進を認めるは Friberg[10] が述べたように吸気中の SO_2 濃度は気管内で薄められ，3 ppm ではほとんど痕跡となり，5 ppm ではきわめて稀薄となるがゆえに前者では何らの影響も認められず，後者では微量の SO_2 がかえって刺激となり，線毛運動の亢進をきたし，排痰速度の初期亢進が生ずるものと思われる．

今，気道排痰能力に密接な関係を有する気道線毛運動におよぼす SO_2 の関係について按ず

るに，SO_2 の気道線毛上皮細胞に直接影響をおよぼす局所作用と吸収による全身作用とが考えられよう，すなわち局所作用については Dalhamn[23]，Frank[26]，山田[29]，牧沢[27]，桂[28] らは局所の炎症変化を，Gross[31]，宮本[30] らは SO_2 低濃度暴露ではアレルギー作用による局所の感受性の増大を，Nadel[31]，Frank[32]，Balchum[33] らは線毛上皮内の受容器への直接局所刺激による遠走神経の反応をそれぞれ報じているが，この内アレルギー作用および遠走神経刺激作用は局所作用と同時に全身作用も含んでいるものと考えられる．全身作用については Dalhamn[23]，Friberg[34]，Frank[32]，Balchum[33]，Amdur[34]，Lee[35]，Speizer[36] らは SO_2 の気道よりの吸収現象を認めており，林[37] は SO_2 反復暴露による低酸素症，血中の硫酸塩および亜硫酸塩の増加に伴う赤血球数の反応を，Nadel[31] は低酸素症および過炭酸症を，越前[38] は骨系統の変化をそれぞれ報じており，Speizer[39]，外山[39]，Frank[40] らは S^{35} の吸収経路，分布，代謝過程などを追跡し，吸収後の組織代謝は今後残された問題であると述べている．他方，線毛上皮細胞の微細構造は Fawcett, Porter[41]，Dalhamn[42]，山本[43] らにより電子顕微鏡的に明らかにされ，Mitochondria（以下「ミ」と略す）が線毛基底部付近に多数集合することが認められ，かかる所見を基として線毛運動の機転については Fawcett[41] らは線毛運動は「ミ」内の酸化反応からのエネルギーによって営まれるものと考え，Robertis[44] らは組織化学的観点から一般細胞においては「ミ」内の Krebs Cycle をはじめ，それと密接な関係を持つ Cytochrom 系酵素が細胞質の好気的代謝に関係すると述べ，山本[42] は細胞の「ミ」が Krebs Cycle のすべてを備えて Oxydative Phosphorylation を行ない，細胞活動の中心であろうと推論していることより考えれば線毛運動は線毛の状態のみでなく「ミ」の状態によっても左右され，またかかる「ミ」の状態は血液性状によって影響されるは容易に予想しうるところであろう．したがってこれらを併せ考えれば SO_2 濃度 5 ppm のごとき低濃度暴露時における気道排塵速度の初期亢進は，主として SO_2 の気道線毛上皮細胞への局所作用による直接刺激に帰因すべく，SO_2 10 ppm 以上の高濃度暴露時の排塵速度の低下は体重減少，血液性状の変化などを併せ考えれば SO_2 の局所作用ももちろん，否定はできないが，それよりもむしろ全身作用が大きな役割を演じ，線毛上皮細胞内の「ミ」に低酸素，無機硫化物濃度の増加，貧血等の血液性状の変化が組織化学的に何らかの影響を与えるためによるものと思われる．しかして日時の経過とともに気道排塵速度が回復に向かうは生体の SO_2 に対する順応現象によるものと考えられ，かかる順応現象については林[45]，佐々木[46]，菅原[47] らもこれを認めており，前田[48] は順応現象は血中予備アルカリの動員能力の増強と肝のガス解毒能力の亢進が最も重要な役割を果し，造血器，網内系もまたこれに関与すると述べている．なお，気道排塵速度が赤血球数，血色素量および Ht 値と高度の相関を示す事実もかかる全身作用の影響を裏書きするものであろう．

血液性状の変化については林[45,49]，田村[51] らは SO_2 の中和に由来する中性塩類の刺激による造血機能の亢進を認め，片山[52]，米山[53]，園田[54] らは赤血球系の減少を，Lee[35] らは血色素量の上昇を，田村[55,56]，前田[48,49] らは白血球系，網内系の機能低下を報じ，対島[56] は末梢血液は初期に変動するも，長期例ではそれぞれ正常値またはそれに近い値に近づくと述べているが，いずれにしろ初期には血液性状に変化をきたし，長期ではこれらが回復に向かうは彼らの等しく認めるところである．私の成績でも暴露 SO_2 濃度は彼らに比して低いが，血液性状の変化はほぼこれに準じ，赤血球数および血色素量は SO_2 5 ppm では初期に軽度の増加を，SO_2 10 ppm 以上では減少を認め，いずれも暴露後 2～4 週頃より回復に向かう．ただし Ht 値は SO_2 20 ppm 暴露時では 5 週後に至るも回復の傾向を認めないが，それ以後の経過を観察していないために回復の有無については明らかでない．しかして SO_2 低濃度暴露時における赤血球数，血色素量などの初期増加は林[49,50]，田村[51] の述べたるがごとく，SO_2 吸収により生じ

た中性塩類の刺激による造血機能亢進に帰因すべく，高濃度暴露時のこれらの減少は Nadel[31]，林[37]，越前[34]，前田[40] らの述べたるがごとく，血中硫酸塩，亜硫酸塩の増加，低酸素症，過炭酸症，骨系統の変化らに帰因する造血機能の減退によるものと思われ，これらの回復は林[37]，佐々木[46]，菅原[47]，前田[40] らの述べたるがごとく，生体の順応現象，すなわち血中予備アルカリの酸性能力の増強，肝のガス解毒能力の亢進，造血器，胸内系の回復に帰因するものと考えられる．なお，白血球数の初期増加は SO_2 5 ppm の低濃度暴露のみならず，SO_2 10 ppm 以上の高濃度暴露時においても生ずるがゆえに，かかる増加は田村[41] らが述べているごとく，呼吸器系の2次的炎症に由来するものと考えられ，その回復は局所ならびに全身の順応によるものと思われる．

第4章　総括ならびに結論

大気汚染が呼吸器系におよぼす影響については汚染物質中の SO_2 がその性質上，また量的にも重要な役割を演ずるは諸家の等しく認めるところである．したがってかかる影響を論ずる上において SO_2 の気道排塵能力に及ぼす影響を究明することは排塵作用が分泌物，異物の排除を司り，呼吸器疾病罹患と密接な関係を有することより考えればきわめて重要なものであろう．しかるに SO_2 の呼吸器系に及ぼす影響については多くの研究報告があるも，SO_2 の気道排塵能力に及ぼす影響を観たものについてはいまだにこれあるを知らない．ここにおいて私は種々な SO_2 濃度の環境に家兎を毎日20分，連日5週にわたって暴露して，SO_2 濃度の排塵能力，体重，血液性状に及ぼす影響を比較観察し，もって大気汚染の呼吸器系に及ぼす機序の一端を明らかにし，大気汚染防止対策の基礎的資料に資せんとして本研究を行なった．

体重 2.9 kg 内外の健康な同一系純雄性白色成熟家兎 88 羽を用い，4 羽を対照群となし，84 羽を 21 羽ずつ暴露 SO_2 濃度 3 ppm, 5 ppm, 10 ppm および 20 ppm の 4 群に分け，暴露箱内で毎日 20 分ずつ，連日 5 週間にわたり，それぞれ所定濃度の SO_2 ガスに暴露し，この間における気道排塵速度の変化を比較観察するとともに体重，赤血球数，血色素量，Ht 値および白血球数の経過を究明した．

研究結果ならびに結論：

1) 気道排塵速度は SO_2 濃度 3 ppm ではほとんど影響されないが，5 ppm 以上の濃度では影響をうける．すなわち SO_2 5 ppm では暴露後 2 週間は亢進し，以後はもとの値に復するが，SO_2 10 ppm および 20 ppm ではいずれも暴露後 3 週間は次第に低下し，以後は回復に向かうも，SO_2 濃度の大なるほど低下が著しく，その回復が遅い．

2) 体重増加は SO_2 濃度 3 ppm ではほとんど影響されず，対照群のそれと同様か，あるいはやや上回るも，SO_2 濃度 5 ppm 以上では体重は暴露後 2～3 週までは次第に減少し，以後は回復に向かう．しかして SO_2 濃度の大なるほど減少が著しく，回復の時期も遅れ，回復度もまた小さい．

3) 赤血球数は SO_2 濃度 3 ppm ではほとんど対照群と差異を認めないが，5 ppm では暴露後 2 週までは初期増加の傾向を認める．SO_2 10 ppm および 20 ppm では暴露後 3 週までは次第に減少し，以後は回復に向かうも，後者は前者に比して減少が著しく，その回復度もきわめて遅い．血色素量および Ht 値の経過もまたこれにほぼ準ずるも，Ht 値は SO_2 5 ppm では初期増加は認め難く，また 20 ppm では暴露 5 週後においても回復の傾向が認められない．

4) 白血球数は SO_2 濃度 3 ppm では対照群とほとんど差異を認めないが，5 ppm 以上の濃度ではいずれも暴露 1 週後には急増し，その回復は SO_2 5 ppm および 10 ppm では 2 週後，20 ppm では 3 週後より認められる．しかして SO_2 濃度の高いほど初期増加が著しく，回復が遅い．

5) 気道排塵速度は赤血球数，血色素量および Ht 値と高度の順相関 ($r=+0.9$) を示すも白血球数との間には相関を認めない．

6) SO_2 濃度 3 ppm では気道排塵能力，血液

性状および体重には影響を認めないが，5 ppm 以上では影響を認め，SO_2 濃度の大なるほどその影響が著しい．すなわち SO_2 5 ppm では気道排塵速度は初期亢進を，赤血球数および血色素量は初期増加を示し，その回復も早いが，SO_2 10 ppm 以上ではかかる亢進，あるいは増加を示さずに減少し，その回復も遅い．

7) 毎日20分連日暴露時の SO_2 濃度の許容限界は 5 ppm と考える．

8) SO_2 低濃度暴露における気道排塵速度の初期亢進は主として SO_2 の気道線毛上皮細胞への局所作用による直接刺激に，赤血球数および血色素量などの初期増加は SO_2 吸収により生じた中性塩類の刺激による造血機能亢進に帰因し，高濃度暴露における気道排塵速度の低下，赤血球数，血色素量などの減少は SO_2 吸収による血中無機硫化物の増加，低酸素症，過炭酸症などによる「ミ」の状態の変化および造血機能の減退に帰因するものと考えられ，これらの回復はいずれも生体の順応現象によるものと思われる．しかして白血球数の初期増加は呼吸器系の2次的炎症に由来し，これが回復は局所ならびに全身の順応によるものと思惟される．

編集するに当たり，終始御懇篤なる御指導ならびに御校閲を賜わった大阪鉱業教授に対し深甚の謝意を表する．なお，本論文要旨は第23回日本公衆衛生学会総会において発表した．

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Über den Einfluß der Verbindungsbildung zwischen schwefliger Säure und Kohlenhydraten auf die SO_2-Bestimmung*

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The Influence of the Reaction Products between SO_2 and Carbohydrates on the Determination of SO_2. *It was found, that the decrease in chemically detectable SO_2 in stored sugar solutions under mild conditions (room-temperature and 60 °C) is partially influenced by the formation of several reaction products, which prevent SO_2 being set free. Under these conditions fructose and sucrose do not react with SO_2. Maltose forms reaction products more readily than glucose. Reaction products of glucose and SO_2 particularly are acids. It was found, that these reaction products exist with and without sulfur. The amount of reaction products is very small.*

Methods for determination of SO_2 were compared. It was found, that the amount of re-detectable SO_2 decreases with increase in sugar concentration. A method is proposed for the rapid determination, at the same time being acceptable for quality control. It was shown, that a colorimetric method for determination of SO_2 in starch products with small amounts of SO_2 is more exact and needs time less than the Monier-Williams method.

(Zusammenfassung siehe Seite 380; Résumé à la page 380)

1. Einleitung

Es ist allgemein bekannt, daß in vielen Zweigen der Lebensmittelverarbeitung schweflige Säure (SO_2) und ihre Salze schon seit langer Zeit als technischer Hilfsstoff und als Konservierungsmittel Verwendung finden. Obwohl die Wirkungsweise der schwefligen Säure auf die einzelnen Lebensmittelinhaltsstoffe noch weitgehend ungeklärt ist, hat der Gesetzgeber auf Grund neuerer ernährungsphysiologischer Erkenntnisse die Tendenz, die Verwendung der schwefligen Säure möglichst einzuschränken.

Über die Reaktionsmöglichkeiten von SO_2 finden sich besonders in der neueren Literatur zahlreiche Angaben, die teilweise widersprüchlich sind. Bereits im Jahre 1898 wird von *Roques* [1] über eine Glucosebisulfit-Additionsverbindung in Wein berichtet, die durch Reaktion der Aldehydgruppe mit SO_2 entsteht. Lange Zeit herrschte Unklarheit darüber, ob es sich bei Reaktionsprodukten von Aldehyd und Bisulfit um α-Hydroxyschwefligsäureester oder um α-Hydroxysulfonsäuren handelt. *Adler* [2] isolierte bei pH 6 zwei verschiedene Sulfonsäuren und bestätigte das Vorhandensein von Sulfon- und Carboxylgruppen in beiden, sowie ihre Stabilität gegenüber heißer Salzsäure und heißem Alkali. Das Vorhandensein eines Schwefligsäureesters konnte er aber nicht ausschließen. Ohne weiteren Nachweis vermutete er, daß auf diesem Wege etwa die Hälfte aller Verbindungen zwischen Kohlenhydrat und SO_2 gebildet wird.

Bravermann [3] stellte fest, daß sich bei 30–35 °C Glucose mit Bisulfit in molarem Verhältnis in wäßriger Lösung innerhalb 3–5 Tagen quantitativ zu Hydroxysulfonat umsetzt. Nach *Ingles* [4] hängen die aus Glucose und Bisulfit gebildeten Produkte stark von den Reaktionsbedingungen ab. Danach entsteht bei niedrigen Temperaturen und einem pH-Wert von 4,5 oben erwähntes Additionsprodukt. Beim Erhitzen einer äquimolaren Lösung von Bisulfit und Glucose auf über 100 °C, also Bedingungen, wie sie auch bei der Bestimmung des SO_2 durch Destillation nach *Monier-Williams* herrschen, erhält man Glucose-6-Sulfat neben Gluconsäure. Das Bisulfit liefert neben entweichendem SO_2 auch Schwefel. Darüberhinaus berichtet er von einer Oxidation der reduzierenden Zucker durch Bisulfit, wenn im Molverhältnis 1:4 gearbeitet wird. *Ingles* [4, 5, 6] weist eine Oxidationswirkung des Bisulfits auch schon bei niedrigeren Temperaturen, als sie von *Hägglund*, *Johnson* und *Urban*

* Nach einem von *W. Nierle* auf der Stärke-Tagung 1970 der Arbeitsgemeinschaft Getreideforschung in Detmold gehaltenen Vortrag.

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[7] berichtet werden, nach. Dabei sollen sich neben Gluconsäure noch mehrere andere Verbindungen bilden, deren Identität aber nicht völlig sichergestellt wurde.

Noch unübersichtlicher werden die Verhältnisse, wenn man auf Stärkehydrolysenprodukte übergeht, da diese trotz intensivster Reinigungsverfahren noch stickstoffhaltige Verbindungen enthalten, die ihrerseits mit SO_2 reagieren könnten. Nach *Hodge* [8] reagieren die Carbonylgruppen der Aldosen mit freien Aminogruppen von Aminosäuren zu Aldosaminen. Durch Amadoriumlagerung bildet sich die 1-Desoxy-1-amino-2-ketose. Durch Wasserabspaltung bilden sich stickstoffhaltige Carbonylverbindungen, die auf verschiedensten Wegen zu Melanoidinen kondensieren. In neueren Arbeiten von Reynolds, Anet und Ingles [9] wird folgendes Schema für die Reaktion von Glucose mit z. B. Glycin vorgeschlagen (Abb. 1).

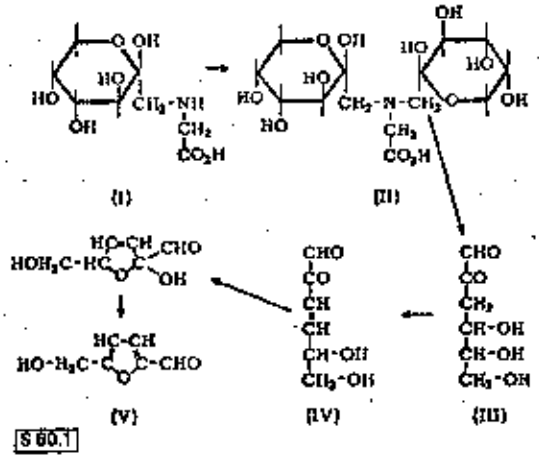

Abbildung 1. Reaktionsschema nach *Reynolds*.

Danach reagiert die Aldose zu Monofructoseglycin, das sich mit einem weiteren Aldosemolekül zum Difructoseglycin verbindet. Im sauren Medium spaltet das Difructoseglycin das Desoxyhexoson ab, welches unter Wasserabspaltung in das 3,4-ungesättigte Desoxyhexoson übergeht. Durch Zyklisierung und weitere Wasserabspaltung im Sauren bildet sich das 5-Hydroxymethylfurfurol (HMF). HMF jedoch liefert nur recht langsam Bräunungsprodukte und reagiert auch nur sehr zögernd mit Aminogruppen. Nach *Sievert* und *Tödt* [10] soll das HMF sehr schnell mit Bisulfit in saurem Medium reagieren.

Aus dem Diketosylamin bildet sich eine große Zahl ungesättigter Carbonylverbindungen, die mit Aminostickstoff schnell zu Bräunungsprodukten reagieren. Mit diesen ungesättigten Carbonylverbindungen soll das SO_2 reagieren und eine Melanoidinbildung verhindern. Diese Reaktionsmöglichkeit des SO_2 könnte z. B. auch bei der Bestimmung des SO_2 in Hydrolysenprodukten nach der Destillationsmethode eine Rolle spielen und zu Verlusten an bestimmbarem SO_2 führen.

In der im folgenden beschriebenen Arbeit finden Verhältnisse, wie sie in technischen Stärkehydrolysaten vorliegen, insofern keine Berücksichtigung, als stets vorhandene „Verunreinigungen" wie Eiweiß- und Zuckerabbauprodukte die zu untersuchenden Reaktionsmöglichkeiten von SO_2 mit reduzierenden Zuckern noch vervielfältigen könnten und damit eine deutliche Aussage unmöglich würde. Daher wurden die folgenden Versuche an Modellösungen mit reinen Zuckern durchgeführt.

2. Reaktion in Modellösungen

Um die Reaktionsbedingungen möglichst übersichtlich und praxisnah zu halten, fanden 50%ige Zuckerlösungen Verwendung, die mit unterschiedlichen Mengen SO_2 versetzt und bei Temperaturen von 20 und 60 °C gut verschlossen gelagert wurden. Diese beiden Temperaturen haben bei der Stärkehydrolyse bzw. -Lagerung eine besondere Bedeutung. Nach den verschiedenen Reinigungsstufen wird das Hydrolysat bei 60 °C von einem Trockensubstanzgehalt von ungefähr 40 % auf einen Gehalt von ca. 80 % eingedampft, wobei Natriumsulfit zugesetzt wird. Um den hochviskosen Sirup pumpfähig zu halten, lagert man ihn bei 60 °C in großen Tanks mehrere Tage.

2.1. *Verhalten von SO_2 bei der Lagerung von Glucoselösungen*

Wie bekannt, ist Natriumsulfit in wäßrigen Lösungen in Abhängigkeit vom pH-Wert instabil und geht innerhalb kurzer Zeit durch Autoxidation in Sulfat über. Es mußte daher untersucht werden, ob und wie Glucose die Beständigkeit des SO_2 in wäßriger Lösung beeinflußt. Zur Klärung dieser Frage wurden Glucoselösungen mit SO_2 versetzt und wie eingangs beschrieben, gelagert. Die Bestimmung des freien SO_2 erfolgte durch Titration mit n/100 Jodlösung, das gebundene SO_2 wurde durch anschließende 10 Minuten lange Einwirkung von 25%iger Kalilauge und nach Ansäuern durch erneute Titration mit n/100 Jodlösung bestimmt.

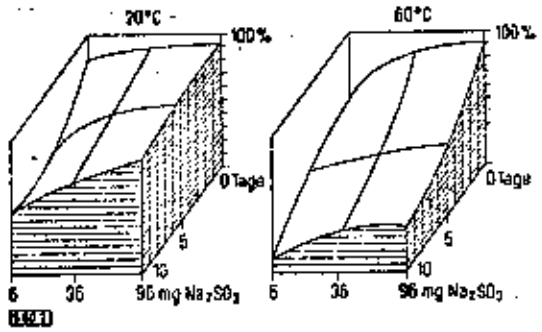

Abbildung 2. Verhalten des Gesamt-SO_2 bei Lagerungstemperaturen von 20 °C und 60 °C.

Die Abbildung 2 zeigt, daß ein erheblicher Unterschied zwischen der Lagerung bei 20 °C und bei 60 °C besteht. Da ein Verdunsten des SO_2 auszuschließen ist, muß angenommen werden, daß die Abnahme des SO_2 und damit die Neigung der Glucose zur Bildung von Additionsverbindungen bei der höheren Temperatur wesentlich größer ist. Dies wird durch die beiden folgenden Abbildungen 3 und 4 erhärtet.

Während bei 20 °C der Gehalt an freiem SO_2 nur geringfügig abnimmt, verschwindet das freie SO_2 im Lauf von

10 Tagen bei 60 °C fast völlig. Bei Berücksichtigung des Analysenfehlers stellt man jedoch fest, daß sowohl bei 20 °C als auch bei 60 °C der Gehalt an reversibel gebun-

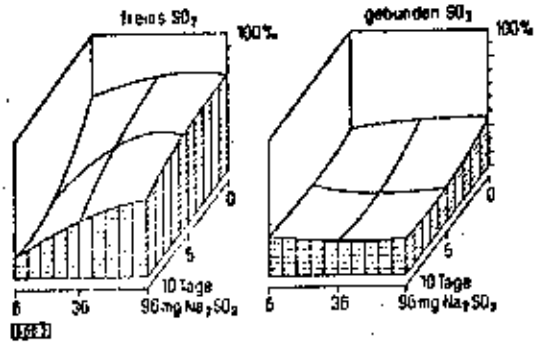

Abbildung 4. Lagerungstemperatur von 60 °C.

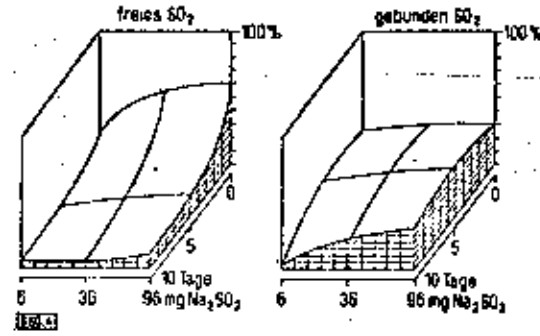

Abbildung 3. Lagerungstemperatur von 20 °C.

denem SO_2 nahezu gleich groß ist und konstant bleibt. Wird aber angenommen, daß das nicht mehr als SO_2 erfaßbare Schwefeldioxid durch Oxidation in Sulfat übergegangen ist, dann müßte die beobachtete Abnahme des freien SO_2 als solche verstanden werden. Durch Einsatz von S-35-markiertem Natriumsulfit ließ sich jedoch nachweisen, daß die Abnahme des analytisch bestimmbaren SO_2 nicht allein eine Oxidation zu Sulfat sein kann, da nach Oxidation des gesamten SO_2 mit Jodlösung und Abtrennung des gebildeten Sulfats mit Bariumchlorid auch im Filtrat des Bariumsulfatniederschlages Radioaktivität gefunden wurde. Unter den beschriebenen Bedingungen finden sich bei Zusatz von 60 mg Natriumsulfit zu 100 ml Glucoselösung etwa 10 % der Aktivität im Filtrat wieder. Dabei bewirken steigende Mengen von SO_2 eine verstärkte Bildung von Reaktionsprodukten.

2.2. *Bildung saurer Verbindungen durch Einwirkung von SO_2*

Unter dem Einfluß von Schwefeldioxid werden in Glucoselösungen in Abhängigkeit von der SO_2-Konzentration saure Verbindungen gebildet. Zu deren Nachweis wurden Glucoselösungen mit unterschiedlichem Gehalt an SO_2 auf pH 4.8 eingestellt und bei 60 °C mehrere Tage gelagert. Die pH-Änderung wurde täglich durch Titration mit Natronlauge ausgeglichen. Aus Abbildung 5 ist der Verbrauch an Natronlauge in Abhängigkeit von der SO_2-Konzentration und der Zeit zu ersehen. Der tägliche Verbrauch an Natronlauge und damit auch die Bildung saurer Verbindungen nimmt plötzlich nach 7 Tagen stark ab. Bei geringerer SO_2-Konzentration wird diese Abnahme schon nach 4 Tagen beobach-

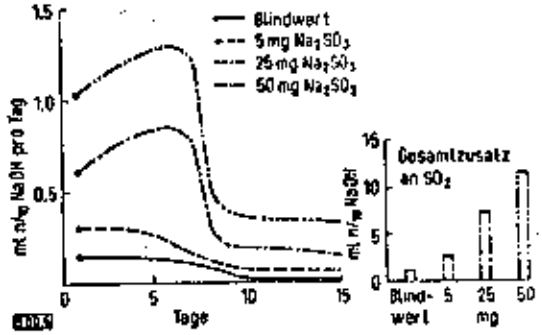

Abbildung 5. Täglicher Verbrauch von NaOH in Abhängigkeit von der Sulfitkonzentration.

tet. Somit besteht ein Zusammenhang zwischen der zugesetzten Menge an Natriumsulfit und dem Verbrauch an Natronlauge. Betrachtet man im Vergleich dazu die Abnahme des freien und gebundenen SO_2 (Abb. 4), so muß man annehmen, daß für die Bildung saurer Verbindungen eine bestimmte Konzentration an freiem SO_2 erforderlich ist, deren Unterschreitung eine Umsetzung von SO_2 mit Glucose nicht mehr zuläßt.

2.3. *Radiochemischer Nachweis von Reaktionen zwischen Zuckern und SO_2*

Über die Reaktionsmöglichkeiten anderer reduzierender und nicht reduzierender Zucker mit SO_2 gibt folgende Untersuchung Auskunft. Zu 50%igen Lösungen von Glucose, Maltose, Galaktose, Saccharose und Fructose wurde S-35-markiertes Natriumsulfit zugesetzt. Der pH-Wert aller Lösungen wurde mit Salzsäure auf pH 4.9 eingestellt. Aus den gutverschlossenen Meßkolben mit 20 °C und 60 °C gelagerten Lösungen wurden in Abständen von 2 Tagen jeweils 10 ml entnommen und diese wie folgt aufgearbeitet. Das Sulfit wurde nach Oxidation als Bariumsulfat entfernt; nach Filtration des gebildeten Niederschlages wurde dieser und auch das Filtrat im low level β-Counter zur Zählung gebracht. Anteile des Filtrates wurden teils mit Natronlauge und teils mit Salzsäure sowie Wasserstoffperoxid 10 min gekocht und nach Zusatz von Trägersulfat mit Bariumchlorid versetzt; die Aktivität der getrockneten Sulfatniederschläge, wie auch die der Filtrate wurden gemessen. In den Filtraten von Saccharose und Fructose fand sich keine Aktivität. Aus dem vorliegenden Zahlenmaterial läßt sich ein deutlicher Unterschied zwischen den drei Zuckern feststellen. Wie die Impulsraten (Tab. 1) zeigen, reagiert Glucose am wenigsten mit SO_2. Überraschenderweise ist bei Maltose zwischen den beiden Lagerungstemperaturen kaum ein Unterschied zu beobachten. Obwohl, wie in der Abbildung 6 gezeigt wird, eine weitgehende Abtrennung von Aktivität, Reaktionsprodukt und Zucker über Sephadex G-10 möglich war, ließ sich eine Anreicherung der in relativ niedrigen Konzentrationen auftretenden Reaktionsprodukte auf diese Weise nicht ermöglichen. Eine Abtrennung der schwefelhaltigen Reaktionsprodukte über Ionenaustauscher war nicht durch-

führbar, da die aktivitätstragenden Verbindungen irreversibel an den Anionenaustauscher gebunden wurden.

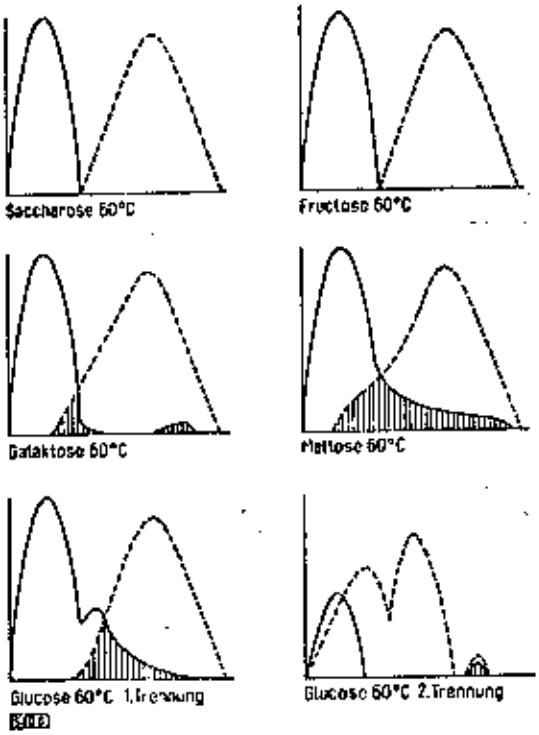

Abbildung 6. Verteilung der Zucker und S-35-Aktivität bei der Trennung an Sephadex.

Als Ordinate ist jeweils die mittels Anthron erhaltene Extinktion sowie die S-35-Aktivität in je 1 ml der einzelnen Fraktionen, als Abszisse sind die einzelnen Fraktionen aufgetragen.

– – – – = Zuckerkonzentration in den Fraktionen,

———— = S-35-Aktivität in den Fraktionen.

Aus den in der Abbildung 6 dargestellten Elutionskurven ist auch zu ersehen, daß nur bei den Aldosen keine völlige Abtrennung zwischen Zucker und S-35-Aktivität zu erreichen war. Gleichzeitig wird auch die große Reaktionsbereitschaft der Aldosen bestätigt, wobei nicht geklärt wurde, ob Maltose selbst oder die im handelsüblichen Produkt zahlreich vorhandenen höheren Zucker bevorzugte Reaktionspartner des SO_2 sind, wie man vielleicht vermuten könnte.

2.4. *Nachweis S-35haltiger Verbindungen*

Aus den Elutionskurven ist allerdings nicht zu erkennen, ob bei der Reaktion nur eine einzige oder mehrere Verbindungen gebildet werden. Die Abbildung eines Dünnschichtchromatogramms einer bei 60 °C mit SO_2 10 Tage gelagerten Glucoselösung zeigt das Vorhandensein mehrerer Kohlenhydrate. Die Lösung wurde unbehandelt, d. h. ohne Entfernen des Sulfits bzw. Sulfats aufgetragen. Vor dem Besprühen wurden die Dünnschichtchromatogramme zur Messung der Radioaktivität mit Röntgenfilm bedeckt. Die Entwicklung des Films ergab die in Abbildung 7 bei „a" aufgezeichneten Flecken. Die Chromatogramme wurden zum Nachweis der Kohlenhydrate mit Anisaldehyd-Schwefelsäure (siehe „b") besprüht. Bei Gegenüberstellung von Autoradiogramm und Chromatogramm fällt auf, daß die aktiven, also schwefelhaltigen Flecken mit dem verwendeten Sprühreagenz nicht reagieren, während an den Stellen des Chromatogramms, an denen chemisch Kohlenhydrate nachweisbar waren, keine Schwärzung des Films erfolgte, also auch keine Schwefelverbindung zu finden war. Die hier beobachtete Bildung einzelner schwefelhaltiger Kohlenstoffverbindungen aus Dextrose unter dem Einfluß von SO_2 wird auch von *Pill Soon Song, Chichester* und *Stadtmann* [11] in einem anderen Zusammenhang erwähnt.

Tabelle 1. Bildung von Reaktionsprodukten in Abhängigkeit von Lagerungszeit und -temperatur.

	20 °C		60 °C	
	0 Tage	10 Tage	1 Tag	10 Tage
Relative Aktivität in 1,0 ml Filtrat in IpM				
Glucose	10	13	110	100
Maltose	12	36	53	51
Galaktose	16	19	108	100

Relative Aktivität im Sulfatniederschlag in IpM

	Oxidation mit Wasserstoffperoxid							
	Säure	Lauge	Säure	Lauge	Säure	Lauge	Säure	Lauge
Glucose	145	178	158	199	196	339	192	270
Maltose	247	271	1026	1023	611	559	650	673
Galaktose	532	437	682	550	838	857	1040	908

Relative Aktivität im zweiten Filtrat in IpM

	Oxidation mit Wasserstoffperoxid							
	Säure	Lauge	Säure	Lauge	Säure	Lauge	Säure	Lauge
Glucose	8,7	7,8	9,8	9,4	75	77	92	61
Maltose	9,5	8,4	24,8	22,2	42	35	46	35
Galaktose	10,2	9,7	12,9	13,2	82	82	68	75

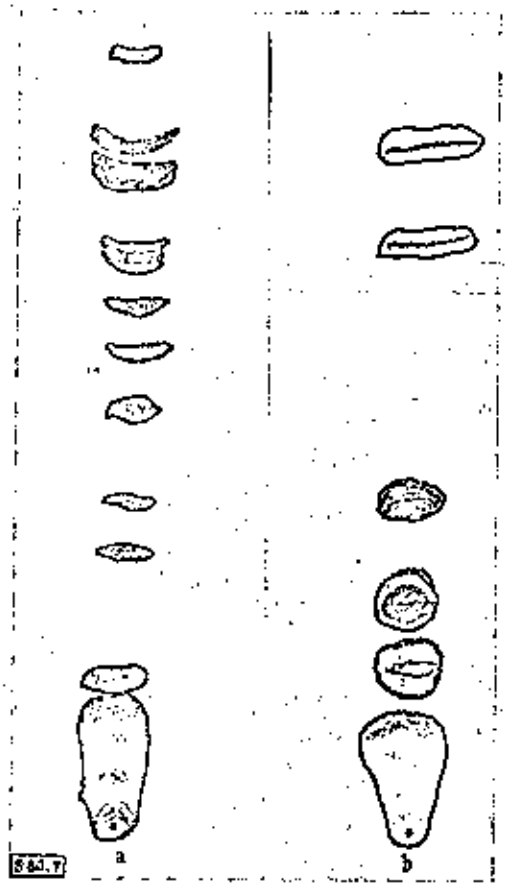

Abbildung 7. a) S-35-aktive Verbindungen (Autoradiogramm); b) Kohlenhydrate (chemisch nachgewiesen).

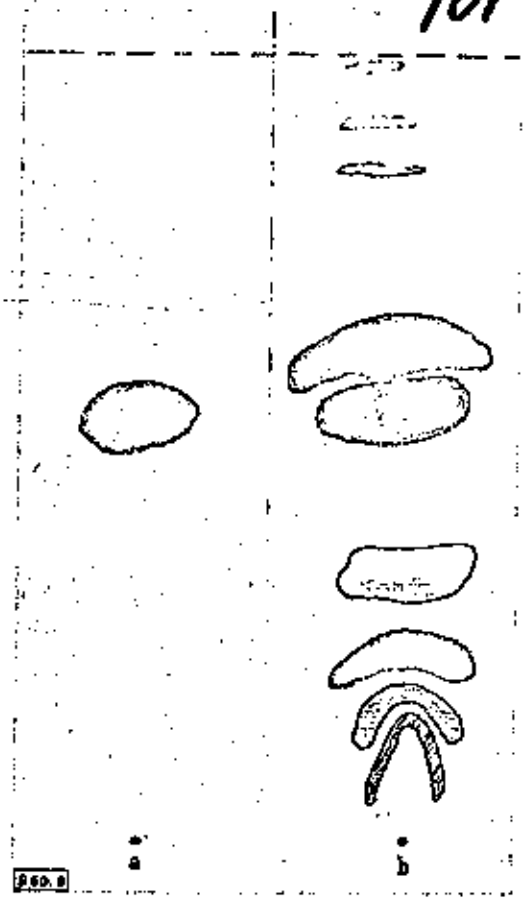

Abbildung 8. Autoradiogramme eines DC-Streifens. a) Glucose C-14; b) Glucose C-14 und SO_2.

2.5. *Nachweis der Umsetzung von Glucose unter der Einwirkung von SO_2 zu anderen Kohlenhydraten*

Die Dünnschichtchromatogramme weisen darauf hin, daß die Umsetzung von SO_2 mit Glucose nicht ausschließlich zu schwefelhaltigen Verbindungen führt, sondern daß vielmehr auch Abbau- bzw. Umsetzungsprodukte des Kohlenhydrates gebildet werden. Diese Vermutung konnte durch ein zusätzlich angefertigtes Autoradiogramm einer bei 60 °C gelagerten C-14-Glucose unter Zusatz von inaktivem Sulfit gestützt werden. Wie aus der Abbildung 8 ersichtlich, werden mindestens 8 C-14-haltige Umsetzungsprodukte beobachtet. Die Vielzahl der so gefundenen Reaktionsprodukte rechtfertigt die Annahme, daß sich bei der Umsetzung von Glucose mit Sulfit auch schon bei sehr milden Bedingungen mehrere Reaktionsabläufe gleichzeitig oder nacheinander vollziehen.

Diese Verhältnisse werden noch viel komplizierter, wenn man von Modellösungen auf die erwähnten technischen Hydrolysenprodukte übergeht. Über das Verhalten des SO_2 während und bei der technischen Stärkehydrolyse, sowie bei der Lagerung von Sirupen soll in einem anderen Zusammenhang ausführlich berichtet werden.

2.6. *Auswirkungen auf die SO_2-Bestimmungen*

Die von *Kröller* [12] geschilderten Schwierigkeiten bei der quantitativen Bestimmung des SO_2 erscheinen nun doch in einem etwas anderen Licht. Nach *Kröller* läßt sich infolge von Oxidationsvorgängen selbst aus reinen Sulfitlösungen mit Schutzgas und Ascorbinsäure keine völlige quantitative Destillation des SO_2 durchführen. Auch eine kolorimetrische Methode, die Reaktion zwischen Pararosanilin, Formaldehyd und SO_2 zu Pararosanilinmethylsulfonsäure zeigt bei Anwesenheit von Glucose unerklärliche SO_2-Verluste. Diese Beobachtungen können durch die im ersten Teil beschriebenen Versuche zu erklären sein. Wir haben unter dem Blickwinkel der Reaktionsmöglichkeiten zwischen SO_2 und Zukkern alle drei üblichen Bestimmungsmethoden für SO_2 miteinander verglichen.

2.6.1. *Kolorimetrische SO_2-Bestimmung*

Geräte

Ausschüttelgerät mit Filterplatte 1 G 3; Zwischenstück mit Rohransatz; *Witt*'scher Topf; 100 ml Meßkolben; Pipetten; Photometer; 1 cm Küvetten.

Chemikalien

0,5 g Parafuchsin (Chroma-Ges. Schmid & Co, Stuttgart-Untertürkheim) mit etwas Wasser 30 min schütteln und auf

100 ml auffüllen und abfiltrieren: 40 ml Filtrat + 60 ml 25%ige HCl auf 1000 ml auffüllen (ca. 5 Tage haltbar); Formaldehyd 0,2%ig (2,5 ml 40%ige Formaldehydlösung auf 500 ml auffüllen); m/100 Na_2CO_3-Lösung (1,0599 g auf 1000 ml auffüllen); Natrium-pyrosulfit $Na_2S_2O_5$.

Eichkurve
Durch entsprechende Verdünnungen werden Lösungen mit 1, 5, 10, 20, 30, 40 und 50 ppm in 100 ml hergestellt. Der Vollständigkeit halber sei an dieser Stelle auch die Bestimmung des SO_2 in Stärken beschrieben.

1,4836 g $Na_2S_2O_5$ entsprechen 1 g SO_2.
1,4836 g $Na_2S_2O_5$ auf 1000 ml ≙ 1 mg SO_2 in 1 ml (I),
10 ml von Lösung I/1000 ml = 10 ppm SO_2 in 1 ml (II),
100 ml von Lösung II/1000 ml = 1 ppm SO_2 in 1 ml (III).

Durchführung der Bestimmung bei Stärke

1 g Maisstärke wird mit 10 ml m/100 Na_2CO_3-Lösung ausgeschüttelt und in 100 ml Meßkolben abgesaugt. Diesen Vorgang wiederholt man 3mal. Das Filtrat wird mit 20 ml Fuchsinlösung und 20 ml 0,2%iger Formaldehydlösung eingefärbt, auf 100 ml aufgefüllt und nach 25 min im Spektralphotometer bei 560 m gemessen. Die Messung erfolgt gegen einen Blindwert, der ebenfalls mit je 20 ml Fuchsin- und Formaldehyd eingefärbt wird. Trübe Lösungen müssen vor der Einfärbung filtriert werden.

Durchführung der Bestimmung in Zuckerlösungen

1 g Sirup in einen 100 ml Meßkolben einwiegen, auflösen, mit 20 ml Fuchsinlösung und 20 ml Formaldehyd anfärben und auffüllen.

Vor der Messung müssen Trübung und Eigenfarbe des Sirups gegen den gleichen Blindwert gemessen werden (evtl. vorher filtrieren).

2.6.2. *Destillation nach* DIN 10 305. *Monier-Williams-Verfahren (ISO-Empfehlung)*

Geräte
Analytische Waage; 1000/ml Dreihals-Destillierkolben mit Normschliff; Kugelkühler 300 NS 29/32 × NS 29/32 DIN 12 581 zylindrischer Tropftrichter, 100 ml Inhalt, mit einem für den Destillierkolben passenden Normschliff, passend für den Destillierkolben; Glasverbindungsstück mit 2 Normschliffen als Verbindung vom oberen Kühlerende zum Hals des Freseniuskolbens; 300 ml Freseniuskolben; Waschflasche B nach DIN 12 596; Kipp'scher Gasentwickler nach DIN 12 485 zur Entwicklung von Kohlendioxid oder Kohlendioxiddruckflasche 10 ml Bürette, Skaleneinteilung 0,02 ml.

Chemikalien
Salzsäure zur Analyse, Dichte 1,18; 0,1 n Natronlauge; Wasserstoffperoxidlösung 3%ig; Bromphenolblauindikator (0,04 g Bromphenolblau werden in 100 ml eines Äthanol-Wassergemisches 1:1 gelöst); Natriumcarbonatlösung (7,5 g Na_2CO_3 werden in 100 ml Wasser gelöst).

Durchführung
Es sind zwei Bestimmungen durchzuführen.
Das vom Kipp'schen Gasentwickler oder der Kohlendioxiddruckflasche erzeugte CO_2 wird zum Entfernen des Chlorwasserstoffes durch eine mit Natriumcarbonatlösung gefüllte Gasflasche geschickt und von dort durch das Gaseinleitungsrohr in den Destillierkolben geleitet.

Der Freseniuskolben wird mit 25 ml H_2O_2-Lösung beschickt und über das Glasverbindungsstück an den Kühler angeschlossen. Durch den Tropftrichter werden zuerst 200 ml dest. Wasser, dann 20 ml HCL in den Destillierkolben gegeben und die Kolbenfüllung nach Abstellen des Kühlwassers für etwa 10 min im Kohlendioxidstrom erhitzt. Je nach dem zu erwartenden SO_2-Gehalt wird eine Menge der Probe auf 1 g genau eingewogen und in etwa der doppelten Menge dest. Wasser gelöst oder dispergiert. Die Lösung bzw. Dispersion wird durch den Tropftrichter, der anschließend mit wenig dest. Wasser nachgespült wird, in den Destillierkolben gegeben.

Anmerkung
Stärkeproben dürfen nicht in die kochende Salzsäurelösung gegeben werden. Der Kohlendioxidstrom wird 20 min durch die Apparatur geleitet, er wird erst dann erhitzt, wenn die gesamte Probendispersion zugegeben ist.

Die Probenzugabe und der Gasstrom müssen gut reguliert werden, um ein Zurücksteigen der Wasserstoffperoxidlösung, Lufteinschlüsse oder ein Anbrennen der Probe zu verhindern.

Die Kolbenfüllung wird 1 Std. unter einem schwachen Kohlendioxidstrom gekocht. Bei beenderer Destillation wird das Kühlwasser abgestellt und der Freseniuskolben entfernt, sobald das Glasverbindungsstück warm zu werden beginnt.

Nach Zusatz von 3 ml Indikatorlösung wird die Lösung mit 0,1 n Natronlauge direkt im Freseniuskolben titriert. Der Umschlag erfolgt von gelb nach blauviolett.

Mit den verwendeten Lösungen wird ohne Zusatz der Probe ein Blindversuch durchgeführt.

Auswertung
Der SO_2-Gehalt in ppm wird nach folgender Zahlenwertgleichung errechnet:

$$SO_2\text{-Gehalt} = \frac{(a-b)\cdot 0{,}0032\cdot 10^6}{E}$$

Hierin bedeuten: E Einwaage in g; a bei der Titration der Probe verbrauchte Menge Natronlauge in ml; b beim Blindversuch verbrauchte Natronlauge in ml.

2.6.3a. *Jodtitration*

Geräte
200er Erlenmeyerkolben; Pipetten; Wasserbad.

Chemikalien
NaOH 10%ig; H_2SO_4 1:3; H_2O_2 3%ig; Stärkelösung 2%ig; Jodlösung 0,02 n.

Durchführung
Je nach dem zu erwartenden SO_2-Gehalt wird eine Menge der Probe auf 4 Stellen nach dem Komma genau eingewogen. Nach Zugabe einiger Eisstücke werden die Proben in ein Wasserbad von ca. 0 °C gestellt. Haben die Lösungen 5 °C erreicht, gibt man 10 ml einer 10%igen NaOH zu und stellt die Proben für weitere 10 min in das Wasserbad. Anschließend werden 10 ml H_2SO_4 (1:3) und 2 ml Stärke-

indikator zugesetzt. Eine Probe dient als Blindwert nach Zugabe von 10 ml H_2O_2 (3%ig). Jede Lösung wird sofort nach der Säurezugabe mit 0,02 n Jodlösung titriert, bis die Blaufärbung 1 Minute lang anhält.

Berechnung
1 ml n/200 Jodlösung = 0,16 mg SO_2; verbrauchte ml n/200 Jodlösung = x mg SO_2.

2.6.3b. Modifizierte Jodtitration

Geräte
wie bei Jodtitration.

Chemikalien
wie bei Jodtitration.

Durchführung
Eine dem zu erwartenden SO_2-Gehalt entsprechende Menge der Probe wird bis zur 4. Stelle nach dem Komma eingewogen. Zu der Probe gibt man 10 ml H_2SO_4 (1:3), 2 ml Stärkeindikator und einige Eisstücke. Eine Probe dient als Blindwert nach Zugabe von 10 ml einer 3%igen H_2O_2-Lösung. Jede Lösung wird sofort mit 0,02 n Jodlösung bis zur Blaufärbung titriert. Die verbrauchten ml der Jodlösung entsprechen umgerechnet dem freien SO_2.

Die austitrierte Probe wird zur Bestimmung des gebundenen SO_2 mit 20 ml 10%iger Natronlauge alkalisch gemacht und nach 10minütigem Stehenlassen wiederum mit 20 ml Schwefelsäure angesäuert. Sodann wird erneut mit 0,02 n-Jodlösung bis zur Blaufärbung titriert. Der Verbrauch an Jodlösung entspricht dem Gehalt an gebundenem SO_2.

2.6.4. Beschreibung der Versuche

Alle folgenden Ergebnisse sind stets Mittelwerte aus mindestens vier Einzelbestimmungen. Zur Feststellung der Fehlerbreiten werden die Versuche mit unterschiedlichen Einwaagen und Verdünnungen durchgeführt.

Solange man reine Lösungen von schwefliger Säure vorliegen hat, sind alle drei Methoden, also kolorimetrische Bestimmung, Destillation und Titration, im Prinzip gleichwertig und gut reproduzierbar, wie aus Tabelle 2 zu ersehen

Tabelle 2. SO_2-Bestimmung in reinen Lösungen (SO_2-Menge in mg/kg).

Gehalt	Dest.	Diff. %	Titr.	Diff. %
505	475	5,9	489	3,2
670	640	4,5	644	3,9
1005	993	1,2	970	3,5
1340	1329	0,8	1295	3,4
1515	1509	1,1	1470	3,0
2690	2690	0	2640	1,6

ist. Sobald aber Glucose vorhanden ist, sind relativ starke Unterschiede zu beobachten.

Wie in der Tabelle 3 gezeigt ist, findet man mit steigenden Mengen von Glucose sowohl bei der kolorimetrischen als auch bei der Destillationsmethode weniger SO_2 wieder. Bei der Titrationsmethode ist dies umgekehrt.

Tabelle 3. Veränderungen des SO_2-Gehaltes in $Na_2S_2O_5$-Lösung nach Zugabe steigender Mengen einer 50%igen Glucoselösung.

Methode	SO_2-Gehalt mg/kg (ohne Glucose) theor.	Glucose-zugabe ml	SO_2-Gehalt mg/kg (mit Glucose)	Abweichung % der Werte theor.
kol.	1010	10	1000	1,0
		20	967	4,3
		30	920	8,9
		40	877	13,2
		50	833	17,5
titr.	1010	10	872	13,7
		20	904	10,5
		30	936	7,3
		40	968	4,2
		50	1000	1,0
dest.		10	929	8,0
		20	897	11,2
		30	849	15,9
		40	801	20,6
		50	769	23,9

Wie ersichtlich, liefert die kolorimetrische Bestimmung die relativ besten Ergebnisse, da sie dem theoretischen Wert am nächsten kommen. Als theoretischer Wert wurde die Menge an SO_2 bezeichnet, die in Form von Natriumpyrosulfit zugegeben wurde. Durch die Glucosezugabe wird ein Teil des Sulfits reversibel und irreversibel an die Glucose gebunden. Letztgenanntes entzieht sich der Bestimmung, wie die steigende Abweichung bei steigender Glucosemenge zeigt. Die übliche Titration mit Jod liefert sehr zweifelhafte Ergebnisse, denn mit zunehmender Glucosemenge nimmt auch der SO_2-Gehalt scheinbar zu. Es ist anzunehmen, daß durch die zugesetzte Natronlauge, die 10 min auf die Zuckerlösung einwirkt, trotz der niedrigen Temperaturen offensichtlich Zersetzungsprodukte gebildet werden, die durch Reduktion von Jod einen gewissen SO_2-Gehalt vortäuschen.

Bei der Destillation beobachtet man eine deutliche Abnahme des SO_2-Gehaltes mit steigender Glucosemenge. Die Abweichung ist jedoch etwas größer als bei der kolorimetrischen Bestimmung. Dies läßt sich wohl auf das 1½stündige Kochen zurückführen. Trotzdem sind kolorimetrische Methode und Destillation recht gut miteinander vergleichbar. Durch die Titration mit Jod erhält man sehr zweifelhafte Ergebnisse. Da diese stark von denen der anderen Methoden abweichen, war zu klären, inwieweit durch Einwirkung von Lauge Jod reduzierende Zerstörungsprodukte gebildet werden. Daher haben wir die Titration modifiziert, d. h. das freie und gebundene SO_2 getrennt nacheinander bestimmt. Als freies SO_2 wird das in saurem Medium bestimmbare Sulfit, als gebundenes SO_2 das durch nachträgliche Laugenbehandlung abspaltbare Sulfit aus der Glucosebisulfit-Additionsverbindung bezeichnet.

In Tabelle 4 sind die nach beiden Titrationsmethoden erhaltenen Ergebnisse gegenübergestellt. Danach unterscheiden sich beide Methoden sehr stark voneinander. Die modifizierte Titration verläuft mit steigenden Mengen von Glucose den vorgenannten Bestimmungsmethoden parallel, d. h. mit

steigender Glucosemenge beobachtet man eine Abnahme im wiedergefundenen SO_2-Gehalt. Dies kann nur bedeuten, daß durch das vorhandene SO_2 unter der Einwirkung von

Tabelle 4. Bestimmung des freien und gebundenen Sulfits (modifizierte Titration) in $Na_2S_2O_5$-Lösung nach Zugabe steigender Mengen einer 50%igen Glucoselösung im Vergleich mit der Normaltitration.

Glucosezugabe ml	SO_2-Gehalt mg/kg theor.	SO_2-Gehalt mg/kg (mit Glucose) Normaltitration	freies SO_2 mg/kg	geb. SO_2 mg/kg	insges. mg/kg	Abweichung der Werte theor.
10	1010	872	944	16	966	5.0
20		904	912	24	936	7.3
30		936	864	32	896	11.3
40		968	824	40	864	14.5
50		1000	784	48	832	17.6

Natronlauge Produkte gebildet werden, die Jod reduzieren und damit schweflige Säure vortäuschen. Auf ein ähnliches Verhalten der freien SO_2 wurde vorher schon einmal hingewiesen.

Mit Hilfe der modifizierten Titration läßt sich beweisen, daß die normale Titration zur SO_2-Bestimmung bei Anwesenheit von Zuckern als Methode zur Betriebskontrolle nicht geeignet ist. Ein direkter Vergleich aller drei Methoden ist nicht völlig korrekt, da nur mit der Titration freies und gebundenes SO_2 bestimmt werden. Nach Abbildung 9 kön-

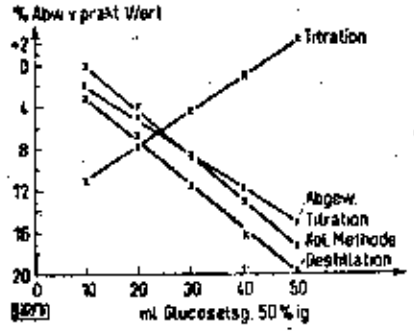

Abbildung 9. Gegenüberstellung verschiedener Methoden zur Bestimmung des SO_2-Gehaltes in glucosehaltigen Lösungen.

nen aber die drei Methoden mit annähernder Genauigkeit miteinander verglichen werden, da der Anteil an gebundenem SO_2 gegenüber dem freien sehr gering ist. Deutlich anders verhält sich die normale Titrationsmethode. Ähnliche Verhältnisse liegen auch bei der Untersuchung von Stärkesirupen vor, doch soll an dieser Stelle darauf nicht näher eingegangen werden.

Zusammenfassend kann gesagt werden, daß je nach den Bedingungen eine mehr oder weniger starke Bildung von Reaktionsprodukten zwischen SO_2 und Kohlenhydraten erfolgt. Aufgrund unserer Untersuchungen scheint die bisher allgemein durchgeführte Betriebskontrolle durch einfache Jodtitration weniger geeignet.

Man sollte vielmehr die modifizierte Titration als Schnellmethode einführen. Aufgrund der vorliegenden Ergebnisse müßte überlegt werden, ob die als Referenzmethode geltende Destillationsmethode nicht durch eine andere schnellere ersetzt oder ergänzt werden könnte, zumal sie bei niedrigen SO_2-Mengen eine gewisse Ungenauigkeit aufweist.

Aufgrund dieser Untersuchungen scheint zur Bestimmung der schwefligen Säure in Stärkehydrolyseprodukten die kolorimetrische Methode zweckmäßiger und bei sehr geringen SO_2-Mengen genauer zu sein als die Destillationsmethode.

Zusammenfassung

Es wird nachgewiesen, daß die Abnahme des Gehaltes an chemisch bestimmbarem SO_2 in lagernden Zuckerlösungen unter milden Bedingungen (Raumtemperatur und 60 °C) zum Teil dadurch bedingt ist, daß sich mehrere Reaktionsprodukte bilden, die eine Abspaltung von SO_2 nicht mehr zulassen. Fructose und Saccharose reagieren unter diesen Bedingungen nicht mit SO_2. Die Neigung zur Verbindungsbildung ist bei Maltose größer als bei Glucose.

Die aus Glucose und SO_2 gebildeten Verbindungen reagieren teils als Säuren, teils sind sie schwefelhaltig oder schwefelfrei. Die Menge der Reaktionsprodukte ist jedoch sehr gering.

Bei Vergleich der Methoden zur Bestimmung von SO_2 zeigt sich, daß mit steigender Zuckermenge der Anteil an wiedergefundenem SO_2 abnimmt. Es wird eine schnelle und in ihrer Genauigkeit ausreichende Methode zur Betriebskontrolle vorgeschlagen. Gleichzeitig wird gezeigt, daß eine kolorimetrische Bestimmung des SO_2 in Stärkeverzuckerungsprodukten bei niedrigen SO_2-Mengen genauere Werte liefert und darüber hinaus bedeutend schneller durchzuführen ist.

Résumé

Influence de la formation d'une combinaison entre l'acide sulfureux et les glucides sur le dosage du SO_2. *La diminution de la teneur en SO_2, dosable chimiquement, dans les solutions de sucres stoquées dans des conditions douces (température ambiante et 60 °C), est partiellement due à la formation de produits de réaction qui ne permettent plus une élimination du SO_2. Dans ces conditions le fructose et le saccharose ne réagissent plus avec le SO_2. La tendance à la formation d'une combinaison est plus importante chez le maltose que chez le glucose. Les composés formés à partir du glucose et du SO_2 réagissent soit comme acide, soit ils contiennent ou non du soufre. Cependant la quantité des produits de réaction reste faible.*

La comparaison des trois méthodes de dosage du SO_2 (dosage colorimétrique, distillation, titration) montre que la proportion du SO_2 retrouvée diminue dans tous les cas, lorsque la quantité de sucre augmente. On propose une méthode de contrôle rapide et suffisamment précise. Il est montré en même temps qu'un dosage colorimétrique du SO_2 présent en faible teneur dans les hydrolysats d'amidon, fournit des valeurs plus précises; de plus, cette méthode est plus rapide que celle utilisée jusqu'à présent dans le contrôle des usines.

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Beitrag zur Standardisierung von Methoden zur Aschebestimmung in Stärken und Hydrolysenprodukten*

Von G. Tagge, Detmold

Contribution to the Standardization of Methods for the Ash Determination in Starches and Hydrolysis Products. *Experiments on ash determination in potato, wheat and maize starch as well as in glucose syrup have shown that the ashing temperature of 525 °C as recommended in various analytical methods at least with the two first mentioned starch species does not produce carbon-free ash and thus cannot lead to accurate results. An ashing temperature of 800 °C is recommended as white pulverous ashes at a minimum variation range of results were thus obtained. With starch hydrolysis products the suitability of the sulphate ash method at 525 °C has been confirmed, provided that ashing time is extended from 2 to 4 hours.*

(Zusammenfassung siehe Seite 384; Résumé à la page 384)

1. Einleitung

In die Standardisierungsarbeiten des ISO/TC 93 (International Organization for Standardization, Technical Committee 93: „Starch Including Derivatives and By-products") soll in Zukunft auch die Erarbeitung von Methoden zur Aschebestimmung einbezogen werden. Im Rahmen des deutschen Fachnormenausschusses „Stärke" wurde inzwischen eine nationale Arbeitsgruppe unter der Federführung des Verfassers gegründet, die neben den internationalen Bemühungen die Entwicklung einer DIN-Norm anstreben wird.

Um diese Arbeiten zu beschleunigen, wurde von *Graefe* [1] die Übernahme von drei Aschebestimmungsmethoden der Corn Industries Research Foundation (CIRF) vorgeschlagen, und zwar für Stärken (CIRF B-8), Glucosesirup (CIRF E-6) und Dextrose (CIRF P-6). Alle drei Methoden schreiben eine Veraschungstemperatur von 525 °C vor, nachdem die Substanz vorher auf offener Flamme verkohlt wurde. Im allgemeinen soll die Veraschung nach zweistündigem Aufenthalt im Muffelofen beendet sein, andernfalls wird eine Unterbrechung vorgenommen, während der das Material mit destilliertem Wasser befeuchtet werden soll.

Unter Bezugnahme auf die erwähnte Veröffentlichung von *Graefe* wies *Müller-Mangold* [2] darauf hin, daß sich die Puder verschiedener Stärkearten gegenüber der nach CIRF B-8 vorgeschriebenen Behandlungsweise sehr unterschiedlich verhalten, so daß in jeder Vorschrift angegeben werden müßte, welche Veraschungstemperatur bei denjenigen Stärken anzuwenden ist, für die 525 °C offensichtlich nicht ausreichen. Nach *Müller-Mangold* geben Mais-, Milo-, Tapioka- und Reisstärkepuder nach zweistündigem Aufenthalt bei 525 °C im Muffelofen weiße oder graue Aschen, die nach einmaligem Anfeuchten, Trocknen und erneutem Erhitzen auf 525 °C keine Kohleteilchen mehr aufweisen. Demgegenüber sollen die Puder von Kartoffel- und Weizenstärke schwarz bleiben, auch wenn die Behandlung mehrmals und gegebenenfalls unter Verwendung von Oxydationsmitteln wiederholt wird.

Auf die Bedeutung der Veraschungstemperatur wurde auch von *Brückner* [3] aufmerksam gemacht, der bei internationalen Gemeinschaftsuntersuchungen zur Bestimmung des Aschegehaltes von Mehlen und Schrot allerdings keinen signifikanten Unterschied zwischen den Ergebnissen bei 600, 800 und 910 °C feststellen konnte. Immerhin beobachtete *Brückner*, daß der Streubereich der Ergebnisse bei Schrot wesentlich größer ist als bei Mehl, ein Hinweis darauf, daß unterschiedliche Materialien unter Umständen verschieden behandelt werden müssen.

Interessant ist die frühere Beobachtung von *Brückner* [4], daß bei Mehlen auch nach vierstündiger Veraschung nur sehr wenig weiße Aschen erhalten werden, daß 16 Std. zwar bei hellen Mehlen einigermaßen ausreichen, bei dunklen Mehlen jedoch wiederum nicht zu zufriedenstellenden Ergebnissen führen. Auch die oxydative Behandlung der bei

* Nr. 3809 der Veröffentlichungen der Bundesforschungsanstalt für Getreideverarbeitung in Berlin und Detmold.

Quaderni Nutriz. 10 (1949)
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Funzione intestinale ed epatica durante alimentazione con polpa di frutta variamente trattata.

Di M. Orlandelli.

(Dall'Istituto di Clinica Pediatrica dell'Università di Bologna diretto dal Prof. Gaetano Salvioli).

La polpa di frutta costituisce dal secondo anno di vita in poi alimento comune consacrato dall'esperienza di decenni.

Alimento comune e non elemento dietetico in quanto di quest'ultimo non ha nessun particolare requisito.

Le marmellate, com'è noto, non sono altro che polpa di frutta trasformata in uno stato fisico più molle, omogeneo, più facilmente digeribile e più ricco dal punto di vista energetico. Unico svantaggio, nei riguardi della polpa fresca, è rappresentato dal depauperamento in vitamine termolabili cui il frutto deve soggiacere qualunque sia il sistema di conservazione e, successivamente, di preparazione della marmellata.

Molte marmellate del commercio sono state ottenute mediante preservazione della frutta dalla fermentazione con anidride solforosa, il che ha valso ad esse l'appellativo erroneo di « solfitate »; appellativo erroneo inquantochè non vengono assolutamente aggiunti solfiti.

C'è chi ha voluto vedere, nonostante un'esperienza di lunghissimi anni, una possibile azione dannosa di tali marmellate, derivante dal processo di solfitazione; tale azione si manifesterebbe sovratutto nei bambini sia malati che, anche, sani.

Onde osservare quanto di vero potesse essere in tale affermazione ho studiato, per incarico del mio Maestro Professor Salvioli,

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Comunicazione al ventesimo Congresso Nazionale di Pediatria.
Lavoro pervenuto in Redazione il 13 Ottobre 1949.

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la tolleranza di tali marmellate nell'infanzia sia in condizioni fisiologiche che, sopratutto, patologiche.

Prima di esporre i miei rilievi ritengo conveniente riprendere alcuni concetti farmacologici con autorità e con chiarezza esposti dal PICCININI al recente V Congresso di Nipiologia.

Per la conservazione della polpa di frutta con anidride solforosa l'aggiunta di questa avviene nella proporzione variabile da 1 a 3 g pro Kg di frutta. Di tale quantitativo una percentuale minima rimane nella marmellata in quanto, essendo l'anidride solforosa un gas volatile anche a temperatura ordinaria, la massima parte di essa viene volatilizzata durante il processo di preparazione della marmellata stessa. Tale percentuale minima è rappresentata da pochi mg pro Kg (30 - 50) ed è notevolmente al di sotto dei valori di 200 mg per litro per i vini e dei 300 mg per litro per gli sciroppi d'uva e di 350 mg per litro dei succhi di frutta, valori ammessi dalla Legislazione sulla vigilanza sanitaria degli alimenti. Comunque i quantitativi di SO_2 presenti nelle marmellate solforate non sono percepibili all'olfatto.

Premesso quanto sopra, passo ad esporre i rilievi che ho raccolto nell'infanzia.

OSSERVAZIONI CLINICHE NELL' INFANZIA.

All'uopo di saggiare se esistono differenze nella tolleranza del bambino alla polpa di frutta variamente trattata, ho usato sia marmellate ottenute da polpa di frutta conservata con la solforazione che marmellate conservate col calore. Le marmellate ottenute da polpa solforata di cui ho disposto per gli esperimenti clinici contenevano quantitativi trascurabili di zolfo (sotto forma di solfiti) pari del resto a quelli notati in ripetute analisi di molti campioni di marmellate solforate di diversa provenienza industriale.

OSSERVAZIONI IN CONDIZIONI FISIOLOGICHE.

A otto bambini sani di età variante dai due ai sei anni è stata somministrata marmellata solforata in ragione di 300 g giornalieri in due volte per la durata di una settimana; lo stesso quantitativo di marmellata non solforata con le stesse modalità e per la stessa durata fu somministrato ad un eguale gruppo di bambini della medesima età.

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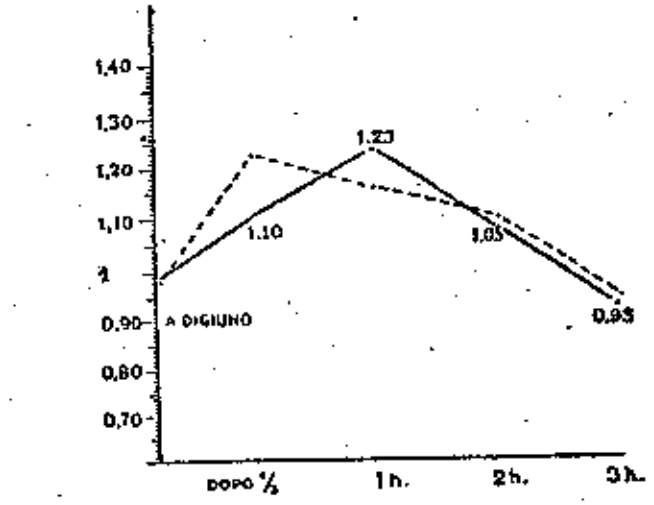
Nello stesso modo ho proceduto con altri due gruppi di bambini di età variabile dai 12 ai 24 mesi, riducendo soltanto il quantitativo giornaliero di marmellata a 200 g.

Non ho notato differenze nella tolleranza intestinale sia dell'un gruppo che dell'altro. Essa si può definire ottima non avendo portato modificazione veruna nel numero delle scariche nè nei caratteri macroscopici e microscopici delle feci (anche la flora non ha subito alcuna modificazione degna di nota).

Del pari nessuna differenza nè variazione ho potuto riscontrare dall'esame delle curve ponderali.

Ho eseguito pure su alcuni soggetti della seconda infanzia curve glicemiche da carico di g 2 pro Kg di marmellata solforata e curve glicemiche da carico di g 2 di marmellata non solforata. Contemporaneamente ho determinato in tali soggetti curve glicemiche da carico di g 1,50 pro Kg di glucosio.

COMASTRI A. di a. 12 — *Soggetto normale.*



Tali curve sono nettamente sovrapponibili e rivelano soltanto una punta massima alla prima ora anzichè alle mezz'ora dalla somministrazione come si nota nelle curve da carico di glucosio. Da ciò si può arguire la buona tolleranza della cellula epatica agli idrati di carbonio introdotti con la marmellata.

Unico rilievo degno di nota è dato dalla comparsa dopo molti giorni di somministrazione, di un senso di riluttanza per l'alimento attribuibile al sapore fortemente dolce, sia nell'uno che nell'altro gruppo dei soggetti che furono sottoposti all'introduzione di 300 g giornalieri.

OSSERVAZIONI IN CONDIZIONI PATOLOGICHE.

Allo scopo di saggiare la tolleranza alla marmellata ottenuta da polpa di frutta solforata di organismi infantili in condizioni patologiche, ho scelto i seguenti malati tutti della seconda e terza infanzia:

5 epatopatie con ittero delle quali 3 agli inizi del processo morboso;

3 soggetti affetti da paratifo B dei quali 2 in fase acuta febbrile;

2 nefritici con discreta iperazotemia:

1 nefrotico.

Ho somministrato a tutti i suddetti malati marmellata solforata eccezion fatta per un itterico, un paratifoso in convalescenza ed un nefritico ai quali ho dato per confronto marmellata non solforata.

La somministrazione è stata protratta per un periodo variabile dai 10 ai 20 giorni, mentre i quantitativi giornalieri hanno oscillato dai 150 ai 300 g.

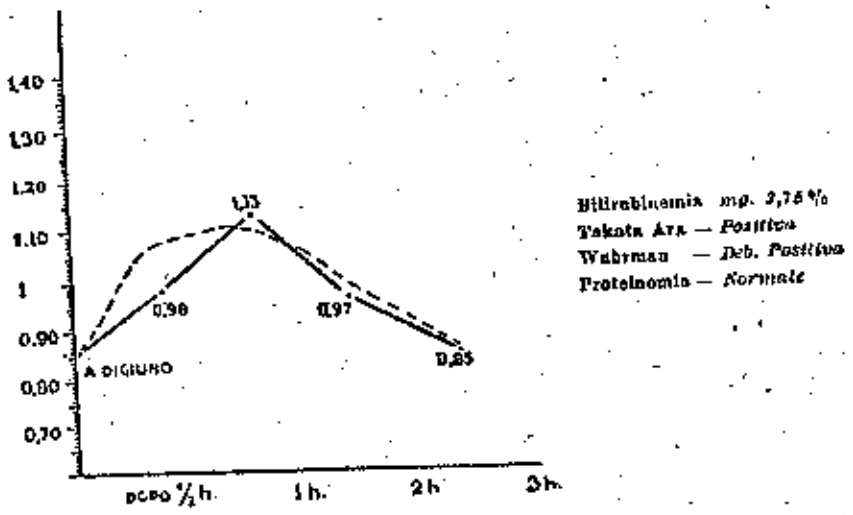
Le osservazioni indubbiamente più interessanti sono quelle notate nei soggetti affetti da epotopatia con ittero, nei quali molte prove di funzionalità epatica rivelavano uno stato di profonda e diffusa sofferenza parenchimale.

In questo gruppo di malati la tolleranza sia intestinale che epatica si è dimostrata buona. Nessun inconveniente si è avuto a lamentare a carico dell'apparato gastroenterico, mentre esami di feci ripetutamente praticati durante il periodo di somministrazione della marmellata non hanno dato rilievi degni di nota.

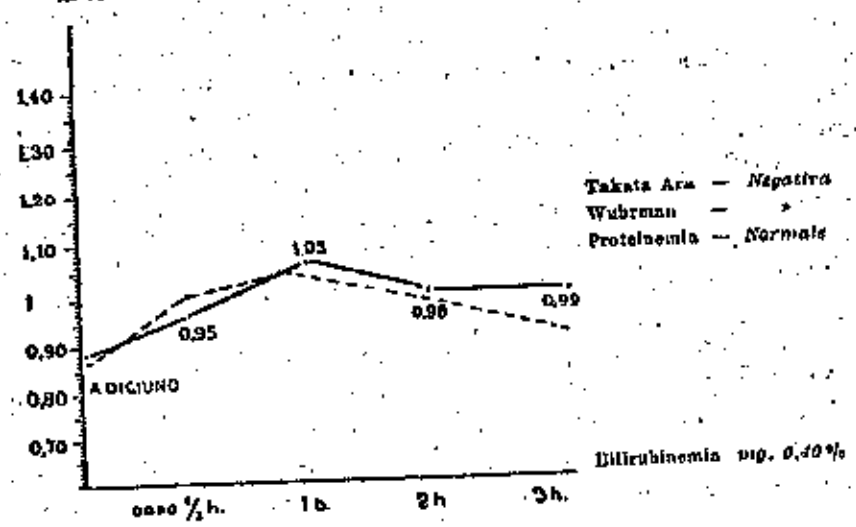
Parimenti la tolleranza della cellula epatica si è dimostrata notevole a giudicare sovratutto dalle curve glicemiche da carico di marmellata (g 2 pro Kg) del tutto simili a quelle praticate in soggetti normali e con la caratteristica più sopra notata di un certo ri-

tardo dell'acme della curva rispetto a quella da carico di glucosio (vedi grafiche).

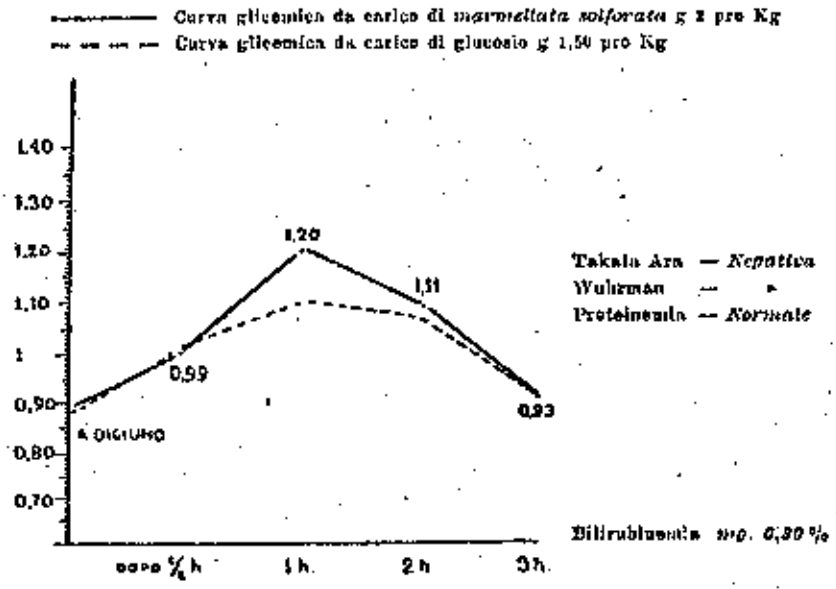
MAGLI R. di a. 7 — *Epatopatia con itterо.*



CIPRESSI F. di a. 8 — *Paratifo B in fase febbrile.*



SEVERI S. di a. 11 — *Paratifo B in fase febbrile.*



Constatazioni non dissimili si possono fare nei riguardi della marmellata non solforata, per quanto questa, è doveroso il notarlo, sia stata introdotta in bambini epatopatici in istato di netto miglioramento dell'affezione morbosa.

Risultati del tutto confortanti si sono pure avuti dalla somministrazione di marmellata solforata durante il decorso di processi paratifosi di media gravità; mette conto di segnalare che, in tali casi, la marmellata rappresentò una parte preponderante nell'alimentazione dei malati. Anche in questi soggetti le curve glicemiche hanno presentato le stesse caratteristiche più sopra segnalate (vedi grafiche).

Analoghe le osservazioni anche nei nefritici in fase iperazotemica e nel soggetto nefrotico. Degno di rilievo è il fatto che nessuna differenza si è notata nei due nefritici all'uno dei quali fu somministrata marmellata solforata e all'altro marmellata non solforata.

Concludendo mi pare si possa affermare che nessuna apprezzabile differenza mi è stato dato notare dal punto di vista della tolleranza infantile tra marmellata solforata e non solforata mentre per quanto riguarda la prima, è opportuno sottolineare che, pure introdotta in bambini malati, e in fase acuta di malattia,

non ha manifestato alcuna azione dannosa sul decorso dell'affezione morbosa anche quando questa interessava precipuamente la cellula epatica o altri importantissimi parenchimi.

RIASSUNTO.

Non si sono notate differenze degne di nota nella tolleranza intestinale, nelle curve ponderali e nelle curve glicemiche da carico, aggiungendo alla dieta di due gruppi di bambini normali, marmellata solforata in un caso e non solforata nell'altro. In bambini affetti da paratifo, da nefrite, da nefrosi, da epatopatie, l'aggiunta alla dieta di marmellata solforata si è dimostrata ben tollerata.

SUMMARY.

No noticeable difference has been observed at all upon the intestinal tolerance, the weight chart and the blood sugar charts by adding sulphated jam or not sulphated jam to the normal child diet.

In children affected by paratyphoid, nephritis, hepatitis, the addition of sulfurated jam to the diet has been well tolerated.

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LONG-TERM EXPOSURE TO LOW CONCENTRATION OF SULFUR DIOXIDE REPORT I EXPERIMENTS WITH MICE

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(Received for publication, 31 July, 1968)

The authors made a series of epidemiological investigations on the effects of air pollution in Yokkaichi on health[1-3], and noticed a highly significant correlation between prevalences of obstructive respiratory diseases and air pollution level, especially the level of sulfur oxides.

However, only with the epidemiological findings, it is still questionable whether sulfur oxides are direct cause of those diseases or not. Many studies performed hitherto have been made in higher concentrations of sulfur dioxide than those occurring in actual air pollution[4].

The present investigation was carried out to study the biological effects of long-time exposure to low concentrations of sulfur dioxide on albino mice by a new method of Teflon permeation tubes which was recently devised for generating sulfur dioxide at constantly low concentrations.

Materials and Methods

The animals used were dd-strain albino mice.

Sulfur dioxide was generated by permeation from Teflon tube containing liquid sulfur dioxide at a certain temperature, diluted with air filterd through activated charcoal pipe at a certain flow rate, and introduced into the exposure chamber.

Three exposure groups, those in about 0.05 ppm, 0.15 ppm and 0.23 ppm sulfur dioxide were designed. Confirmation of the concentrations were made by p-rosaniline method.

Experimental animals of the 0.05 ppm and 0.15 ppm exposure groups were killed after 3

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months of exposure. Those of the 0.23 ppm exposure groups were killed after 6 weeks. Histological examinations of the lung, heart, spleen, kidney and liver were made.

Results

Increase of average body weight of the 0.05 ppm (average 0.05, range 0.02–0.06) exposure group was almost identical with that of the control group (Fig. 1).

Increase of average body weight of the 0.15 ppm (average 0.15, range 0.12–0.18) exposure group was generally inferior to that of the control group, and 4 out of 10 exposed mice died within the experiment-term. The first one was killed by

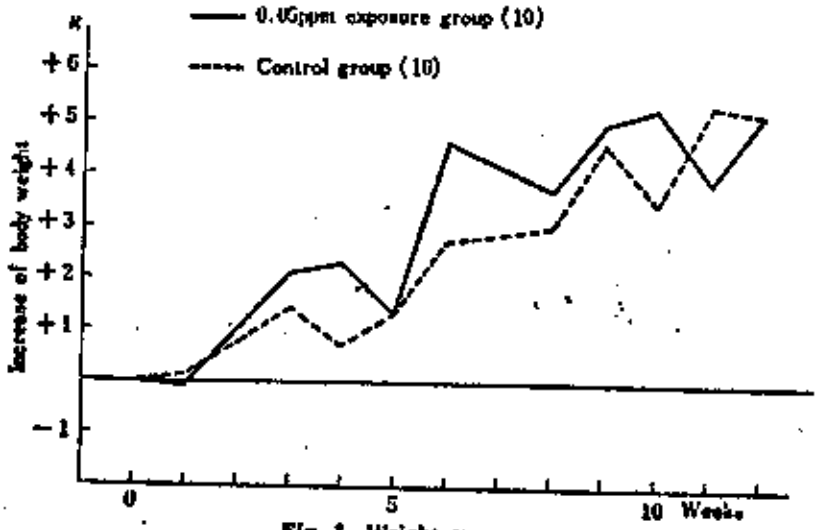

Fig. 1 Weight curves.

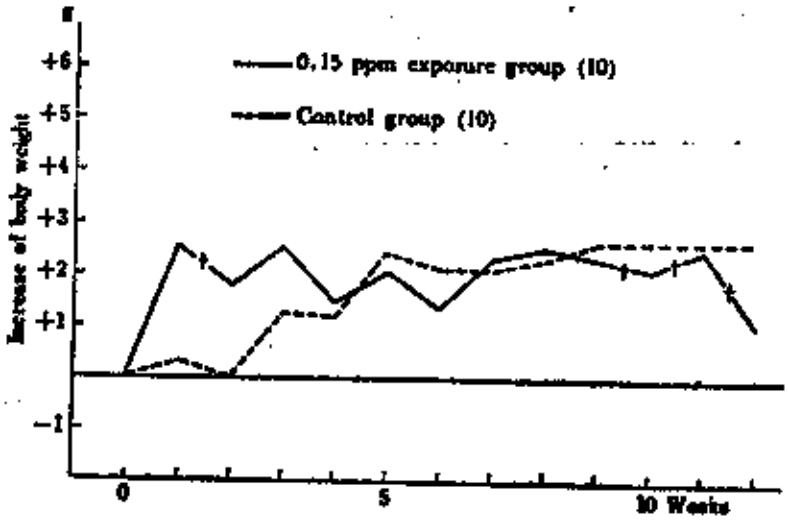

Fig. 2 Weight curves.

accident, and the others were dead from the exposure (Fig. 2).

Between increase of average body weight of the 0.23 ppm (average 0.23, range 0.20–0.25) exposure group and that of the control group, significant differences were recognized, and 4 in 15 mice of this group died within the experiment-term (Fig. 3).

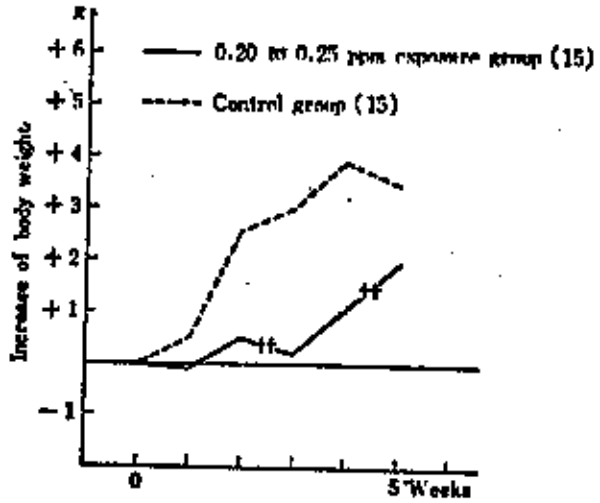

Fig. 3. Weight curves

On macroscopical examination, a significant change was observed in the lungs, the surface of which was dark red (Fig. 4–1).

This finding was observed in one third of mice of the 0.23 ppm exposure group, and in a half of mice of the 0.15 ppm exposure group, but not in mice of the 0.05 ppm exposure group.

On microscopical examination of the lungs which showed dark red appearance, congestion and hemorrhage were observed, and infiltration of inflammatory cells was also noticed. In addition, thickening of smooth muscle layers of the blood vessels and bronchi were observed in the mice of the 0.15 ppm exposure group (Figs. 4–3, 4).

Formation of multiple abscesses was a remarkable finding in the mice died from the exposure in these groups (Figs. 4–5, 6), and in part of living mice similar formation of lung abscesses was also observed in less degree.

Histological examination of other organs revealed a slight congestion and hypertrophy of lymph follicles in the spleeen and infiltration of inflammatory cells in the heart and liver.

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Discussion

This report is the first setp of experimental observation with animals. It is clear that we cannot discuss the whole mechanism of effects of air pollution from these results, but it is noticeable that the respiratory organ of mice, at least, is impared by long-term exposure to sulfur dioxide at low concentrations similar to those in actual air pollution.

It is stated by Catcott that their susceptibility to various air pollutants varies[7] with animal species. So similar experiemnts with albino rats, guinea pigs and rabbits are now in progress in our laboratory.

Summary

Exposure of albino mice to 0.15 ppm sulfur dioxide for 3 months and 0.23 ppm for 6 weeks produced remarkable congestion and hemorrhage of the lungs. Thickening of smooth muscle cells of the blood vessels and bronchi were observed in the former group.

Formation of multiple abscesses was also a remarkable finding in the mice dead from the exposure, and in part of living mice similar slight formation of lung abscesses was also observed.

This study was sponsored by the Ministry of Health and the Authorities of Mie Prefecture and Yokkaichi City. We would like to express our sincere thanks to Prof. K. Izutsu in the Department of Pathology of the present University for his kind help in the present work.

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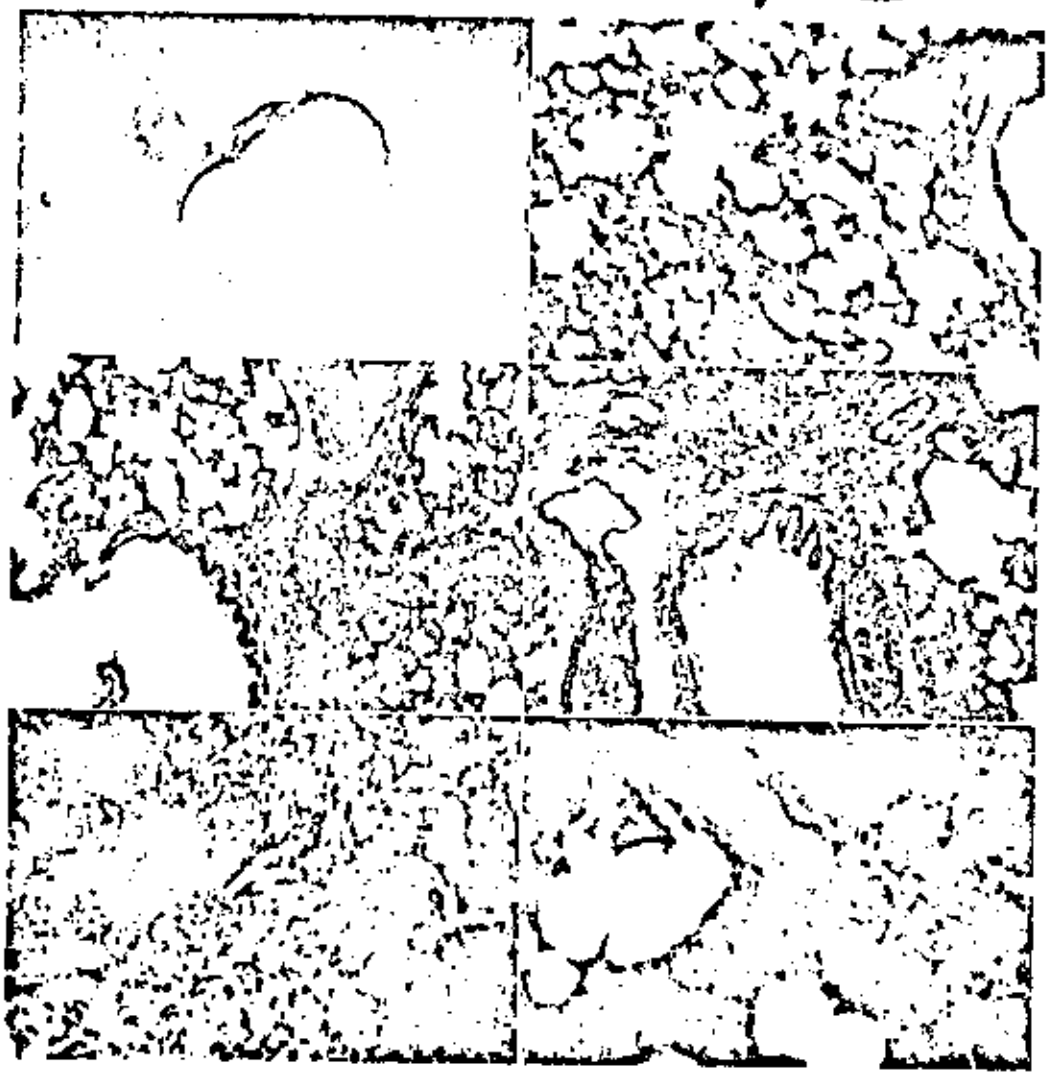

1. Macroscopical feature of the lung of the 0.15 ppm exposure mice.
2. Histological feature of the lung of the control mice.
3, 4. Histological feature of the lung of the 0.15 ppm exposure mice.
5, 6. Histological feature of the lung of the mice died from the 0.15 ppm exposure.

J. Ass. Offic. Anal. Chem. 50 (1967)

...ative results for aldehydes in ... sulfite method; ... C ...)/100 ml

Wine Sample Number		
2	3	4
6.04	12.78	37.45[a]
6.38	12.70	34.76
6.05	12.32	35.20[b]
5.83	12.79	36.13
6.21	12.60	35.55
5.28	11.11	29.37
6.43	12.78	37.60[c]
6.20	13.75	34.10[d]
4.95	12.30	33.78
6.05	12.56	32.90
6.15	12.73	35.27[f]
0.19	0.41	1.58

...sample instead of 50 ml.
...om breakage in transit.
...ted by Coll. 6 are uniformly low ... mean), and a very low value for ...en by Coll. 9; these values were ...an and standard deviation calcu-

... same as Sample 3 with added ...mg CH_3CHO/100 ml.

However, only four of the ... used a smaller sample; two ...orators (Colls. 1 and 7) ob... ...plete recovery.

...as ...ribed reflects the free ...nt ... any aldehyde tem... with bisulfite ion already ... wine; the method does not ...ed aldehydes. In other words, ...ts "free" aldehydes as op... ...l" when the latter term is ...e acetals. Distillation is car... presence of the alkaline borax ...ent extraneous aldehyde for... ...he action of acids on sugars ...ng heating (3).

...aldehyde and ethyl alcohol ...mbine to form acetal at the ... (7), the percentage in the ...s only about 1% for table ...ng about 12 vol. % of alco... ...3% for dessert and aperitif ...ng about 20 vol. % alcohol) ...se percentages of aldehydes ...d be still less for wines con... ...iable aldehydes in the bisul... ...e. Therefore, it was not con... ...al to determine acetals apart

from free aldehydes for wines. However, the determination could be made by the method of Guymon and Crowell for free aldehydes and for acetal in extract-containing brandies (3).

The addition of EDTA (disodium salt) greatly improves the precision of this procedure over earlier direct bisulfite methods.

It is recommended that the method for aldehydes in wine be adopted as official, first action.

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E. A. Crowell, Department of Viticulture and Enology, University of California, Davis, Calif.

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Simultaneous Determination of Volatile Acids and Sulfur Dioxide in Alcoholic Beverages by Microdiffusion

By JOSEPH L. OWADES and JOSEPH M. DONO (Rheingold Breweries, Inc., 36 Forrest St., Brooklyn, N.Y. 11206)

A new technique has been developed for determining both volatile acid and sulfur dioxide in beer, wines, and distilled liquors. The new method avoids the troublesome distillation common to other methods by use of microdiffusion in an especially modified diffusion cell. Small samples are required and many replicates can be performed in one day. Results were in excellent agreement with the AOAC method for wine, and precision was very good.

Sulfur dioxide and other volatile acids have long been recognized as important components of alcoholic beverages, either as concomitants of fermentation or as inhibitors of undesirable reactions. The plethora of methods for their determination is a testament of their significance.

We have applied the principle of microdiffusion to the analysis of alcoholic beverages for both volatile acids and sulfur diox-

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ide. Although the latter compound was not the original object of the investigation, the need to eliminate it as an interference pointed the way to a method for its determination. The technique of microdiffusion has been applied to the analysis of volatile acids in beer and wines (1, 2).

All current methods for volatile acids utilize distillation (3), as do the accepted methods for sulfur dioxide (4). We have found that distillation of beer continues to yield volatile acids and that any end point is somewhat arbitrary and capricious.

Microdiffusion does not suffer from this defect. The diffusion is conducted in a Conway cell (5), especially modified for this analysis (Fig. 1) at 55–60°C. The time for diffusion varies with the beverage analyzed, from 6 hours for wines to 16 hours for beer. The diffusion is assisted by the effective dehydration of the sample in the cell during the analysis. The volatile acids are trapped in a buffer solution in the center section of the cell and titrated directly in the cell (Fig. 2). The sample size required is less than 1 ml, and as many as 50 determinations can be performed in a day.

Interference from sulfur dioxide may either be accepted to give total volatile acids or eliminated to give volatile acids exclusive of sulfur dioxide. The sulfur dioxide is removed by complexing it with a mercury salt in the microdiffusion cell. Concurrent analyses, with and without removal of sulfur dioxide, serve as the means for determining this ubiquitous compound.

METHODS

Apparatus

(a) *Micrometer microburet.*

(b) *Microsyringe.*—1 ml/inch (or 1 µl/division).

(c) *Diffusion cell.*—Modified plastic Conway diffusion cell constructed of white polypropylene with clear polystyrene cover. Dimensions: i.d., 59 mm; diam. of central chamber, 35 mm. Outermost annular chamber is fitted with 1/16" soft gum rubber gasket. Clear polystyrene cover is externally coated with reflective aluminum lacquer. A pinch clamp was used to hermetically seal cover. See Fig. 1.

(*Note:* All above equipment is available from Labtician Products Co., 190-04 90th Ave., Hollis, N.Y.)

Reagents

(a) *Sodium citrate stock solution, 1N.*—Dissolve 9.8 g sodium citrate.$2H_2O$ in ca 50 ml distilled water and make to 100 ml.

(b) *Sodium citrate, 0.05N.*—Just before use, add 5 ml stock solution, (a), to 100 ml volumetric flask and make to volume with distilled water; resulting 0.05N solution is enough for ca 50 determinations. Prepare solution fresh daily.

(c) *Barium hydroxide solution, 0.01N.*—Place a few hundred ml freshly boiled distilled water in 1 L volumetric flask, add 1.58 g barium hydroxide.$8H_2O$, dissolve, and fill to mark with freshly boiled distilled water. Store in tightly-stoppered bottle.

Standardize solution as follows: Rapidly filter ca 100 ml thru Whatman No. 1 filter paper. Titrate clear filtrate against 10 ml portion

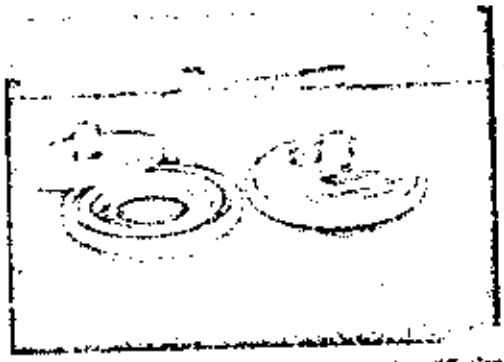


Fig. 1—Modified Conway cell for microdiffusion analysis.

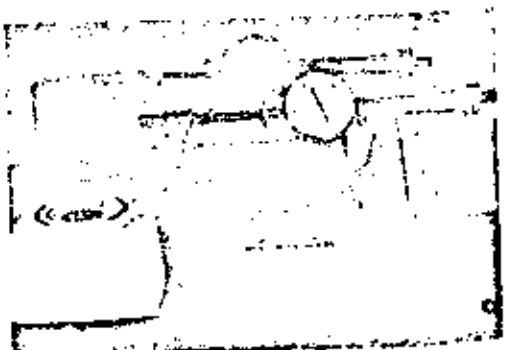


Fig. 2—Titration assembly for microdiffusion analysis.

This paper was presented at the Eightieth Annual Meeting of the Association of Official Analytical Chemists, Oct. 10–13, 1966, at Washington, D.C.

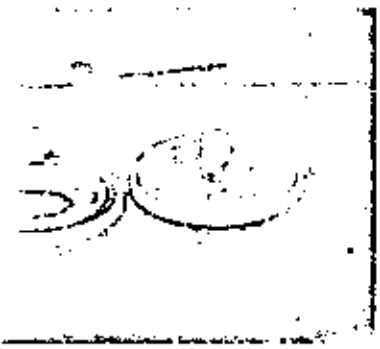
...mi... lacquer. A pinch clamp ...ally seal cover. See Fig. 1. ...e equipment is available ... Products Co., 190-04 99th Ave.,

citrate stock solution, 1N.— ...g sodium citrate.$2H_2O$ in ca 50 ...ater and make to 100 ml.

citrate, 0.05N.—Just before use, ...k solution, (a), to 100 ml volu... ...nd make to volume with distilled ...ng 0.05N solution is enough for ...inations. Prepare solution fresh

hydroxide solution, 0.01N.— ... hundred ml freshly boiled dis... ... 1 L volumetric flask, add 1.58 g ...oxide.$8H_2O$, dissolve, and fill to ...eshly boiled distilled water. Store ...pered bottle.

... solution as follows: Rapidly fil... ... thru Whatman No. 1 filter paper. ... filtrate against 10 ml portion



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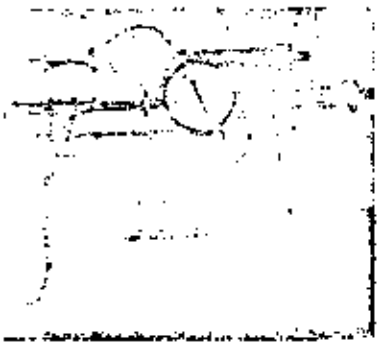

...on assembly for microdiffusion analysis.

potassium acid phthalate solution, (h), using 2-3 drops phenolphthalein, (f). Standardize daily.

$N = (g\ KHC_8H_4O_4 \times 10)/(ml\ Ba(OH)_2 \times 204.228)$.

(d) *Phosphoric acid solution, 10%.*—Place ca 50 ml distilled water in 100 ml volumetric flask, add 11.8 ml 85% phosphoric acid (analyzed reagent, ACS) and make to volume.

(e) *Mercuric oxide solution.*—Dissolve 1 g red mercuric oxide, HgO, (analyzed reagent, ACS) in 100 ml 10% sulfuric acid.

(f) *Indicator solution.*—Dissolve 1 g phenolphthalein in 100 ml neutral 95% alcohol.

(g) *Nonionic neutral wetting agent.*—Place 0.25 ml NPX Tergitol (Union Carbide Corp., New York, N.Y.), or equivalent, in 1 L volumetric flask and make to volume with distilled water.

(h) *Potassium acid phthalate solution.*—Place few hundred ml of distilled water in 1 L volumetric flask and add 2.000 g potassium acid phthalate (NBS) previously dried at 120°C in circulating air oven for 2 hr. Dissolve and fill to mark with distilled water.

Total Volatile Acidity

Pipet 10 ml beverage sample into 50 ml Erlenmeyer flask and add 1 ml 10% phosphoric acid solution, (d). If sample contains CO_2, decarbonate by filtering thru Whatman No. 3 filter paper at room temperature before diluting.

With aid of small spatula, evenly distribute 6-9 g anhydrous sodium sulfate into outer, annular chamber of modified Conway cell. Carefully pipet known amount of acidified sample onto sodium sulfate. (For samples containing < 200 ppm of volatile acids, 1 ml sample is satisfactory; for samples containing > 200 ppm, 0.5 ml sample is required.) Pipet 2 ml 0.05N sodium citrate solution, (b), into central chamber along with 2 drops 0.025% NPX Tergitol wetting agent, (g). Prepare blank with distilled water in identical manner.

Place sealed units in 55-60°C air oven for 11-19 hr. To prevent uneven heating of cells, adequately insulate metal shelves in oven with several sheets of asbestos.

After 16-19 hr, remove samples and let cool to room temperature for 15-20 min. without removing clamps. (If samples cannot be titrated at this time, freeze until used.) (Cells which show droplets of condensed liquid on upper lid will give low results. Cells with insufficient quantity of Na_2SO_4 will have liquid in outer annular chamber; these will also give low values.) Titrate samples with 0.01N barium hydroxide solution, (c), using 1 drop phenolphthalein, (f). Titrate directly in central chamber of the cell, using syringe microburet and fine glass stirring rod to mix sample. (Atmospheres laden with CO_2 will interfere with the titration and should be avoided.)

(*Note:* With wines, if results are desired within one working day, shorten diffusion time to 6 hr; 80-85% recovery is acceptable or can be corrected for.)

g Acetic acid/1 ml sample = $[(X - B) \times N \times 60.0]/[S \times (10/11) \times 10]$,

where X = ml $Ba(OH)_2$ used for sample; B = ml $Ba(OH)_2$ used for blank; N = exact normality of $Ba(OH)_2$; and S = ml sample used.

Volatile Acidity (Exclusive of SO_2)

Pipet 10 ml sample into 50 ml Erlenmeyer flask and add 1 ml mercuric oxide solution, (e).

Proceed as for determination of total volatile acidity.

g Acetic acid/100 ml sample = $[(Y - B) \times N \times 60.0]/[S \times (10/11) \times 10]$,

where Y = ml $Ba(OH)_2$ used for sample; B = ml $Ba(OH)_2$ used for blank; N = exact normality of $Ba(OH)_2$; and S = ml sample used.

ppm SO_2 (by difference) = $[(X - Y) \times N \times 64.0 \times 1000]/[S \times (10/11)]$,

where X = ml $Ba(OH)_2$ used for total volatile acidity; Y = ml used for total volatile fatty acids; N = exact normality of $Ba(OH)_2$; and S = ml sample used.

Results and Discussion

Recoveries of acetic acid added to beers and wines by microdiffusion are shown in Table 1. Recovery of sulfur dioxide added to beer is shown in Table 2.

These results were compared with the AOAC method on some representative wines (Table 3). Results for sulfur dioxide determination on a domestic red wine and a domestic white wine were 75.1 and 122.5 ppm SO_2, respectively, by the Monier-Williams method and 74.5 and 132.0 ppm SO_2, respectively, by the proposed microdiffusion method.

Microdiffusion results on some commercial beers and wines are shown in Table 4; the precision of replicates is also included. Volatile acidity recoveries from an imported

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Table 1. Recovery of acetic acid added to beer (ppm) and wines (g/100 ml)

Sample	Volatile Acidity	HOAc Added	HOAc Found	HOAc Recovered	% Recovery
Beer					
A	62.4	29.4	92.1	29.7	101
B	53.0	58.8	109.0	56.0	96
C	56.1	60.4	117.4	61.3	101
D	56.7	60.4	115.4	58.7	97
E	64.7	60.4	123.8	59.1	98
F	59.1	100.3	153.1	94.0	94
G	60.2	100.3	166.2	106.0	106
Wine					
California Burgundy	0.051	0.024	0.086	0.025	104.0
California Burgundy	0.056	0.024	0.079	0.023	96.0
California Burgundy	0.028	0.024	0.053	0.025	104.0
California Gamay	0.043	0.024	0.065	0.022	92.0
N.Y. State Sauterne	0.023	0.029	0.052	0.029	100.0

Table 2. Recovery of sulfur dioxide added to beer

Beer	Volatile Acidity Exclusive of SO_2: HOAc, ppm	SO_2 Added, ppm	Total Volatile Acidity:* HOAc, ppm	SO_2 Recovered, ppm	% Recovery
A	76.5	46.2	116.6	42.9	92.9
B	72.8	47.0	118.6	50.5	107.4
C	76.5	61.6	132.8	60.2	97.7
D	76.5	77.0	151.0	79.5	103.2
E	76.5	92.0	167.1	96.8	105.2
F	72.8	93.0	156.2	89.6	96.3
G	78.4	107.8	178.8	109.2	101.3
H	78.4	186.4	230.0	167.3	90.0

* The volatile acidity shown was corrected for the actual SO_2 content in the beers (5.6 ppm).

Table 3. Comparison of methods for total volatile acidity: g HOAc/100 ml

Sample	Proposed Method	AOAC Method
California Sherry	0.039	0.039
Apple Wine A	0.201	0.207
Apple Wine B	0.081	0.084
N.Y. State Concord	0.042	0.046
Blackberry	0.035	0.041
N.Y. State White Grape Wine	0.027	0.030

Table 4. Recovery of volatile acids in beer (ppm) and wines (g/100 ml)*

Sample	HOAc Recovery
Beer	
A	27.2; 25.2
B	113.8; 118.5
C	98.5; 98.5
D	105.1; 106.0
E	55.2; 54.3
F	68.0; 68.6
G	100.2; 103.0
Wine	
California Port A	0.017; 0.017; 0.016
California Port B	0.020; 0.020; 0.021
California Burgundy A	0.056; 0.056; 0.057
California Burgundy B	0.025; 0.025; 0.026
California Beaujolais	0.037; 0.037; 0.037
Imported Burgundy	0.056; 0.054; 0.057
N.Y. State Burgundy	0.028; 0.026; 0.026

* Exclusive of SO_2.

Scotch and a domestic Bourbon were 90.0 and 595.0 ppm HOAc, respectively.

Results for total volatile acids and for sulfur dioxide on some wines are shown in Table 5.

A method has been developed for determining volatile acids in which sulfur dioxide is either eliminated as an interference or is

and wines (g/100 ml)

Recovered	% Recovery
29.7	101
56.0	96
61.3	101
58.7	97
59.1	98
94.0	94
106.0	106

0.025	104.0
0.023	96.0
0.025	104.0
0.022	92.0
0.029	100.0

ed to beer

SO_2 Recovered, ppm	% Recovery
42.9	92.9
50.5	107.4
60.2	97.7
79.5	103.2
96.8	105.2
89.6	96.3
109.2	101.3
167.3	90.0

nt in beers (5.6 ppm).

covery of volatile acids in beer (ppm) and wines (g/100 ml)[a]

	HOAc Recovery
Beer	
	27.2; 25.2
	113.8; 118.5
	98.5; 98.5
	105.1; 106.0
	55.2; 54.3
	68.0; 68.5
	100.2; 103.0
Wine	
A	0.017; 0.017; 0.016
B	0.020; 0.020; 0.021
undy A	0.056; 0.056; 0.057
undy B	0.025; 0.025; 0.025
alais	0.037; 0.037; 0.037
undy	0.056; 0.054; 0.057
undy	0.028; 0.026; 0.028

SO_2.

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Table 5. Determination of total volatile acidity and sulfur dioxide in wine

Wine	Total Volatile Acidity, g HOAc/100 ml	Sulfur Dioxide, ppm (by difference)
California Burgundy A	0.061	27.4
California Burgundy B	0.028	15.1
California Beaujolais	0.043	37.0
Imported Burgundy C	0.065	45.2
California Sauterne A	0.043	87.7
California Sauterne B	0.036	89.1
N.Y. State Sauterne	0.057	79.5
California Sauterne C	0.027	38.4

actually determined concurrently. To avoid distillation, the method uses microdiffusion. It has been applied to beer, wine, and distilled liquors and could probably be applied to other foods as well. The sample size required is small and the time for the analysis is short enough to permit many determinations in one day.

Results agree well with the AOAC method for wines and can serve for beer determinations in which present methods are not accurate enough for general practical use.

Acknowledgment

The cooperation of Monroe Coven of the Monarch Wine Company, Brooklyn, New York is gratefully acknowledged.

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Use of Gas Chromatography in Distillery Research and Control

By G. I. deBECZE, H. F. SMITH, and T. E. VAUGHN (Research Laboratories, Schenley Distillers, Inc., Lawrenceburg, Ind. 47025)

Twelve volatile components commonly present in many whiskies can be determined directly by gas chromatography on four columns under specified conditions. Separation and measurement of higher alcohols, separation of acetone from ethyl formate, measurements of four esters present, and proof of the absence of several components previously suspected to be present in distilled alcoholic beverages are described. Numerical values corresponding to the peaks are given for the components. Gas chromatograms combined with conventional chemical analysis give the best identification of bottled goods.

Gas-liquid chromatography (GLC) has only recently been applied to the investigation of distilled alcoholic beverages, and the related literature is not extensive. Ethyl alcohol was quantitatively determined in wines with acetone as an internal standard (1, 2). Several investigators used gas chromatography to analyze spirits (3–8), and others used it to determine various components in whisky and various fermented materials (9–20).

Gas chromatography was first used in our laboratory to investigate distilled alcoholic beverages in 1959. This paper reports progress on the use of gas chromatography for analysis, comparison, and identification of distilled alcoholic beverages, and for the investigation of intermediates.

Proceedings were defined for the measurements of twelve volatile components commonly present in whiskies. Several homologs, previously measured as one component by conventional analysis, were identified and quantitatively determined; e.g., in addition to ethyl acetate, ethyl esters of acids other than acetic acid were individually measured.

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The Effect of Sulfur Dioxide and Ozone on Acetylcholinesterase

Alice Y. S. Pan, M.D., M.S.C.H., and Zygmunt Jegier, D.Sc., Montreal

Using a newly devised method of calculating very small kinetic differences, it was established that both sulfur dioxide and ozone inhibit the enzymic activity of acetylcholinesterase (AChE) in vitro. Starting from approximately 4 ppm for SO_2 and 0.3 ppm for O_3, the relative activity of the exposed AChE can be derived from the formula: $y = 100 - bx^c$, where y is the relative enzymic activity and x is the concentration of the pollutant.

VARIOUS studies have shown that ozone and nitrogen dioxide produce enzyme alterations,[1,2] but to our knowledge no work has been reported in this field using sulfur dioxide as the toxic agent. The study presented here was primarily designed to investigate the effect of SO_2 upon the enzyme acetylcholinesterase (AChE) in vitro.

In the case of O_3, it is already known that a concentration of 8 ppm can decrease AChE levels in the erythrocytes of mice[3]; for this reason, it was thought to be of interest to compare the relative effects of SO_2 and O_3 upon this same enzyme.

Materials and Methods

Reagents—The enzyme was AChE obtained from bovine erythrocytes type I; 50-mol units were dissolved in 500 ml of 0.154M sodium chloride and divided into eight aliquots, thus providing material for four experiments (eight exposure solutions: four test samples and four controls).

The substrate was 0.01M acetylcholine iodide (AChI) in 0.154M of NaCl. It was determined that, under our experimental conditions, the optimal substrate concentration was 1.4×10^{-3}M. Fresh substrate solution was prepared for each experiment and kept ice-cold while in use.

The titration solution was 0.1M of sodium hydroxide. Twice-distilled water free of carbon dioxide was used and the molarity was checked with potassium hydrogen phthalate in the usual manner.

Methods—The enzymatic activity of AChE was determined by measuring the rates of hydrolysis of the substrate by the pH stat method.

The titration apparatus consisted of an automatic titrator, a recorder, an autoburette equipped with a 0.250-ml syringe, and a microtitration assembly including glass and mercurous chloride electrodes, a magnetic stirrer, titration vessels, and a thermostatic jacket that maintained the temperature of the enzyme solution at 25 C.

The enzyme solutions (exposed sample and control) were first adjusted to pH 7.5 manually, and the substrate was added when the relay clicked at the first indication of a slight decrease of pH below 7.4; the kinetic run lasted about three minutes.

Gassing experiments were conducted by introducing SO_2 or O_3 at a constant rate into a uniform flow of air, part of which was diverted into gas washing bottles containing the enzyme solution.

Sulfur dioxide (anhydrous) was infused by means of a variable speed syringe pump; throughout the gassing period, the concentrations were assayed with an SO_2 analyzer using the continuous electroconductivity technique.

Ozone was produced by passing a controlled flow of O_2 through a commercial ozone generator. The ozone meter (a microcoulomb ozone sensor) assaying the O_3 concentration was also

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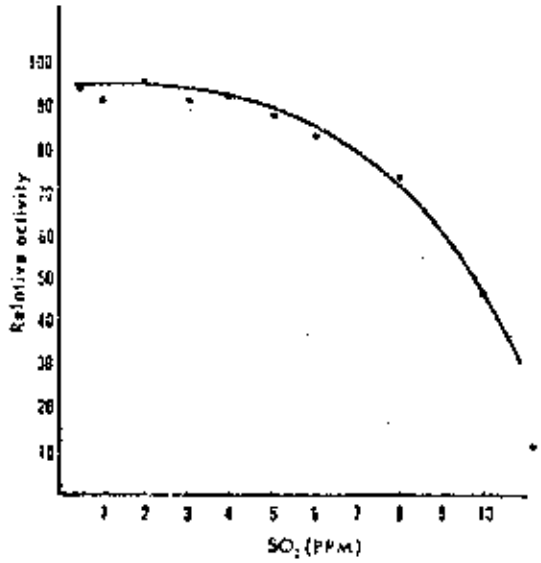

Fig 3.—Dose-response curve for SO_2. Each point is mean of 24 differences (four experiments), all of which were statistically significant ($P < 0.01$).

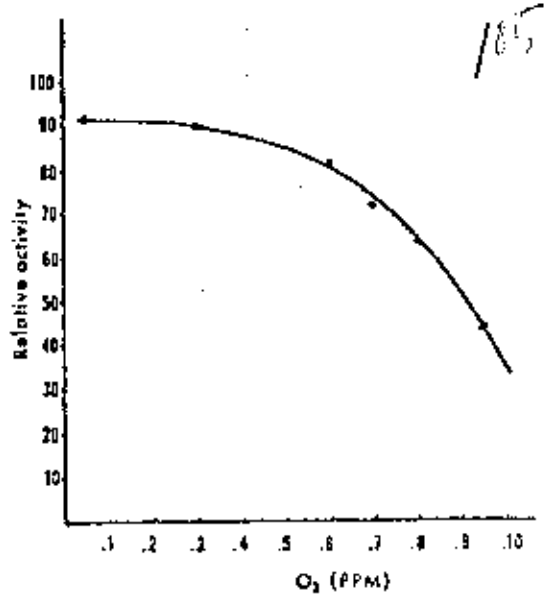

Fig 4.—Dose-response curve for O_3. Each point is mean of 18 differences (three experiments), all of which were statistically significant ($P < 0.01$).

Table 1.—*pH Variations After Exposure to SO_2 and O_3**

Pollutants	Concentrations (ppm)	pH	SD	No. of Samples
SO_2	0.5	6.50	0.13	24
	1.0	6.70	1.30	24
	2.0	6.20	0.80	24
	3.0	6.00	0.23	24
	4.0	5.90	0.09	24
	5.0	5.70	0.51	24
	6.0	5.49	0.11	18
	8.0	5.11	0.06	18
	10.0	4.77	0.09	18
O_3	0.05	6.49	0.07	18
	0.30	6.50	0.07	18
	0.60	6.50	0.06	18
	0.70	6.40	0.12	18
	0.80	6.30	0.22	18
	0.95	6.20	0.26	18

* The unexposed enzyme solution had a pH of 6.8.

Table 2.—*Statistical Data*

Pollutants	Concentrations (ppm)	Degrees of Freedom	P
SO_2	0.5-5.0	23	0.01
	6.0-10.0	17	0.005
O_3	0.05-0.30	17	0.01
	0.60-0.95	17	0.005

$$\frac{1}{V} = \frac{Km}{V} \cdot \frac{1}{[S]} + \frac{1}{V}, \text{ where}$$

Km is the reciprocal of the affinity of AChE for AChI; *v*, the velocity of the hydrolysis of AChI; *V*, the maximum rate at which the reaction can proceed; and [S], the final concentration of AChI. Km was found to be 3.8 × 10^{-4}M AChI (Fig 2).

The same graphical presentation was used to determine the type of inhibitor to which SO_2 pertained. Both intercepts changed after the enzyme had been exposed to 4 ppm SO_2 for one hour. Since both *V* and *Km* were modified, it appears that SO_2 could be classified as a "mixed type" inhibitor. In such a case, it is not possible to determine the inhibition constant *Ki* by graphical methods.

Before exposure, the pH of the enzyme solution was 6.8. No change in pH was observed in the controls bubbled with air, whereas the pH determination was slightly decreased in samples exposed to O_3 and markedly decreased in those exposed to SO_2. Table 1 shows the gradual decrease of pH as SO_2 concentration increased. As stated under "Methods," each titration sample was adjusted to pH 7.4 before the substrate was added.

In order to compare the different degrees of inhibition of AChE activity at various concentrations of SO_2 and O_3, the velocity of the reaction of the control was arbitrarily taken as 100, and the reaction rate of the inhibited enzyme was calculated from the expression:

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$$100 - \left(\frac{a - x}{a} \cdot 100\right),$$

where a is the rate of reaction of the control and x is the rate of reaction of the enzyme gassed with "c" ppm of SO_2 or O_3. The dose-response curves are shown in Fig 3 and 4. The uninhibited rate of the reaction, as calculated from the amount of NaOH used for the titration of the sample, was 7.6 nanomols/second (SD = 0.09). The statistical data are shown in Table 2. The general formula applied to paired experiments was used.

Comment

Our intention was to work only with concentrations of SO_2 such as can be found in ambient atmospheres; however, in order to establish a conclusive correlation between the degree of enzyme inhibition and the amount of SO_2 to which the enzyme solution was exposed, higher pollutant concentrations were also used.

From the dose-response curves obtained, it appears that both SO_2 and O_3 must reach a certain threshold dose before a direct relationship between the degree of inhibition of the enzyme and the concentration of the pollutant can be established. Under our experimental conditions (one hour gassing into 60 ml of enzyme solution containing 6 μmol units of bovine erythrocyte AChE), the threshold doses were attained with approximately 4 ppm SO_2 and 0.3 ppm O_3. Below these concentrations, the degree of inhibition of enzymic activity was small and the results were erratic for SO_2 exposures; but from then on, the relative enzymic activity plotted versus the concentration of the pollutant gave exponential curves having the general form: $y = 100 - be^{x}$, where y is the relative enzymic activity and x is the pollutant concentration. The value of b was found to be 2.5 for SO_2 and 4.3 for O_3.

The basic mechanism of AChE activity inhibition by SO_2 has not been identified, but the possibility that SO_2 acts through the intermediary of $NaHSO_3$ was considered. The latter, in concentrations equivalent to the final concentration of SO_2 in the AChE solution after one hour of exposure to 2.8 and 4.1 ppm SO_2, was added directly to the enzyme solution and allowed to operate for one hour. The results of this limited experiment showed that although $NaHSO_3$ did decrease AChE activity, its inhibitory effect was weaker than that of SO_2.

Animal experiments conducted with SO_2 labelled with radioactive sulfur (^{35}S)[13] have shown that a small portion of the SO_2 absorbed by the upper airways enters the blood; ^{35}S has been found in the plasma, the cellular compartments, and many of the tissues of the body.[13] Although no direct extrapolation can be made from in vitro studies to in vivo experiments, the results of our study suggest that the bronchoconstriction induced by SO_2 both in animals and man[7] might be mediated by the action of SO_2 upon AChE.

Ozone is known to react with proteins.[9] It is thought to inactivate an important constituent of the active site of AChE: the amino acid serine.[9]

The effect of O_3 upon erythrocyte AChE in vivo is possibly mediated by lipid peroxidation.[10] More detailed experimentation will have to be undertaken before any tentative explanation of the mode of action of SO_2 and O_3 upon AChE can be offered.

Bendrick Nowak and Pierre Bernier provided technical assistance in this study.

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INCIDENCE OF LUNG TUMOURS IN LX MICE EXPOSED TO (1) FREE RADICALS; (2) SO_2

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Although the spontaneous incidence of pulmonary adenoma varies greatly between line-bred strains of mice, they all show a higher species susceptibility to this very characteristic tumour than other species of small rodent kept under similar laboratory conditions. Moreover, some chemical carcinogens for mouse lung, e.g. urethane and polycyclic hydrocarbons, induce similar tumours to those of spontaneous origin, but at an earlier age and in greater numbers than are found in untreated controls. Thus, the mechanism of carcinogenesis in the mouse lung appears to be very complex, involving unidentified factors responsible for the spontaneous tumours, in addition to controlled experimental factors.

A close association between the occurrence of subpleural alveolar hyperplasia and neoplasia and the presence of engorged lymphatics filled with apparently normal lymphocytes in the affected sector of the lung, has been reported (Peacock and Peacock, 1956). While the significance of this association is not clear, it might be of aetiological importance. Occasionally such engorged lymphatics are seen in the normal subpleural lymphatic sites without any epithelial hyperplasia, in apparently healthy lungs, and these may be regarded as physiological.

On the other hand, in our experience subpleural alveolar hyperplasia is rarely observed in the absence of such lymphatic engorgement. Clearly, in a single section or in a series of sections taken from a single lung, one cannot form an adequate impression of the duration of such lymphatic engorgement; but it seems possible that if it is maintained for more than a certain length of time, alveolar hyperplasia of the associated overlying epithelium may follow automatically, and this in its turn may provide a suitable site for the action of potential carcinogens.

It seemed desirable, therefore, to devise experiments which would create areas of subpleural lymphatic engorgement and maintain them for a known length of time, and observe the possible effects of such treatment on the subsequent incidence of hyperplastic and neoplastic lesions in the lung. With this in mind, we chose two non-cumulative inorganic irritants capable of being administered as airborne pollutants, namely free radicals and sulphur dioxide.

MATERIALS AND METHODS

Free radicals were chosen partly because they are present in cigarette smoke, which is associated with cancer of the lung in man, and also because free radicals are generated in the electrostatic air filter which was used in previous experiments on the possible influence of airborne soot as a potential carcinogen for the mouse lung (Peacock, 1962). Sulphur dioxide was chosen because of its presence in

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urban atmospheric pollution and because it is a gaseous and non-cumulative irritant which is easily controlled.

Mice of the LX colony, bred in this laboratory from stock kindly supplied by Dr. Bloom, were used because they are known to be highly susceptible to the induction of lung adenoma in response to urethane, and because they are heterozygous though selectively bred for large size (Bloom, 1964).

To house the experimental mice, two identical perspex chambers, each measuring 50 × 40 × 85 cm. and of approximately 180 litres' capacity, were used. These chambers were equipped with two perspex shelves, each of which could carry four standard mouse boxes; 12 boxes in all. The chambers were closed by sliding doors and had input ventilation tubes of 5 cm. diameter and similar output openings on one side of each cage. No intercommunication was possible between the two chambers.

The mice were segregated by sex and were kept in galvanised boxes each containing not more than five mice, usually members of one litter. Food pellets (Diet 41) and tap water in plastic bottles, were constantly available.

Group 1: 41 Male and 39 female, three-month-old LX mice born at about the same date as the experimental groups, were kept in the main animal house in similar boxes.

Group 2: 30 male and 30 female LX mice, three months old, were kept in Chamber 1, which was equipped with thin copper sleeve electrodes, 1·25 cm. broad and 6 mm. apart, fitted around the input ventilation duct. The lower electrode was connected to the active terminal of a diathermy unit giving an output of 1·5 kw at 2 kv. The upper electrode was earthed.

The technique for free radical reproduction was that of radio-frequency discharge (Shaw, 1960). The apparatus was switched on for 3 hours daily from Monday to Friday inclusive throughout the experiment.

Group 3: 35 male and 30 female LX mice, three months old, were kept in Chamber 2 under similar conditions to those in Group 2, except that the ventilation duct was equipped with a side tube through which SO_2 could be added to the input air.

By preliminary pilot trials a rate of 20 ml. per minute for 5 minutes (500 p.p.m.) was found to be well tolerated, and this dosage was given daily from Monday to Friday throughout the experiment.

All animals were inspected daily by the technical staff throughout the experiment, and sick animals were reported, and were examined by one of us (P.R.P.) and if considered to be seriously ill were killed by chloroform and immediately autopsied. Despite this, some animals were found dead and were autopsied as soon as possible after discovery. Most survivors over two years old were killed and immediately autopsied.

The procedure adopted was described in detail in a previous communication from this laboratory (Peacock and Peacock, 1966). Suspicious lesions in any organ were processed for histology. The lungs were inflated with 1% formalin and removed intact for further inspection. The heart was removed and all obvious lung lesions noted. The lungs were then compressed between the lid and inverted base of a plastic petri dish and examined from both sides by transmitted light. In this way, lesions of less than 1 mm. diameter can be seen by the naked eye as translucent spots. All such lesions were processed for serial sectioning, stained by Van Geison or Mallory and Gomori's aldehyde fuchsin elastic stain, in

order to distinguish between alveolar hyperplasia and neoplasia, in which condition the elastica is defective.

RESULTS

Only mice that survived for three hundred days or more are considered in assessing the results because no primary tumours of the lung were seen in LX mice below this age. The essential details of experimental data, autopsy and histology for each mouse, and the site and degree of neoplasia, when present, are given in Tables I to III.

In order to give some numerical value to the degree of neoplasia in the lungs, one (+) was awarded for each mm. of tumour diameter for the largest tumour in any one mouse. A mouse with one tumour of 3 mm. and several smaller tumours, scores (3), and so on. Admittedly this method under-estimates the differences between a single tumour of 1 mm. or less diameter discovered during histological examination of an apparently normal lung and a mass of tumours which caused death or serious respiratory embarrassment. However, the method of attempting to count individual tumours is thought to be less satisfactory because a number of small tumours may become confluent and so the number of recorded tends to grow less as the tumours grow larger.

The incidence of primary lung hyperplasia and neoplasia is shown in Tables IV and V. Since the degree of neoplasia is debatable all neoplastic lesions are included under Adenoma in Table IV, (see Discussion). It will be seen that in the mice exposed to SO_2 there is an increased incidence of primary neoplasia in the lung of both sexes, from 31% to 51% in males and from 17% to 43% in females.

In those exposed to free radicals there was an increase of approximately 10% in males and 6% in females. Primary lung tumours in males occurred about twice as frequently as in females in groups 1 and 2.

DISCUSSION

The anatomical and histological grading shows about equal distribution between tumours of subpleural and other origin in males of groups 1 and 2, and in females of group 3; in the other groups subpleural exceeded other-site tumours.

A number of factors which cannot easily be quantitated must be considered in assessing these results. Thus, the grading of neoplasia as carcinoma or adenoma is not always easy. Lung tumours in mice rarely metastasise outside the thorax and even large tumours replacing a whole lobe may show no invasive tendency. We classify as carcinoma only those tumours which invade blood vessels (fig. 6) or other organs, though in many adenomata atypical growth suggesting malignancy may be found.

For this reason all primary tumours of the lung are included under the heading "Adenoma" in Table IV and some of these also appear under "Primary Carcinoma" and under "Hyperplasia".

All the "Primary carcinoma" mice also had adenoma, but some "Hyperplasia" mice had no neoplastic lesions.

The distinction between hyperplasia and neoplasia of the pulmonary alveolar epithelium is somewhat arbitrary since both types of lesion are often found together or in different parts of the same lung. Inevitably therefore subjective judgment plays a part in assessing the degree of neoplasia.

In general, the group exposed to SO_2 had more and large primary lung tumours

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than untreated controls, and at an earlier age. Those exposed to free radicals showed an incidence of lung tumours intermediate between the SO_2 and control group.

The occurrences of primary carcinoma of the lung in females was limited to the SO_2 group. Fewer of the mice exposed to SO_2 were free from detectable primary neoplastic lung lesions than in the other two groups. In many animals several stages of hyperplasia and neoplasia were present together at autopsy. In heterozygous animals, genetic factors may determine variation in the effects of environmental carcinogens and might be held responsible for the higher incidence of lung tumours in the SO_2 group. Against this probability is the fact that tumours of other organs, notably the liver in males and the lymphatic system in females, show no such group distinction. In Table VI a comparison is made of the actual and percentage incidence of hepatoma and lymphomatoses (including leukaemia and lymphosarcoma), on the same basis as that used in Table V. For ease of comparison the results for lung and liver tumours are shown in the form of a histogram (Fig. 1).

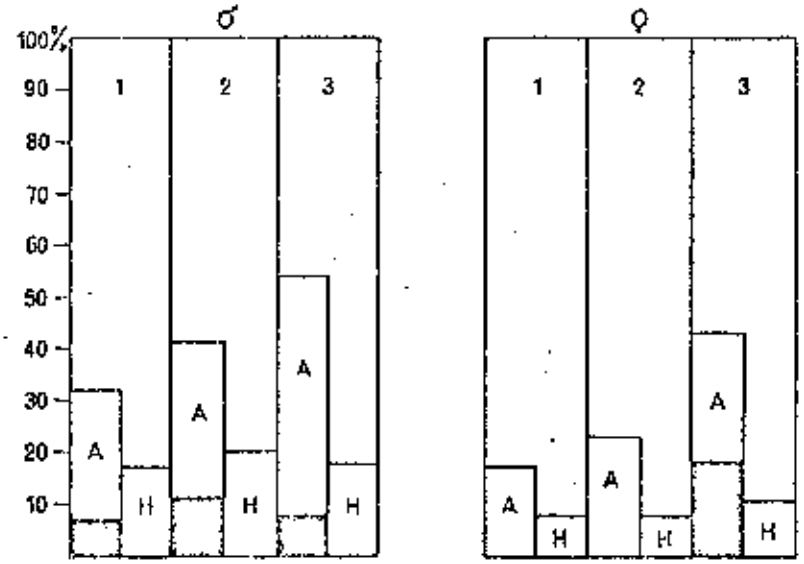

FIG. 1.—Histogram showing percentage distribution of pulmonary papillary adenoma A and hepatoma H; histologically malignant tumours shown in black. Figures at head of columns correspond with Groups 1, 2, and 3.

It is clear that there is no significant variation in the distribution of hepatoma between the three groups, but that the incidence in the males is more than twice that in the females. The distribution of lymphomatosis shows a greater susceptibility of females in all groups, but no apparent relationship to experimental conditions. The higher incidence of lymphomatosis in male control mice may be related to their lower incidence of lung tumours, but with the small number involved (5/35) it is not thought to be aetiologically significant.

Independent statistical analysis of the figures in Tables IV–VI by our colleague Dr. S. Iversen shows that such a distribution of tumours could be a matter of chance.

TABLE I.—*Controls*

No.	Weight (g.)	Age at death (days)		Lung* 1	2	3	4	Autopsy (A) and Histology (B)
Males:								
1450/2	45	323		−	−	−	−	(A) Haemorrhagic mass in right axilla. (B) Haemorrhagic lymphoma.
1451/1	45	360		−	−	−	−	(A) and (B) Haemorrhagic enteritis; N.A.F. in lungs.
1425/1	39	393		−	−	−	−	(A) and (B) Enteritis and pneumonia.
1328/4	50	411		−	−	−	−	(A) Large pale liver. (B) Lymphomatosis.
1392/2	80	K.426		−	−	−	−	(A) Perineal tumour; multiple metastases. (B) Anaplastic sarcoma.
1452/2	35	K.460		−	−	−	−	(A) Poor condition; tumour in liver; stone in bladder; pneumonia. (B) Hepatoma; pneumonia.
1452/3	30	K.494		−	−	−	−	(A) and (B) Collapsed and congested lung.
1328/5	45	495	(1)†	+	−	+	−	(A) Ulcer of right ear; multifocal lung tumours. (B) Squamous carcinoma *in situ* of ear; subpleural papillary adenoma and alveolar hyperplasia.
1452/4	40	K.499	(12)	+	+	−	−	(A) Poor condition; large central liver tumour; large tumour left lobe of lung, small tumour right azygos lobe. (B) Hepatoma; multifocal papillary adenoma.
1461/2	35	K.511	(5)	+	+	−	−	(A) Abscess of right eye; large tumour invading heart and diaphragm. (B) Papillary adenoma and carcinoma.
1450/1	42	534		−	−	−	−	(A) Multiple nodules in liver. (B) Lymphomatosis.
1539/1	45	K.548		−	−	−	−	(A) Very large liver. (B) Leukosarcoma.
1542/2	40	571		−	−	−	−	(A) Subcutaneous abscess of left groin; liver tumour; lung congested. (B) Hepatoma; lung congested.
1450/2	28	571	(12)	+	+	−	−	(A) Multifocal tumour of right middle lobe of lung. (B) Multifocal papillary adenoma and carcinoma.
1539/2	65	K.594		−	−	−	+	(A) Good condition, N.A.F. (B) Other site alveolar hyperplasia in lung.
1329/5	45	596		−	−	−	−	(A) Large central liver tumour (5 g.); lung congested. (B) Hepatoma; lung congested.
1539/3	56	K.608	(3)	+	+	−	−	(A) Good condition; tumour (5 mm.) right middle lobe of lung. (B) Multifocal papillary adenoma.
1457/5	75	K.640		−	−	−	−	(A) Good condition but distended bladder (20 ml.). (B) Lungs N.A.F.
1459/3	24	K.645		−	−	−	−	(A) Kyphosis; left lobe collapsed. (B) Interstitial pneumonia.
1327/1	47	K.645		−	−	−	−	(A) and (B) Good condition; N.A.F.
1327/2	45	K.645		−	−	−	−	(A) and (B) Good condition; N.A.F.
1459/4	48	651		−	−	−	−	(A) and (B) Autolysis; N.A.F. in lung.
1459/5	45	K.658		−	−	−	−	(A) and (B) Distended bladder; N.A.F. in lung.
1450/3	50	K.679	(2)	+	−	−	−	(A) Multifocal tumours in lungs. (B) Subpleural papillary adenoma.
1450/4	65	K.679	(1)	+	−	−	−	(A) Tumour (4 mm.) right upper lobe of lung; tumour (1 cm.) in liver. (B) Subpleural papillary adenoma; hepatoma.
1450/5	70	K.679	(3)	−	+	+	−	(A) Tumour (3 mm.) right middle lobe of lung and smaller lesions. (B) Other site papillary adenoma and subpleural alveolar hyperplasia.
1460/6	70	K.679	(2)	−	+	−	−	(A) Multifocal tumour right middle and right azygos lobes of lung. (B) Other site papillary adenoma.
1542/3	35	K.696		−	−	−	−	(A) Mesenteric tumour; soft stones in bladder. (B) Haemangioma.
1456/2	38	708	(2)	+	+	−	−	(A) Retention; stone in bladder; tumour in left lung. (B) Multifocal papillary adenoma.
1538/4	52	714		−	−	−	−	(A) and (B) Good condition; N.A.F. in lungs.
1456/3	40	730	(1)	+	−	−	−	(A) Stone in bladder; hydronephrosis; tumour right lobe of lung. (B) Subpleural papillary adenoma.
1456/4	50	730		−	−	−	−	(A) and (B) Stone in bladder; N.A.F. in lung.
1456/5	40	730		−	−	−	−	(A) and (B) Stone in bladder; N.A.F. in lung.
1329/1	45	K.813		−	−	−	−	(A) Tumour on right side of neck; tumour in liver. (B) Lymphoma; hepatoma.
1329/4	35	873		−	−	−	−	(A) and (B) Traumatic rupture of heart; lung collapsed.

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No.	Weight (g.)	Age at death (days)		Lung* 1	2	3	4	Autopsy (A) and Histology (B)
Females								
1453/1	30	317		—	—	—	—	(A) and (B) Haemorrhagic gastroenteritis; autolytic; N.A.F. in lung.
1541/1	20	367		—	—	—	—	(A) and (B) Autolytic; lung congested.
1451/1	52	436		—	—	—	—	(A) and (B) Right side pyometra.
1330/1	35	473		—	—	—	—	(A) Cystitis; many soft stones. (B) Chronic cystitis.
1460/1	45	K.491		—	—	—	—	(A) and (B) Poor condition; ruptured pyometra; mesenteric abscesses; hepatoma.
1330/2	45	K.517		—	—	—	+	(A) Multiple tumours in subcutaneous tissue and muscles of thorax and limbs. (B) Lymphosarcoma and other site alveolar hyperplasia.
1462/2	25	532	(11)	+	+	—	—	(A) Poor condition, large tumour in left lobe of lung. (B) Multifocal papillary adenoma.
1540/1	45	535		—	—	—	—	(A) and (B) Pyometra; lungs N.A.F.
1453/2	30	K.552		—	—	—	—	(A) Multifocal infarcts; large mass in left uterus. (B) Septic infarcts and septic endometritis.
1462/3	55	561		—	—	—	—	(A) Left pyometra; tumour in left ovary; nodules in liver. (B) Lymphosarcoma.
1538/1	50	K.578		—	—	—	—	(A) and (B) Purulent cystitis; lungs congested.
1331/5	45	K.581	(3)	+	—	—	—	(A) Ascites; multifocal tumour of liver omentum lymph nodes; lung tumour. (B) Lymphatic leukaemia; lymphosarcoma; subpleural papillary adenoma.
1541/3	40	K.606		—	—	—	—	(A) Large liver tumour right lobe. (B) Hepatoma.
1460/2	40	K.615		—	—	—	—	(A) and (B) Good condition; N.A.F.
1330/3	50	K.622		—	—	—	—	(A) Haemorrhagic ascites; large nodular liver; tumour of right uterus. (B) Lymphatic leukaemia; lymphosarcoma.
1541/2	20	626		—	—	—	—	(A) and (B) Lungs congested.
1455/3	50	K.631		—	—	—	—	(A) Large tumours involving abdominal viscera and mediastinum. (B) Multifocal lymphosarcoma.
1541/4	25	K.632	(1)	+	+	—	—	(A) Enteritis; tumour in right lower lobe of lung. (B) Multifocal papillary adenoma.
1458/1	33	K.641		—	—	—	+	(A) Bilateral hydrosalpynx; tumour of right lower lobe of lung. (B) Small other site alveolar hyperplasia.
1455/4	50	K.646		—	—	—	—	(A) Good condition; large uterus; congested lung. (B) Endometriosis.
1458/2	35	K.648		—	—	—	—	(A) Good condition; tumour in left ovary and uterus. (B) Squamous carcinoma of uterus.
1458/3	45	K.643		—	—	—	—	(A) and (B) Good condition; hydrosalpynx.
1540/2	45	K.658		—	—	—	—	(A) and (B) Good condition; very fat; N.A.F.
1538/3	47	K.678		—	—	—	—	(A) and (B) Good condition; hydrosalpynx; papilloma of forestomach; lung lesions. (B) Papilloma of forestomach; desquamative alveolar hyperplasia.
1451/3	53	K.679	(4)	+	—	+	—	(A) Good condition; hydrosalpynx; tumour in right upper lobe of lung. (B) Subpleural papillary adenoma and alveolar hyperplasia.
1330/5	52	680		—	—	—	—	(A) Ascites; multifocal liver tumours; haemorrhage salivary gland. (B) Lymphomatosis.
1538/4	50	K.724		—	—	—	—	(A) Tumour of back and ulcerated tumour of right groin. (B) Multifocal myxosarcoma.
1540/3	30	724		—	—	—	—	(A) and (B) Good condition; lungs congested.
1540/4	35	724	(4)	+	—	—	—	(A) Good condition; tumour (4 mm.) in right lower lobe of lung. (B) Subpleural papillary adenoma.
1330/4	18	761		—	—	—	—	(A) and (B) Autolytic; N.A.F.

* Primary Lung Lesions:—Column 1—Papillary adenoma of subpleural origin.
2—Papillary adenoma of other sites.
3—Alveolar hyperplasia of subpleural origin.
4—Alveolar hyperplasia of other sites.

† Figures in brackets show diameter in mm. of largest tumour.

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TABLE II.—*Free Radicals*

No.	Weight (g.)	Age at death (days)	Duration of experiment (days)		Lung* 1	2	3	4	Autopsy (A) and histology (B)
Males:									
1321/2	75	319	234		−	−	−	−	(A) Large hepatic tumour; stone in hypertrophic bladder. (B) Hepatoma.
1357/1	35	421	385		−	−	−	−	(A) and (B) Necrotic penis, cystitis, pyelitis.
1323/4	50	K.435	347	(3)	+	+	−	−	(A) Tumour of antrum and orbit; multifocal lung tumours. (B) Squamous carcinoma of antrum; papillary adenoma.
1361/1	50	440	422	(2)	+	+	−	−	(A) Liver tumour; large multifocal lung tumours. (B) Hepatoma; subpleural and other site papillary adenoma and carcinoma.
1325/5	65	464	316		−	−	−	−	(A) and (B) Autolytic dark lung; patchy pneumonia.
1360/1	65	470	441		−	−	−	−	(A) Generalized lymphatic tumours; nodes liver and spleen. (B) Leukaemia.
1323/3	40	477	389						(A) Autolytic; N.A.F. in lungs.
1323/1	50	495	417		−	−	−	−	(A) Intestinal obstruction by caecal tumour. (B) Anaplastic adenocarcinoma of caecum.
1325/2	60	K.574	486		−	−	−	−	(A) and (B). Traumatic ulcers (fighting); N.A.F. in lung.
1325/4	70	K.574	486		−	−	−	−	(A) and (B). Traumatic ulcers (fighting); oedema of lung.
1360/2	60	584	534	(1)	−	+	−	−	(A) Necrotic penis; distended bladder; pale mottled liver; lung tumour. (B) Tyzzer's disease; other site papillary adenoma.
1325/1	35	609	524	(11)	+	+	−	−	(A) Large tumours in right lower lung; pneumonia. (B) Multifocal papillary adenoma and carcinoma.
1321/1	55	610	525	(5)	+	+	−	−	(A) Stone in distended bladder; multifocal lung tumour. (B) Subpleural and other site papillary adenoma.
1325/3	55	K.636	548		−	−	−	−	(A) Abdominal swelling, torsion of distended bladder; liver tumour. (B) Hepatoma.
1357/2	25	669	633		−	−	−	−	(A) Huge liver tumour. (B) Hepatoma.
1360/3	40	K.669	649	(3)	+	+	−	−	(A) Skin tumour right ear; tumour right middle lobe. (B) Necrotic squamous carcinoma; subpleural and other site papillary adenoma.
1361/2	40	684	616	(10)	+	+	−	−	(A) Multifocal lung tumour involving ribs and diaphragm. (B) Multifocal papillary adenoma and carcinoma.
1361/3	60	691	673	(2)	+	−	+	−	(A) Bladder obstructed by stone; huge liver tumour. (B) Hepatoma.
1323/2	30	694	606		−	−	−	−	(A) and (B) Urethral stone obstruction; N.A.F. in lung.
1361/5	55	K.714	696	(2)	+	−	+	−	(A) Traumatic ulcers (fighting); bladder stone; multifocal tumour in lung. (B) Subpleural papillary adenoma and alveolar hyperplasia.
1361/4	50	K.740	728	(3)	+	−	+	−	(A) Multifocal lung tumour. (B) Subpleural papillary adenoma and alveolar hyperplasia.
1321/5	33	K.750	662		−	−	−	−	(A) and (B) Nasal abscess; N.A.F. in lung.
1360/4	35	K.757	726		−	−	−	−	(A) and (B) N.A.F.
1360/5	48	K.757	728		−	−	−	−	(A) and (B) N.A.F.
1357/3	27	K.773	737	(8)	+	+	+	+	(A) Huge tumour right middle lobe of lung and multifocal tumours in other lobes. (B) Subpleural and other site papillary adenoma and alveolar hyperplasia.
1357/4	28	K.773	737		−	−	−	−	(A) Liver tumour. (B) Hepatoma.
1357/5	29	K.773	737		−	−	−	−	(A) N.A.F. (B) Lungs: perivascular lymphatic engorgement.
1321/3	50	K.805	729	(3)	+	+	−	−	(A) Ulcer of right axilla; multifocal tumour right upper and right lower lobes of lung. (B) Subpleural and other site papillary adenoma.
1321/4	35	847	702	(2)	−	+	−	+	(A) Chronic nephritis; tumour right lower and left lung. (B) Other site papillary adenoma and alveolar hyperplasia.

No.	Weight (g.)	Age at death (days)	Duration of experiment (days)		Lung* 1	2	3	4	Autopsy (A) and histology (B)
Females:									
1324/3	35	396	311	(2)	—	+	—	—	(A) Large tumour right upper lobe of lung; tumour right mandible; tumour of thymus. (B) Well differentiated papillary adenoma; lymphoma; squamous carcinoma.
1322/2	55	K.403	318		—	—	—	—	(A) Anasarca; diffuse pelvic tumour; metastases in liver. (B) Anaplastic sarcoma of subcutaneous tissues; lymphosarcoma of parametrium.
1358/1	30	408	372		—	—	—	—	(A) and (B) Acute enteritis and hepatitis. N.A.F. in lung.
1356/1	25	434	399		—	—	—	—	(A) Bilateral hydronephrosis; vesical stone.
1359/1	35	478	449		—	—	—	—	(A) Cyst left side of neck; liver tumour. (B) Hygroma in lymph node; trabecular hepatoma.
1326/1	33	491	403		—	—	—	—	(A) Ascites; pneumonia. (B) Pneumonia—autolytic.
1324/2	40	536	448		—	—	—	—	(A) and (B) Haemorrhagic ascites and visceral lymphomatosis.
1356/2	40	K.539	504		—	—	—	—	(A) Proptosis right side; large bladder stone; pelvic tumour. (B) Spindle cell sarcoma of vagina; metastasis in orbit.
1326/2	52	545	452		—	—	—	—	(A) Ascites and pleural effusion; lung collapsed. (B) Lymphomatosis; leukaemia.
1324/5	50	K.649	564	(1)	+	+	—	+	(A) Ascites; huge liver and spleen; pyometra; multifocal lung tumour (B) Leukaemia; subpleural and other site papillary adenoma; other site alveolar hyperplasia.
1359/3	43	695	667		—	—	—	+	(A) Chronic peritonitis and pyometra; tumour left lung. (B) Other site alveolar hyperplasia.
1356/3	35+	761	666		—	—	—	—	(A) Tumour left lung. (B) Desquamating alveolar hyperplasia; perivascular lymphomatitis.
1326/3	23	714	629	(1)	+	—	+	—	(A) Enteritis; lung tumours. (B) Subpleural papillary adenoma in area of alveolar hyperplasia.
1358/2	45	716	680		—	—	—	—	(A) and (B) Pyometra; multifocal liver abscesses; N.A.F. in lung.
1324/1	55	K.721	633		—	—	—	—	(A) Mammary tumour R.2. (B) Pleomorphic carcinoma.
1359/4	35	K.724	695		—	—	—	—	(A) Tumour right uteral origin; metastases in liver and lung. (B) Osteogenic sarcoma.
1359/2	15	725	696		—	—	—	—	(A) and (B) Autolytic; N.A.F. in lungs.
1359/5	36	K.725	696		—	—	—	—	(A) and (B) Septic endometritis.
1324/4	39	749	661		—	—	—	—	(A) and (B) Chylous ascites; visceral lymphomatosis.
1358/3	30	751	715		—	—	—	—	(A) Pendulous tumour right liver lobe; lesions in lungs. (B) Trabecular hepatoma; oedema of lungs.
1322/3	55	754	660		—	—	—	—	(A) Ascites; large mottled liver. (B) Leukaemia.
1358/4	25	K.773	737	(1)	+	—	—	—	(A) Subpleural lung tumours. (B) Small subpleural papillary adenoma.
1358/5	40	K.773	737		—	—	—	—	(A) and (B) Good condition; no lesions found.
1322/5	40	783	695	(6)	+	—	—	—	(A) Haemothorax; tumour left lung; uterine tumours L > R. (B) Subpleural papillary adenoma; adenocarcinoma of uterus; metastases in lung.
1356/4	27	K.792	757		—	—	—	—	(A) and (B) Good condition; no lesions found.
1356/5	28	K.792	757		—	—	—	—	(A) Good condition; lesion left lung. (B) Perivascular lymphatic engorgement.
1322/4	25	K.798	710		—	—	—	—	(A) Uterine tumour; ovarian cysts; mediastinal lymphoma. (B) Adenocarcinoma of uterus; lymphomatosis.
1322/1	45	K.833	745		—	—	—	—	(A) Tumour right side vulva; paraplegia. (B) Squamous papilloma and carcinoma of vulva.
1326/5	50	832	797	(3)	+	+	—	—	(A) Multifocal tumour right middle and left lobes; liver enlarged; right ovarian cyst; endometriosis. (B) Subpleural and other site papillary adenoma; leukaemia; endometriosis.
1326/4	35	K.896	808	(5)	+	—	—	—	(A) Tumour in left lung. (B) Subpleural papillary adenoma.

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TABLE III.—SO_2

No.	Weight (g.)	Age at death (days)	Duration of experiment (days)		Lung* 1	2	3	4	Autopsy (A) and Histology (B)
Males:									
1355/1	100	346	311	(2)	+	—	—	—	(A) Very fat; bladder stone; fibrinous peritonitis; 2 tumours left lung. (B) Subpleural papillary adenoma.
1355/2	25	300	325	(2)	+	+	—	—	(A) Autolytic; tumour right middle and left lung. (B) Pneumonia and papillary adenoma.
1355/3	35	444	379		—	—	—	—	(A) and (B) Autolytic; lung congested and oedematous.
1351/1	45	498	463	(3)	+	+	—	+	(A) Bladder distended; turbid urine; liver tumour; multifocal lung tumour. (B) Trabecular hepatoma; multifocal adenoma and hyperplasia.
1319/1	60	515	430		—	—	—	—	(A) and (B) Acute oedema of lung; pyelonephritis; stones in bladder.
1351/2	65	535	500		—	—	—	—	(A) and (B) Autolytic; N.A.F. in lungs; soft stones in bladder; pyelonephritis.
1372/1	40	548	489		—	—	—	—	(A) and (B) Autolytic; N.A.F. in lung.
1353/1	40	567	532	(2)	+	+	—	—	(A) Tumour of liver; distended bladder; tumour of left lobe of lung. (B) Trabecular hepatoma; multifocal papillary adenoma.
1319/2	30	600	512	(2)	+	+	—	—	(A) Multifocal liver tumours; tumours right lower lobe of lung. (B) Trabecular hepatoma, papillary adenoma.
1319/5	55	603	515		—	—	—	—	(A) Traumatic abscess (fighting); distended bladder; liver tumour. (B) Trabecular hepatoma.
1355/4	25	K.603	538	(3)	+	—	+	—	(A) Poor condition; lung lesions. (B) Subpleural lymphangitis; alveolar desquamation; subpleural papillary adenoma and alveolar hyperplasia.
1355/5	30	K.603	538		—	—	—	—	(A) and (B) Poor condition; N.A.F. in lungs.
1353/2	50	K.632	597		—	—	—	—	(A) and (B) Severe trauma (fighting); N.A.F. in lungs.
1372/2	30	K.658	601		—	—	—	—	(A) and (B) Left pyonephrosis; right pyelonephritis; lung congested.
1315/4	50	674	589		—	—	—	—	(A) Turbid pleural effusion; mediastinal tumours. (B) Lymphomatosis.
1353/3	60	K.686	651	(8)	+	+	—	—	(A) Distended bladder; stone in urethra; liver tumour; tumour left lobe of lung. (B) Trabecular hepatoma; multifocal papillary adenoma and carcinoma.
1315/5	40	K.701	616		—	—	—	—	(A) Phimosis; full bladder; lesions in lung. (B) Desquamating alveolar hyperplasia.
1319/3	30	719	631		—	—	—	—	(A) Autolytic. (B) Oedema of lung.
1315/3	35	K.741	706	(5)	+	—	—	—	(A) Trauma (fighting); cystitis; lung tumours. (B) Subpleural papillary adenoma.
1351/4	47	K.741	706	(4)	+	—	—	—	(A) Trauma (fighting); tumour in left lung. (B) Subpleural papillary adenoma.
1319/4	44	K.749	664	(3)	+	—	+	—	(A) Nasal and subcutaneous abscess; multifocal lesions in lungs. (B) Subpleural papillary adenoma; alveolar hyperplasia.
1372/3	36	K.756	700	(4)	—	+	—	—	(A) Patchy pneumonitis; tumour right upper lobe of lung. (B) Large other site papillary adenoma.
1372/4	32	K.756	600		—	—	—	—	(A) Poor condition. (B) Subpleural lymphangitis.
1353/4	50	K.791	756	(5)	+	+	+	+	(A) Good condition; tumour right middle lobe of lung. (B) Subpleural and other site papillary adenoma; alveolar hyperplasia.
1353/5	50	K.791	756	(2)	+	+	+	+	(A) Good condition; tumour right lower and left lobes of lungs; mesenteric tumour. (B) Papillary adenoma and alveolar hyperplasia; angioma.
1351/5	30	K.791	756	(5)	+	+	—	—	(A) Large tumour right lower lobe of lung; cystic mesenteric nodes. (B) Multifocal papillary adenoma and carcinoma.
1315/1	35	K.896	808	(3)	+	—	—	—	(A) Good condition; tumour left lobe of lung. (B) Subpleural papillary adenoma.
1315/2	30	K.896	808		[illegible]				[illegible]

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No.	Weight (g.)	Age at death (days)	Duration of experiment (days)		Lungs 1	2	3	4	Autopsy (A) and Histology (B)
Females:									
1320/4	70	316	231		−	−	−	−	(A) Large thymus. (B) Lymphomatosis.
1316/1	60	K.325	237		−	−	−	−	(A) Large mammary tumour R.3. (B) Cystic mammary adenocarcinoma.
1318/3	55	371	230		−	−	−	−	(A) Anasarca; large liver; tumour of uterine cervicus. (B) Lymphomatosis.
1318/4	25	403	316	(18)	+	+	−	−	(A) Huge tumour right upper lobe of lung compressing heart. (B) Multifocal carcinoma of lung.
1320/3	65	406	321		−	−	−	−	(A) and (B) Left pneumonia.
1352/1	40	445	330		−	−	−	−	(A) and (B) Autolytic; lungs congested.
1318/5	40	454	306		−	−	−	−	(A) Pyometra; lung lesions. (B) Oedema of lung; pyaemic abscesses.
1316/2	70	558	470	(2)	−	+	−	−	(A) Anasarca; haemorrhage in peritoneum; large liver tumour; right uterine tumour. (B) Sarcoma of uterus; other site papillary adenoma.
1320/5	27	K.593	503	(3)	+	+	−	−	(A) Multifocal tumour of right lower lobe of lung. (B) Subpleural and other site papillary adenoma and carcinoma.
1354/1	30	K.623	587	(3)	+	−	−	−	(A) Pericarditis; large tumour right middle lobe of lung; mesenteric cysts; hygroma. (B) Subpleural papillary adenoma.
1352/2	35	625	560	(2)	+	+	−	−	(A) Tumour in left lung; pedunculated liver tumour. (B) Haemangiolipoma; multifocal papillary adenoma.
1350/5	50	641	551	(1)	+	+	−	−	(A) Multifocal lung tumour. (B) Multifocal papillary adenoma.
1318/1	65	642	554		−	−	−	−	(A) Small scarred liver. (B) Hepatocellular carcinoma; metastases in lung.
1350/1	35	K.643	553	(12)	+	+	−	−	(A) Huge tumour left lung. (B) Multifocal papillary adenoma and carcinoma.
1352/3	20	K.655	590		−	−	−	−	(A) Enlargement of mediastinal nodes. (B) Lymphatic engorgement.
1316/4	55	671	586	(3)	−	−	+	+	(A) Large abdominal tumour involving uterus; lungs congested. (B) Lymphomatosis; congestion and alveolar hyperplasia.
1350/3	40	733	643	(1)	+	−	+	−	(A) Small tumours right middle lobe of lung; right ovarian cyst. (B) Subpleural papillary adenomas.
1320/1	40	757	672	(5)	−	+	−	−	(A) Tumour right hind leg; papilloma of forestomach; large tumour right middle lobe of lung. (B) Fibrosarcoma of leg; other site papillary adenoma.
1352/4	50	K.757	692		−	−	−	−	(A) Large liver tumour (4g); lungs congested. (B) Heptoma.
1350/2	59	760	670		−	−	−	−	(A) Multifocal liver tumour; tumour left uterine horn. (B) Adenocarcinoma of uterus; metastasis in liver.
1320/2	45	K.771	686	(1)	+	+	−	−	(A) Turbid ascites; liver tumour; mediastinal tumours; small lung tumour. (B) Hepatocellular carcinoma; mediastinal metastases; subpleural and other site papillary adenoma.
1354/2	65	K.776	741		−	−	−	−	(A) Ascites; tumour of pylorus; tumour right upper lobe of lung. (B) Lymphosarcoma.
1316/5	25	K.779	693	(1)	−	+	−	−	(A) Poor condition; tumour right azygos and left lobes of lung. (B) Other site papillary adenoma.
1354/3	27	789	754		−	−	−	−	(A) Massive pneumonia.
1354/4	35	K.791	756		−	−	−	−	(A) and (B) Good condition. N.A.F.
1354/5	45	K.791	756	(2)	+	−	+	+	(A) Huge purple liver; tumour right azygos lobe of lung. (B) Extramedullary haemopoiesis; papillary adenoma and alveolar hyperplasia.
1352/5	45	K.791	756		−	−	−	−	(A) Good condition; uterine tumours. (B) Endometriosis.
1318/2	40	792	707	(10)	+	+	−	−	(A) Ovarian cyst; huge tumour right azygos and right middle lobes of lung. (B) Multifocal papillary adenoma and carcinoma.
1350/4	20	K.847	757		−	−	−	−	(A) and (B) Poor condition; pale kidneys; N.A.F. in lung.
1316/3	30	K.896	808		−	−	−	−	(A) and (B) Uterine mucocele; N.A.F. in lung.

TABLE IV.—*Actual Number and (%) of LX mice over 300 Days Old With or Without Primary Lung Neoplasia or Hyperplasia*

		No lung lesion	Primary carcinoma	Adenoma	Hyperplasia
Group 1 (Control)	♂	24/35 (69%)	2/35 (6%)	11/35 (31%)	3/35 (9%)
	♀	23/30 (77%)		5/30 (17%)	3/30 (10%)
Group 2 (Free radical)	♂	16/29 (55%)	3/29 (10%)	12/29 (41%)	4/29 (14%)
	♀	22/30 (73%)		7/30 (23%)	3/30 (10%)
Group 3 (Sulphur dioxide)	♂	13/28 (46%)	2/28 (7%)	15/28 (54%)	5/28 (18%)
	♀	16/30 (53%)	4/30 (13%)	13/30 (43%)	3/30 (10%)

TABLE V.—*Site Incidence of Tumours/Mice at Risk*

		Subpleural	Other sites
Group 1 (Control)	♂	9/35 (26%)	7/35 (20%)
	♀	5/30 (17%)	2/30 (7%)
Group 2 (Free radical)	♂	10/29 (35%)	10/29 (35%)
	♀	6/30 (20%)	3/30 (10%)
Group 3 (Sulphur dioxide)	♂	14/28 (50%)	9/28 (32%)
	♀	10/30 (33%)	10/30 (33%)

TABLE VI.—*Actual Numbers and (%) of LX Mice Over 300 Days Old with Hepatoma and/or Lymphomatosis*

		Hepatoma	Lymphomatosis
Group 1 (Control)	♂	6/35 (17%)	5/35 (14%)
	♀	2/30 (7%)	8/30 (27%)
Group 2 (Free radical)	♂	6/29 (20%)	1/29 (3%)
	♀	2/30 (7%)	9/30 (30%)
Group 3 (Sulphur dioxide)	♂	5/28 (18%)	1/28 (4%)
	♀	3/30 (10%)	4/30 (13%)

EXPLANATION OF PLATES

FIG. 2.—♂ LX 1317/2, age 96 days. Died after accidental exposure for 5 hours to SO_2, 500 p.p.m. Lungs showed patchy congestion. H. & E. ×100.

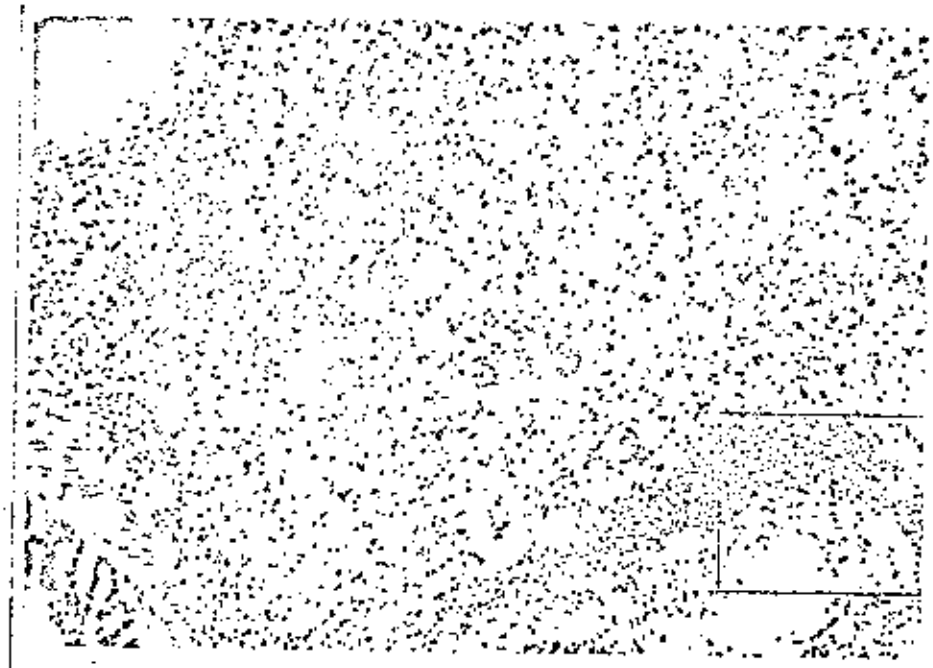
FIG. 3.—Same mouse as above marked are enlarged. A small branch of bronchial artery showing oedema and periarterial lymphangitis. H. & E. ×350. (Nuclear detail emphasized by red filter).

FIG. 4.—♀ LX 1320/5, age 593 days; SO_2, 503 days. Killed in poor condition. Depressed sternum; multifocal tumour (5 mm. diameter) in base of right lower lobe. Section shows adjacent foci of alveolar hyperplasia (R) and papillary adenoma (L), separated by compressed alveoli and lymphatics engorged with lymphocytes. Mallory and Gomori's aldehyde fuchsin. ×85.

FIG. 5.—Enlargement of above, shows intact elastin in hyperplastic focus (R) and defective or absent elastin in neoplastic focus (L). Note lymphatics engorged with apparently normal lymphocytes and absence of other leucocytes. Contrast with Fig. 3. ×250.

FIG. 6.—♀ LX 1318/1, age 403 days; SO_2, 318 days. Killed in poor condition. Right side of thorax distended by large tumour in right upper lobe compressing other right lobes and displacing heart and depressing and invading right dome of diaphragm. No remote metastases found. Section shows anaplastic papillary adenocarcinoma invading venule. H. E. ×250.

All above photographed on Pan F film with green filter.



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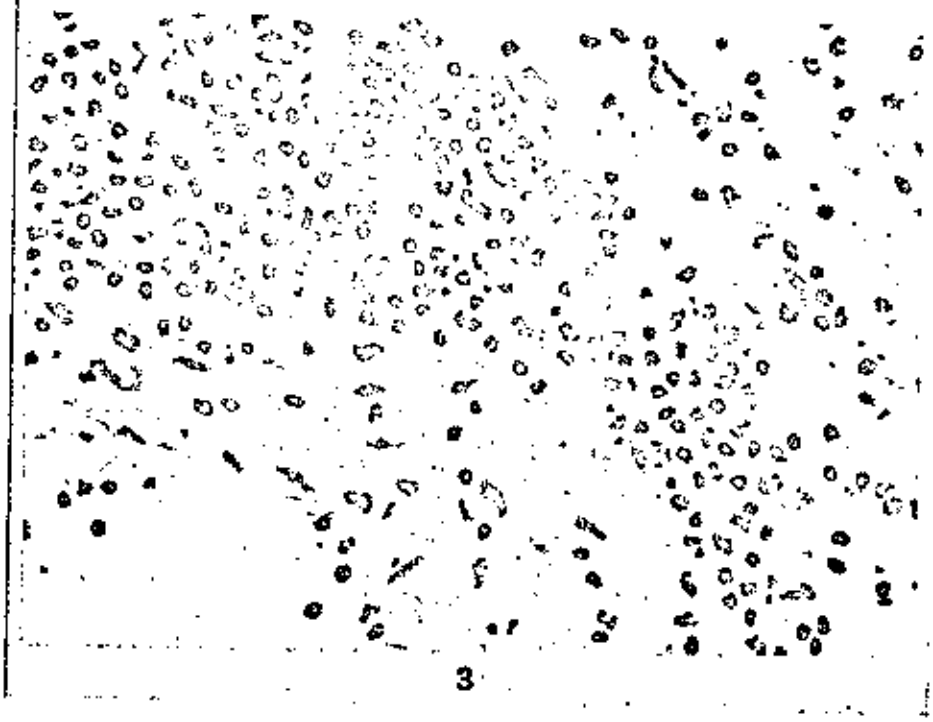

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Peacock and Spence.

However, tumours occurred earlier in both experimental groups, the incidence of lung tumours was about twice as high in mice of both sexes exposed to SO_2 as in controls, and in the females there were as many malignant tumours in the SO_2 group as there were adenomata in the controls.

Malignancy was observed only in the larger tumours and presumably occurred as a progressive development in a tumour which had already reached a diameter of about 5 mm., and by implication had been growing for some time. From the presence of such large tumours in the SO_2 group, generally at an earlier age than in the controls, it is concluded that the experimental conditions accelerated the onset of neoplasia in susceptible mice. The results are consistent with a positive effect of exposure to SO_2 and to a lesser extent, of exposure to free radicals.

Thus it appears that the LX population contains a resistant moiety of about 50% which is unaffected by the experimental conditions.

In the controls about 70% of males and 80% of females live to old age without developing spontaneous lung tumours while the remainder show varying susceptibility.

How does the chemically simple gas SO_2 induce primary lung tumours in susceptible mice?

Early pilot experiments showed that with toxic exposure to SO_2 death might occur within an hour or two from acute oedema and congestion of the lung. In less severe cases which survived for several days or were killed at various intervals from hours to weeks after exposure, the most constant features were inter-alveolar congestion and oedema and intra-alveolar exudate accompanied by lymphangitis of subpleural and interstitial lymphatics. In the early acute inflammatory stages many polymorphs were present along with lymphocytes in these lymphatics (Figs. 2 and 3), but in later stages apparently normal lymphocytes persisted in the engorged lymphatics, unaccompanied by evidence of chronic inflammatory reaction and particularly in immediate juxtaposition to areas of alveolar epithelial hyperplasia and neoplasia (Fig. 4 and 5).

It has been observed previously in other experiments that such lymphatic engorgement is found fairly regularly associated with subpleural alveolar hyperplasia and neoplasia (Peacock and Peacock, 1966). This association of lymphatic engorgement with local alveolar hyperplasia and neoplasia has not yet been explained. It might represent a defence reaction which, in these cases, must have failed to prevent progressive neoplasia; or it might be an aftermath of inflammatory lymphangitis which favours, in some way, local hyperplasia of adjacent alveolar epithelium.

It is suggested that clinically subtoxic exposure to SO_2 causes such lymphatic engorgement and associated alveolar hyperplasia which, in the mouse, appear to predispose to further progression to neoplasia (Fig. 4 and 5). The action of free radicals is inconclusive.

It is concluded that the increased incidence of primary lung tumours in LX mice of both sexes in Group 3 is a consequence of the initial essentially inflammatory reaction to SO_2, followed by a state of apparent tolerance, which accelerates the inherent tendency of these mice to develop lung tumours spontaneously but does not justify the classification of SO_2 as a chemical carcinogen as generally understood.

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SUMMARY

Three comparable groups of LX mice of both sexes were examined for primary lung tumours and other lesions. Group 1, untreated controls; Group 2 exposed to inhalation of free radicals; and Group 3 exposed to inhalation of SO_2.

No lung tumours were observed in mice below 300days of age and only those which survived this age are considered in assessing the results.

An increased incidence of primary lung tumours in both sexes exposed to SO_2 was approximately doubled as compared with controls; carcinoma of the lung in females was observed only in those exposed to SO_2. There was a slight increase in lung tumours in both sexes exposed to free radicals. The incidence of hepatoma and lymphomatosis, the next most frequent tumours in controls, was unaffected by the experimental conditions. There was an association between persistent lymphatic engorgement and alveolar hyperplasia and the development of progressive neoplasia, papillary adenoma and carcinoma, in the lungs of mice in all groups. Repeated exposure to SO_2 apparently accelerated the unexplained sequence of events which leads to the growth of spontaneous lung tumours in the mouse.

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A direct titrimetric method for the rapid estimation of sulphur dioxide in fresh pork sausage

D. PEARSON AND T. S. A. WONG

Summary

A method for the rapid estimation of sulphur dioxide in fresh pork sausage has been developed. A good correlation was obtained between Shipton's modification of Monier-Williams' reference method and the recommended procedure based on Potter's titration method for dehydrated cabbages. The method is applied to a sausage macerate which is twice titrated with iodine. Hydrogen peroxide is used as a specific oxidizing agent for the sulphur dioxide.

Introduction

The Preservatives in Food Regulations 1962 (S.I 1962 No. 1532) permit the presence of up to 450 ppm sulphur dioxide in fresh sausage. In practice it is usually incorporated in the mix as metabisulphite, but for the purpose of the regulations the preservative is calculated as the total sulphur dioxide (w/w). As the amount of preservative present falls rapidly, the determination in sausages has to be carried out in the factory as soon as possible after production. Conventionally the total sulphur dioxide is determined volumetrically after distillation with acid and trapping the gas produced in an oxidizing agent. For reasons of convenience and rapidity it would be useful if a method were available in which the determination is carried out on an extract or macerate without recourse to distillation.

Procedures based on that described by Monier-Williams (1927) are normally used for reference purposes. These involve reflux distillation of the sample in an inert atmosphere with acid and trapping the sulphur dioxide produced in hydrogen peroxide in which it is oxidized to sulphuric acid. The amount of acid produced is then titrated with alkali. If necessary, the result can be confirmed gravimetrically by precipitation as barium sulphate. The method has been modernized by Thrasher (1961, 1966), and Shipton (1954) in particular also paid special attention to shortening the time required for the determination. For more rapid control purposes, however, many workers employ downward distillation of the sulphur dioxide, which is titrated directly with

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iodine as it distils over into water containing starch (Preservatives Determination Committee, 1928).

The more rapid methods described involve the determination of sulphur dioxide on an extract of the food rather than a distillate. These are mostly based on a fuchsin-formaldehyde colorimetric procedure (Joslyn, 1955; Carruthers, Heaney & Oldfield, 1965) or titration with iodine (Ripper), such procedures have been mainly applied to vegetable and fruit products, especially fruit pulps and wines, and also sugar. The fact that neutral sodium sulphite does not combine with carbonyl compounds and that the hydroxysulphonic acid compounds are rapidly decomposed on treatment with alkali were used by Ripper as the basis for the determination of total sulphur dioxide in wine by direct iodine titration. This involved a cold digestion with caustic alkali and then, after acidification, rapid titration with 0·02 N iodine using starch as indicator. The addition of acetone (Bennett & Donovan, 1943; Prater *et al.*, 1944) and formaldehyde (Ponting & Johnson, 1945; Potter & Hendel, 1951) reduced errors caused by the reduction of iodine by substances other than sulphur dioxide. Prater's method involving two titrations with iodine was adapted for use with dehydrated cabbage by Potter (1954). Titration of one aliquot of the sample extract gives a measure of the total reducing substances. Instead of adding acetone (cf. Prater *et al.*, 1944) to the other aliquot, hydrogen peroxide was employed to oxidize sulphite to sulphate. It was also claimed that compared with other methods, the end-point was sharper.

The factors affecting the accuracy and reproducibility of the rapid iodometric titration for the direct determination of sulphur dioxide have been discussed by Joslyn & Braverman (1954). Apart from a tentative method described in a privately circulated BFMIRA Report (Anderton & Locke, 1956), no procedures for the rapid direct determination of sulphur dioxide in meat products were found during the authors' literature search. The purpose of this paper was to develop a method for determining the total sulphur dioxide on a water extract of fresh sausage such that it gave results in good agreement with a reference distillation procedure.

Experimental

I. *Reference method involving distillation*

The Shipton modification of the Monier-Williams method was employed (Pearson, 1970).

II. *Rapid methods applied to macerate of sample*

The various methods were applied to 10% and 20% extracts made by macerating fresh sausage samples with water and filtering (see Method II(c) below).

(a) *Rosaniline—formaldehyde colorimetric method.* The method devised for sugar (Carruthers *et al.*, 1965) was applied to the filtrate.

(b) *Direct titration with iodine.* The sample filtrate was digested in the cold for 15 min with alkali, acidified and the 'total' sulphur dioxide titrated with 0·05 N iodine.

(c) *Potter's iodine titration method.*

Reagents

0·05 N iodine
5 N hydrochloric acid
5 N sodium hydroxide
3% hydrogen peroxide
1% starch solution

Procedure

The fresh sausage sample was prepared by mincing and mixing in a pestle and mortar. 50 g comminuted sample was blended for 90 sec with 250 ml water in a mechanical blender (MSE Atomix) and filtered as rapidly as possible through a large fluted filter paper. Into each of two 250 ml conical flasks A and B were pipetted 75 ml of the 20% extract, followed by 75 ml water and 5 ml of 5 N sodium hydroxide. The solution was stirred gently using a magnetic stirrer, avoiding beating air into the solution and then allowed to stand for exactly 20 min.

To A was added 7·0 ml of 5 N hydrochloric acid and 10 ml of starch solution and, stirring gently, the liquid was titrated immediately with 0·05 N iodine to a definite blue colour (A–ml).

To B was added 7·0 ml of 5 N hydrochloric acid, 2 ml of 3% hydrogen peroxide and 10 ml of starch solution and, stirring gently, the liquid was titrated immediately with 0·05 N iodine to a definite blue colour (B–ml).

$$\text{Sulphur dioxide in sample (ppm)} = \frac{250\,(A-B) \times 1600}{75 \times \text{weight of sample taken}}$$

$$= 107\,(A-B) \text{ if } 50 \text{ g is taken.}$$

Results and discussion

Preliminary findings

All results of rapid methods were compared against Shipton's reference procedure, which gave recoveries of 96–98% SO_2 when tested with sulphite solution.

The acid-bleached fuchsin—formaldehyde colorimetric method (IIa) gave much higher results than the figures given by the reference method. The reaction is interfered

with by a large number of substances and it was difficult to assess accurately the blank.

Direct titration of the extract with iodine gave very high results whereas after digestion with alkali and acidification (method IIb) fairly reasonable and comparable figures were obtained in spite of the rather poor end-point. It was apparent, however, that the blank varied considerably according to the particular sausage recipe employed. The method could only be considered satisfactory if a reliable blank were known. This would apply in routine factory control where products manufactured to the same recipe are examined continually.

The above difficulties were largely overcome by titrating the extract using Potter's method IIc and the results obtained were comparable with those given by the reference method. The method was therefore submitted to further investigation by considering factors which might influence the results with a view to getting results as close to the reference figures as possible. All the following factors were examined in relation to pork sausage extracts.

Effect of the time of maceration

A sample of fresh sausage was submitted to various maceration times ranging from ½ to 2 min. From the results in Table I, it would appear that the 'optimum' time of maceration is 1½ min. Presumably lower times gave incomplete extraction and larger periods resulted in losses due to oxidation, probably encouraged by heat and the beating of air into the liquid. A maceration time of 1½ min is therefore recommended.

TABLE 1. Effect of maceration time on the recovery of sulphur dioxide using Potter's titration

Maceration Time (min)	Volume of 0·05 N iodine used (ml) Extract	Blank	Sulphur dioxide ppm
½	3·1	0·5	277
1	3·4	0·6	299
1½	3·5	0·65	304
2	3·8	1·1	288

Effect of the time of digestion with alkali

Fresh sausage samples were examined by Potter's method using cold alkali digestion times of 10 and 20 min and by Shipton's reference procedure. From the results in Table 2, it is apparent that cold digestion for 20 min gave values closer to those obtained with the reference method. In view of the alkaline pH, losses due to oxidation should be minimal and the differences are more likely to be due to the time required to release the 'bound' sulphur dioxide. Cold digestion for 20 min with alkali is therefore recommended.

TABLE 2. Effect of time of digestion of pork sausage extracts with alkali on the recovery of sulphur dioxide using Potter's titration

Sample no.	Digestion time of 10 min. Volume of 0·05 N iodine (ml)		Sulphur dioxide ppm	Digestion time of 20 min Volume of 0·05 N iodine (ml)		Sulphur dioxide ppm	Sulphur dioxide using reference method ppm
	Extract	Blank		Extract	Blank		
1	3·5	0·6	309	3·4	0·6	299	307
2	3·4	0·6	299	3·3	0·5	299	318
3	3·8	0·7	331	3·9	0·7	341	339
4	4·0	0·6	363	4·1	0·6	373	378

Effect of other factors

The use of the other binding agents such as formaldehyde and acetone produced unsatisfactory end-points in the titration. In order to save time in routine control and reduce possible losses of the preservative some samples were filtered through muslin. Although the filtration was much quicker than when paper was used, it was very difficult to decide on the position of the end-point. This was presumably due to the effect of the interfering solid matter which would pass through muslin.

Evaluation of results using the rapid method

The results obtained on twelve samples of fresh pork sausage are quoted in Table 3. From statistical analysis the means and ranges of values for the two methods are as follows:

Reference method: 341 ± 28 ppm (coeff. of variation 8·22%)
Rapid method: 336 ± 27 ppm (coeff. of variation 8·02%)

Applying the t-test, t = 2·081. There is good correlation between the two methods, and there is only about 5% probability of the results of the rapid procedure not being comparable with those using the reference technique. The recovery from sulphite solutions using the rapid method was 100%. The method gave high results, however, where the sample had badly decomposed. This corresponds to previous findings with dehydrated vegetables which had undergone lengthy storage.

Conclusions

The total sulphur dioxide of fresh pork sausage can be rapidly determined by applying Potter's titration method to the sample macerate. The results obtained are in good agreement with those from the reference method. By dispensing with the distillation

step it is possible for the analyst to cope with several samples simultaneously. Although the method does not appear to be applicable to badly deteriorated samples this is unlikely to be a serious disadvantage in practice as the factory control chemist is normally only concerned with freshly prepared choppings.

TABLE 3. Comparison of results obtained for the determination of sulphur dioxide in twelve samples of fresh sausage using the recommended rapid method and the reference procedure

Sample no.	Reference method		Rapid method		
	Volume of 0·05 N NaOH used (ml)	Sulphur dioxide ppm	Volume of 0·05 N iodine used (ml) Extract	Blank	Sulphur dioxide ppm
5	10·4	333	3·8	0·7	331
			3·8	0·6	341
6	10·8	346	3·8	0·7	331
			3·8	0·7	331
7	10·6	339	3·9	0·6	352
			3·9	0·7	341
8	10·6	339	3·8	0·7	331
			3·9	0·7	346
9	9·95	318	3·4	0·6	299
			3·3	0·5	299
10	9·6	307	3·5	0·6	309
			3·4	0·6	290
11	8·9	285	3·3	0·6	288
			3·3	0·6	288
12	12·3	394	4·2	0·6	384
			4·3	0·7	384
13	11·8	378	4·0	0·6	363
			4·1	0·6	373
14	11·0	352	4·0	0·7	352
			4·0	0·7	352
15	11·1	355	4·0	0·7	352
			4·0	0·7	352
16	10·7	342	3·7	0·6	331
			3·7	0·6	331

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Über die Einwirkung von Sulfit auf einige DPN hydrierende Enzyme

Von

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Mit 3 Textabbildungen

(Eingegangen am 14. Juni 1956)

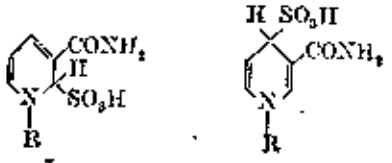
Während der in unserem Laboratorium laufenden Untersuchungen über die Milchsäuredehydrogenase aus Herzmuskel wurde bei der Wirksamkeitsbestimmung reinster Fraktionen die Beobachtung gemacht, daß die Umsatzzahlen der Brenztraubensäure-Hydrierung mit DPNH[1] stark schwankende Werte ergaben. So erhielt man mit einem enzymatisch dargestellten DPNH-Präparat den in der Literatur angegebenen Wert von etwa 30000 Mol/Min/100000 g Enzym, während ein mit Dithionit reduziertes DPN (OHLMEYER [10]) nur etwa die Hälfte dieser Zahl erreichte. Hier mußte ein Hemmstoff vorhanden sein, in welchem wir das aus dem Dithionit entstehende Sulfit-Ion vermuteten. Es wurden deshalb Versuche angestellt, ob sich die Reaktion BTS + DPNH + $H^{\oplus}$ ⇌ MS + $DPN^{\oplus}$ durch Zusatz von Sulfit hemmen läßt. Hierzu setzten wir zum System aus krist. MDH + BTS + enzymatisch dargestelltem DPNH bei pH 7,2 kleine Mengen Sulfit und erreichten schon mit 2,5 γ Sulfit pro ml Testlösung eine Hemmung um 50%.

Es stellte sich aber heraus, daß für diesen Hemmeffekt nicht eine Reaktion des hydrierten Coenzyms mit Sulfit verantwortlich ist, sondern eine winzige Menge von DPN, die in allen enzymatisch erhaltenen DPNH-Präparaten vorhanden ist. Gab man nämlich zum obigen Ansatz von vornherein zusätzliches DPN zu, so stieg der Hemmeffekt sehr deutlich an. Auch die Rückreaktion, die sich bei pH 7,2 in Anwesenheit von viel Enzym und viel Lactat im Anfangsverlauf gut messen läßt, erwies sich als äußerst stark durch Sulfit hemmbar. Hieraus war der Schluß zu ziehen, daß die beobachtete Wirkung des Sulfits auf einer Reaktion mit DPN beruhen mußte.

Ein Additionsprodukt aus Sulfit und DPN wurde 1938 von MEYERHOF, OHLMEYER u. MÖHLE [7] spektroskopisch beobachtet, für das von

[1] Folgende Abkürzungen werden benützt: Milchsäuredehydrogenase MDH, Diphospho-pyridin-nucleotid DPN, hydriertes Diphospho-pyridin-nucleotid DPNH, Brenztraubensäure BTS, Milchsäure MS.

Kuhn die Struktur einer „Aldehyd-bisulfit-Verbindung" (Ia) vorgeschlagen wurde. Das Addukt zeigt im Gegensatz zu DPN eine starke Absorption bei 320 mμ. Diese läßt sich in einer verdünnten Lösung beider Komponenten ($2 \cdot 10^{-4}$ m an Sulfit bzw. $5 \cdot 10^{-5}$ m an DPN) nicht erkennen. Bei hoher Verdünnung ist nämlich die Additionsverbindung



Ia Ib

nahezu völlig in die Komponenten dissoziiert. Eine spektrophotometrische Bestimmung der Gleichgewichtskonstanten k lieferte den immerhin recht großen Wert von $k = 0{,}5 \cdot 10^5$. Dieser Berechnung liegt ein molarer Extinktionskoeffizient von $\varepsilon = 4{,}8 \cdot 10^6$ cm²/Mol bei 320 mμ zugrunde, den wir bei 200fach molarem Überschuß von Sulfit über DPN bestimmt haben. Bei 340 mμ betrug $\varepsilon = 3{,}5 \cdot 10^6$, das ist etwa der von Colowick [3] angegebene Wert ($3{,}36 \cdot 10^6$). Fügt man zu einer Lösung von DPN und Sulfit, die wegen der nahezu vollständigen Dissoziation bei 320 mμ fast keine Absorption zeigt, steigende Menge krist. MDH, so taucht exakt im Bereich der Absorption des DPN-Sulfitaddukts momentan eine Absorptionsbande auf, deren Intensität in weitem Bereich proportional mit der Proteinmenge zunimmt (Abb. 1).

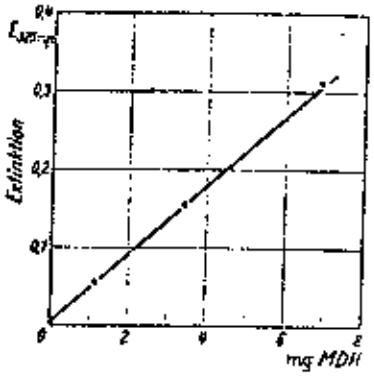


Abb. 1. Abhängigkeit der Menge des DPN-Sulfit-MDH-Komplexes von der Protein-Konzentration. Zu einer Lösung von $6 \cdot 10^{-4}$ Mol Sulfit und $6 \cdot 10^{-7}$ Mol DPN in 3 ml 0,1 m Phosphatpuffer pH 7,2 wurden steigende Mengen kristallisierter MDH gegeben. Nach kurzem Umschütteln wurde die Extinktion bei 320 mμ abgelesen und von diesem Wert die entsprechende DPN-Sulfit- bzw. Enzym-Sulfit-Extinktion abgezogen

Gleichzeitig nimmt, wie bei dem eingehender untersuchten DPN-CN-Addukt (Colowick [3]), die Extinktion der Hauptbande von 260 mμ ab, da der an dieser Stelle von Pyridiniumteil der Molekel herrührende Absorptionsbeitrag zurückgeht. In Abb. 2 sind die Absorptionskurven der DPN-Sulfitlösung ohne und mit Protein neben derjenigen der Additionsverbindung aufgezeichnet.

Hieraus geht hervor, daß erstens das Sulfit zusammen mit DPN in Form seines Addukts an das Protein gebunden wird. Diesem Addukt ist wohl analog neueren Ergebnissen über die Struktur des DPNH und der DPN-CN-Verbindung, die einer Hydro-pyridin-4-sulfonsäure (I b)

Einwirkun

zuzuschreiben. Zw
fischen Protein ei
dargestellte strukt

Die Frage nach
ließ sich ebenfalls
wurde zu einer an

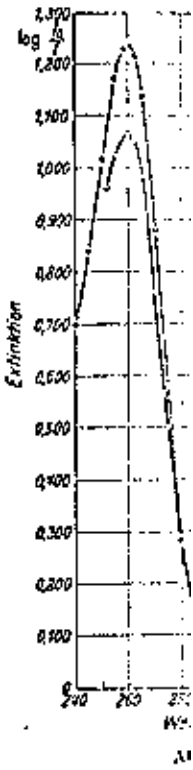
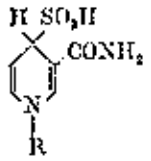


Abb. 2. Absorptionsspek
1,7 · 10⁻⁴ Mol DPN in 3
in 3 ml 0,1 m Phosphat
84 in 3 ml 0,1 m Phosph

eines großen mol
steigenden Menge
Sulfit pro ml Me
Protein-freien An
tionalen Anstieg
Selbst die 4fache
erhöhen. In Gegen
die doppelte Ext

:yd-bisulfit-Verbindung" (Ia) vorge-
:n Gegensatz zu DPN eine starke Ab-
h in einer verdünnten Lösung beider
: bzw. $5 \cdot 10^{-5}$ m an DPN) nicht er-
st nämlich die Additionsverbindung



Ib

. dissoziiert. Eine spektrophotometri-
:tskonstanten k lieferte den immerhin
5. Dieser Berechnung liegt ein molarer
$\cdot 10^6$ cm²/Mol bei 320 mμ zugrunde,
, wir bei 200fach molarem Überschuß
Sulfit über DPN bestimmt haben.
340 mμ betrug $\varepsilon = 3{,}5 \cdot 10^6$, das ist
a der von COLOWICK [3] angegebene
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Gleichzeitig nimmt, wie bei dem ein-
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:OLOWICK [3]), die Extinktion der
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:ser Stelle von Pyridiniumteil der
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:orptionskurven der DPN-Sulfirlösung
:nigen der Additionsverbindung auf-

:ns das Sulfit zusammen mit DPN in
:tein gebunden wird. Diesem Addukt
:sen über die Struktur des DPNH und
:ner Hydro-pyridin-4-sulfonsäure (I b)

zuzuschreiben. Zweitens muß die Affinität der Sulfonsäure zum spezifischen Protein eine außerordentlich große sein, da sonst die in Abb. 1 dargestellte strikte Proportionalität nicht zu erklären wäre.

Die Frage nach der Anzahl der vom Protein gebundenen Moleküle ließ sich ebenfalls spektrophotometrisch beantworten. Zu diesem Zweck wurde zu einer ausreichend konzentrierten MDH-Lösung in Gegenwart

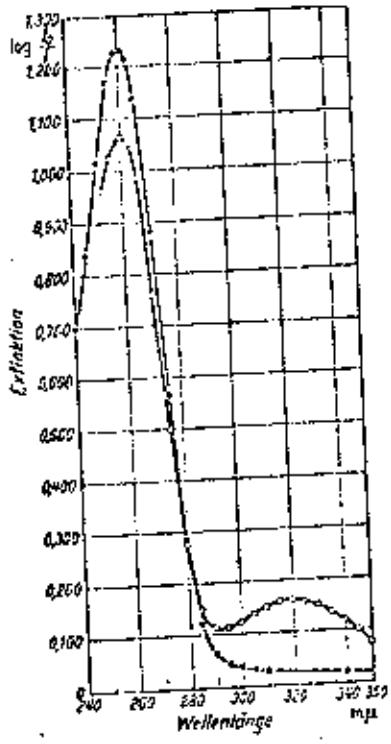


Abb. 2a

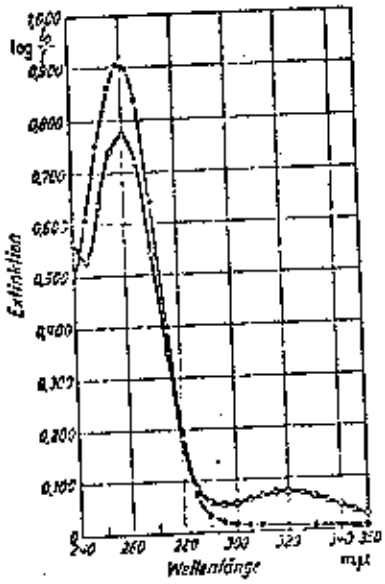


Abb. 2b

Abb. 2. Absorptionsspektren des DPN-Sulfit-Addukts mit und ohne MDH. a ohne Protein ●——● $1{,}7 \cdot 10^{-4}$ Mol DPN in 3 ml 0,1 m Phosphatpuffer pH 7,2; ○——○ $1{,}7 \cdot 10^{-4}$ Mol DPN und $9 \cdot 10^{-4}$ Sulfit in 3 ml 0,1 m Phosphatpuffer pH 7,2. b mit Protein ●——● $1{,}25 \cdot 10^{-4}$ Mol DPN und $8 \cdot 10^{-4}$ Mol Sulfit in 3 ml 0,1 m Phosphatpuffer pH 7,2; ○——○ $1{,}25 \cdot 10^{-4}$ Mol DPN, $8 \cdot 10^{-4}$ Mol Sulfit und 1,75 mg MDH in 3 ml 0,1 m Phosphatpuffer pH 7,2

eines großen molaren Überschusses von DPN Sulfit in kleinsten, ansteigenden Mengen zugesetzt. Bereits bei der Konzentration von 1 γ Sulfit pro ml Meßlösung betrug die Extinktionszunahme gegenüber dem Protein-freien Ansatz bei 320 mμ 0,032, um nach einem direkt proportionalen Anstieg bei 4 γ den Sättigungswert von 0,072 zu erreichen. Selbst die 4fache Sulfitmenge vermochte diesen Betrag nicht mehr zu erhöhen. In Gegenwart der doppelten Menge MDH wird in gleicher Weise die doppelte Extinktion (0,144) gefunden. Aus den Verhältnissen von

eingesetzten Protein und beobachteter Extinktion bei Sättigung läßt sich die Anzahl gebundener DPN-Sulfitmoleküle recht genau zu 4 auf 1 Mol Eiweiß (MG 135 000) bestimmen.

Auf Grund von stöchiometrischen Hemmversuchen mit p-Chlormercuribenzoat am gleichen Enzym kommt Neilands [8] zu einer Zahl von 2 DPN-Molekeln, die pro Mol Eiweiß gebunden werden.

Nahe bis an den Bereich der Sättigung wird das gesamte Sulfit in Form des Addukts an Protein gebunden. So beträgt bei Zugabe von 3 γ Sulfit die Extinktionszunahme wie gesagt 0,032, was 10,4 γ DPN-Sulfit pro Ansatz entspricht; das ist nahezu die theoretisch mögliche aus 3 γ Sulfit durch Addition an DPN sich bildende Adduktmenge

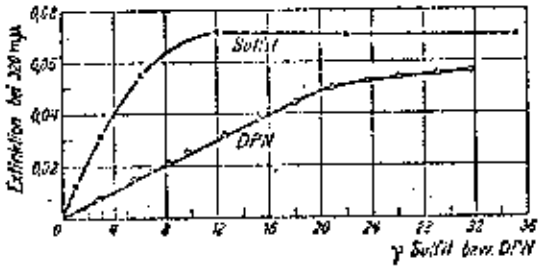


Abb. 2. Abhängigkeit der Extinktion des DPN-Sulfit-MDH-Komplexes von der Sulfit- bzw. DPN-Konzentration. Im ersten Fall (——) enthält die Meßlösung 1,2 · 10^{-5} Mol DPN und 1,78 mg krist. MDH in 3,2 ml 0,1 m Phosphatpuffer pH 7,2. (——) 5 · 10^{-7} Mol Sulfit und 1,78 mg MDH in 3,2 ml 0,1 m Phosphatpuffer pH 7,2

(17,8 γ). Dieselben quantitativen Verhältnisse findet man auch, wenn man umgekehrt die Sulfitkonzentration auf einem konstanten hohen Wert hält und das DPN in kleinen Mengen sukzessive zugibt (Abb. 3).

Um Näheres über die Stabilität des DPN-Sulfit-Enzymkomplexes zu erfahren, wurde ein vorläufiger Dialysierversuch angestellt. Bei 15 Std Dialyse des gesättigten Komplexes gegen die 100fache Menge Phosphatpuffer (pH 7,2) sank die Extinktion auf die Hälfte ab.

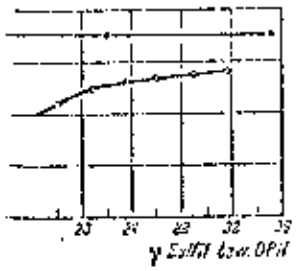
Wir haben uns bemüht, auf spektroskopischem Weg einen etwa entstehenden analogen Komplex aus DPN-CN mit MDH bei pH 7,2 nachzuweisen, da die Bildung eines DPN-CN-Addukts ohne Enzym bei pH 10 durch Meyerhof [7] und später von Colowick u. Mitarb. [3] in seinem charakteristischen Spektrum beschrieben wurde. Es zeigte sich jedoch, daß selbst in Gegenwart einer 15fach höheren CN^--Konzentration, verglichen mit dem Sulfit, keinerlei Absorption im Bereich von 300—350 mμ wahrzunehmen war. Dies stimmt mit der Wirkungslosigkeit des Cyanids als Hemmstoff bei der enzymatischen BTS-Hydrierung überein.

Auch am *Hydroxylamin* liegen gleichartige Untersuchungen von Karlan u. Mitarb. [4, 5] vor, welche zeigten, daß die Alkoholdehydrogenasen

...teter Extinktion bei Sättigung läßt ...-Sulfitmolekel recht genau zu 4 auf ...men.

...hen Hemmversuchen mit p-Chlor... kommt Neilands [8] zu einer Zahl ... Eiweiß gebunden werden.

...sättigung wird das gesamte Sulfit in ...bunden. So beträgt bei Zugabe von ... wie gesagt 0,032, was 16,4 γ DPN-... ist nahezu die theoretisch mögliche ... DPN sich bildende Adduktmenge



...-Sulfit-MDH-Komplexes von der Sulfit- bzw. DPN-... Meßlösung 1,2 · 10⁻⁴ Mol DPN und 1,75 mg krist. MDH ... Sulfit und 1,75 mg MDH in 3,2 ml 0,1 m ... 7,2

Verhältnisse findet man auch, wenn ...tration auf einem konstanten hohen ... Mengen sukzessive zugibt (Abb. 3).

...ät des DPN-Sulfit-Enzymkomplexes ...ger Dialysierversuch angestellt. Bei ...omplexes gegen die 100fache Menge ... Extinktion auf die Hälfte ab.

... spektroskopischem Weg einen etwa ... aus DPN-CN mit MDH bei p_H 7,2 ...es DPN-CN-Addukts ohne Enzym bei ... später von Colowick u. Mitarb. [3] ...ktrum beschrieben wurde. Es zeigte ...art einer 15fach höheren CN^--Konzen..., keinerlei Absorption im Bereich von ... Dies stimmt mit der Wirkungslosig...ei der enzymatischen BTS-Hydrierung

... gleichartige Untersuchungen von Kap... zeigten, daß die Alkoholdehydrogenasen

aus Hefe (Hefe-ADH), noch viel stärker die aus Leber (Leber-ADH) durch Hydroxylamin gehemmt werden. Am Leberenzym konnte die Bindung von DPN + Hydroxylamin im UV durch eine starke Absorptionsbande bei 300 mμ sichtbar gemacht werden. Wir haben ebenfalls Hydroxylamin als Hemmstoff der MDH geprüft und nur eine sehr schwache Wirkung gefunden; während 2,5 γ Sulfit pro ml Testlösung, wie oben beschrieben, sofort die Reaktionsgeschwindigkeit auf die Hälfte herabsetzten, bewirkt die 100fach molare Menge an Hydroxylamin nur eine Hemmung um 20%. Im UV-Spektrum war selbst bei Anwesenheit von 1 mg Hydroxylamin keine Veränderung des Spektrums von MDH + DPN wahrzunehmen.

Wir untersuchten die Wirkung des Sulfits an weiteren DPN-spezifischen Enzymen: Einen Hemmeffekt ähnlicher Größenordnung konnten wir bisher nur bei der Äpfelsäure-Dehydrogenase feststellen. Mit 5 γ Sulfit pro ml Testlösung wurde eine Hemmung der Hydrierungsgeschwindigkeit von Oxalessigsäure um 33% beobachtet. Viel weniger empfindlich ist Glutaminsäure-Dehydrogenase und α-Glycerophosphat-Dehydrogenase. Die Leber-ADH[1] zeigt ebenfalls eine geringe, aber deutliche Hemmbarkeit. Hier kommt man mit 50 γ Sulfit pro ml Testlösung zu einer Hemmung um 70%. Das analoge Enzym aus Hefe ist im Gegensatz dazu gegen Sulfit völlig unempfindlich. Dasselbe gilt für die Glycerinaldehyd-3-phosphat-Dehydrogenase (PGDH). In Übereinstimmung mit den Hemmbefunden findet man bei den unempfindlichen Proteinen keine Extinktionszunahme bei 320 mμ, wenn Sulfit und DPN in der Lösung vorhanden sind.

Die äußerst große Empfindlichkeit gegenüber Sulfit, die wir bisher an 2 Schlüsselenzymen des intermediären Stoffwechsels dartun konnten, gibt vielleicht eine Erklärung für die altbekannte, desinfizierende Eigenschaft der schwefligen Säure. Mit seleniger Säure wurde keine Hemmwirkung auf MDH beobachtet.

Diskussion

Aus den Ergebnissen der Hemmversuche und aus den spektroskopischen Messungen an unserem und an anderen Laboratorien scheinen sich bis heute mehrere Gruppen von Dehydrogenasen voneinander abzuheben. Die typischen gegen Jodacetat empfindlichen (Hefe-ADH und PGDH) werden durch Sulfit nicht gehemmt, mäßig jedoch durch Hydroxylamin. Glutaminsäure-DH und Glycerophosphat-DH werden mäßig durch Sulfit, stärker durch Hydroxylamin gehemmt. Die Leber-ADH, die äußerst empfindlich gegen Hydroxylamin ist, wird durch Jodacetat nicht, durch Sulfit und Cyanid mäßig gehemmt. Die gegen Sulfit hoch-

[1] Wir danken Herrn Privatdoz. Dr. O. Wieland, München, herzlich für die Überlassung einer Probe des krist. Enzyms.

empfindlichen Enzyme (Herzmuskel-MDH und Äpfelsäure-Dehydrogenase) lassen sich nur wenig durch Hydroxylamin vergiften.

In Tab. 1 sind diese Unterschiede übersichtlich zusammengestellt.

Tabelle 1*. *Die Wirkung von Hydroxylamin, Sulfit und Jodacetat auf verschiedene Dehydrogenasen*

Enzym	Hydroxylamin	Sulfit	Jodacetat**
Hefe-ADH	+++	0	+
PGDH	+	0	+
Glutamins.-Dehydr.	+++	++	0
Glycero-phosph.-Dehydr.	+++	++	0
Leber-ADH	++++	++	0
Äpfels.-Dehydr.	+***	+++	0
Herzmuskel-MDH	+***	++++	0

* Die Testlösungen enthielten je nach Umsatzzahl der Enzyme zwischen 0,1—3 γ Protein pro ml. Da die untersuchten Hemmstoffe stets in einer mehr als 1000fach mehrfachen Konzentration, bezogen auf Enzym, zur Anwendung kamen, spielt eine Variation des Eiweißgehaltes zur Beurteilung des Hemmeffektes keine grundsätzliche Rolle. Die Proteinkonzentrationen wurden so gewählt, daß ohne Hemmstoff etwa $3 \cdot 10^{-6}$ Mol Substrat pro ml Testlösung in der Minute umgesetzt wurden (T = 25°).

0 = 25% Hemmung mit > 500 γ Hemmstoff
+ = 25% Hemmung mit 50—100 γ Hemmstoff
++ = 25% Hemmung mit 20— 50 γ Hemmstoff
+++ = 25% Hemmung mit 2— 10 γ Hemmstoff
++++ = 25% Hemmung mit < 2 γ Hemmstoff

** Mit Enzym 5 min bei 0° vorinkubiert.

*** Hemmung wohl durch Reaktion mit dem Substrat verursacht.

Diese bedeutenden Differenzen scheinen uns sehr für verschiedene Wirkungsmechanismen der Dehydrogenasen zu sprechen. Die Rolle der wohl in allen diesen Enzymen vorhandenen SH-Gruppen und des in der Mehrzahl aufgefundenen Zinks [14, 15, 16, 17, 18] wird hierdurch eher schwerer verständlich.

Beschreibung der Versuche

MDH aus Herzmuskeln wurde nach der Methode von Straub [12] isoliert und zweimal umkristallisiert. 70% reines DPN wurde von der Firma C. F. Boehringer & Söhne, Mannheim geliefert. Enzymatische Darstellung von DPNH: Das nach Nygaard u. Thomsen [14] dargestellte DPNH enthielt noch große Mengen des Bicarbonat-Carbonat-Puffers (ca. 20% Gehalt an DPNH). Um diese Verunreinigungen abzutrennen, lösten wir je 150 mg Rohprodukt in 5 ml Methanol, zentrifugierten den Rückstand ab und fällten mit 32 ml Äthanol und 25 ml Äther bei 0°C aus dem Überstand das hydrierte Coenzym in einer Reinheit, die der des Ausgangsprodukts entsprach (70%). Ausbeute 40—50%. Alle Teste wurden in einer

uskel-MDH und Äpfelsäure-Dehydr.
rch Hydroxylamin vergiften.

chiede übersichtlich zusammengestellt.

Hydroxylamin, Sulfit und Jodacetat
ne Dehydrogenasen

	Hydroxylamin	Sulfit	Jodacetat**
	+++	0	+
	+	0	+
	+++	++	0
	+++	++	0
	++++	++	0
	+***	+++	0
	+***	++++	0

nach Umsatzzahl der Enzyme zwischen
rsuchten Hemmstoffe stets in einer mehr als
rzogen auf Enzym, zur Anwendung kamen,
es zur Beurteilung des Hemmeffektes keine
zentrationen wurden so gewählt, daß ohne
pro ml Testlösung in der Minute umgesetzt

mit > 500 γ Hemmstoff
50—100 γ Hemmstoff
20— 50 γ Hemmstoff
ung mit 2— 10 γ Hemmstoff
ung mit < 2 γ Hemmstoff
ubiert.

n mit dem Substrat verursacht.

scheinen uns sehr für verschiedene
hydrogenasen zu sprechen. Die Rolle der
handenen SH-Gruppen und des in der
4, 15, 16, 17, 18] wird hierdurch eher

ng der Versuche

der Methode von STRAUB [12] isoliert und
PN wurde von der Firma C. F. Boehringer
tische Darstellung von DPNH: Das nach
o DPNH enthielt noch große Mengen des
Gehalt an DPNH). Um diese Verunreini-
ng Rohprodukt in 5 ml Methanol, zentri-
n mit 32 ml Äthanol und 25 ml Äther bei
coenzym in einer Reinheit, die der des Aus-
ente 40—50%. Alle Teste wurden in einer

Temperaturcuvette bei 25°C durchgeführt. Die Testzusammensetzung war nach den für die Herzmuskel-MDH optimalen Bedingungen:
1,9 ml 0,1 m Phosphatpuffer pH 7,2; 0,03 ml 0,05 m ITS und 0,05 ml DPNH = $1 \cdot 10^{-7}$ Mol DPNH.

Die Reaktion wurde durch Zugabe von 0,02 ml einer entsprechend verdünnten MDH-Lösung gestartet. Gemessen wurde die Abnahme der Extinktion bei 366 mμ im Lauf der 1. min.

Die Testzusammensetzung für die MS-Dehydrierung war:
1,78 ml 0,1 m Phosphatpuffer pH 7,2; 0,2 ml 0,5 m MS-Lösung; 0,02 ml DPN = $1,5 \cdot 10^{-6}$ Mol DPN.

Es wurde die Zeit für die Extinktionszunahme der DPNH-Bande um 0,030 bei 366 mμ gestoppt.

Kristallisierte Alkoholdehydrogenase aus Hefe isolierten wir nach der Methode von RACKER [11]. Die Testzusammensetzung enthielt:
0,1 ml Acetaldehyd = $2,3 \cdot 10^{-6}$ Mol; 0,07 ml DPNH = $0,4 \cdot 10^{-7}$ Mol; 3,03 ml 0,1 m Phosphatpuffer pH 7,2.

Hier wie auch in den folgenden Enzymreaktionen wurde ebenfalls die Reaktion durch 0,02 ml einer verd. Enzymlösung ausgelöst und nach dem Beispiel der ITS-Hydrierung kinetisch verfolgt. Die Leber-ADH war aus Pferdeleber nach BONNICHSEN [2] dargestellt.

Testzusammensetzung:
1,01 ml 0,1 m Phosphatpuffer pH 7,2; 0,04 ml DPNH = $4 \cdot 10^{-7}$ Mol; 0,03 ml 0,5 m Acetaldehyd.

Die α-Glycerophosphat-Dehydrogenase kristallisierten wir nach der Methode von BÜCHER u. Mitarb. [1].

Testzusammensetzung:
0,07 ml DPNH = $0,4 \cdot 10^{-7}$ Mol; 0,1 ml Triosephosphat-Lösung ≈ $3,2 \cdot 10^{-7}$ Mol Dioxyacetonphosphat; 3,12 ml 0,1 m Phosphatpuffer pH 7,2. Die Triosephosphatlösung wurde aus Fructose-1,6-diphosphat mit Hilfe von Aldolase nach der Vorschrift von MEYERHOF [9] dargestellt.

Die Äpfelsäure-Dehydrogenase wurde nach der Methode von STRAUB [13] isoliert.

Testzusammensetzung:
1,91 ml 0,1 m Phosphatpuffer pH 7,2; 0,02 ml Oxalessigsäure = $1 \cdot 10^{-6}$ Mol; 0,05 ml DPNH = $4 \cdot 10^{-7}$ Mol.

Die Glutaminsäure-Dehydrogenase lieferte uns die Firma C. F. Boehringer & Söhne, Mannheim.

Testzusammensetzung:
1,81 ml 0,1 m Phosphatpuffer pH 7,2; 0,1 ml 0,1 m α-Ketoglutarsäure; 0,02 ml m Ammonium-acetat, 0,05 ml DPNH = $4 \cdot 10^{-7}$ Mol.

Die 3-Phosphoglycerinaldehyd-Dehydrogenase bezogen wir von der Firma C. F. Boehringer & Söhne, Mannheim. Die Testzusammensetzung war für die Hinreaktion: 0,04 ml DPN = $2 \cdot 10^{-7}$ Mol; 0,04 ml Triosephosphat-Lösung = $5 \cdot 10^{-7}$ Mol Phosphoglycerinaldehyd; 0,06 ml Arseniat = $6 \cdot 10^{-6}$ Mol; 1,86 ml 0,05 m Triäthanolamin-HCl-Puffer pH 7,5.

Für die Rückreaktion benützten wir folgende Testlösung:
2,2 ml 0,05 m Triäthanolamin-HCl-Puffer pH 7,5; 0,07 ml 0,5 m $MgSO_4$; 0,1 ml 0,1 m Hydrazin; 0,08 ml 3-Phosphoglycerinsäure = $1,2 \cdot 10^{-6}$ Mol; 0,05 DPNH = $6 \cdot 10^{-7}$ Mol; 0,2 ml ATP = $1,7 \cdot 10^{-6}$ Mol; 10 γ Kinase.

Die Reaktion wurde ausgelöst durch 20 γ Dehydrogenase.

Wir danken der Firma C. H. Boehringer & Sohn, Ingelheim, für die Unterstützung dieser Arbeit.

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Biochemische Zeitschrif

Über die

Aus dem hi

Bekanntlich best
löslichen Proteinen,
haben eine Reihe a
Keratine, resistent
tinen aber durch i
Mengen von jodierte
sonders starke Mol
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Spongins noch völli

Aus dem Mittelmeer
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Bestimmung der He
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lare Galaktose-Manno
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mit 5 ml n HCl im Ko
Zugabe von 5 ml n N
Flüssigkeit wurde der
Hexuronsäurereakti
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(From the Institute of Occupational Health, Helsinki, Finland)

SKIN ALLERGY TO SIMPLE GASEOUS SULPHUR COMPOUNDS

By

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Helsinki

Allergy to sulphur and to its simplest compounds seems to be very rare. In several wellknown monographs the opinion has been expressed that neither sulphur itself nor the sulphides ever cause eczema (Bonnevie) and that, as far as the etiology of occupational dermatitis is concerned, hydrogen sulphide and carbon disulphide are practically out of the question (Bering & Zitzke). In accordance with these statements I have not found in the literature any report on skin allergy to simple gaseous sulphur compounds. Therefore two cases observed by me are reported in the following:

Case 1. (Rec. N: 228 1948). Master builder in a sulphite cellulose factory, male, 30 years. Had previously suffered from infectious diseases only. In August 1948, itching, red patches and wheals with white borders developed on his palms and later on other areas of the skin. In addition he suffered from headache. The patient noticed that the wheals only appeared when he was working and then not until afternoon or evening. The lesions disappeared almost totally when the patient was two days away from work.

The patient's work place was near a reservoir into which hot waste liquor from the sulphite spirit mill is taken every three hours. At this time sulphur dioxide spreads into the immediate surroundings. The patient was thus exposed to the gas, especially when the wind was blowing from the reservoir.

Nov. 1, 1948: on presentation there were no pathological changes on the skin. Routine patch tests (20 substances).—Nov. 3, 1948: Intracutaneous tests: routine series (24 extracts)—, sulphur dioxide 1:100,000 0.01 ml—, 1:10,000 0.01 ml—, 1:1000 0.01 ml + (weak, probably unspecific reaction).

Nov. 4–Dec. 13, 1948: the patient made observations on variations of his skin trouble at his work place. He remained well as long as he was away from

work. After starting work he acquired pruritic wheals even on the first evening. During 6 days the patient tried to avoid the gas and when exposed to it used a gasmask. This time he remained well. Three other trials without the gasmask resulted in a recurrence on the first evening, once, or on the second, twice. By the following morning the patient had usually recovered. When the factory was not in operation for two weeks, there was no eruption.

Clinical exposure tests: Dec. 14, 1948: the patient was exposed to chlorine gas, 2 minutes above a vessel in which chlorine was developed. No eruption. Jan. 22, 1949: 10-11 a.m. The patient was exposed to sulphur dioxide above a vessel. At 9 p.m. small wheals on the neck, Jan. 27, 1949: 10-11 a.m. the patient was exposed to sulphur dioxide in a room, 39.8 PPM or 0.004 vol. per cent. At 6 p.m. urticarial wheals on the trunk and on the extremities. The eruption grew worse during the next 9 hours, after which it gradually disappeared.

This was a case of urticaria caused by sulphur dioxide. The positive clinical exposure test in a chamber with sulphur dioxide in a concentration of 0.004 vol. per cent for one hour showed that relatively slight exposure to this gas was sufficient to cause the urticarial wheals. The maximum allowable concentration to which a normal person can be exposed without toxic effect continuously for 8 hours daily is 0.001 vol. per cent.

Case 2. Rec. N: 360/1953. Woman clerk, 42 years. She had suffered from malaria in 1945, from nephritis in 1945 and 1947, and from polyarthritis in 1949. In 1952 she was treated for scabies with some "sulphur ointment" and acquired a universal dermatitis, which lasted about 20 days. In 1951 after blowing flowers of sulphur onto a rose she developed a patchy eruption on the face. This subsided in two to three days.

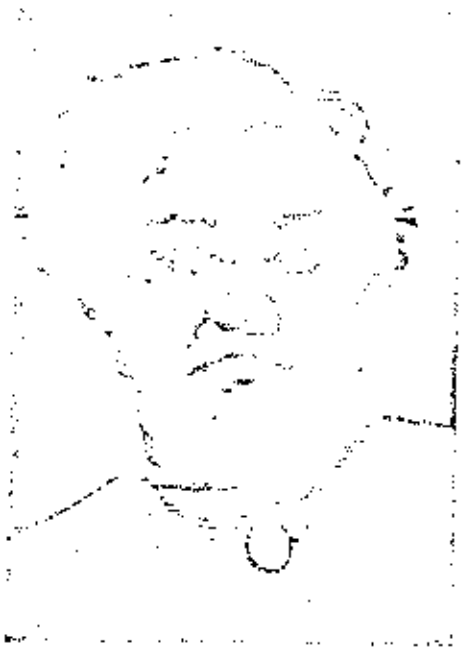
Nov. 3 and 6, and Dec. 1 and 10, 1953: in the mornings itching on the face, neck and forearms, on which sites a patchy eruption developed during the afternoons. In addition irritation of the eyes and headache. Her husband also had these latter symptoms but no skin changes. The eruption usually disappeared in 4–5 days.

A new drainpipe from a rayon factory had recently been built near the house where the patient was living, and she had noticed the smell of rotten eggs in the rooms, the strength of which varied, probably according to the wind.

Dec. 14, 1953: when consulting the Institute of Occupational Health for the first time, the patient had patches of reddish, scaling dermatitis on the eyelids, neck, arms and chest.

Routine patch tests: formalin 4 % ++++. Sulphur powder (Techn.) 100 % +++, 10 % +—, 5 % +++, 1 % ++(+), 0.1 % —. Carbon disulphide 20 % ++, 10 % +(+), 5 % +?, 1 % —. Tiolan ointment ++(+). Hydro-

gen sulphide 0.334 % (saturated aqueous solution)—. Alb-, Nyl-, Blood count Hb 14.1/91, E 4.60, L 0.90, L 6.000, N 57.5, Eo 2.5, Ba 0.5, Mon 6.5, Ly 33.0. During the few days when the tests were being made, the skin changes disappeared, and the patient returned home. After the first night there, she felt itching on the face in the morning, and the next morning the eruption recurred. Since it was aggravated in the following days, the patient moved to a



boarding-house. During the 23 days there she remained well.

Jan. 20, 1954: at 10 a.m. exposure to hydrogen sulphide 0.0015 vol. per cent for 10 minutes in a chamber. Immediately after the exposure a slight headache, and about 4 hours later itching on the face, neck and shoulders. Jan. 21, 1954: at 10 a.m. mild inflammatory changes on the skin areas mentioned above. New exposure to hydrogen sulphide 0.0014 vol. per cent for 20 minutes. The headache immediately became worse, there was vomiting, and oedematous, patchy and in part diffuse dermatitis developed on the face and neck at 12 o'clock. Jan. 22, 1954: the dermatitis was aggravated (Fig.). During these days the patient had fever up to 38.5° C. Jan. 23–26, 1954: Cortisone tab. 25 mg. X 4–2. Jan. 27, 1954: the skin changes had disappeared almost totally.

In a chemical analysis of the air in the patient's room 0.0004 vol. per cent hydrogen sulphide and 0.0001 vol. per cent carbon disulphide were found.

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In this case the patient was evidently sensitized to sulphur by an antiscabietic treatment. Subsequently she had an attack of dermatitis due to the flowers of sulphur. When a new drainpipe from the rayon factory was built near her home, she again acquired dermatitis of the exposed parts probably due to a low concentration of hydrogen sulphide (and carbon disulphide?) in the rooms, especially when the wind was suitable. That the patient was sensitized at least to sulphur itself was indicated by the patch test, which was positive even for a one per cent concentration, whereas the 8 control persons did not react even at 60 per cent. The positive patch test to carbon disulphide, 5–10 per cent against 20–50 per cent or more in the controls, and to "Tiolan", as well as the positive exposure test with hydrogen sulphide may also be due to hypersensitivity to sulphur itself. The patient was also sensitized to formalin, probably owing to previous use of antiperspirants for the axillae. This, however, can scarcely have any bearing on the attacks of dermatitis, during which the axillae were unaffected.

The patch test was negative to hydrogen sulphide, but the test concentration, a saturated aqueous solution, also was low. Anyhow the hypersensitivity of the patient can be considered to be high, because exposure to such a low concentration as 0,0012–0,0015 vol. per cent of hydrogen sulphide in the air for 10–20 minutes was sufficient to cause a violent attack of dermatitis on the exposed areas of the skin. The maximum allowable concentration (8 hours daily) for hydrogen sulphide and carbon disulphide is 0,002 vol. per cent, i.e. higher than those in the patient's room and those used in these experiments.

Sulphur is widely used for unspecific desensitization in various allergic states, which serves to emphasize its non-allergenic nature. In fact, as was mentioned earlier in this paper, sulphur is regarded as a noneczematogenic element. It has, however, a particular, irritating effect on the skin, which becomes dry and "craquelliert". Bonnevie, who used 25 per cent sulphur in lanolin for a patch test, saw never a positive

reaction, whereas Zurhelle has reported striking hypersensitivity to sulphur (as about 5 per cent ointment or even as "Sulfoderm" powder).

Similarly simple sulphides and hydrosulphides are regarded as nonallergenic (Bonnevie). On the other hand thiokol is an example of a polysulphide which (even as the fumes) rather frequently sensitizes the skin (Bonnevie, Pirilä). Another, simpler sulphur compound with a high sensitizing index is dixanthogene, which is used for antiseabietic treatment (Pirilä & Kilpiö, Polano). Sensitization to persulphates, which are used for the bleaching of wheat flour, is well known, and sodium sulphite and sulphur subchloride can also cause eczema (Bering & Zitzke, Bonnevie). Thioglycolic acid, which is commonly used in cold waving solutions, only rarely sensitizes the skin, and I have seen only two positive reactions of allergic type to it. On the other hand a newer cold wave preparation, thioglycerine, is an active sensitizer (Gasser, Burckhardt). The majority of the other eczematogenic compounds containing sulphur have relatively high molecular weights and often also ring structures. Examples are many medicaments, such as the sulphonamides, dimethylthianthrene ("Mitigal"), tetramethylsulphides, etc. The latter are also used as antioxidants and, like them, some other sulphur-containing vulcanizing agents can also sensitize the skin (Bonnevie & Marcussen etc.).

Thus, whereas probably the majority of the eczematogenic sulphur compounds have relatively complicated chemical structures, some fairly simple sulphur compounds can also sensitize the skin, according to the literature. This report serves to show that even very simple, gaseous sulphur compounds and sulphur itself are not ruled out as etiologic factors in urticaria and allergic eczema.

In the cases reported in this paper the gaseous sulphur compounds occurred as air contaminants in the patients' professional or home environments. With increasing industrilisation, atmospheric pollution, the excessive concentration of foreign matter in the air, has increased health hazards, especially

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in great industrial centers. The aim of this report is to stress that gases and other air contaminants must be taken into consideration as etiologic factors in skin diseases also.

SUMMARY

One case of urticaria due to sulphur dioxide and one case of dermatitis due to hydrogen sulphide are reported. Clinical exposure tests with sulphur dioxide and hydrogen sulphide gave positive results in the respective cases.

In the first case exposure to 0.004 vol. per cent sulphur dioxide for one hour was sufficient to cause urticarial wheals. The maximum allowable concentration (for normal persons continuously 8 hours daily) is 0.001 vol. per cent. In the second case exposure to 0.0012—0.0015 vol. per cent hydrogen sulphide for 20 minutes caused a violent attack of dermatitis on the exposed areas of the skin. The maximum allowable concentration for hydrogen sulphide is 0.002 vol. per cent. In this case, patch tests with 1 per cent sulphur and 5 per cent carbon disulphide were also positive.

The sensitizing powers of various sulphur compounds are discussed. The health hazards due to air pollution, especially in great industrial centers, are pointed out. It is stressed that gases and other air contaminants must be taken into consideration as etiologic factors in skin diseases also.

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ISTITUTO DI FARMACOLOGIA SPERIMENTALE DELLA R. UNIVERSITÀ DI PISA
DIRETTORE: PROF. ITALO SIMON

Dott. ARNALDO PIVA

RICERCHE FARMACOLOGICHE SUL SOLFITO DI SODIO

I) RAGIONE DELLA RICERCA.

Il solfito di sodio è un farmaco che ha una storia. Dopo essere stato portato alle stelle come rimedio insuperabile di ogni male, oggi non solo è completamente dimenticato, ma l'azione farmacologica del farmaco ci è assai poco nota; ci mancano le conoscenze più elementari sulla sua azione. Non conosciamo la dose minima letale, l'azione sul cuore e sul respiro, la fenomenologia che dà la morte, senza contare le altre ricerche più fini che si possono fare e che conviene forse praticare per rivalutare, nei limiti del giusto e del lecito, l'applicazione dei solfiti in terapia perchè la loro attività antisettica indubbia, la buona tolleranza dell'organismo animale verso di essi ci fanno nascere il dubbio che l'ostracismo ad essi dato in terapia sia più dovuto ad una reazione all'abuso imponderato piuttosto che al danno apportato in ogni caso dal rimedio.

Con questi concetti, persuaso d'una verità fondamentale, che l'applicazione terapeutica dei farmaci debba essere preceduta da uno studio farmacologico preciso, ho voluto studiare la tossicità del solfito di sodio e il meccanismo col quale produce la morte negli animali.

Le mie esperienze si dividono in due gruppi: azione generale, meccanismo della morte.

II) Azione generale del solfito di sodio e dose minima letale lontana.

Ho determinato la dose minima letale lontana per via endovenosa nei conigli ai quali iniettavo nella vena giugulare destra una soluzione normale di solfito di sodio (gr. 126.088 in un litro).

Ho cominciato ad iniettare delle dosi che non uccidevano i conigli e mano mano sono salito a dosi che li uccidevano sempre più rapidamente.

Avverto che degli animali venuti a morte ho praticata la autopsia ed ho pigliato i pezzi degli organi per gli esami istologici.

Riporto le esperienze:

1ª ESPERIENZA — 5 febbraio 1932, mattino.

Coniglio bianco-nero di gr. 1500.

N

Iniettati cc. 7 di soluzione — (gr. eq. 0.0846 per Kgr.).

I

Inizio dell'iniezione: 11.23'30".

A cc. 7 si è sospesa l'iniezione perchè i battiti cardiaci, sebbene validi, erano rallentatissimi ed accennavano ad un arresto definitivo del cuore.

Terminata l'iniezione alle ore 11.35'30" (tempo impiegato: 12 minuti).

Slegato, l'animale si riprese presto; in principio rifiutò il cibo, ma poi mangiò e stette bene.

2ª ESPERIENZA — 5 febbraio 1932, pomeriggio.

Coniglio rosso di gr. 1500.

N

Iniettati cc. 12 di soluzione — (gr. eq. 0.0080 per Kgr.).

I

Inizio dell'iniezione ore 15.15.

A cc. 5 il coniglio è scosso da convulsioni che si attenuano con la sospensione dell'iniezione per 1'; cuore sempre valido.

A cc. 10, il cuore è sempre valido, ma si nota un accentuato rallentamento dei suoi battiti per cui si sospende l'iniezione per 5'.

A cc. 10.5 è sospesa di nuovo l'iniezione per 3' e così pure a cc. 11.

Terminata l'iniezione alle ore 15.48: (tempo impiegato: 33 minuti).

Slegato, l'animale è in cattive condizioni, presenta respiro raro, superficiale e in 5' muore.

AUTOPSIA.

Polmoni di colore bruno-roseo; cuore flacido, arrestato in diastole; fegato, reni ed altri visceri normali.

ESAME ISTOLOGICO DEGLI ORGANI.

Reni: congestione dei capillari glomerulari e della midollare.

Polmoni: alveoli e piccoli bronchi ripieni di stravasi emorragici. Globuli rossi non deformati.

Miocardio: numerose emorragie capillari fra le fibre, che si presentano normali.

Fegato: parenchima e cellule epatiche normali.

3ª ESPERIENZA — 5 febbraio 1932, pomeriggio.

Coniglio bianco-nero di gr. 1400.

N

Iniettati cc. 8.4 di soluzione — (gr. eq. 0.0060 per Kgr.).

I

Inizio dell'iniezione: ore 17.

A cc. 5.5 i battiti del cuore sono scesi a 160, respiro 52.

A cc. 7, iniettati in 13.30' compaiono convulsioni; i battiti del cuore sono scesi a 96 con notevole aritmia. Polipnea: respiro superficiale.

A cc. 8.4, iniettati in 31', il coniglio è scosso da contrazioni tonico-cloniche e dopo poco, slegato, muore.

Terminata l'iniezione alle ore 17.31' (tempo impiegato: 31 minuti).

AUTOPSIA.

Cuore flaccido, arrestato in diastole. Polmoni di colore bruno-roseo: reni di forma e consistenza normale, corticale leggermente iperemica, midollare pallida. Fegato succulento con qualche nodulo di coccidiosi, cistifellea distesa da bile piuttosto pallida, vescica ripiena di urine gialla torbida.

ESAME ISTOLOGICO DEGLI ORGANI.

Reni: capillari glomerulari e della midollare notevolmente congesti. - Epiteli dei tuboli normali.

Polmoni: emorragie recenti nei bronchi piccoli e negli alveoli.

Miocardio: nessuna alterazione della fibra miocardica; qualche leggera emorragia capillare.

Fegato: normale.

4ª ESPERIENZA — 6 febbraio 1932, mattino.

Coniglio bianco-grigio di gr. 1200.

N

Iniettati cc. 6 di soluzione — (gr. eq. 0.0050 per Kgr.).

I

Inizio dell'iniezione: ore 11.15.

A cc. 4 iniettati in 15', il cuore è sempre valido mentre il respiro si è fatto un pò dispnoico e l'animale è preso da convulsioni.

A cc. 5 iniettati in 20', il coniglio è calmo, con cuore valido, ma coi battiti diminuiti di numero, respirazione superficiale e dispnoica.

A cc. 5.5, in 25, il cuore è sempre valido ma con 132 battiti, respiro 80 dispnoico, alcune convulsioni.

Terminata l'iniezione alle ore 11,45 (tempo impiegato: 30 minuti).

Il coniglio, slegato, sta bene e mangia.

5ª ESPERIENZA — 8 febbraio 1932, mattino.

Coniglio bigio di gr. 1450.

N

Iniettati cc. 8 di soluzione — (gr. eq. 0.0055 per Kgr.).

I

Inizio dell'iniezione: ore 11.15.

A cc. 4 iniettati in 15', cuore valido, respiro normale.

A cc. 5.2 il coniglio è preso da convulsioni che terminano con la sospensione dell'iniezione per 1'; respiro dispnoico.

A cc. 7.4 iniettati in 28'. L'animale presenta respiro superficiale dispnoico, il cuore ha 140 battiti.

Alle ore 10.45', termine dell'iniezione, il cuore presenta 92 battiti.

Tempo impiegato per l'iniezione: 30 minuti.

Slegato, l'animale è molto depresso, è coricato su di un fianco ed ha 60 respiri. Dopo 15 minuti presenta paralisi prima degli arti inferiori, poi completa e in 25' muore.

AUTOPSIA.

Cuore arrestato in diastole, polmoni bruno-rosei di aspetto normale, fegato succulento, reni leggermente ipermici, vescica non molto distesa da urina giallo torbida.

ESAME ISTOLOGICO DEGLI ORGANI.

Reni: i capillari della midollare verso la corticale sono congesti. Il calibro degli epiteli tubulari è normale.

Polmoni: alveoli e piccoli bronchi infarciti di globuli rossi stravasati.

Miocardio: normale.

Fegato: normale.

6ª ESPERIENZA — 9 febbraio 1932, mattino.

Coniglio bigio di gr. 1400.

N

Iniettati cc. 7.4 di soluzione — (gr. eq. 0.0053 per Kgr.).

I

Inizio dell'iniezione: ore 11.45.

A cc. 5 il coniglio è calmo, con cuore valido e respiro un pò dispnoico.

A cc. 7 il cuore è notevolmente rallentato (120 battiti) e presenta aritmia.

Alle ore 12.15 l'iniezione è terminata (tempo impiegato: 30 minuti).

Il coniglio slegato sta bene e mangia subito.

7ª ESPERIENZA — 11 febbraio 1933 mattino.

Coniglio bianco grigio di gr. 1500.

Iniettati cc. 8.1 (gr. eq. 0.0054 per Kgr.).

Inizio dell'iniezione: ore 10.30.

A cc. 4.5 pur presentandosi un respiro un pò dispnoico, il cuore è valido con ritmo normale.

A cc. 6 iniettati in 30', il coniglio è calmo con cuore valido, ritmo normale, respiro dispnoico.

A cc. 7, i battiti del cuore sono notevolmente diminuiti.

Termine dell'iniezione: ore 11.15 (tempo impiegato: 45 minuti).

Il coniglio, slegato, mangia e sta bene.

8ª ESPERIENZA — 12 febbraio 1932 mattino.

Coniglio grigio di gr. 1000.

Iniettati cc. 5.6 (gr. eq. 0.0056 per Kgr.).

Inizio dell'iniezione: ore 11.45.

Già a cc. 2.4 i battiti del cuore sono diminuiti, e il respiro è un pò dispnoico.

A cc. 4, iniettati in 40', cuore sempre valido, battiti diminuiti e respiro superficiale nettamente dispnoico.

A cc. 5 i battiti sono discesi a 64, aritmici.

A cc. 5.4 i battiti sono 33.

Termine dell'iniezione: ore 12.45 (tempo impiegato: 60 minuti).

L'animale, slegato, presenta paralisi degli arti anteriori, quindi, dopo 10 minuti, anche i posteriori sono paralizzati.

Ad un tratto si rianima ma poi respira più faticosamente e presenta contrazioni tonico-cloniche agli arti posteriori; in 35' muore.

AUTOPSIA.

Nel cavo pleurico scarsissima quantità di liquido.

Polmoni di aspetto e colorito normali, cuore floscio in diastole. Fegato di aspetto normale, la corticale dei reni è un pò congesta.

ESAME ISTOLOGICO DEGLI ORGANI.

Reni: notevole congestione dei capillari glomerulari e dei capillari della midollare nella parte che confina con la corticale. Gli epiteli dei tubuli di calibro normale.

Polmoni: nel lume dei piccoli bronchi si nota presenza di stravasi emorragici recenti e così pure negli alveoli si nota la presenza di numerosi globuli rossi.

Miocardio: la fibra miocardica appare normale; si notano emorragie recenti dei capillari.

Fegato: normali il parenchima e le cellule epatiche.

Dobbiamo ora considerare i risultati delle esperienze da vari punti di vista: da quello della dose minima letale, da quello della sintomatologia, da quello che riflette la causa della morte.

Incominciamo col fissare la dose minima letale.

Nel nostro Istituto si costuma determinare la dose minima letale lontana, cioè la più piccola dose di un farmaco che, iniettato nelle vene di un animale, lo uccide non immediatamente. Le regioni per le quali questo metodo di determinazione è preferito agli altri, sono esposte ampiamente in parecchi lavori.

La seguente tabella I^a ci dà un quadro preciso di ciò che è la dose minima letale di solfito di sodio.

TABELLA I^a

Num. d'ord.	Num. dell'esperim.	Peso del coniglio in gr.	cc. iniettato	gr. equivalenti per Kg.	Dur. infes. in m.'	Esito
1	I	1500	7	0,0046	12	vive
2	IV	1200	6	0,0050	30	"
3	VI	1100	7.4	0,0053	30	"
4	VII	1560	8.1	0,0054	45	"
5	V	1450	8	0,0055	30	muore in 25'
6	VIII	1000	5.6	0,0056	60	" " 35'
7	III	1400	8.4	0,0060	31	" " 10'
8	II	1500	12	0,0080	33	" " 5'

Esaminando le cifre della tabelle si rileva che fino a gr. equivalenti 0.0054 per Kgr. l'animale, se sopravvive, sta, in seguito, perfettamente bene; con la dose di gr. equivalenti 0.0055 l'animale muore.

Gli animali che sopravvivono di più durano in vita da 25 a 85 minuti dopo l'iniezione e non è possibile ottenere una sopravvivenza maggiore. Questo fatto che, a primo aspetto, colpisce un poco, appare invece perfettamente logico quando si consideri che il solfito nell'organismo viene in buona parte ossidato in solfato.

Ora è chiaro che quella parte che si ossida in solfato è perfet-

tamente inattiva, o poco attiva, mentre tutta l'attività tossica si deve porre a carico di quella che rimane come solfito. Fino a gr. equivalenti 0.0054 per Kgr. la quantità di solfito che rimane immodificato è insufficiente ad uccidere l'animale; con la dose di gr. equivalenti 0.0055 per Kgr. si tocca la dose che uccide. A questo punto bisogna ammettere che si sia raggiunto la quantità minima di solfito capace di indurre una modificazione permanente, irreversibile, incompatibile con la vita, di organi fondamentali e, poichè la morte interviene in un tempo massimo oscillante tra 25 e 35 minuti, è chiaro che l'anione solforoso produce rapidamente tali modificazioni e che non è possibile avere la morte preceduta da una sopravvivenza più lunga degli animali.

Rimane perciò fissato che la dose minima letale lontana di solfito di sodio è uguale a gr. equivalenti 0.0055 per Kgr. e rimane pure fissato che il solfito uccide rapidamente gli animali in quanto le lesioni prodotte dall'anione solforoso sugli organi raggiungono rapidamente quel grado che è incompatibile con la vita.

Rimane ora da considerare il risultato dell'autopsia e dell'esame anatomo-patologico.

L'autopsia ci ha dato sempre un cuore flaccido, arrestato in diastole, polmoni di colore bruno-roseo di aspetto normale, cavo pleurico con poca quantità di liquido, reni di forma e consistenza normale con corticale leggermente iperemica. Il fegato era succulento; la cistifellea ripiena di bile piuttosto pallida e la vescica turgida per orina torbidiccia, di color giallo chiaro. L'esame anatomo patologico praticato sul cuore reni, fegato e polmoni non ci ha dato reperti dei quali si possano trarre molte conclusioni. Infatti, eccetto che congestione dei capillari glomerulari e della midollare nei reni ed emorragie diffuse negli alveoli e piccoli bronchi polmonari, nulla l'altro di notevole si è potuto osservare.

In complesso posso affermare che le lesioni trovate negli organi della vita vegetativa non sono tali da giustificar la morte, nè permettono di pensare che possano contribuire all'esito letale accelerando in qualche modo il quadro finale dovuto alla lesione della funzione degli organi presiedenti al respiro e alla circolazione.

Poichè è noto che il solfito di sodio emolizza « *in vitro* » i globuli rossi del sangue ho fatto esperienze volte a cercare se la morte

potesse essere dovuta all'emolisi. Le esperienze furono praticate su sangue di coniglio estratto dalla carotide e defibrinato subito, circa mezz'ora dopo l'estrazione del sangue dai vasi e alla temperatura di 37° C.

Mi servii di una soluzione di solfito isotonica con una soluzione di cloruro di sodio al 0.9 % che, secondo quello che si rileva dalle Tabelle di Landolt-Bornstein, è eguale a 2.94 %.

Le esperienze sono raccolte nelle seguenti due tabelle II e III.

TABELLA II ª (*)

Provetta N.	1	2	3	4	5	6	7	8	9	10	11
NaCl 0.9% cc.	10	9	8	7	6	5	4	3	2	1	0
Na_2SO_3 2.01%	0	1	2	3	4	5	6	7	8	9	10
gr. di solfito in 100 cc. di sol.	0	0.201	0.588	0.882	1.176	1.470	1.764	2.058	2.352	2.646	2.940
ore 0.30'	Non si riscontra nessuna modificazione										
" 1											
" 2	E' incominciata la sedimentazione: il liquido sovrastante è incoloro.									+	
" 6	—	—	—	—	±	±	±	+	+	+	+
" 16	—	—	—	±	±	±	+	+	+	+	+
" 24	—	—	±	±	±	+	+	+	+	+	+
" 28	—	+	+	+	+	+	+	+	+	+	+

(*) Il segno — indica assenza di emolisi, il + più emolisi, il segno ± traccia di emolisi.

Da queste risulta che, con soluzioni della concentrazione oscillanti da gr. 0.0588 % (Tabella III ª) a gr. 2.940 % (Tabella II ª) non si ottiene emolisi in vitro neppure dopo due ore.

A questo punto bisogna fare un confronto fra le dosi di solfito che determinano la morte e queste dosi, che, in vitro, non riescono ad emolizzare i globuli rossi neppure in due ore, tenendo presente che la morte dell'animale interviene dopo 35 minuti.

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TABELLA III^a

Provetta N.	1	2	3	4	5	6	7	8	9	10	11
NaCl 0.9 % cc.		9.8	9.6	9.4	9.2	9	8.8	8.6	8.4	8.2	8
Na_2SO_4 2.94 %	0	0.2	0.4	0.6	0.8	1	1.2	1.4	1.6	1.8	2
gr. di solfito in 100 cc. di soluz.	0	0.0588	0.1176	0.1764	0.2352	0.2940	0.3528	0.4116	0.4704	0.5292	0.5880
ore 0.30'											
" 1	Non si riscontra alcuna modificazione										
" 2	E' incominciata la sedimentazione: il liquido sovrastante è incolore.										
" 6	—	—	—	—	—	—	±	±	±	±	±
" 16	—	—	—	—	—	—	±	±	±	±	+
" 24	—	±	±	±	±	±	±	+	+	+	+
" 28	—	±	±	±	±	+	+	+	+	+	+

Allora si vede che, mentre l'animale muore con gr. equivalenti 0.0055 per Kgr. corporeo in 25 minuti, l'emolisi in vitro non si ottiene neppure dopo due ore con una dose quadrupla.

Il che ci dà la dimostrazione che l'azione emolitica del solfito di sodio sul sangue, che si può dimostrare in vitro, non interviene per nulla nel meccanismo della morte.

III) Meccanismo con cui il solfito produce la morte.

Passiamo ora ad esaminare la sintomatologia prima degli animali sopravvissuti, poi di quelli morti. Negli animali sopravvissuti i fatti salienti osservati sono alterazioni gravi delle funzioni circolatorie (forte rallentamento dei battiti cardiaci) e respiratoria (dispnea), disturbi che, dopo qualche tempo, si attenuano o scompaiono senza lasciare conseguenze apparenti.

Negli animali che vengono a morte si osservano gravi irregolarità degli apparati respiratorio e circolatorio che, in un certo momento, cessano di funzionare. Intervengono allora contrazioni to-

nico-cloniche che caratterizzano il quadro finale dell'avvelenamento.

Per illustrare meglio questo quadro dell'avvelenamento ho fatto alcune esperienze nelle quali prendevo contemporaneamente il tracciato del respiro e della pressione.

Esse dimostrarono che cuore e respiro si spengono quasi contemporaneamente, sebbene la funzione respiratoria sia forse quella che cede per ultima.

IV) Conclusioni.

I. - La dose minima letale lontana per via endovenosa del solfito di sodio è di gr. equivalenti 0.0055 per Kgr.

II. - La morte interviene per azione diretta e contemporanea del farmaco sui centri respiratori e circolatori.

III. - L'esame anatomo-patologico non ha dimostrato alcuna lesione importante negli organi della vita vegetativa.

IV. - L'azione emolitica del farmaco non ha importanza nel determinismo della morte.

RIASSUNTO

La dose minima mortale a distanza per via endovenosa del solfito di sodio nel coniglio è di g. eq. 0,0055 per Kgr. La morte è dovuta all'azione diretta e contemporanea dei due farmaci sui centri respiratori e circolatori.

RÉSUMÉ

La dose minime a distance par injection intraveineuse du sulfite neutre de sodium dans le lapin est de g. eq. 0,0055 par Kgr. La morte arrive par l'action directe et contemporaine des deux médicaments sur les centres respiratoires et circulatoires.

ZUSAMMENFASSUNG

Die kleinste Minimal langwirkende tödliche Dosis durch Veneneinspritzung für Natriumsolfit im Kaninchen ist gr. eq. 0,0055 pro kilo. Der Tod ist bedingt durch die direkte u. gleichzeitige Wirkung beider Arzneinen auch die respirationischen u. zyrcolatorischen zentren.

SUMMARY

The minimal lethal dose in the distance of sodium sulphit for intravenous injection in the rabbit is: eq. gr. 0,0055 pro Kilogram.

Dead is due to the direct and contemporany action of both the drugs on the centres of respiration and bload circulation.

Alkalimetric Determination of Sulphur Dioxide in Wine

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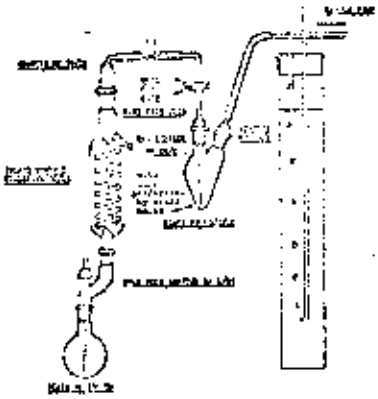
By B. C. Rankine and K. F. Pocock, the Australian Wine Research Institute, Adelaide.



A knowledge of the sulphur dioxide content of wine is essential for modern winemaking, and in particular, it is important to know the level of free sulphur dioxide in white table wines, because this is responsible for the germicidal and anti-oxidant action. When sulphur dioxide is added to wine it combines with acetaldehyde, various carbonyl compounds, and to a much lesser extent, with glucose. If sufficient is added, portion remains uncombined and this is termed free sulphur dioxide. The quantity bound is of little or no value as germicide or anti-oxidant.

The various methods for determining sulphur dioxide in wines have been reviewed by Deibner (1). The current iodometric methods (Ripper) are used for measuring total, i.e., free and combined sulphur dioxide, by distillation, and free by direct titration. However, the determination of free sulphur dioxide by this method is not accurate, and in wines to which ascorbic acid or other iodine-reducing compounds have been added, the results are quite misleading. Since free sulphur dioxide is the critical determination, the current iodometric methods are not sufficient.

A more accurate method for determining free, combined and total sulphur dioxide has appeared in European literature. It has the major advantage that free sulphur dioxide can be determined accurately in white wines, and ascorbic acid does not affect the determination. The method will measure total, but not free sulphur dioxide in red wines.

The method was developed by Paul (2, 3) in Austria, and other workers (4, 5, 6) have confirmed its general suitability. The method has been examined in detail at the Australian Wine Research Institute, with a view to using it for research on sulphur dioxide in wine and for recommending it to the Australian wine industry. The method was reported in 1962 (7) in this Journal, and the present paper gives an account of developments in the method which have taken place in the intervening years. The method is now widely used in the Australian wine industry, and it was felt that a review of the method would be of benefit to the industry, particularly on account of the increased demand for table wines and the greater importance being placed on free sulphur dioxide in white wines.

Principle

The wine sample is acidified with phosphoric acid to approximately pH 1, which converts the free sulphite to undissociated sulphur dioxide making it more volatile, and prevents the dissociation of bound sulphur dioxide at room temperature. The dissolved sulphur dioxide is then removed from the solution by bubbling with a current of air drawn through the flask by a water-jet pump. The liberated sulphur dioxide is carried by the air stream through an efficient water-cooled condenser, which condenses any volatile acid removed from the wine, and is then bubbled into excess dilute hydrogen peroxide containing an acid-base indicator. There it is oxidised to sulphuric acid, which is titrated with dilute standard alkali. The amount of alkali used is proportional to the free sulphur dioxide present in the wine.

The combined sulphur dioxide on the same sample can then be determined by renewing the neutralised hydrogen peroxide-indicator solution and aspirating again while the acidified wine is gently boiled, which liberates the combined sulphur dioxide. The total sulphur dioxide can be determined in one operation by omitting the aspiration at room temperature.

Apparatus

A modified Lieb-Zacherl apparatus is used, which can be constructed from Quickfit interchangeable glassware, obtainable from most laboratory suppliers. A sketch is included to show the design required. The Quickfit components and their catalogue numbers, according to the 1966 Catalogue, are as follows:—

- 1 only 100 ml. medium-necked round-bottom flask with B24 socket (FR100/3M). Smear inside of neck below the joint with silicone grease to prevent frothing.
- 1 only swan-neck adaptor with B24 joints (MA6/33), the air-leak tube can be made from a piece of glass tubing drawn out at one end to an internal diameter of approximately 2.5 mm.
- 1 only Inland Revenue condenser with B24 joints (C6/13).
- 1 only B24 cone adaptor (MF/10/3B).
- 1 only ball and cone joint and clip (MS13, FS13, JC13).*
- 1 only gas bubbling tube with a B14 joint (MF15/1) with the tube extending to the bottom of the pear-shaped flask and ending in a small bulb perforated with holes, or a similar arrangement for dispersing the gas.*
- 1 only 50 ml. pear-shaped two-neck flask with B14 sockets (FP50/1/1A).
- 1 only B14 cone-to-tubing adaptor (MF10/1).

The components marked * need to be made up according to the sketch by a glassblower. A 10 ml. microburette with a two-way stopcock and miscellaneous glassware are also needed. The complete equipment is available from certain of the chemical supply companies in South Australia.

The tall cylinder in the sketch is a vacuum regulator for the water-jet pump. It consists of a water-filled cylinder about 2 ft. high fitted with a two-holed rubber stopper, through which passes a sliding glass tube about 2 ft. long and a glass T piece to which the pump is connected. The depth of immersion of the glass tube determines the vacuum, when bubbles issue from the lower end during operation of the water pump. The vacuum can be varied at will by raising or lowering the sliding glass tube.

Solutions

1. Indicator — 0.100 g. methyl red and 0.050 g. methylene blue made to 100 ml. with 50 per cent alcohol.

2. Hydrogen peroxide 0.3 per cent — take 10 ml. of 10 volume 3 per cent hydrogen peroxide and dilute to 100 ml. with distilled water. Prepare fresh daily. Keep stock solution in refrigerator, since it decomposes fairly quickly at room temperature. The strength of the hydrogen peroxide can be checked if necessary. The method for doing this is described below.

3. Phosphoric acid 25 per cent — take 280 mls. of A.R. phosphoric acid (90 per cent) and make to 1 litre with distilled water.

4. Standard sodium hydroxide N/100 — dissolve 0.41 g. of A.R. sodium hydroxide in recently boiled and cooled

distilled water and make to 1 litre. Place in a 1 litre bottle fitted with a two-holed stopper. Attach a soda-lime tube to one hole and to the other, a glass tube extending to the bottom of the bottle. Attach plastic tubing to this tube and connect to the two-way tap of a 10 ml. microburette. The 1 litre reservoir is placed on a shelf higher than the top of the burette to give the necessary head for filling. Attach a soda-lime tube to the top of the burette, then standardise against N/100 acid.

Method

Free SO2

1. Pipette 10 ml. of 0.3 per cent hydrogen peroxide into the 50 ml. pear-shaped flask. Add 3 drops indicator, which should turn purple. Adjust colour to olive-green with the dilute sodium hydroxide and connect the flask to the bubbling tube and vacuum lead.

2. Pipette 20 ml. wine and 10 ml. of 25 per cent phosphoric acid into the 100 ml. round-bottom flask and connect to the apparatus. Run cold water at a fast rate through the condenser.

3. Turn on the water-jet pump and allow air to be drawn through the apparatus at approximately 1 litre per minute. The indicator in the hydrogen peroxide solution should turn purple. Continue aspiration at this flow-rate for 10 minutes.

4. Remove the pear-shaped flask, rinse the bubbler with distilled water into the flask, and titrate the contents with standard sodium hydroxide to the initial olive-green colour. 1 ml. N/100 NaOH = 0.32 mg. SO2. For 20 ml. wine the titration figure in mls. multiplied by 16 = parts per million free SO2.

Combined SO2.

Combined sulphur dioxide can then be determined on the same sample by replacing the hydrogen peroxide-indicator solution and aspirating for 10 minutes at a flow rate of 1 litre per minute while the contents of the round-bottom flask are maintained gently boiling. The heating should be carried out with a small flame 4-5 cm. high from a micro-burner and without a wire gauze so that the point of the flame is in contact with the flask. The calculation is the same.

Total SO2

Total sulphur dioxide can be determined in the one operation by aspirating the wine whilst boiling, which releases the bound as well as the free sulphur dioxide.

Discussion

Acetic acid below 1.2 grams per litre in the wine has negligible effect, providing an efficient water condenser is used, but above this figure a trace may be carried over, causing slightly high results. If an efficient water condenser is not used a large titration error can occur, e.g. if all the acetic acid in a wine containing 0.5 gram per litre was carried over, it would use 16.7 mls. of N/100 sodium hydroxide, equivalent to 267 p.p.m. SO2. If the wine contains over 1.2 grams per litre of volatile acid a blank determination is desirable. This is carried out by adding a few drops of concentrated hydrogen peroxide to the acidified wine before distillation to oxidise the sulphur dioxide present. The value thus obtained is then subtracted from the value obtained when no peroxide is added to the wine. Since the actual quantity of sulphur dioxide determined is small, a very dilute standard alkali is used, and care must be taken in general manipulation. The method is not a rough approximation but a precision semi-micro method.

The method is designed so that the amount of standard alkali in a 10 ml. burette can adequately cover the range of free sulphur dioxide normally expected in wine. If combined or total sulphur dioxide above 160 parts per million is to be measured, the quantities of wine and phosphoric acid should be halved.

Adequate hydrogen peroxide is present to oxidise all the sulphur dioxide which would be expected in any wine, e.g. for 30 parts per million free sulphur dioxide there is of the order of 100 fold excess of hydrogen peroxide. Since hydrogen peroxide is unstable at room temperature the stock solution should be kept in the refrigerator. If there is any doubt about the strength of the solution, it can be easily checked as follows:

Prepare N/10 potassium permanganate solution by adding 3.2 g. KMnO4 to 900 ml. distilled water, boil for 15 minutes, cool and make to 1 litre with distilled water. Store in an amber bottle. Pipette 1.0 ml. of the stock 10 volume hydrogen peroxide solution into a 200 ml. conical flask containing approximately 80 mls. distilled water, add 5 ml. dilute sulphuric acid (1 acid + 5 water) and 10 ml. of N/10 potassium permanganate and mix. The pink colour should disappear. If it remains the peroxide has lost at least half its strength and should be discarded.

We have found that the flow rate of air used to aspirate the sulphur dioxide from the wine is important. At a flow rate of 1 litre per minute an aspiration time of ten minutes (with the four stills in use at the Institute) is sufficient to aspirate all the free or combined sulphur dioxide. If a slower flow rate is used or the passage of air is restricted due to restrictions in the glass bubblers, a longer aspiration time is required.

The flow rate can be measured with a simple gas rotameter (a slightly tapered calibrated glass cylinder containing an indicator ball) connected to the air inlet tube in the round-bottom flask, or by displacement of water through the water-jet pump. In this case a graduated flask filled with water and fitted with an inlet and outlet tube, the latter reaching to the bottom of the flask, is inserted in the vacuum line near the water-jet pump. The rate at which the vessel empties indicates the flow rate of air.

Whilst a flow rate of 1 litre per minute is desirable, it need not be precise and in fact can vary by up to 50 per cent. However, with very low air flow the aspiration is incomplete in the time allowed, and with very rapid flow rates the recovery may be low because the sulphur dioxide is not in contact with the peroxide solution for sufficient length of time. Wineries are advised to check on flow rate and time of aspiration to ensure that all the sulphur dioxide is being aspirated.

Following the work of Burroughs and Sparks (6), we have examined the effect of aspirating with nitrogen gas instead of air, and using high instead of low vacuum. Neither of these procedures appeared to offer advantages over the existing method.

The temperature at which the free sulphur dioxide is measured is important, since the equilibrium between free and bound sulphur dioxide is temperature dependent and as the temperature rises more sulphur dioxide becomes free. The temperature in the wine during aspiration should be below 20 deg. C, and on a hot day in a winery laboratory it may be necessary to cool the flasks during aspiration.

It is not possible at present to measure free sulphur dioxide in red wines by this method, nor indeed by any method with which we are aware. The reason is that some sulphur dioxide is bound to red pigments in the wine (8), and this

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is released when the wine is acidified prior to aspiration, giving rise to spurious free sulphur dioxide. Efforts to overcome pigment effect by altering the pH of acidification, and rate and duration of aspiration, have been unsuccessful.

Summary

An alkalimetric method of determining sulphur dioxide in wines is described, which can be used to determine free sulphur dioxide in white wines, including those containing ascorbic acid. It will also measure total sulphur dioxide in both white and red wines, and is more accurate than the iodometric method.

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NEW LENNONS HOTEL PLANNED

Federal Hotels Ltd. proposes to build an hotel of international standard on a site near that of Lennons Hotel, Brisbane. It is planned to be a tower of accommodation units built on a four-storey hotel base, and is expected to contain about 300 bedrooms. There will be three basement floors, on the two lower ones of which parking for 120 to 130 cars will be provided, and the upper basement will contain bar and grill rooms. The ground floor also will have bars, lounges, and the main reception area for hotel guests.

On the first floor there will be a ballroom and other function facilities; the second floor will contain the main dining areas, with cocktail and lounge areas; and the third floor will be the main administrative section, with offices and some conference rooms. A large heated swimming pool and guest facilities, including a grill bar, are planned for the roof area. On two levels of the building — in the top basement and on the ground floor — there will be shopping facilities.

In commenting on the project, the chairman of Federal Hotels Ltd., Mr. Greg Farrell, said some of the basic concepts could come under review when final planning took place. This would be reached in about six months, and the hotel would be built within two years of the planning's completion.

Purchase of the present Lennons Hotel is said to be under negotiation by the Brisbane City Council for expansion of its accommodation, but it is understood that, irrespective of whatever may transpire in this respect, building of the new hotel will go on.

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Frau *Marianne Pohl*, MTA am Hygienischen Institut, gilt unser besonderer Dank für ihre sorgsame, gewissenhafte und erfahrene Assistenz.

Anschrift des Verfassers:
Prof. Dr. G. Firnau, Dr. U. Strahlmann
2 Hamburg 36, Gorch-Fock-Wall 15

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Beitrag zur Bestimmung und Beurteilung des Azetaldehyds bzw. der an Azetaldehyd gebundenen schwefligen Säure im Wein

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Von H. Rebelein

Der wichtigste Bindungspartner der schwefligen Säure (SO_2) im Wein ist der Azetaldehyd. An ihn ist im Normalfall die Hauptmenge des im Wein enthaltenen SO_2 gebunden (70 % bis 90 %). Im Hinblick auf das berechtigte Bestreben, die Menge des SO_2 im Wein auf das unbedingt erforderliche Mindestmaß zu beschränken, muß man mit *Kielhöfer*[1]) einig gehen, der schon 1963 geäußert hat, „daß es notwendig ist bei der Weinbereitung vor allem darauf zu achten, daß dieser Anteil der gebundenen SO_2 möglichst niedrig bleibt. Das wiederum bedeutet, daß der Gehalt an Azetaldehyd des Weines möglichst gering sein muß". *Kielhöfer* hat sich in seiner bemerkenswerten Arbeit eingehend mit den verschiedenen Bindungsformen des SO_2 im Wein beschäftigt. Er unterscheidet 3 Bindungsformen: Das an Azetaldehyd gebundene SO_2, kurz azetaldehyd-schweflige Säure genannt, das an Glukose gebundene SO_2 und das an sonstige Weinbestandteile gebundene SO_2, von ihm Rest-SO_2 genannt.

Nach seinen Feststellungen ist das an Glukose gebundene SO_2 für Tischweine, auch wenn sie noch viel unvergorenen Zucker enthalten, praktisch bedeutungslos. Es bewegt sich bei süßen Tischweinen in der Größenordnung von 0 bis etwa 2 bis 4 mg/l. Relativ große Bedeutung ist nach *Kielhöfer* dagegen der restschwefligen Säure zuzumessen. Diese kann bei edelfaulem und sauerfaulem Lesegut das an Azetaldehyd gebundene SO_2 sogar übertreffen. Weine aus sauerfaulem Lesegut, welche wegen ihres hohen Gehaltes an Stoffen, die für die Bindung der restschwefligen Säure verantwortlich sind, häufig überschwefelt sind, werden von *Kielhöfer* als „Schwefelfresser" bezeichnet.

Die Kenntnis der verschiedenen Bindungsformen des SO_2 läßt im Einzelfall Aussagen darüber zu, ob eine Überschwefelung auf eine unsachgemäße Kellerbehandlung zurückzuführen ist oder ob der Wein wegen eines von Natur aus bedingten anormalen Mehrbedarfes an SO_2 zwangsläufig mit einer zunächst den zulässigen Grenzwert übersteigenden hohen Schwefelzugabe ausgebaut werden mußte.

Es liegt auf der Hand, daß in Beanstandungsfällen eine Überschwefelung von z. B. 360 mg/l gesamtschwefliger Säure unterschiedlich beurteilt werden muß, je nachdem, ob die 360 mg/l sich z. B. aus 40 mg/l freier, 40 mg/l restschwefliger Säure und 280 mg/l azetaldehyd-schwefliger Säure zusammensetzen (was in der Regel beweisen würde, daß mit dem untauglichen Mittel einer wiederholten Nachschwefelung versucht worden ist, einen restsüßen Wein gärungsstabil zu halten) oder ob eine Überschwefelung nur deshalb eingetreten ist, weil von den 360 mg/l gesamtschwefliger Säure allein z. B. 200 mg/l zur Bindung von Faulstoffen erforderlich gewesen sind.

An der Staatlichen Chemischen Untersuchungsanstalt Würzburg wird deshalb seit Erscheinen der *Kielhöfer*'schen Arbeit in jedem Falle einer festgestellten Überschwefelung eine Differenzierung der einzelnen Bindungsformen des SO_2 durchgeführt.

Für die Bestimmung des Azetaldehydes benutzten wir bis jetzt die Methode von *Jaulmes* und *Espezel*[2]).

Dabei wird der Azetaldehyd durch Destillation abgetrennt und in einer Vorlage wieder in azetaldehyd-schweflige Säure übergeführt. Durch Differenztitration mit n/40 Jodlösung wird die azetaldehyd-schweflige Säure ermittelt. Das Verfahren arbeitet zuverlässig, immerhin ist es als Destillationsverfahren zum mindesten im Routinebetrieb der Überwachung für Serienbestimmungen nicht be-

sonders geeignet, abgesehen von dem sehr großen Lösungsmittelverbrauch, den eine Bestimmung beansprucht.

Wir waren deshalb bemüht, einen einfacheren Weg zur Bestimmung des an SO_2 gebundenen Azetaldehyds bzw. des an Azetaldehyd gebundenen SO_2 zu finden. Ein solcher bot sich in der von uns bereits beim Butylenglykol[1]) und bei der Milchsäure[1]) zu quantitativen kolorimetrischen Bestimmungsmethoden ausgearbeiteten Violettfärbung an, welche Azetaldehyd mit Piperidin und Nitroprussidnatrium gibt. Allerdings konnte eine derartige Methode nur dann den gewünschten arbeitstechnischen Vorteil gewährleisten, wenn der Umweg der Destillation vermieden werden konnte.

Aus früheren Arbeiten[2]) wußten wir bereits, daß der an SO_2 gebundene Azetaldehyd mit Piperidin und Nitroprussidnatrium in der gleichen Weise reagiert wie freier Azetaldehyd. So lag der Gedanke nahe, den an schweflige Säure gebundenen Azetaldehyd im Wein, der zuvor mit Kohle entfärbt worden ist, direkt der Reaktion mit Piperidin und Nitroprussidnatrium zu unterziehen.

Unsere nachfolgend beschriebenen Versuche zeigten, daß dies möglich ist.

Wir stellten zunächst eine Lösung mit bekanntem Gehalt an azetaldehyd-schwefliger Säure her: ca. 1 ml frisch destillierter Azetaldehyd wurde in einem mit ca. 500 ml Wasser beschickten 1-l-Meßkolben mit einem mäßigen Überschuß an schwefliger Säure versetzt, zum Liter aufgefüllt und über Nacht stehen gelassen. Der genaue Azetaldehydgehalt der Lösung wurde dann nach *P. Jaulmes — Espezel* bestimmt. Von dieser Grundlösung wurden passende Verdünnungen hergestellt. Je 2,0 ml der Lösungen wurden mit 5,0 ml einer 0,4 %igen Nitroprussidnatriumlösung und 5,0 ml verdünntem Piperidin versetzt. Die Intensität der entstehenden Violettfärbung wurde kolorimetrisch gemessen.

Es ergab sich eine Gerade (Abb. 1).

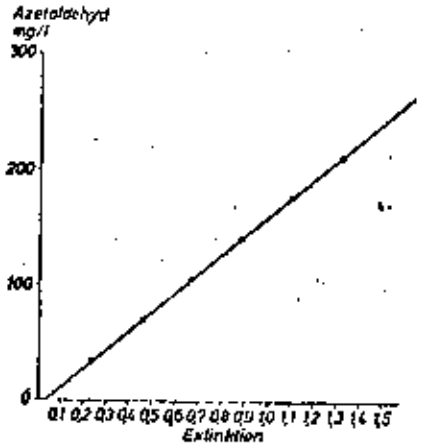

Abb. 1.

Zur Bestimmung des Azetaldehydes im Wein wurden ca. 25 ml Wein mit 2 g Aktivkohle entfärbt. 2,0 ml des Filtrates wurden der oben beschriebenen Reaktion unterworfen. Mit den Extinktionen wurde aus der mit reiner azetaldehydschwefliger Säure aufgestellten Eichgeraden der Azetaldehydgehalt des Weines entnommen. Gleichzeitig wurde der Azetaldehydgehalt nach der Methode *P. Jaulmes — Espezel* bestimmt.

In Tabelle 1 sind die nach beiden Verfahren erhaltenen Werte einander gegenübergestellt.

Tabelle 1
Vergleichende Azetaldehyd-Bestimmungen

Art des Weines	mg/l Azetaldehyd, kolorimetrisch bestimmt	mg/l Azetaldehyd nach Jaulmes und Espezel bestimmt
ausländ. Rotwein	156	154
ausländ. Rotwein	83	84
ausländ. Weißwein	105	105
ausländ. Weißwein	240	238
deutscher Weißwein	54	55
deutscher Weißwein	40	40
deutscher Weißwein	65	62
Dessertwein Muskateller	62	60
Wermutwein	72	68
roter Brennwein	46*)	46
weißer Brennwein	94*)	98

* nach Zusatz von SO_2; enthält freien Azetaldehyd

Die beiden Bestimmungsmethoden zeigen eine befriedigende Übereinstimmung. Gegenüber der bisherigen Destillationsmethode besitzt die kolorimetrische Methode den Vorteil der Schnelligkeit. Eine Bestimmung beansprucht nur einige Minuten. Serienmäßige Ansätze sind möglich und verkürzen die Bestimmungszeit noch erheblich. Lediglich bei Weinen, die bereits freien Azetaldehyd enthalten, liefert die kolorimetrische Methode niedrigere Werte. Anscheinend wird freier Azetaldehyd von Aktivkohle zum Teil absorbiert. Führt man aber den freien Azetaldehyd vorher in azetaldehyd-schweflige Säure über, dann erhält man auch in diesen Fällen gut übereinstimmende Werte. Die Anwesenheit von Zucker, Alkohol und sonstigen Inhaltsstoffen des Weines stört offensichtlich unter den im Wein vorliegenden Bedingungen nicht, wie die auch bei Dessertweinen, Wermut- und Brennweinen gut übereinstimmenden Werte beweisen. Wir hatten zuvor Versuche mit reinen verdünnten Lösungen von azetaldehyd-schwefliger Säure durchgeführt, denen jeweils Glukose bzw. Fruktose, Saccharose, Glycerin, Alkohol oder Säuremischungen (letztere auf einen im Wein vorliegenden pH-Wert teilneutralisiert) zugesetzt worden waren. Hierbei hatte sich gezeigt, daß die Anwesenheit von Alkohol, Glycerin und gepufferten Säuren keine Änderung der Extinktionswerte hervorrief. Durch Zusatz steigender Zuckermengen waren die Extinktionen allerdings angestiegen. Deshalb war zunächst eine Korrektur der Werte in Abhängigkeit vom Zuckergehalt in Betracht gezogen worden. Die zahlreichen, in der Tabelle nicht vollständig wiedergegebenen Parallelversuche zwischen der kolorimetrischen und der Destillationsmethode zeigten aber, daß unter den Milieubedingungen des Weines selbst bei hohen Zuckerwerten keine merkliche Beeinflussung der Extinktion eintrat.

Bei der Ausarbeitung der Methode mußten wir feststellen, daß das Alter der verdünnten Piperidinlösung aus noch nicht ganz erklärlichen Gründen einen Einfluß auf die Extinktionshöhe ausübte. Wird die verdünnte Piperidinlösung jedoch unmittelbar vor der Bestimmung bereitet, dann erhält man jederzeit reproduzierbare Werte. Wir erhielten mit einer im Dunkeln aufbewahrten Lösung von azetaldehyd-schwefliger Säure und einer gleichfalls im Dunkeln aufbewahrten 0,4 %igen Nitroprussidnatriumlösung während eines Zeitraumes von 6 Wochen stets die gleiche Extinktion, wenn die Reaktion mit einer unmittelbar frisch bereiteten verdünnten Piperidinlösung vorge-

nommen wurde. Die gleichen Lösungen ergaben aber bei Verwendung älterer verdünnter Piperidinlösungen Extiktionswerte, welche mit steigendem Alter der Piperidinlösung anstiegen: So ergab eine 14 Tage alte Piperidinlösung bereits um 10 % höhere Werte. Möglicherweise ist die Intensität der Farbreaktion vom Hydratationszustand der verdünnten Piperidinlösung abhängig. Es wäre denkbar, daß die Einstellung eines stabilen Hydratationszustandes nach der Mischung von Piperidin mit Wasser längere Zeit beansprucht. Bei unseren früheren, auf der Azetaldehyd-Reaktion mit Nitroprussidnatrium und Piperidin aufgebauten Methoden konnte dieser Effekt weitgehend dadurch ausgeschaltet werden, daß wir die Reaktionslösung, deren Milieu wegen der vorangegangenen Aufarbeitung des Weines stets gleich war, mit Natriumazetat pufferten, so daß auch mit alten Piperidinlösungen vergleichbare Werte erzielt werden konnten.

Bei der im vorliegenden Falle im Wein direkt vorgenommenen Reaktion mußten wir jedoch feststellen, daß die stabilisierende Wirkung des Natriumazetates in dem wechselnden Zucker-, Säure- und Alkohol-Milieu der Weine versagte. Wir wählten deshalb den Ausweg, die Piperidinlösung vor jeder Bestimmung frisch herzustellen.

Die Reproduzierbarkeit der Werte ist sehr gut. Sehr deutlich lassen sich sowohl die Reproduzierbarkeit wie auch die praktische Unabhängigkeit der Reaktion vom Zucker-, Alkoholgehalt und sonstigen Inhaltsstoffen des Weines am Beispiel einer Trockenbeerenauslese zeigen, die einen Zuckergehalt von 267 g/l und einen Alkoholgehalt von 56 g/l aufwies. In der Verdünnung 1 + 1 wurden 460 mg Azetaldehyd festgestellt, in der Verdünnung 1 + 7 lieferte die Bestimmung 458 mg/l.

Arbeitsvorschrift:

A) erforderliche Ausrüstung

1. *Reagenzien*
   Nitroprussidnatrium z. A.
   Piperidin z. A.
   Aktivkohle zur Entfärbung
2. *Gerätschaften*
   Pipetten 2,0; 5,0 ml
   Erlenmeyerkolben 100, 200 ml
   Trichter
   Harte Faltenfilter
   Photometer
   Küvetten 1 cm
3. *Lösungen*
   verdünntes Piperidin: 2,0 ml Piperidin werden jeweils unmittelbar vor der Bestimmung mit 20,0 ml Wasser vermischt
   0,4 %iges Nitroprussidnatrium: 1 g pulverisiertes Nitroprussidnatrium wird in einem 250 ml Meßkolben in Wasser gelöst und zur Marke aufgefüllt

B) Ausführung der Bestimmung

Ca. 25 ml Wein werden in einem Erlenmeyerkölbchen mit ca. 2 g Aktivkohle versetzt. Man schwenkt einige Sekunden kräftig um, läßt 2 min stehen und filtriert klar durch ein hartes Faltenfilter.

Zu 2,0 ml des farblosen Filtrates gibt man in einem Erlenmeyerkölbchen unter Umschwenken 5,0 ml 0,4 %ige Nitroprussidnatriumlösung und 5,0 ml verdünnte Piperidinlösung. Das Reaktionsgemisch wird sofort in einer 1 cm-Küvette bei 570 nm gegen Luft photometrisch gemessen. Als Meßwert gilt das Maximum der Extinktion, das nach etwa 50 sec. auftritt. Der im Wein enthaltene Azetaldehydgehalt wird einer Eichkurve entnommen, die nach der unten ausgeführten Vorschrift aufgestellt worden ist.

Der Azetaldehydgehalt multipliziert mit dem Faktor 1,65 ergibt die an Azetaldehyd gebundene schweflige Säure.

Die sogenannte Rest-schweflige Säure wird erhalten, indem von der gesamten schwefligen Säure die freie schweflige Säure und die azetaldehyd-schweflige Säure abgezogen werden.

Enthält der Wein ausnahmsweise freien, nicht an schweflige Säure gebundenen Azetaldehyd, dann muß vor Durchführung der Bestimmung der freie Azetaldehyd erst in azetaldehyd-schweflige Säure übergeführt werden. Hierzu versetzt man einen Teil des Weines mit einem kleinen Überschuß an freier schwefliger Säure und läßt ihn vor Durchführung der Bestimmung einige Stunden stehen.

C) Aufstellung der Eichkurve

a) Herstellung einer Lösung von azetaldehyd-schwefliger Säure mit bekanntem Gehalt an Azetaldehyd

1. *Reagenzien*
   Azetaldehyd zur Synthese
   conc. Salzsäure z. A.
   Borsäure z. A.
2. *Lösungen*
   n-Lauge
   n/10 Jodlösung
   5- bis 6 %ige Lösung von schwefliger Säure
   verdünnte Salzsäure: 25 ml conc. HCl mit Wasser auf 100 ml aufgefüllt
   Alkalilösung: 8,75 g Borsäure werden mit 400 ml n-Lauge aufgelöst und mit Wasser auf 1 l aufgefüllt

Man bestimmt den SO_2-Gehalt einer 5- bis 6 %igen Lösung von schwefliger Säure durch Titration einer abgemessenen Menge mit n/10 Jodlösung. Dann gibt man in einen 1 l-Meßkolben soviel Milliliter der SO_2-Lösung als 1500 mg SO_2 entsprechen. Durch einen Trichter gibt man dann in den Meßkolben ca. 1 ml frisch destillierten, in einer Kältemischung aufgefangenen Azetaldehyd. Man füllt zur Marke auf und mischt. Die so hergestellte Grundlösung läßt man über Nacht stehen. Durch stellt man den genauen Azetaldehydgehalt der so hergestellten Lösung von azetaldehyd-schwefliger Säure wie folgt fest:

50 ml der Grundlösung werden in einem 500 ml Erlenmeyerkolben mit 10 ml verdünnter Salzsäure versetzt. Nach Zugabe von 100 ml Wasser und Stärkelösung wird die freie schweflige Säure mit n/10 Jodlösung bis zur schwachen Blaufärbung titriert. Danach werden 100 ml Alkalilösung zugesetzt, wobei die blaue Färbung verschwindet. Die nun freigesetzte azetaldehyd-schweflige Säure wird mit n/10 Jodlösung bis zur schwachen Blaufärbung titriert. Verbrauch a ml n/10 Jodlösung. Die Grundlösung enthält dann a · 44,05 mg Azetaldehyd im Liter.

b) Eichkurve

Je 5,0; 10,0; 15,0; 20,0 und 25,0 ml der Grundlösung werden in 100 ml Meßkölbchen mit Wasser zur Marke aufgefüllt und gemischt. Diese Verdünnungen entsprechen Weinen mit einem größenordnungsmäßigen Gehalt an Azetaldehyd von ca. 40, 80, 120, 160 und 200 mg/l; der genaue Gehalt der Verdünnungen muß aufgrund des ermittelten Azetaldehydgehaltes der Grundlösung berechnet werden.

Je 2,0 ml der Verdünnungen werden der unter B) beschriebenen Bestimmung unterworfen. Die erhaltenen Extiktionen ergeben, gegen die entsprechenden Azetaldehydwerte der Verdünnungen aufgetragen, eine Gerade, welche nicht durch den Nullpunkt geht.

In Tabelle 2 ist das Ergebnis unserer bisherigen Untersuchungen über den Azetaldehydgehalt bzw. die an Azetaldehyd gebundene schweflige Säure von in- und ausländischen Weinen zusammengestellt.

Die Zahlenwerte der Tabelle 2 decken sich weitgehend mit den Werten, die aus der *Kielhöfer*'schen Mitteilung abgelesen werden können. *Kielhöfer* führt in seinem Bericht die Werte von 12 Weinen an. Läßt man drei von ihm mitangeführte Ausnahmefälle (ein Wein aus edelfaulem, ein Wein aus sauerfaulem Lesegut und ein Versuch mit zugesetztem Azetaldehyd) weg, dann ergeben sich folgende Mittelwerte:

Freies SO_2 mg/l	15	(0—29)
Gesamt-SO_2 mg/l	134	(46—245)
Azetaldehyd-SO_2 mg/l	94	(46—163)
Rest-SO_2 mg/l	25	(0—64)
Azetaldehyd mg/l	65	(32—126)

Tabelle 2

Durchschnittswerte (Streubereich) der freien und gesamten schwefligen Säure, der azetaldehyd-schwefligen Säure, der restschwefligen Säure und des Azetaldehyds von Weinen

	31 durchgegorene Weine des Handels mit Zuckergehalten unter 2 g/l (in- u. ausländ. Weine)	28 Weine des Handels mit Restzucker (in- u. ausländ. Weine)	31 durchgegorene Weine des sauerfaulen Jahrganges 1968 (fränkische Weine)
Gesamt-SO_2 mg/l	129 (37—318)	307 (120—515)	203 (62—357)
Freie SO_2 mg/l	24 (6—66)	34 (7—65)	33 (10—61)
Azetaldehyd-SO_2 mg/l	82 (42—223)	232 (96—440)	100 (12—181)
Rest-SO_2 mg/l	23 (0—52)	41 (7—81)	70 (10—226)
Azetaldehyd mg/l	57 (17—158)	160 (66—303)	69 (29—125)
Zucker g/l	—	22 (3—69)	—

Die Weine der Tabelle 2 sind in 3 Gruppen aufgeteilt, welche sich in charakteristischer Weise unterscheiden:

Gruppe 1 (Durchgegorene Weine):

Die gesamte schweflige Säure besitzt im Durchschnitt eine Größenordnung, welcher der vor einem Jahrzehnt noch für ausreichend angesehene Grenzwert von 200 mg/l gesamter schwefliger Säure gerecht würde. In der gleichen Größenordnung liegen die von *Kielhöfer* mitgeteilten Werte. Bei diesen Weinen macht die azetaldehyd-schweflige Säure im Durchschnitt 78 % der gebundenen schwefligen Säure aus; bei *Kielhöfer* errechnen sich 79 %. Die restschweflige Säure beansprucht somit im Mittel nur 22 % der gebundenen schwefligen Säure (bei *Kielhöfer* 21 %).

Gruppe 2 (restzuckerhaltige Weine):

Der Durchschnitt der gesamtschwefligen Säure ist gegenüber der 1. Gruppe um 178 mg/l angestiegen. Dieser Anstieg geht fast ausschließlich zu Lasten der an Azetaldehyd gebundenen schwefligen Säure (Anstieg = 150 mg/l). Auch hier entfällt der Hauptanteil der gebundenen schwefligen Säure auf die azetaldehyd-schweflige Säure (85 %).

Gruppe 3 (durchgegorene Weine aus einem sauerfaulen Jahrgang (1968):

Hier ist der absolute Wert der restschwefligen Säure gegenüber normalen durchgegorenen Weinen im Durchschnitt auf das Dreifache angestiegen. Der Prozentsatz der azetaldehyd-schwefligen Säure ist dementsprechend von 78 % auf 59 % gesunken, während der Anteil der restschwefligen Säure von 21 % auf 41 % der gebundenen schwefligen Säure angestiegen ist. In einem Falle bestehen sogar 69 % der gebundenen schwefligen Säure (226 mg/l absolut) aus restschwefliger Säure. Dieser Wein hatte einen Gehalt an gesamter schwefliger Säure von 357 mg/l, war also im üblichen Sinne überschwefelt. Eine diesbezügliche Beanstandung wäre aber in Kenntnis der Bindungsformen hier völlig ungerechtfertigt, da der zur Überschwefelung führende natürliche Mehrbedarf an schwefliger Säure ursächlich durch eine vom Winzer nicht zu verantwortende anormale Beschaffenheit des Lesegutes bedingt war.

Im höchsten Maße interessant dürften in diesem Zusammenhang die in Abbildung 2 graphisch dargestellten Beobachtungen bei restsüßen Weinen sein. In Abb. 2 wurde die azetaldehyd-schweflige Säure in Abhängigkeit vom jeweiligen Restzuckergehalt der Weine aufgetragen. Eine Proportionalität zwischen dem Zuckergehalt und dem Bedarf solcher restsüßer Weine an SO_2 (deren Restsüße ausnahmslos keine echte Restsüße war, sondern durch Zusatz von Süßreserve bewirkt worden sein muß, wie die dabei festgestellten Glukose : Fruktose-Verhältnisse von nahezu 50 : 50 be-

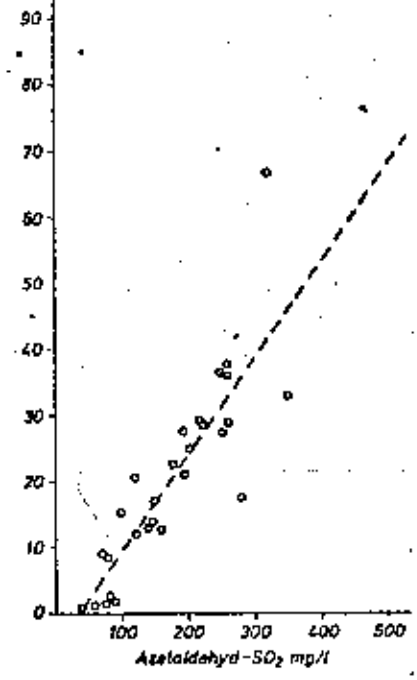

Abb. 2.

weisen) ist unverkennbar. Interessant ist vielleicht auch der Hinweis, daß die Verlängerung der Proportionalitäts-Geraden in Abb. 2 bei ca. 150 bis 200 g/l Zucker zu einem Gehalt an Azetaldehyd-SO_2 von ca. 1500 mg/l führen würde.

Als Erklärung für die in Abb. 2 zu erkennende Proportionalität bieten sich zwei Denkmöglichkeiten an.

a) Man könnte daran denken, daß der Gärungsdruck mit steigendem Zuckergehalt größer wird, so daß eine größere Menge des Gärungszwischenproduktes Azetaldehyd durch SO_2 abgefangen werden muß, damit die Umwandlung des Azetaldehydes in Alkohol und damit die initiale Einleitung des eigentlichen Gärprozesses nicht erfolgt. Diese Deutung

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würde zu der Erfahrungstatsache passen, daß zum sogenannten Totschwefeln von Traubenmosten, das in südlichen Ländern geübt wird, ca. 1300 bis 1500 mg/l schwefliger Säure notwendig sind[6]). Dies wäre aber die Menge, zu welcher auch die Verlängerung der Geraden der Abb. 2 bei einem Zuckergehalt von etwa 150 bis 200 g/l führen würde.

b) Man könnte die Proportionalität aber auch damit deuten, daß die Süßreserven bereits totgeschwefelt waren, was bedeuten würde, daß sie einen Gehalt an gesamtschwefliger Säure von 800 bis 1200 mg/l aufweisen müßten. Dann wäre verständlich, daß mit steigender Zugabe von Süßreserven, d. h. von unvergorenem Zucker, auch die Menge der bei totgeschwefeltem Lesegut an Azetaldehyd gebundenen schwefligen Säure in derartigen süß gehaltenen Weinen ansteigen müßte.

Für die aus Abbildung 2 zu ziehenden *praktischen* Schlußfolgerungen kann es aber dahingestellt bleiben, ob der eine oder andere Erklärungsversuch zutrifft oder noch weitere Deutungen in Betracht zu ziehen sind; wie immer man die erwähnte Beobachtung auch deuten mag, die sowohl aus Abbildung 2 wie auch aus Tabelle 2 hervorgehende Erfahrungstatsache bleibt bestehen, daß der SO_2-Gehalt (und zwar ausschließlich die an Azetaldehyd gebundene schweflige Säure) eines Weines um so größer wird, je mehr Restzucker er enthält. An Hand der Abbildung 2 könnte man beinahe vorhersagen, welchen Gehalt an azetaldehydschwefliger Säure und damit auch an Gesamt-SO_2 ein Wein mit einem bestimmten Restzuckergehalt zwangsläufig besitzen wird.

Nachdem durch die Arbeiten von *Krämer* und Mitarbeitern[7]) dargelegt worden ist, daß auch die an Azetaldehyd gebundene SO_2 in ihrer toxischen Wirkung nicht wesentlich anders zu beurteilen ist als die freie SO_2, kann wohl kaum ein Zweifel daran bestehen, daß das Bestreben dahin gehen muß, die schweflige Säure im Wein so niedrig wie möglich zu halten.

Im Hinblick auf die nach den obigen Ausführungen bei im Verkehr befindlichen restsüßen Weinen beobachtbare Abhängigkeit des SO_2-Gehaltes von der Menge des Restzuckers dürfte deshalb die Frage berechtigt sein, ob Bestrebungen, den Restzuckergehalt von Tischweinen zu erhöhen, in Zukunft weiterhin ausschließlich nur unter wirtschaftlichen Gesichtspunkten diskutiert werden sollen, oder ob nicht in erster Linie dabei an die mit einer Erhöhung des Restzuckergehaltes einhergehende Erhöhung der schwefligen Säure gedacht werden sollte.

Nach unserem Ermessen kommt also der Bestimmung des Azetaldehydes bzw. der azetaldehyd schwefligen Säure und der damit möglichen Differenzierung der einzelnen Bindungsformen der SO_2 im Wein eine weitaus größere Bedeutung zu, als ihr bisher beigemessen worden ist. Sie ist nicht nur für eine gerechtere Beurteilung von faktischen Überschwefelungen von Bedeutung, sondern sollte auch im Hinblick auf die Sammlung eines weitgespannten statistischen Materials über die Abhängigkeit der Menge der an Azetaldehyd gebundenen schwefligen Säure vom Restzuckergehalt routinemäßig in die Untersuchung einbezogen werden. Mit Hilfe der beschriebenen Schnellmethode wäre eine solche routinemäßige Bestimmung keine Erschwernis der chemischen Untersuchung.

Zusammenfassung

Es wird eine Schnellmethode zur Bestimmung des Azetaldehydes bzw. der an Azetaldehyd gebundenen SO_2 im Wein beschrieben. Die Bestimmung erfolgt im vorher entfärbten Wein direkt kolorimetrisch auf der Basis der bekannten Farbreaktion zwischen Azetaldehyd, Piperidin und Nitroprusssäurenatrium. Sie dauert nur einige Minuten. Serienmäßige Ansätze sind möglich.

Vergleiche zwischen dieser Schnellmethode und der von *Jaulmes* und *Espezel* beschriebenen Destillationsmethode ergaben gut übereinstimmende Werte.

Die Bedeutung, welche der Bestimmung des Azetaldehydes und der damit möglichen Differenzierung der einzelnen Bindungsformen des SO_2 zukommt, wird an Hand von 90 untersuchten Weinen diskutiert. 3 Gruppen von Weinen werden unterschieden: durchgegorene Weine, restsüße Weine und Weine aus faulem Lesegut: Bei restsüßen Weinen geht der wesentlich erhöhte Gehalt an gesamter SO_2 fast ausschließlich zu Lasten der azetaldehydgebundenen SO_2. Es besteht eine deutliche Proportionalität zwischen dem Restzuckergehalt und der Menge des an Azetaldehyd gebundenen SO_2.

Bei Weinen aus faulem Lesegut wird die Erhöhung des gesamten SO_2 fast ausschließlich durch die sogenannte rest-schweflige Säure bewirkt. Hier liegt demnach ein durch die Kellerbehandlung kaum beeinflußbarer natürlicher Mehrbedarf an SO_2 vor, der u. U. zu einer Überschreitung der gesetzlich festgelegten Grenzwerte führen kann. In solchen Fällen kann die Differenzierung der Bindungsformen des SO_2 zu einer sachgerechteren Beurteilung von Überschwefelungen beitragen.

Summary

The article describes a speedy method to determine acetaldehyde and the SO_2 tied to acetaldehyde, in wine. The analysis is done directly, in previously decolorized wine, on the basis of the well-known colour reaction between acetaldehyde, piperidine and sodium nitroprusside. It takes only minutes, and series preparations are possible.

Comparisons between this quick method and the distillation process described by *Jaulmes* and *Espezel* have yielded figures of good congruity.

The importance of determining the acetaldehyde, which makes it possible to differentiate between the individual linkage types of the SO_2, is discussed on the basis of 90 wine samples analysed. These samples were split up into three groups: fully fermented wines, residual-sugar wines, and wines made from rotten fruit. In residual-sugar wines, the considerably increased total SO_2 content is almost exclusively an increase in acetaldehyde-linked SO_2. There is a distinct proportionality between the amount of residual sugar and the amount of acetaldehyde-linked SO_2.

In wines made from rotten fruit, the increase in total SO_2 is almost exclusively brought about by the remaining sulfur dioxide. This would suggest a natural surplus demand for SO_2 that can hardly be controlled through the cellar treatment and may possibly lead to contents beyond the limits specified by law. In such cases, a differentiation between SO_2 linkage types may help to view excessive sulfur contents more pertinently.

Résumé

L'article décrit une méthode rapide pour la détermination de l'acétaldéhyde, ou le SO_2 lié à l'acétaldéhyde, contenu dans le vin. La détermination s'effectue directement dans le vin préalablement décoloré par colorimétrie sur la base de la réaction colorée connue entre acétaldéhyde, pipéridine et sodium nitroprusséque. Elle ne dure que quelques minutes. Des essais en série sont possibles.

Des comparaisons entre cette méthode rapide et la méthode de distillation décrite par *Jaulmes* et *Espezel* ont démontré des résultats correspondants.

L'importance à attribuer à la détermination de l'acétaldéhyde pour différencier les différents types de liaisons de SO_2 est discutée par référence à 90 sortes de vins examinés, où distingue trois différents groupes de vins: des vins entièrement fermentés, des vins doux à sucre résiduel et des vins produits à partir des raisins pourris. Dans les vins doux à sucre résiduel, le contenu total en SO_2 est considérablement élevé, presque entièrement au détriment de SO_2 lié à l'acétaldéhyde. Il y a là une proportionnalité

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très nette entre le contenu en sucre résiduel et la quantité de SO_2 lié à l'acétaldéhyde.

Dans les vins produits à partir des raisins pourris, l'augmentation totale en SO_2 est presque exclusivement due à l'acide dit sulfureux résiduel. Ici, il s'agit donc d'un besoin supplémentaire naturel en SO_2 peut initiemgalde par le traitement en cave, qui peut aussi, selon les circonstances, à surpasser les limites légales. Dans ces cas, la distinction des différentes formes de liaisons de SO_2 peut contribuer à une appréciation plus juste de la sulfuration.

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Zur Restmengen-Bestimmung von Acrylnitril in Lebensmitteln

Von Ernst Kröller

Zum Entwesen von Mühlen, Speichern und Lebensmittelbetrieben werden vorzugsweise Begasungsmittel eingesetzt, zu denen nach Abs. B I e des Pflanzenschutzmittelverzeichnisses der Biologischen Bundesanstalt[1]) u. a. das Acrylnitril gehört. Dieser Stoff ist stark toxisch und wird deshalb in der Giftabteilung I der Giftverordnung der Länder eingeordnet. Die Anwendung erfolgt als Reinsubstanz („Ventox®") oder als 20%ige Lösung in Tetrachlorkohlenstoff („Acrylon®") in Spezialapparaturen, die eine gleichmäßige Verdampfung und Verteilung im Raum bewirken. Die Acrylnitrilkonzentration liegt im allgemeinen zwischen 25 und 40 g/m³, die Einwirkungszeit beträgt 48 Std. Begast wird eine große Anzahl von Lebensmitteln und Drogen sowie Mühlen und leere Speicherräume. Nach der Begasung wird der Hauptteil des Wirkstoffes durch Belüften verflüchtigt, während je nach Art des Lebensmittels verschieden große Restmengen dann verbleiben. Da z. Z. noch kein zuverlässiges Verfahren zu ihrer Bestimmung besteht, wurde noch kein Toleranzwert für Acrylnitril in die Höchstmengen VO — Pflanzenschutz —[2]) aufgenommen. Bis dahin wird nur eine Restmenge von 0,01 ppm Acrylnitril in Lebensmitteln geduldet. Im Hinblick auf die Festlegung eines Toleranzwertes wurde eine empfindliche Bestimmungsmethode für die lebensmittelchemische Überwachung ausgearbeitet, worüber im folgenden berichtet wird.

A) *Voruntersuchungen*

I. Eigenschaften und chemisches Verhalten.

Acrylnitril (Vinylcyanid), $CH_2=CH-CN$ ist eine farblose Flüssigkeit von charakteristischem Geruch, deren Siedepunkt bei 77,6° C liegt, Sp. −− − 82° C. Seine Flüchtigkeit bei 20° C beträgt 500 mg/l und der Dampfdruck 174 Torr. Die relative Gasdichte des Dampfes (Luft = 1) liegt bei 1,83. Mit einem Flammpunkt von 0° C ist es sehr leicht entzündlich (Zündgrenzen zwischen 3 und 17 Vol-%). In reinem Zustand ist Acrylnitril nicht haltbar und polymerisiert oft explosionsartig. Antioxydantien wirken stabilisierend. Es ist sehr reaktionsfähig (z. B. „Cyanäthylierungen") und stark toxisch (3) (MAK-Wert = 20 ppm [45 mg/m³]). Mit vielen organischen Lösungsmitteln ist es mischbar, in Wasser ist es zu etwa 7,5 % löslich.

II. Versuche zur analytischen Bestimmung.

Die ersten Untersuchungen zur Analytik des Acrylnitrils erfolgten im Hinblick auf seine Bestimmung als Monomerenanteil in Polyacrylnitril-Kunststoffen. Es wurden hierzu u. a. polarographische[4]) und papierchromatographische[5]) Methoden, die UV-Spektralphotometrie bei ~ 210 nm[6]) und IR-Photometrie[7]) herangezogen. Die Empfindlichkeit dieser Verfahren ist jedoch meist so gering, daß die in Lebensmitteln noch geduldeten µg-Mengen damit nicht mehr erfaßt werden können. Es mußte deshalb eine andere — weitaus empfindlichere — Reaktion gefunden werden, mit der im µg-Bereich noch zuverlässige Bestimmungen möglich sind.

Hierbei wurde beobachtet, daß bei der Einwirkung von Brom auf Acrylnitrillösungen eine Verbindung entsteht, die mit Pyridin-Benzidinlösung einen rot- bis rotbraunen Farbstoff liefert. Die Überprüfung dieser Reaktion ergab, daß die Farbstoffbildung dem Lambert-Beer'schen Gesetz gehorcht. Es stellte sich jedoch heraus, daß bei gleicher Acrylnitrilmenge die an verschiedenen Tagen angesetzte Farbreaktion zu verschiedenen Ergebnissen führt. Die Erklärung dafür wurde in einer starken Lichtabhängigkeit des Bromierungsvorganges gefunden. In völliger Dunkelheit erfolgt mit Brom keine Reaktion, während in hellem Sonnenlicht eine starke Bromierung stattfindet. Es liegt somit eine photochemische Reaktion zwischen Acrylnitril und Brom vor, bei der u. a. aus der Nitrilgruppe quantitativ Bromcyan (CNBr) entsteht. Dieses reagiert dann leicht mit Pyridin und Benzidin zu einem roten Azomethinfarbstoff mit einem Absorptionsmaximum von 524 bis 525 nm, der z. B. zur quantitativen Bestimmung der Blausäure dienen kann[8]). Bei der Erprobung verschiedener Lichtquellen erwies sich eine Quecksilberdampflampe für den stöchiometrischen Verlauf der Bromierung am geeignetsten. Versuche zum Ersatz des Broms durch andere Halogenierungsmittel (z. B. JCl, JCl_3, JBr_3, J_2) schlugen fehl. Die eingesetzte Brommenge hatte auf den Reaktionsverlauf keinen Einfluß. Nach diesen Ergebnissen konnten im Bereich von 1 bis 20 µg Acrylnitril reproduzierbare Werte erhalten werden.

Als nächstes wurde die Abtrennung des Acrylnitrils aus Lebensmitteln untersucht. Dabei zeigte sich, daß eine Extraktion mit leicht flüchtigen Lösungsmitteln wie u. a. Methylenchlorid, Diäthyläther oder Pentan nicht möglich ist, da sich das Acrylnitril mit diesen Stoffen sehr leicht beim Einengen mitverflüchtigt. Erfolgreicher gestaltete sich eine destillative Abtrennung durch Erwärmen des Lebensmittels im Stickstoffstrom, jedoch treten dabei hohe Verluste durch Polymerisation des Acrylnitrils an der großen Oberfläche der Probe auf. Diese Verluste lassen sich jedoch weitgehend vermeiden, wenn die Destillation in Gegenwart eines hochsiedenden Lösungsmittels erfolgt. Hierzu eignet sich

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THE SYSTEMIC AND LOCAL EFFECTS OF THYOGLYCOLIC ACID AND BISULPHITE COMPOUNDS

By Frederick Reiss, M. D.,* and Bruno Gerstl, M. D.**

The physical and chemical properties of keratin are closely related to the presence of cystine cross-linkages in the protein. Goddard and Michaelis[1] found that sodium thioglycolates dissolve wool at a pH of 11 or higher, opening cystine linkages according to the following equation:

$R\,SSR + 2\,HSCH_2COOH \rightarrow 2RSH + (SCH_2COOH)_2$. This reaction with thioglycolate is reported to be also present at a lower pH[2] but only small amounts of keratin go into solution[3] at pH 9.5 or lower.

Sodium bisulphite opens cystine linkage according to the following equation: $R\,SSR + H_2SO_3 \rightarrow R\,SH + R\,SSO_3H$. The optimum conditions for bond openings are pH of 5.5-6.0 and concentration of 2M[4]. These reactions indicate that the substances mentioned above may be harmful when brought in contact with live tissue.

Thioglycolates are used in industry for dehairing hides and in processing wool. They are also applied cosmetically as depilating creams and in making "cold permanent waves". Bisulphites and sulphur dioxides are employed as bleaching and preserving agents in food and textile industries, as well as for treatment of parasitic skin diseases. The wide use of these compounds made an investigation of their biological effects desirable.

The toxicity of thioglycolate was found incidental to other studies. Hill and Lewis[5] noted that 330 mg. of sodium thioglycolate given by mouth were fatal to a two kg. rabbit. Cohen[6] reported that 100 mg. of thioglycolate per kg. animal killed a rabbit within 24 hours.

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Rost and Franz[7] reported that 3-4 ml. of a M-aqueous solution of sodium bisulphite (3-400 mg.) given intravenously were lethal for rabbits. The death of the animals seemed to be due to paralysis of the vascular and respiratory centers and occurred within a short time after the injection. If the animal survived the first hour after the injection, later adverse effects could not be ascertained by clinical observation. On repeated injection of smaller doses, no toxic effect could be observed and apparently no accumulation took place. Oral doses as high as 0.4 gm. per kg. were tolerated.

Materials and Methods

Experiments Concerning Systemic Effect

Thioglycolic acid (Eastman Kodak) was prepared as 0.6 M-aqueous solution of soduim thioglycolate of pH 9.6-9.7. Sodium bisulphite C.P. was dissolved in water and the pH adjusted to 5.85 with NaOH and the concentrations arbitrarily selected at 0.6 M and 1.8 M. Bisulphite was also employed as salt of ethanolamine which, as an organic cation, should render the compound more lipoid soluble. It was prepared by passing SO_2 into an aqueous solution of ethanolamine at a low temperature. The pH then was adjusted to 5.85 and the concentration to 0.6 M. The bisulphite solutions rejected were 0.6 M in each instance.

For toxicity tests mice and rabbits were used. Albino mice of 22-25 gm. were injected intraperitoneally and observed for gross changes such as convulsions and survival rates. The rabbits were mature animals kept on purina diet with addition of fresh lettuce and with free access to water. The solutions were injected into the earvein and the blood pressure was subsequently determined on the central artery of the ear by means of a rubber pellot.

Experiments Concerning Effects on Skin

For studying the effect on the skin, white rabbits and pigs were used. The respective areas were shaved with an electric clipper 24 hours prior to the experiment. Three methods of a application were tried.

A. A surgical sponge measuring 1.5 x 1.5 inches was soaked with the respective solution, re-wetted repeatedly during the experiment and kept in intimate contact with the skin.

B. The solution was sprayed on the rabbit skin by means of an atoomizer The distance of the nozzle from the skin was 8 inches.

C. The fluid tested was applied by injecting it into rubber cups that had been fastened to the rabbit skin with Johnsons liquid

adhesive. The inside diameter of the cup was 14 mm. and the height 1.5 mm. and the content approximately 0.40 ml. To exert pressure on the skin, slightly more fluid (0.5 ml.) than the capacity of the cup was injected. The method is similar to that of Draize et al.[8]

In all groups the treated area of skin was excised immediately after termination of the experiment and fixed in 10% formalin. Histologic sections were stained with haematoxylin-eosin or by Massons' method.

I. Systemic Effects

Five groups of three mice each were injected intraperitoneally with 0.3, 0.2, 0.1 and 0.05 ml. of 0.6 M thioglycolate. The animals injected with 0.2 or 0.3 ml. became irritable, jumped up to the cover of the cage but quieted down in about 45 minutes. All animals were dead 3 hours after injection. One of the 3 animals injected with 0.1 ml. died 18 hours after injection while the other two and the mice injected with 0.05 ml. survived. The results obtained with intravenous injections of thioglycolate in rabbits, summarized in Table I, were similar to those of Cohen.[5] The absence of methemoglobin formation is noteworthy. For the production of its toxic effect, thioglycolate requires apparently a prolonged time resulting in death 18 to 48 hours after injection.

Mice injected intraperitoneally with 0.5, 0.4 or 0.3 ml. of a 0.6 M bisulphite solution died within a few minutes. Of a group of 5 mice injected with 0.2 ml., two died shortly after injection while three survived. Four mice injected with 0.1 ml. of the same solution also survived.

Six rabbits were injected intravenously with ethanolamine bisulphite in amounts from 0.57 to 1.45 millimols per kg. rabbit. All animals receiving one millimol or more per kg. showed rapid fall of blood pressure, clonic convulsions and spontaneous defecation, terminating in death within a few minutes after injection. Rabbits injected with 0.87 millimols bisulphite per kg. or less showed slight or no effect on blood pressure and recovered soon without subsequent effect. In another experiment, sodium bisulphite instead of ethanolamine bisulphite was used. Its toxicity was identical (1.02 millimols per Kg.) to that of the ethanolamine solution indicating that the ethanolamine did not have an enhancing effect.

Microscopic study of the parenchymatous organs (heart, lung,

liver, kidneys and spleen) of these rabbits failed to reveal any morphological changes.

Two other rabbits of similar weight were injected intravenously with 0.6 M ethanolamine bisulphite solution three times weekly for a period of three weeks. The total amount injected was 13.2 millimol, or 2.92 millimols per kg. body weight. Blood counts and haemoglobin determinations were made prior to the start and at weekly intervals. A slight increase of polymorphonuclear cells was observed in both animals throughout the period of injections. No further effect was seen. The rabbits were sacrificed 14 days after the last injection. Another rabbit was injected similarly with corresponding molar amounts of thioglycolate. Gross and microscopic study of the organs failed to reveal any changes that could be correlated with injections.

II. Local Effects on Rabbit Skin

A. Sodium thioglycolate:

Two rabbits were treated with thioglycolate (1.8 M) by method A. The soaked sponges were applied for 30 and 180 minutes. Microscopic sections of the skin treated for 30 minutes revealed that the upper layer of the stratum corneum was largely missing and only necrotic fragments of it were atrached to the preserved basal layer. The cortex of the extracutaneous part of the hair also showed marked fragmentation. No cellular reaction of epidermis or cutis, however, was found.

Microscopic preparations of the skin treated for three hours showed that the upper layer of the epidermis was replaced by clumps and irregular shaped pieces of pink-staining material. The basal cells were flattened in some instances. No cellular infiltration was observed.

B. Sodium Bisulphite:

Sodium bisulphite was applied to 8 rabbits by methods A and C. The results obtained are summarized in Table II.

Two rabbits (#37 and #39) were treated by method B. The skin of rabbit #37 was sprayed with 500 ml. of 0.6 M sodium bisulphite solution and the procedure repeated 24 hours later. There were no gross changes of the skin. Microscopic sections revealed moderate lamellated hyperkeratosis. No cellular infiltrations or edema were found. Rabbit #39 was treated with 3600 ml. of a 0.6 M bisulphite solution in mono-ethanolamine. The skin showed

small hemorrhages and edema at the end of treatment. Histologic examination revealed several foci of necrosis and polymorphonuclear infiltration in the epidermis. Edema of the cutis and polymorphonuclear infiltration were noted in the upper layer of the corium. The observation should be noted here that the pH of the spray when collected had shifted to 3.0. This would indicate oxidation of the bisulphite to the more acid sodium bisulphate and some of the changes may be due, therefore, to the sulphites at an acid reaction.

III. Local Effects on Pig Skin

In this experiment, a molar solution of sodium bisulphite of pH 5.85 and a molar solution of sodium thioglycolate of pH 9.8 were applied on corresponding sides of the shaved abdominal skin of a 45 pound female pig employing method A for 30 and 180 minutes. Bisulphite after 30 minutes and 3 hours respectively and thioglycolate after 30 minutes had no visible effect on the skin except moderate hyperemia. Thioglycolate after a 3 hour application disclosed not only an intense hyperemia and edema but also spotty hemorrhages.

Microscopically the skin treated with thyoglycolic acid for 30 minutes showed edema and partial vacuolization of some epidermal cells with moderate polymorphonuclear infiltration and localized edema of the pars papillaris of the cutis. The horny layer of the adjacent area was thinner than usual and loosely laminated. After 3 hours of application this agent produced acidophilic discoloration of the cytoplasm of the epidermal cells similar to that seen in erythema multiforme. Furthermore there was pyknosis and karryorrhexis of nuclei as well as loss of the upper epidermal layer at several points. Complete destruction of epidermis and necrosis of the upper layer of the cutis (Fig. 1) was noted for a distance of about 3 mm. In the preserved parts of the epidermis, moderate polymorphonuclear infiltration as well as hyperchromasia of the nuclei of epidermal cells was found.

The skin treated with sodium bisulphite for 30 minutes showed an intact epidermis with a compact keratin layer. Slight edema of the pars papillaris of the cutis and occasional splitting of collagen fibers were noted. A few minimal foci of polymorphonuclear infiltration were seen in the epidermis (Fig. 2) of the skin treated for three hours with bisulphite. The keratin layer was more laminated and detached than in the sections obtained after 30 minutes of treatment.

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Fig. 1. Skin of pig treated with a M solution of sodium thioglycolate for 3 hours (method A) showing loss of epidermal layers at several points due to severe necrosis. Localized edema in the upper cutis. H & E x 157.

Fig. 2. Skin of pig treated with a M solution of sodium bisulphite for 3 hours (method A) showing an intact epidermis. Slight edema of the pars papillaris of the cutis with splitting of collagenous fibers. H & E x 200.

Discussion

Studies concerning the systemic effects of thioglycolic acid and sodium bisulphite show both substances to be capable of producing death of the experimental animal when given in relatively large doses. When tested in mice, the toxicity of the two compounds was of about the same order. The mechanism of their action, however, differs. The neurological disturbances observed after injection of thioglycolic acid and the absence of lesions in heart and parenchymatous organs even by microscopic examination indicate a late effect upon the central nervous system probably somewhere in the motor area. As reported by Hills and Lewis[7] thioglycolic acid is oxidized in the animal body and eliminated. When the amount injected surpasses the oxidizing capacity of the red blood cells, damage upon the nerve tissue becomes apparent.

Sodium bisulphite in adequate doses exerts an instantaneous effect upon the central nervous system as indicated by clonic convulsions, and particularly upon the cardiovascular center with consequent rapid fall of blood pressure.

The minimal lethal dose of thioglycolic acid was found to be between 1.00 and 1.85 millimols per kg. rabbit, that of bisulphite, either as sodium or ethanolamine salt, as 1.02 millimols.

Sodium thioglycolate has been known to be a keratolytic agent (Hoshall, 9) and its external application could be presumed to be followed by changes of the upper epidermal layer. The experiments reported confirm this assumption.

Although the skin of rabbit has been recommended repeatedly for investigations of this nature the relative delicacy of its epidermal layer made it advisable to employ another animal species whose epidermal architecture would be more comparable to that of human skin. The necrotizing effect upon the epidermis and extension of the lesion into the deeper layers of skin, however, were in pigs even more obvious than in rabbit skin (Fig. 2).

Sodium bisulphite both in aqueous solution and dissolved in ethanolamine impaired the integument; and superficial necrosis was seen when its was applied for a prolonged period upon rabbit skin (Table 2). It is, however, distinctly less harmful than thioglycolic acid as evidenced by the experiments on pig skin (Fig. 2). An almost intact skin was found after a 30 minutes application of bisulphite, while degeneration and vacuolization of epidermal cells, as well as polymorphonuclear infiltration was noted after thioglycolic acid treatment of identical duration.

A comparison of the results of a 3 hour treatment reveals that a few minimal foci of polymorphonuclear infiltration of the epi-

dermis and lamination and detachment of the keratin layer represented the effect of the bisulphite, while thioglycolic acid produced extensive necrosis (Fig. 1) and severe degenerative and cellular reaction of epidermis and cutis.

Summary

1. The minimal lethal dose of thioglycolic acid for 22-25 gm. white mice was 0.1 cc. and that of sodium bisulphite 0.2 cc. of a 0.6 M solution.
2. The minimal lethal dose of thioglycolic acid and bisulphite respectively for rabbits was 1.00 to 1.85 and 1.02 millimols per kg. animal. The toxicity of the sodium salt equalled that of the ethanolamine salt.
3. Thioglycolic acid given parenterally is toxic to the central nervous system. It requires for its effect a period of 18 to 48 hours. Bisulphite administered parenterally in larger doses has an immediate toxic effect probably by blocking the oxidation mechanism.
4. Both substances upon local application produce changes of the skin which are noticeable histologically after as short an interval as 30 minutes in both rabbits and pigs. The lesions due to bisulphite are slight while those induced by thioglycolic acid in comparable experiments consist of destruction of the epidermis and necrosis of the upper layer of the cutis.

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TABLE I

Rabbit No.	Rabbit Weight in Grams	Compound injected	Total amount in milliods	Amount per kg. in milliods	Observation Table
XXI	1825	Thioglycolic acid	5.00	2.75	Labored respiration for 17 minutes. No convulsions. Rabbit was well 25 minutes after injection. Blood pressure variations not more than 15 mm. Rabbit was found dead 24 hours after injection in a state of advanced post-mortem autolysis. No microscopic sections taken.
XX	1860	"	4.00	2.15	Respiration labored for 16 minutes after injection. Hyperemia of visible vessels. Rabbit then recovered. No decided change of blood pressure. On the following day rabbit was very quiet. Upon attempt to put it upon its feet, paralysis of the front legs and weakness of the hind legs was noted. General reactions of rabbit sluggish. Rabbit found dead on second day. Advanced autolysis as seen in microscopic sections, prevented interpretation.
LIV	2700	"	5.00	1.85	No convulsions or other reactions noted during first 60 minutes after injection. Rabbit found dead 18 hours later. Microscopic sections did not reveal any changes of the heart. In the lungs, passive congestion was noted. Liver and spleen sections showed only post-mortem autolysis. An occasional glomerulus with a granular pink-staining exudate within the capsular space was seen in kidney sections.
LV	2700	"	7.50	2.77	Injected in two portions on 2 subsequent days. Animal became quiet after 2nd injection. No convulsions. 6 hours later comatose. 10 cc. of blood were withdrawn from the heart for determination of methemoglobin. Rabbit died after cardiac puncture. Microscopically, a few round cells with pyenotic nuclei were found in the sinusoids of bronchial lymph nodes and also in the spleen. Liver free of change. Glomerular capillaries congested, small round-cell exudate within glomeruli. A few glomeruli showed an hemorrhagic exudate. Spectroscopic examination of the blood failed to reveal the presence of methemoglobin.
LI	2830	"	2.83	1.0	No changes; survived.
LII	2820	"	2.44	0.87	No changes; survived.

TABLE II

EFFECT OF BISULPHITE SOLUTIONS ON RABBIT SKIN (Methods A and C)

Ion and Concentration	Duration of Exposure	Method	Rabbit No.	Histology
ulphite (1.8 Mol.) in onoethanolamine	30 min.	A	24	Slight to marked lamellary splitting of the horny layer with change of staining quality. Pyknosis of nuclei noted in stratum germinativum in a few instances.
ulphite (1.8 Mol.) in onoethanolamine	4 & 5 hrs.	C	5	Stratum corneum is slightly detached. Corium shows slight edema. Small amount of hemorrhagic exudate and minimal polymorphonuclear infiltration of the cutis.
$NaHSO_3$ (1.8 Mol.) in aqueous olution	5 hrs.	C	7	Stratum corneum is detached from the epidermis. Slight or no edema and an occasional polymorphonuclear cell found in the corium.
ulphite (1.8 M) in onoethanolamine	9 hrs.	C	1	Disintegration of epidermal cells at one point with intensive polymorphonuclear reaction. Edema of corium and small foci of polymorphonuclear infiltration.
hysiological saline	9 hrs.	C	1	No changes.
ulphite (1.8 Mol.) in onoethanolamine	20 hrs.	C	94	Small epidermal erosion and polymorphonuclear infiltration of corium.
$NaHSO_3$ (1.8 Mol.) in aqueous olution	20 hrs.	C	94	Changes similar to those produced by $NaHSO_3$ in monoethanolamine solution (Rabbit 94) but less extensive.
ulphite (1.8) Mol.) in onoethanolamine	24 hrs.	C	94	Necrosis of all layers of epidermis. Abscess formation with intensive polymorphonuclear reaction in all layers of cutis.
$NaHSO_3$ (1.8 Mol.) in aqueous olution	24 hrs.	C	94	Changes similar to those produced by $NaHSO_3$ monoethanolamine (Rabbit 94 & 7).
ulphite (1.8 Mol.) in onoethanolamine	72 hrs.	C	90	Sloughing off of epidermis with ulcer formation. Intensive polymorphonuclear infiltration and slight edema of cutis.
$NaHSO_3$ (1.8 Mol.) in aqueous olution	72 hrs.	C	97	Lesions similar to those induced by $NaHSO_3$ in Monoethanolamine but less intensive. No ulcer formation.
hysiological saline	72 hrs.	C	90 97	Minimal amount of polymorphonuclear infiltration in upper layer of corium.

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SULFUR DIOXIDE POISONING AS A CAUSE OF ASTHMA*

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MANY factors, such as cold, exercise, and overeating, have been recognized as nonspecific precipitating causes of bronchial asthma in individuals with a predisposed hereditary constitutional disposition. In all probability the inhalation of noxious gases, such as coal gas and sulfur dioxide gas, as well as the inhalation of gasoline vapors and fresh paint may be included in the same category of nonspecific precipitating causes.

Cooke[2] believed, on clinical grounds, that there may be a certain specificity in the inhalation of sulfur dioxide fumes, principally because this gas initiates attacks in relatively few asthmatic individuals, and in those who have been free from asthmatic attacks for a long time. However, there is no immunologic proof of the specificity of sulfur dioxide fumes in initiating attacks of bronchial asthma. It appears more likely, particularly in patients who have been exposed to a heavy concentration of sulfur dioxide fumes, that the primary effects are the result of an inflammatory, destructive lesion in the upper respiratory tract. The respiratory mucous membrane may be so altered that the bacteria already present or those inhaled multiply and gain access to underlying tissue to cause a suppurative bronchitis, as indicated in Case 1. Individuals who are constitutionally predisposed may respond to the products of the bacteria and develop a bacterial hypersensitivity, with a clinical symptom complex of bronchial asthma.

The inhalation of sulfur dioxide is very irritating to the upper respiratory tract. Even in low concentrations (0.001 per cent to 0.003 per cent)[3] it will cause sneezing, lacrimation, and a cough in most people. However, individuals who have been exposed to the sulfur dioxide fumes over a long period of time develop a certain degree of resistance, so that they can tolerate two or three times the above-mentioned concentration in the air, with little or no symptomatology.

Inhalation of sulfur dioxide gas has a deleterious effect on animals and human beings, and deaths have been recorded, following exposure of a few hours.[3] When sulfur dioxide is inhaled, contact with moisture transforms the gas to sulfurous acid which is rapidly oxidized to sul-

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furic acid. The gas is highly irritating, and in severe cases destructive lesions of the upper respiratory tract, such as bronchopneumonia and pulmonary edema, have occurred.

The effects of chronic exposure to sulfur dioxide have been studied. A group of 100 individuals, working in an electric refrigerator manufacturing plant, was investigated and compared with a healthy normal group of 100 people.[4] In the exposed group there was observed a diminution in the sense of taste and smell, an increase in the incidence of pharyngitis and tonsillitis, and increased fatigability and dyspnea on exertion.

Little cognizance of the role of sulfur dioxide as an exciting factor in attacks of bronchial asthma has been taken. No complete description of a case of asthma resulting from exposure to sulfur dioxide fumes had appeared in the literature until last year, when Dowling[5] described a case occurring in a repairman of electric refrigerators.

Within the past year we have studied the following three cases of bronchial asthma in men who had been working about electric refrigerators and who had been exposed to sulfur dioxide fumes.

CASE 1.—H. L., male, 32 years of age, was employed for ten years as a repairman of electric refrigerators. On a number of occasions he had been exposed to the fumes of sulfur dioxide from leaking refrigerators, and each time he had the usual reaction, consisting of fits of coughing, sneezing, and a running nose. Five days before admission to the hospital he was exposed to an unusually large concentration of leaking sulfur dioxide fumes, overcome with a severe attack of sneezing and coughing, and for the first time experienced breathlessness, with marked expiratory difficulty typical of bronchial asthma. He was seen by me at this time and referred to the Mount Sinai Hospital for further observation.

Physical examination disclosed a well-developed and well-nourished young adult male, with moderate cyanosis of the lips and fingernails. Respirations were labored and accompanied by audible wheezing. The chest was hyperresonant, and there was prolonged expiration with sibilant and sonorous rales. The remainder of the physical examination was entirely negative. Laboratory findings were negative.

While in the hospital he ran a rather stormy course, culminating in status asthmaticus. He was placed in an oxygen tent and helium was administered, but with only little improvement. He gradually improved, and began to respond to adrenalin injections, but frequent diurnal and nocturnal attacks of asthma persisted. These were accompanied by expectoration of large amounts of thick tenacious sputum which on a number of occasions was blood streaked. When he was somewhat improved, a bronchoscopic examination was performed. This revealed a large amount of cheesy secretion in both bronchi. Smears obtained from the exudate showed a great number of polymorphonuclear leucocytes, and occasional gram-negative and gram-positive diplococci. Search for acid-fast bacilli was without result. He remained in the hospital for the following two months with only slight improvement in the number of his attacks. X-ray of the chest on several occasions was always negative. Skin tests by the intradermal method were completely negative. At present, six months after the onset of his illness, he still has an occasional attack of asthma which responds well to adrenalin injections.

CASE 2.—I. G., male, 29 years of age, was employed as a repairman of electric refrigerators. He had never been previously ill. There was no family history or

personal history of allergic manifestations. He had been exposed to small amounts of sulfur dioxide fumes from leaking refrigerators for a number of months, and noticed the usual symptoms of running nose and burning sensation in the throat. One day after exposure to an unusually large amount of sulfur dioxide fumes from a leaking refrigerator, he experienced his first attack of difficulty in breathing. From that time on numerous typical attacks of bronchial asthma occurred. X-rays of his chest and sinuses were negative. Skin tests by the intradermal method revealed markedly positive reactions to chicken feathers and ragweed pollen, and moderately positive reactions to orris root, duck epithelium, dog epithelium, and rabbit epithelium.

He was observed for the entire following year, during which time he had frequent severe attacks of asthma which responded well to adrenalin injections. Since the patient observed no real improvement in his condition, he became discouraged and no longer sought medical advice.

CASE 3.—P. D., male, 37 years of age, was employed as a handy man in an apartment house. He had never been previously ill. There was no family history of allergy. Periodically, however, he had noticed a few urticarial wheals which he attributed to the eating of eggs. As part of his duties it was his job to keep the electric refrigerators in good repair and also to paint them with Duco enamel. He had frequently noticed some irritation of his eyes, running of his nose, coughing, and mild wheezing after exposure to leaking refrigerators. On one occasion after he had spent some time in an ill-ventilated cellar painting the boxes with Duco enamel, irritation of his nose and chest became especially noticeable. Despite these effects, he continued with his work. After some months, he again spent considerable time painting the refrigerators with Duco enamel. Following this, the previous mild wheezing became worse, and was accompanied by dizziness and weakness. He continued at his work for about ten days, but his attacks of respiratory difficulty became so severe that he had to enter the hospital.

On admission he was moderately cyanotic and dyspneic. Respirations were 24 a minute. He coughed spasmodically and brought up small amounts of thick tenacious sputum. There was normal pulmonary resonance throughout both lungs. Numerous sibilant and sonorous râles could be heard throughout both sides of the chest; prolonged labored respiration was noted. The remainder of the physical examination was completely negative. Laboratory findings were negative.

Skin tests by the intradermal method were completely negative, except for a slight reaction to egg white. Chest x-ray revealed no abnormalities in the lungs and a moderate enlargement of the left ventricle of the heart. While in the hospital, he continued to improve and was discharged twelve days after admission, still having moderate attacks of asthma. He was observed by his private physician for a few months, during which time he showed no further improvement. He became discouraged and discontinued his visits to the doctor.

This case is not as clear-cut as the other two, since there is the additional factor of Duco enamel, which is a complex mixture containing a number of acetates of alcohol, among them, amyl, propyl, and butyl. From the history of the case, it is apparent that the sulfur dioxide caused mild attacks of asthma, and the severe attacks were precipitated by the additional sensitivity to some element in the Duco enamel.

CONCLUSIONS

1. Three cases of prolonged bronchial asthma following exposure to sulfur dioxide fumes from leaking refrigerators are reported.

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2. Sulfur dioxide refrigerants represent an industrial hazard, particularly for individuals with an allergic background.

3. An individual with a family history of allergy or manifestations of illness due to hypersensitivity, should not seek employment in an occupation in which he may be exposed to sulfur dioxide fumes.

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Neuropathologic changes in a case of sulfite oxidase deficiency

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RECENTLY, Laster and associates[1,2] described the symptomatology and clinical course of a 32-month-old male infant suffering from a previously undefined metabolic disorder, characterized by sulfite oxidase deficiency.[1-3] Before elucidation of this disorder, three other siblings in the child's family had died during the neonatal period or during early infancy. In the present case autopsy revealed significant pathologic changes confined almost exclusively to the nervous system, a finding in keeping with the child's symptoms of severe motor and mental dysfunction. The following report describes these neuropathologic alterations.

CASE HISTORY

Since the patient's past history, clinical course, and biochemical abnormality have already been described in detail,[1,2] only a summary is included herein.

Past history. The infant weighed 3.9 kg. at birth and displayed hyperextension of head and back and mild tremor of the extremities. Motor dysfunction progressed steadily and by one month muscular rigidity was obvious in all extremities and he frequently assumed an opisthotonic posture with hyperextension of neck and back, flexed elbows, and extended lower extremities. Because of dietary intolerance, eggs and meat were gradually eliminated from his diet, and because of difficulties in swallowing, liquids were also withheld. By one year the child spent most of his time in an opisthotonic position. Nystagmus on lateral gaze and bilateral subluxation of the lenses were noted. An encephalogram suggested a focal disturbance in the left hemisphere. He suffered from intermittent respiratory infections. He never turned over, sat up, talked, or gave any evidence of recognizing people or objects.

Admission findings. The child was admitted to the National Institutes of Health at 30 months of age. He weighed 10.9 kg. and was 81 cm. long. His skin was yellow. A hemangioma, 1 cm. in diameter, was present in the scalp. He maintained the opisthotonic position except for brief moments. There was a striking increase in muscle tone with decorticate posture in the upper extremities and decerebrate posture in the lower. Rarely, rhythmic jerking movements were present in the upper extremities, right more than left. Deep tendon reflexes were increased and sustained. Clonus could be elicited in all extremities. The Babinski sign was present. Loud noises or painful stimuli elicited increased rigidity. He appeared to be blind, his eyes making wandering, apparently purposeless, movements. EEG revealed epileptiform discharges over the left hemisphere and evidence of diffuse, severe brain damage more marked on the left. Laboratory studies revealed a mild iron deficiency anemia and an elevated concentration of carotenoids in the serum.

Course. During his forty-five days in the hospital the patient began to have episodes of relative unresponsiveness accompanied by a marked decrease in muscle rigidity and associated with sinus bradycardia and sinus arrhythmia. Episodes of respiratory distress were also noted, beginning on the thirty-ninth hospital day. These lasted several hours and appeared intermittently and with increased severity on subsequent days. On the forty-fifth day he suddenly became cyanotic, sweaty, and flaccid. Pulse rate was 80 beats per minute, systolic blood pressure determined by palpation was 125 mm. Hg, temperature was 36.4° C., and respirations were 30 per minute. Moderate bronchospasm developed and was followed by cardiorespiratory arrest.

Sulfite oxidase deficiency. The patient's urine contained virtually no inorganic sulfate but displayed greatly elevated concentrations of sulfite, S-sulfo L-cysteine, and thiosulfate. No sulfite oxidase was demonstrable postmortem in liver, brain, or kidney, although this enzyme was readily de-

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Fig. 1. Marked hydrocephalus with severe atrophy of white matter and atrophy of cortical tissue in scattered gyri. A cystic lesion is present in the white matter of the frontal lobe (arrow).

monstrable postmortem in tissues from control subjects.[8] The concentrations of methionine-activating enzyme, cystathionine synthase, and cystathinase were determined in either brain, kidney, or liver and were always within normal limits.[8]

The deficiency of sulfite oxidase in the tissues was thought to explain the virtual absence of sulfate from the urine.[4] The increased concentrations of sulfite, S-sulfo-L-cysteine, and thiosulfate in the urine were thought to represent secondary manifestations of the oxidase deficiency.[4]

GENERAL AUTOPSY

The lungs displayed focal pulmonary edema, generalized congestion of intraalveolar capillaries, and a focal hemorrhage in the left lower lobe. The kidneys displayed focal microscopic calcifications in their papillae. Sections of deltoid muscle displayed atrophic muscle fibers. The skin was yellow and a hemangioma was present in the scalp. No significant changes were demonstrable in other organs.

NEUROPATHOLOGY

Gross examination. The brain weighed 470 gm. (normal for this age, 1,200 gm.). The meninges were transparent. Scattered sulci were deeper and wider than usual, accentuating the gyral pattern. Within these areas gyri appeared smaller than normal and displayed a granular surface. Gyri over the convexity of the left parietal lobe were most affected and this portion of the brain was depressed. Half the right hemisphere was removed for chemical analysis and the remaining brain was fixed in 10% formalin.

Examination of the fixed brain revealed considerable dilatation of the lateral ventricles (Fig. 1) and slight dilatation of the third ventricle. The aqueduct and fourth ventricle were of normal size. The ependyma was not remarkable except for an oval defect in the wall of the left lateral ventricle. The defect was 11 mm. long, 3 mm. wide, and 2 mm. deep and was surrounded by a pale yellow zone 1 mm. thick.

The white matter of the cerebral hemispheres was markedly diminished in width (Fig. 1) and within the deep white matter there were scattered small cysts up to 1 cm. in diameter. These were widely separated from one another and were sometimes traversed by a web-like mesh of residual tissue. Also in the deep white matter were areas of brown discoloration. Throughout the hemispheres the arcuate fibers stood out and appeared relatively free of alteration.

The cortical gyri varied greatly in appearance. Many appeared normal, but there were scattered

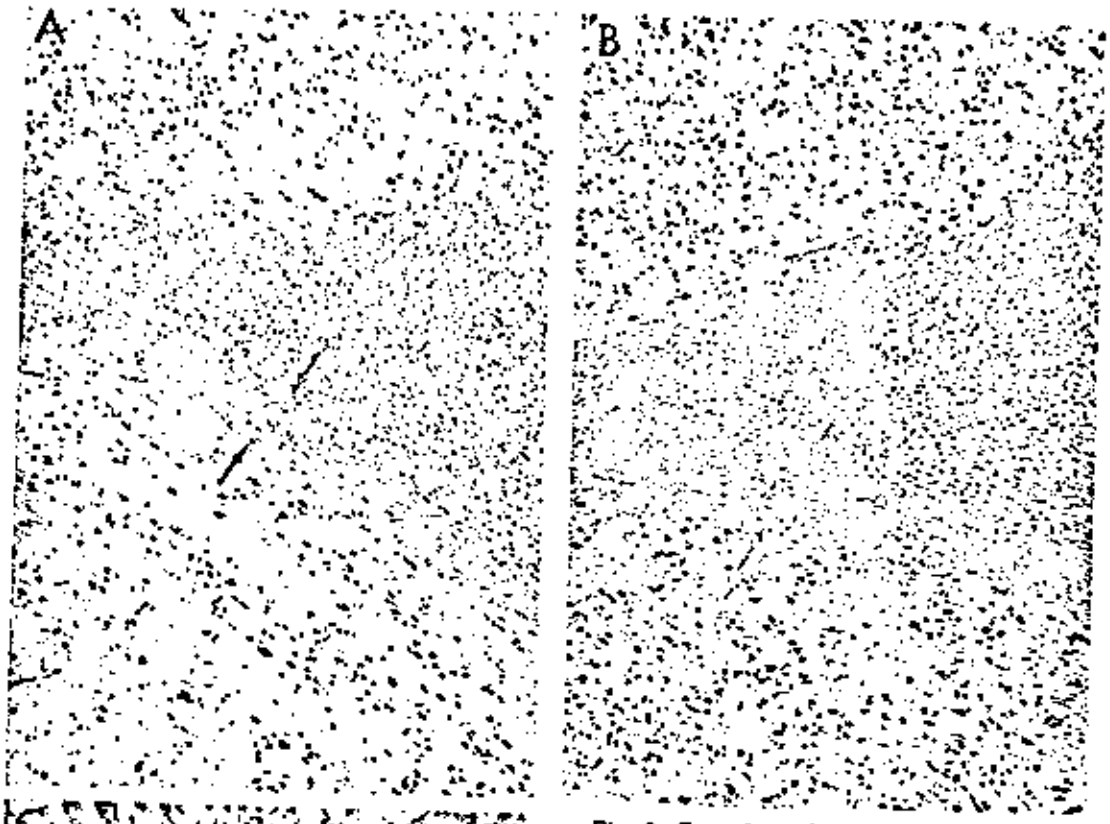

Fig. 2. Great loss of myelin (A, luxol fast blue-PAS, × 75) and axons (B, Bodian, × 85) in deep white matter between intact cortical tissue. The zone of arcuate fibers is relatively well preserved, particularly adjacent to the lower cortical region where it is delineated by arrows. (C) Bodian stain reveals many preserved axons in arcuate zone to left of arrows, with virtual absence of axons in deeper white matter to the right (× 650).

gyri that were shrunken and had a granular surface. The sulci between these gyri were deeper and wider than usual. Several of the affected gyri displayed a yellow discoloration of their cut surface. The cortical alterations appeared most marked in the parietal area and were least apparent in the frontal lobes (Fig. 1). They were not as widespread as the alterations of white matter.

The basal ganglia and thalamus were markedly atrophied and displayed irregular areas of cavitation which did not encroach on the surrounding hemispheric white matter. The cerebellum displayed cavitation of the dentate nuclei and the roof nuclei. These cystic lesions extended for a small distance into surrounding cerebellar white matter.

No abnormalities were recognized in the spinal cord.

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Fig. 3. [A] Cyst (x) is bounded by dense mass of astrocytic fibers and is surrounded by rarefied white matter separated from cortex by relatively preserved zone of arcuate fibers (arrows). [B] Astrocytic fibers bounding cyst (x). PTAH, × 40, × 650.

Microscopic examination. Paraffin-embedded tissue was cut at 8 μ. Hematoxylin and eosin was employed as a routine stain and the nature of structural abnormalities was further elucidated by additional histological techniques referred to in the text.

The meninges revealed slight focal thickening in sections of cerebrum and basis pontis. Vessels throughout the brain had a normal appearance except for slight focal fibrosis of some vessels observed in or near the lesions described below. The ependymal defect, recognized grossly in the wall of the left lateral ventricle, was lined by flattened cells and the surrounding tissue was free of pigment. Scattered, small, ependymal granulations were found in the wall of the fourth ventricle.

Major lesions in the case occurred both in white and gray matter. The white matter lesions throughout the central nervous system will be described first.

Within the cerebrum the white matter contained lesions of varying age and extent. There was a diffuse and marked loss of myelin in the deep white matter, best recognized in sections stained with luxol blue-periodic acid–Schiff (LB-PAS). This myelin loss sometimes involved the zone of arcuate fibers but often this zone was less affected (Fig. 2A). The Bodian stain for axons revealed a great loss of axons in the deep white matter, but this loss was usually not as great as the myelin loss and, again, the arcuate fibers were better preserved (Fig. 2B and 2C). The number of oligodendroglia was greatly reduced throughout

the white matter and throughout the white matter there were scattered macrophages containing PAS-positive granules. These granules were sudanophilic in paraffin-embedded material subsequently stained with Sudan black B. The granules also stained with oil red O in frozen sections of formalin-fixed material. The macrophages tended to cluster in perivascular spaces. Throughout the white matter a diffuse astrocytosis was evident. Astrocytic fibers were well stained with phosphotungstic acid hematoxylin (PTAH). These fibers formed a loose mesh except in scattered areas where especially dense tangles of astrocytic fibers were observed. These areas bore no particular relationship to the cystic lesions described below. Occasional microscopic foci of basophilic mineral deposits were scattered through the white matter.

Cystic lesions were found, widely separated, throughout the deep white matter. When they were close to cortex the cysts were sharply demarcated from the latter by the relatively preserved arcuate zone (Fig. 3A). These cysts were empty except for occasional vessels with adherent glial fibers. The margins of the cyst sometimes were demarcated by a dense zone of astrocytic fibers (Fig. 3B).

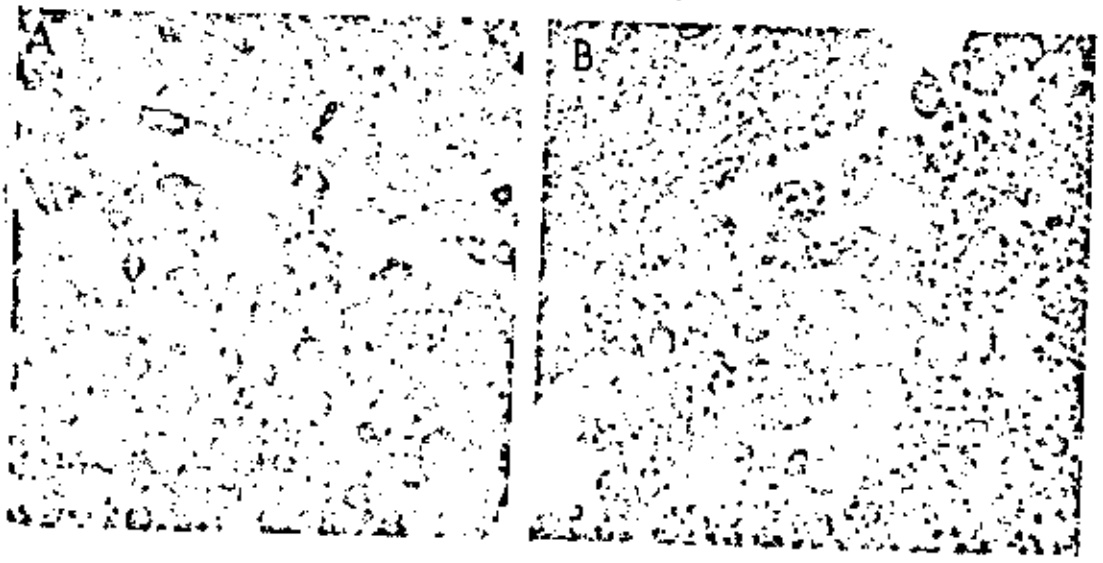
In addition to empty cavities, microscopic foci of necrosis were found in which the destroyed tissue was replaced by collections of macrophages containing PAS-positive and sudanophilic granules (Fig. 4).

The diffuse changes in cerebral white matter were found in all lobes of the cerebrum as were the frank cysts. The necrotic foci containing macrophages were noted in blocks from randomly selected regions of the frontal and parietal lobes. The white matter elsewhere in the central nervous system was not remarkable except for moderate myelin loss in the cerebellar folia and in the corticospinal tracts of the basis pontis and in the pyramidal tracts of the medulla and spinal cord (Fig. 5). The Bodian stain revealed slight axon loss in the pyramidal tract.

In addition to the alterations in white matter, severe, but less diffuse, changes in gray matter were found in several regions of the brain. The cortex itself displayed several types of lesions. In scattered gyri there was a diffuse loss of neurons of variable severity with accompanying astrocytosis. In some gyri very large numbers of astrocytes were crowded together in the cortical tissue, but more often the atrophic gyri (Fig. 6A) displayed a more moderate increase in the number of astrocytes whose processes stained well with PTAH (Figs. 6B and 6C). The astrocytic fibers formed a mesh of variable density and were often most prominent in the subpial zone (Fig. 6B). Basophilic mineral deposits were sometimes seen in the deeper cortical layers. Still other gyri revealed focal areas of necrosis manifest by zones of rarefaction and containing scattered neurons, macrophages, astrocytes, and numerous small blood vessels. These focal zones of necrosis were often found at the base of a sulcus or were symmetrically arranged on either side of the sulcus. Occasionally, they were found in a laminar configuration. Rarely, a laminar zone of degeneration was found manifest only by a linear group of astrocytes and their processes.

In addition to the lesions in cortical gray matter, severe and essentially symmetrical lesions were found in the basal ganglia, thalami, and cerebellar nuclei. The pallidum contained several small cysts with a dense surrounding meshwork of glial fibers best seen with PTAH (Figs. 7A and 7B). The area of gliosis extended into the internal capsule.

Fig. 4. Cystic lesion in deep white matter contains macrophages within which are granules stained by PAS [A, × 650] and sudan black [B, × 650].



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The putamen also contained dense foci of gliosis and neuronal loss, which were more prominent in its anterior portions. In addition to empty cysts, occasional collections of macrophages were found replacing foci of necrotic tissue in the basal ganglia.

The thalami were largely replaced by scattered cysts (Fig. 8). Some of these contained only a few blood vessels with adherent glial fibers. Other cysts displayed areas to which thin interconnecting trabeculae of remaining tissue imparted a honeycombed appearance (Fig. 8). Tissue adjacent to the cysts displayed marked loss of neurons and intense astrocytosis. Adjacent to the thalamic cysts there were foci of mineral deposits, some of which were encrusted neurons. These deposits stained intensely with alizarin red for calcium and weakly with Perl's stain for iron.

The cyst formation and gliosis in basal ganglia and thalamus did not extend into the surrounding tissue of the cerebral hemispheres. In the cerebellum, however, cavitary lesions in the dentate nucleus did encroach to a slight extent on the surrounding white matter. Glial proliferation was present in the adjacent tissue. Scattered cerebellar folia also displayed lesions. In some foci there was almost complete loss of granular cells and Purkinje cells with astrocytosis in the molecular layer, but most folia were better preserved and displayed only slight Purkinje cell loss. Nevertheless, numerous axonal swellings were seen throughout the granular layer of all folia.

Elsewhere in the nervous system there were only slight lesions in scattered nuclear masses. There was mild gliosis and loss of neurons in the colliculi and in the pontine nuclei. There was moderate loss of neurons in the inferior olivary nuclei. The ventral horns of the spinal cord were not remarkable except for a segment of the medial cell column in the right anterior horn at the rostral end of the cervical cord. In this zone the motor neurons displayed signs of retrograde reaction or so-called central chromatolysis.

The dorsal and ventral roots adjacent to the cord in cervical, thoracic, lumbar, and sacral segments were well myelinated and appeared unremarkable. The spinal ganglia and peripheral nerves were not examined.

The optic nerves displayed virtual absence of myelin and marked loss of axons. There was a marked loss of cells in the ganglion layer of the retina and a narrowing of the nerve fiber layer with loss of axons. Bilateral ectopia lentis was present.

DISCUSSION

Severe lesions were present in both the white and gray matter of the cerebral hemispheres but were more diffuse in the former. Lesions were also marked in the basal ganglia, thalami, gray matter of the cerebellar folia and cerebellar nuclei. Descending degeneration of the corticospinal tracts was recognized

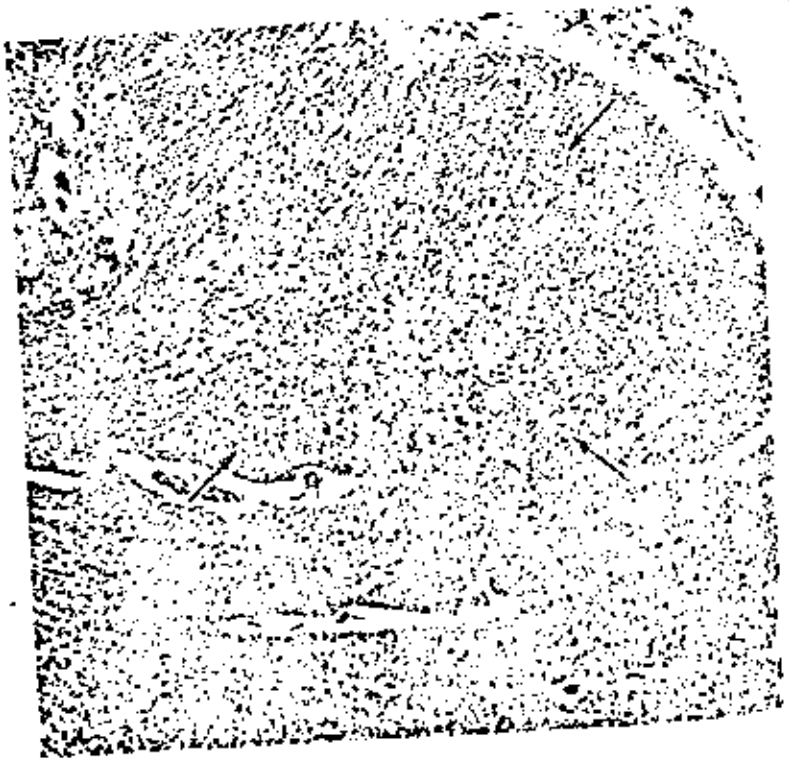

Fig. 5. Spinal cord, thoracic segment, with degeneration of pyramidal tract delineated by arrows. Luxol fast blue-PAS, × 75.

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in the brainstem and spinal cord. These lesions were of variable age.

In the deep white matter, the marked severity and widespread distribution of the myelin and axonal losses, together with the diffuse proliferation of astrocytic fibers, suggest that these alterations were initiated long before death. In addition, old cystic lesions were found in the white matter and in the thalami, basal ganglia, and cerebellar nuclei. The cerebral cortex also displayed old lesions, consisting of shrunken gyri in which there was virtual absence of neurons and replacement of tissue by astrocytes with their processes. Occasionally, older lesions in the cortex took a different form and consisted only of a laminary zone of astrocytosis.

In addition to the older areas of damage, the cortex, basal ganglia, and white matter displayed more recent lesions. In the cortex there were a few very recent foci of necrosis, sometimes arranged in a laminar pattern. Cortical lesions of intermediate age were also seen, marked by zones of rarefaction, astrocytosis, and apparent vascular proliferation. These were sometimes distributed at the base of a sulcus or on either side of the same sulcus. In the basal ganglia recent lesions consisted of necrotic foci filled with macrophages. Similar foci were observed scattered throughout the deep white matter. In addition, lipid-laden macrophages were more diffusely distributed throughout the white matter and accumulated in perivascular spaces, suggesting that widespread destruction of the scant, remaining myelin may have continued until the time of death.

The variable age of these lesions, in addition to their distribution and diverse morphologic appearance, suggests that they have different etiologies which may or may not be related to each other or to the deficiency of sulfite oxidase. There is no evidence of a malformation, of infection, or of neoplasm. Since the child was abnormal at birth, we may presume an injury either in utero or at delivery. The history does not delineate such an episode at either period of time. The oldest lesions in the case are surrounded by a dense glial reaction suggesting that they occurred after the fifth month of gestation when astrocytes have developed.[1] By one year of age the symptoms appear to have been fully developed. The older lesions in the case could certainly have originated in the first year of life. The

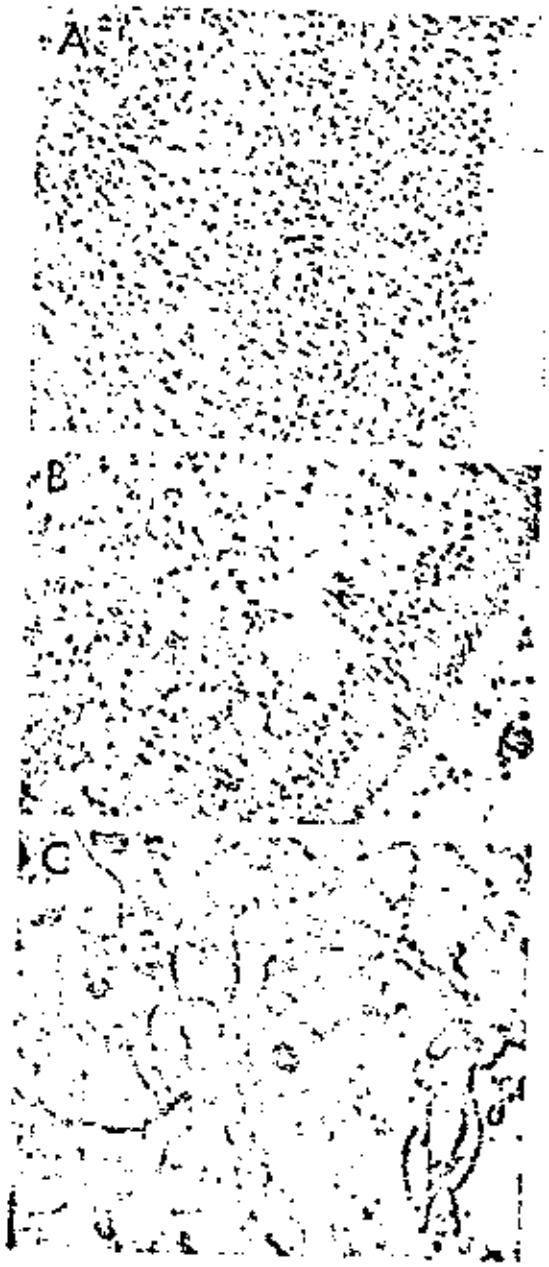

Fig. 6. [A] Cortical tissue has disarranged appearance in atrophic gyrus. Hematoxylin and eosin, × 120. [B] There is marked loss of neurons with replacement by astrocytes and their processes. Glial fibers are most dense adjacent to pial surface on right. PTAH, × 120. [C] Higher magnification of astrocytes deep within cortex PTAH, × 650.

more recent lesions, and especially those of laminar configuration in the cortex, may be related to periods of anoxia associated with the intermittent respiratory distress noted during his terminal hospitalization. Lesions of intermediate age, particularly around the base of sulci or symmetrically arranged on either side of the same sulcus, may also be related to anoxia. Such lesions are sometimes seen following seizures; however, in the present case, frank seizure activity appears to have been questionable and confined to rare, rhythmic movements of the upper extremities.

The diffuse loss of myelin and the gliosis in the deeper white matter and the relative sparing of the arcuate fibers are characteristic of some leukodystrophies, and particularly of diffuse sclerosis (Schilder's disease).[5] The presence of sudanophilic lipid in macrophages diffusely distributed in the white matter is also characteristic of this condition; however, the latter findings can occur wherever myelin breaks down and are frequent nonspecific findings in the cerebral atrophies and encephalomalacias of infancy and childhood.[6] Moreover, the cortical and thalamic lesions are not characteristic of diffuse sclerosis. It is intriguing, however, to note that the present case is characterized by disordered sulphur metabolism and that recent studies do implicate sulphur metabolism in the production or maintenance of myelin. Thus, the metachromatic and globoid forms of leukodystrophy have been characterized by deficiencies of enzymes important in the metabolism of brain sulfatides, but no evidence has been reported implicating abnormal levels of sulfite oxidase in these diseases.[7,8] Sulfatide analysis was carried out in the present case, and no abnormalities were found in the ratio of sulfatides to other brain lipids or to the wet weight of the brain.[9] Moreover, the lesions in the white matter are not those of the above-mentioned leukodystrophies.

Cerebral edema is another process which appears to affect the white matter of the cerebral hemispheres. Moreover, edema preferentially attacks the deep white matter and alters the zone of arcuate fibers to a lesser extent. In addition, myelin breakdown has been reported during the development of changes produced by edema with the production of sudanophilic breakdown products which are

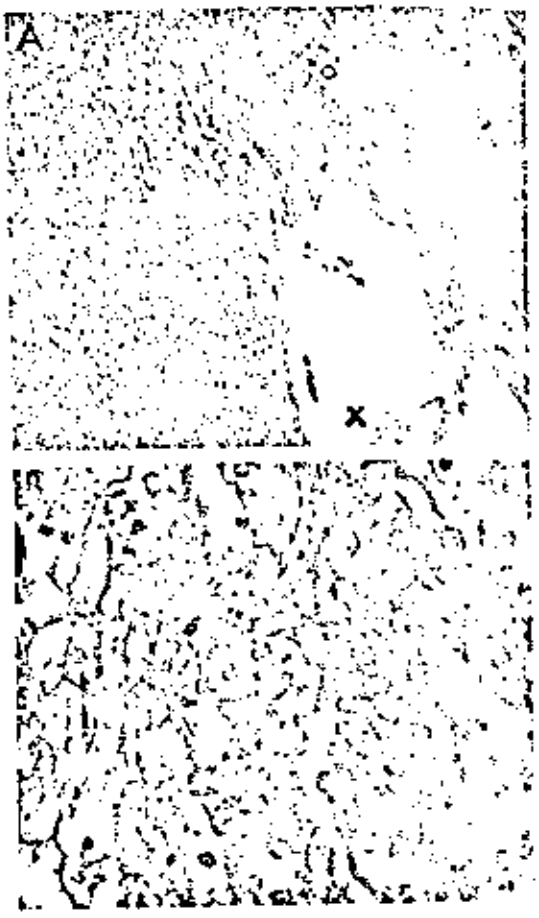

Fig. 7. [A] Cyst (x) in basal ganglia, with adjacent zone of glial proliferation. PTA11. × 40. [B] Glial meshwork at higher magnification PTA11, × 240.

taken up by macrophages.[10] Furthermore, it has been suggested that edema can ultimately lead to cavitation of the deeper white matter[11-13] and that cavitary lesions in the white matter of young children may represent the end result of edema.[13] However, the published evidence relating cystic lesions and severe myelin breakdown to antecedent edema rests largely on an examination of adult cases, while the causes and histologic consequences of edema in children require further elucidation. In any event, edema itself is a nonspecific consequence of many antecedent conditions and if it were responsible for the destruction of white matter in the present case, one would still have to define the initial cause of the edema, the cause of the lesions elsewhere in

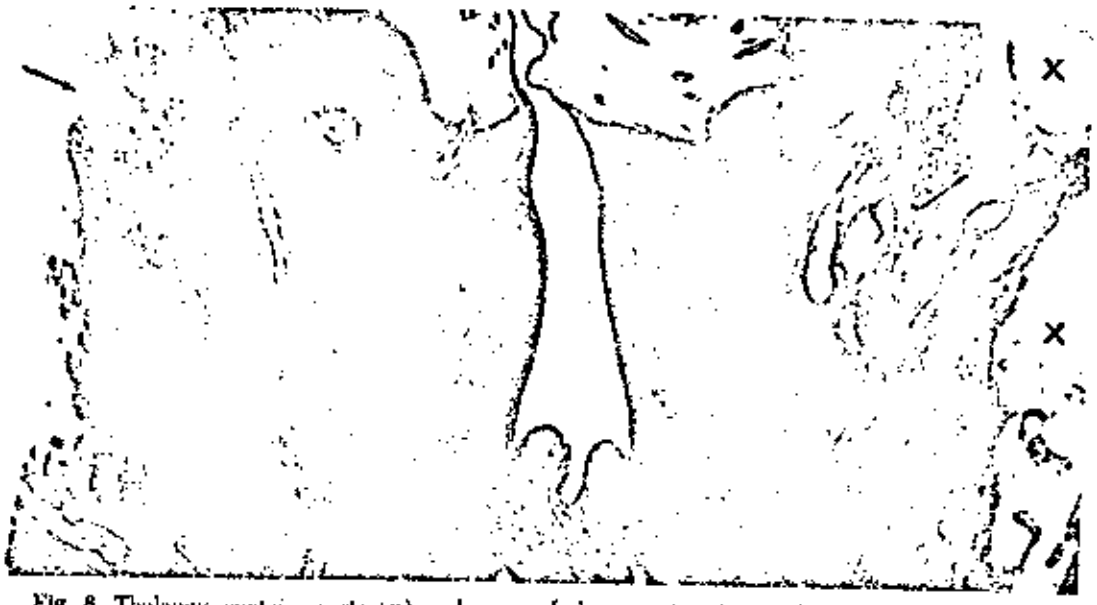

Fig. 8. Thalamus contains cysts (x) and areas of degeneration (arrows) bridged by a honeycomb network of remaining tissue. Extensive dark areas are dense zones of astrocytosis. PTAH, × 5.

the brain, or the possible relationship of the lesions to the sulfite oxidase deficiency.

The difficulties in relating the anatomic findings to a specific process or to an underlying biochemical defect are further accentuated by our knowledge of the patient's 7 siblings, 3 of whom died at three days, four days, and five weeks of age after displaying evidence of neurologic dysfunction. If the patient and his 3 afflicted siblings were all affected by sulfite oxidase deficiency, they might be expected to share a significant neuropathologic lesion. Instead, the findings vary greatly from case to case, with a marked similarity appearing only between the 2 cases that had survived for the longest period of time. The child surviving for three days had a congested brain which was not examined histologically. In the brain of the child who survived for four days, alterations were only recognized after microscopic examination and consisted of hypertrophic astrocytes distributed throughout the white matter and foci of calcification in thalamus and basal ganglia. The sibling who survived for five weeks displayed neuropathologic lesions most closely resembling those of the present case. This sibling's brain revealed marked loss of myelin and axons in the white matter of the cerebral hemispheres and areas of softening in the cerebral cortex and white matter. Cysts were visible on gross examination of the basal ganglia and the hemispheric white matter. The spinal cord displayed degeneration of the pyramidal tracts, which, as in the present case, was probably secondary to severe cerebral damage. Although there is a striking resemblance between the findings in this last sibling and those of the present case, important differences were also manifest. Unlike the situation in the present case, the cortical lesions in this sibling were contiguous with those in the white matter, sparing of the arcuate zone was not recognized, and the cysts were often located immediately adjacent to the overlying cortex. Thus, the sibling who survived for five weeks more closely resembles the classical description of cystic encephalomalacia of infancy than does the present case. Indeed, the contiguity of cortical and white matter lesions in typical cases of cystic encephalomalacia led to the suggestion that the degenerative process spread directly from white matter to cortex.[8] In the present case such a sequence of events appears improbable because there was no necessary topographical relationship between lesions in the white and gray matter and because lesions in the white matter were often separated from those in

adjacent cortical gyri by a relatively spared zone of arcuate fibers.

Because of the neuropathologic differences between the various dead siblings, there is no morphologic support for the suggestion that all siblings shared a common enzymatic deficiency. However, this hypothesis is not disproved by the morphologic evidence because the differences between cases may reflect variability in the duration of illness and variability in the viscisitudes of postnatal life, which might produce secondary changes in one case but not in another. Moreover, an enzymatic defect could conceivably cause neurologic dysfunction without manifesting itself as a specific and readily recognizable morphologic lesion. Therefore, the significance of one or more of the findings in the present case can only be evaluated through comparison with other cases in which a similar biochemical defect has been demonstrated. Since the clinical findings in this case resemble those in more typical cases of cystic encephalomalacia of infancy and since such cases are often grouped in the cerebral palsy category,[6] it may be useful to screen patients with cerebral palsy in an effort to detect sulfite oxidase deficiency. A spot test for urinary sulfite has been suggested as a useful screening procedure.[2,14] If similarly affected individuals are discovered, subsequent postmortem examination may reveal the relationship of the pathologic findings described herein to sulfite oxidase deficiency.

SUMMARY

Neuropathologic findings are presented in a child with a newly defined metabolic error consisting of sulfite oxidase deficiency. The deep cerebral white matter displayed marked, diffuse loss of myelin, moderate to marked loss of axons, and pronounced proliferation of glial fibers. In addition, multiple, but well-separated, cysts of varying age were present in the deep white matter. The arcuate fibers were less affected and often separated the more rarefied deep white matter from the overlying cortex, which also contained numerous scattered areas of damage. These varied from old and complete glial scarring of individual gyri to smaller foci of recent softening. Cystic and gliotic lesions were also found in basal ganglia, thalamus, and cerebellum. The nature and distribution of lesions differed somewhat from that of typical cystic encephalomalacia of infancy and was not that of a leukodystrophy. The exact relationship, if any, of the lesions to sulfite oxidase deficiency remains to be elucidated.

Albert Geldermann, M.D., performed the autopsy; Richard S. Smith, M.D., Ophthalmology Branch, NINDB, performed the examination of the eyes and permitted review of the slides; Abner Golden, M.D., Georgetown University Medical School, Washington, D.C., supplied information about the first sibling; and Paul J. Anderson, M.D., Mount Sinai Hospital, N.Y., supplied information pertaining to the second and third siblings and permitted a review of the slides of the latter.

The author thanks Drs. L. Laster, S. H. Mudd, and F. Irreverre for their assistance in preparing this manuscript, and Dr. L. Thomas for placing the pathologic material at the author's disposal.

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Analyst 56: 451-2 (1931)

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Notes.

The Editor wishes to point out that the pages of the Journal are open for the inclusion of short notes dealing with analytical practice and kindred matters. Such notes are submitted to the Publication Committee in the usual manner.

OSMIUM TETROXIDE POISONING.

I HAVE been unable to find any record of fatal cases due to exposure to the fumes of osmium tetroxide, but indirectly one case of fatal poisoning has come to my notice. This case was referred to by Mr. C. M. Hole in an article appearing in the *Jewelers' Circular*. My interest lies particularly in determining the pathology both of the acute condition and of chronic poisoning, with the expectation of subsequently developing some method of neutralising the effects.

It may not be generally known that inhaling the fumes of osmium tetroxide in high concentration is a very dangerous procedure; and, if the effect on human beings is at all analogous to that on experimental animals, it would seem probable that some cases of fatal poisoning must have occurred in the past. In animals, at least, the cause of death is a pneumonia which appears very shortly after their exposure to the fumes.

Any information that can be given me in this connection will be deeply appreciated.

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THE POSSIBLE EFFECT OF SULPHUR DIOXIDE WHEN USED AS A PRESERVATIVE FOR DRIED FRUITS, ETC.

IN the course of routine work on products preserved with sulphur dioxide, and more particularly with fruit juices, the presence of hydrogen sulphide has made itself evident to the users by its odour and the flavour of the juices after dilution.

Under some conditions the presence of hydrogen sulphide was suspected in dried fruits also, and it was in connection with the examination of some hundreds of such samples that the following information was gathered.

For the prevention of the growth of moulds and the elimination of living organisms, it is necessary to "sulphur" such fruits as raisins, sultanas, apricots, peaches, apples and pears before they are marketed, and, for the preservation of fruit juices, either sulphur dioxide or benzoic acid may be employed.

In the course of work undertaken in Spain, France, Italy and Greece, I have been able to reduce the proportions of sulphur dioxide to a minimum without diminishing the market value or keeping qualities of the dried fruits, but the moist pulps need different treatment, and the existing Regulations permit the use of larger proportions. It is in these products, which are used for the preparations of syrups and "soft" drinks, that the effect of sulphur dioxide is most pronounced.

In order to demonstrate the possible state or situation of the sulphur dioxide in dried fruits, the following examination was made of a shipment of sultanas:

Sulphur dioxide before washing	..	375 parts per million.
" " after washing	..	320 " " "
" " in the washings	..	45 " " "

This seems to indicate plainly that most of the gas is present as an actual compound of the aldoses with the sulphite.

It might be argued that such a compound was entirely harmless to consumers of such fruit, but such a contention would not hold good if the dried fruit were used for making, say, artificial wines, mince meat or sauces, and the same disability is attached to the use of fruit pulps containing sulphite, either added purposely or left in barrels accidentally after cleaning, for I have found that a yeast fermentation becomes possible in diluted solutions; and, when that is active, the sulphur compounds are reduced to hydrogen sulphide.

To prove that this was possible, I expressed the juice from some Almeria grapes, sterilised it by boiling, and divided it up in flasks. To one, sulphur dioxide was added in the form of sodium bisulphite to approximately the proportion of 750 parts per million, as permitted in the Regulations. To another nothing was added. Both were then inoculated with a platinum wire dipped in an active growth of yeast. Other flasks were used as controls, and the neck of each was covered with a piece of lead paper and an inverted beaker.

After 24 hours at 65° F. an active fermentation was set up in the plain juice, but nothing had commenced in those containing sulphur dioxide. These were then diluted with more sterilised juice and left for several days under the same conditions, the results being as follows:

Flasks, to which no sulphur dioxide had been added, produced no coloration whatever on the lead paper, but with those containing the diminished proportion of sulphur dioxide, active fermentation had started, and the lead papers were marked with a perfectly defined black disc on an unstained white background.

Some difficulty was experienced in obtaining dried fruit free from sulphur dioxide for similar tests, but such samples as I have tested and found free from it yield no blackening whatever with lead paper when they are suitably mixed with water and fermented.

A fermentation test for the presence of sulphur dioxide, is too complicated for routine work, but I found that "sulphured" fruit and "sulphited" pulps have the disability that, when moistened or diluted with syrup, they develop an objectionable amount of hydrogen sulphide if once fermentation by yeast is allowed to commence.

It is necessary, therefore, to prevent the possibility of conditions which will permit fermentation, and, among other precautions, such products should be examined for the presence of active yeast cells; and if such are found present, the syrups or mixtures should be pasteurised before bottling.

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THE DETERMINATION OF PHOSPHORUS IN STEEL, ALLOY STEELS AND CAST IRON.

MR. ETHERIDGE, in his paper on "The Determination of Phosphorus in Steel, etc." (ANALYST, 1931, 14), begins by remarking that "Phosphorus is oxidised to orthophosphoric acid in nitric acid solution," and on page 16 he remarks that "oxidation

Z. Lebensm.-Unters.-Forsch. 103 (1956)

...en Methodik sind weitere Ver-
... unter Zusatz von Cofermenten,
...ten, notwendig. Hinweise auf
...dete (kompetitive) Hemmung
...kierung des Succinodehydrase,
...Zitronensäure- und C_4-Dicarbon-
... umgesetzt werden, geht aus
...n mit Methylenblau und Tri-
...uche, insbesondere die Hemm-
...ängs in reifenden Sauermilch-
...enzyme katalysiert werden. Die
...ebenden: die von der Mikrobien-
...se der Käsesuspensionen nimmt
...lerungen im Bereich der Käse-
...ndlich. Daraus folgt, daß die
...ebot und Substratkonzentration
...den nur Prolin, Glutaminsäure,
...diesen Säuren wiederum lediglich
...sant, als Aminosäuren im
...menge der bei der Reifung
...ammenwaterkäse scheint der Abbau
...anderen Wegen zu verlaufen[1].
...Käsefertigungen erfolgen, können
...ziehung gebracht werden. Die
...nd normaler Reifungsführungen.
... nicht auf Hemmwirkungen des
...z Atmung salzfreien Käses un-
...e sogar eine schwache Atmungs-
...en Divergenzen in der Atmungs-
...ren, daß Zusatz von Kochsalz
... Mikrobienentwicklung und damit
...ls dies bei anormaler Reifung der
...anismen im Wachstum gehemmt.
...men, geht aus Mitteilungen von
Penicillium camemberti beträcht-
...einkeimwesen jedoch nicht.
...anormilchkäse nicht ausschließlich
... verschiedene spezifische De-
...wicklung in Erscheinung tretende
...n des Wasserstoffes lassen sich
...äre an einen Zerfall von Ameisen-
...er *Coli*-Gärung, einem Spezialfall
...ch nicht völlig geklärten Butter-
... wurde. Im letztgenannten Fall
...*idium* in Aktion. Ferner besteht
...sen die STICKLAND-Reaktion ab-
... tionierungen zwischen einer

... ntnote 33, 535 (1950); ref. nach

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bzw. zwei Aminosäuren vor sich gehen[1]. Als Reaktionsprodukte entstehen Fettsäuren, Kohlensäure, Ammoniak und Wasserstoff. Derartige Umsetzungen sind besonders bei Buttersäurebildnern für eine Reihe von Aminosäuren beobachtet worden.

Zusammenfassung

Im Verlauf normaler und anormaler Reifung von Sauermilchkäse wurden Atmung und Gärung untersucht. Die *Atmung* des Käses ist im p_H-Bereich zwischen 5 und 8 nur wenig von der Wasserstoffionen-Aktivität abhängig; sie besitzt einen Temperaturkoeffizienten von 1,4—1,7. Zu Beginn der Reifung ist sie niedrig, steigt nach 6—8 Tagen zum Maximum an und nimmt dann wieder etwa bis zum Anfangswert ab. Anormale Reifungsführung zeigt in allen Phasen höhere Aktivität als normal gereifter Käse. Der respiratorische Quotient pendelt zwischen den Werten 0,8—0,9 und ist in beiden Reifungsführungen praktisch gleich. Unter aeroben Verhältnissen treten keine nennenswerten Decarboxylierungen auf. Blausäure, Azid und Penicillin hemmen die Atmung stark, Kochsalz aktiviert schwach. Von zahlreichen, hinsichtlich ihrer atmungssteigernden Wirkung an „gewaschenen" Käsesuspensionen geprüften Substraten waren folgende aktiv: Glucose, DL-Milchsäure, Bernsteinsäure, Essigsäure, Äthanol, L-Glutaminsäure, L-Prolin, L-Lysin, DL-Phenylalanin und DL-Alanin. Die stärkste Wirkung riefen DL-Milchsäure, Glucose und DL-Alanin hervor. Die *Gärung* setzt erst beim Atmungsmaximum des Käses ein, ist im Vergleich zur Atmung gering und für die Hauptreifung ohne ausschlaggebende Bedeutung. Die unter anaeroben Bedingungen auftretende CO_2-Entwicklung wird von einer H_2-Bildung begleitet. Die Gärung ist unabhängig von dem geprüften Temperaturbereich und zeigt ein eng begrenztes, zwischen p_H 6 und 7 liegendes Maximum.

Die Schweflige Säure des Weines in hygienischer Sicht

Von

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Mit 3 Textabbildungen

(Eingegangen am 1. Dezember 1955)

Von altersher verwendet man in der Weinkellerwirtschaft Schweflige Säure. Die Fässer werden, bevor Most oder Wein eingefüllt wird, „eingebrannt", indem durch das Spundloch vorher entzündete Schwefelschnitten, Schwefelfäden oder Schwefelringe eingehängt werden. Außerdem werden Aufbrennrohre oder Schwefelverbrennungsapparate verwendet, mit denen in kurzer Zeit große Mengen SO_2 hergestellt werden.

Die Gewohnheit, das „Einbrennen" mittels elementaren Schwefels hat sich sehr wahrscheinlich historisch aus der Gewohnheit des römischen Altertums, mit Schwefel oder glühender Holzkohle Gewürze, Kräuter oder Samen im Weinfaß zu verbrennen, entwickelt[2]. Damals pflegte man keine naturreinen, sondern fast stets nur „gewürzte" Weine zu trinken. Im Mittelalter wurde auch bei uns noch der Wein gewürzt, schließlich kam der „Gewürzbrand" aus der Mode, wurde sogar gesetzlich verboten und übrigblieb von dieser Weinbehandlungsmethode nur der Schwefel, da man die konservierende und reduzierende Wirkung der Schwefligen Säure als vorteilhaft, vor allem für den Weißwein, erkannte.

[1] JANKE, A.: Arch. Mikrobiol. 15, 472 (1951). — FRANKE, W.: Angew. Chem. 52, 706 (1939).
[2] BASSERMANN-JORDAN, F. VON: Geschichte des Weinbaues. Frankfurt: Keller 1907; Dtsch. Weinbau 20, 9 (1941). — KRETSER, E.: Geisenheimer Lindenbl. v. 18. 11. 1955.

Im Mittelalter begann die allmähliche Bevorzugung des Weißweines vor dem Rotwein. Der Schwefel war prädestiniert den Weißwein „weiß" zu erhalten, während er ohne Schwefel allzuleicht hochfarben und rahm wird.

Mit der Zunahme des Weißweingenusses und des Schwefelns der Weißweine begannen Diskussionen, ob die Schweflige Säure für die menschliche Gesundheit unbedenklich ist. Die Diskussion darüber ist bis heute nicht verstummt. Die folgenden Ausführungen haben den Zweck, einen Beitrag zur Klärung dieser Frage zu leisten.

Gesetzliche Beschränkungen

In Deutschland regelte das Weingesetz von 1909 und 1930 mit Artikel 7 der Ausführungsverordnung vom 16. Juli 1932 die Anwendung der Schwefligen Säure im Wein wie folgt:

„Gestattet ist das Schwefeln mittels folgender Verfahren, sofern hier nur kleine Mengen Schwefliger Säure oder Schwefelsäure in die Flüssigkeit gelangen:

a) Verbrennen von Schwefel oder Schwefelschnitten mit Ausnahme von gewürzhaltigem Schwefel,

b) Verwendung von reiner gasförmiger Schwefliger Säure,

c) Verwendung von mindestens fünf von Hundert Schwefeldioxyd enthaltenden Lösungen reiner gasförmiger Schwefliger Säure in destilliertem Wasser,

d) Verwendung von technisch reinem Kaliumpyrosulfit, auch in Tablettenform."

Das deutsche Weingesetz hat keine zahlenmäßige Begrenzung der Schwefligen Säure vorgenommen. Es spricht lediglich von „kleinen Mengen". Daraus ergaben sich bald Unstimmigkeiten.

Die deutschen Lebensmittelchemiker kamen daher im Einverständnis mit ärztlichen Sachverständigen (Reichsgesundheitsamt) überein, unter „kleinen Mengen" 200 mg Gesamt-SO_2 je l zu verstehen, wovon 50 mg in freiem Zustand vorliegen dürfen. Trockenbeerenauslesen stellen eine Ausnahme dar. Diese Empfehlungen der Lebensmittelchemiker bildete die Grundlage eines preußischen Ministerialerlasses vom 30. August 1912. Danach wurde in der Praxis der staatlichen Weinkontrolle verfahren, indem normale deutsche Weine mit SO_2-Gehalten über 200 mg/l beanstandet und die Hersteller bestraft wurden. Welche Mengen Schweflige Säure die einzelnen Staaten im Wein erlauben, zeigt die Tab. 1.

Tabelle 1. *Zugelassene Mengen an Schwefliger Säure in verschiedenen Ländern*

Land	Höchstgrenze für gesamte Schweflige Säure in mg/l	Höchstgrenze für freie Schweflige Säure in mg/l
Argentinien	200	—
Brasilien	350	50
Bulgarien	200	20
Deutschland: Wein	200	50
Traubensüßmost	125	—
Obstsüßmost	80	—
England	450	—
Frankreich	450	100
Griechenland	450	—
Italien	200	20
Jugoslawien	200	20
Österreich	350	100
Portugal	350	—
Rumänien: Weine	350	—
Moste u. Dessertweine	500	—
Schweiz: Landweine	400	40
Luxusweine	450	100
Spanien	450	100
Südafrikanische Union: trockene Weine	200	21
Süßweine	357	32
Ungarn	200	—
USA	350	70

Die Tabelle zeigt, daß die Grenzwerte für Schweflige Säure von 200—500 mg/l schwanken. In Frankreich, Griechenland, Österreich und Spanien ist ein Spielraum von + 10% SO_2 vorgesehen. In Frankreich darf der Wein also bis 500 mg SO_2/l enthalten. Diese hohen SO_2-Gehalte wurden lediglich den süßen, weißen Bordeauxweinen (*Graves, Hautes Sauternes* usw.) zuliebe gestattet. Rumänien und die Südafrikanische Union machen in sehr vernünftiger Weise einen Unterschied

Weißweines vor dem Rotwein. Der
..en, während er ohne Schwefel alizu-

des Schwefelns der Weißweine
für die menschliche Gesundheit
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..niker bildete die Grundlage eines
..in der Praxis der staatlichen
..Gehalten über 200 mg/l beanstandet und die Hersteller bestraft wurden. Welche Mengen Schweflige Säure die einzelnen Staaten im Wein erlauben, zeigt die Tab. 1.

Die Tabelle zeigt, daß die Grenzwerte für Schweflige Säure von 200—500 mg/l schwanken. In Frankreich, Griechenland, Österreich und Spanien ist ein Spielraum von + 10% SO_2 vorgesehen. In Frankreich darf der Wein also bis 500 mg SO_2/l enthalten. Diese hohen SO_2-Gehalte wurden lediglich den süßen, weißen Bordeauxweinen (*Graves, Hautes Sauternes* usw.) zuliebe gestattet. In Rumänien und die Südafrikanische Union machen in sehr vernünftiger Weise einen Unterschied

zwischen trockenen und süßen Weinen. SO_2-Mengen bis 500 mg/l werden in der Weinliteratur (mit ganz wenigen Ausnahmen) als hygienisch völlig unbedenklich hingestellt. Dabei werden stets die Arbeiten der Ärztekommission von Bordeaux 1910 und des Reichsgesundheitsamtes Berlin 1912 als Beweise angeführt. Allein schon der Umstand, daß diese ärztlichen Gutachten nun schon 45 Jahre alt sind, macht es fraglich, ob ein Operieren mit diesen Argumenten aus der Zeit von 1910—1912 noch gerechtfertigt ist.

Gutachtliche Stellungnahmen

Den Anhängern der „Harmlosigkeitstheorie" stehen aber angefangen von den Stadtvätern der Reichsstadt Köln im 15. Jahrhundert, welche das Schwefeln verboten, weil „dadurch die Natur des Menschen belästigt und der Trinker in Krankheit gebracht werde", bis zum heutigen Tage Tausende von Menschen gegenüber, welche behaupten, daß sie beim Genuß von reichlich geschwefeltem oder überschwefeltem Wein Blutandrang im Kopf, Brennen im Hals oder Kopfschmerzen bekämen. Dabei handelt es sich keineswegs nur um Laien, sondern es sind Weinkenner, Weinwissenschaftler, Mediziner und sonstige naturwissenschaftlich Gebildete darunter. Es sei nur eine international anerkannte Kapazität der Weinchemie, der 1935 verstorbene Professor C. VON DER HEIDE, genannt, der in der 9. Auflage des „Nessler" auf die Diskrepanz zwischen dem Ärztegutachten von Bordeaux und der praktischen Erfahrung hinwies[1].

Bis in die allerneueste Zeit hinein[2] wird aber immer noch das nun schon 45 Jahre zurückliegende *Gutachten der Ärztekommission von Bordeaux* dafür angeführt, daß selbst 650 mg SO_2 im Wein keinerlei Störungen des Allgemeinbefindens oder des Stoffwechsels verursachen würden.

Es lohnt sich, dieses berühmte Bordelaiser Ärztegutachten aus dem Jahre 1910 genauer zu studieren[3].

Eine Gruppe von Weininteressenten hatte einen Versuch mit 8 Personen, die 30 Tage in einem Krankenhaus unter ärztlicher Aufsicht lebten und dabei verschieden große Mengen stark und normal geschwefelten Weines trinken mußten, finanziert. Eine Ärztegruppe hat die 8 Versuchspersonen täglich befragt und auf das Allgemeinbefinden, Verdauung, Blutdruck, Körpertemperatur, Eiweiß- und Stickstoffgehalt des Urins, Schlaf, Reflexe usw. untersucht. Besonders aufmerksam wurden die 4 Versuchspersonen beobachtet, die täglich 1½ l eines 650 mg SO_2 enthaltenden Weines tranken.

Aber das einzige, was diese Personengruppe von der anderen, die normal, d. h. schwach geschwefelten Wein trank, in den Untersuchungen nach 3 Tagen unterschied, war eine Vermehrung ihrer Erythrocyten. Dieser Befund wurde von dem Ärztekollegium dahingehend gedeutet, daß der Genuß stark geschwefelten Weines nicht nur keinerlei gesundheitsschädlichen, sondern sogar gesundheitsfördernde (eben die Vermehrung der roten Blutkörperchen) Wirkungen erkennen ließ.

Nach unserem heutigen Wissen ist beim Genuß von stark geschwefeltem Wein von entscheidender Bedeutung, welcher Säuregrad im Magensaft der Versuchspersonen vorliegt. Wir finden aber in dem Bordelaiser Ärztegutachten nichts darüber, ob Magenausheberungen und eine Bestimmung der Säureverhältnisse der Magensäfte der 8 Versuchspersonen vorgenommen worden sind.

Sodann ist beim Genuß von stark geschwefeltem Wein zu berücksichtigen, ob die gleichzeitig genossenen Speisen die Acidितätsverhältnisse des Magensaftes verändern.

[1] HEIDE, C. v. D.: In J. NESSLER, Die Bereitung, Pflege und Untersuchung des Weines. S. 307 Stuttgart 1930.
[2] VOGT, R.: Weinchemie und Weinanalyse. Stuttgart 1950.
[3] GAUTHIER, J.: J. Ann. Falsificat. 3, 226—235 (1910).

Auf deutscher Seite führte um dieselbe Zeit das *Kaiserliche Gesundheitsamt in Berlin* Versuche an Kaninchen, Ratten und Meerschweinchen durch, denen peroral und parenteral Schweflige Säure bis zu letalen Dosen verabreicht wurden. Diese Dosen wurden vom Körpergewicht der Versuchstiere auf das normale Körpergewicht des Menschen umgerechnet. Dabei ergab sich, daß die in Weinen enthaltenden SO_2-Mengen für den menschlichen Körper unschädlich sind. Diese Art der Beweisführung kann nicht mehr als stichhaltig angesehen werden.

Es ist daher angebracht, die Frage nach der hygienischen Wirkung der Schwefligen Säure des Weines erneut aufzuwerfen und mit neuzeitlichen Methoden zu prüfen.

Versuche

In einer Weingegend, in der natürlich von der Bevölkerung mehr Wein getrunken wird als anderswo, gibt es häufig Gelegenheit, Menschen anzutreffen, die zu viel Wein auf einmal getrunken haben und daher mehr oder minder stark berauscht sind. Nicht immer merkt man den starken Weingenuß am Benehmen der Leute; denn es gibt stets Menschen, die von Natur oder durch Gewöhnung sehr viel vertragen. Aber zu reichlichen Weingenuß kann man stets an der Ausatmungsluft feststellen.

Es bleibt in einer Weingegend nicht aus, daß man in den Bereich des Atemgeruches eines Menschen gerät. Dabei ist mir immer wieder aufgefallen, daß es Menschen gibt, deren Atem ausgesprochen und stark nach SO_2 riecht, während bei anderen Menschen, die den gleichen Wein in gleicher Menge getrunken hatten, die SO_2-Komponente nicht zu bemerken ist.

Auf Grund dieser Beobachtungen zog ich den Schluß, daß in den Mägen der einzelnen Menschen die gebundene Schweflige Säure des Weines mehr oder weniger leicht freigesetzt wird. Daß im menschlichen Magen die gebundene Schweflige Säure in die „freie" Säure zurückverwandelt wird, darauf hat schon von der Heide im Jahre 1930 aufmerksam gemacht. Darüber, ob und in welchem Maß dies der Fall ist und welche Faktoren dabei eine Rolle spielen, lagen bisher keine Angaben vor. Einige Versuche sollen hierüber jetzt Auskunft geben.

Versuch mit Magenaushebern

Eine Versuchsperson, die nur Tee als Frühstück eingenommen hatte, trank vormittags 200 ml eines Weines, dessen Gehalt an gesamter und freier Schwefliger Säure unmittelbar vorher bestimmt worden war. Nach 15—30 min wurde per Gummischlauch Magensaft entnommen, sofort filtriert und das Verhältnis von freiem zu gebundenem SO_2 in der Saftprobe jodometrisch bestimmt.

Schon beim ersten Versuch zeigte sich, daß sich im menschlichen Magen dieses Verhältnis ändert. Waren z. B. im dargebotenen Wein 26% des Gesamt-SO_2 im freien Zustand, so war bei dem im Magensaft wiedergefundenen Gesamt-SO_2 nach 15 bis 30 min der Anteil an freiem SO_2 30—32%. Derartige Magenausheberungen wurden insgesamt 3mal an 3 verschiedenen Tagen durchgeführt. Das dritte Mal hatte die Versuchsperson die Vereinbarung vergessen, nur Tee zu frühstücken und hatte zum Tee ein Weißbrot gegessen. Dies war sofort bei der Titration des freien und gesamten SO_2 zu merken, indem der prozentuale Anteil des freien SO_2 im ausgehobenen Magensaft nicht zu-, sondern leicht abgenommen hatte. Daraus ist ersichtlich, daß nach dem Genuß von stärkehaltigen Speisen im Magen eine Bindung der freien Schwefligen Säure eintreten kann.

Versuche mit Modellmagensäften

Magenausheberungen sind nicht so ergiebig, daß man mit dem gewonnenen Magensaft Serienuntersuchungen durchführen könnte. Daher wurden solche mit

künstlichen Magensäften angesetzt. Die Zusammensetzung der künstlichen Magensäfte war folgende:

a) *Normacid:* 780 ml 0,1 n-KH_2PO_4 + 90 ml 2 n-HCl auf 6 l aufgefüllt,
b) *Anacid:* 1200 ml 0,1 n-KH_2PO_4 + 35 ml 2 n-NaOH auf 6 l aufgefüllt,
c) *Subacid:* 1200 ml 0,1 n-KH_2PO_4 + 25 ml 2 n-NaOH auf 6 l aufgefüllt,
d) *Hyperacid:* 900 ml 0,1 n-KH_2PO_4 + 210 ml 2 n-HCl auf 6 l aufgefüllt.

Der Gehalt an freier HCl wurde mit 0,1 n-NaOH für 100 ml Magensaft mit Dimethylaminoazobenzol und die Gesamtacidität mit Phenolphthalein als Indicator bestimmt. Wird bei den graphischen Darstellungen das Verhältnis der freien Säure zur Gesamtacidität eines Magensaftes mit 9/25 angegeben, so heißt das, daß 100 ml des betreffenden Magensaftes 9 ml 0,1 n-NaOH mit Dimethylaminoazobenzol als Indicator und 25 ml NaOH mit Phenolphthalein als Indicator verbrauchten.

Es wurde folgendermaßen vorgegangen:

1. Vor dem Versuch wurde der Gehalt an freiem und gesamtem SO_2 des betreffenden Weines nach den in der Weinchemie üblichen Methoden jodometrisch bestimmt.

2. Wein und Modellmagensaft wurden im Verhältnis 1:1 gemischt und in einem HOEPPLER-Thermostaten bei 37° C gehalten.

3. Um den Einfluß der menschlichen Körpertemperatur auf das Verhältnis zwischen freiem und gesamtem SO_2 allein zu studieren, wurde gleichzeitig Wein ohne Zusatz von Magensaft bei 37° C gehalten.

4. Im Abstand von 15—30 min wurde jeweils jodometrisch der Anteil des freien SO_2 im Wein bzw. Wein-Magensaft-Gemisch bestimmt. Die SO_2-Werte in den graphischen Darstellungen beziehen sich auf den unverdünnten Wein.

Die ersten Serienversuche mit Modellmagensäften waren überraschend. Wir hatten erwartet, daß in den hyperaciden Magensäften die stärkste Aufspaltung der gebundenen Schwefligen Säure eintreten würde. Indessen trat, so oft wir auch wiederholten, die stärkste Zunahme an freiem SO_2 in anaciden und subaciden Magensäften ein.

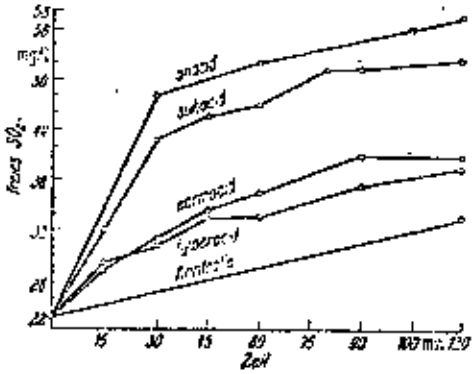

Abb. 1. *Freie Schweflige Säure bei verschiedener Acidität des Magensaftes bei Hautes Sauternes* (p_H 3,12; freie SO_2 22 mg/l; ges. SO_2 295 mg/l); anacider Magensaft —12/23, subacider Magensaft —9/23; normacider Magensaft 22/40, hyperacider Magensaft 36/56; Erläuterung vgl. Text

Abb. 1 zeigt das Ergebnis eines Versuches mit 4 Magensäften und einem Hautes Sauternes-Wein, der für einen weißen Bordeauxwein relativ niedrige SO_2-Werte aufwies; denn es sind im Handel oft weiße Bordeauxweine mit SO_2-Gehalten hart an den Grenzzahlen des französischen Weingesetzes (450 mg Gesamt-SO_2, 100 mg freies SO_2) und nicht selten sogar darüber anzutreffen.

Diese Diagramme zeigen:

1. daß allein die menschliche Körpertemperatur im Laufe von 2 Std. den Gehalt an freiem SO_2 von 22 mg/l auf 40 mg/l, d. h. um rund 74% steigert,

2. im subaciden Magensaft nimmt der Gehalt an freiem SO_2 sogar von 22 mg/l auf 50 mg, d. h. um 127% und im anaciden Magensaft sogar um 151% zu.

Gesetzt der Fall, der weiße Bordeaux hätte den nach dem französischen Weingesetz erlaubten Gehalt von 100 mg freiem SO_2 gehabt, so wären im Laufe von 2 Std. im anaciden Magen daraus insgesamt 251 mg freies SO_2 geworden.

Warum tritt aber im hyperaciden Magensaft nur eine Steigerung von 74% und im anaciden Magensaft eine solche von 151% ein?

Diese uns anfänglich unerklärliche Erscheinung findet jedoch ihre einfache Erklärung in der Tatsache, auf die bereits von der Heide[1] hinwies, daß die Geschwindigkeit des Zerfalls der gebundenen Schwefligen Säure in saurer Lösung ganz außerordentlich verlangsamt wird. Besonders bei der glucoseschwefligen Säure — und in süßen Weinen liegt die gebundene Schweflige Säure hauptsächlich in dieser Form vor — ist dies nach von der Heide der Fall.

In Süßweinen ist der an Glucose gebundene Anteil der Schwefligen Säure besonders groß. Daher begann in dem Samoswein (Abb. 2) und in dem süßen italienischen Muskatellerwein (Abb. 3) die Bildung von freiem SO_2 sofort beim Mischen mit dem anaciden und subaciden Magensaft — im Samoswein stieg innerhalb von 60 min der Gehalt an freiem SO_2 um das Doppelte, im Muskatellerwein sogar um das 3,5-fache.

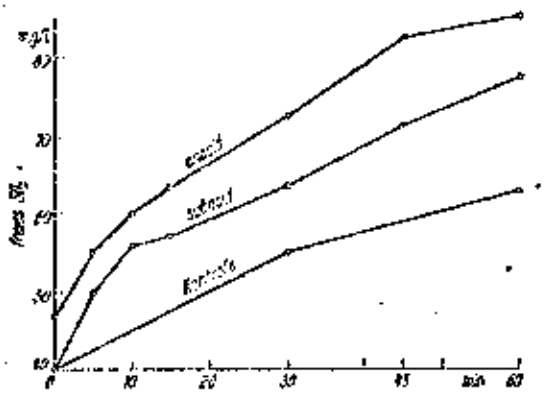

Abb. 2. *Freie Schweflige Säure bei verschiedener Acidität des Magensaftes bei Samoswein* (p_H 3,52; freie SO_2 40 mg/l; ges. SO_2 230 mg/l); anacider Magensaft —12/23, subacider Magensaft +9/25

Immer, wenn ein Süßwein getestet wurde, konnte dieselbe Erscheinung festgestellt werden, nämlich eine spontane und sehr rasche Zunahme von freiem SO_2. Diese Feststellung ist deswegen wichtig, weil die Weinkellerwirte in dem Glauben, daß lediglich ein Zuviel an freiem SO_2 vom Weintrinker lästig empfunden wird, fast allgemein nur der *freien* Schwefligen Säure, nicht aber der *gebundenen* Säure Aufmerksamkeit schenken. Wie die in Abb. 1—3 wiedergegebenen Fälle zeigen, können ausländische Süßweine mit relativ niedrigem Gehalt an freiem SO_2 in anaciden und subaciden Magensäften eine geradezu stürmische Entbindung von SO_2 bewirken.

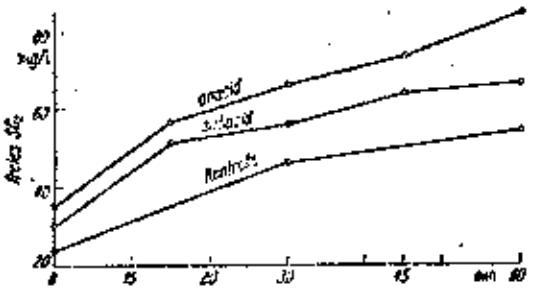

Abb. 3. *Freie Schweflige Säure bei verschiedener Acidität des Magensaftes bei süßem Muskatellerwein* (p_H 3,5; freies SO_2 23 mg/l; ges. SO_2 240 mg/l); anacider Magensaft —12/23; subacider Magensaft +9/25

Es zeigte sich, daß deutsche Weine sich im Prinzip ebenso verhalten; sie haben jedoch mit Ausnahme von Beerenauslesen keinen so hohen Zuckergehalt.

Als allgemeine Regel kann für deutsche Weißweine gelten: *je höher der Zuckergehalt um so höher der SO_2-Gehalt.* Vom Verbraucher werden heute Weine mit einer sog. „Restsüße" bevorzugt. Der Erzeuger sucht diesem Verbraucherwunsch nachzukommen und glaubt oft, mit kräftigen Schwefelgaben den Restzucker erhalten zu können. Man beobachtet seit Bestehen des Wunsches nach Weinen mit einer Restsüße in Deutschland eine Tendenz zu übermäßiger Anwendung von Schwefliger Säure.

[1] Heide, C. v. d., u. F. Schmitthenner: Der Wein. S. 147. Braunschweig: Vieweg & Sohn 1922.

...ndet jedoch ihre einfache Erklä-
... hinwies, daß die Geschwindig-
... in saurer Lösung ganz außer-
...oseschwefligen Säure — und in
...hauptsächlich in dieser Form vor

...il der Schwefligen Säure beson-
...) und in dem süßen italienischen
...katellerwein (Abb. 3) die Bildung
... freiem SO_2 sofort beim Mischen
... dem anaciden und subaciden
...gensaft — im Samoswein stieg
...erhalb von 60 min der Gehalt an
...em SO_2 um das Doppelte, im
...katellerwein sogar um das 3,5-
...he.

Immer, wenn ein Süßwein ge-
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...eute Weine mit einer sog. „Rest-
...n Verbraucherwunsch nachzukom-
...ben den Restzucker erhalten ...
...ches nach Weinen mit einer Restsäu-
... Anwendung von Schwefliger Säure

... S. 147. Braunschweig: Vieweg & Sohn

Zur Erläuterung der oben angeführten Zucker-SO_2-Regel und der Größenordnungen an freiem und gebundenem SO_2-, die in deutschen Weinen angetroffen werden können, seien einige Analysenbelege angeführt.

Tabelle 2. *Schweflige Säure-Gehalte einiger Weine nach* K. Herwig[1]

Weinherkunft	Zucker g/l	Schweflige Säure gesamt mg/l	frei mg/l	gebunden mg/l
1934er Rüdesheimer	2,5	132	54	78
1934er Moselwein	1,7	170	68	102
1950er Niersteiner	5,2	233	30	203
1950er Niersteiner	5,7	313	39	274
1949er Liebfraumilch	13,4	211	61	150
1949er Liebfraumilch	32,6	238	90	148
1934er Geisenheimer	36,0	296	114	182

Relativ hohe SO_2-Gehalte können auch, wie K. Hennig zeigte, Schaumweine haben. Es hat sich nach dem Kriege in Deutschland immer mehr eingebürgert, dem Schaumwein bei der Zugabe des sog. Expeditionslikörs SO_2 mitzugeben. Da die Schaumweine 18—40 g Zucker enthalten, wird die Schweflige Säure in starkem Maße als glucoseschweflige Säure gebunden. Diese merkt man beim Genuß zunächst nicht, sie wird aber im Magen aufgespalten.

Aus den 1952 von K. Hennig[2] veröffentlichten Analysen deutscher und französischer Schaumweine der Nachkriegszeit müssen wir leider feststellen, daß die deutschen Nachkriegsschaumweine 3—4mal mehr SO_2 aufweisen als die getesteten französischen Schaumweine, wodurch natürlich ihre Bekömmlichkeit auch für Kranke und Rekonvaleszenten vermindert wird.

Tabelle 3. *Gehalt verschiedener Schaumweine an SO_2*

Gruppe	Schaumweine	Gehalt an SO_2 frei mg/l	gebunden mg/l	insgesamt mg/l
I	6 deutsche Schaumweine, hergestellt ausschließlich aus deutschen Grundweinen mit Jahrgangs- u. Herkunftsbezeichnungen	3—69	100—182	105—223
II	6 deutsche Markensekte, hergestellt aus Grundweinen deutscher und französischer Herkunft	2—18	62—173	66—180
III	8 billigere Schaumweine, sog. „Hausmarken"	2— 8	95—133	80—135
IV	französischer Marken-Champagner	2— 4	18—51	20—53

Daß die deutschen Weine, wenn sie gebundene, vor allem glucoseschweflige Säure enthalten, sich in den Modellmagensäften genau so verhalten wie ausländische Weine, belegen Versuche mit einem 1950er Deidesheimer und 1951er Oppenheimer Wein[3].

Besprechung der Ergebnisse

Die vorliegenden Versuche zeigen, daß für die Beurteilung der Bekömmlichkeit eines Weines nicht allein sein Gehalt an *freier* Schwefliger Säure, sondern auch an

[1] Herwig, K.: Dtsch. Wein-Ztg. 88, 66 (1952)
[2] Hennig, K.: Dtsch. Wein-Ztg. 88, 341—346 (1952).
[3] Schanderl, H.: Wein u. Rebe 35, Nr. 20 u. 21 (1953).

gebundener Schwefliger Säure maßgebend ist; denn von dieser werden hauptsächlich *anacide* und *subacide* Menschen irritiert. Wenn sie nach dem Genuß von Weißweinen oder Schaumweinen über Magenschmerzen und Brennen im Hals klagen, so hat dies seinen Grund darin, daß in ihren Mägen sofort nach dem Weingenuß eine lebhafte Aufspaltung der glucoseschwefligen Säure einsetzt. Das gasförmige Schwefeldioxyd ist es, das das Brennen und im Mund einen intensiven „Schwefelgeschmack" erzeugt.

Die Befunde an Modellmagensäften beweisen die Richtigkeit dessen, was schon 1922 von der Heide ausgeführt hat:

„Selbst der Einwand, daß ja die schweflige Säure im Wein größtenteils in „gebundener" Form vorliege, in der sie gesundheitsunschädlich sei, ist nicht durchschlagend, weil im Magen die gebundene schweflige Säure allmählich in die „freie" Säure zurückverwandelt wird und diese nun ihre Giftwirkung entfalten kann."

Die vorliegenden Versuche, gewonnen an Modellmagensäften, zeigen, daß vor allem die subaciden und anaciden Menschen vor dem Genuß reichlich geschwefelten Weines gewarnt werden müssen. Darüber hinaus bilden sie Gegenargumente gegen die bei Kellerwirtschaftlern weit verbreitete Tendenz, den Gehalt an SO_2 der Weine zu bagatellisieren und in jedem Fall als hygienisch harmlos hinzustellen. Die Befunde mögen dazu beitragen, das Bestreben, noch höhere SO_2-Gaben als bisher zu erlauben, abzubremsen. Im Gegenteil müssen wir bestrebt sein, nur so viel SO_2 im Wein anzuwenden, als aus technischen Gründen unbedingt erforderlich ist. Ferner müssen alle Möglichkeiten, SO_2 durch physiologisch unbedenkliche Stoffe zu ersetzen, geprüft werden. In diesem Zusammenhang sei besonders auf Ascorbinsäure (Vitamin C) hingewiesen, mit der nach Koch[1] ein besserer Oxydationsschutz erreicht werden kann als mit Schwefliger Säure.

Zusammenfassung

1. Das berühmte Ärztegutachten von Bordeaux (1910), über die hygienische Harmlosigkeit der Schwefligen Säure im Wein wird einer Kritik unterzogen.

2. Es konnte einesteils in nativem Magensaft, andererseits in Serienversuchen mit Modellmagensäften nachgewiesen werden, daß bei der Körpertemperatur von 37° C. vor allem in anaciden und subaciden Magensäften, eine rasche und starke Entbindung von freiem SO_2 aus gebundener Schwefliger Säure, insbesondere aus glucoseschwefliger Säure, stattfindet.

3. Aus diesen Gründen sind reichlich geschwefelte Weine für anacide und subacide Menschen nicht bekömmlich.

Die Bestimmung der Trockenmasse in Emulsionslikören mit infraroten Strahlern

Von

J. Korrász

*Mitteilung aus Budapest Főváros Vegyészeti és Élelmiszervizsgáló Intézete**

(Eingegangen am 15. November 1955)

Bei der Bestimmung der Trockenmasse von Emulsionslikören, besonders der mit Eier bereiteten, wie z. B. „Eierbrandy" tritt während einer längeren Erhitzung bis 100° C Zersetzung ein, so daß wir bei scheinbarer Gewichtskonstanz falsche Resultate erhalten können. Selbst durch Trocknung mit Sand kann der durch Zersetzung

[1] Koch, J.: Dtsch. Wein-Ztg. 89, 344 u. 356 (1953).

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Sulfur Dioxide-Induced Lymphocyte Defects in Human Peripheral Blood Cultures

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Human lymphocyte cultures were prepared from peripheral blood of healthy males by a standard technique utilizing phytohemagglutinin. Nine cultures were established in each of five experiments. One, designated control, was allowed to grow without interruption for 3 days. Each experimental culture received a single exposure to 100 cc of 5.7 ppm SO_2 in air by bubbling the gas through the culture medium at either 0, 1, 2, or 3 days of incubation. An additional four cultures received an identical amount of the same quality of air minus the SO_2 at each time interval. All cultures were harvested as smears and hypotonic spread preparations at 72 hours after treatment for 3 hour with colchicine and ^{3}H-thymidine. Statistical analysis revealed significantly reduced cell growth (enlargement), DNA synthesis, and mitotic indices in the cultures exposed to sulfur dioxide at initiation of incubation compared to controls and air-treated cultures. In addition, chromosome abnormalities, mainly in the form of clumping, were observed in the SO_2-treated cultures. Lymphocyte damage as caused by sulfur dioxide exposure is offered as one explanation for the reduced immunological responses observed in laboratory animals after inhalation of the gas *in vivo*.

That prolonged or repeated exposure to various concentrations of sulfur dioxide can be damaging or injurious to metals, vegetation, laboratory animals and human beings, is well documented. Extensive review articles on this subject have been presented by Greenwald (1951) and more recently by the United States Public Health Service (1967).[1] Whereas numerous clinical and epidemiological reports have appeared in the literature, little is known of the effects of sulfur dioxide at the cellular level. Peacock and Spence (1937) noted an increased incidence of lung tumors in mice exposed to SO_2, and Lamb and Reid (1968) observed cellular changes in bronchial epithelium of rats similarly treated. Thompson and Pace (1962) showed that exposure of established tissue culture cell lines to 5–25 ppm of SO_2 8 hours a day for 5 consecutive days was lethal to varying percentages of cells. Exposure to 5 ppm for 2 consecutive days, however, apparently produced no changes, although no attempt was made to determine if sublethal cellular damage occurred.

Several investigations have demonstrated reduced immunological functions in animals exposed to sulfur dioxide. Ronzani (1908) noted that rabbits and guinea pigs exposed to 500 ppm of SO_2 6–7 hours a day for a month demonstrated reduced resistance to various microorganisms and reduced production of antibodies and agglutinins. Similar observations were published by Navrotskii in 1959. More

[1] "Air Quality Criteria for Sulfur Oxides," U. S. Department of Health, Education, and Welfare, Public Health Service, Washington, D. C. (1967).

recently, Ardelean *et al.* (1967), who studied rabbits exposed 7 hours daily for 113 days to just 7 ppm of SO_2, reported that the gas inhibited the formation of agglutinins. They concluded that sulfur dioxide exerts both a local irritative effect and, since it penetrates into the general circulation, a general action on the mechanisms of immunogenesis. That inhaled sulfur dioxide travels in the blood stream is supported by the work of Litkens (1955) and Lee and Danner (1966). It was thus felt that human lymphocytes *in vitro* provided an ideal system in which to study the effects of moderate concentrations of SO_2 on blood cells involved in immune function.

MATERIALS AND METHODS

Lymphocytes obtained from peripheral blood of three normal males, 22–32 years of age, were cultured in Eagle's minimal essential medium with 20% human plasma, phytohemagglutinin (Difco PHA-P) and antibiotic according to modifications of the technique of Moorhead *et al.* (1960). Nine 5-cc cultures, each with equivalent cell densities, were established in each of five experiments. One of these, designated control, was allowed to grow without interruption for 3 days. Each experimental culture received a single exposure to 100 cc of 5.7 ppm SO_2 in air (Matheson) by bubbling the gas through the culture medium at a slow rate (over a period of 1 hour) at either zero, 1, 2, or 3 days of incubation. To test for possible mechanical damage to the cells caused by this procedure, an additional four cultures received an identical amount of the same quality of air (Matheson) minus the SO_2 at each time interval. All cultures were harvested as both smears and hypotonic spread preparations at 72 hours after treatment for 1 hour with 0.1 μg/ml of colchicine and 1 μCi/ml of ^{3}H-thymidine (New England Nuclear, sp act 6.7 Ci/mmole). Radioautographs were prepared by dipping the slides in liquid Kodak NTB2 emulsion followed by 3 days' exposure at 4°C. The slides were stained with MacNeal's tetachrome.

Lymphocytes incubated in short-term culture with PHA undergo a series of changes termed "blastogenesis" culminating in mitosis (see review article by Robbins, 1964). The following criteria were therefore employed to assess lymphocyte response to the sulfur dioxide-air mixture and to air alone compared with controls: (1) Enlargement from the predominantly small lymphocyte population of peripheral blood to medium or large "blastoid" cells. In order to be considered enlarged, a cell had to possess a minimum diameter of 12 μ measured in any direction; (2) the percentage of total cells which incorporated ^{3}H-thymidine, and hence were synthesizing DNA, during the period the isotope was available; and (3) the mitotic index, or percentage of total cells in karyokinesis after a 1-hour exposure to colchicine. In addition, chromosome spreads were analyzed for mean chromosome counts and for the possibility of aberrations caused by the sulfur dioxide or by mechanical disruption.

RESULTS

Morphological Observations

Figure 1 is a radioautograph of a smear prepared from a control culture showing typical cellular response to PHA. The majority of cells present have enlarged

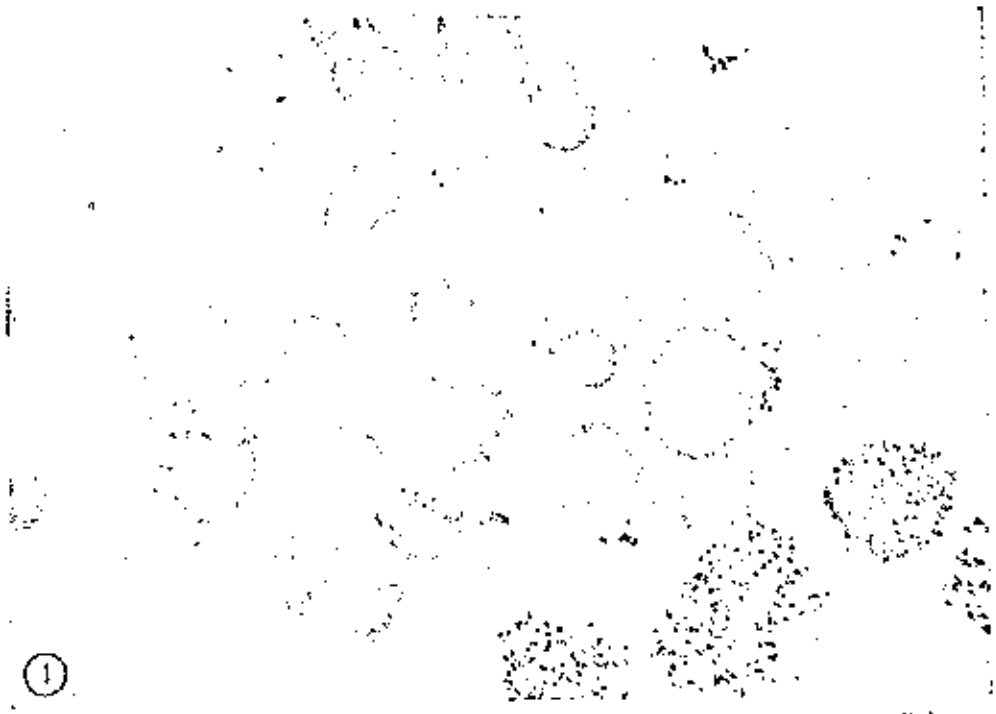

FIG. 1. Radioautograph of a smear from a control culture showing typical cellular response to phytohemagglutinin. ×1350.

from small lymphocytes. The percentage of DNA-synthesizing cells (indicated by the incorporation of radioactive thymidine) is comparable to figures reported in the literature (Cooper *et al.*, 1963; Mackinney *et al.*, 1962; and Michalowski, 1963). Two distinct mitotic figures are evident.

Figure 2 is from a culture which received 100 cc of air at the initiation of incubation. Although cell enlargement and labeling are evident, the field possesses cellular debris, and the two mitotic figures are not so distinct as in controls. Figure 3, however, represents a culture through which air was bubbled after 2 days of incubation, and with the exception of slightly condensed mitotic figures, the cells appear essentially normal. These micrographs suggest that mechanical damage to cells does occur in cultures which received air during the initial period of incubation, but that once growth is established, such an effect is negligible.

Figure 4 is taken from a culture exposed to the same amount of a mixture of 5.7 ppm SO_2 in air at the initiation of incubation. The cells, although technically enlarged, are pyknotic and very deeply stained. Two of the cells in this field are labeled, although the grains are difficult to discern from the densely stained chromatin, and the mitotic figure in the center of the field is abnormally condensed. Such morphological evidence of reduced cellular response decreased progressively in the cultures exposed to SO_2 at 1, 2, and 3 days of incubation.

A normal human metaphase plate containing the full chromosome complement is shown in Fig. 5. This micrograph was taken from a culture which received air at day 1 of incubation. Figure 6, photographed at a lower power, is a field

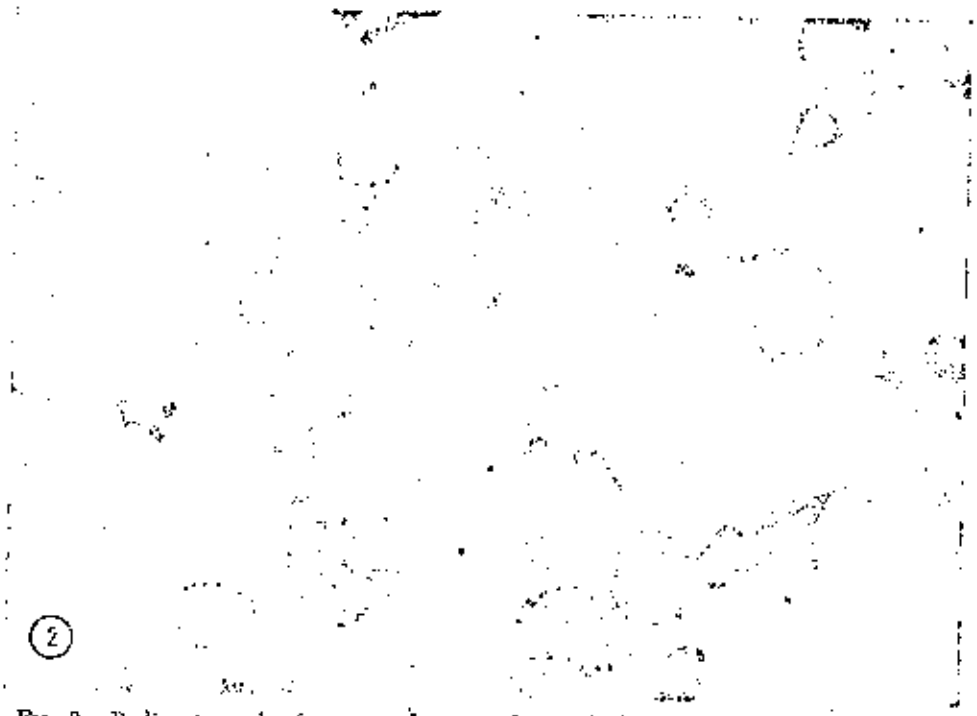

FIG. 2. Radioautograph of a smear from a culture which received air at the initiation of incubation. Cellular debris is present. ×1350.

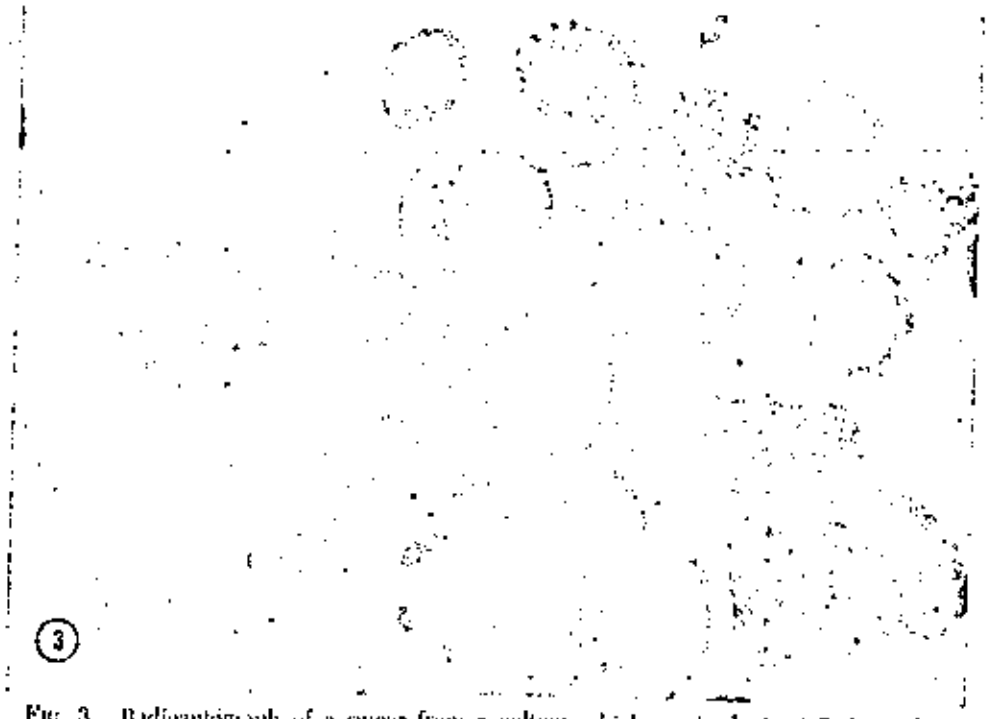

FIG. 3. Radioautograph of a smear from a culture which received air at 2 days of incubation. Cellular morphology is essentially comparable to control cultures. ×1350.

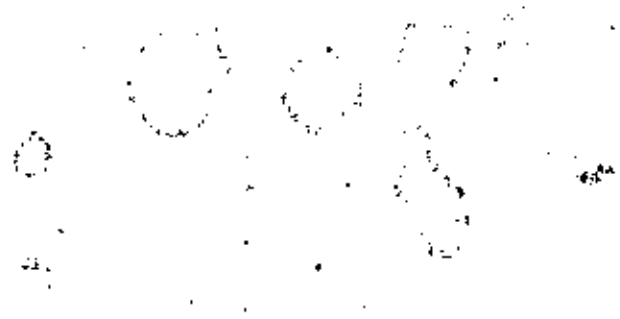

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FIG. 4. Radioautograph of a smear from a culture which received SO_2 at the initiation of incubation. Lymphocyte size is greatly reduced. The cells are pyknotic, and the mitotic figure is abnormally condensed. ×1350.

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FIG. 5. A normal human metaphase plate containing the full chromosome complement. Taken from a culture which received air at day 1 of incubation. ×2400.

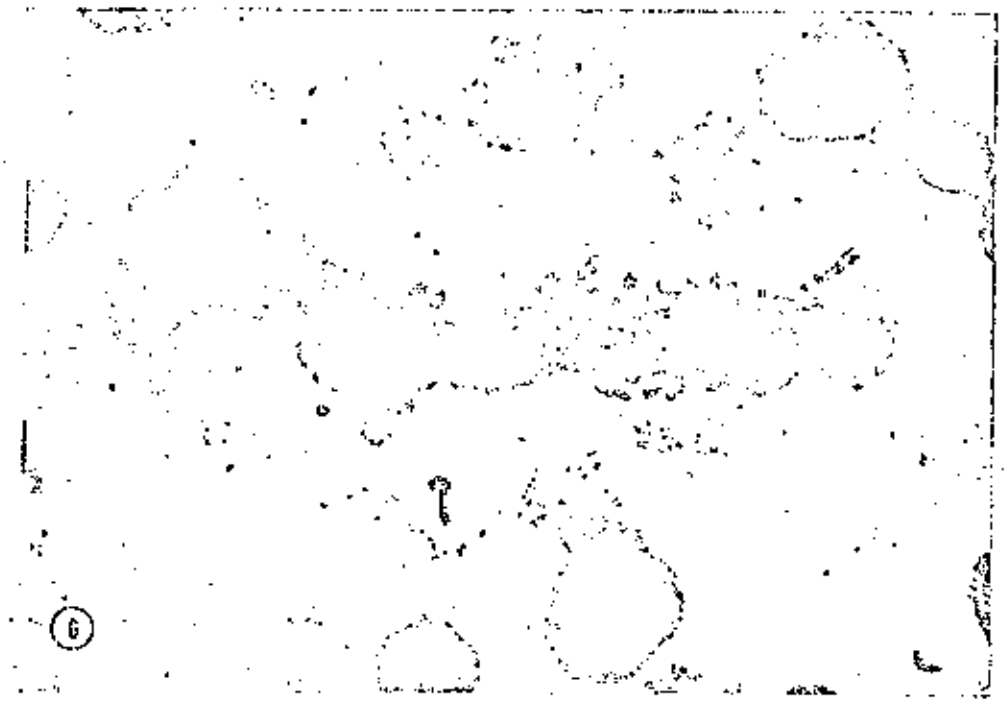

FIG. 6. Hypotonic spreads from a culture which received SO_2 at the initiation of incubation. The chromosomes are clumped and fuzzy. Note the free chromosome some distance from the nearest spread. ×1500.

taken from a culture which received SO_2 at the initiation of incubation. In this case, the chromosomes are clumped and possess an unusually dense core with fuzzy edges, giving the impression of being out of focus. Despite the clumping, a free chromosome is evident some distance from the nearest hypotonic spread.

Quantitative Analysis

The percentages of enlarged, labeled, and dividing cells were determined for each of the nine treatments (control; air at 0, 1, 2, or 3 days; and SO_2 at 0, 1, 2, or 3 days) per experiment. Up to 10,000 cells were counted on masked slides for each treatment. In addition, the following measurements were made (also from masked slides): (1) diameters of 75 randomly chosen lymphocytes per treatment; and (2) chromosome counts on 140 cells from hypotonically treated cultures. The figures were subjected to an analysis of variance, and an F test was made for each criterion of response to determine differences between treatment means. When there were differences, the least significant difference (LSD) was calculated at the 5% significance level (see Table 1). A difference between two or more means which was greater than the LSD was considered significant, and therefore was accepted as having been produced by the treatment employed.

Enlargement. Figure 7 demonstrates the mean percentages of enlarged cells in cultures which received air or SO_2 at 0, 1, 2, or 3 days of incubation compared to controls (see also Table 1). It is apparent that the data are very similar for the

TABLE I

MEAN PERCENTAGES OF ENLARGED CELLS (INCLUDING DIAMETERS), DNA-SYNTHESIZING, AND DIVIDING CELLS FROM CONTROL, AIR-TREATED, AND SO_2-TREATED CULTURES[a]

	Enlargement (%)	Cell diameter (μ)	DNA synthesis (%)	Mitotic index (%)
Control	80.03	23.90	52.16	1.54
Air				
Day 0	81.20	23.10	44.81	1.18
Day 1	81.58	23.67	51.69	1.58
Day 2	77.94	23.37	53.03	1.54
Day 3	83.11	23.73	51.06	1.32
SO_2				
Day 0	79.69	18.83	36.39	0.71
Day 1	86.48	23.50	45.40	1.02
Day 2	85.47	24.40	55.97	1.09
Day 3	85.17	23.63	56.71	1.15
LSD	6.74	2.37	10.40	0.51

[a] The least significant difference (LSD) is listed in each case at the bottom of the table. Results of five experiments.

three categories of cultures at each time interval, although those treated with SO_2 at days 1, 2, and 3 demonstrate slightly elevated levels of enlargement. On the other hand, since a diameter of only 12 μ was established as a criterion of enlargement, it was felt that whereas the percentages of enlarged cells were similar for each set of cultures, variation may have existed among actual cell

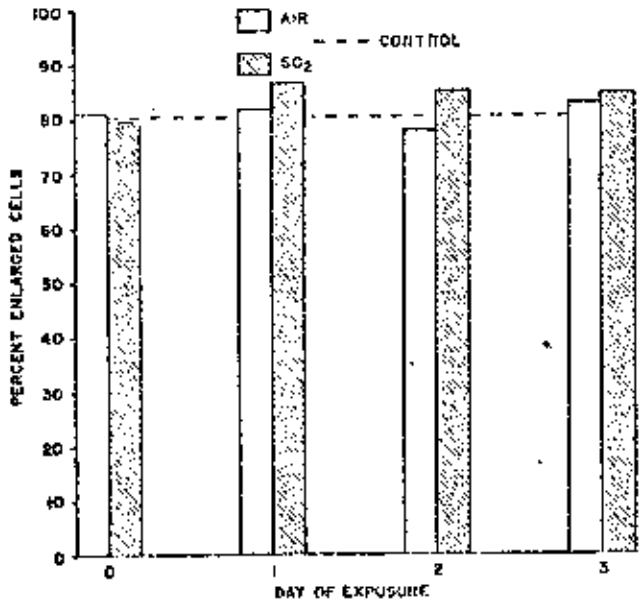

FIG. 7. Percentages of enlarged cells from cultures exposed to air or SO_2 at 0, 1, 2, and 3 days of incubation compared to controls.

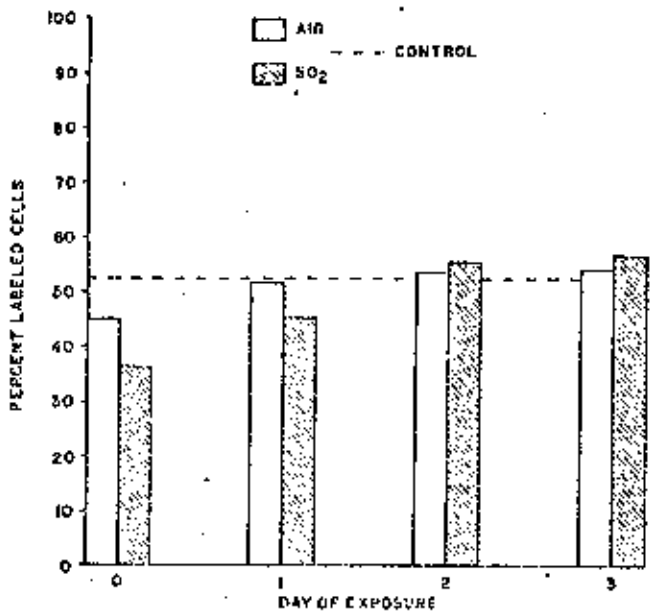

FIG. 8. Percentages of DNA-synthesizing cells from cultures exposed to air or SO_2 at 0, 1, 2, and 3 days of incubation compared to controls.

sizes. This was prompted by comparison of cell sizes in Fig. 4 (SO_2 at initiation of incubation) with those of controls. Whereas the cells in Fig. 4 are technically "enlarged," it is apparent that they are smaller than control cells. From Table 1, it can be seen that the mean diameter of cells exposed to SO_2 at the initiation of incubation is significantly smaller than those of cells in the other 8 categories.

DNA synthesis. Table 1 and Fig. 8 demonstrate the mean percentages of labeled cells which were observed in the various cultures. It is readily apparent that the percentage of DNA-synthesizing cells in cultures which received SO_2 at the initiation of incubation is significantly reduced compared to all others except that which received air at the same time and that which received SO_2 at day 1.

Mitotic index. From Table 1 and Fig. 9 it can be observed that the mean mitotic index for cultures which received SO_2 at the initiation of incubation is significantly lower than controls and all but one of the air-treated cultures. The figures for the cultures which received air are less consistent, but in each case they are higher than those for cultures which received SO_2 at the same intervals.

Chromosome counts. The mean number of chromosomes in randomly chosen hypotonic spreads from control, air-treated, and SO_2-treated cultures are presented in Table 2. Whereas the mean for SO_2-treated cultures is lower than that for controls or air-treated cultures, the reduction in chromosome count is not statistically significant. On the other hand, whereas over 86% of control spreads contained the full chromosome complement, less than half of the SO_2-treated spreads possessed all 46 chromosomes. With the exception of chromosome number, all of the control and air-treated spreads were judged normal as far as chromosome structure and spacing were concerned. Approximately 20% of the spreads of SO_2-treated cultures, however, were considered to be abnormal, mainly in the

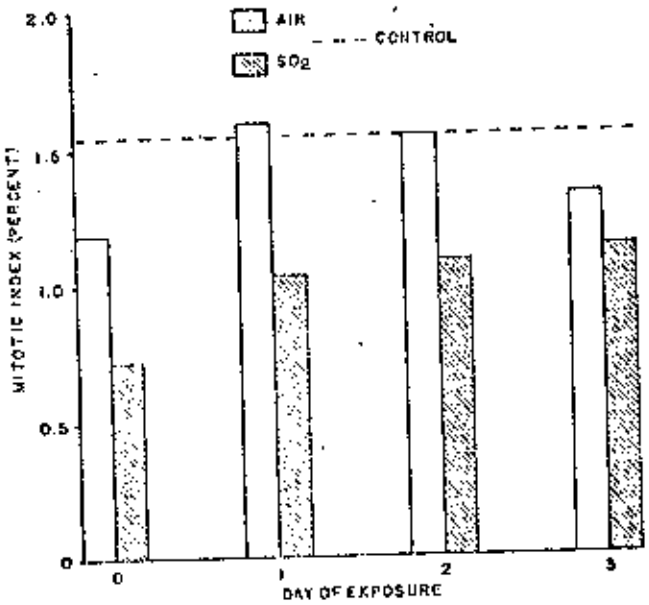

FIG. 9. Percentages of dividing cells from cultures exposed to air or SO_2 at 0, 1, 2, and 3 days of incubation compared to controls.

form of clumping and fuzziness observed in Fig. 6. Only one chromosome break was observed, this from an air-treated culture.

DISCUSSION

From the preceding data, it can be concluded that administration of a 5.7 ppm SO_2-air mixture, especially during the initiation of culture incubation, produced severe defects in lymphocyte kinetics *in vitro*. These defects were manifested as reductions in cell size, in DNA synthesis and in mitosis. Cells which were exposed to air alone at the initiation of incubation also demonstrated reduced DNA synthetic and mitotic capabilities, but to a considerably less degree than those exposed to sulfur dioxide. In contrast to control or air-treated cultures, those exposed to SO_2 also demonstrated chromosomal abnormalities. These observations suggest that whereas air bubbles most likely inflict mechanical damage on lymphocytes exposed at culture initiation, damage caused by sulfur dioxide is both quantitatively more severe and qualitatively different. In addition, the lympho-

TABLE 2

CHROMOSOME ANALYSES FROM CONTROL, AIR-TREATED, AND SO_2-TREATED CULTURES

	No. of spreads	Mean no. of chromosomes	With full complement (%)	Abnormal spreads (No.)	Abnormal spreads (%)
Controls	30	45.03	86.67	0	0.0
Air	44	45.07	61.36	0	0.0
SO_2	66	43.82	46.97	13	19.70

cytes were more sensitive to the SO_2-air mixture than to air alone during the later intervals of exposure as evidenced by reduced mitotic indices.

The mechanism of action of sulfur dioxide on lymphocytes is at present open to speculation. Changes in pH of the culture media upon exposure to air or SO_2 were minimal and equivalent. The initial pH of the culture media was 7.0. This approached 6.6 as incubation progressed, which is evidence of metabolic activity of the cells and, therefore, indicative of good culture growth. The pH of cultures exposed to either air or SO_2 momentarily approached 7.2, but thereafter gradually returned to slightly acidic, which indicated that the cells experienced partial recovery from the treatment. The conclusion that altered pH could not have been the limiting factor in lymphocyte response (since both the air-treated and SO_2-treated cultures demonstrated the same minor change) is supported by the findings of Thompson and Face (1962).

It is well established that DNA synthesis in cultured human lymphocytes is minimal to nonexistent prior to 24 hours of incubation (Bender and Prescott, 1962; Cooper *et al.*, 1963; and Michalowski, 1963). It is interesting to note that in the present investigation, the lymphocytes were most susceptible to damage by sulfur dioxide before DNA synthesis was actively initiated. Damage to cells exposed to the gas after 48 or 72 hours of incubation was insignificant compared to lymphocytes in control cultures. This observation suggests that sulfur dioxide preferentially affects cells in preparation for DNA synthesis, but not those in which "blastogenesis" is well established. Lymphocyte response in certain immunological reactions *in vivo* is similar to the *in vitro* response to PHA (Robbins, 1964). Thus, reduction in cultured lymphocyte response upon exposure to SO_2 may offer one explanation for the reduced immunological responses observed in animals exposed to the gas *in vivo* (see introductory material). Interference with normal nucleic acid metabolism by sulfur dioxide has also been reported by Shapiro (1970) who observed that sodium bisulfite (the chemical form of SO_2 in the blood) altered the genetic code of yeast.

In addition to depressing DNA synthesis, sulfur dioxide also severely inhibited lymphocyte enlargement when applied at the initiation of incubation, and reduced the mitotic index at all intervals of exposure. The slightly increased percentages of enlarged cells in cultures exposed to SO_2 at days 1, 2, and 3 of incubation may reflect the fact that cell division was repressed. Thus, whereas the lymphocytes exposed at these intervals grew in size to control values, less of them divided, resulting in a proportionately larger percentage of enlarged cells. It cannot presently be determined if the abnormal chromosome spreads observed in SO_2-treated cultures were the result of a direct action by the sulfur dioxide, or simply an indication of inferior cellular growth. The high percentage of spreads which lacked the full chromosome complement, as well as the reduced mitotic indices, however, suggest that the SO_2 may have somehow interfered with the normal structure and/or function of the mitotic apparatus.

The concentration of sulfur dioxide used in these experiments was within the range of short-term exposures recorded near point sources (smelters, coal-fired power plants) in the U. S. and elsewhere.[1] Whereas the active exposure to the gas lasted only an hour, some of the sulfur dioxide may have been converted to

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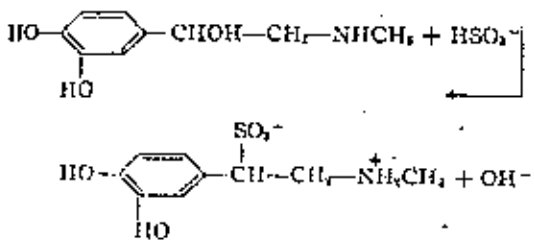
A Note on the Preparation of 1-(3,4-Dihydroxyphenyl)-2-methylaminoethane Sulfonic Acid from Epinephrine*

By LOUIS C. SCHROETER† and TAKERU HIGUCHI

KINETIC STUDIES (1, 2, 3) indicated that epinephrine reacts with sodium bisulfite or sulfite solution to yield a zwitter-ionic sulfonate:



The preparative value of this reaction and comprehensive physical constants of the product and its acetyl derivative have not been previously reported.

EXPERIMENTAL

Epinephrine hydrochloride, 0.025 mole (5.492 Gm.) and sodium bisulfite, 0.025 mole (2.602 Gm.) were dissolved in about 50 ml. distilled water; the pH was adjusted to 6.8 by the addition of standard sodium hydroxide. The solution was introduced into a three-necked flask fitted with a dropping funnel, reflux condenser, and a tube for flushing the solution with nitrogen. The reaction mixture (ca. 80 ml.) was refluxed for six hours while keeping it flushed with nitrogen. Standard hydrochloric acid (1 N) was added periodically to maintain pH range 6.7 to 6.9. After refluxing, excess water (ca. 65 ml.) was distilled from the solution. The white crystalline precipitate which separated on cooling the solution was recrystallized from 1 N hydrochloric acid and twice from distilled water. Yield: 5.28 Gm. (85.3%).

Anal.—Calcd. for $C_9H_{13}NO_6S$: C, 43.71; H, 5.29; N, 5.66; O, 32.36; S, 12.97. Found: C, 43.72; H, 5.79; N, 5.50; O, 31.90; S, 13.05. The product melted with decomposition at 263° (Kofler hot stage) and exhibited the following optical crystallographic properties: system, orthorhombic; class, dipyramidal; refractive indexes, $\alpha = 1.541$, $\beta = 1.650$, $\gamma = 1.676$; optic sign, negative; $2V = 55°$ (calculated from α, β, and γ). X-ray powder diffraction data shown in Table I were obtained using copper radiation with a nickel filter (1.5405 Ångstroms). The pKa determined in water was 8.95. Ultraviolet spectrum in water shows maxima at 228 and 278 mμ; $\epsilon_{228} = 15,650$, $\epsilon_{278} = 2,850$.

1-(3,4-Diacetoxyphenyl)-2-methylaminoethane Sulfonic Acid.—Acetylation of 1-(3,4-dihydroxyphenyl)-2-methylaminoethane sulfonic acid was accomplished by suspending 2 mM (495 mg.) of the latter in 3.0 ml. glacial acetic acid. Two milliliters acetic anhydride and two drops concentrated hydrochloric acid were added to the solution and the latter refluxed for about twenty minutes. The white crystalline precipitate which separated on cooling the solution was recrystallized from glacial acetic acid. Yield: 413 mg. (62.5%).

TABLE I.—X-RAY POWDER DIFFRACTION DATA 1-(3,4-DIHYDROXYPHENYL)-2-METHYLAMINOETHANE SULFONIC ACID

d, A	I/I_1	d, A	I/I_1
8.34	0.43	3.64	0.32
7.40	0.48	3.52	0.26
6.18	0.90	3.40	1.00
5.58	0.85	3.33	0.34
5.29	0.87	3.21	0.46
4.69	0.35	3.09	0.29
4.58	0.33	2.81	0.58
4.32	0.60	2.65	0.39
4.17	0.41	2.44	0.34
4.01	0.82	2.24	0.31
3.75	0.83	..	..

TABLE II.—X-RAY POWDER DIFFRACTION DATA 1-(3,4-DIACETOXYPHENYL)-2-METHYLAMINOETHANE SULFONIC ACID

d, A	I/I_1	d, A	I/I_1
15.22	0.85	4.73	0.58
9.29	0.77	4.59	0.43
7.48	0.75	4.27	0.63
6.23	1.00	3.91	0.64
5.83	0.81	3.75	0.76
5.47	0.55	3.53	0.55
5.04	0.91	3.36	0.39
4.90	0.56	3.13	0.38
..	..	3.06	0.47

Anal.—Calcd. for $C_{13}H_{19}NO_8S$: C, 47.13; H, 5.13; N, 4.23; O, 33.80; S, 9.68. Found: C, 47.21; H, 5.16; N, 3.93; O, 33.07; S, 9.48. Acetyl determination: theory, 25.98%. Found, 26.71%. The derivative melted 212 to 213° (Kofler hot stage) and exhibited the following crystallographic properties: system, triclinic; refractive indexes, $\alpha = 1.480$, $\beta = 1.502$, $\gamma = 1.562$; optic sign, positive; $2V = 65°$ (calculated from α, β, and γ). X-ray powder diffraction data shown in Table II were obtained using copper radiation with a nickel filter (1.5405 Ångstroms). The pKa determined in water was 8.30. Ultraviolet spectrum in water shows maxima at 263 and 269 mμ with a shoulder at 215 mμ: $\epsilon_{263} = 8,900$, $\epsilon_{269} = 411$.

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* Received December 2, 1959, from the School of Pharmacy, University of Wisconsin, Madison and the Research Laboratories, The Upjohn Co., Kalamazoo, Mich.
† Research Associate, The Upjohn Co.
The authors wish to thank Dr. John W. Shell, The Upjohn Co., for helpful crystallographic suggestions and Dr. William A. Stroick, The Upjohn Co., and members of his staff, for analyses.

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122

E. Seeber

Fremdstoffe in Lebensmitteln

Kalkuliertes Risiko unserer Zeit

Die bei Lebensmitteln immer höher gesteckten Ansprüche in der Verbrauchererwartung können hinsichtlich Angebotsformen, Haltbarkeit, Zeitersparnis sowie Preisgestaltung nur mit Hilfe einer Vielzahl fremder Stoffe in der Lebensmittelwirtschaft realisiert werden.

Während früher das reine, naturbelassene Lebensmittel das Normalerzeugnis darstellte, haben sich in den letzten zwei bis drei Jahrzehnten die Verhältnisse nahezu umgekehrt. Um die hieraus resultierenden eventuellen neuen Gefahrenquellen für den Verbraucher möglichst auszuschließen, hat der Gesetzgeber in dem Änderungsgesetz zum Lebensmittelgesetz von 1958 die Verwendung fremder Stoffe allgemein dem Verbotsprinzip unterworfen. Es besagt, daß sie nur dann Lebensmitteln zugesetzt werden dürfen, wenn sie hierfür ausdrücklich zugelassen sind. Wesentlichste Kriterien hierbei sind zwei Gesichtspunkte, nämlich ihre gesundheitliche Unbedenklichkeit sowie ihr technologisches Erfordernis. Darüber hinaus hat man die Empfehlungen der Deutschen Forschungsgemeinschaft berücksichtigt, wonach Hauptnahrungsmittel, wie Brot, Butter, Milch, Fleisch u. a., nicht mit fremden Stoffen hergestellt werden dürfen.

Zur einwandfreien Information des Verbrauchers ist in erster Linie der Hersteller, aber auch jeder Händler verpflichtet, die Verwendung fremder Stoffe in Lebensmitteln kenntlich zu machen, so zum Beispiel mit den Worten „mit Farbstoff", „mit Konservierungsstoff Sorbinsäure", „geschwefelt bzw. stark geschwefelt", „mit Phosphaten" u. a.

Nachdruck aus BGI 63 (1970).

Was versteht man nun unter Fremdstoffen im Sinne des Lebensmittelgesetzes? Nach § 4a Abs. 2 LMG sind diese Verbindungen Stoffe, die dazu bestimmt sind, mitverzehrt zu werden, die aber keinen Gehalt an verdaulichen Kohlenhydraten, Fetten und Eiweißen enthalten, das heißt also Stoffe, die keinen ernährungsphysiologischen Nährwert besitzen. Hierzu gehören vor allem die Konservierungsstoffe, die zur Haltbarmachung der Lebensmittel dienen, sodann die Farbstoffe als Schönungsmittel, auch die zur Oberflächenbehandlung von Obst benutzten Stoffe sowie diejenigen Stoffe, die bei der Aufbewahrung (zum Beispiel Verpackungsmaterial) in die Lebensmittel hineingelangen können. Zur besseren Übersicht über die genannten Verbindungen fremder Stoffe sei folgende Einteilung gebracht, die allerdings keinen Anspruch auf Vollständigkeit hat.

1. Farbstoffe

Ihre Verwendung ist geregelt in der Verordnung über färbende Stoffe vom 19. 12. 1959; sie dienen als Schönungsmittel von Lebensmitteln, sind toxikologisch eingehend geprüft und gesundheitlich praktisch unbedenklich.

2. Konservierungsstoffe

Ihre Verwendung ist geregelt in der Verordnung über konservierende Stoffe vom 19. 12. 1959; sie dienen zur Haltbarmachung von Lebensmitteln und sind toxikologisch praktisch unbedenklich.

3. Oberflächenbehandlungsmittel

Ihr Zusatz bei der Behandlung von Früchten und Fruchterzeugnissen ist geregelt in der Fruchtbehandlungs-Verordnung vom 19. 12. 1959; sie sind nur unter der Kennzeichnungspflicht mit den Worten zum Beispiel „mit Orthophenylphenol, Schale nicht zum Verzehr geeignet" verkehrsfähig. Unter Beachtung dieser Vorschrift sind sie gesundheitlich praktisch risikofrei.

4. Antioxydantien

Hierbei handelt es sich um eine heterogene Stoffgruppe, die das Ranzigwerden von Speisefetten und Ölen verhindert. Bei der einen Gruppe handelt es sich um Vitamine zweier verschiedener Typen, der Askorbinsäure (Vitamin C) und der Tokopherole (Vitamin E), die neben ihrer antioxydativen Wirkung gleichzeitig einen wünschenswerten ernährungsphysiologischen Zweck erfüllen; sie finden daher breite Verwendung in der Margarineherstellung. Bei der anderen Gruppe, die bei uns bisher nicht zugelassen ist, aber in den EWG-Mitgliedstaaten verwendet wird, handelt es sich um phenolische, das heißt ringförmige Substanzen — den Gallaten —, wie Butylhydroxyanisol (BHA) und Butylhydroxytoluol (BHT), die Vitamin B_1 zerstö-

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ren sollen sowie zu Lebervergrößerungen in Tierversuchen geführt haben. Die Bundesregierung wird versuchen, daß diese Stoffgruppe bei den Angleichungsbestrebungen unserer lebensmittelrechtlichen Vorschriften nicht zugelassen wird.

5. Emulgatoren, Stabilisatoren, Geliermittel, Dickungs- und Standmittel

Ihre Verwendung ist überwiegend geregelt in der Allgemeinen Fremdstoff-Verordnung vom 19. 12. 1959; sie dienen zur Erreichung einer bestimmten physikalischen Beschaffenheit der Lebensmittel und werden weitgehend verwendet bei Mayonnaisen, Soßen und Speiseeis; toxikologisch sind sie praktisch risikofrei.

6. Säuren und deren Salze

Ihre Verwendung ist geregelt in der Allgemeinen Fremdstoff-Verordnung vom 19. 12. 1959; als Azetate, Zitrate, Tartrate finden ihre Salze weitgehende Verwendung bei der Käseherstellung; sie sind toxikologisch unbedenklich.

7. Aromasubstanzen und ihre Verstärker (Essenzen)

Die Verwendung dieser Stoffe ist geregelt in der Essenzen-Verordnung vom 19. 12. 1959; sie finden breite Anwendung bei Brausen, Puddings, Kunstspeiseeis, Zuckerwaren, Kaugummi u. a.; toxikologisch sind sie bis auf das kürzlich umstrittene Kumarin praktisch risikofrei; von den Aromenverstärkern, die in den EWG-Mitgliedstaaten Verwendung finden, wäre die Glutaminsäure evtl. toxikologisch von Bedeutung, die für die Krankheitssymptome der sogenannten Chinaessenkrankheit für verantwortlich angesehen wird; die Untersuchungen hierzu sind jedoch noch nicht abgeschlossen.

8. Schwefeldioxid

Die Verwendung von Schwefeldioxid und dessen Salze (Sulfite) ist geregelt in der Verordnung über die Verwendung von Schwefeldioxid vom 13. 8. 1969; die konservierenden wie bleichenden Eigenschaften von SO_2 finden breite technologische Anwendung. Auf seine möglichen toxikologischen Risiken soll anschließend eingegangen werden.

9. Phosphate

Sie finden eine weitverbreitete Verwendung in der Lebensmitteltechnologie, besonders bei nichttierischen Erzeugnissen, und sind hier geregelt in der Allgemeinen Fremdstoff-Verordnung vom 19. 12. 1959; nach Neuzulassung als Kutterhilfsmittel bei Fleischerzeugnissen sind sie zusätzlich geregelt unter Kennzeichnungszwang in der Änderungs-Verordnung zur Fleisch-Verordnung vom 25. 8. 1969.

Zunächst muß die Fragestellung erörtert werden, was der Begriff „gesundheitliches Risiko" bedeutet. Bekanntlich findet sich für nahezu jede Substanz ein toxikologischer Dosisbereich, selbst für Stoffe, die als unbedenklich angesehen werden, wie zum Beispiel Kochsalz. Grundsätzlich kann daher festgestellt werden, daß die Mehrzahl der fremden Stoffe in Lebensmitteln weder eine akute noch eine chronische Toxizität im Bereich der gesetzlich festgelegten Höchstmengen aufweisen. Die Prüfung der Toxizität erfolgt in der Regel im Tierversuch, ähnlich wie bei den Arzneimitteln. Während Arzneimittel jedoch nur kurzfristig beim Menschen gegeben werden, ist der Mensch auf den Verzehr von Lebensmitteln während der Zeit seines gesamten Lebens angewiesen. Dieser „Langzeitversuch" ist es, der eine abschließende Beurteilung eines fremden Stoffs so besonders schwierig macht. Deshalb spricht man nicht von direkt „unschädlichen" Substanzen, sondern vorsichtshalber von Stoffen, die zur Zeit „duldbar" sind.

Im Jahr 1969 haben zwei Substanzen im Rahmen des Zulassungsverfahrens in der Verordnungsgebung besonders lebhafte Debatten in den Sachverständigengremien ausgelöst, nämlich Schwefeldioxid und Phosphate:

Schwefeldioxid ist ein fremder Stoff, der technologisch hervorragende konservierende, bakterienblütende und bleichende Wirkung besitzt, der jedoch toxikologisch nicht ganz unbedenklich ist. Deshalb hat der Gesetzgeber 1969 eine eigene Verordnung über Schwefeldioxid bei seiner Verwendung in Lebensmitteln verabschiedet. Im Gegensatz zu früher ist danach der Hersteller verpflichtet, alle Lebensmittel mit einem höheren Schwefeldioxidgehalt als 500 mg/kg, die zum Rohverzehr geeignet sind, mit den Worten „stark geschwefelt" kenntlich zu machen. In der Regel handelt es sich hierbei jedoch um Lebensmittel, die nicht so oft und dann nur in kleineren Mengen verzehrt werden, wie zum Beispiel Meerrettich in zubereiteter Form, Rosinen, getrocknete Aprikosen, Birnen u. ä. Ein Erhitzen solcher Lebensmittel vor dem Verzehr wie Backen oder Kochen wirken sich insofern günstig aus, als sich hierbei der größte Teil des Schwefeldioxids verflüchtigt.

Dennoch handelt es sich toxikologisch um zwei Probleme:

1. Schwefeldioxid zerstört schnell eines unserer wichtigsten Vitamine, nämlich Vitamin B_1, das ohnehin schon jetzt zu knapp in der heute üblichen Nahrungszufuhr vorhanden ist. Ein Genuß geschwefelter Lebensmittel sollte daher sorgfältiger als bisher geprüft werden, wobei ein Rohverzehr — das heißt ohne Erhitzen — möglichst zu vermeiden ist.

2. Schwefeldioxid verursacht eine lokale Reizwirkung, wodurch Ma-
1312 genschleimhautentzündungen begünstigt werden. Es ist daher nicht

verwunderlich, daß magenempfindliche Menschen gerade gute Weinsorten schlecht vertragen, da die besten Weine zum Unterschied zu anderen geschwefelten Weinsorten und Lebensmitteln ohne Kenntlichmachung die höchsten Schwefeldioxidmengen aufweisen. Deshalb hat der Gesetzgeber die Industrie beauftragt, andere Verfahren zu entwickeln, durch die SO_2 eines Tages entbehrlich wird.

Phosphate stellen eine andere Gruppe fremder Stoffe dar, die nicht in unbegrenzten Mengen verzehrt werden können. Nach den Bestimmungen der Allgemeinen Fremdstoff-Verordnung sind sie ebenfalls ohne Kenntlichmachungszwang als Zusatz erlaubt bei der Schmelzkäsezubereitung, bei Kondensmilch, bei koffeinhaltigen Erfrischungsgetränken u. a. Mit der Neuzulassung zu Fleischerzeugnissen, besonders bei der Brühwurstherstellung als Kutterhilfsmittel, entbrannte die Frage nach dem erforderlichen Bilanzausgleich. Hierunter versteht man allgemein, ob die Phosphatzufuhr durch diese Maßnahme überzogen werden könnte, was letztlich im Langzeitversuch Störungen des Mineralstoffwechsels zu Folge haben könnte. Spätfolgen mit Entkalkungen des Skelettsystems bei gleichzeitig gesteigerter Nierensteinbildung sind bei langdauerndem „leeren Phosphatüberschuß" theoretisch nicht ausgeschlossen. Nach übereinstimmenden Aussagen der Experten seien jedoch unter den derzeitigen Verzehrsgewohnheiten des Verbrauchers keine derartigen Schäden zu befürchten. Deshalb wurden mit dieser Neuzulassung auch nur bestimmte Phosphatsalze bei gleichzeitiger Höchstmengenbegrenzung zugelassen.

Beide Beispiele zeigen, wie komplex die Verhältnisse bei der Beurteilung möglicher gesundheitlicher Risiken durch Fremdstoffe sind. Andererseits ist klar, daß auf die Verwendung von Fremdstoffen bei Lebensmitteln nicht mehr verzichtet werden kann: Angesichts der Bevölkerungsexpansion und des Hungers in vielen Teilen der Welt kommt ihnen das Verdienst zu, weitgehend Fehlproduktionen sowie Verderb von Lebensmitteln auszuschließen. Ebenso wäre unser derzeitiges Lebensmittelangebot, besonders die Vielzahl der Halbfertigprodukte, die dem Verbraucher ein wirtschaftlich rentables Enderzeugnis garantieren, ohne Fremdstoffzusätze nicht denkbar. Die Zulassung der Fremdstoffe in Lebensmitteln muß man daher als „kalkuliertes Risiko" unserer Zeit hinnehmen.

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CIENCES

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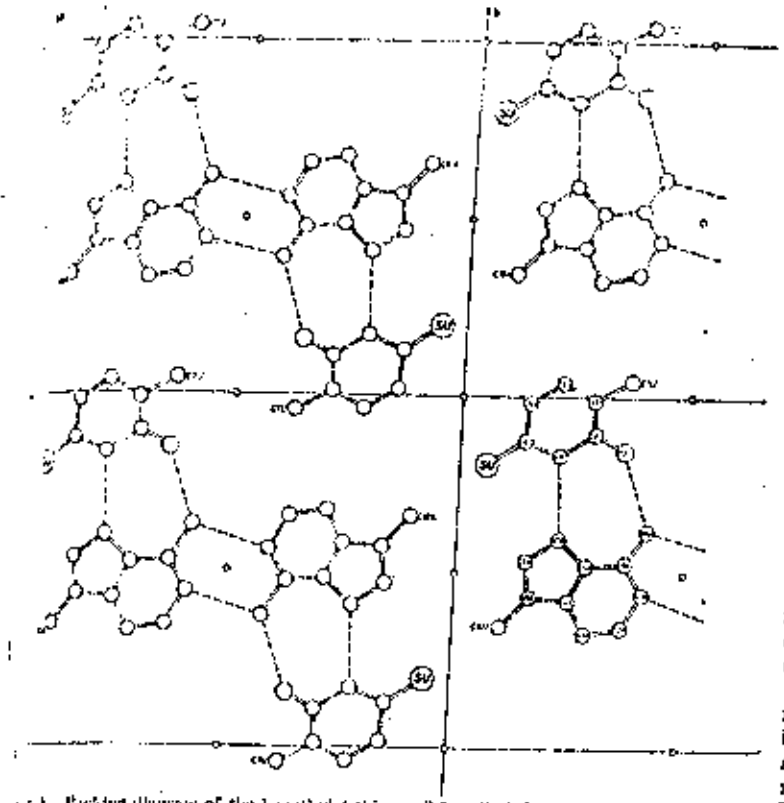

Fig. 1. Packing diagram of the 1-methyl-4-thiouracil·9-methyladenine complex projected down a*.

This is, however, not the only effect which determines the geometry of these complexes, for in the 1-methyl-4-thiouracil·9-methyl-adenine base pair the regular Hoogsteen scheme applied[11]. One would assume therefore that in the 1-methyl-4-thiouracil·9-methyladenine complex, the formation of a hydrogen bond involving uracil O2 instead of S4 is favoured for two reasons. The sulphur atom is less electronegative than the oxygen atom, that is the NH . . . S hydrogen bond is weaker than an NH . . . O bond[12]; furthermore, the packing arrangement might prefer this type of hydrogen bonding.

We thank Professor F. Cramer for his interest and support and Professor D. Moatz, Universität Braunschweig, for making available his Siemens diffractometer.

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Specific Deamination of RNA by Sodium Bisulphite

The ability of nitrous acid to deaminate the cytosine residues of nucleic acids to uracil has made it valuable both as a mutagen[1,2] and as an aid to studies of the structure[3] and function[4,5] of nucleic acids. Its use is, however, complicated by the fact that it also deaminates the adenine and guanine residues in nucleic acids[6,7]. Guanine, the reaction of which is least desirable for mutagenic purposes, is usually the base most reactive to nitrous acid[8]. In addition, nitrous acid causes the formation of nitro compounds[8], the cross-linking of DNA (ref. 9), the destruction of guanine residues[10], and other side reactions which diminish its usefulness.

We recently reported that sodium bisulphite can be used to deaminate cytosine derivatives, by the route illustrated in Fig. 1 (ref. 11). The cytosine derivative is converted quickly by sodium bisulphite, at pH 5, to an addition compound which readily deaminates, on standing, to the corresponding uracil addition compound. The latter is converted to uracil by adjustment of the pH to 9, and removal of excess bisulphite. Adenine and guanine derivatives do not react in these conditions. We now wish to report the successful application of this procedure to the specific deamination of the cytosine residues of yeast RNA.

To a solution of 5·0 mg of sodium yeast ribonucleate (Schwarz BioResearch, Orangeburg) (purified by HCl precipitation at 5° C, and washing with ethanol and ether) in 0·5 ml. of H_2O was added 118 mg of $NaHSO_3$. The pH was adjusted to 5 by addition of concentrated HCl, and the reaction was allowed to proceed as indicated in Table 1. The pH was then adjusted to 9 with 1 M NaOH. The RNA was separated from the bisulphite by chromatography on 'Sephadex G-25M' (Pharmacia, Piscataway). For analysis of composition, the RNA was hydrolysed at 37° C with 10 ml. of 1 M KOH for 24 h. The solution was neutralized to pH 6·5 with $HClO_4$, cooled at 4° C for 16 h, and the precipitated $KClO_4$ was removed by filtration. The nucleotide mixture was then fractionated on a column of Amberlite 'CG-400' resin (formate), using an exponential gradient in formate (for details see ref. 12). The column is capable of resolving the four major natural ribonucleoside 2′-(3′)-phosphates, as well as inosine 2′-(3′)-phosphate and xanthosine 2′-(3′)-phosphate. The peaks eluted were identified by their position and by their R_f (thin-layer chromatography) in a solvent made of 13·8 g $NaH_2PO_4 \cdot H_2O$ dissolved in 900 ml. of H_2O. The pH of this solvent was adjusted to 6·8 with H_3PO_4, the volume was diluted to 1,000 ml. with H_2O, and 200 g of $(NH_4)_2SO_4$ and 20 ml. of 1-propanol were added. The R_f values observed for the 2′-(3′)-phosphates of adenosine, guanosine, uridine and cytidine, respectively, were 0·31, 0·52, 0·76 and 0·86. The quantitative amounts of each

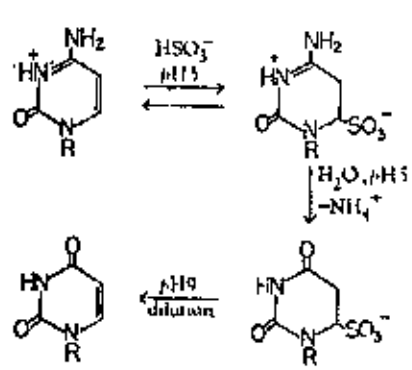

Fig. 1. Deamination of cytosine derivatives by bisulphite

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peak eluted from the column were determined by a spectrophotometric method[12].

The results of four deamination runs, in various conditions, are summarized in Table 1. No new reaction products were observed, and all of the adenine and guanine nucleotides were recovered (within experimental error). A varying amount of deamination of the cytosine residues of the RNA to uracil was observed. An initial portion of the cytosines reacted rapidly. The rate of reaction then slowed down, and finally levelled off when 90 per cent of the cytosines was deaminated. This is not surprising, considering the heterogeneous nature of yeast RNA. It suggests that the reactivity of cytosine residue to bisulphite is strongly influenced by the secondary structure of the RNA. The altered composition of the RNA in run 4 was reflected in its ultraviolet spectrum. The ratio A_{250}/A_{260} for the deaminated RNA (*p*H 2) was 0·44 (λ_{max} = 258) whereas the corresponding ratio for the starting RNA was 0·62 (λ_{max} = 260).

Table 1. ANALYSIS OF BISULPHITE-MODIFIED RNA

Run	Conditions	Nucleotides isolated (moles × 10⁴) Cp	Up	Ap	Gp	Deamination of Cp (per cent)
1	Unmodified RNA	2·3	3·1	2·8	3·2	
2	15 h, 25° C	1·3	4·3	2·5	3·1	45
3	24 h, 37° C	0·75	4·8	2·6	3·2	68
4	84 h, 37° C	0·20	5·2	2·7	3·3	91
5	108 h, 37° C	0·21	5·7	2·9	3·4	92

The abbreviations Cp, Up, Ap, and Gp refer to the 2′-(3′)-phosphates of cytidine, uridine, adenosine, and guanosine, respectively.

Preliminary experiments with DNA confirm the dependence of cytosine reactivity on nucleic acid secondary structure. In the conditions of run 5, the cytosines of single stranded phage F_1 DNA were completely converted to uracils, while those of double stranded calf thymus DNA were essentially unreacted. Thus sodium bisulphite should be of value for the chemical modification of nucleic acids, and may replace nitrous acid in many of its uses.

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Inhibition of Vaccinia Virus Growth by 1-Methyl-4-aminopiperazine

RIFAMPICIN inhibits the replication of poxviruses[1,2]; experiments with other rifamycin derivatives suggested that the hydrazone side-chain of rifampicin was essential to its antiviral activity[3]. We describe here the action of a characteristic component of this side-chain, 1-methyl-4-aminopiperazine, and compare it with that of the analogue of rifampicin, 3-formylrifamycin SV (FSV), another compound, 1-methyl-4-benzylidenaminopiperazine, which contains the hydrazone side-chain but the rifamycin moiety.

The activities of these drugs, together with twenty-seven other rifamycin derivatives, were screened, plating eighty plaque forming units (p.f.u.) of vaccinia viruses on primary cultures of chick embryo fibroblasts. After the adsorption period, the cell sheet was overlaid with nutrient medium in agar containing 800–100 μg of each drug. With vaccinia virus, methylaminopiperazine and methylbenzylidenaminopiperazine completely inhibited plaque formation at 200–300 μg/ml. The second drug was slightly toxic to the cells at 800 μg/ml. At twice the limiting dose of toxicity, none of the drugs inhibited plaque formation by fowl plague, Newcastle disease, pseudorabies and herpes type 2 viruses. Some of the drugs were highly toxic: the cells did not stain with neutral red after 4 days' treatment with 50–100 μg of 4-benzylidenazo-1-deoxyrifamycin S (BDS), the O-benzyloxime of 3-formylrifamycin SV (BOF), 4-(1-methyl-2-imidazolinylidiazo)-4-deoxyrifamycin SV (MID) or 1′-benzyl-2-methyl-3′-acetylpyrrolo-(3,2-C)-4-deoxyrifamycin S (BAP).

In cells treated for 1 h with 200–400 μg/ml. of the compounds ^{14}C-uridine incorporation was inhibited by 90 per cent, and the incorporation of a hydrolysate of ^{14}C-protein was only reduced by 40–50 per cent. Methylbenzylidenaminopiperazine also inhibited uridine incorporation but its action was more easily reversed by washing the cells; methylaminopiperazine had no effect. Similar results were obtained when *in vitro* transcription with treated nuclei was investigated (Table 1).

Although the activity of polymerase aggregate was not influenced by methylaminopiperazine in the assay mixture *in vitro*, treatment of whole cells with methylamino-

Table 1. ACTION OF VARIOUS INHIBITORS (400 μg/ml.) ON INCORPORATION OF ^{3}H-UTP INTO ACID PRECIPITABLE MATERIAL OF SONICATED NUCLEI FROM CHICK EMBRYO FIBROBLASTS

	Incorporation (d.p.m.) Number	Per cent of control
No drug	1,424	
BDS	348	24
MID	191	13
FSV	1,506	106
Rifampicin	1,293	91
Methylaminopiperazine	1,924	135
Methylbenzylidenaminopiperazine	174	12
BAP	522	36
BOF	624	44

The components of the assay mixture were added in the following order: (a) 0·7 ml. of a solution containing 80 μmoles of Tris-HCl (*p*H 8·0), 2·5 μmoles of $MnCl_2$, 1 μmole each of CTP and GTP, 2 μmoles of ATP, 0·1 μmole of UTP, 5 μmoles of phosphoenolpyruvate and 0·01 M mercaptoethanol; (b) 400 μg of one inhibitor; (c) 2 μCi (corresponding to 0·0013 μmole) of ^{3}H-UTP and 20 μg of pyruvate kinase; (d) 0·3 ml. of sonicated nuclei obtained from 8×10^7 cells, according to the method of Bach and Johnson[4]. After incubation for 30 min at 37° C, trichloroacetic insoluble radioactivity was determined by standard procedures.

Table 2. ACTION OF METHYLAMINOPIPERAZINE ON WHOLE CELLS AND ON BROKEN CELLS

	Incorporation of ^{3}H-UTP by sonicated nuclei (d.p.m.) Number	Per cent of control
No inhibitor	2,808	
Methylaminopiperazine (800 μg/ml.)		
(a) On suspended whole cells, for 2 h at 37° C	928	32
(b) On suspended whole cells, for 30 min at 37° C	522	18
(c) On suspended whole cells, for 30 min at 0° C in distilled water	456	16
(d) On broken cells, immediately after treatment with the Dounce homogenizer	2,745	95
(e) On the enzyme aggregate, in the assay mixture before addition of ^{3}H-UTP	2,650	95

5×10^6 Embryo fibroblasts were suspended in Eagle's medium without calcium and with 0·5 per cent gelatinine plus 2·5 per cent calf serum. The suspension was divided into six aliquots which were agitated for 2 h at 37° C; samples (a) and (b) were treated with the drug for 2 h and the last 30 min, respectively. All the samples were then centrifuged and the sediments collected in ice-cold distilled water. The drug was then added to sample (c) after 30 min at 0° C and treatment with 0·25 per cent "Trypsin A-Difco", the cells were disrupted with six strokes of a Dounce homogenizer and the drug was added to sample (d). The nuclei were isolated, sonicated, and assayed for polymerase activity as in Table 1. In one mixture (e), the drug was added just before ^{3}H-UTP.

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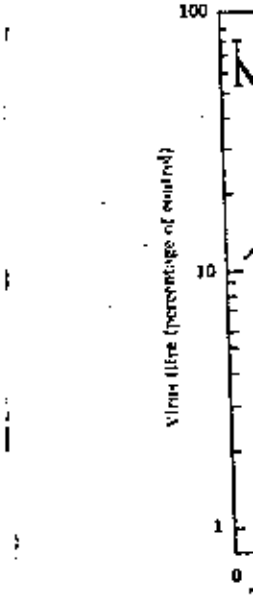

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4 and 6 as well as of 3 and 7 show superimposable fragmentation patterns, with base peaks at *m/e* 135 (*cf.* 13) in each case. Differences in peak intensities are negligible except for a stronger peak at *m/e* 302 in 6 and *m/e* 260 in 7, both corresponding to $M^+ - 28$.

The expected stereochemistry of the photoproducts of 2 and 5, based on mechanistic analogy with the photolysis products of α,β-epoxy ketones,[2b,3] is shown in structures 4, 6, and 8. Work designed for detailed proofs of these structures is currently in progress.[14]

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(14) Satisfactory elemental analyses and spectral data were obtained for all new crystalline compounds. The products 6, 8, 9, and 11, which could not be obtained in crystalline form, were carefully purified by column and preparative thin layer chromatography (2 mm, precoated Merck silica gel plates; one spot for each compound). The resulting samples gave satisfactory empirical formulas (high-resolution mass spectra) and spectral data.

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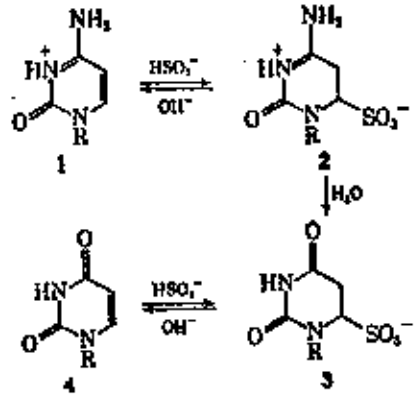
Reactions of Uracil and Cytosine Derivatives with Sodium Bisulfite. A Specific Deamination Method

Sir:

An important feature of the chemistry of uracil and cytosine is the susceptibility of their ring systems to nucleophilic attack at the 5,6 double bond. A number of synthetic transformations of uracil nucleosides have recently been shown to proceed through the intramolecular addition of a sugar substituent to that double bond.[1-4] It is believed that the transient addition of an external nucleophile is involved in deuterium exchange processes of uracil,[5] in the mechanism of cleavage of uracil by hydroxylamine,[6] and in the mode of action of thymidine synthetase.[7] We now wish to report that sodium bisulfite adds reversibly to the 5,6-double bond of uracil and its nucleosides, and that the favorable equilibrium with this reagent permits their complete conversion, under physiological conditions, to the dihydrouracil derivatives 3. This reaction is reversed in basic solution. Cytosine derivatives react similarly and are converted by sodium bisulfite to saturated adducts of structure 2. This conversion is analogous to the initial step of the reaction of the potent mutagen, hydroxylamine, with cytosine derivatives.[8] Unlike



a, R = β-D-2-deoxyribofuranosyl
b, R = β-D-ribofuranosyl
c, R = H

hydroxylamine, however, bisulfite does not react further to displace the amino group of the adduct. Rather, 2 deaminates upon standing in aqueous solution to give 3. Adjustment of the pH of the solution to 10 converts 3 to the uracil derivative 4. This sequence constitutes a method for the specific deamination of cytosine derivatives, under mild conditions, in the presence of the other common nucleic acid components. Adenosine and guanosine do not react with sodium bisulfite. The above reaction sequence is comparable to that involved in the hydrolytic deamination of cytidine by hot, aqueous, acidic buffer.[9-11] In that case, however, the equilibrium to the adduct corresponding to 2 is unfavorable, this adduct is present in very low concentration, and the conditions required by the reaction are too vigorous for it to be useful for preparative or mutagenic purposes.[12]

The ultraviolet absorbtion of uracil was rapidly lost after a sample was dissolved in a 1 *M* solution of $NaHSO_3$ at pH 7 ($t_{1/2}$ = 9.7 min at 24°). No ultraviolet-absorbing spots were observed when the reaction mixture was examined by thin layer chromatography on cellulose. After exposure of the plate to ammonia, however, a single ultraviolet-absorbing spot [R_f 0.33 in 2-propanol–water (70:30)] was detected. (When a portion of the reaction mixture was brought to pH 10 with ammonia, uracil was regenerated.) The product was isolated by crystallization from the reaction mixture and analyzed as a monohydrate of $C_4H_5N_2O_5SNa$. Its infrared spectrum (KBr) contained peaks at 8.20, 8.44, and 9.44 μ, which are attributed to a sulfonate group.[13] The nmr spectrum (D_2O) lacked the uracil low-field H-5 and H-6 absorbances, exhibiting instead multiplets at τ 5.55 (one proton) and 6.93 (two protons). This resembled the spectra of known 6-substituted dihydrouracils[14] and was consistent with structure 3 (R = H) for the product of the reaction. Cytosine derivatives showed a similar reactivity to sodium bisulfite. The unstable adducts 2 have not yet been isolated in pure

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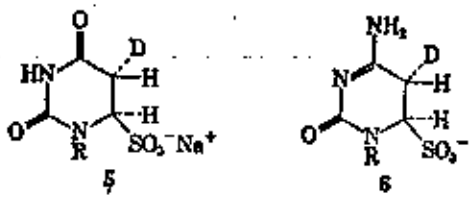
form, and their structural assignment is based upon the information below. Dilution (1:10) of a solution of 2a in 2.26 *M* $NaHSO_3$, pH 5.5, caused it to revert to deoxycytidine. Adjustment of the pH of the original solution to 10 had the same effect. Data on the nmr spectra of the adducts 5a, 5b, 6a, and 6b, prepared in D_2O, are given in Table I. The H-5 and H-6 protons,

Table I

Compd	Chemical shifts, τ, in D_2O		
	H-5	H-6	H-1′
5a	6.96	5.05	4.10 (t), 4.30 (t)
5b	6.98	5.05	4.35 (d), 4.65 (d)
6a	6.78	5.06	4.00 (t), 4.40 (t)
6b	6.76	5.06	4.40 (d), 4.68 (d)

in each case, appeared as moderately sharp singlets ($J_{5,6}$ < 3 cps). The small coupling constants were consistent with diequatorial positions for the 5 and 6 protons.[14] This indicated that the addition of the sulfite group and the deuteron was stereoselective and *trans*.



5 6

a, R = β-D-2′-deoxyribofuranosyl
b, R = β-D-ribofuranosyl

Upon addition of alkali to the reaction mixtures containing 5a, 5b, 6a, and 6b, the spectra of the parent uracil and cytosine nucleosides reappeared. No deuterium incorporation had taken place, demonstrating that the elimination also proceeded with *trans* stereochemistry. The H-1′ protons of the adducts 5a, 5b, 6a, and 6b appeared as pairs of doublets or triplets. This was attributed to the existence of the adducts as mixtures of two diastereomers. Similar observations have been made in the cases of the photohydrates of uridine and uridine 5′-phosphate.[15,16]

The kinetics and equilibrium of the uracil (0.02 *M*)–sodium bisulfite (1 *M*) reaction were studied at 24° by ultraviolet spectroscopy. The equilibrium was completely in favor of 3c at pH <8, then fell off sharply to 50% 3c at pH 9.25 and 0% 3c at pH >10.2. The following expression was derived (neglecting activity coefficients and the slight consumption of bisulfite in the formation of 3c): $K_e = [1]([H^+] + K_s)([H^+] + K_u)/([\text{uracil}][H^+]^2)$. The values of K_s and K_u (the dissociation constants for bisulfite and uracil) were determined by titration to be 3.7×10^{-7} and 5.2×10^{-10}, respectively, at the ionic strength of the reaction mixture. By substituting these values and the experimental equilibrium data into the above equation, a consistent value of 1.3×10^3 was calculated for K_e, the equilibrium constant. The pseudo-first-order rate constant, K, for the forward reaction rose in inverse proportion to $[H^+]$ in the pH range 2–6. It reached a maximum at pH 7–8 and fell off with a further rise in pH. This could be explained by the assumption that the reaction involved a rate-determining attack by sulfite ion upon neutral uracil. The following expression was der $K = K^*K_s[H^+]/\{([H^+] + K_s)([H^+] + K_u)\}$, wher is a pH-independent rate constant. Using a val 1.7×10^{-3} sec^{-1} for K^*, excellent agreement wa tained between the observed rates and those calcu from this equation. A comparison of the reaction of uracil and several of its derivatives was made a 7.2 (24°, 1 *M* $NaHSO_3$). The observed values (s of K were: uracil 1.4×10^{-3}; uridine $9.5 \times$ deoxyuridine 6.8×10^{-4}; uridine 5′-phosphate $\times 10^{-4}$. In the case of thymine, the overall equilib for its reversible reaction with bisulfite was found t less favorable. At 24°, in 1 *M* $NaHSO_3$, a maxi loss of 40% of its ultraviolet absorbtion was obse in the pH range 4–6. The equilibrium constant estimated at 0.8.

The variation of the equilibrium of the reaction. oxycytidine + $NaHSO_3 \rightleftharpoons$ 2a, with pD was studie nmr in 2.26 *M* $NaHSO_3$ in D_2O solution, 37°, using *M* (initial) deoxycytidine. The following values obtained: pD <6, 100% 2a; pD 6.6, 71% 2a; 7.0, 55% 2a; pD 7.4, 33% 2a; pD 7.7, 26% 2a; >8.2, 0% 2a. At a given pD, it was found that lo temperature favored the formation of 2a. Thus, at 7.4, 6°, 67% of 2b was present. The rate of the forw reaction was too rapid for convenient measuremen 37°, pD 4 or higher, under the above conditions. half-life of 8 min was observed at pD 3.0, and one o min was obtained at pD 2.7. This decline of the r with decreasing pD resembled the situation obser with the uracil–bisulfite reaction and suggested t the rate-determining step may involve addition by sul ion.

Upon standing in aqueous solution, the cytosine ducts 2 deaminated to the corresponding uracil addu 3. This was demonstrable by thin layer chromatog phy, nmr spectroscopy, and the isolation of 3c from cytosine–bisulfite reaction mixture.

The deamination reaction (2a → 3a) was studi This was done in unbuffered bisulfite solutions (adjusted with DCl) in D_2O at 37°, over the pD ra 3.0 to 6.6. A maximum at pD 5.8 was observed, k 3.0×10^{-4} sec^{-1}, which fell off to $k = 8.6 \times$ at pD 6.6 and 7.8×10^{-5} sec^{-1} at pD 4.9. At pD no appreciable deamination was observed, unless citr buffer (of the same pD) was added. These results su gest the involvement of the protonated species 2a, and catalysis by buffer anions.

To demonstrate the preparative utility of the reactio 50 mg of deoxycytidine was allowed to react with 2 mg of sodium bisulfite in 1 ml of H_2O at 25°. Af 14 hr of reaction, the excess bisulfite was removed precipitation with $Ba(OH)_2$ and the solution was ne tralized with a cation exchange resin. Crystallizatio of the reaction mixture afforded a 94% yield of deox uridine. In a similar fashion, 1 mg of cytidylyl-(3′–5 adenosine was converted to a single product, identic in R_f and ultraviolet spectrum with uridylyl-(3′–5 adenosine.

Sodium bisulfite should be a reagent of considerab utility in nucleic acid chemistry both for its ability convert uracil and cytosine derivatives to the saturat adducts 2 and 3 and for its catalysis of the deaminatio of cytosine to uracil. There has been considerab interest in methods for converting uracil to saturat

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derivatives for analytical reasons and because of the occurrence of dihydrouracil in tRNA.[17-19] A similar transformation is involved in the well-known photohydration reaction of uracil,[20,21] which has important consequences for the functioning of nucleic acids.[22,23]

In addition to its synthetic use in nucleotide chemistry, we believe that sodium bisulfite should be of value as a reference mutagen. It is the first reagent to be described that can *specifically* convert one common, naturally occurring nucleic acid heterocycle to another. It should finally be noted that sodium bisulfite is a food additive and sulfur dioxide is an atmospheric pollutant. It now seems probable that these substances may constitute a genetic hazard to living organisms.

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Carbon–Carbon and Carbon–Hydrogen Bond Length–Bond Overlap Correlations

Sir:

The well-known environmental changes in single carbon–carbon bond lengths have been attributed to differences in hybridization of carbon atoms.[1] The bonds have been classified into sp^n–sp^m types (n, m being 1, 2, 3) and a rule is found[2] that carbon–carbon single bond distance decreases by 0.04 Å when one of the participating carbon atoms changes the hybridization type from sp^3 to sp^2, or sp^2 to sp. However, there is sufficient evidence in the literature to indicate that useful correlations may be obtained when the hybridization in molecules is not restricted to the special cases sp, sp^2, and sp^3. For example, it has been found that J-(^{13}C–H), assumed to depend mainly on the percentage of s character in the bonds,[3] of the bridgehead carbon atom of norbornane is much larger than that of the usual sp^2–sp^3 bond.[4] Similarly, the enhanced acidity and reduced reactivity of aryl positions adjacent to fused strained rings toward electrophilic substitution indicate that the atomic orbitals of the fused aryl carbon used to construct the strained ring have higher p character than the simple sp^2 hybrids suggest.[5] The theoretical approaches also suggest models which do not restrict the hybridization parameters (exponent n in sp^n) to integers. Thus the hybrids calculated by the maximum overlap approximation,[6] which are of the general sp^n (n = a noninteger) form, lead to correlations of bond energy,[7] chemical shift,[8] or spin–spin coupling (^{13}C–H)[9] with hybridization. Other theoretical approaches, like local orbital prediction of the hybridization based on the CNDO/2 MO wave functions, also lead to noninteger hybrid parameters.[10] On the other hand, an estimate of the s:p ratio from experimental bond lengths, following the idea that hybrid radius is a function of hybridization,[11] was made, and noninteger hybrid parameters were suggested.[12] All this indicates that the restriction of hybridization parameters to integers is neither justified nor useful in approximate descriptions of the bonding in molecules.

Here we considered various single C–C and C–H bonds and established a correlation between the bond lengths and corresponding bond overlaps based on hybrids with noninteger values of the exponent n, m. It is found that hybridization characterized by noninteger exponents obtained by the maximum overlap approximation can account for C–C and C–H bond length variations and be useful in predictions of the environmental changes of single C–C bonds in molecules for which the experimental data are not complete or available. Bond overlaps depend on the amount of electron density in the region between the atoms forming the bond and are expected, therefore, to be a useful bond index, especially for similar bonds, when bonded electrons are approximately equally bound to their own atoms, and there is no high polarity of bonds.[13,14] This is, for example, a justification for Coulson's well-known definition of bond orders,[15] and is implied in various semiempirical methods in which overlap or integrals proportional to overlap are allowed to depend on the bond length.[16,17]

Bond overlap for some 20 hydrocarbons for which experimental bond lengths are known have been evaluated. Hybrid orbitals characterizing the bonds have been obtained by the method of maximum overlap. The method of calculation has been previously described in detail.[6] The computations were not directly based on the variational theorem in which an energy expression is minimized with respect to a set of parameters. Instead, a basis set consisting of local hybrid orbitals was assumed, and parameters specifying the hybridization were varied to maximize the total overlap. Briefly, in the method we search for optimal parameters a_i, b_i, of individual hybrids ψ_i =

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Toxicological Evaluation of some Combinations of Food Preservatives

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Abstract—Short- and long-term studies are reported in which preservatives were administered orally, singly or in pairs, to rats and mice. Daily doses of nisin (2 or 4 mg/kg), sorbic acid (40 or 80 mg/kg), benzoic acid (40 or 80 mg/kg) and sodium bisulphite (80 or 160 mg/kg), and mixtures of benzoic acid/sodium bisulphite (40/80 or 80/160 mg/kg) and sorbic acid/nisin (40/2 or 80/4 mg/kg) were given to mice or rats for 3, 17 or 18 months, and in a further test nisin (0·4–400 mg/kg/day), sorbic acid (10 mg/kg/day) and a polymeric impurity derived from the sorbic acid were tested in mice for 2 months. In addition to the effects of these substances on growth and survival, haematology and blood chemistry (in rats), kidney function (in rats) and reproduction (in mice), the effects of certain stress factors, including dietary restriction, low temperature and carbon tetrachloride administration, on the potential toxicity of the preservatives were studied in both species.

In general, these doses showed some adverse effects in both rats and mice, particularly on growth, survival and susceptibility to stress, but sorbic acid appeared to be the least toxic. The effects of the benzoic acid/sodium bisulphite mixture were generally greater than those of the compounds given singly, while the reverse was the case with the sorbic acid/nisin mixture. The antibiotic, nisin, increased the growth rate when given alone. The authors' recommendation that the use of benzoate and sulphite as preservatives should be further restricted and that sorbic acid should be used instead where possible is being followed in the USSR.

INTRODUCTION

Despite the development of newer techniques, chemical preservatives still retain their economic importance for extending the shelf-life of foods and conserving their nutritional value. Until recently sulphites and benzoates found fairly wide use in the USSR as food preservatives, but now their use is restricted as much as possible. Limited amounts of sulphurous acid are permitted in certain foods, including some wines, jams, fruit candies, starch, tomato puree, dried fruits, fruit juices and some fresh and semi-processed fruits. Benzoic acid is also used, for preserving gelatin, fruit candy and paste, pastilles, margarine, egg mayonnaise, salmon caviare, spiced sprats and some berries and fruit products, and sodium benzoate is permitted in fruit juices, fish preserves and margarine.

Recently sorbic acid and the antibiotic, nisin, have been proposed for extensive use as preservatives, but their safety-in-use had to be established before they could be added to the existing list of permitted food additives. It was decided, therefore, that the technological and economic questions concerning these materials should be studied at the All Union Institute of the Food Canning Industry, while we undertook the toxicological studies.

Toxicological data are available for the administration of each of these compounds individually to experimental animals (Joint FAO/WHO Expert Committee on Food Additives, 1962), but not for their administration in combination. According to the literature, sodium bisulphite fed at dietary levels of 0·05% for up to 2 yr had no adverse effects but levels of 0·1% or higher reduced weight gain and induced histopathological changes in some organs (Fitzhugh, Knudsen & Nelson, 1946). The authors attributed these effects to avitaminosis B_1 following destruction of this vitamin in the diet by the sodium bisulphite. Intubated doses of up to 2·5 g sodium bisulphite given daily to dogs for up to 419 days had no toxic effects. Sodium benzoate had no adverse effects in rats given dietary levels of up to 4% for 90 days, but levels of 8% increased mortality, reduced weight gain and caused hypertrophy of the liver and kidneys (Deuel, Alfin-Slater, Weil & Smyth, 1954). No toxic effects occurred in four generations of rats fed 0·5 or 1·0% in the diet (Kloekebusch & Lang, 1960). High levels of protein in the rat diet have a protective action against this preservative (Lang, 1961). Sorbic acid had no toxic effects when fed in the diet of rats at levels of up to 8% for 90 or 1000 days (Deuel *et al.* 1954; Lang, 1960). No toxic effects were detected in rats fed 0·1–0·3% nisin in the diet, with cheese, for periods of 12 wk–1 yr, survival, weight gain, reproduction indices, haematology, gastro-intestinal and kidney function and organ histology being within the limits of the control animals (Hawley, 1958; Frazer, 1960).

Since mixtures of food additives are ingested in a normal diet, it was considered appropriate to carry out short- and long-term oral toxicity tests in rats and mice on combinations of benzoic acid and sodium bisulphite and of sorbic acid and nisin, as well as on the individual compounds.

EXPERIMENTAL

Materials. Benzoic acid and sodium bisulphite were analytical-grade reagents obtained commercially. Sorbic acid, obtained commercially, contained an unknown polymeric impurity (0·02–0·07% ashed residue) which was insoluble in water, sparingly soluble in acids or alkali and moderately soluble in chloroform. Nisin with an activity of 1×10^6 IU/g was purchased from Aplin & Barrett Ltd., Yeovil, Somerset, UK. It was of largely unknown composition but contained a stabilizer.

Animals. Both sexes of cross-bred white mice and Wistar rats were used, the initial body weights varying according to the nature of the experiment. Mice were housed in groups of 10–25 and rats in groups of five.

Experimental design and conduct

2-Month study in mice. Groups of 25 male and 25 female mice (initial body weight 8–10 g) received for 2 months by oral intubation daily doses of 0·4, 4·0 or 400 mg nisin/kg/day (approximately equivalent to 0·002, 0·02 and 2·0% in human food). Groups of 25 male and 25 female mice (initial body weight 15–20 g) received the same doses of nisin for a similar period. Sorbic acid (40 mg/kg/day) and the polymeric impurity obtained from it (0·8 or 800 mg/kg/day) were given to similar groups of male and female mice from each body-weight range. The mice were observed for general condition and behaviour, and survival, food consumption and weight gain were recorded daily. Tests were carried out to determine the effects of hunger, physical stress and poisoning with carbon tetrachloride on the test animals, compared with untreated controls.

3-Month study in mice. Groups of 50 male and 50 female mice (initial body weight 8–10 g)

received, by oral intubation, sorbic acid or benzoic acid (in doses of 80 mg/kg/day), sodium bisulphite (160 mg/kg/day) or nisin (4 mg/kg/day). Similar groups received mixtures of the same levels of benzoic acid and sodium bisulphite and of sorbic acid and nisin. The observations and tests performed on these mice were the same as those described for the 2-month study, except that a co-carcinogenicity test with Ehrlich ascites carcinoma was added.

17-Month study in mice. Groups of 25 male and 25 female mice (initial body weight 10–15 g) and groups of 25 male and 25 female mice (initial body weight 16–20 g) were given sorbic acid or benzoic acid in doses of 40 mg/kg/day, sodium bisulphite (80 mg/kg/day) or nisin (2 mg/kg/day). Similar groups received mixtures of the same levels of benzoic acid and sodium bisulphite and of sorbic acid and nisin. The test compounds were fed in a paste prior to the main feed, feed and water were provided *ad lib.* and a control group received only the basic diet. The parameters studied on the animals in this test were those listed for the 2-month study, except that poisoning by carbon tetrachloride was not studied and in addition organ weights were recorded. After 8 months on the preservative-combination or control diets some animals were mated and reproduction was studied over five generations. The weight gain of the litters after weaning (at 8–10 g body weight) was recorded for 3-5 months.

18-Month study in rats. Groups of ten male and ten female rats (initial body weight 100–120 g) received sorbic acid or benzoic acid (40 mg/kg/day), sodium bisulphite (80 mg/kg/day) or nisin (2 mg/kg/day). Groups of 50 male and 50 female rats (initial body weight 100–120 g) received sorbic acid (40 mg/kg/day) in combination with nisin (2 mg/kg/day), or benzoic acid (40 mg/kg/day) in combination with sodium bisulphite (80 mg/kg/day). Test compounds were fed in a paste prior to the main feed, while control groups received the basic diet alone. Food and water were provided *ad lib.* Food and water consumption, weight gain and the effects of stress factors were recorded. blood morphology was studied and the following were estimated: titre of serum complement, by our micro-modification of the classical method; phagocytic activity of leucocytes, by the classical method of Kost and Stenco as described by Predtechenskii (1959), serum-ceruloplasmin level by Ravin's method (Petrin'kina, 1959); blood alkalinity (Panisyak, 1952); blood ketones by Netelson's method (Todorov, 1960); blood morphology; erythrocyte sedimentation rate, by a modification of Efmann's method (Mukhorina, 1962); C-reactive protein in serum (Pashinin, 1962).

Effect of stress factors on preservative toxicity in mice and rats

Food restriction. At the termination of the feeding studies, surviving mice were subjected to a restricted dietary intake for up to 5 days, the degree of restriction being 50% after the 2-month, 90% after the 3-month and 100% after the 17-month test. Rats (ten/group) were starved for up to 13 days. Both rats and mice received water *ad lib.* and administration of the preservatives was continued at the same dose levels by oral intubation. Weight loss, mortality rate and, in the case of the rats, the rate of weight-recovery after the return of survivors to a normal diet, were recorded.

Swimming test. All mice surviving at the end of the 2- and 17-month studies were fitted with a 2-g weight on the tail and left to swim in a water bath at 15–18°C. The time until they sank exhausted was recorded.

Low temperature. Groups of 15 rats from those fed combinations of preservatives were exposed to cold (−2–+8°C) for 12–24 hr at the end of the 18-month study.

Centrifugation. To study the functional efficiency of the rat's circulatory-respiratory system

after the prolonged consumption of mixtures of preservatives, groups of 15 rats were subjected to a rotation rate of 90–300 revs/min for 15–30 min.

Carbon tetrachloride detoxification test. This test was carried out to determine the effect of feeding preservatives on the detoxifying action of the liver. Groups of 7–32 mice that had received single preservatives for 2 or 3 months were given carbon tetrachloride by oral intubation in a single dose of 0·1 ml/mouse in sunflower oil (1:1 solution). After receiving one of the combinations of preservatives for 18 months groups of 9–15 rats were treated with orally-intubated carbon tetrachloride either in a single dose of 0·6 ml/rat or in daily doses of 0·2–0·3 ml/rat for 15 days. The mortality rate in these animals was compared with those from the feeding-test control groups given similar doses of solvent.

Kidney function test. Groups of 15 rats that had been given combinations of preservatives were given a kidney function test at the end of the 18-month period of feeding. A saturated solution of K_2HPO_4 was administered in doses of 4 ml/rat twice daily for 6 wk, and haematological studies and urinary protein determinations were carried out.

RESULTS

2-Month study in mice

Weight gain after 15, 30 and 60 days on test showed little divergence from the controls in any group, except in males receiving 0·4, 4·0 or 400 mg nisin/kg/day, in which the weight gain was significantly increased in the group with the lower initial body weight, and in males and females of the group with the higher initial body weight given the polymeric impurity from sorbic acid in a dose of 800 mg/kg. Survival and food consumption in the test groups did not differ significantly from those of the controls. The effect of food restriction on body weight and survival is shown in Table 1. The weight loss was highest in groups receiving the higher doses of nisin and the lower level of polymeric impurity, but survival time was highest in the two groups given the impurity. The same tendency was found in the effects of physical stress and carbon tetrachloride poisoning (Table 2), with mice given nisin being more susceptible than the animals of the other groups, and those given sorbic acid or the lower level of sorbic acid impurity showing a lower degree of mortality than the controls.

3-Month study in mice

Highest mortality rates at 2·5 months were recorded in mice given nisin or the benzoic acid/sodium bisulphite combination (Table 3). Animals given benzoic acid or sodium bisulphite separately showed about twice as high a survival rate as was seen with the combination of the two.

With the exception of males of the nisin group, test animals did not grow as well as the controls at 3 months, but this finding was not associated with a reduced feed intake (Table 4). The weight gain of mice fed the benzoic acid/sodium bisulphite combination was higher than that of mice fed either of the components, whereas in the case of nisin and sorbic acid the reverse was the case.

Under conditions of restricted feed intake mice on benzoic acid, the benzoic acid/sodium bisulphite combination or nisin showed the highest mortality rate (Table 1). The effect of carbon tetrachloride on the mortality of mice fed the preservatives for 3 months can be seen from Table 2. Mice given benzoic acid or sodium bisulphite showed a higher mortality rate than controls but those given sorbic acid were not susceptible to the dose of carbon tetrachloride given. The results of the co-carcinogenicity test are reported below.

Table 1. *Effect of dietary restriction on body weight and survival of preservative-treated mice*

Test compound(s)	Daily intake of preservative (mg/kg)	Group size*	Mortality (%) after 5 days on restricted diet	Weight loss† (%)	Days required to regain lost weight	Survival of fasted animals (days)
50% Food restriction after 2 months on preservative treatment						
None (control)	—	28	43	20		3·37
Nisin	0·4	58	34·5	19·5		3·5
	4·0	54	48·4	24·4		2·7
	400·0	46	70	25·0		3·7
Sorbic	40·0	42	19	14·6		3·5
Sorbic acid impurity	0·8	32	37·5	27·8		5·2
	800·0	28	21·4	20·0		4·25
90% Food restriction after 3 months on preservative treatment						
None (control)	—	16	56·3	41·2		4·6
Benzoic acid	80	14	85·7	31·7		4·3
Sodium bisulphite	160	14	57·2	36·9		4·5
Sorbic acid	80	14	55·7	41·5		4·5
Nisin	4	13	84·6	34·7		4·1
Benzoic acid/sodium bisulphite	80/160	12	83·3	37·9		4·2
Sorbic acid/nisin	80/4	14	64·3	43·8		5·4
100% Food restriction after 17 months on preservative treatment						
None (control)	—	32	12·5	17·8	1·6	
Benzoic acid	40	12	50·0	26·0	2·7	
Sodium bisulphite	80	17	0·0	17·8	2·3	
Sorbic acid	40	10	30·0	26·4	2·1	
Benzoic acid/sodium bisulphite	40/80	33	51·5	22·4	2·6	
Sorbic acid/nisin	40/2	40	7·5	16·7	2·0	

* Values for males and females are pooled together.
Groups of mice were used with body weights in the range 23·5–33·3 g before feed restriction.

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Table 2. *Effect of 0·1 ml carbon tetrachloride on the mortality of preservative-treated mice*

Test compound	Daily intake (mg/kg)	Mortality (%)
None (control)*	—	28·6
Benzoic acid*	80	62·5
Sodium bisulphite*	160	45·0
Sorbic acid*	80	0·0
Control†	—	22·3
Nisin†	0·4	46·7
	4·0	56·4
	400·0	70·0
Sorbic acid†	40·0	14·8
Sorbic acid impurity†	0·8	0·0
	800·0	26·6

* Groups of ten mice fed the control or preservative diet for 3 months.
† Groups of 7–32 mice fed the control or preservative diet for 2 months.
The effects of carbon tetrachloride were not tested on mice fed the combinations of preservatives.

Table 3. *Survival rate at 2·5 and 8 months in mice given preservatives orally, alone or in combination*

Test compound(s)	Daily intake (mg/kg)	Survival (%) in Males at month 2·5*	Males at month 8†	Males at month 8‡	Females at month 2·5*	Females at month 8†	Females at month 8†
None (control)	—	60	72·5	78·5	62	—	—
Benzoic acid	80	68			68		
Sodium bisulphite	160	70			68		
Sorbic acid	80	98			52		
Nisin	4	12			32		
Benzoic acid/sodium bisulphite	80/160	30			38		
	40/80		44·4	35·4		28·5	55·3
Sorbic acid/nisin	80/4	48			74		
	40/2		22·4	9·4		37·9	73·0

* Groups initially comprised 50 male or 50 female mice, weighing 8–10 g in either case.
† Groups initially comprised 9–29 mice of both sexes, weighing 10–15 g. Controls were males only.
‡ Groups initially comprised 26–56 mice of both sexes, weighing 16–25 g. Controls were males only.

17-Month study in mice

Growth and survival. The survival rate of these mice at 8 months can be seen in Table 3. Animals in the control groups had a better survival rate than those receiving the preservative

combinations. The poorest survival was in males receiving sorbic acid in combination with nisin, but this group registered the greatest weight gain as shown in the reproduction study data (Table 5). This was especially marked in the group with initial body weights between 10 and 15 g. The weights of liver, kidney and testes relative to body weight in mice sacrificed at the end of the test period were lower in the group given sorbic acid alone than in all the other groups.

Reproduction study. In the four generations of offspring, the weight gain of the litters was studied for 3·5 months after weaning (at 8–10 g body weight) and the effects of the preservative combinations on the weight of the animals at 3·5 months are shown in Table 5. Those fed sorbic acid in combination with nisin grew better on the whole than mice given benzoic acid in combination with sodium bisulphite.

Effects of stress. In the swimming test a better time was recorded for the groups of mice given sorbic acid alone or in combination with nisin than for mice given benzoic acid alone or in combination with sodium bisulphite. The effect of feed restriction on body weight and survival can be seen in Table 1. Percentage mortality after 5 days of feed restriction was highest in groups receiving benzoic acid alone or in combination with sodium bisulphite. The time required to regain lost weight was also slightly greater in these groups.

Tumour incidence. After the feeding of preservative diets for 17 months, 8/100 mice in the benzoic acid/sodium bisulphite group of the first generation and 1/8 in the third generation of the same group were found to have malignant tumours. No tumours were found in the control or sorbic acid/nisin groups. In connexion with these findings we decided to study the possible co-carcinogenicity of the preservatives using the tumour transplantation test. Erlich ascites mouse carcinoma was transplanted intraperitoneally into mice which had been fed for 3-months on the test diets. The tumour growth was greatest in mice that had received sodium bisulphite, and was less marked in mice given bisulphite in combination with benzoic acid or benzoic acid alone. The tumour growth in mice given sorbic acid alone or in combination with nisin was comparable with that in the control group.

Long-term effects in rats

Rats fed the sorbic acid/nisin mixture grew better than those fed benzoic acid/sodium bisulphite over an 18-month period (Table 6). The titre of serum complement and the phagocytic activity of the leucocytes were also higher in the former than in the latter group, but the level of serum ceruloplasmin was higher in the latter than in the former. Cold stress, centrifugation and kidney loading with K_2HPO_4 had most effect in rats given the benzoic acid/sodium bisulphite combination.

Blood alkalinity, C-reactive protein levels and blood morphology were comparable in all groups including the controls. But the erythrocyte sedimentation rate was increased in rats fed the benzoic acid/sodium bisulphite combination, in which group the level of blood ketones was 1·97 mg/100 ml, compared with 2·5 mg/100 ml in the control group and 3·4 mg/100 ml in the sorbic acid/nisin group.

The results in Table 7 indicate that rats fed benzoic acid for 18 months developed some tolerance to a lethal dose of the compound given terminally. Rats on a sorbic acid/nisin diet tolerated carbon tetrachloride better than did those on the sodium bisulphite/benzoic acid diet. In the latter group, 20% of the animals showed signs of haemorrhagic keratoconjunctivitis, which developed 2 months after the beginning of the study. The effects of starvation (with water available *ad lib.*) were inconclusive, rats in all the test groups surviving longer than the controls.

Table 4. *Mean values of weight gain and daily intake of feed and water in mice given preservatives orally, singly or in combination for 3 months*

Test compound(s)	Daily intake (mg/kg)	Weight gain (% of initial body weight)		Daily feed intake (g)		Daily water intake (ml)	
		Males	Females	Males	Females	Males	Females
None (control)	—	199	167	2·33	2·81	3·67	4·14
Benzoic acid	80	125	118	2·57	2·65	3·85	3·52
Sodium bisulphite	160	143	124	2·43	1·98	3·33	3·13
Sorbic acid	80	145	144	2·17	2·13	3·86	3·31
Nisin	4	227	143	3·35	4·31	5·92	4·34
Benzoic acid/sodium bisulphite	80/160	174	141	3·24	2·32	3·54	2·63
Sorbic acid/nisin	80/4	129	115	2·34	2·48	3·32	4·42

Groups of 50 males and 50 females (initial body weight 8–10 g) were used.
Feed and water consumption were recorded daily throughout the test period.

Table 5. *Weight gain at 3·5 months in four generations of mice given combinations of preservatives from weaning to mating*

Test compounds	Dosage level (mg/kg)	Weight gain (% weight at weaning) at 3·5 months for generation							
		F_1		F_2		F_3		F_4	
		Males	Females	Males	Females	Males	Females	Males	Females
None (control)	—	218	200	147	118	228	212	106	80·5
Benzoic acid/sodium bisulphite	40/80	150	145	103	99	180	217	171	212
Sorbic acid/nisin	40/2	257	202	138	118	219	199	242	187

Table 6. *Mean values of weight gain and daily intake of feed and water in rats fed various preservatives singly or in combination for 18 months*

Test compound(s)	Daily intake mg/kg)	Weight gain at 18 months (% of initial body weight)		Daily feed intake (g)		Daily water intake (ml)	
		Males	Females	Males	Females	Males	Females
None (control)	—	203·49	168·8	17·2	9·6	37·8	26·4
Benzoic acid	40			14·8	13·1	28·6	26·3
Sodium bisulphite	80			14·5	9·8	32·0	25·7
Sorbic acid	40			13·6	9·7	38·7	27·3
Nisin	2			16·8	9·2	39·5	32·6
Benzoic acid/sodium bisulphite	40/80	164·8	151·8				
Sorbic acid/nisin	40/2	253·7	203·0				

Weight gain was estimated in groups of 50 rats (initial body weight 100–120 g) and feed and water intakes in groups of ten.

Table 7. *Effect of carbon tetrachloride and sodium bisulphite and benzoate on mortality in rats fed preservatives singly or in combination for 18 months*

Test compound(s) fed for 18 months	Secondary compound and dose		Mortality (%)
None (control)	Sodium benzoate (mg/kg)	3600	100
Sorbic acid/nisin		3400	71·5
Sodium bisulphite/benzoic acid		3400	100
Benzoic acid		4000	25
None (control)	Sodium bisulphite (mg/kg)	860	100
		430	100
Sorbic acid/nisin		860	66·6
		430	33·3
Sodium bisulphite/benzoic acid		860	100
		430	60
Sorbic acid/nisin	Carbon tetrachloride (ml)	0·6	33·3
		0·2	11·0
Sodium bisulphite/benzoic acid		0·6	60·0
		0·2	27·0

Groups of 3–15 rats were used.

DISCUSSION

The data presented suggest that the preservatives tested had some slight adverse effects on rats or mice in a dosage range equivalent to the maximum levels at which these preservatives may occur in the human diet. The toxic effects of benzoic acid and sodium bisulphite when administered in combination appeared to be synergistic or additive to some extent, while there were indications that the reverse was the case with the sorbic acid/nisin combination. This is an important consideration in the control of the use of preservatives for extending the shelf-life of food for human consumption. In our experiments the sorbic acid showed the lowest toxicity and changes that were recorded in some tests were not considered to be pathological. Moreover, this unsaturated fatty acid has some beneficial effect on the animal organism, being not only a good preservative for food but a nutritional additive with some features of the vitamins. Therefore it was considered reasonable to recommend to the Food Hygiene Department of the Ministry of Health of the USSR a maximum restriction on the permitted list of foods preserved with sodium bisulphite (and other compounds of sulphurous acid) and with benzoates. This recommendation has been accepted.

As the data obtained for nisin were not convincing, it was decided to carry out a further study with nisin manufactured in the Soviet Union. This was recently completed (Shillinger, Bogoroditskaya & Osipova, 1969) and the home-produced nisin was found to be less toxic than the imported. However nisin is an antibiotic, a fact confirmed by our own studies (particularly the finding of weight stimulation in the test animals) and its use as a food preservative should therefore be limited. The high content of nisin occurring naturally in some dairy products is not a sound reason for unrestricted use of this compound as a food preservative, but there is some case for using nisin in combination with sorbic acid, as we have recommended for some products. In the present situation it is considered reasonable to restrict the use of benzoates and sulphites at the expense of extending the use of sorbates, a trend which is at present being followed in the USSR.

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Evaluation toxicologique de certaines combinaisons de produits de conservation des aliments

Résumé—L'article décrit des études à court et à long terme, au cours desquelles des produits de conservation ont été administrés par voie orale, isolément ou par paires, à des rats et à des souris. Des rats ou des souris ont reçu pendant 3, 17 ou 18 mois des doses quotidiennes de nisine (2 ou 4 mg/kg), d'acide sorbique (40 ou 80 mg/kg), d'acide benzoïque (40 ou 80 mg/kg) ou de bisulfite de sodium (80 ou 160 mg/kg) ou de mélanges d'acide benzoïque et de bisulfite de sodium (40/80 ou 80/160 mg/kg) et de mélanges d'acide sorbique et de nisine (40/2 ou 80/4 mg/kg). La nisine (0,4–400 mg/kg/jour), l'acide sorbique (40 mg/kg/jour) et une impureté polymère dérivée de l'acide sorbique ont été testés pendant 2 mois sur des souris au cours d'un autre essai. En plus des effets de ces substances sur la croissance et sur la survie, sur l'hématologie et la chimie du sang (chez les rats), sur la fonction rénale (chez les rats) et sur la reproduction (chez les souris), on a étudié, chez les deux espèces animales, les effets de certains facteurs de stress: restriction alimentaire, basse température et administration de tétrachlorure de carbone, sur la toxicité potentielle des produits.

D'une manière générale, les doses précitées ont eu certains effets nocifs, autant chez les rats que chez les souris, plus particulièrement sur la croissance, la survie et la sensibilité au stress. L'acide sorbique s'est révélé le moins toxique. Les effets du mélange d'acide benzoïque et de bisulfite de sodium étaient généralement plus prononcés que ceux de ces produits administrés séparément, mais l'inverse a été observé pour le mélange d'acide sorbique et de nisine. Administré seul, l'antibiotique qu'est la nisine accélérait la croissance chez les rats. On recommande de restreindre davantage l'emploi du benzoate et du sulfite comme produits de conservation des aliments et de les remplacer autant que possible par l'acide sorbique. Cette recommendation est déjà suivie en URSS.

Toxikologische Prüfung einiger Kombinationen von Konservierungsmitteln für Nahrungsmittel

Zusammenfassung.—Es wird über kurz- und langzeitige Untersuchungen berichtet, bei denen Konservierungsmittel einzeln oder paarweise Ratten und Mäusen oral verabreicht wurden. Tägliche Dosen von Nisin (2 oder 4 mg/kg), Sorbinsäure (40 oder 80 mg/kg), Benzoesäure (40 oder 80 mg/kg) und Natriumbisulfit (80 oder 160 mg/kg) und Mischungen von Benzoesäure/Natriumbisulfit (40/80 oder 80/160 mg/kg) und Sorbinsäure/Nisin (40/2 oder 80/4 mg/kg) wurden 3, 17 oder 18 Monate Mäusen oder Ratten verabreicht, und bei einem weiteren Versuch wurden 2 Monate lang Nisin (0,4—400 mg/kg/Tag), Sorbinsäure (40 mg/kg/Tag) und eine aus der Sorbinsäure isolierte polymere Verunreinigung an Mäuse verabreicht. Ausser den Wirkungen dieser Substanzen auf das Wachstum und das Überleben, die Hämatologie und Blutchemie (bei Ratten), die Nierenfunktion (bei Ratten) und die Fortpflanzung (bei Mäusen) wurden die Wirkungen bestimmter Stressfaktoren einschliesslich der Futtereinschränkung, der Einwirkung niedriger Temperaturen und der Anwendung von Tetrakohlenstoff auf die mögliche Toxizität der Konservierungsmittel bei beiden Species untersucht.

Im allgemeinen zeigten diese Dosen einige nachteilige Wirkungen bei Ratten und Mäusen, besonders auf das Wachstum, das Überleben und die Stressempfindlichkeit, jedoch schien die Sorbinsäure am wenigsten toxisch zu sein. Die Wirkungen der Benzoesäure/Natriumbisulfit-Mischung war im allgemeinen grösser als die der einzeln verabreichten Verbindungen, während es im Fall der Sorbinsäure/Nisin-Mischung gerade umgekehrt war. Das Antibioticum Nisin erhöhte die Wachstumsgeschwindigkeit bei Einzelverabreichung. Der Autor empfiehlt, die Verwendung von Benzoat und Sulfit als Konservierungsmittel weiter einzuschränken. Stattdessen sollte wie in der UdSSR Sorbinsäure stets verwendet werden, wenn es möglich ist.

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to the electron beam. The appearance of the solid when immediately exposed to the beam was the same as described above and did not change with further exposure.

Based on the above evidence, we can conclude that the individual particle of amorphous calcium phosphate appears as a spheroid with an electron translucent center and with an average outer diameter of 200Å. While the particle diameters are rather uniform under the conditions used in this paper, changes in particle size with variations in chemical preparatory conditions are presently under investigation. Preliminary specific surface measurements of the amorphous phase as obtained by both low angle X-ray scattering (9) and low temperature nitrogen adsorption (10) indicate that the shell of the spheroid may be porous, at least to sorption gases, although these pores are not visible in the electron microscope.

The formation of the amorphous calcium phosphate is a kinetic phenomenon since this compound is unstable in solution and will spontaneously convert into crystalline hydroxyapatite (5). The observation that critical supersaturation must be achieved before instantaneous separation of amorphous calcium phosphate from solution occurs suggests that the kinetics of formation is controlled by homogeneous nucleation. The theory of homogeneous nucleation (11) predicts that nuclei would assume spherical shapes to minimize the positive free energy of interface formation. The particles of crystalline calcium phosphates do not assume spherical forms but are facet-shaped polyhedra, generally needles or plates. Therefore, it is possible that the noncrystalline mode of development occurs because it allows for the formation of spherical surfaces, and consequently can proceed along a path of minimum surface energy.

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Inactivation of Histidine Deaminase by Carbonyl Reagents[1]

In this communication we report that histidine deaminase (histidine ammonia lyase, E.C. 4.3.1.3), an enzyme which catalyzes the conversion of histidine to ammonia and urocanic acid, is inactivated by carbonyl reagents. This suggests that an enzyme-bound carbonyl group is involved in the catalytic process. The reagents which inactivate are $NaBH_4$, phenylhydrazine, $NaHSO_3$, NH_2OH, and KCN. The inactivation by KCN has previously been reported (1). When the inactivation is carried out with $NaBH_4(^3H)$, tritium is incorporated into the protein (approximately 1 atom/100 mg of protein assuming no isotope effect) and is not removed by extensive dialysis, which suggests that the reduction results in the formation of a carbon-hydrogen bond. The reaction of the enzyme with the carbonyl reagents was carried out under the following conditions: 5-7 × 10^5 units (1) of enzyme, 0.15 M carbonyl reagents, and 0.05

TABLE I
Inhibition of Histidine Deaminase by KCN

Additions to reaction mixture*		Relative reaction rate
Histidine	0.010 M	100
Histidine	0.010 M	90
Histidine KCN	0.040 M 0.060 M	52
Histidine KCN	0.010 M 0.020 M	72
Histidine KCN	0.010 M 0.060 M	21

* Reaction conditions are those used in the standard assay.

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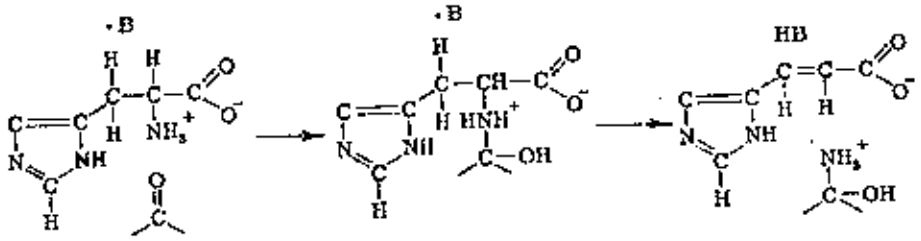
M Tris-acetate buffer, pH 8.6, in a total volume of 0.10 ml were allowed to react for 10 minutes at 25° except for the reaction with $NaBH_4$, which contained 0.25 M $NaBH_4$ and 0.15 M potassium phosphate buffer, pH 7.5. In all cases, the reaction mixture was then diluted 2500-fold and assayed for enzyme activity within 15 minutes. NH_4OH, $NaBH_4$, phenylhydrazine, and $NaHSO_3$ produced total inhibition. With KCN, no inhibition is observed after dilution, but when KCN is added directly to the assay mixture, inhibition is obtained. The inhibition is reversed by substrate and increases with increasing concentration of inhibitor. Typical results obtained with KCN are shown in Table I.

These results suggest that histidine deaminase contains an aldehydic or ketonic carbonyl group which is required for catalytic activity. This carbonyl group can participate in the catalytic process in the following way:



According to this mechanism the amino group of the substrate adds to the carbonyl group of the enzyme to form a protonated carbinolamine or possibly a protonated Schiff base. The interaction between the carbonyl group and the amino group, regardless of its exact nature, results in the formation of a better leaving group and facilitates the elimination. It has been shown that the p*K* of carbinolamines is two to three units lower than that of the parent amine (2). The deamination of histidine, according to the proposed mechanism, leads to the formation of urocanic acid and an adduct of ammonia and the carbonyl group associated with the enzyme. The formation of a covalent compound between ammonia and the enzyme provides an explanation for the observation that, under certain conditions, the dissociation of ammonia from the enzyme is rate-limiting (3).

The role assigned to the carbonyl group in the mechanism proposed here represents a new function for carbonyl groups in enzymic reactions, since carbonyl groups are generally thought to act as electron sinks. Pyridoxal phosphate generally provides carbonyl groups, which constitute part of the catalytic center of enzymes. However, available evidence suggests that histidine deaminase does not contain pyridoxal phosphate. The spectrum of the enzyme (at a concentration of 2 mg protein/ml) did not show any absorptions characteristic of pyridoxal, and $NaBH_4$ reduction produced no significant spectral changes above 190 mμ. The enzyme used in these experiments was at least 50% pure as judged by acrylamide gel electrophoresis. No inactivation was obtained by irradiation with ultraviolet light (4) or by dialysis against cysteine (5). At no point could the enzyme activity be enhanced or restored by addition of pyridoxal phosphate. It is tentatively concluded that the carbonyl group is furnished by a tightly bound cofactor other than pyridoxal or by part of the enzyme protein.

The enzyme was prepared from *Pseudomonas* sp. (ATCC 11299b) by a modification (6) of a previously published procedure (3). The specific activity of the purified enzyme was 115,000 units/mg. Assays were carried out under the following conditions at 25°: dithiothreitol, 2×10^{-3} M; $MnSO_4$, 10^{-4} M; histidine, 4×10^{-2} M; and Tris-acetate buffer, pH 8.6, 2×10^{-2} M. The appearance of urocanic acid is measured by the change in optical density at 277 mμ (1). Under these assay conditions the rate is linear for at least 5 minutes. Enzyme units are those defined previously (1).

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22 (1950)

Table II. Specific Gravity of Foreign and Domestic Oil Shales

Sample No.	Source	Modified Fischer Oil Yield, Gal./Ton	Specific Gravity, 60°/60° F.
51	South Africa	23.3	1.645
52		45.0	1.691
53		45.0	1.627
54		48.8	1.614
55		99.5	1.376
56	Australia	26.2	1.679
57		52.9	1.633
58		119.2	1.301
59		179.9	1.244
60		147.2	1.256
61	Brazil	31.3	1.962
62		33.6	2.016
63	Manchuria	25.1	1.909
64		26.3	1.924
65		35.6	1.840
66		48.1	1.840
67		55.0	1.857
68		66.8	1.820
69	Indiana	4.8	2.316
70		6.6	2.289
71		9.7	2.216
72		12.4	2.146
73		14.3	2.2.2
74	Kansas	10.9	2.190
75		8.1	2.207
76	Kentucky	5.6	2.267
77		11.5	2.232
78		16.4	2.114
79	Nevada	10.2	2.356
80		22.8	2.072
81	Ohio	7.1	2.490
82		9.8	2.352
83		14.7	2.222
84	Tennessee	62.8	1.670
85	Texas	8.7	2.379
86		7.8	2.332
87	Wyoming	7.4	2.354
88		39.8	1.969

Table II gives the oil yields and specific gravities of 38 oil shales from four foreign countries and eight states other than Colorado. These data show that the numerical value of the oil yield-specific gravity relationship for the specified Colorado oil shales is not applicable to all oil shales; however, they indicate that such relationships may be obtained for oil shales from other specific areas. Furthermore, this rapid method of estimating the oil yield of shale may be useful for routine control purposes where time and equipment will not allow the determination of oil yields by the modified Fischer assay.

SUMMARY

The relationship between the oil yield by the modified Fischer assay and the specific gravity of certain Colorado oil shales provides a practical means of estimating the oil yields of other oil shales from the same area. The oil yields estimated from the specific gravities of samples from the Green River formation in the Bureau of Mines oil-shale mine, Rifle, Colo., differed from the actual oil yields of the shales by a maximum of 3.0 gallons of oil per ton and by an average of 1.2 gallons of oil per ton of shale. The numerical value of the oil yield-specific gravity relationship for these Colorado oil shales was not applicable to all oil shales. However, it indicated that similar relationships may be obtained for oil shales from other specific areas, and this method of estimating the oil yield of oil shale may be adapted to rapid routine testing of oil shales where time and equipment will not allow assays to be made by the more reliable modified Fischer-retort assay method.

This report represents the results of work done under a cooperative agreement between the Bureau of Mines, United States Department of the Interior, and the University of Wyoming.

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Acid-Bleached Fuchsin Solution as Analytical Reagent

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DILUTED solutions of basic fuchsin, which are decolorized with sulfuric acid and to which formaldehyde is added after bleaching, have been recommended as a quantitative reagent for sulfur dioxide and thiols. The reagent is much less sensitive for thiosulfate. Other substances than thiols interfere with the sulfur dioxide reaction. Their reactions are less sensitive and slower for comparable concentrations than those of the thiols, but they are also more difficult to mask. The masking of thiols or thiosulfate can be achieved with mercuric chloride or with the chlorides of platinum and palladium(II). The mercury-precipitated thiols and thiosulfates must be removed by filtration or centrifugation before the sulfur dioxide test is carried out; otherwise the sulfur dioxide reagent will react with the precipitate. The mercuric thiosulfate precipitate is even more reactive than thiosulfate itself, whereas the platinum and palladium precipitates are not reactive or much less so.

Grant (*1*) was the first to point out unknown substances which interfere with the sulfur dioxide reaction and which, unlike the thiols, cannot be removed by masking with mercuric chloride. He removed the mercuric thiol precipitates by centrifugation before adding the sulfur dioxide reagent, which can be kept for a long time by working with two stock solutions.

Stock Solution I is made from 225 ml. of water, 22 ml. of concentrated sulfuric acid, 4 ml. of basic fuchsin (3% alcoholic solution), or 8 ml. of fuchsin in the case of Grant's quantitative procedure. The solution is made up with water to 400 ml. and aged 3 days. The precipitate which forms is decanted or filtered off, or, better still, the fresh solution is shaken with 150 ml. of carbon tetrachloride, when the colloidal precipitate goes into the interface. This purification procedure with the aid of a separation funnel is useful under all similar circumstances.

Stock Solution II is made by adding 5 ml. of 40% formaldehyde, to water to make 100 ml.

Reagent. Before use, 10 parts of I are mixed with 1 part of II. A sensitive reagent for sulfurous acid is obtained by taking 0 parts of II.

Grant (*1*) makes the interesting statement that the blanks of the tissues which he tested for sulfur dioxide give a slightly positive color reaction, although "it seems unlikely that it represents any naturally occurring sulfur dioxide." The author's experiments with gelatin, for which he introduced the test, have also shown interferences by gelatin, even in the case of Eastman chemically-pure gelatin and other gelatins that contained no sulfur dioxide. The interfering gelatin reaction is, however, much less sensitive and much slower than the reaction with sulfur dioxide. It could be still further minimized and almost inhibited by plati-

...m chloride, and although no amino acids present in gelatin were found to give the interfering reaction, cysteine and tryptophan were recorded as interfering with the sulfur dioxide test. The tryptophan trace reaction is, however, very slow. Enediols like ascorbic acid and reductone and o-dihydroxybenzenes like chlorogenic, caffeic, and ethyl hydrocaffeate, were found to give strong but slow to very slow color reactions with the reagent. These reactions, unlike the quick but fading thiol reaction of cysteine, are not inhibited by mercuric or platinum chlorides, and it can therefore be assumed that untreated fruit juices or products which give the color reaction very distinctly, even in the presence of platinum or mercuric chloride, contain ascorbic acid, an enediol, or o-dihydroxybenzene derivatives. The reaction with proteins needs further investigation.

The presence of anionic wetting agents can also interfere with all the reactions which are given by acid-bleached fuchsin-formaldehyde (sulfur dioxide, thiols, enediols, o-dihydroxybenzenes, sulfinic acids), but the anionic wetting agents are easily detected because they react slowly with Stock Solution I, and do not depend on the presence of formaldehyde.

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Microdetermination of Iodine in Plant Material

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THE simplicity inherent in the spectrophotometric procedure used by Gross, Wood, and McHargue (*1*) for the determination of iodine in biological materials makes it well suited for the routine examination of agricultural crops. However, certain modifications which make it possible to determine smaller amounts of iodine extend greatly the usefulness of this procedure.

Table I. Determination of Iodine

Iodine, γ/5 ML	% Transmittance at 575 mμ
0.10	98.7
0.25	92.0
0.50	84.0
0.60	70.0
1.00	41.0
2.00	17.0
5.00	17.0

EXPERIMENTAL

By decreasing the final volume from 50 to 5 ml., 0.1 microgram of iodine can easily be detected when a Model DU Beckman quartz spectrophotometer is used with standard Corex cells having a 1-cm. light path. However, the color developed is not suitably stable if the concentrations of the reagents are maintained at the level used by Gross, Wood, and McHargue. The chief difficulty appears to be associated with a slow decomposition of the potassium iodide, resulting in a gradual increase in the amount of starch-iodide chromogen formed. This difficulty is partially overcome by greatly decreasing the amount of potassium iodide used. Further improvement is obtained by substituting phosphoric acid for sulfuric acid in order to maintain the pH of the solution at approximately 2.5. These changes do not significantly decrease the sensitivity of the starch to iodine and the peak of maximum light absorption remains at 575 mμ. The color is stable for an indefinite time if the solution is kept in a bath of ice water until transmittance readings are ready to be made. Cooling also increases the sensitivity of the starch iodide reaction, especially at the lower concentrations of iodine. Potato starch appears to be slightly better than arrowroot starch, because the sol is less opalescent.

PROCEDURE

A sample of dry plant material weighing 1 gram will be sufficient in most cases. Oxidize the sample in the usual manner, using 10 ml. of 10 *N* chromium trioxide solution and 50 ml. of concentrated sulfuric acid. After cooling, the digestion mixture add 50 ml. of distilled water and 2 or 3 large chips of porous tile or alundum.

Collect the distillate in a 50-ml. Pyrex beaker containing 1 ml. of 0.2 *N* sodium hydroxide. Five milliliters of 30% phosphorus acid are sufficient to reduce the excess chromium trioxide completely and ensure recovery of the iodine present. Collect exactly 35 ml. of distillate.

After the distillate has been evaporated to 5 or 10 ml., prepare a reference solution in another 50-ml. Pyrex beaker by adding 1 ml. of 0.2 *N* sodium hydroxide and 5 to 10 ml. of water. Oxidize the iodides as described by Gross, but use 1 or 2 drops of 0.2 *M* potassium permanganate followed by 3 drops of 25% phosphoric acid, and continue the evaporation to a final volume of 2 or 3 ml.

Transfer the contents of the beakers to small graduated cylinders or test tubes marked at 5 ml. Immerse them in a beaker of ice water for 2 minutes. Add one drop of 5% potassium iodide, mix, and add 10 drops of 0.25% potato starch. Mix thoroughly, dilute to the 5-ml. mark, mix again, and immerse in the ice water. Allow the tubes to remain in the ice water at least 2 minutes before reading the transmittance of the unknown against the reference solution. Make readings at 575 mμ in a cell having a 1-cm. light path.

A curve prepared in the following manner indicates that the starch-iodide chromogen follows Beer's law over the range of 0.1 to 5.0 micrograms of iodine. Standard solutions of potassium iodate containing from 0.10 to 5.00 micrograms of iodine and 1 ml. of 0.2 *N* sodium hydroxide are treated as described above for an unknown, except that distillation and the treatments prior to distillation are omitted. When the micrograms of iodine per 5 ml. are plotted against the logarithm of the per cent transmittance a straight line is obtained which passes through 100% transmittance at zero microgram of iodine. Table I shows the results obtained in this laboratory with a Model DU Beckman spectrophotometer.

Table II. Recovery and Reproducibility Tests

Material	Known Iodine Content γ	Found by This Method γ	P.p.m.
Standard iodine solution	0.20	0.24	...
Standard iodine solution	0.50	0.49	...
10 ml. of 10 *N* CrO₃	...	0.18	...
30 ml. of 10 *N* CrO₃	...	0.55	...
Wheat straw I, 1-gram sample	...	1.32	0.264
Wheat straw I, 1-gram sample	...	0.23	0.230
Wheat straw II, 1-gram sample	...	2.85	2.850

If commercial chromium trioxide is used in this procedure, it is necessary to make a blank correction or to prepare a reference curve by carrying the standard iodine solutions through the same

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Sterekhova
Н. П. СТЕРЕХОВА.

ЯВЛЕНИЯ ОБЩЕТОКСИЧЕСКОГО ДЕЙСТВИЯ СЕРНИСТОГО АНГИДРИДА ПО ДАННЫМ КЛИНИЧЕСКИХ И ЭКСПЕРИМЕНТАЛЬНЫХ НАБЛЮДЕНИЙ.

Сернистый газ является одним из наиболее распространенных вредных промышленных газов, загрязняющих атмосферу не только производственных помещений, но и городов.

В условиях нашей бурно развивающейся социалистической промышленности вопрос о токсикологии этого газа имеет большое гигиеническое значение.

Большинство исследователей относят сернистый ангидрид к группе, так называемых, раздражающих газов, вызывающих воспалительные изменения верхних, а при больших концентрациях — и глубоких дыхательных путей (Тер-Газаров, Пахомычева и др.).

Отметив патологические изменения со стороны органов пищеварительного тракта в виде порчи зубов, повреждений слизистой желудка, вплоть до образования язвы, Шафранова, Шифрин, Тер-Газаров и др. склонны объяснить эти изменения местным прижигающим действием сернистого газа.

Н. В. Лазарев в руководстве по токсикологии 1951 г. также относит SO_2 к раздражающим газам, и полагает, что вопрос об общетоксическом действии его не ясен.

В зарубежной литературе имеются указания о благоприятном влиянии сернистого газа на заболевания верхних дыхательных путей и туберкулезный процесс в легких. Реакционная сущность этих взглядов не подлежит сомнению, и их несостоятельность доказана экспериментальными исследованиями Левина и статистическими данными сотрудника нашего Института доцента В. А. Литкенса о повышенной заболеваемости туберкулезом и гриппом рабочих, подвергающихся воздействию сернистого ангидрида.

Заслуживают пристального внимания наблюдения Гадаскиной о том, что в организме задерживается до 66% поступившего в дыхательные пути газа. Сидоренков обнаружил присутствие SO_2 в циркулирующей крови, что нашло подтверждение и в наших наблюдениях. Сидоренков, а также Никулина и сотр. отметили существенные изменения в обмене веществ и ферментативных процессах под влиянием сернистого газа. Все это позволяет подойти к изучению токсикологии сернистого газа с позиций общетоксического действия.

Первым этапом настоящей работы было исследование функционального состояния печени у рабочих, подвергающихся воздействию сернистого газа в производственных условиях медеплавильного завода, где, наряду с неблагоприятными метеорологическими условиями, концентрации SO_2 на отдельных рабочих местах значительно превышают предельно допустимые.

Одна часть рабочих подверглась обследованию в учреждениях медсанчасти завода, другая, с наиболее выраженными изменениями со стороны печени, была вызвана в клинику Института для углубленного обследования. Данные о распределении этих групп рабочих по полу, возрасту и стажу приведены в таблице № 1.

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Таблица № 1

Данные о распространении обследованных по полу, возрасту и стажу

Возраст	Стаж работы						Общее к-во обследованных по возрасту
	до 5 л.		с 6 до 15 л.		с 16 и больше		
	м	ж	м	ж	м	ж	
До 35 л.	1	—	4	1	—	—	6
С 36 до 45 л	—	—	6	—	9	3	18
С 46 л. и выше	—	—	—	—	4	2	6
Общее к-во по стажу	1		11		18		30

Из таблицы видно, что все обследованные имели значительный стаж работы на данном предприятии. Из предъявленных жалоб на первый план выступали указания на боли в эпигастральной области и в области правого подреберья. Боли носили тупой характер («ноющие», «чувство тяжести») и часто появлялись после приема жирной пищи. Нередки также жалобы на плохой аппетит и тошноту. Можно было отметить, что с увеличением стажа работы учащаются и эти жалобы.

На втором по частоте месте были жалобы на кашель, одышку и боли в груди.

Объективно обнаруживалась большая частота поражений верхних дыхательных путей в виде атрофических, реже гипертрофических ринита, фарингита и ларингита.

Наряду с изменениями верхних дыхательных путей, отмечены явления бронхита (5 чел.), начальные явления эмфиземы легких, сухого плеврита и у 3-х рабочих — очаговый туберкулез легких в фазе уплотнения.

Небольшая часть рабочих предъявляла жалобы на неприятные ощущения в области сердца и сердцебиения. Клиническое обследование и электрокардиография обнаружили у многих из них явления дистрофии миокарда.

Хронические формы желудочно-кишечной патологии наблюдались у половины всех обследованных. Из них:

гиперацидный гастрит — в 5 случаях;
анацидный гастрит — в 6 случаях;
язвенная болезнь 12-перстной кишки — 3 случая;
отметить зависимость формы гастрита от стажа не удалось.

С целью исследования состояния желчевыводящих путей проведено дуоденальное зондирование, которое выявило большое количество слизи, деформированных лейкоцитов, солей билирубина кальция по всех порциях желчи.

У большинства обследованных (25 чел.) отмечено значительное увеличение печени. Исследования функции ее по отдельным лабораторным показателям выявили существенные патологические отклонения. Так, качественная реакция на уробилин в моче была положительной и резко положительной почти у всех рабочих этой группы. У 23 из 30 обследованных содержание билирубина крови оказалось повышенным. Качественная реакция Ван-ден-Берга во всех случаях показала прямой (печеночный) билирубин.

С целью исследования углеводной функции печени мы пользовались глюкозной нагрузкой.

Таблица № 2

Величина гипергликемического показателя

Величина гиперглик. показат.	от 1,0 до 1,5	от 1,6 до 2,0	от 2,0 и выше
Количество обследованных	3	12	12

Как видно из таблицы № 2, в 24 из 27 случаев гипергликемический показатель был выше нормы.

По своему характеру большинство гипергликемических кривых отличалось от таких кривых у здоровых людей.

Особенность их заключалась в двухфазности подъема сахара после нагрузки, при этом фазе вторичного подъема предшествовала фаза падения сахара до исходного уровня и ниже его.

У 8 человек уровень сахара крови и через 2,5 часа оставался выше исходного.

На рисунке № 1 представлено графическое изображение нескольких кривых.

Таким образом, в ответ на сахарную нагрузку большинство обследованных реагировало резким подъемом уровня сахара в крови и несколько атипичными колебаниями величин его в течение исследования.

Флокуляционная проба Такта-Ара и коагуляционная проба Вельтмана являются показателем состояния образующихся в печени белковых коллоидов крови.

Постановка этих реакций у всей группы рабочих показала патологическое удлинение коагуляционной ленты у большинства и патологическое значение флокуляционной пробы у 50% всех обследованных.

По своей интенсивности эти изменения имели различную выраженность, что зависело от степени нарушения функций печени. На основании данных этих лабораторных исследований возможно было заключить о начальном и обратимом характере таких нарушений у многих рабочих.

Условиями восстановления нарушенных функций печени было не только прекращение воздействия сернистого газа, но и применение курса инсулиново-глюкозной терапии.

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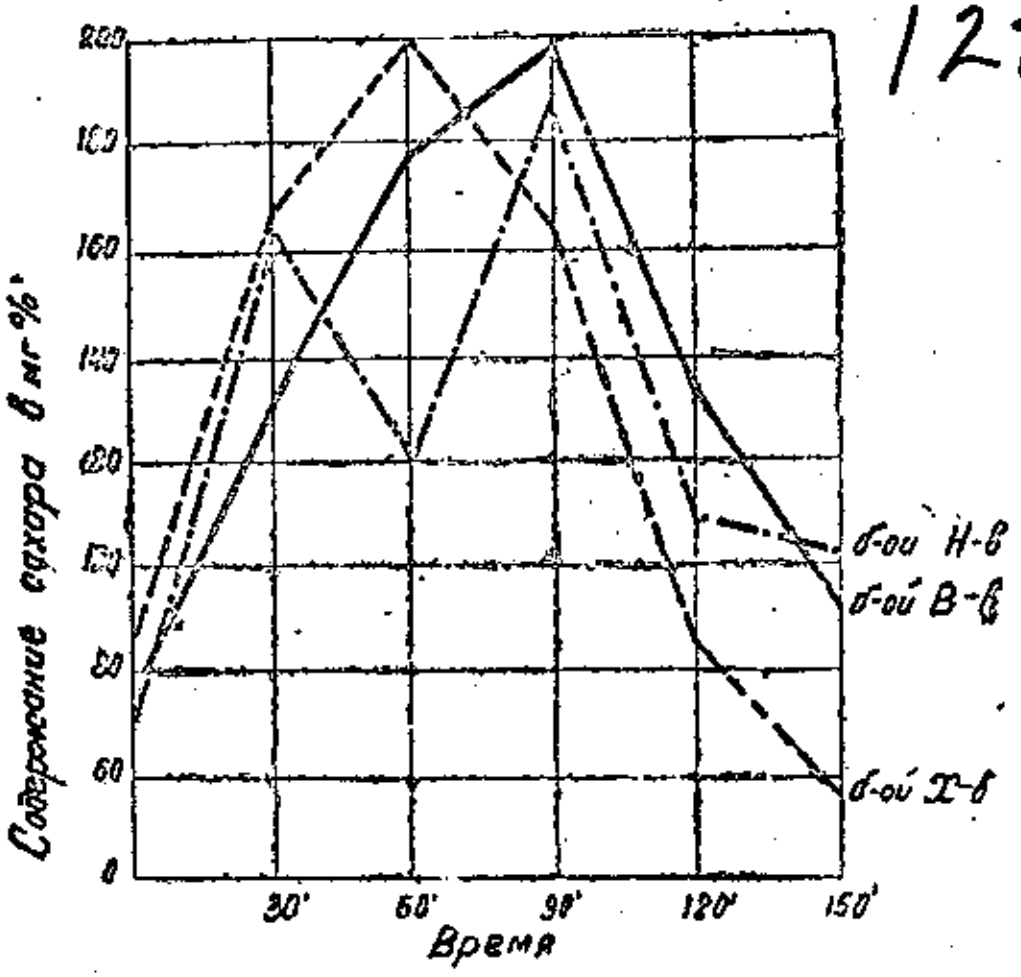

Рис. 1.

Эти изменения функционального состояния печени можно рассматривать, как частное выражение общего действия сернистого ангидрида. В этом нас убеждает и тот факт, что параллелизм между длительностью воздействия сернистого ангидрида (стаж работы) и степенью нарушения функции печени отсутствует.

Изменения белкового и углеводного обмена при отравлении сернистым ангидридом (И. В. Сидоренков) и данные наших наблюдений являются, разумеется, выражением не только прямого, но и непрямого действия SO_2 на рефлекторных и гуморальных путях.

Говоря о явлениях общетоксического действия этого газа, следует указать интересные наблюдения Кочановой. При добавлении к изолированной крови животных бисульфита натрия она обнаружила повышение образования молочной кислоты в процессе расщепления углеводов.

Это явление можно было объяснить связыванием бисульфитом некоторых промежуточных продуктов расщепления углеводов (типа альдо- или кетосоединений).

Исходя из этих данных, можно думать, что в первичных токсикодинамических реакциях сернистого ангидрида с субстратом происходит связывание этих веществ.

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Поэтому следующим этапом нашей работы явилось определение бисульфитсвязывающих соединений в крови и цистина в моче у рабочих, подвергающихся хроническому воздействию SO_2 в производственных условиях. Помимо этого, намечалось исследование этих показателей у животных после внутривенного введения им бисульфита натрия.

Эти исследования у рабочих были проведены непосредственно в предприятии по истечении 4 часов их обычной производственной работы. Данные общеклинического обследования были рассмотрены ранее.

Таблица № 3

Биохимические показатели у рабочих

Кровь		Моча					
содержание бисульфитсвяз. веществ в мг%		содержание цистина в мг/литр		содержание закат O_2 в мг/литр		содержание креатинина в мг/литр	
до 6 мг%	с 6,5 до 15,0 мг%	до 500 мг/л	с 500,0 до 2500,0 мг/л	до 13 мг/л	выше 13 мг/л	до 1,5 мг/л	с 1,6 до 2,0 мг/л
7 обсл.	22 обсл.	15 обсл.	14 обсл.	2 обсл.	12 обсл.	13 обсл.	1 обсл.

Как видно из таблицы № 3, у большей части обследованных (в 22 из 29 случаев) отмечено повышение количества бисульфитсвязывающих веществ крови от 6,5 до 14,7 мг% при норме 4—6 мг%.

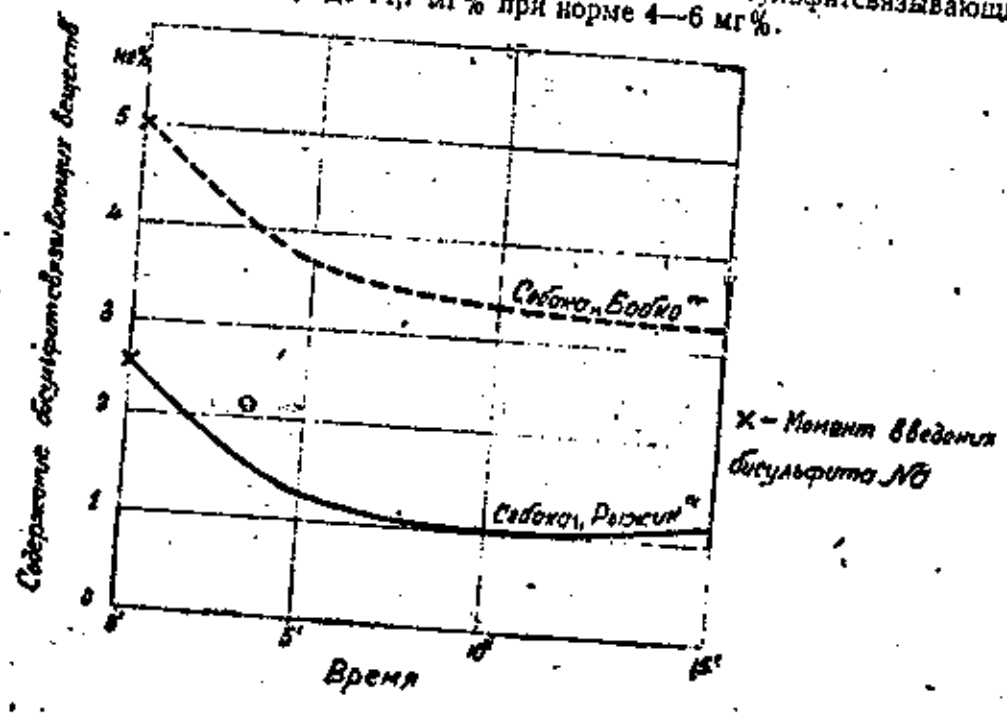

Рис. 2.

Почти во всех случаях имеется снижение выделения креатинина в расчете на 1 литр мочи, количество же выделяемого цистина даже при учете большой амплитуды физиологических колебаний у значительного числа

рабочих было повышенным. То же отмечается и со стороны вакат-кислорода. Содержание сахара, молочной кислоты в крови и фосфатов в моче не изменено.

Экспериментальные исследования проводились на 5 собаках. Поставлено 12 опытов. Методика постановки опыта состояла в следующем:

У животных определялось исходное количество бисульфитсвязывающих субстанций и глютатиона в крови, после чего вводился бисульфит натрия из расчета 70 мг на кг веса тела собаки. Через 5, 10, 15 минут после введения вновь определялось содержание бисульфитсвязывающих веществ и глютатиона в венозной крови.

Следует отметить, что у всех животных после введения бисульфита натрия наблюдались токсические явления — двигательное беспокойство, резкая одышка, обильная саливация, у некоторых — непроизвольное мочеиспускание.

Во всех проведенных опытах отмечено закономерное снижение бисульфитсвязывающих субстанций, окисленной части и общего количества глютатиона. (См. рис. 2 и 3).

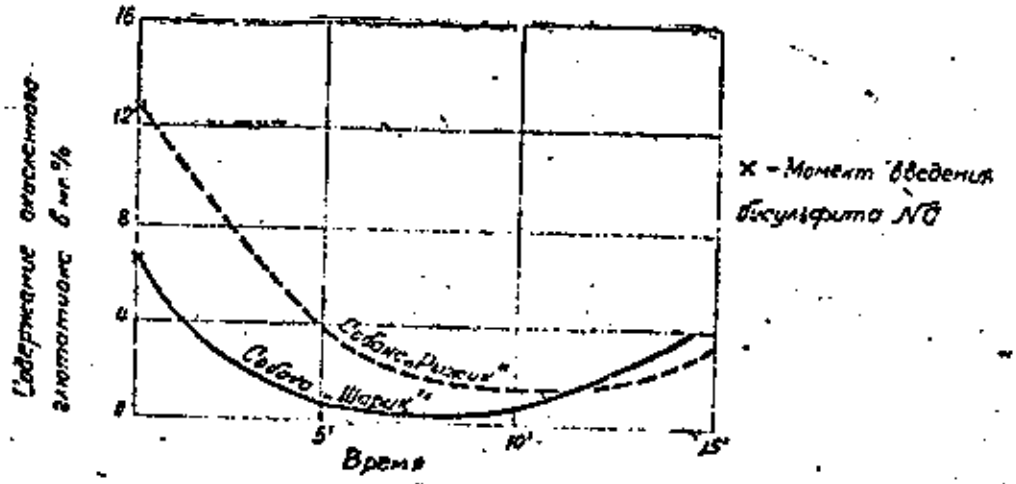

Рис. 3.

Таким образом, в результате проведенной работы можно сделать следующие выводы:

1. Полученные данные клинико-лабораторных исследований указывают на существенные отклонения углеводной и пигментной функции печени, а также — на изменение состояния коллоидов крови у подавляющего числа обследованных, подвергающихся профессиональному воздействию сернистого ангидрида.

2. Эти изменения можно рассматривать, как явления общего действия сернистого газа.

3. Выражением нарушений обмена веществ при экспериментальном отравлении бисульфитом могут быть увеличение содержания бисульфитсвязывающих субстанций, которое можно объяснить компенсаторными изменениями тканевого обмена; увеличение выделения цистина с мочей и повышение вакат-кислорода мочи.

4. Несмотря на малочисленность группы обследованных рабочих можно заключить, что страдание хроническим заболеванием печени является противопоказанием к поступлению на работу, связанную с воздействием сернистого газа.

5. При установлении начальных и обратимых нарушениях функций печени у рабочего необходим временный вывод его из сферы действия профессиональной вредности и применение глюкозо-инсулиновой терапии при богатой углеводами диэте.

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A Sensitive Direct Colorimetric Technique for the Determination of Traces of Sulfur Dioxide in Beer

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THE PRECISE DETERMINATION of sulfur dioxide has been of interest to the brewing industry for many years. Some of the older, less sensitive methods are, of course, no longer of value because of the extremely low levels that exist in present beers. The method used most frequently today in the brewing industry for the determination of sulfur dioxide in beer is the Monier-Williams distillation procedure as described in *Methods of Analysis of the A.O.A.C.* (1). This rather cumbersome and insensitive procedure has been applied to beer for want of a better technique. The A.S.B.C. has no accepted method for the determination of sulfur dioxide.

Excellent reviews of the chemistry of sulfur dioxide in foods and the various methods for its determination have recently been published (6, 9) and need not be repeated here. However, the literature pertinent to the colorimetric reaction used in the method proposed in this paper will be reviewed.

The reaction between the decolorized dyestuff, fuchsin, and aldehydes has been known for many years and for a long time has been the basis for a commonly employed colorimetric test for aldehydes. In 1942 Steigman (10), by modifying the conditions of this reaction, proposed the technique as a sensitive colorimetric test for sulfites. Since the publication of this paper, others have appeared in which this reaction is used, with modifications, for the determination of sulfur dioxide in animal tissues, blood and fruit (7), gas (2), air (12, 13), grape juice (5), and wines.[1, 2] It is surprising that this apparently very sensitive reaction has not received some attention in a search for a procedure applicable to beer. Our interest in the subject was stimulated some years ago by our research associated with the effects of ascorbic acid compounds on taste stability and the extent to which sulfites in beer may influence flavor properties. A sensitive procedure which would differentiate slight differences in SO_2 content for such a purpose was obviously much needed.

A colorimetric method applied directly to wines appears to have been worked out by La Rosa and Joslyn whose unpublished data are referred to in the Joslyn and Braverman review article (9). Apparently sulfur dioxide is used more extensively and at much higher levels in the wine industry than in beer. Dupaigne (5) used the fuchsin color reaction for the determination of sulfur dioxide in grape juice, but his method involved distillation and application of the color test to the distillate.

A recent paper on the determination of traces of sulfur dioxide in the atmosphere by West and Gaeke (13) introduced several noteworthy contributions to this procedure. They substituted para-rosaniline hydrochloride as the dyestuff for the less pure fuchsin in the preparation of their color reagent. They also studied and used in their procedure the disulfitomercurate ion, the complex formed between sulfur dioxide and mercury. This complex is extremely stable and nonoxidizable and yet fully yields its sulfur dioxide when treated with the color reagent.

Sulfur dioxide readily forms complexes of varying degrees of stability with many substances existing in foods, and these complexes are discussed in the reviews previously cited (6, 9). Sulfites complex readily with carbonyl compounds, with materials having unsaturated bonds, and with amines. Since beer contains a wide variety of such compounds as sugars, metabolic products of the fermentation, and nitrogen-containing scission products, it could

[1] Unpublished work by W. V. LaRosa, 1950.
[2] Unpublished work by M. A. Joslyn, 1953.

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red that of the traces of sulfur
at may find their way into beer,
dable proportion might be in the
state. The pH of beer is in a
vorable for such complexing to
e. When complexed in this fashi-
ur dioxide becomes more stable
ion, and any antiseptic qualities
exert at much higher concentra-
n found in beer would be greatly
The complexing of the sulfite by
constituents would further reduce
e weak antioxidant properties but
ance its reaction in the production
in flavor characteristics. Uncom-
sulfite ions in water at the same
vels as the total sulfur dioxide
nd in beer are reported to be be-
human taste threshold (3).

omplex formed with the carbonyl
nds in beer is probably of the type
alpha-hydroxysulfonic acid. These
es have certain specific stabilities
pH of beer but decompose at high
pH's to regenerate the original car-
compound and the sulfur dioxide.
ction may be somewhat slow at
mperature and at acid pH's but is
pid at high pH's. These complexes
have certain specific stabilities to
reagents and there even may be a
tion for the sulfur dioxide among
ferent complexing agents normally
in beer.

method presented in this paper, it
seen, is able to differentiate be-
the free (uncomplexed) and the
(complexed) sulfur dioxide. It has
ther advantage that it is conducted
on a small sample of beer without
cessity for a distillation procedure.
sults obtained are reproducible and
rably more reliable and sensitive in
nges below 5 p.p.m. than the
-Williams method. In this range
larly the latter method is inade-

rovements in analytical methods
extend sensitivities and make poss-
ne separation of constituents former-
resolvable often change our patterns
king. The differentiation of the sul-
beer into the free and bound sulfite
s simple determination poses an in-
ng question as to what should be
ered the actual sulfite content of
ls only the free and unbound sulfite
considered, or should the bound sul-
fite which no longer reacts like sulfite also
be included? Methods in present use, such
as the Monier-Williams, appear to give the
total sulfur dioxide content which includes
both the free and the complexed forms.
The aeration procedure of Brenner,
Owades, and Tazio (3) appears to give
intermediate values comprising the free
and some of the bound SO_2.

Briefly, our method involves measuring
three 10-ml. portions of beer into three
100-ml. volumetric flasks. The contents of
one flask are diluted to volume and an
aliquot is used directly for the color de-
velopment, giving a reading on the readily
reacting SO_2 bodies that we term the
"Free-SO_2." The beer in the second flask
is hydrolyzed with 0.1*N* sodium hydroxide
in the presence of mercury to decomplex
all the SO_2 bound by beer constituents.
A color reading on an aliquot of this solu-
tion gives the "Total-SO_2." The contents
of the third flask are oxidized with a few
drops of 0.05*N* iodine, and a color reading
on this sample gives a blank correction for
the calculations. In each case the color
reaction is conducted simply by placing
an aliquot of the diluted sample solution
in a 50-ml. volumetric flask, adding 5 ml.
of the color reagent and 5 ml. of formalde-
hyde solution, making up to volume, and
allowing the color to develop for 30 min-
utes at 25°C. and then taking a reading
on the solution in a photometer.

We use the improved para-rosaniline
color reagent, and the complexing proper-
ties of the mercuric ion are employed to
stabilize the calibration standards and the
decomplexed SO_2. The intensity of the
color developed is proportional to the con-
centration in the range up to about 30μg.
under the conditions of color development
as shown in Fig. 1.

After considerable preliminary experi-
mentation, the following details of the
method were worked out:

Method

Reagents. Acid-Decolorized Para-Rosani-
line Color Reagent: Transfer 100 mg. of
para-rosaniline hydrochloride to a 250-ml.
volumetric flask. Dissolve in approximately
200 ml. of water and add 40 ml. of (1 + 1)
HCl; dilute to mark. Let stand about 15
minutes at room temperature before use.

Formaldehyde Solution: Dilute 5 ml. of
40% formaldehyde solution to 1 liter with
water.

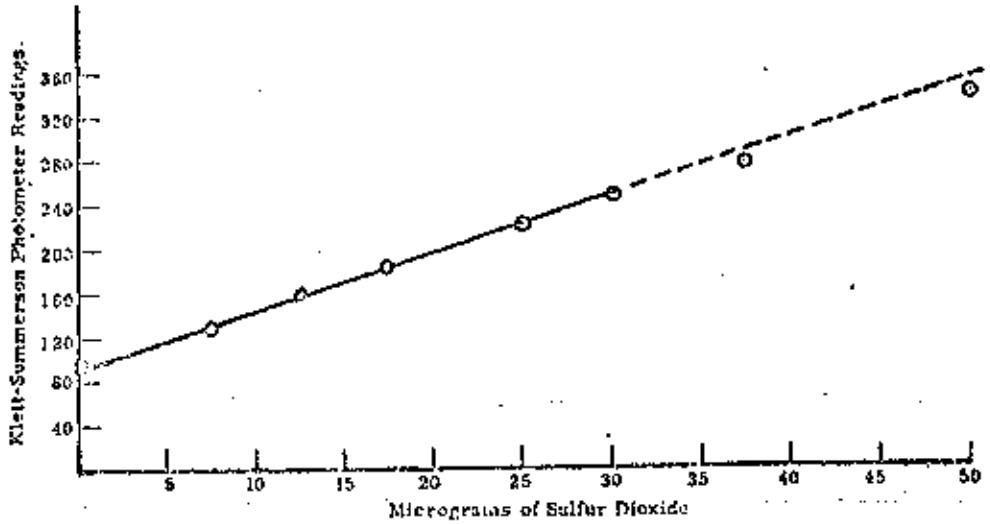

Fig. 1. Typical calibration curve.

Mercury Stabilizing Solution: Dissolve 27.2 g. of mercuric chloride and 11.7 g. of sodium chloride in water and dilute to 1 liter.

0.1*N* Sodium hydroxide

0.1*N* Sulfuric acid

Starch Indicator: Prepare an approximately 1% soluble starch solution.

0.05*N* Iodine solution

Procedure. Free-SO_2: Measure accurately 10 ml. of beer sample into 100-ml. volumetric flask and make up to volume with water. Pipet 25 ml. of this diluted sample into a 50-ml. volumetric flask. Add 5 ml. of the color reagent, mix; add 5 ml. of the formaldehyde solution, mix, and make up to volume with water. Place this flask in a water bath at 25° C. and hold for 20 minutes. Read the color developed in a photometer using a wave length of the light in the neighborhood of about 550 mμ. If a filter photometer is employed for the color reading, use a filter with maximum transmission in the green region.

Total-SO_2: Pipet 2 ml. of the Mercury Stabilizing Solution and 5 ml. of 0.1*N* sulfuric acid into a 100-ml. volumetric flask. Measure accurately 10 ml. of the beer sample into this flask. Add 15 ml. of 0.1*N* sodium hydroxide solution and mix. Allow to stand for about 15 seconds and then add 10 ml. of 0.1*N* sulfuric acid. The bound sulfite has now been decomplexed from the beer constituents and exists as the disulfitomercurate ion. Make up to volume with water and pipet 25 ml. of this solution into a 50-ml. volumetric flask for the color development. The color development is conducted as given above under "Free-SO_2."

Blank Determination: Measure 10 ml. of beer sample into 100-ml. volumetric flask and add 0.5 ml. of the starch solution. Carefully add the iodine solution drop by drop, from a 1-ml. pipet, until a permanent pale bluish color remains. Add one drop further to give a slight excess. Dilute to volume with water. Allow to stand until the blue color fades. Then pipet a 25-ml. aliquot of the colorless solution into a 50-ml. volumetric flask and conduct the color development as given above under "Free-SO_2."

Calculations. For Free-SO_2, subtract the photometer reading of the iodine blank solution from the photometer readings obtained on the Free-SO_2 color solution. Multiply this difference by the calibration factor, converting photometer readings into μg. of SO_2, and calculate to p.p.m. based on charge used.

The Total-SO_2 is obtained in a similar manner by subtracting the photometer readings of the iodine blank solution from the photometer readings of the Total-SO_2 color solution. Multiply the difference by the calibration factor and calculate p.p.m. in the same way.

The Bound-SO_2 may be obtained by subtracting the Free-SO_2 from the Total-SO_2. These calculations may be expressed in the following equations:

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$$\text{Free-}SO_2\ \text{p.p.m.} = \frac{(\text{P.R. Free-}SO_2 - \text{P.R.I}_2\ \text{blank}) \times \text{factor}}{\text{ml. beer in color flask}}$$

$$\text{Total-}SO_2\ \text{p.p.m.} = \frac{(\text{P.R. Total-}SO_2 - \text{P.R.I}_2\ \text{blank}) \times \text{factor}}{\text{ml. beer in color flask}}$$

Bound-SO_2 p.p.m. = p.p.m. Total-SO_2 − p.p.m. Free-SO_2

Notes. Do not degas the beer before sampling. Cool the beer to avoid excessive gas evolution. Use a trace of hexyl alcohol to control any foam during the sampling operation.

The charges of beer suggested in the above method are suitable for most present beers. However, the initial charge of beer or the aliquot taken for color development may be varied, depending on the level of SO_2 encountered in particular samples.

The color reagent and the formaldehyde reagent are stable for at least 3 weeks. If a sediment forms in the color reagent, the clear supernatant liquid may be decanted and used. Sometimes a haze may be encountered in the color solution which may give an erroneously high photometer reading. This interference may be eliminated by centrifuging the color solution immediately before reading in the photometer.

The color readings on the iodine blanks will usually be found to be low and uniform. Therefore, when conducting tests on a series of similar beers it may not be necessary to conduct blank tests on all samples.

Calibration. Sodium bisulfite, $NaHSO_3$, is used for preparing the calibration curve. The bisulfite should be assayed with standard iodine solution before use. Chemically pure sodium bisulfite usually contains 56 to 58% SO_2. Prepare a solution of sodium bisulfite such that 1 ml. = 10 mg. SO_2 (approximately 8.60 to 8.95 g. $NaHSO_3$ for 500 ml., depending upon the assay).

To a 500-ml. volumetric flask add 100 ml. of the Mercury Stabilizing Solution. With an accurate 10-ml. pipet, add to this flask 1 ml. of the sodium bisulfite solution and make up to volume; 1 ml. of this solution is equivalent to 20 μg. of SO_2. This solution may be further diluted for use in the preparation of the calibration standard, if desired.

Into a series of 100-ml. volumetric flasks, measure out 10-ml. portions of a beer sample (preferably one with low Free-SO_2). Add to these flasks containing 10 ml. of beer the following levels of the diluted bisulfite standard: 0, 20, 40, 60, 80, 100, 120, and 140 μg. of SO_2. Dilute to volume with water and transfer 25-ml. aliquots to 50-ml. volumetric flasks. Add 5 ml. of the color reagent, swirl to mix, and add 5 ml. formaldehyde solution. Mix, dilute to volume with water, mix thoroughly, and hold at 25°C. for 20 minutes. Read these solutions in the photometer, using a wave length of approximately 550mμ or a green filter and a cell size to give photometer readings of acceptable precision (the cell size is the same as that which will be used in the actual SO_2 determinations).

The photometer readings obtained from this series of calibration solutions, if plotted on linear graph paper (photometer readings vs. concentration), should give a straight line. Calculate the calibration factor converting photometer readings into μg. SO_2 contained in the 25-ml. aliquot used in the color development flask. If the same charge of sample is always used, the calibration factor may be calculated to convert directly into p.p.m. SO_2. Fig. 1 is a typical calibration curve.

Experimental

Reproducibility. The color development procedure gives a highly reproducible color reading as shown in Table I, which reports

TABLE I

Reproducibility of Results

Sample		Free-SO_2		Total-SO_2		
		Photometer Readings	Parts per Million	Photometer Readings	Parts per Million	Bound Photometer Readings
Beer I	a	93	1.0	181	8.1	77
	b	90	1.0	181	8.1	77
	c	87	0.8	181	8.1	77
	d	89	0.8	185	8.1	78
Beer II	a	97	1.4	201	9.5	79
	b	96	1.3	201	9.5	79
	c	96	1.3	202	9.3	79
	d	93	1.2	202	9.3	79

quadruplicate check results on two beers. In fact, on the iodine blank tests, the readings are so constant that once the iodine blank level is established it is usually not necessary to run the blank tests on all beer samples. This eliminates possibly one-third of the tests. Blank tests may be

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checked only on occasional samples or if the beer color is dark or unusual.

Interferences. Sulfhydryl compounds and enediols have been previously cited (7, 11) as giving colorations with the dye reagent. Cysteine may be present in beers at trace levels and H_2S at extremely low trace levels. Isoascorbic acid or its sodium salt, an enediol, may be added to beers at levels up to about 100 p.p.m. We tested the possible interference of these materials at levels much beyond those normally to be expected in beer. The results of these tests as recorded in Table II show that no essential interference with the color development was encountered. Slight increases of a fraction of 1 p.p.m. as Free-SO_2 were obtained with the abnormally high increment of cysteine. At normal levels no interference would be encountered.

TABLE II

Effect of Cysteine, H_2S and Isona on SO_2 Results

Addition	Free-SO_2 Photometer Readings	Free-SO_2 Parts per Million	Total-SO_2 Photometer Readings	Total-SO_2 Parts per Million	L Blank Photometer Readings
			Beer III		
As-is	90	1.0	181	8.1	77
1000 p.p.m. Cysteine	100	1.7	183	8.0	77
Approx. 10 p.p.m. H_2S	89	0.9	185	8.2	77
80 p.p.m. Isona[b]	88	0.8	181	8.1	77
1000 p.p.m. Isona[b]	88	0.8	...[a]	...	77
			Beer IV		
As-is	97	1.4	202	9.3	79
1000 p.p.m. Cysteine	105	2.1	195	8.9	77
Approx. 10 p.p.m. H_2S	95	1.4	200	9.3	77
80 p.p.m. Isona[b]	90	0.9	197	9.0	78
1000 p.p.m. Isona[b]	90	0.9	...[a]	...	77

[a] Colloidal opalescence formed.
[b] Sodium isoascorbate.

Recovery of added SO_2 and Comparison with Monier-Williams Method. When traces of SO_2 are added to beer, a portion is rapidly complexed and this added SO_2 is not completely recoverable when tested by the Free-SO_2 modification of this test.

In addition to the apparent loss due to the formation of complexes, there may be also an actual irreversible destruction of the SO_2 by oxidation. The rate at which this oxidation takes place is dependent upon numerous factors, the state of oxidation of the beer, the amount of oxygen present either dissolved or in the head space, the levels of catalytically active metals present in the beer, and the presence of other reducing agents, to name a few. The added SO_2 existing in the complexed state in the beer is recoverable by the Total-SO_2 modification of the test, but the SO_2 which is oxidized is not. Since both the complexing and oxidation factors are operating at the same time, tests showing only the recovery of added SO_2 may not give a true picture of the precision of the method. Therefore, during recovery tests, the method was checked against the recoveries obtained by the Monier-Williams method on the same sample bottles.

TABLE III

Recovery of Added SO_2 and Comparison with Monier-Williams Method[a]

Beer Sample	SO_2 Added	Free-SO_2 Recovery of Added SO_2			Total-SO_2 Recovery of Added SO_2			Monier-Williams Method Recovery of Added SO_2			Total SO_2 as Percent of M-W
	p.p.m.	p.p.m.	p.p.m.	%	p.p.m.	p.p.m.	%	p.p.m.	p.p.m.	%	
V	...	2.9	...	...	9.9	...	...	9.8	...	...	101
V	5	1.7	1.8	36	11.5	1.6	92	15.0	...	101	97
VI	...	1.5	...	...	10.6	...	...	10.1	...	...	105
VI	10	6.2	4.7	47	19.6	9.0	90	20.2	10.1	101	97
VII	...	0.5	...	...	5.8	...	...	5.5	...	...	105
VII	15	5.0	4.5	30	16.9	11.1	11	18.0	12.5	83	94
VIII	...	1.1	...	...	8.1	...	...	8.7	...	...	100
VIII	20	12.2	10.8	61	29.3	20.6	103	30.1	21.7	100	96

[a] Beers standing about 1 day at 5°C. after addition of SO_2 before test.

Table III compares the results on four beers with and without added SO_2. On the beers to which SO_2 was not added, the Free-SO_2 results are quite low, amounting to between only 14 and 29% of the Total-SO_2. The colorimetric Total-SO_2 figures on both the sample with and the sample without the added SO_2 check very well with the Monier-Williams results, varying between 94 and 105% of the Monier-Williams distillation figures. The recoveries of the added SO_2 by the colorimetric procedure are good, within 90 to 103% of the added amounts. On Beer VII low recoveries were experienced by both methods, indicating that some of the added SO_2 had been irrecoverably lost, probably by oxidation. The recoveries of the added SO_2 are somewhat higher by the distillation procedure than by the colorimetric technique.

The precision of the Monier-Williams method is not good in the range of SO_2 now encountered in beers. Differences of only 0.1 ml. in the titration are equivalent to about 1 p.p.m. SO_2. Preliminary tests

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indicate that substantial improvement in sensitivity and precision is possible in the Monier-Williams technique if the colorimetric procedure is substituted for the volumetric or gravimetric test now used. This would be of importance when the Monier-Williams method is used for food products which, unlike beer, may require a distillation to obtain an extract suitable for colorimetric reading. Instead of using hydrogen peroxide to receive the distillate, the "Mercury Stabilizing Solution" should be employed. Our tests also indicate that the distillation of the SO_2 in beer is essentially complete in 30 minutes.

The Complexing of SO_2 by Beer Constituents. Beer contains many carbonyl compounds and materials with unsaturated bonds reported to act as complexing agents. Sulfur dioxide may react with the carbohydrates present, and traces of other carbonyl compounds may enter the beer as by-products of the fermentation. Further, the pH of beer is in a range especially favorable for these complexing reactions to take place.

The complexes of SO_2 with the various beer constituents have certain specific stabilities depending upon the pH, temperature, oxidation levels, and the presence and concentrations of the various complexing agents. Here again we can imagine the beer as a complicated, labile system in which the free and complexed SO_2 levels are in a state of flux with competition among the various complexing agents for the available SO_2. The system, at any one temperature, tends to stabilize with the SO_2 bound among the complexes of the highest stability.

The SO_2 bound by the various complexing agents in beer does not directly react with the color reagent, but it may be readily decomplexed and then made available for color development. Most of the SO_2 normally found in beer is in the complexed state and only a small fraction may be termed "Free."

At the pH of beer, the rate of complexing of SO_2 is fairly rapid. Most of the data in the literature relative to the SO_2 complexes are generally at levels greatly in excess of the trace levels found in beer. To develop some data under conditions pertinent to beer, some preliminary model experiments were conducted. Aqueous solutions buffered at pH 4.5, containing alcohol, glucose, or dextrin, were prepared. Approximately 25 p.p.m. of SO_2 were added and the Free- and Total-SO_2 determined on each solution as quickly as possible. The solutions were then allowed to stand and the tests repeated at intervals. The results are presented in Table IV. As was expected, there was only slight or no complexing in the pure alcohol solution. However, in the glucose and dextrin solutions, both containing carbonyl groups, complexing with a corresponding drop in the Free-SO_2 content was evidenced in the first test period after standing only 1.5 hours. On further standing, the extent of complex formation did not increase. In the case of dextrin there was an apparent drop in the Bound-SO_2 on standing, but this may have been due to retrogradation and precipitation of dextrin noticed at the 5°C. storage temperatures with concomitant loss of available carbonyl groups. In any case, it appears that the complexing of these traces of SO_2 is rapid and reaches a maximum in less than 1.5 hours at room temperature. The Total-SO_2 figures in Table IV are interesting in that there were no apparent losses of SO_2 during the entire period of the test, even though the solutions were saturated with dissolved oxygen and exposed to large head spaces of air. In water, at the pH of beer, these traces of SO_2 are not very effective reducing agents with respect to atmospheric oxygen.

TABLE IV

Rate and Extent of Complexing of Traces of SO_2 by Different Beer Constituents[a]

Material	Initial			After 1.5 Hours[b]			After 18 Hours			After 3 Days			After 7 Days		
	Free SO_2	Total SO_2	Bound	Free SO_2	Total SO_2	Bound	Free SO_2	Total SO_2	Bound	Free SO_2	Total SO_2	Bound	Free SO_2	Total SO_2	Bound
			%			%			%			%			%
Water-control	26.4	26.8	1	27.7	26.0	nil	26.1	27.2	3	27.4	26.5	nil	24.8	21.8	nil
4% Alcohol	25.0	26.4	5	25.5	25.9	2	26.1	25.9	nil	24.6	25.0	2	23.5	23.5	nil
4% Glucose	25.0	26.6	7	24.9	26.3	18	21.9	23.6	14	21.0	23.9	19	22.0	26.6	18
4% Dextrin	25.0	26.7	6	18.2	26.7	32	23.1[c]	25.8	10	21.3[c]	23.1	12	21.8[c]	25.4	11

[a] 4% Solutions of the C.P. grade materials prepared in 0.1 M aqueous $NaH_2PO_4 \cdot H_2O$ solution to buffer pH at 4.5, 25 p.p.m. of SO_2 added. Results expressed as table as p.p.m. SO_2.

[b] This storage period was at room temperature (25°C.). The others were after storage in a refrigerator (5°C.) to avoid biological changes.

[c] The dextrin solution had retrograded in these tests, as shown by the formation of a precipitate.

The extent of binding of the SO_2 in the above tests indicates that the carbohydrates may not be the primary complexing agents for SO_2 in beer, but that other more active materials may be present. Small amounts of carbonyl compounds are normally formed as by-products of the fermentation. Compounds of low molecular weight, such as acetaldehyde and other aldehydes, acetylmethylcarbinol, acetol, and many others possibly existing in trace quantities in beer, may much more strongly bind the SO_2 than do the carbohydrates. Burger, Gleuister, and Becker (1) reported aldehydes in beer in the range of 3 to 11 p.p.m.

Acetaldehyde is one of the intermediates in alcoholic fermentation and readily forms a complex with SO_2. An acetaldehyde-bisulfite complex may be prepared as a white crystalline material and is commonly available. It is readily soluble in water or beer, but only a fraction of its sulfite content exists in the "free" form, titratable with iodine. Solutions of acetaldehyde-bisulfite in water and in beer, calculated to contain 25 p.p.m. SO_2, were prepared, and the Free- and Total-SO_2 were determined. The results are tabulated in Table V and show that even in very dilute solutions in water or beer, a high proportion of the added sulfite remains in the complexed state with acetaldehyde.

To see the extent of the SO_2 complexing by volatile materials of low molecular weight which may be present in beer, some beers were distilled under the conditions used for the A.S.B.C. alcohol determination, and the distillates were collected. The combined distillates were buffered to pH 4.5 with sodium phosphate and tested at intervals for Free- and Total-SO_2 with and without the addition of approximately 25 p.p.m. SO_2. During the distillation, no precautions were taken to avoid polymerization or oxidation reactions, so the distillate may not have contained all of the original volatile materials present in the beer.

The results are tabulated in Table VI. In the first place, some SO_2 from the sulfite present in the beer had distilled over and when the tests were made (about 1 day after the distillate was collected) this sulfite was nearly completely in the complexed form. When approximately 25 p.p.m. of SO_2 were added to this distillate and it was immediately tested, about 13% of the added SO_2 was complexed in the short time required to pipet out the samples before adding the color reagent. After a few hours 33% of the added SO_2 or 45% of the Total-SO_2 was complexed by these traces of low boiling compounds distilling along with the alcohol. These tests would indicate that the low-molecular-weight congeners of the fermentation are probably quite active in the complexing of the SO_2 normally present in beer.

Mercury ions form a very stable complex with SO_2. However, this complex differs from the complexes present in beer in that the mercury complex reacts completely with the color reagent to give colors equivalent to the SO_2 present in the complex. In fact, mercury ions when added to beer form so stable a complex

TABLE V

Tests on Acetaldehyde-Sodium Bisulfite Complex[a]

Medium	Calculated Amount of SO_2 Added[b]	Free-SO_2	Total-SO_2	Bound
	p.p.m.	p.p.m.	p.p.m.	%
Water	nil	nil	nil	..
	10	2.6	9.6	73
	20	5.0	17.7	72
Beer IS	nil	1.1	7.2	84
	10	2.3	17.1	87
	20	2.9	26.1	89
Beer X[c]	nil	..	8.8	..
	10	..	16.7	..
	15	..	19.7	..
	20	..	21.7	..

[a] The acetaldehyde-sodium bisulfite used contained, by iodine titration, 5% Free SO_2.
[b] Added as acetaldehyde-sodium bisulfite complex. Tests conducted within a few hours of preparation of samples.
[c] Tests on beer X were conducted by the Monier-Williams method.

TABLE VI

Complexing of Traces of SO_2 by Low-Boiling Fermentation By-Products

Materials	Initial Test Free SO_2	Initial Test Total SO_2	Initial Test Bound	After 5 Hours Free SO_2	After 5 Hours Total SO_2	After 5 Hours Bound	After 1.5 Days Free SO_2	After 1.5 Days Total SO_2	After 1.5 Days Bound
			%			%			%
Ethyl alcohol, 4% pure	0.2	0.3	..	0.0	0.3	..	0.2	0.2	..
Beer distillate[b]	0.3	7.3	96	0.0	6.6	100	0.1	6.1	98
Ethyl alcohol, 4% pure, plus 25 p.p.m. SO_2	26.1	26.1	nil	26.1	26.1	nil	26.1	26.1	nil
Beer distillate plus 25 p.p.m. SO_2	22.9	31.9	28 13[c]	17.7	31.9	45 33[c]	17.9	31.9	44 32[c]

[a] $NaH_2PO_4 \cdot H_2O$, 0.1G., added to all solutions to buffer pH at 4.5. Samples stored at 5°C.
[b] Composite of distillates from several beers distilled according to method for alcohol determination, Beer-4, ASBC Methods.
[c] Percent of Added SO_2 bound.

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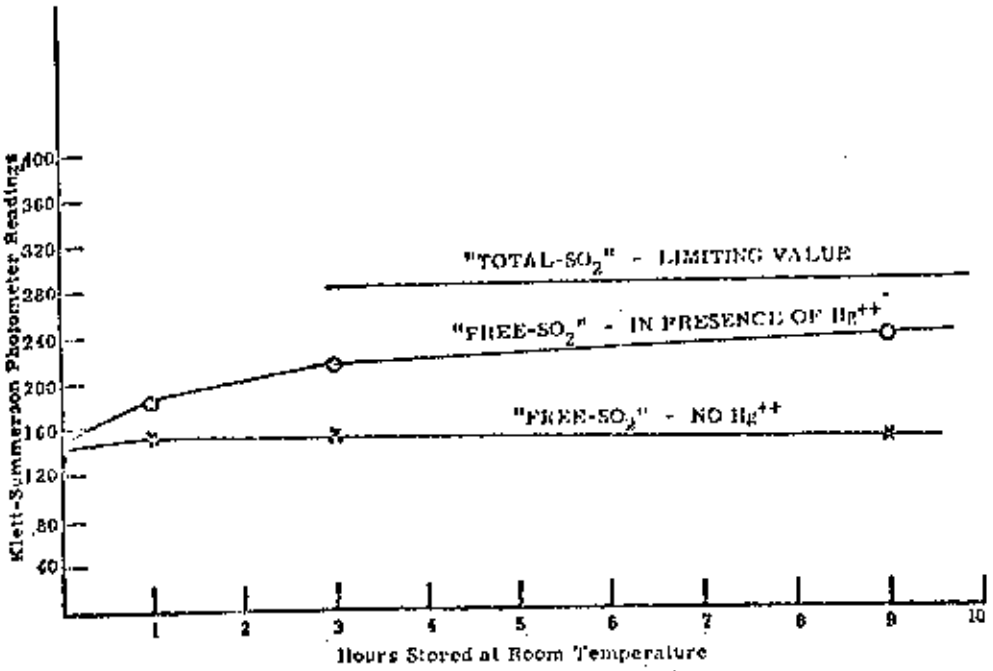

Fig. 2. Effect of mercury ions on the Free-SO_2 determination, showing the tendency of Hg^{++} ions to "decomplex" the beer-bound SO_2.

with SO_2 that the beer-bound-SO_2 tends to be decomplexed. This reaction is somewhat slow at room temperature and may reach an equilibrium before complete decomplexing is attained. This is shown in the curves of Fig. 2, which represents "Free-SO_2" tests on a beer at intervals both in the presence and absence of mercury ions.

The SO_2 bound by beer constituents may be liberated by increasing the pH with 0.1*N* sodium hydroxide by the method suggested for wine (6) to obtain Total-SO_2 results. However, when this alkali is neutralized during the test, some of the liberated SO_2 rapidly reverts again to the beer-bound form, and low erratic Total-SO_2 figures are obtained. We found that when this alkaline hydrolysis is conducted in the presence of mercuric ions, the alkali-liberated SO_2 is complexed by the mercury and prevented from reverting to the unavailable beer-bound form. By conducting the hydrolysis in this fashion, complete recovery of the beer-complexed SO_2 is obtained.

Effect of Oxidation. An experiment was set up to determine the effect of oxidation on the SO_2 content of beers and the relative reactivity of the Free and Bound forms. Two beers from the same brewery were used and to one, sodium isoascorbate (Isona) was added at the rate of 2 lb. per 100 bbl. Each of these beers was tested as-is and with the addition of 10 p.p.m. of SO_2. All beers were set up to contain two levels of air in the bottles; low air, about 0.5 ml., and a fairly high level averaging about 6 ml. per bottle. All the samples were tested for Free and Total-SO_2 immediately after being set up and after 6 and 20 days' storage at room temperature.

The results obtained are tabulated in Table VII. The traces of sulfur dioxide are oxidized only slowly at room temperature even on the samples having 10 p.p.m. of added SO_2 and 6.0 ml. of air in the bottles. It appears that the Free-SO_2 is the more reactive fraction and that the Bound-SO_2 may act as a reservoir to slowly replenish the Free-SO_2 as it is consumed. On the samples containing the added SO_2, the drop in Free-SO_2 is not wholly due to oxidation; some is removed by complexing with beer constituents, as can be seen by the increase in Bound-SO_2 at 6 days. Possibly, if the samples had been tested earlier, say after 1 or 2 days, this increase in Bound-SO_2 might have been much greater. These trends are more clearly seen in the curves of Fig. 3, which illus-

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TABLE VII

Effect of Oxidation on SO_2 in Beer[a]

Beer No.	Added SO_2	Approx. Air Content of Bottles	Total SO_2 0 Days	Total SO_2 6 Days	Total SO_2 20 Days	Free SO_2 0 Days	Free SO_2 6 Days	Free SO_2 20 Days	Bound SO_2 0 Days	Bound SO_2 6 Days	Bound SO_2 20 Days
	ppm	ml/12 oz.	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm	ppm
XI	none	0.5	12.7	12.0	10.8	5.1	2.6	2.0	9.6	9.2	8.8
XI	10	0.5	22.0	19.0	17.6	10.5	6.3	5.3	11.5	12.7	12.3
XII[b]	none	0.5	9.5	10.1	9.3	1.1	1.7	1.1	8.4	8.4	8.2
XII[b]	10	0.5	19.4	16.1	13.5	6.1	1.5	2.8	11.0	13.6	10.5
XI	none	6.0	12.8	10.7	6.1	3.0	2.5	1.7	9.8	8.2	4.4
XI	10	6.0	21.9	16.0	12.0	10.7	6.6	2.7	11.2	11.4	9.3
XII[b]	none	6.0	9.5	6.4	6.9	1.6	0.8	0.9	7.9	5.6	6.0
XII[b]	10	6.0	19.1	15.7	8.0	9.0	1.3	0.0	9.6	11.1	7.2

[a] Stored at room temperature.
[b] Beer XII contained Isona (sodium iso-carbate), 2 lb./100 bbl.

trates the data for the beers with 10 p.p.m. SO_2 addition and the 6.0 ml. of air in the bottles. Similar curves are obtained for the samples containing 0.5 ml. air, except that the curves are not as steep because of the milder oxidizing conditions.

Sulfur Dioxide Content of Market Beers. The Free- and Total-SO_2 was determined on a series of beers from the United States and other countries. The results are tabulated in Tables VIII and IX. All the beers examined have only trace levels of sulfur dioxide. For the U. S. samples, the Total-SO_2 varied from 2.0 p.p.m. to 15.7 p.p.m. with 59 to 94% in the bound form. The SO_2 in the beers from outside the U. S. also was in a similar low range. The table also contains other analytical data, where available, as determined on other bottles of the same lot of beer.

Summary

A sensitive direct colorimetric method for the determination of traces of sulfur dioxide in beers is presented. The color reaction depends upon the red coloration formed when acid-decolorized para-rosaniline and formaldehyde are treated with traces of sulfites. The color developed is proportional to the concentration of sulfites in the range up to about 80 μg., thus giving a very sensitive test. No interference is encountered from cysteine, H_2S, or enediol compounds at levels much beyond that normally encountered in beer.

The reproducibility of color development is good, and the method is able to distinguish between the Free-SO_2 and the SO_2 bound or complexed with various beer constituents. Good recoveries of added SO_2 were obtained, and the Total-SO_2 results checked well with those obtained by the

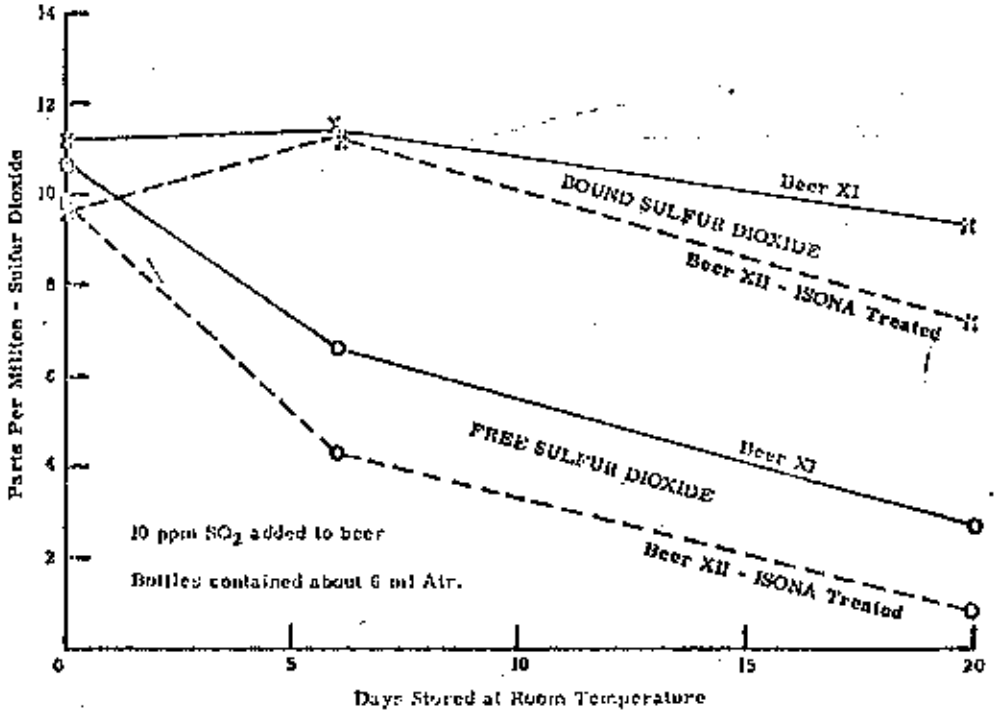

Fig. 8. Typical effects of oxidation on the free and bound sulfur dioxide in beer.

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TABLE VIII
Sulfur Dioxide in U. S. Beers

Area and Beer No.	Sulfur Dioxide Free	Total	Bound	Average Air Level	HT	Fe
	p.p.m.	p.p.m.	%	ml.		
East						
1	1.5	15.7	91	1.3	225	0.6
2	3.0	10.9	73	1.3	270	0.10
3	2.5	12.0	79	1.2	500	0.10
4	0.8	8.0	90	1.5	0	0.20
5	1.1	13.8	91	1.0	925	0.05
6	2.6	9.5	73	3.0	1050	0.15
Midwest						
7	2.7	10.1	73	1.0	600	0.15
8	0.4	3.6	89	1.3	75	0.10
9	0.0	3.0	73	2.0	2100	0.20
10	0.3	3.6	91	1.1	1250	0.05
11	3.1	8.2	59	1.0	325	0.10
12	0.6	2.0	70	..	1050	...
West						
13	0.9	8.0	89	0.6	0	0.20
14	0.0	5.5	89	0.5	350	0.05
15	1.9	8.0	76	1.3	1000	0.05
16	1.1	7.9	85	1.9	1050	0.20

TABLE IX
Sulfur Dioxide in Non-U. S. Beers

Beer No.	Country	Sulfur Dioxide Free	Total	Bound	Air Level	HT	Fe
		p.p.m.	p.p.m.	%	ml.		
17	Canada	0.2	2.7	93	1.0	675	<0.05
18	Canada	7.6	16.2	53	1.5	120	0.10
19	Mexico	0.1	1.0	90	2.0	330	0.05
20	Mexico	2.2	10.5	71	1.2	360	0.20
21	Panama	0.3	5.4	95	2.5	110	0.25
22	Venezuela	0.0	0.0	93	..	...	...
23	Colombia	0.7	8.3	92	..	...	...
24	Cuba	0.3	2.9	96	1.5	1150	0.30
25	Puerto Rico	0.6	5.3	84	1.0	0	<0.30
26	England	0.0	1.7	100	..	...	...
27	Holland	0.2	4.7	96	..	...	...
28	Denmark	0.5	4.7	89	..	...	...
29	W. Germany	0.3	4.1	89	..	...	...
30	Japan	0.0	1.1	100	..	...	...
31	Israel	0.1	12.9	97	..	...	...

Monier-Williams distillation technique.

Model experiments were set up to demonstrate the complexing properties of some beer constituents, and indications were obtained that some low boiling constituents in the alcohol distillate fraction of beer are quite active in binding SO_2.

The Free- and Total-SO_2 was determined on a series of beers after storage with and without added air in the bottle. Sulfur dioxide appears to react only slowly with molecular oxygen.

A series of beers from the United States and other countries were examined for their SO_2 content. It was found that the beers have uniformly low SO_2 levels and that the major portion of the SO_2 is in the complexed state.

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THE EFFECTS OF SULPHUR DIOXIDE UPON ESTABLISHED CELL LINES CULTIVATED IN VITRO[1]

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Abstract

Studies have been made on the effects of SO_2 and its salts on strain L, mouse liver, and HeLa cells. Of the cell lines tested, the HeLa cells seemed to be more sensitive to SO_2 and its salts than the cells of mouse origin.

Cells cultivated in "biological" medium grow in concentrations of gaseous SO_2 up to 2000 p.p.m., although somewhat inhibited. Cells subjected to a concentration of 500 p.p.m. in this medium are not affected greatly and their growth is comparable to those cells in control cultures.

The addition of various salts of SO_2 (Na_2SO_4, Na_2SO_3, and $NaHSO_3$) in concentrations from 10 to 200 mg% produced responses ranging from complete inhibition of growth (by 200 mg% $NaHSO_3$) to apparent stimulation of growth by some concentrations of Na_2SO_4. Toxicity of these salts was in the order of $NaHSO_3 > Na_2SO_3 > Na_2SO_4$.

When cells in vitro are directly exposed to SO_2 in specially designed culture flasks, strain L cells are apparently able to tolerate 5 p.p.m. SO_2 for five 8-hour exposure intervals, provided a "recovery" period follows each exposure.

Certain components of serum seem to play a very important role as protective agents in modifying the effect of gaseous SO_2, possibly by combination.

Introduction

The problems arising from an ever increasing degree of air pollution are well known (1). The chemical nature of many of these "smogs" has been fairly well established (2, 3, 4). In a number of instances where serious health impairments have been experienced, gaseous sulphur dioxide has been cited as one of the major contaminants of the air (5, 6, 7). This is not surprising when it is realized that more than 1,000,000 tons of sulphur are discharged from industrial chimneys each year in the United States.

In spite of its recognition as an important air pollutant, very little is known about its mode of action upon living tissue other than as a "respiratory irritant". An excellent review of the effect of sulphur dioxide upon a variety of mammals has been published by Greenwald (8). The possibility of the use of in vitro studies to supplement this type of information as to the role of air pollutants in health has been suggested by several investigators (9, 10, 11).

It is believed that many of the difficulties encountered in eliciting measurable responses of whole animals to low concentrations of air pollutants might be eliminated through the use of the tissue culture method. The advantages of this method for cytotoxic studies has been shown previously (12). Further, since all total body reactions to adverse conditions must ultimately be traced to the cellular level, it was expected that useful information as to the mecha-

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Contribution from the Institute for Cellular Research, University of Nebraska, Lincoln, Nebraska. These investigations were supported by grants from the U.S. Public Health Service (Grant No. 6139-C2) and the Tobacco Industry Research Council.

nism of action of sulphur dioxide might be ascertained via observations on tissue cells in vitro.

The following investigations are a part of a series of studies carried out in our laboratories in an effort to gain fundamental knowledge concerning air pollutants and their action upon cells.

Materials and Methods

Three cell lines were employed in these investigations: strain L cells (NCTC 929), a well-known mouse fibroblast; mouse liver cells (NCTC 1769) derived from the parenchymal epithelium of the liver; and HeLa cells derived from an epithelial carcinoma of the human cervix.

Two different types of media were employed: one, commonly termed a "biological" medium, consisted of 40% balanced salt solution, 40% horse serum, and 20% chick embryo extract (1:1); the other, referred to as a semi-synthetic medium, was composed of Earle's NCTC 109 (13) supplemented with 10% horse serum.

Replicate cultures were prepared as described by Pace and Aftonomos (14). The cultures were subjected to SO_2 by one of three approaches: (*a*) triweekly exposures to the pollutant, immediately following medium change, by flushing each Carrel flask (D-3.5) with the desired concentration of SO_2; (*b*) continuous exposure of the cells to salts of SO_2 (Na_2SO_4, Na_2SO_3, and $NaHSO_3$) in concentrations ranging from 10 to 200 mg%; (*c*) direct exposure of the cells to the gas for specific time intervals in a specially designed flask described previously (15).

The effect of SO_2 was ascertained by observing changes in growth of the exposed cells as compared to control cultures. Cell numbers were determined at selected time intervals using one of two methods of analysis: (*a*) the nuclei enumeration method of Sanford (16); or (*b*) the Coulter Electronic Cell Counter (17).

From time to time, viability tests were made using erythrosin B as the staining agent (18). The readings were taken within a 15-minute period since proper timing seems to be critical in vital staining. If the cells are exposed to the stain for too long, erroneous results may be observed (18).

Results

(A) Effects of High Concentrations of SO_2 upon Cells in "Biological" Medium

When strain L cells were exposed to 2000 p.p.m. SO_2, growth was strongly inhibited, as shown in Table I. In fact, practically no growth was observed in the SO_2-treated cells as compared to a more than 12-fold increase in the control cultures after 12 days' exposure. Similarly, 1000 p.p.m. SO_2 inhibited growth of strain L cells by about 38%, although 500 p.p.m. did not seem to affect growth to any great extent. In fact, except for the 8th day, L cells exposed to 500 p.p.m. SO_2 did as well as, or better than, the controls.

Apparently, because of the high buffering potential of the 40% serum as well as the added buffers in the saline part of the medium, the change in pH was

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TABLE I

Effect of SO_2 on strain L, liver, and HeLa cells cultivated in "biological" medium (Each culture was flushed with the desired concentration of gas for 30 seconds every 48 hours. Six experiments were carried out with the strain L cells, three with the liver cells, and four with HeLa cells)

Time of exposure in days	Concentration of SO_2 in p.p.m.			
	0	500	1000	2000
	Number of cells in millions per ml			
	Strain L cells			
0	.370	.370	.370	.370
2	.519	.634	.577	.301
4	1.559	1.671	1.164	.397
6	2.233	2.407	1.520	.361
8	3.220	3.037	1.836	.385
10	3.701	3.791	2.188	.314
12	4.538	4.542	2.775	.423
	Mouse liver cells			
0	.360	.360	.360	.360
2	.747	.703	.636	.600
4	1.617	1.373	1.343	1.103
6	2.417	2.167	2.123	1.270
8	3.717	4.100	2.516	1.740
10	4.650	5.120	2.900	1.983
12	5.750	5.250	2.350	1.600
	HeLa cells			
0	.340	.340	.340	.340
2	.563	.878	.560	.414
4	1.313	1.518	1.662	.400
6	2.315	2.713	1.763	.619
8	3.313	4.013	2.163	.700
10	4.353	5.488	2.475	.833
12	4.700	6.000	2.375	.695

relatively slight, except in the very highest concentration (2000 p.p.m. SO_2).

Several interesting phenomena were observed when liver cells were exposed to SO_2. Even at 2000 p.p.m. the cells tolerated the SO_2 for a considerable period. Although cell growth was affected, a marked inhibition (about 50%) was not noted until the 6th day. At 1000 p.p.m., inhibition reached 50% on the 10th day, but at a concentration of 500 p.p.m., little, if any, deleterious effects were observed.

The inhibiting effect of 2000 p.p.m. SO_2 on HeLa cells was very marked. During the 12-day interval, the maximum population increased less than 2.5-fold, while the control cultures showed a 13.8-fold increase. When exposed to 1000 p.p.m. SO_2, HeLa cells were also inhibited; by the 12th day, the cell population was approximately half that of the control cultures. At 500 p.p.m., cell growth was stimulated; after 12 days' exposure it was noticeably greater than that of the controls.

Similar experiments were carried out employing the semisynthetic medium which contained only 10% horse serum. Under these circumstances, much lower concentrations of gaseous SO_2 were required to produce growth inhibition.

In general, concentrations of 200, 100, and 50 p.p.m. SO_2 showed effects similar to those observed with 2000, 1000, and 500 p.p.m. SO_2 in "biological medium". There was also a considerably reduced buffering capacity with the lower serum concentration. Thus cells exposed to 200 p.p.m. SO_2 in semisynthetic medium did have a slight pH shift to an acid condition (pH 6.8).

(B) Effects of Salts of SO_2 (Na_2SO_4, Na_2SO_3, and $NaHSO_3$)

1. Results with Na_2SO_4

Exposure of strain L cells to 200 or 100 mg% Na_2SO_4 resulted in very little inhibition during the first 7 days as shown in Table II. In 50 mg% or 10 mg%, growth was approximately comparable to that in the controls.

TABLE II

The effect of Na_2SO_4 on strain L, mouse liver, and HeLa cells (Culture media were changed 3 times weekly. All cell counts are the average of three experiments. Three sample flasks were randomly selected at each specified time; thus each value represents the total of nine cultures)

Time of exposure in days	Concentration of Na_2SO_4 in mg%				
	0	10	50	100	200
	Number of cells in millions per ml				
	Strain L cells				
0	.218	.218	.218	.218	.218
3	.489	.482	.472	.428	.373
5	1.003	1.013	.956	.889	.741
7	1.461	1.601	1.483	1.487	1.291
9	1.785	1.830	1.751	1.100	1.293
	Mouse liver cells				
0	.492	.492	.492	.492	.492
2	1.180	1.037	1.083	.961	.937
5	1.320	1.603	1.835	1.625	1.559
7	2.722	2.638	3.218	2.810	1.793
9	2.625	3.300	2.600	3.300	1.950
	HeLa cells				
0	.350	.350	.350	.350	.350
2	.794	.582	.692	.555	.535
5	1.182	1.029	.730	.455	.609

Growth of mouse liver cells was inhibited by 200 mg% Na_2SO_4, from 14% on the 1st day to as much as 25% on the 7th day. In 100 mg%, 50 mg%, and 10 mg% Na_2SO_4, there was an increase in cell numbers over the controls.

HeLa cells proved to be quite sensitive to the higher concentrations of Na_2SO_4. After 5 days, growth was inhibited in all concentrations.

2. Results with Na_2SO_3

Sodium sulphite had a very pronounced effect on all cell lines. The results of these experiments are presented in Table III. There was no proliferation whatever in the cells subjected to 200 mg%. This was also true for HeLa cells in 100 mg% Na_2SO_3. Increase in cell number was noted in strain L and liver cells at this concentration although the increase was rather slight as compared

TABLE III

The effect of Na_2SO_3 on strain L, mouse liver, and HeLa cells
(Culture media were changed 3 times weekly. All cell counts are the average of three experiments. Three sample flasks were randomly selected at each specified time; thus each value represents the total of nine cultures)

Time of exposure in days	Concentration of Na_2SO_3, in mg%				
	0	10	50	100	200
	Number of cells in millions per ml				
	Strain L cells				
0	.218	.218	.218	.218	.218
3	.486	.434	.406	.277	.163
5	1.003	.873	.861	.463	.138
7	1.461	1.708	1.291	.819	.095
9	1.785	1.669	1.470	.728	.067
	Mouse liver cells				
0	.492	.492	.492	.492	.492
2	1.180	.856	1.151	.855	.469
5	1.820	1.219	1.721	1.453	.444
7	2.722	1.234	2.673	1.132	.273
9	2.625	1.450	3.475	2.200	.110
	HeLa cells				
0	.350	.350	.350	.350	.350
2	.794	.541	.333	.308	.117
5	1.182	.485	.413	.285	.100

to the controls. HeLa cells seemed to be most sensitive to Na_2SO_3, but some growth was noted in 50 mg% and 10 mg%. Strain L cells were also inhibited in 50 mg%, but proliferation in 10 mg% Na_2SO_3 was comparable to that in the control cultures. Liver cells, however, actually proliferated to a greater extent in 50 mg% Na_2SO_3 than they did under control conditions although growth was inhibited in 10 mg%. This apparent inconsistency was found in all the Na_2SO_3 experiments with liver cells.

3. Results with $NaHSO_3$

The results obtained following exposure of the cells to various concentrations of $NaHSO_3$ are presented in Table IV. In 200 mg% the cells of all three lines soon died. This was also true for HeLa cells in 100 mg% $NaHSO_3$. Growth of strain L and liver cells in 100 mg% $NaHSO_3$ was inhibited. This also obtains for strain L and HeLa cells in 50 mg% and liver and HeLa in 10 mg%. Mouse liver cells and strain L cells after 9 days' exposure to 50 mg% and 10 mg% $NaHSO_3$, respectively, had proliferated at least as much as the controls. The liver cells when exposed to 50 mg% $NaHSO_3$ responded in a manner similar to that observed in 50 mg% Na_2SO_3; i.e., growth was actually greater than that of the controls although a lower concentration (10 mg%) was inhibitory.

(C) The Effect of Direct Exposure to Several Concentrations of SO_2 on Strain L Cells

The cells were exposed to SO_2 in specially designed flasks as described previously (15). The control cultures were treated in the same manner except that

TABLE IV

Effect of $NaHSO_3$ on strain L, liver, and HeLa cells
(Culture media were changed 3 times weekly. All cell counts are the average of three experiments. Three sample flasks were randomly selected at each specified time; thus each value represents the total of nine cultures)

Time of exposure in days	Concentration of $NaHSO_3$, in mg%				
	0	10	50	100	200
	Number of cells in millions per ml				
	Strain L cells				
0	.197	.197	.197	.197	.197
3	.478	.478	.425	.238	.021
5	1.180	1.091	.770	.289	dead
7	1.785	1.726	1.139	.485	dead
9	1.920	2.152	1.565	.377	dead
	Mouse liver cells				
0	.492	.492	.492	.492	.492
2	1.180	.886	1.010	.730	.112
5	1.820	1.371	1.614	.787	.073
7	2.722	2.035	2.331	.518	.440
9	2.625	2.000	2.865	.520	dead
	HeLa cells				
0	.350	.350	.350	.350	.350
2	.791	.556	.462	.222	.096
5	1.182	.430	.390	.185	.015

the usual 95% air + 5% CO_2 mixture flowed through the flasks instead of SO_2 mixtures.

1. Results with 25 p.p.m. SO_2

In the first series of experiments, the cells were subjected to 25 p.p.m. SO_2 for 8-hour intervals during each day. Each exposure was followed by a 16-hour "recovery" period. The flow rate through each of the flasks was 150 ml per minute. Once each hour the cells were rinsed for 30 seconds with balanced salt solution. In this series (Table V, A) a single 8-hour exposure to SO_2 inhibited growth as compared to controls and resulted in a large number of detached cells. Detached cells were also found in the control cultures, but not in very great numbers. Staining with erythrosin B (18) indicated that only 10% of the detached cells in the SO_2-treated cultures were living, while 90% of those in the controls were still viable.

In a second series of experiments using 25 p.p.m. SO_2 the test cultures were exposed for 8 hours on 2 successive days, each exposure followed by a 16-hour "recovery" period. At the end of the 2nd day, all of the cells treated with SO_2 were dead and those which remained attached to the flasks were "fixed". On the other hand, practically all the attached cells in the controls were living, as were 93% of those that had detached.

2. Results with 10 p.p.m. SO_2

In another group of experiments on strain L cells, the concentration of SO_2 was reduced to 10 p.p.m. and the results are presented in Table VI. In the first

TABLE V

Effect of 25 p.p.m. SO_2 on strain L cells

(These cells were exposed continuously for 8 hours and then returned to control culture conditions for 16 hours before termination of experiment or re-exposure to the test condition. The numbers below represent the number of cells per ml and is an average of 10 separate cultures. All tests were carried out in medium 199 + 10% horse serum)

Experimental conditions	Number of cells per ml	
	Controls	25 p.p.m. SO_2
(A) Exposed 8 hours (1 day)		
Initial cell number	206,100	206,100
Final number of attached cells	385,250	240,650
Number of detached cells	16,800	72,550
Number of dead cells	1,680	65,300
Number of living cells	400,370	246,900
(B) Exposed 8 hours (2 days in succession)		
Initial cell number	198,700	198,700
Final number of attached cells	621,250	96,950
Number of detached cells	24,850	137,850
Number of dead cells	1,725	137,850
Number of living cells	644,375	96,950*

*Cells appeared to be fixed.

TABLE VI

Effect of 10 p.p.m. SO_2 on strain L cells

(These cells were exposed continuously for 8 hours and then returned to control culture conditions for 16 hours before termination of experiment or re-exposure to the test condition. The numbers below represent the number of cells per ml and is an average of 15 separate cultures)

Experimental conditions	Number of cells per ml	
	Controls	10 p.p.m. SO_2
(A) Exposed 8 hours (1 day)		
Initial cell number	235,450	235,450
Final number of attached cells	738,200	649,150
Number of detached cells	78,450	96,250
Number of dead cells	7,840	86,630
Number of living cells	808,810	658,770
(B) Exposed 8 hours (3 days in succession)		
Initial cell number	204,150	204,150
Final number of attached cells	847,350	584,600
Number of detached cells	75,650	127,400
Number of dead cells	3,785	116,910
Number of living cells	919,215	594,090
(C) Exposed 8 hours (5 days in succession)		
Initial cell number	196,200	196,200
Final number of attached cells	984,200	301,450
Number of detached cells	65,600	154,050
Number of dead cells	6,000	145,055
Number of living cells	1,043,800	310,445

series of experiments, in which a single, 8-hour exposure to 10 p.p.m. SO_2 was used, there was a slight tendency toward a decreased proliferation rate. Although the number of detached cells was only 20% greater than for the controls, all but 10% of them were dead. In the controls, 90% of the detached cells

were still viable. In the second series of experiments, 8-hour exposures with 16-hour "recovery" periods were employed for 3 days in succession. In these experiments cell proliferation was reduced to about 65% that of control cultures. Although 95% of the detached cells in the controls were still living, 92% of those in the SO_2-treated cultures were dead.

In a third series of this group (Table VI, C), 10 p.p.m. SO_2 was employed for 8 hours per day, 5 days in succession. Cell proliferation became progressively poorer in these cultures during the 5 days of the experiment, finally reaching about 30% of that observed in the control cultures. Again, although there were only a few dead cells among those that had detached in the controls, most of the detached cells in the test cultures were dead.

3. Results with 5 p.p.m. SO_2

When 5 p.p.m. SO_2 passed through the culture flasks during an 8-hour period for 2 days in succession (Table VII, A) there was no significant difference in cell numbers produced as compared to control cultures. Strain L cells apparently can readily tolerate the 8-hour exposure to 5 p.p.m. SO_2 for 2 days in succession.

TABLE VII

Effect of 5 p.p.m. SO_2 on strain L cells

(These cells were exposed continuously for 8 hours and then returned to control culture conditions for 16 hours before termination of experiment or re-exposure to the test condition. The numbers below represent the number of cells per ml and is an average of 10 separate cultures)

Experimental conditions	Number of cells per ml	
	Controls	5 p.p.m. SO_2
(A) Exposed 8 hours (2 days in succession)		
Initial cell number	436,200	436,200
Final number of attached cells	821,300	800,450
Number of detached cells	20,550	25,950
Number of dead cells	2,050	22,350
Number of living cells	839,800	804,050
(B) Exposed 8 hours (5 days in succession)		
Initial cell number	351,200	351,200
Final number of attached cells	968,700	721,150
Number of detached cells	64,150	83,750
Number of dead cells	7,830	62,000
Number of living cells	1,025,020	743,000

After a succession of 8-hour exposures for 5 days, growth was about 72% that of the controls. It is interesting to note that although the number of detached cells did not differ greatly between control and experimental cultures, most of those found in the latter were dead.

Discussion

Greenwald concludes his review (8) with a statement to the effect that SO_2 can neither be accused of, nor absolved from, responsibility for the serious health hazards of the London smog of 1952 and the Donora episode of a few

years earlier, as well as other incidents. Even though its role as a hazard in air pollution remains open to question, the toxicity of SO_2 is well known. It is felt that further knowledge on the action of this substance on living cells could lead to a better understanding in reference to its role as an air pollutant. In recent years the tissue culture method has become well established as a research tool for the study of cytotoxicity (12, 19, 20). Hence, it is evident that its use in the study of air pollution would be recognized and some of its application has already been reported (10, 11, 15).

In the observations noted here, concentrations of 2000, 1000, and 500 p.p.m. SO_2 were arbitrarily chosen with the expectation that an immediate toxic effect would be observed. It was somewhat surprising, therefore, to find that even in 2000 p.p.m. growth occurred in all three cell lines, although markedly inhibited.

In 500 p.p.m. SO_2, proliferation of strain L and liver cells compared favorably with those in control cultures; in contrast, growth of HeLa cells exceeded that in the control cultures (Table I).

In view of the relatively high concentrations required to produce cytotoxic effects on these cells, efforts were made to determine the relative affinity of the culture medium for the gas. When a semisynthetic culture medium (NCTC 109+10% horse serum) was employed under similar conditions, much lower concentrations of SO_2 were required to produce marked inhibition. This suggests that the higher serum content (40% in "biological" medium as compared to 10% in semisynthetic medium) might play an important role in the protection of cells from SO_2 toxicity.

In the semisynthetic medium, concentrations of 200, 100, and 50 p.p.m. SO_2 produced results roughly comparable to those obtained with 2000, 1000, and 500 p.p.m., respectively, in "biological" medium. This leads to the suggestion that in "biological" medium the gas combines with some constituent or constituents of the serum portion.

In order to test this hypothesis further, experiments were run to ascertain what effects, if any, might be observed if certain salts of SO_2, rather than SO_2 itself, are added to the culture medium. Since the medium is in effect a physiological salt solution supplemented with vitamins, amino acids, etc., the sodium salts of SO_2 were studied. Of all salt concentrations prepared, only the 200 mg% $NaHSO_3$ when added to the culture medium caused a shift of pH beyond the ranges for normal cell growth. This is probably due to the relatively high buffering capacity of the bicarbonate system in NCTC No. 109. Thus it is felt that, with the possible exception of the cells exposed to 200 mg% $NaHSO_3$, the modifications of growth rates as noted in this investigation is not attributable to pH differences.

The addition of Na_2SO_4 (Table II) to the culture medium produced several interesting phenomena. It was found that the HeLa cells (of human epithelial origin) were more sensitive to Na_2SO_4 than either of the mouse cell types (fibroblast and epithelial) which seemed to benefit from the addition of as much

as 100 mg% Na_2SO_4 for at least 7 days. In the case of mouse liver cells, growth was increased over that of controls in 100 mg%, 50 mg%, and 10 mg% by the 7th day. The addition, however, of as much as 200 mg% Na_2SO_4 definitely inhibited cell proliferation in all cell lines.

The Na_2SO_3 (Table III) was of no benefit to any of the three cell lines tested. At only one concentration did any of the experimental cells equal in number or exceed those of control cultures, liver cells on the 9th day in 50 mg%. As was observed with Na_2SO_4, the HeLa cells were more sensitive to Na_2SO_3 than the strain L or liver.

The marked toxic effect of 200 mg% $NaHSO_3$ (Table IV) may possibly be explained on the basis of pH, since this amount of the salt lowered the pH of the nutrient medium to approximately 6.8. This pH would probably not kill the cells (at least, not as quickly as noted in these experiments), but might conceivably reduce cell proliferation. As was observed under other circumstances, the different cell lines varied in their sensitivity to the effects of $NaHSO_3$ at this concentration. At 100 mg% $NaHSO_3$, growth in all cell lines was strongly retarded. Growth in HeLa cells was completely inhibited.

In 10 mg% and 50 mg% $NaHSO_3$ the same phenomenon observed with the other salts was noted, namely, the greater proliferation of liver cells in 50 mg% than in 10 mg%. At present, this phenomenon is not understandable. It is inconceivable that a substance should have a greater inhibitory effect at a low (10 mg%) than at a high concentration (50 mg%).

The results obtained with various salts of SO_2 suggest that the toxic action of this gas upon the cells in vitro is perhaps mediated through some other pathway than by combination to produce the several salts. Richter (21) reported the formation of ferritin and the induction of fine structure changes in HeLa cells within 4 hours after the addition of ferrous sulphate in concentrations as low as 5 gamma per ml. No efforts were made to determine the presence of ferritin under our experimental conditions. The iron concentration in our culture media is rather low, being present only in the added serum. Therefore, in view of the very small amounts of ferrous sulphate reported by Richter to be needed for ferritin formation, this mechanism might exist and should perhaps be explored.

Pace (22) and Mast and Pace (23, 24) studied the role of sulphur on growth and respiration of certain protozoan cells. In most cases, very low concentrations of the sulphur salts could stimulate cell growth but at higher concentrations the same salts became quite inhibitory. The general principle seems applicable here.

Since the possibility still existed that the SO_2 gas could react or combine with components in the medium (either "biological" or semisynthetic) in some way other than by direct salt formation, another series of experiments were undertaken. In these experiments the SO_2 was brought into closer contact with the cell monolayer by using a physiologically balanced salt solution, as described in a previous report (15). Under these circumstances it is evident from the

results that 25 p.p.m. SO_2 is quite toxic to the cells used in these tests. In fact, within 2 days all strain L cells subjected to it were dead. On the other hand, when exposed (8 hours per day) to 10 p.p.m. or 5 p.p.m. SO_2, strain L cells were still proliferating after 5 days, but the rate is reduced considerably.

Thus from the results of these studies on SO_2, several important phenomena have been observed. Firstly, in the presence of a relatively high serum content, such as found in "biological" media, cells cultivated in vitro are apparently "protected" from rather high concentrations of SO_2, presumably owing to the combination of the gas with organic constituents of the medium. Therefore, whatever the effect of the SO_2 on proliferation of cells in vitro, it is apparently not mediated through the direct action of increased salt concentrations upon the cells. Secondly, of the three cell lines reported, HeLa cells were more sensitive to SO_2 than those of mouse origin. This would seem to support the general thesis that humans are more sensitive to SO_2 than other mammals (8). Thirdly, in none of the conditions reported here did there seem to be even a suggestion of the development of tolerance, as such, by the cells following repeated exposures, although this phenomenon has been reported for the whole animal (8). Finally, these studies would indicate the feasibility of the use of tissue culture methods for long-term studies on the effects of low concentrations of air pollutants upon cells.

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1. Vom medizinisch-psychologischen, arbeitspsychologischen und arbeitshygienischen Standpunkt dürfte zur wirksamen Fluktuationsbekämpfung eine vertiefte Analyse der Werktätigen mit folgenden Ausscheidungsgründen förderlich sein: Betriebswechsel, eigener Wunsch, gesundheitliche Gründe, Aufhebungsvertrag, Haft, familiäre Gründe und fristlose Entlassungen. Diese Kategorien umfassen in unserem Untersuchungsgut 45 % aller Ausgeschiedenen.

2. Für die Betriebe besonders belastend scheinen uns die Frühausscheidungen zu sein. Darunter verstehen wir Werktätige, die innerhalb von 3 Monaten nach ihrer Einstellung den Betrieb wieder verlassen. Wir schlagen vor, diese Werktätigen dem klinischen Psychologen zu einer abschließenden Untersuchung vorzustellen.

3. Durch psychodiagnostische Eignungsuntersuchungen könnte die Fluktuationsquote gesenkt werden, da solchen Untersuchungen im Hinblick auf die Fluktuation ein prophylaktischer Wert zukommen kann. Dazu müßten aber die personellen, apparativen und räumlichen Ausstattungen der betriebspoliklinischen psychotechnischen Labors wesentlich verbessert werden.

4. Aufschlüsselung der ausgeschiedenen Werktätigen auf die Kostenstellen und Bezugnahme dieser Werktätigen auf die Gesamtzahl der Beschäftigten je Kostenstelle. Auf diese Weise erhält man vergleichbare Werte, die die Berechnung und den Vergleich der Abgängerquote für einzelne Betriebsteile erlauben. Damit lassen sich Schwerpunkte für die Leitungs-, Erziehungs- und Kaderarbeit erkennen.

5. Für Sicherheitsinspektionen, Kaderabteilungen und Betriebsakademien dürften auch Analysen bedeutsam sein, die die Fluktuation unter dem Gesichtspunkt der Dauer der Betriebszugehörigkeit betrachten.

6. Nicht unwichtig dürfte die Aufschlüsselung der Abgänge nach dem fachlich-beruflichen Qualifikationsniveau sein.

7. Berechnung der betrieblichen und volkswirtschaftlichen Verluste, die durch vermeidbare Fluktuation entstehen.

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Aus der Silikoseklinik Sofia

Verlaufsbeobachtungen des Säure-Basen-Gleichgewichts bei Schwefeldioxideinwirkung

Von D. TSCHARAKTSCHIEV, L. GRESCHEWA und S. RASCHEV

Zusammenfassung

Zur Frage der Azidose durch Schwefeldioxid wurden bei 45 Ratten nach subakuter Einwirkung von Schwefeldioxid untersucht:

das aktuelle pH, der aktuelle pCO_2, das Standardbikarbonat, das aktuelle Bikarbonat, die Pufferbasen, der Basenüberschuß, die Total-CO_2 im arteriellen Blut.

Im Urin untersuchten wir pH, Titrationsazidität und Ammoniakmenge. Es wurden Veränderungen im Sinne einer metabolischen Azidose in der Periode unmittelbar nach der SO_2-Einwirkung (3. und 24. Stunde) festgestellt.

Diese Abweichungen bilden sich verhältnismäßig schnell zurück, so daß am 3. Tag eine fast völlige Kompensation festgestellt werden konnte.

Резюме

Для выявления ацидоза, вызванного сернистым ангидридом после подострого воздействия сернистого ангидрида у 45 крыс было исследовано содержание актуального pH, актуального pCO_2, стандартного бикарбоната, актуального бикарбоната, буферных баз, избыток баз, тотального — CO_2 в артериальной кровли. В моче мы исследовали pH, титрованную кислотность и количество аммиака. При этом были установлены изменения в смысле метаболического ацидоза сразу же после воздействия SO_2 (3-й и 24-й час.).

Эти отклонения подвергаются относительно быстро обратному развитию, так что на третий день можно было констатировать полную компенсацию.

Summary

The question of acidosis caused by sulphur dioxide was examined in 45 rats following the subacute effect of sulphur dioxide:

the actual pH, the actual pCO_2, the standard bicarbonate, the actual bicarbonate, the buffer bases, the base surplus, the total CO_2 in the arterial blood.

In the urine we examined pH, titration acidity and ammonia quantity. The authors detected changes expressed by a metabolic acidosis in the period immediately after the SO_2-effect (3rd and 24th hour).

These deviations renormalize naturally relatively quickly. Thus, an almost complete compensation could be observed on the 3rd day.

Schwefeldioxid hat eine vielseitige Einwirkung auf den Organismus. An den Atmungswegen und Lungen ruft es Bronchialspasmen, Tracheobronchitis, Pneumosklerosen u. a. hervor (10, 1, 3, 4). Außerdem hat es eine allgemeine toxische Wirkung über die Metabolitprozesse. Der Vitamin-C-Stoffwechsel wird gestört (2), der Brenztraubensäurespiegel im Blut erhöht sich, die Oxy-

dationsprozesse werden inhibiert und die glykolytischen Prozesse verstärkt (11), die Enzymaktivität wird verändert (9, 8, 6).

Unter Berücksichtigung der Veränderungen in den Atmungsorganen und in den Metabolitprozessen sind Veränderungen im Säure-Basen-Gleichgewicht des Organismus zu vermuten.

Eigene Beobachtungen an Arbeitern, die einer chronischen Schwefeldioxideinwirkung unterliegen, sprechen in dieser Richtung. Die Klärung des Wesens der genannten Abweichungen ist eine Voraussetzung für ihre Korrektur (6).

Zu diesem Zweck haben wir das Säure-Basen-Gleichgewicht bei Ratten nach subakuter Schwefeldioxideinwirkung untersucht und dabei besonders auf Veränderungen in den ersten Stunden und Tagen nach der Vergiftung geachtet.

Arbeitsmethode

Die Untersuchungen wurden an männlichen weißen Ratten (n = 45) vor und nach Schwefeldioxideinwirkung durchgeführt. Schwefeldioxid wurde einmalig während 4 Stunden bei einer mittleren Dosis von 0,27 mg/l angewendet. Die Tiere (Mittelgewicht 250 ± 20 g) wurden im Vivarium bei folgendem Fütterungsregime gehalten: 1,56 g Eiweiß, 0,64 g Fette und 9,08 g Kohlenhydrate, das 49,87 Kalorien täglich je Ratte sicherte und saurer Charakter (0,36 % als Essigsäure) hatte.

Vor der Vergiftung und 24 und 72 Stunden nach der Vergiftung wurden folgende Meßgrößen bestimmt: im Arterienblut aktuelles pH, aktueller pCO_2, Standardbikarbonat, aktuelles Bikarbonat, Pufferbasen, Überschuß von Basen und totale CO_2; im Urin pH, titrierte Säure und Ammoniak.

Ergebnisse

Unsere Angaben zeigen bedeutende Veränderungen des Säuren-Basen-Gleichgewichts bei den mit Schwefeldioxid vergifteten Ratten. Im Vergleich mit dem Ausgangsniveau fällt das akuelle Blut-pH von 7,333 auf 7,291, der aktuelle pCO_2 von 31,0 auf 23,0 mm Quecksilber, das Standardbikarbonat von 17,9 auf 14,2 m val/l, der Überschuß von Basen von 8,2 auf −14,0 m val/l.

Die Veränderungen sind signifikant und die statistische Zuverlässigkeit beträgt in mehreren Fällen über 99,0 % ($p < 0,01$, Tabelle 1).

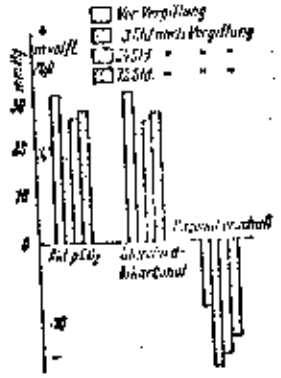

Abb. 1
Verlaufsbeobachtungen der aktuellen pCO_2, Standardbikarbonat und Basenüberschuß vor und nach Vergiftung

Tabelle 1 Veränderungen der Säure-Basen-Meßgrößen im Blut vor und nach Vergiftung

Meßgrößen (im arter. Blut)	Vor Vergiftung	Nach Vergiftung	P
Akt. pH	7,333	7,291	0,01
Akt. pCO_2 mm Hg	31,0	23,0	0,01
Standardbikarbonat in val/l Plasma	17,9	14,2	0,001
Akt. Bikarbonat in val/l Plasma	16,0	11,0	0,001
Pufferbasen in val/l Blut	40,4	31,0	0,001
Basenüberschuß in val/l Blut	− 8,2	− 14,0	0,001
Total CO_2 in val/l Plasma	16,9	11,8	0,001

Tabelle 2 Verlaufsbeobachtungen der Säure-Basen-Meßgrößen im Blut vor und 3, 24 und 72 Stunden nach Vergiftung

Meßgrößen (im arteriellen Blut)	Vor Vergiftung	3 Std. nach Vergiftung	24 Std. nach Vergiftung	72 Std. nach Vergiftung
Akt. pH	7,333	7,301	7,296	7,298
Akt. pCO_2 mm Hg	31,0	18,0	25,0	27,0
Standardbikarbonat in val/l Plasma	17,9	12,0	14,6	13,4
Akt. Bikarbonat in val/l Plasma	16,0	9,1	11,6	12,8
Pufferbasen in val/l Plasma	40,4	31,7	34,3	36,7
Basenüberschuß in val/l Blut	− 8,2	− 15,7	− 14,1	− 11,4
Total CO_2 in val/l Plasma	16,8	8,7	12,2	13,6

Tabelle 3 Säure-Basen-Meßgrößen im Harn vor und nach 24 und 72 Stunden nach der Vergiftung

Meßgrößen (Harn)	Vor Vergiftung	24 Std. nach Vergiftung	72 Std. nach Vergiftung
pH	6,31	5,62	6,10
Tit. Säure ml 0,1 n NaOH/24 Std.	0,32	1,25	0,80
NH_4^+ mg/24 Std.	4,17	16,04	2,32

Die niedrigsten Werte wurden in der 3. und 24. Stunde nach der Vergiftung festgestellt, und am 3. Tag (72. Stunde) zeigt sich eine Neigung zur Normalisierung der Meßgrößen, aber ohne daß die Normalwerte erreicht werden (Tabelle 2, Abb. 1).

Im Urin ist das pH nach 24 Stunden erniedrigt, die titrierte Säure und Ammoniak sind stark erhöht. Am Ende der Untersuchung werden pH und die Ammoniakausscheidung normal, während die titrierte Säure noch immer erhöht bleibt, aber nicht so ausgeprägt wie unmittelbar nach der Vergiftung (Tabelle 3).

Beurteilung

Die Herabsetzung von pH im Arterienblut und im Urin der vergifteten Tiere ist ein Hinweis auf die Azidoseentwicklung. Die Azidose ist nicht kompensiert, weil der Unterschied im Vergleich mit dem aktuellen pH im Blut vor der Vergiftung bedeutend ist ($p < 0,01$).

Die niedrigeren Bikarbonat- und Pufferbasenwerte charakterisieren die Azidose als metabolisch. Das bestätigen auch die Urinbefunde. Erhöhung der Titrationssäure und des Ammoniaks. Die Menge des Kohlendioxids im Blut sinkt kompensatorisch, reicht aber nicht aus und bei der aktuellen pH im Blut tritt eine Veränderung ein. Die Veränderungen im Säure-Basen-Gleichgewicht und der Regulationsmechanismen sind am bedeutendsten unmittelbar nach der Vergiftung und zeigen eine Neigung zur Kompensation am Ende der Beobachtungsperiode (3. Tag). Die Kompensation ist aber noch nicht vollständig.

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Schlußfolgerungen

1. Subakute Vergiftung mit Schwefeldioxid bewirkt eine primäre metabolische Azidose mit sekundärer Herabsetzung des Kohlendioxids.

2. Die Azidose ist nicht kompensiert wie das erniedrigte aktuelle pH im Arterienblut zeigt. Die kompensatorische Lungen- und Nierenregulation reicht nicht aus.

3. Die Abweichungen sind in den ersten Stunden nach der Vergiftung am stärksten ausgeprägt. Am Ende der Beobachtung zeigen sie eine Tendenz zur Normalisierung.

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Chlorkohlenwasserstoff-Pestizide
Toxizität

661.41:661.716:632.9
615.9

Aus dem Deutschen Zentralinstitut für Arbeitsmedizin (Direktor: OMR Dr. *H.-G. Häublein*)

Klinische Beobachtungen zur chronischen Toxizität der Chlorkohlenwasserstoff-Pestizide

Von W. SCHÜTTMANN

Zusammenfassung

Während über die akute Toxizität der Chlorkohlenwasserstoff-Pestizide DDT und HCH Klarheit herrscht, gilt die Frage ihrer chronischen Giftwirkungen zum Teil noch als umstritten. Es wird über eigene klinische Beobachtungen chronischer Gesundheitsschäden berichtet, die nach beruflichem Kontakt mit diesen Mitteln bei der Produktion oder Anwendung aufgetreten sind. Es handelt sich um aplastische Anämien bzw. Pannyelophthisen, um chronische Leberschäden im Sinne der Hepatosen und ihrer Folgezustände und um neurologische Krankheitsbilder. Alle diese chronischen Gesundheitsschäden sind unter Expositionsbedingungen aufgetreten, bei denen eine Überschreitung der MAK-Werte anzunehmen war. Erkrankungen durch DDT und HCH bei der Allgemeinbevölkerung im Gefolge der Rückstandsbildung in Nahrungsmitteln wurden bisher nicht beobachtet. Ein generelles Verbot dieser Mittel erscheint nicht berechtigt. Jedoch ist eine Einschränkung des DDT zugunsten anderer Pestizide und die Bereitstellung rückstandsfreier Lebensmittel für Säuglinge, Kleinkinder und Leberkranke zu fordern.

Резюме

В то время как острая токсичность пестицидов ДДТ и гексахлорана, хлорпроизводных углеводородов яда, вопрос хронических действий их для считается еще неразрешенным. В статье описываются клинические наблюдения хронических повреждений здоровья, возникших после профессионального контакта с этими средствами в ходе их производства или при их применении. Речь идет о апластической анемии или об апластических нарушениях костного мозга, о хронических повреждениях печени и гепатическом синдроме и о их последствиях, и о неврологических заболеваниях. Все эти хронические повреждения здоровья возникли в таких условиях, когда по всей вероятности имело место превышение максимально допустимой концентрации газов или летучих растворителей в воздухе рабочего помещения.

Среди населения не наблюдалось заболеваний, вызванных ДДТ и гексахлораном вследствие образования остатков в пищевых продуктах.

Мы считаем поэтому неоправданным всеобщее запрещение этих средств. Однако необходимо требовать ограничения ДДТ в пользу других пестицидов и предоставления пищевых продуктов свободных от остатков для новорожденных, детей ясельного возраста и для больных, страдающих заболеваниями печени.

Summary

Whereas the acute toxicity of the chlorocarbonic hydrogen pesticides DDT and HCH has been cleared up, the question of their chronic toxic effects is partly still the subject of controversial discussions. The author report on their own clinical observations of chronic health injuries caused after the vocational contact with these substances at their production or application. These injuries are aplastic anaemias or pannyelophthises, chronic liver injuries, such as hepatoses and their consequences, as well as neurological syndromes. All these chronic health injuries arose under condition of exposure where an exceeding of the maximally admissible concentration values was to be supposed. Diseases caused by DDT and HCH with the general population as a consequence of residue formation in food have not yet been observed. A general prohibition of these substances does not appear to be justified. A limitation of DDT in favour of other pesticides and placing at the disposal of foodstuffs free from residues for babies, young infants and liver patients should be demanded.

1939 entdeckte *Paul Müller* in Basel die insektizide Wirksamkeit des DDT. Damit begann die Ära der synthetischen Kontaktinsektizide aus der Reihe der Chlorkohlenwasserstoffe. Ihrem Einsatz bei der Bekämpfung von Krankheitserreger übertragenden Insekten und von Pflanzenschädlingen verdanken Millionen Menschen ihr Leben. Seit Einführung dieser Mittel wird die Boden-

Новые основные принципы селекции карпа,
*Авторы: Лайош

Ezekből következik, hogy ha meg akarjuk találni a nagyértékű, fehérjedús takarmánymennyiség gazdaságos kihasználásának módját, úgy

1. meg kell állapítani a hulladék toxikus hatását és ki kell kutatni a veszélytelen takarmányozás lehetőségeit és

2. meg kell találni a víz eltávolításának leggazdaságosabb módszerét, hogy a nyersen fel nem etethető mennyiségből tárolható takarmány készülhessen.

A kénessavtartalom tekintetében eddig az volt a felfogás, hogy annak ártalmas hatásától tartani nem kell, mert *az állat a rossz szagú takarmányból csak oly kis mennyiséget hajlandó elfogyasztani, hogy abból ártalom nem származhat.*

Hatodik hónapja folyó etetési kísérleteim ezt a feltevést megdöntötték. Amikor nehéz takarmánybeszerzési viszonyok következtében anyakocák és süldők máról-holnapra csak ilyen hulladékot kaphattak, 1—2 napi húzódozás után minden állat mohón ette a méregtartalmú takarmányt. Azóta pedig annyira megszerették (és annyira nem törődnek a kénes szaggal), hogy mikor az állományt — a takarmánykihasználás kikutatására — több csoportra osztottam, az egyik csoport, amelyik kontroll céljából nem kapott többé a hulladékból, csak néhány napos szoktatás után volt hajlandó a *méregnélküli* takarmányt oly jóízűen és oly mennyiségben fogyasztani, ahogy azt azelőtt a kénes szagú takarmánnyal tette.

A szakirodalom igen kevés adatot nyújt arra, hogy a kéndioxid, illetve a kénessav perorális felvétele milyen ártalmakat idéz elő. Hogy a gáz alakjában az állati szervezetbe jutó kéndioxid a légutak megletegedésére és sokszor elhullásra vezet, arra sok adat van, de hogy a tápcsatornán felvett kénessav milyen ártalmat okoz, arra a szakirodalomban csupán egy adatot találtam: *Kionka* japán tudós közel ötven év előtti észleletét.

Tizenhat évvel ezelőtt *Berke Péter* és *Bocsó József* végzett kénessav tartalmú takarmánnyal etetési kísérletet Magyaróváron. Ők kénessav tartalmú szárított répaszeletet etettek és több, különféle állattal 5—48 napon át folytatott etetés eredményeként azt állapították meg, hogy a felvett kénessav *semmiféle hátrányos befolyást nem gyakorolt az állatok étvágyára, közérzetére és emésztésére.*

Egyéb ártalomra a kísérlet nem terjedt ki.

Ezzel szemben *Kionka* 1902-ben azt az észleletet hozta nyilvánosságra, hogy kutyák által hosszabb időn át felvett, aránylag kis napi adagú kénessav *terhes állatoknál jelentős ártalmakat okoz:* a foetus pusztulását, elvetélést és a világra jött állatok életképtelenségét.

Kionka észlelete arra adott ösztönzést, hogy meglehetősen extrém kísérlettel próbáljuk felderíteni, milyen összefüggés van a kénessav és az ily takarmányt fogyasztó sertések körében jelentkező gyakori elvetélés között.

Ezekkel az elvetélésekkel mindeddig nem sokat törődtek. Különféle más okra, legtöbbször *Bang*-fertőzésre vezették azokat vissza.

Három különféle származású és jellegű, egészséges anyakocával kezdtük meg a kísérletet.

Az egyik egy 14 hónapos, 150 kg súlyú, előhasi berkshirei koca, a második egy egyesztendős, 124 kilós, ugyancsak előhasi, durva-simaszőrű, fekete, ú. n. háerkai fajta volt, a harmadik pedig egy kétéves, 170 kg súlyú, másodszor ülő yorkshire.

Az első kocát 1949 szeptember 3-án, a másodikat szeptember 22-én, a harmadikat október 25-én húgattuk be.

Az etetési kísérlet 1949 november 3-án kezdődött

Előtte az állatok takarmánylisztből, extrahált napraforgódarából és nyers répaszeletből álló keveréket kaptak.

November 3-án minden átmenet nélkül olyan keményítőgyári, *szűrőprésen áthajtott* moslékot kezdtünk etetni, amelyben kb 50 % szárazanyag volt.

Néhány nap alatt a kocák elérték azt a fejadagot, napi 3 kg-ot, amellyel

0·560 kg nyers proteinhez,
0·256 kg nyers zsírhoz és
1·075 kg keményítőhöz jutottak.

Ebben a takarmányban felvettek fejenként és naponként

0·460 kg emészthető fehérjét
1·770 kg keményítőértéket, ezenkívül
256 mg szabad kénessavat és
1660 mg össz. kénessavat.

Az állatok ezt a takarmányt egy-két napi szoktatás után jó étvággyal fogyasztották. Emésztési zavar nem jelentkezett.

A november 8-án és december 8-án végzett súlymérés szerint az eltelt egy hónap alatt

az első koca ..... 6·4 kg
a második koca .. 6·2 kg
a harmadik koca .. 8·2 kg súlygyarapodást

ért el.

Az első koca december 26-án, vemhessége 112. (!) napján lecellett. Hét malacának átlagos alomsúlya 1·36 kg, a kiegyenlítettsége 94·5 % volt.

A világra jöttük után semmi rendellenességet sem mutató malacokon később bágyadtság jelei mutatkoztak és ez lassan letargiává fejlődött. Ezért életük 30. órájában 30 köbcentiméter tízszázalékos szőlőcukor oldatot kaptak, bőr alá fecskendezve. Reggelre, mielőtt még megérték volna életük 48. óráját, mind a hét malacot kimúlva találtuk.

Az *Állategészségügyi Intézet* boncolási lelete szerint mind a hét hullán szeptikus gyomor- és bélgyulladás volt megállapítható.

Ebből a leletből *Bang*-fertőzésre kellett következtetni, de a vérvizsgálat negatív leletet adott. Így — a fentemlített *Kionka*-észlelet alapján — csak arra kellett következtetni, hogy a kénessav okozta a malacok pusztulását, amelyet az anyjuk, vemhességének második felére eső 54 napon keresztül felvett.

Erre az alapos gyanúra tekintettel 1950 január 1-től a másik két kocának már nem adtunk többet a kénessavas takarmányból.

A második koca 1950 január 16-ra virradóra, vemhessége 116. napján ellett. Reggel az anya mellett hat malachullát találtunk.

A boncolás itt is szeptikus gyomor- és bélgyulladást eredményezett. Egyik hulla tüdeje légtelen volt, ez tehát halva jött a világra. A *Bang*-vérvizsgálat itt is negatív volt. Így mostmár kétségtelennek látszott, hogy az anya 58 napon át

tartó kénessav felvétele folytán pusztultak el a malacok és a mérgező hatást már nem tudta elhárítani az, hogy a vemhesség utolsó 16 napján az anyaállat már nem kapott többé kénessavat.

A harmadik koca február 20-án, 118 napra ellett és 16 malacot hozott a világra. Ez az anya vemhességének első 67 napján kapott kénessavas takarmányt, a további 71 napon át már nem jutott méreganyaghoz.

A sok malac közül kettőt mi pusztítottunk el, néhányat agyonnyomott az anyja, de a többi makkegészséges. Nyolcheles korukban 14·0 kg volt az átlagsúlyuk.

Ezeket a jelenségeket összevetve megállapítható, hogy a kénessav a fogamzásra és *a vemhesség első felében* a magzatok fejlődésére ártalmas hatást nem gyakorol, a vemhesség további időszakában adagolt kénessavtartalmú takarmány azonban elpusztítja, illetve életképtelenné teszi a magzatot. A vemhesség utolsó 16 napján már nem tudja a szervezet kiheverni azt a mérgezést, amelyet a vemhesség megelőző, 58 napig tartó szakában szenvedett.

A gyakorlati kiértékelés szempontjából figyelembe esik, hogy a kísérleti állatok legfeljebb csak negyedannyi kénessavhoz jutottak, mint amennyit a keményítőgyárak által forgalomba hozott friss moslék etetése esetén kaptak volna.

A gyárak olyan moslékot hoznak forgalomba, amelynek szárazanyagtartalma 15—25% között változik, a kísérletünkhöz viszont — külön utánjárással szerzett — olyan moslékot használtunk, amelynek szárazanyagtartalma 50 % volt. Mi minden kg *feletetett szárazanyaggal* csupán 1·0 kg kénessav tartalmú vizet vittünk az állat tápcsatornájába, a 20% szárazanyagtartalmú moslékot etető sertéstartó pedig ennek négyszeresét.

Ha a sertéstartó az általa feletetett takarmánynak *csupán negyedrészét* nyújtja *friss* keményítőgyári moslék formájában, már akkor is annyi méreganyagot ad az állatnak, amennyit mi adtunk a kísérleti állataiknak, már akkor is olyan veszélynek tette ki az állatát és annak ivadékait, mint amilyennek mi kitettük a miénket.

Pontos számvetés megdöbbentő képet adna arról hogy mennyi kárt okozott a magyar állattenyésztésnek az a sok moslék, amelyet a négy magyar keményítőgyár ebben a gazdasági évben a sertéstartók *vemhes állatai* számára kiszolgáltatott.

*

Korosításra tartott sertések takarmányozásánál nem ilyen sötét a helyzet. Ebben az irányban folytatott — a fentivel hasonlóan extrém-kísérlet megnyugtató eredményeket adott.

Erre a kísérletre *egy alomból* származó nyolc darab öthónapos york süldőt állítottunk be.

Ezek, a beállítás alkalmával 42 kg átlagsúlyú süldők, az első hónap alatt olyan, a fentiekhez hasonló *préselt* moslékot kaptak, amelyeknek napi fejadagjában, napi 2 kg-ban, volt:

1·440 kg szárazanyag,
0·383 kg nyers protein,
0·175 kg nyers zsír,
0·745 kg keményítő.

Ebben felvettek fejenként és naponként:
0·314 kg emészthető fehérjét és
1·230 kg keményítőértéket, ezenkívül
176 mg szabad kénessavat és
1130 mg össz. kénessavat.

Egyhónapi etetés után megejtett súlymérés felderítette, hogy a takarmánykihasználás igen alacsony volt, az állatok fejenként és naponként (átlagban) mindössze 214 gr súllyal gyarapodtak.

A második kísérleti hónapban a süldők a fenti fejadagnak 120 százalékát kapták és — a nyálképzés fokozása érdekében — még 200 gr darált tengericsutkát kevertünk a napi fejadaghoz. Ebben a hónapban már sokkal jobb volt a takarmánykihasználás, a süldők (átlagban) napi 400 gr súlygyarapodást értek el.

*

Tehénnel folytatott etetési kísérlet* a tejválasztás tekintetében adott kedvezőtlen eredményt.

Egy lapályfajta, frissfejős tehén szabályszerűen kiszámított abrakjába naponként 6·0 kg körülírt moslékot adtunk. (A tápértékét természetesen beszámítottuk a szabványba.)

Ezzel az etetéssel a tehén naponta 653 mg szabad és 4.267 mg össz. kénessavhoz jutott.

A kísérlet megindításának hetében a napi tejhozam 22 liter volt. Ez pár nap multa csökkenni kezdett és ez a csökkenés a harmadik héten napi nyolcliteres tejhozamnál állott meg.

A második hónapban a tehén kénessavmentes takarmányt kapott. A tejhozam lassan emelkedni kezdett, de 16 liter fölé nem emelkedett.

A harmadik hónapban a tehén újból az első havi takarmányt kapta, napi 22 literes tejszolgáltatásra kiszámított mennyiségben. A 16 literes napi hozam 10 literre esett.

A negyedik — megint csak kénessavmentes — hónapban újból emelkedett a tejhozam, de csak 13 litert ért el.

Ezek szerint — ha egy állattal elért eredményt figyelembe lehet venni — *a kénessavtartalmú takarmány etetése jelentékenyen csökkenti a tejelválasztást.*

*

A fent leírt észleleteket nem lehet végleges és teljes eredményeknek tekinteni. A kezdett munkát sokféle irányba ki kell még terjeszteni (pl. a kénessav hatása apaállatok szaporító képességére, korosításra tartott sertések takarmányértékesítésére, stb.). Ahhoz azonban elegendőek, hogy irányt mutassanak. Ez az irány pedig az, hogy *sürgősen be kell szüntetni a kénessavas moslék forgalomba hozatalát* és meg kell kezdeni a tartósított és méregtelenített keményítőgyári kukoricamoslék készítését.

Ezért a tartósítás legalkalmasabb módozatait is ki kellett kutatni.

A tartósításra vonatkozó kísérletek abból álltak, hogy kis mennyiségekkel, hevenyészve összeállított berendezésekben kipróbáltuk azokat a bevált vízelvonó eljárásokat, amelyeket külföldi, korszerűen berendezett gyárak alkalmaznak.

A kísérletek kielégítő eredményeket adtak. A légszáraz állapotig menő vízelvonás nem csak azt eredményezte, hogy olyan szemcsés szerkezetű

* Kevésbé kielégítő eredmény, mert csak egy tehénnel végzett kísérlet. Szerk.

anyagot kaptunk, amely éppen úgy tárolható és eltartható, mint pl. a szemes tengeri, hanem azt is, hogy *az anyagban levő kénessav majdnem teljes egésze eltávozott* és így gyakorlatilag teljesen méregtelen takarmányt kaptunk.

A leggazdaságosabb eljárás megkeresésénél a hulladék természetéből kellett kiindulni.

A keményítő gyártásánál a szemestengerit *kéndioxid* tartalmú vízben áztatják, hogy a keletkező kénessav a kukoricaszem szarószerű részeit megpuhítsa és a bezárt keményítőszemcsék ilyen feltárás következtében könnyebben kinyerhetők legyenek.

A kénessavoldat a kukorica szerves anyagával többé-kevésbé labilis komplexvegyületeket alkot. Ezeknek mérgező hatása az állatoknál akkor jelentkezik, amikor az alapjában nem mérgező kénessavas sókból a mérgező *nátriumbiszulfit* lehasad. *Franz, Jakobj, Kerp,* és *Walbaum* német kutatók már évtizedekkel ezelőtt kikutatták e komplexek viselkedését és munkájuk nyomán alakult ki a keményítőgyári törköly és moslék feldolgozásának akkori technológiája: az anyagból a vizet *folytonos keverés közben elfőzték.*

A mi kutatásunk abból indult ki, hogy ez a szárítási és egyben mérgetlenítési eljárás takarmánygazdálkodási szempontból nem alkalmas, mert *drága.* A termikus vízeltávolítás a jelen esetben — amikor 10% szárazanyag tartalmú hulladékról van szó — olyan sok hőenergiát igényel, hogy annak költségét és hozzá a folytonos keverési energia költségét állati takarmány nem bírja el.[1])

Az eddigi eljárás költségeivel szemben jelentős csökkenés érhető el azzal, ha a kénessav tartalmú víznek egyik részét a termikus eljárásnál sokkal olcsóbb *mechanikai művelettel* távolítjuk el és csak az így el nem távolítható vízmennyiség eltávolításánál kerül alkalmazásra a drága *termikus módszer.*

A problémát egyszerű vízeltávolítási kérdéssé redukálja az a körülmény, hogy a mérgetlenítéshez sincs szükség az eddig alkalmazott *állandó keverésre* és közvetett gőzzel történő, 100 C°-nál magasabb hőmérsékleten végrehajtott *elfőzésre.* A kénessavas komplexek vizes oldatai ugyanis már szobahőmérsékleten is könnyen komponenseikre esnek szét, a nagy higítás folytán nagy a disszociáció és ha a kedvező feltételek adva vannak, a kéndioxid igen könnyen és igen rövid idő alatt elillan.

A keményítőgyári hulladéknál ezek a kedvező feltételek részben adva vannak, részben néhány egyszerű berendezés felállításával megteremthetők.

Tudvalevő, hogy a keményítő gyártásánál kétféle hulladék keletkezik: a *törköly* és a *moslék.*

A kénessavas vízben áztatott, megpuhult kukoricát megőrlik és az őrleményt hő vízmennyiséggel keverve szitára viszik. A lisztfinomságú, pépessé vált őrleményrészt a víz keresztülmossa a szitán és ez hosszú, majdnem vízszintes asztalokon ülepedésre kerül. Ami leülepszik belőle: az a keményítő, ami az ülepítő asztalról, vagy tálcáról továbbfolyik: az a *moslék.*

A szitán át nem jutott őrleményrész, amely főleg a héj és csíra durva szemecséiből áll, a szita tetején tovább sodródik. Ez a *törköly.*

(E két elnevezést érdemes tisztázni, mert a magyar terminológia igen zavaros, felcserélí a két elnevezést és más megjelöléseket *(glutin, léha, hempli, sárga,* stb.) is használ.)

A laza szerkezetű törkölyről a víz egy felállítandó szita segítségével olcsón leszűrhető. Ha utána centrifugálunk, a törkölyben olyan kevés víz marad, hogy az jelentéktelen költséggel kiszárítható. A manipuláció alatt a törkölyt a levegő átjárja, az anyagszemcsék felületének összege igen nagy, így a kéndioxid eltávozása gyors és erőteljes. *A kiszárított anyag gyakorlatilag kénessavmentes.*

A moslék más szemszerkezetű és így más feldolgozást kíván. Ülepítéssel az eredetileg 5—15 százalékos szárazanyagtartalom csupán 30—35%-ra emelhető. Az így kapott kenőcsszerű anyagot — a szemcsék őrlési finomsága szerint — vagy centrifugával, vagy szűrőpréssel kell tovább víztelenítení. Az ily módon nyert, 55—65% szárazanyagtartalmú anyagban levő víz már csak termikus úton távolítható el.

A négy magyar gyár egyikében sem alkalmazzák a víz és a méreganyag eltávolításának ezt a gazdaságos módszerét.

A gyárak egyáltalán nem sokat törődnek a víz eltávolításával, inkább arra törekednek, hogy minél több vizes moslékot és törkölyt adjanak el. Nyári dologidőben, amikor csökken a kereslet a csak névleg olcsó, de felmerülő magas szállítási költségek miatt igen drága moslék iránt, az anyag egy része elpusztul. Ennek elkerülése érdekében oly irányban kísérleteztek, hogy állami gazdaságokat próbáltak nyers moslék átvételére kényszeríteni.

Ez a takarmánygazdálkodási szempontból káros állapot két okra vezethető vissza. Az egyik az, hogy *a hulladék feldolgozására szolgáló berendezések hiányosak és korszerűtlenek:* csak a termikus víztelenítésre nyújtanak lehetőséget.[2])

A másik ok az ármegállapításban keresendő.

Az ipari takarmányok árának megállapításánál annak idején — igen helyesen — azok takarmányértékéből és némely kiviteli célra alkalmas takarmánynál (pl. a sörélesztőnél) az exportparitásból indultak ki. A nyers és szárított moslék ily módon megállapított eladási ára között oly csekély különbség van, hogy az nem fedezi a mai ócska berendezésekkel elvégezhető szárítás önköltségeit.

Ezért nem törődnek a keményítőgyárak a nyers moslék kénessav tartalmával és az abból előállható állattenyésztési károsodással. *A nyers moslék értékesítése effektív bevétel jelent, a szárítás pedig ráfizetést jelentene.*

Takarmánygazdálkodási szempontból, az állattenyésztés érdekében azonban a mai állapot gyökeres változást kíván. A mérgezési lehetőségek kiküszöbölése és nagyértékű takarmánymennyiség biztosítása érdekében vagy *fel kell emelni a szárított termék árát* olyan szintre, hogy a gyárak haszonnal, vagy legalább is ráfizetés nélkül száríthassanak, vagy pedig *át kell térni a víztelenítésnek fejlettebb kocszerű és a termikusnál sokkal olcsóbb módszerére.*

Nem lehet kétséges, hogy melyik a helyesebb megoldás.

[1]) Nem tartozik ugyan szorosan ide, de meg kell ülepíteni, hogy a sörélesztőnél kapcsolódó a helyzet.

[2]) A sörélesztőnél a termikus szárítás magas költsége az exportlehetőséget veszélyezteti, ami szépen fogalmak jelent nagy kárát.

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Рациональное использование кукурузной барды крахмальной промышленности.

Автор: *Имре Вираг*

Резюме.

Крахмальная индустрия Венгрии обрабатывающая кукурузу, ежегодно продуцирует великое количество отбросов, богатое в белках, соотвеcтвующее 3500 тоннам корма. Использование его не так происходит, как это требуют интересы животноводства. Причина затруднений заключается в том, что отбросы содержат много сернистой кислоты и воды.

В связи с кормами содержающими сернистую кислоту до последних времен существовало то мнение, что отравления животных не нужно боятся, так как из корма, имеющего неприятный запах животное и так много не съедает. Автор на основании опытов констатирует, что это мнение является ошибочным, так как проголодавшее животное из жидкого корма съедает такое количество которое может ему повредить.

Насчет того какие токсические действия вызывает пероральный (через рот) прием сернистой кислоты, в международной научной литературе мало данных. Японский ученый Киоиса у собак наблюдал аборт, венгерские исследователи Берке и Боч при экспериментах с 48-ми дневным кормлением не замечали токсических действий.

Автору стало заметным, что у многих свиноматок съедавших барду из крахмальных заводов наступил аборт. Поэтому он с тремя свиноматками начал кормальные эксперименты. Все три животные получали обезводненную прессованием барду которая содерживала 50% сухого вещества; этим кормом каждое животное получали ежедневно в поголовно 256 мгр. свободной и 1660 мгр. общей сернистой кислоты.

Первая свиноматка получала из этого корма во второй половине беременности в течение 54 дней вплоть до дня опороса. Все семь поросят родившиеся живыми в течение 48 часов погибли.

Вторая свиноматка получала данный корм в 43—100-ых днях беременности (в течение 58 дней). Родилось шесть поросят: пять живых, одно мертвое, но в течение нескольких часов все погибли.

Третья свиноматка получала сернистокислотный корм в первых 67 днях беременности. С поросятами этой матки не было никакой беды, и в 8-ми недельном возрасте все были здоровыми.

Ни одна из трех свиноматок не была заражена Бангом, таким образом наблюдавшееся явление могла причинить только сернистая кислота.

Содержимые для племенной цели отъемыши в течение почти шести месяцев получали корм содержающий сернистую кислоту (176 мгр. свободной и 1130 мгр. общей сернистой кислоты) и никакого вреда на них не оказывалось. Соответственно своему возрасту развивались они хорошо.

С одной коровой проводили эксперимент таким образом, что в раздаче 6 кг на день одни месяц ей давали барду, один же месяц ей не давали барды содержащей сернистую кислоту. В этом количестве содержалось 653 мгр свободной и 4267 мгр общей сернистой кислоты. Сернистая кислота снижала продукцию молока. В первых месяцах она немного повышалась, но в результате четырех месяцев продукция с начальных 22 литров упала на ежедневные 13 литров.

Венгерские заводы не приготовляют сушеную барду, так как они устроены только на термическое сушение старой системы. Это же ведет к высокой себестоимости продукции, и цена готового корма в таком случае будет высокой цены установленной органом власти. Автор при своих опытах с современными сушильными методами приходит к выводу, что большую часть воды необходимо удалить помощью центрифуги, сжимания и центрифуги, а только остающуюся воду нужно удалить термическим путем. Этим методом можно приготовить из отбросов крах мальных заводов дешевый, устойчивый и безвредный корм.

Utilisation rationnelle de la lavure de maïs des amidonneries.

Par Imre Virág.

Résumé.

Les amidonneries hongroises travaillant le maïs produisent des déchets riches en albumine dans une quantité annuelle correspondant à 3500 tonnes de fourrage. L'utilisation de ce sous-produit précieux ne se fait pas comme l'exigent les intérêts de l'élevage. Les difficultés sont causées par la grande teneur des déchets en acide sulfureux et en eau.

Jusqu'à présent l'opinion générale concernant les fourrages à une teneur d'acide sulfureux tenait qu'il ne faut pas craindre l'empoisonnement des animaux car ils ne consomment que peu du fourrage malodorant. Par ses recherches l'auteur a constaté que cet avis est faux car l'animal qui a faim consume autant du fourrage à une teneur de poison qui lui peut nuire.

Dans la littérature spéciale internationale on ne trouve que peu de données concernant l'effet toxique de la réception perorale de l'acide sulfureux. Le savant japonais Kioaka observa des avortements chez des chiens, les savants hongrois Berke et Bocsó pendant leurs expériences de 48 jours n'observèrent aucun effet toxique.

L'auteur fut frappé par le fait que de nombreux porcs consumant de la lavure des amidonneries avortèrent. Il commença donc des expériences d'alimentation avec trois truies. Tous les trois animaux obtenaient une lavure déssechée par pressage contenant 50 p. c. de matière sèche. Par ce fourrage chaque animal reçut par jour et par tête 256 mg d'acide sulfureux libre et 1660 mg d'acide sulfureux total.

L'une des truies fut alimentée de ce fourrage dans la seconde moitié de sa gestation pendant 54 jours jusqu'au jour de la mise bas. Ses sept cochons nés vivant périrent tous en 48 heures.

La seconde truie obtint du fourrage les 43—100 jours de la gestation (durant 58 jours). Elle mit bas six cochons, cinq vivant, un mort, mais en quelques heures tous périrent.

La troisième truie obtint du fourrage à acide sulfureux les 67 premiers jours de la gestation. Rien n'est arrivé à ses cochons, à l'âge de huit semaines il étaient sains tous.

Toutes les trois truies sont exemptes de l'infection Bang et ainsi la cause des phénomènes observés ne pouvait être autre chose que l'acide sulfureux.

A des porcelets élevés aux fins de reproduction et nourris presque durant six mois d'un fourrage à acide sulfureux (à une teneur d'acide sulfureux libre de 176 mg et d'acide sulfureux total de 1130 mg) aucun dommage ne s'est manifesté. Ils se développèrent bien conformément à leur âge.

Une expérience fut effectuée d'une vache de sorte que par mois à tour de rôle elle reçut et ne reçut pas de lavure à acide sulfureux dans une quantité de 6·0 kg par jour. Cette lavure contenait 653 mg d'acide sulfureux libre et 4267 mg d'acide sulfureux total. L'acide sulfureux diminuait le rendement de lait qui augmentait de nouveau quelque peu dans les mois exempts de poison, mais en fin de compte le rendement de lait originellement de 22 liter par jour diminuait à 13 liter par jour.

Les usines hongroises ne produisent pas de lavure déssechée, n'étant installées qu'au sechage thermique d'un système ancien. Ça résulte à des frais de revient élevés et le prix du fourrage serait plus élevé que celui fixé par le tarif officiel. Sur la base de ses expériences effectuées par des procédés modernes de sechage auteur est parvenu à la conclusion que la plus grande partie de l'eau doit être éloignée par filtrage, pressage et par centrifugation et seulement le reste par voie thermique. Par ce procédé on peut produire des déchets des amidonneries un fourrage bon marché, durable et exempt de poison.

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26:59 (1956)

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КАФЕДРА ОБЩЕЙ ГИГИЕНЫ ЛСГМИ
Зав. кафедрой — член-корр. АМН СССР, проф. Р. А. Бибаева

ВЛИЯНИЕ СЕРНИСТОГО ГАЗА НА ОРГАНИЗМ

Н. В. Волкова

Директивы XX съезда Коммунистической партии Советского Союза по шестому пятилетнему плану развития СССР на 1956—1960 гг. определили дальнейший рост промышленных предприятий, жилищного строительства, улучшение коммунального и бытового обслуживания населения городов, рабочих поселков и городского благоустройства.

Ярким выражением заботы партии и правительства о здоровье населения являются постановления о мерах борьбы с загрязнением атмосферного воздуха и об улучшении санитарно-гигиенических условий населенных мест. Эти постановления решают одну из важнейших гигиенических проблем — проблему оздоровления внешней среды.

В этой связи особое значение приобретает вопрос изучения реакции организма на длительное воздействие факторов внешней среды малой интенсивности, поскольку здоровье населения находится в прямой зависимости от внешней среды. С увеличением строительства промышленных предприятий и потребления ими каменного угля значительно возросло задымление городов. Одновременно с ростом количества твердых загрязнений в воздухе происходит и увеличение количества газообразных сернистых соединений. Из всех газов, загрязняющих атмосферный воздух населенных мест, наибольшее распространение и значение имеет сернистый газ. Наличие сернистого газа в воздухе промышленных городов настолько характерно, что его считают показателем «порчи» атмосферного воздуха.

Исследования советских авторов по загрязнению атмосферного воздуха показали, что среднегодовые концентрации сернистого газа в отдельных городах колеблются от 0,17 до 0,51 мг/м³, максимальные среднесуточные концентрации достигают 0,94—2,2 мг/м³. Максимальные разовые концентрации, особенно вокруг крупных промышленных объектов, могут достигать 20 мг/м³ и выше. Такие концентрации сернистого газа,

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конечно, не могут быть безразличными для здоровья населения, особенно, если учесть длительность их влияния.

Наиболее крупными трудами в этой области являются работы Ленинградской научно-исследовательской лаборатории коммунальной гигиены и кафедры общей гигиены ЛСГМИ, выполненные под руководством Р. А. Бабаянца, и комиссии Государственной санитарной инспекции под руководством В. А. Рязанова, на основании которых были предложены допустимые нормы загрязнения атмосферного воздуха. Эти работы являются первым этапом в изучении и нормировании концентраций сернистого газа в городском воздухе.

Вторым этапом в разрешении этой проблемы является в настоящее время физиологическое обоснование предложенных норм путем применения чувствительных физиологических методов, прежде всего метода условных рефлексов.

Экспериментальное изучение токсичности сернистого газа проводилось на основе клинических наблюдений за течением этой интоксикации, а также с точки зрения влияния больших концентраций этого газа на функции различных органов и систем в остром опыте.

Работами советских авторов (М. М. Никулина, Ц. Д. Гетман, М. Н. Марголина, 1934; И. В. Сидоренков, 1952; А. В. Сакиин, 1954) доказано, что сернистый газ, помимо местного раздражающего действия, обладает значительным резорбтивным влиянием, вызывая резкие сдвиги в биохимических процессах организма.

Хроническое действие сернистого газа на организм изучалось путем наблюдения над рабочими промышленных предприятий (А. Е. Тер-Газаров, 1926; Н. К. Шифрин, 1926; Rostoski und Crecelius, 1930; Kehoe, Machle, Kitzmiller, 1932; В. А. Литкенс, 1951; Н. П. Стерехова, 1954 и др.). При этом наблюдались, наряду с изменениями в дыхательной и пищеварительной системах, существенные отклонения углеводной и пигментной функций печени, а также показателей крови.

Существование хронического отравления сернистым газом подтверждается не только наблюдением над людьми, подвергавшимися воздействию этого газа в производственных условиях, но и в экспериментах на животных (Kisskall K., 1904; Ronsani E., 1908; Vedder and Armstrong, 1933 и др.). Опыты показали, что сернистый газ вызывает падение веса животных, изменение красной крови, а также снижает сопротивляемость организма к инфекциям.

Однако следует заметить, что все исследователи изучали в основном влияние высоких, почти не встречающихся или редко встречающихся в воздухе открытых мест, концентраций сернистого газа.

Ввиду огромной важности проблемы оздоровления внешней среды и отсутствия экспериментальных данных о хроническом

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действии небольших концентраций сернистого газа на организм, данная работа была поставлена с целью экспериментальной проверки токсичности этих концентраций и накопления данных для обоснования предельно допустимых концентраций сернистого газа в атмосферном воздухе промышленных городов.

Задача состояла в исследовании на экспериментальных животных хронического влияния небольших концентраций сернистого газа на высшие отделы центральной нервной системы, на дыхание, кровь, вес животных и патолого-гистологическую картину верхних дыхательных путей.

В наших исследованиях мы руководствовались основным положением учения И. П. Павлова о решающем значении высшей нервной деятельности при установлении влияния факторов внешней среды на организм.

Учитывая, что взаимоотношения организма с внешней средой осуществляются с помощью безусловных и условных рефлексов, а, с другой стороны, значительную чувствительность метода условных рефлексов, мы считали необходимым попытаться установить минимальные концентрации сернистого газа, действующие на центральную нервную систему.

Изучение пищевых двигательных условных рефлексов проводилось на белых крысах по усовершенствованной методике Л. П. Котляревского (1951), позволяющей иметь объективное представление о величине латентного периода условного рефлекса по записи на кимографической ленте. Установка, применявшаяся нами для исследования пищевых двигательных условных рефлексов, состояла из деревянной камеры с зеркалом, через которое экспериментатор наблюдал за поведением животных, аппарата для дачи условных сигналов, устройства для регистрации условных реакций.

Условные раздражители, применявшиеся нами при изучении условнорефлекторной деятельности крыс, были отнесены к звуковому анализатору. До затравки у подопытных крыс были выработаны положительные (на тон № 1 — 1200 гц, прерывистый 0,5 сек.) и отрицательные (на тон № 2 — 630 гц, длинный) двигательные пищевые условные рефлексы. Во время опытов тон № 1 повторялся 9 раз, а тон № 2 — 2 раза с интервалами последовательно в 1 и 2 мин. Длительность изолированного действия условного раздражителя равнялась 5 сек. Через 5 сек. условный раздражитель подкреплялся безусловным (пищей). Общее время действия условного раздражителя было равно 10 сек. После выработки прочных положительных условных рефлексов были выработаны тормозные условные рефлексы на тон № 2, время действия которого равнялось 10 сек., при этом подкрепление пищей не производилось.

Всего было проведено более 2000 опытов. После выработки прочных положительных и отрицательных условных рефлексов крысы были подвергнуты хронической интоксикации в газовой камере объемом 210 л.

Перед началом хронического опыта в течение нескольких дней был отрегулирован режим работы камеры. Хроническое воздействие сернистого газа на животных было начато только после того, как в камере была получена необходимая для работы концентрация газа.

Все подопытные животные были разделены на три группы, соответствующие трем сериям опытов. Каждая серия крыс подвергалось хроническому воздействию сернистого газа в течение полутора месяцев, по 3,5 часа в день.

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Действие сернистого газа на условнорефлекторную деятельность

Первая серия опытов. В первую серию входили крысы № 1, 7 и 8. Во время опыта концентрация сернистого газа в газовой камере колебалась от 16 до 24 мг/м³ (в среднем 20 мг/м³). До затравки у всех крыс этой серии были выработаны прочные условные рефлексы, величина скрытого периода условного рефлекса была относительно постоянной, дифференцировка — абсолютной. Для примера приводим кимограммы некоторых опытов с крысами этой серии (рис. 1).

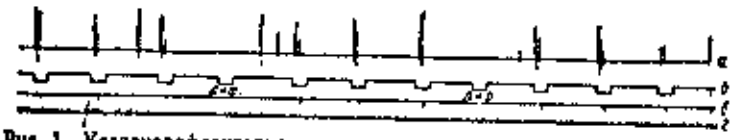

Рис. 1. Условнорефлекторная деятельность у крысы № 1 до начала работы в газовой камере (опыт № 173, 23/IV 1954 г.).
а — условная реакция животного; б — условный раздражитель; в — безусловный раздражитель; г — отметка времени.

Из приведенной кимограммы видно, что у крысы № 1 условнорефлекторная реакция до затравки — прочная, крыса открывала дверцу задолго до дачи безусловного раздражителя (пищи). Латентный период условного рефлекса находился в пределах 1,4—2,6 сек. На тон № 2 (дифференцировка) крыса не реагировала.

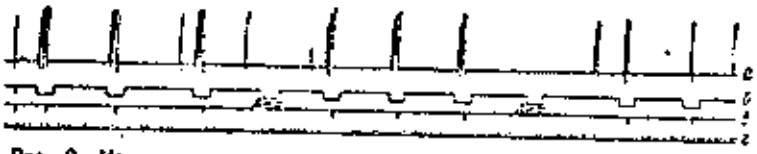

Рис. 2. Изменение положительного условного рефлекса на тон № 1 у крысы № 8 (обозначения те же, что на рис. 1).

После окончания затравки крысы поступали в камеру для условных рефлексов, где производились наблюдения за изменениями в центральной нервной системе, наступившими в результате воздействия сернистого газа.

Уже первый день затравки показал, что концентрация сернистого газа 20 мг/м³ оказывает влияние на условнорефлекторную деятельность крыс. Первые изменения были отмечены со стороны положительного условного рефлекса на тон № 1 (рис. 2).

У всех крыс этой серии в первый день затравки отмечалось удлинение реакции на отдельные условные раздражители.

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Латентный период у крысы № 1 увеличился до 4 сек., у крысы № 7 — до 3,6 сек., у крысы № 8 — до 3—5 сек. Хотя увеличения латентного периода условного рефлекса были единичными, они обращали на себя внимание величиной, которая до затравки не отмечалась. Дифференцировка в первый день затравки продолжала оставаться абсолютной.

По мере продолжения опытов в газовой камере наблюдалось дальнейшее углубление нарушений со стороны центральной нервной системы. Увеличение латентного периода стало значительным и постоянным. На рис. 3 показаны изменения латентного периода в секундах у крысы № 1, наступившие после затравки, по сравнению с латентным периодом, имевшим место до затравки.

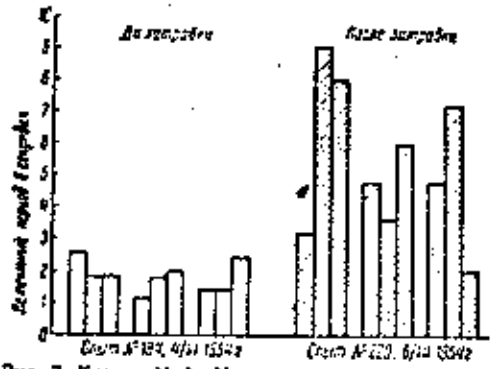

Рис. 3. Крыса № 1. Нарушение условнорефлекторной деятельности при концентрации SO_2 20 мг/м³.

Латентный период условного рефлекса до затравки у этой крысы (опыт № 194) был в пределах 1,2—2,6 сек., а после затравки (опыт № 220) — в пределах 2—9 сек. Таким образом, после затравки сернистым газом в концентрации 20 мг/м³ наступило резкое удлинение условнорефлекторной реакции (почти в 3,5 раза).

Изменения условнорефлекторной деятельности у крысы № 7 были аналогичными. Если до затравки латентный период условного рефлекса был в пределах 0,4—2,8 сек., то после затравки он увеличился до 1—8 сек., т. е. почти в 3 раза.

Латентный период условного рефлекса у крысы № 8 увеличился также почти в 3 раза — с 0,8—2,2 сек. до 1,6—6 сек.

На рис. 4 приведены, на примере крысы № 1, результаты изменения величины латентного периода условного рефлекса у крыс данной серии при воздействии сернистого газа в концентрации 20 мг/м³. Нижняя кривая показывает изменения латентного периода условного рефлекса за пять опытов до за-

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травки, а верхняя кривая — те же изменения за пять опытов во время затравки сернистым газом. Средняя величина латентного периода условного рефлекса у этой крысы увеличилась в два с лишним раза (с 1,7 до 3,9 сек.).

В табл. 1, 2 и 3 приведены результаты трех опытов с целью сопоставления и иллюстрации изменений условнорефлекторной деятельности у крыс данной серии при концентрации сернистого газа 20 мг/м³.

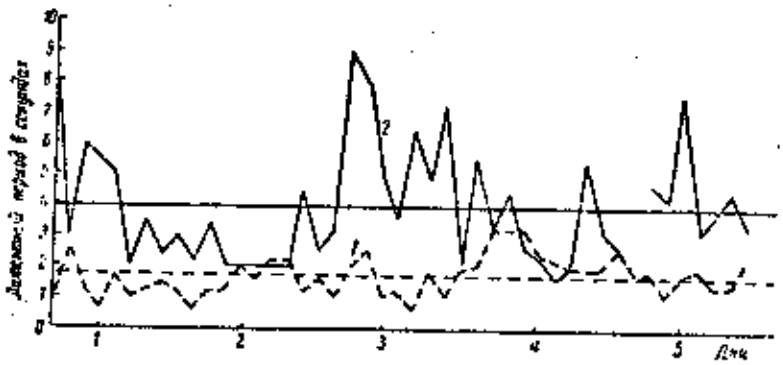

Рис. 4. Изменения латентного периода условного рефлекса в динамике (5 опытов до и после затравки) при концентрации SO_2 20 мг/м³.
1 — до затравки, 2 — после затравки.

У крысы № 7 (табл. 1) латентный период условного рефлекса до затравки колебался в пределах 0,4—2,2 сек., причем $^4/_5$ всех условных реакций имели скрытый период до 1 сек. Уже в первый день затравки отмечалось увеличение амплитуды колебания латентного периода от 0,4 до 3,6 сек., при этом только $^1/_5$ всех условных реакций имела скрытый период до 1 сек. В опыте № 237, т. е. на 31-й день затравки, увеличение латентного периода было значительным и стойким, скрытый период колебался от 1,4 до 8 сек., что в два с лишним раза больше, чем в первый день и в 3,5 раза больше, чем до затравки.

В той же последовательности приведены в табл. 2 результаты трех опытов, полученные у крысы № 8. До затравки латентный период условного рефлекса у этой крысы был в пределах 1—2,2 сек., в первый же день затравки он резко увеличился (до 5 сек.). Если в первый день затравки наблюдалось единичные случаи увеличения латентного периода (на 7 и 8-й условные раздражители), то на 24-й день отмечалось значительное повышение латентного периода вплоть до частичного выпадения условного рефлекса.

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Таблица 1

Изменения условнорефлекторной деятельности у крысы № 7 при концентрации SO_2 20 мг/м³

Время дня раздражителя от начала опыта	Раздражитель (тон)	№ раздражителя	Время изолированного действия раздражителя (сек.)	Общее время действия раздражителя (сек.)	Латентный период условного рефлекса (сек.)	Натуральный условный рефлекс
Опыт № 200, 28 мая 1954 г. (до затравки)						
9.43	№ 1	1887	5	10	1,2	+
44	№ 1	1888	5	10	1	+
46	№ 1	1889	5	10	1,4	+
47	№ 2	343			+	
49	№ 1	1890	5	10	1,2	+
50	№ 1	1891	5	10	0,6	+
52	№ 1	1892	5	10	0,6	+
53	№ 2	344			+	
55	№ 1	1893	5	10	0,4	+
56	№ 1	1894	5	10	2,2	+
58	№ 1	1895	5	10	0,6	+
Опыт № 205, 4 июня 1954 г. (первый день затравки)						
13.43	№ 1	1940	5	10	1,4	+
44	№ 1	1941	5	10	2	+
46	№ 1	1942	5	10	3,6	+
47	№ 2	355			+	
49	№ 1	1943	5	10	0,4	+
50	№ 1	1944	5	10	1	+
52	№ 1	1945	5	10	1,2	+
53	№ 2	356			+	
55	№ 1	1946	5	10	1	+
56	№ 1	1947	5	10	2,4	+
58	№ 1	1948	5	10	2,4	+
Опыт № 237, 12 июля 1954 г. (31-й день затравки)						
12.04	№ 1	2219	5	10	4	+
05	№ 1	2220	5	10	2,2	+
07	№ 1	2221	5	10	2	+
08	№ 2	417			+	
10	№ 1	2222	5	10	4,4	+
11	№ 1	2223	5	10	2,6	+
13	№ 1	2224	5	10	4,8	+
14	№ 2	418			+	
16	№ 1	2225	5	10	1,4	+
17	№ 1	2226	5	10	6	+
19	№ 1	2227	5	10	5,6	+

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Таблица 2

Изменения условнорефлекторной деятельности у крысы № 8 при концентрации SO_2 20 мг/м³

Время дачи раздражителя от начала опыта	Раздражитель (тон)	№ раздражителя	Время изолированного действия раздражителя (сек.)	Общее время действия раздражителя (сек.)	Латентный период условного рефлекса (сек.)	Натуральный условный рефлекс
Опыт № 130. 17 мая 1954 г. (до затравки)						
9.25	№ 1	1765	5	10	1	+
26	№ 1	1766	5	10	1,8	++
28	№ 1	1767	5	10	2	+
29	№ 2	325			+	
31	№ 1	1768	5	10	1,2	+
32	№ 1	1769	5	10	1,6	++
34	№ 1	1770	5	10	1,8	+
35	№ 2	326			+	
37	№ 1	1771	5	10	2	+
38	№ 1	1772	5	10	1,8	++
40	№ 1	1773	5	10	2,2	+
Опыт № 201. 4 июня 1954 г. (первый день затравки)						
15.25	№ 1	1864	5	10	1	+
26	№ 1	1865	5	10	1,4	++
28	№ 1	1866	5	10	1	+
30	№ 2	347			+	
32	№ 1	1867	5	10	1,2	+
33	№ 1	1868	5	10	1	++
35	№ 1	1869	5	10	1,8	+
36	№ 2	348			+	
38	№ 1	1870	5	10	3	+
39	№ 1	1871	5	10	5	++
41	№ 1	1872	5	10	1	+
Опыт № 224. 3 июля 1954 г. (24-й день затравки)						
11.25	№ 1	2070	5	10	2,4	+
26	№ 1	2071	5	10	2,6	++
28	№ 1	2072	5	10	2	+
29	№ 2	393			+	
31	№ 1	2073	5	10	5,2	+
32	№ 1	2074	5	10	3,6	++
34	№ 1	2075	5	10	3,4	+
35	№ 2	394			+	
37	№ 1	2076	5	10	—	+
38	№ 1	2077	5	10	4,6	++
40	№ 1	2078	5	10	4,6	+

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У крысы № 1 (табл. 3) так же, как и у крыс № 7 и 8, первые изменения условнорефлекторной деятельности наступали в первый день затравки. Если в опыте № 173 (до затравки) латентный период был 1,4—2,6 сек., то в первый день он увеличился до 4 сек., причем латентный период больше чем 1,6 сек. встречался в этом опыте в 1,5 раза чаще. На восьмой день затравки (опыт № 202), помимо значительного увеличения латентного периода, наблюдалось частичное выпадение условного рефлекса. Наступившие изменения в условнорефлекторной деятельности данной крысы стойко держались в течение всей экспозиции, что видно из кимограммы (рис. 5), где представлены результаты опыта № 222 на 28-й день затравки.

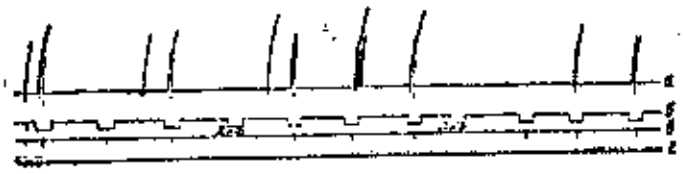

Рис. 5. Крыса № 1. Опыт № 222, 8.VII 1954 г. Частичное выпадение условного рефлекса.

а — условная реакция животного; б — условный раздражитель; в — безусловный раздражитель; г — отметка времени.

На основании анализа табл. 1, 2 и 3 и рис. 5 можно прийти к заключению, что у всех трех крыс первой серии, подвергавшихся воздействию сернистого газа в концентрации 20 мг/м³, первые изменения условнорефлекторной деятельности наступали в первый день затравки. По мере продолжения опытов отмечалось стойкое понижение корковой возбудимости вплоть до исчезновения условных рефлексов.

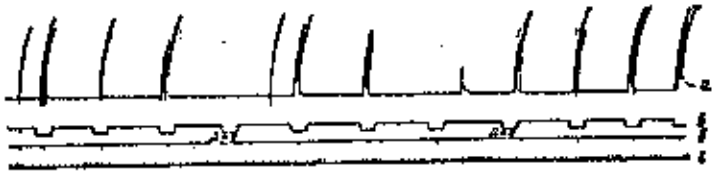

Рис. 6. Крыса № 7. Опыт № 232, 6.VII 1954 г. Удлинение латентного периода с единичными выпадениями условного рефлекса и растормаживанием дифференцировки (обозначения те же, что на рис. 5).

Помимо серьезных нарушений положительных условных рефлексов, в процессе затравки наступали также изменения и со стороны отрицательных условных рефлексов, выражавшиеся в растормаживании дифференцировок (рис. 6).

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Таблица 3

Изменение условнорефлекторной деятельности у крысы № 1 при концентрации SO_2 20 мг/м³

Время дачи раздражителя от начала опыта	Раздражитель (тон)	№ раздражителя	Время изолированного действия раздражителя (сек.)	Общее время действия раздражителя (сек.)	Латентный период условного рефлекса (сек.)	Натуральный условный рефлекс
Опыт № 173, 23 апреля 1954 г. (до затравки)						
14.30	№ 1	1620	5	10	1,4	+
31	№ 1	1621	5	10	2,6	++
33	№ 1	1622	5	10	1,6	+
34	№ 2	295			+	
36	№ 1	1623	5	10	1,6	+
37	№ 1	1624	5	10	1,4	++
39	№ 1	1625	5	10	1,8	+
40	№ 2	296			+	
42	№ 1	1626	5	10	1,4	+
43	№ 1	1627	5	10	1,4	++
45	№ 1	1628	5	10	1,8	+
Опыт № 195, 4 июня 1954 г. (первый день затравки)						
16.00	№ 1	1807	5	10	1,4	+
01	№ 1	1808	5	10	2	++
03	№ 1	1809	5	10	1,8	+
04	№ 2	336			+	
06	№ 1	1810	5	10	1,2	+
07	№ 1	1811	5	10	0,8	+++
09	№ 1	1812	5	10	1,6	+
10	№ 2	337			+	
12	№ 1	1813	5	10	2	+
13	№ 1	1814	5	10	2,4	+++
15	№ 1	1815	5	10	4	++
Опыт № 202, 15 июня 1954 г. (8-й день затравки)						
16.10	№ 1	1879	5	10	—	+
11	№ 1	1880	5	10	—	+++
13	№ 1	1881	5	10	—	++
14	№ 2	352			+	
16*	№ 1	1882	5	10	6	+
17	№ 1	1883	5	10	—	+++
19	№ 1	1884	5	10	—	++
20	№ 2	353			+	
22	№ 1	1885	5	10	1	+
23	№ 1	1886	5	10	3	+++
25	№ 1	1887	5	10	3	++

* Затравка не взята.

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Таким образом, результаты первой серии опытом показывают следующее.

1. Концентрация сернистого газа 20 мг/м³ установленная ГОСТ как предельно допустимая для воздуха цехов, не является безразличной, так как вызывает нарушение условнорефлекторной деятельности.

2. Изменения условнорефлекторной деятельности наступали в первый день затравки, при этом прежде всего страдал положительный условный рефлекс на тон № 1.

3. По мере продолжения опытов отмечалось стойкое понижение корковой возбудимости вплоть до исчезновения условных рефлексов.

4. Латентный период условного рефлекса в отдельных опытах увеличился в 3—3,5 раза, средняя величина латентного периода увеличилась в два с лишним раза.

5. Изменения со стороны отрицательных условных рефлексов выражались нарушением дифференцировок.

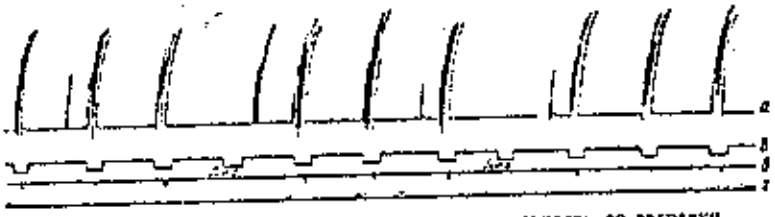

Рис. 7. Крыса № 2. Условнорефлекторная деятельность до затравки.

Вторая серия опытов. Во вторую серию входили крысы № 2, 3 и 6. Концентрация сернистого газа в камере колебалась в пределах 3—7 мг/м³ (в среднем 5 мг/м³). До воздействия сернистого газа во время опыта у всех животных этой серии было отмечено активное поведение, прочные условные рефлексы и нулевая дифференцировка.

На рис. 7 представлена кимографическая запись опыта до затравки у крысы № 2. Как видно из кимограммы, условные рефлексы у этой крысы были выработаны прочно, условнорефлекторная реакция — живая, латентный период был в пределах 1,5 сек., дифференцировка была нулевой.

В первый день опыта в газовой камере, при концентрации сернистого газа 5 мг/м³ и экспозиции 3,5 часа, не удалось заметить каких-либо изменений условнорефлекторной деятельности крыс; латентный период условного рефлекса оставался в тех же пределах, а дифференцировка — нулевой (рис. 8).

Условнорефлекторная деятельность у крысы № 2 в первый день затравки, как это видно на рис. 8, не изменилась. Латентный период продолжал оставаться в пределах 1,5 сек. Изменений со стороны дифференцировки не наступало.

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У крысы № 3 той же серии в первый день затравки также не отмечалось нарушений условнорефлекторной деятельности. Латентный период оставался почти в тех же пределах, что и до затравки (0,6—1,2 сек.). Аналогичной была картина и у крысы № 6. Латентный период условного рефлекса оставался в тех

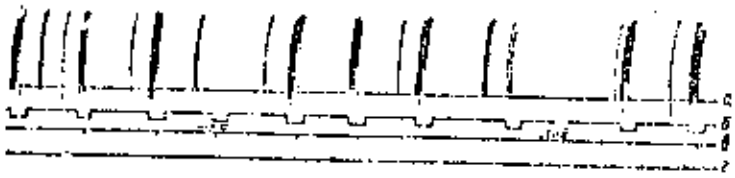

Рис. 8. Крыса № 2. Условнорефлекторная деятельность в первый день затравки.

же пределах (0,6—1,4 сек.), изменений со стороны дифференцировки не наблюдалось.

По мере продолжения опытов в газовой камере первые изменения, как и в первой серии опытов, были отмечены со стороны положительного условного рефлекса на тон № 1. Однако эти изменения наступали не сразу, а на пятый (крысы № 3 и 6) и шестой день (крыса № 2) со дня затравки (табл. 4).

Таблица 4

Изменение условнорефлекторной деятельности у крысы № 6 при концентрации SO_2 5 мг/м³

Время дня раздражителя от начала опыта	Раздражитель (тон)	№ раздражителя	Время изолированного действия раздражителя (сек.)	Общее время действия раздражителя (сек.)	Латентный период условного рефлекса (сек.)	Натуральный условный рефлекс
Опыт № 213, 6 июля 1954 г., 5-й день затравки)						
15.50	№ 1	2018	5	10	0,6	+
51	№ 1	2019	5	10	1,6	++
52	№ 1	2020	5	10	1,4	+
53	№ 2	373		10	+	
55	№ 1	2021	5	10	0,6	+
56	№ 1	2022	5	10	3	+++
58	№ 1	2023	5	10	1,6	+
55	№ 2	374		10	+	
16.01	№ 1	2024	5	10	1	+
02	№ 1	2025	5	10	3,4	++
04	№ 1	2026	5	10	2	+

В табл. 4 приведен протокол опыта № 213 с крысой № 6, из которого видно, что на пятый день затравки у данной крысы

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отмечались единичные случаи увеличения латентного периода условного рефлекса до 3—3,4 сек., что ранее не отмечалось.

Начиная с пятого и шестого дня затравки, у всех крыс данной серии наблюдалось довольно значительное увеличение латентного периода условного рефлекса. Если на пятый день затравки это увеличение было единичным, то в дальнейшем до конца опыта оно носило постоянный характер. На рис. 9 представлены записи, иллюстрирующие эти изменения.

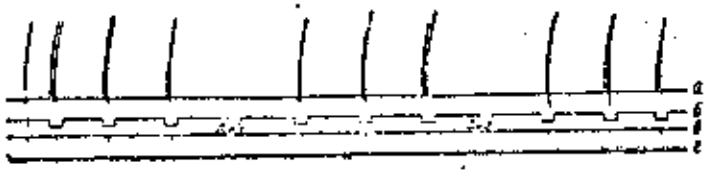

Рис. 9. Крыса № 2. Удлинение латентного периода условного рефлекса при концентрации SO_2 5 мг/м³.

В результате воздействия сернистого газа в концентрации 5 мг/м³ латентный период условного рефлекса у крысы № 2 увеличился до 5 сек., что видно из рис. 9. При этих же условиях у крысы № 3 латентный период условного рефлекса увеличился до 4,6 сек., а у крысы № 6 — до 6,2 сек. Изменений со стороны дифференцировки не наблюдалось. Эти нарушения становятся более наглядными при графическом изображении изменений латентного периода условного рефлекса до и после затравки (рис. 10).

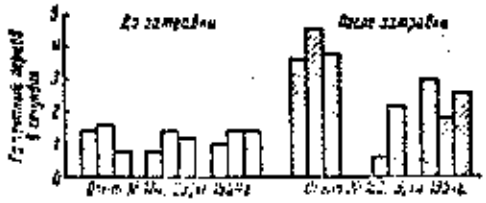

Рис. 10. Крыса № 3. Нарушение условнорефлекторной деятельности при концентрации SO_2 5 мг/м³.

На рис. 10 представлены изменения латентного периода условного рефлекса в секундах до и после затравки у крысы № 3. Как видно из рисунка, латентный период до затравки (опыт № 174) был 0,6—1,6 сек., а на шестой день затравки (опыт № 182) он увеличился в 2,5 раза. Кроме того, наблюдалось единичное выпадение условного рефлекса. У крыс № 2 и 6 этой же серии латентный период увеличился в 2 раза.

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Нарушения условнорефлекторной деятельности у крыс данной серии, наступавшие обычно на пятый и шестой день затравки, в последующем носили волнообразный характер (рис. 11).

На рис. 11 приведены, на примере крысы № 3, изменения латентного периода условного рефлекса у крыс данной серии за пять опытов до и после затравки. Нижняя кривая показывает изменения латентного периода до затравки, а верхняя

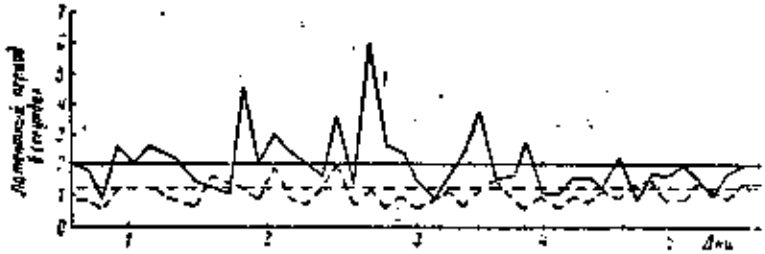

Рис. 11. Крыса № 3. Изменение латентного периода условного рефлекса в динамике при концентрации сернистого газа 5 мг/м³.
1 — после затравки; 2 — до затравки.

показывает те же изменения после затравки. На рисунке видно, что верхняя кривая носит волнообразный характер: временами то поднимается до 4—6 сек., то опускается до 2—3 сек. Средняя величина латентного периода у этой крысы (верхняя горизонтальная линия) увеличилась почти в 2 раза, т. е. с 1,2 до 2 сек.

Помимо удлинения латентного периода, временами отмечалось полное выпадение условного рефлекса, дифференцировка продолжала оставаться нулевой (рис. 12).

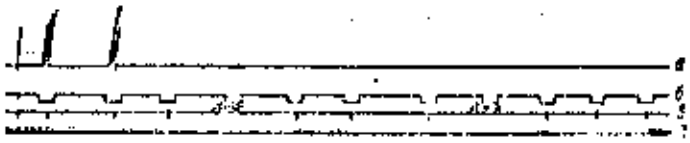

Рис. 12. Крыса № 2. Выпадение условного рефлекса у крысы № 2 при концентрации SO_2 5 мг/м³.

На кимограмме (рис. 12) представлены глубокие изменения в корковых процессах, наступившие у крысы № 2 в результате затравки сернистым газом в концентрации 5 мг/м³. Как видно из записи, на условные раздражители крыса не реагировала, в то время как до затравки у этой крысы были выработаны прочные условные рефлексы (рис. 7).

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Таблица 5

Изменение условнорефлекторной деятельности у крысы № 6 при концентрации сернистого газа 5 мг/м³

Время зачи раздражителя от начала опыта	Раздражитель (тон)	№ раздражителя	Время изолированного действия раздражителя (сек.)	Общее время действия раздражителя (сек.)	Латентный период условного рефлекса (сек.)	Натуральный условный рефлекс
Опыт № 206, 27 июня 1954 г. (до затравки)						
15.10	№ 1	1955	5	10	1	+
11	№ 1	1956	5	10	0,6	+
13	№ 1	1957	5	10	0,4	+
14	№ 2	359			+	
16	№ 1	1958	5	10	1,6	+
17	№ 1	1959	5	10	1,2	+
19	№ 1	1960	5	10	0,6	+
20	№ 2	360			+	
22	№ 1	1961	5	10	0,8	+
23	№ 1	1962	5	10	1	+
25	№ 1	1963	5	10	0,8	+
Опыт № 209, 1 июля 1954 г. (первый день затравки)						
16.02	№ 1	1991	5	10	0,6	+
03	№ 1	1992	5	10	0,6	+
05	№ 1	1993	5	10	1,4	+
06	№ 2	367			+	
08	№ 1	1994	5	10	1	+
09	№ 1	1995	5	10	0,6	+
11	№ 1	1996	5	10	1,4	+
12	№ 2	368			+	
14	№ 1	1997	5	10	0,8	+
15	№ 1	1998	5	10	1,0	+
17	№ 1	1999	5	10	0,8	+
Опыт № 219, 13 июля 1954 г. (11-й день затравки)						
12.05	№ 1	2072	5	10	2,6	+
06	№ 1	2073	5	10	2,6	+
08	№ 1	2074	5	10	—	+
09	№ 2	385			+	
11	№ 1	2075	5	10	—	+
12	№ 1	2076	5	10	—	+
14	№ 1	2077	5	10	—	+
15	№ 2	386			+	
17	№ 1	2078	5	10	—	+
18	№ 1	2079	5	10	—	+
20	№ 1	2080	5	10	—	+

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Подобные изменения были отмечены и у крысы № 6 той же серии, что говорит о резком снижении корковой возбудимости, как следствия воздействия относительно малой концентрации сернистого газа (5 мг/м³).

В табл. 5 приведены результаты трех опытов с крысой № 6 для иллюстрации динамики нарушений условнорефлекторной деятельности при концентрации SO_2 5 мг/м³. Если в опыте № 206 (до затравки) латентный период у этой крысы был в пределах 0,4—1,6 сек. и в тех же пределах продолжал оставаться в первый день затравки, то уже на одиннадцатый день было отмечено увеличение латентного периода вплоть до полного выпадения условного рефлекса.

Аналогичные изменения наблюдались и у крысы № 3 (табл. 6). Латентный период до затравки у этой крысы был в пределах 0,6—1,2 сек.; в первый день затравки изменения условнорефлекторной деятельности не наступило, латентный период был попрежнему 0,6—1,2 сек., но уже на восьмой день затравки наблюдалось значительное увеличение латентного периода с единичными выпадениями условного рефлекса.

У крысы № 2 (табл. 7) данной серии нарушения условнорефлекторной деятельности наступали в том же порядке, что и у крыс № 3 и 6. Латентный период до затравки был в пределах 0,6—1,4 сек., в тех же пределах он оставался и в первый день затравки, а на девятый день отмечалось значительное увеличение латентного периода (до 4,2 сек.). Нарушений со стороны дифференцировки не наблюдалось.

Анализ результатов второй серии опытов позволяет сделать следующее заключение.

1. Сернистый газ в концентрации 5 мг/м³ при хроническом действии оказывает токсический эффект, вызывая серьезные нарушения со стороны центральной нервной системы.

2. Первые изменения условнорефлекторной деятельности наступали со стороны положительных условных рефлексов на 5—6-й день затравки.

3. Наблюдавшиеся в течение затравки изменения условных рефлексов, носили волнообразный характер.

4. Латентный период условного рефлекса в отдельных опытах увеличивался в 2—2,5 раза, средняя величина латентного периода условного рефлекса увеличилась почти в 2 раза.

5. Помимо увеличения латентного периода, наблюдалось частичное выпадение условных рефлексов.

6. Нарушений со стороны дифференцировки не отмечалось.

Третья серия опытов. В третью серию опытов вошли крысы № 4, 5 и 9. Концентрация сернистого газа в камере колебалась от 1 до 4 мг/м³ (в среднем 2,5 мг/м³). Все взятые в опыт крысы были здоровыми, положительные и отрицательные условные рефлексы были выработаны прочно. После выработки прочных положительных и отрицательных условных рефлексов

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Таблица 5

Изменение условнорефлекторной деятельности у крысы № 3 при концентрации сернистого газа 5 мг/м³

Время начала раздражителя от начала опыта	Раздражитель (тон)	№ раздражителя	Время реального действия раздражителя (сек.)	Общее время действия раздражителя (сек.)	Латентный период условного рефлекса (сек.)	Натуральный условный рефлекс
Опыт № 170, 22 июня 1954 г. (до затравки)						
9.30	№ 1	1596	5	10	0,8	+
31	№ 1	1597	5	10	0,8	+
33	№ 1	1598	5	10	0,6	+
34	№ 2	287			+	
36	№ 1	1599	5	10	1,2	+
37	№ 1	1600	5	10	1,2	+
39	№ 1	1601	5	10	1,2	+
40	№ 2	288			+	
42	№ 1	1602	5	10	1	+
43	№ 1	1603	5	10	0,8	+
45	№ 1	1604	5	10	0,6	+
Опыт № 175, 1 июля 1954 г. (первый день затравки)						
16.20	№ 1	1641	5	10	0,6	+
21	№ 1	1642	5	10	0,8	+
23	№ 1	1643	5	10	0,6	+
24	№ 2	297			+	
26	№ 1	1644	5	10	0,6	+
27	№ 1	1645	5	10	1,2	+
29	№ 1	1646	5	10	0,6	+
30	№ 2	298			+	
32	№ 1	1647	5	10	1,2	+
33	№ 1	1648	5	10	0,8	+
35	№ 1	1649	5	10	1	+
Опыт № 182, 9 июля 1954 г. (8-й день затравки)						
16.05	№ 1	1704	5	10	3,6	+
06	№ 1	1705	5	10	4,4	+
08	№ 1	1706	5	10	3,8	+
09	№ 2	311			+	
11	№ 1	1707	5	10	–	
12	№ 1	1708	5	10	0,6	+
14	№ 1	1709	5	10	2,2	+
15	№ 2	312			+	
17	№ 1	1710	5	10	3	+
18	№ 1	1711	5	10	1,6	+
20	№ 1	1712	5	10	2,6	+

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Таблица 7

Изменение условнорефлекторной деятельности у крысы № 2 при концентрации сернистого газа 5 мг/м³

Время дачи раздражителя от начала опыта	Раздражитель (тон)	№ раздражителя	Время изолированного действия раздражителя (сек.)	Общее время действия раздражителя (сек.)	Латентный период условного рефлекса (сек.)	Натуральный условный рефлекс
Опыт № 173. 28 июня 1954 г. (до затравки)						
13.55	№ 1	1623	5	10	0,6	+
56	№ 1	1624	5	10	0,6	+
58	№ 1	1625	5	10	0,8	+
59	№ 2	293			+	
14.01	№ 1	1626	5	10	1,4	+
02	№ 1	1627	5	10	1,4	+
04	№ 1	1628	5	10	0,6	+
05	№ 2	294			+	
07	№ 1	1629	5	10	1,2	+
09	№ 1	1630	5	10	1	+
10	№ 1	1631	5	10	1,4	+
Опыт № 175. 1 июля 1954 г. (первый день затравки)						
16.38	№ 1	1641	5	10	0,8	+
39	№ 1	1642	5	10	0,8	+
41	№ 1	1643	5	10	1,0	+
42	№ 2	297			+	
44	№ 1	1644	5	10	1,4	+
45	№ 1	1645	5	10	1,0	+
47	№ 1	1646	5	10	1,0	+
48	№ 1	1647	5	10	1,4	+
50	№ 2	298			+	
51	№ 1	1648	5	10	0,4	+
53	№ 1	1649	5	10	1,0	+
54	№ 1	1650	5	10	1,0	+
Опыт № 183. 10 июля 1954 г. (9-й день затравки)						
12.43	№ 1	1713	5	10	3	+
44	№ 1	1714	5	10	3,2	+
46	№ 1	1715	5	10	2,8	+
47	№ 2	313			+	
49	№ 1	1716	5	10	3,6	+
50	№ 1	1717	5	10	4,2	+
52	№ 1	1718	5	10	4	+
53	№ 2	314			+	
55	№ 1	1719	5	10	3,6	+
56	№ 1	1720	5	10	3,6	+
58	№ 1	1721	5	10	3,8	+

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все крысы подвергались хроническому воздействию сернистого газа в концентрации 2,5 мг/м³ по 3,5 часа в день.

У всех крыс данной серии как в первый день затравки, так и в последующие дни серьезных нарушений со стороны условнорефлекторной деятельности отмечено не было. Латентный период условного рефлекса после затравки оставался в тех же пределах, как и до затравки. Нарушения дифференцировок также при этом не наблюдалось.

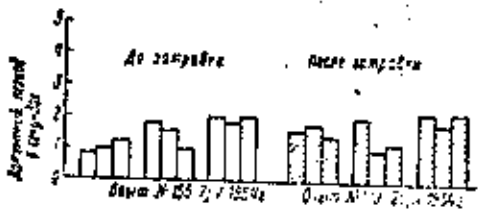

Рис. 13. Крыса № 5. Латентный период условного рефлекса до и после затравки SO_2 при концентрации 2,5 мг/м³.

На рис. 13 показано изменение латентного периода условного рефлекса у крысы № 5 до и после затравки. Латентный период у этой крысы до затравки был в пределах 0,8—2 сек. и после затравки (опыт № 170) почти не изменился (1—2,2 сек.).

Как видно из табл. 8, где приведены результаты трех опытов с крысой № 4, латентный период в опыте № 156 (до затравки) колебался в пределах 0,8—1,4 сек., в тех же пределах он оставался и в первый день затравки, а на 16-й день было отмечено увеличение амплитуды колебания латентного периода на 0,2 сек. в ту и другую сторону. У этой же крысы и на 13-й день затравки латентный период находился в тех же пределах (0,6—1,4 сек.).

У крысы № 5 этой же серии (табл. 9) латентный период до затравки был в пределах 0,4—2,4 сек., а в первый день и девятый день затравки — 0,8—2,2 и 0,8—2,4 сек., т. е. уменьшение амплитуды колебания на 0,4 сек. Аналогичной была картина и у крысы № 9 (табл. 10), латентный период до затравки у которой был в пределах 0,6—3 сек., а после затравки наблюдалось уменьшение амплитуды колебания всего лишь на 0,2 сек.

Анализ табл. 8, 9 и 10 показывает, что условнорефлекторная деятельность у всех крыс этой серии в процессе хронической затравки сернистым газом в концентрации 2,5 мг/м³ не нарушалась. Имевшее место в отдельных опытах увеличение или уменьшение амплитуды колебания латентного периода на 0,2 сек. нами не принималось во внимание, так как это могло

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Таблица 8

Условнорефлекторная деятельность у крысы № 4 при концентрации сернистого газа 2,5 мг/м³

Время дачи раздражителя от начала опыта	Раздражитель (тон)	№ раздражителя	Время индивидуального действия раздражителя (сек.)	Общее время действия раздражителя (сек.)	Латентный период условного рефлекса (сек.)	Натуральный условный рефлекс
Опыт № 156, 8 апреля 1954 г. (до затравки)						
12.20	№ 1	1470	5	10	1	+
21	№ 1	1471	5	10	1	+
23	№ 1	1472	5	10	1	+
24	№ 2	257			+	
26	№ 1	1473	5	10	0,8	+
27	№ 1	1474	5	10	0,8	+
29	№ 1	1475	5	10	1	+
30	№ 2	258			+	
32	№ 1	1476	5	10	1	+
33	№ 1	1477	5	10	1,4	+
35	№ 1	1478	5	10	1	+
Опыт № 157, 9 апреля 1954 г. (первый день затравки)						
13.15	№ 1	1479	5	10	0,8	+
16	№ 1	1480	5	10	1	+
18	№ 1	1481	5	10	1	+
19	№ 2	259			+	
21	№ 1	1482	5	10	1,4	+
22	№ 1	1483	5	10	1	+
24	№ 1	1484	5	10	1,2	+
25	№ 2	260			+	
27	№ 1	1485	5	10	0,8	+
28	№ 1	1486	5	10	0,8	+
30	№ 1	1487	5	10	1,4	+
Опыт № 172, 27 апреля 1954 г. (16-й день затравки)						
13.15	№ 1	1613	5	10	1	+
16	№ 1	1614	5	10	0,6	+
18	№ 1	1615	5	10	1	+
19	№ 2	289			+	
21	№ 1	1616	5	10	1,6	+
22	№ 1	1617	5	10	1,4	+
24	№ 1	1618	5	10	1,6	+
25	№ 2	290			+	
27	№ 1	1619	5	10	1	+
28	№ 1	1620	5	10	0,6	+
30	№ 1	1621	5	10	1	+

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Таблица 9

Условнорефлекторная деятельность у крысы № 5 при концентрации сернистого газа 2,5 мг/м³

Время дания раздражителя от начала опыта	Раздражитель (тон)	№ раздражителя	Время подкрепленного действия раздражителя (сек.)	Общее время действия раздражителя (сек.)	Латентный период условного рефлекса (сек.)	Натуральная условная рефлексия
Опыт № 154, 6 апреля 1954 г. (до затравки)						
12.01	№ 1	1438	5	10	2,2	+
02	№ 1	1439	5	10	1	+
04	№ 1	1440	5	10	1,2	+
05	№ 2	266			+	
07	№ 1	1441	5	10	1,8	+
08	№ 1	1442	5	10	2,4	+
10	№ 1	1443	5	10	1,8	+
11	№ 2	267			+	
13	№ 1	1444	5	10	0,8	+
14	№ 1	1445	5	10	1,2	+
16	№ 1	1446	5	10	0,4	+
Опыт № 156, 8 апреля 1954 г. (первый день затравки)						
12.40	№ 1	1456	5	10	2,0	+
41	№ 1	1457	5	10	0,8	+
43	№ 1	1458	5	10	1,6	+
44	№ 2	258			+	
46	№ 1	1459	5	10	2	+
47	№ 1	1460	5	10	2,2	+
49	№ 1	1461	5	10	1,8	+
50	№ 2	259			+	
52	№ 1	1462	5	10	1	+
53	№ 1	1463	5	10	1,2	+
55	№ 1	1464	5	10	0,8	+
Опыт № 164, 17 апреля 1954 г. (9-й день затравки)						
12.40	№ 1	1528	5	10	0,8	+
41	№ 1	1529	5	10	1	+
43	№ 1	1530	5	10	1,1	+
44	№ 2	274			+	
46	№ 1	1531	5	10	1	+
47	№ 1	1532	5	10	1,4	+
49	№ 1	1533	5	10	1	+
50	№ 2	275			+	
52	№ 1	1534	5	10	2,4	+
53	№ 1	1535	5	10	1,4	+
55	№ 1	1536	5	10	1,2	+

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Таблица 10

Условнорефлекторная деятельность у крысы № 9 при концентрации сернистого газа 2,5 мг/м³

Время дня раздражителя от начала опыта	Раздражитель (тон)	№ раздражителя	Время изолированного действия раздражителя (сек.)	Общее время действия раздражителя (сек.)	Латентный период условного рефлекса (сек.)	Натуральный условный рефлекс
Опыт № 160, 7 апреля 1954 г. (до затравки)						
12.57	№ 1	1499	5	10	1	+
58	№ 1	1500	5	10	2,2	+
13.00	№ 1	1501	5	10	1,6	+
01	№ 2	268			+	
03	№ 1	1502	5	10	0,6	+
04	№ 1	1503	5	10	2	+
06	№ 1	1504	5	10	1,6	+
07	№ 2	269			+	
09	№ 1	1505	5	10	1,2	+
10	№ 1	1506	5	10	1,2	+
12	№ 1	1507	5	10	3	+
Опыт № 162, 9 апреля 1954 г. (первый день затравки)						
12.56	№ 1	1517	5	10	1,4	+
59	№ 1	1518	5	10	2,4	+
13.01	№ 1	1519	5	10	2	+
02	№ 2	272			+	
04	№ 1	1520	5	10	0,8	+
05	№ 1	1521	5	10	1,6	+
07	№ 1	1522	5	10	1,6	+
08	№ 2	273			+	
10	№ 1	1524	5	10	1,2	+
11	№ 1	1525	5	10	1,4	+
13	№ 1	1526	5	10	2,8	+
Опыт № 167, 16 апреля 1954 г. (8-й день затравки)						
12.58	№ 1	1562	5	10	0,8	+
59	№ 1	1563	5	10	2	+
13.01	№ 1	1564	5	10	2	+
02	№ 2	281			+	
04	№ 1	1565	5	10	2	+
05	№ 1	1566	5	10	2	+
07	№ 1	1567	5	10	1	+
08	№ 2	282			+	
10	№ 1	1568	5	10	1,4	+
11	№ 1	1569	5	10	3	+
13	№ 1	1570	5	10	2,8	+

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быть в пределах погрешности опыта. Кроме того, в этом же нас убеждала и средняя величина латентного периода, которая после затравки не изменилась.

Таким образом, результаты третьей серии опытов позволяют сделать заключение, что сернистый газ в концентрации 2,5 мг/м³ при хроническом его действии не оказывает влияния на условнорефлекторную деятельность: латентный период в результате затравки не изменялся, изменения со стороны дифференцировок отсутствовали.

Результаты наших опытов позволяют полагать, что сернистый газ в концентрациях, начиная с 3—7 мг/м³, при длительном его действии вызывает угнетение процессов возбуждения в коре головного мозга, которая реагирует понижением условных двигательных рефлексов.

К сожалению, мы не могли сравнить наши результаты с литературными данными ввиду отсутствия последних. Однако указания П. В. Сидоренкова (1952), что сернистый газ вызывает угнетение дыхания нервной ткани, а также Ф. И. Дубровской (1954) о снижении световой чувствительности глаза при косвенном воздействии сернистого ангидрида на кору головного мозга, как нам кажется, находится в некотором соответствии с полученными нами данными.

Действие сернистого газа на дыхательную функцию

Изучение влияния сернистого газа на дыхание проводилось на 3 кроликах (самцах) весом 3—3,5 кг. Всего было произведено 20 опытов. Предварительно кролика фиксировали в станке, после чего ему надевали специальную маску с клапанами. Вдыхательный клапан маски через резиновую трубку соединялся с камерой, где создавалась испытываемая концентрация сернистого газа, выдыхательный клапан оставался свободным и сообщался непосредственно с помещением. Через отросток, расположенный на передней поверхности маски, последняя сообщалась с капсулой Марея и записывающим приспособлением. Запись велась на закопченной ленте при быстром вращении кимографа таким образом, что кролик не видел подключения газа, включения кимографа и других движений рук экспериментатора.

При концентрации сернистого газа 20 мг/м³, как видно на записи (рис. 14), у кроликов наступали резкие изменения дыхания. Уже через 1—2 секунды после подключения газа наблюдалось резкое увеличение глубины дыхания с явным преобладанием выдоха. Число дыханий при этом почти не увеличивалось. До подключения газа число дыханий в 1 минуту было 37, после подключения газа — 39.

При концентрации сернистого газа 5 мг/м³ так же, как и при 20 мг/м³, в момент подключения газа наблюдался резкий выдох,

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после чего наступало значительное увеличение глубины дыхания (рис. 15). Число дыханий при этом увеличивалось с 37 до 39—40 в 1 минуту.

Наименьшая концентрация сернистого газа, при которой наблюдались изменения со стороны дыхания, была 2 мг/м³, что видно из приводимой записи (рис. 16).

Рис. 14. Увеличение глубины дыхания у кролика при концентрации SO_2 20 мг/м³.

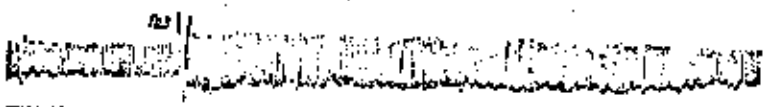

Рис. 15. Увеличение глубины дыхания у кролика при концентрации SO_2 5 мг/м³.

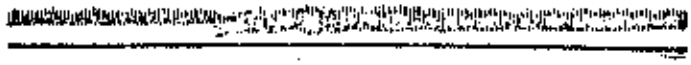

Рис. 16. Увеличение глубины дыхания у кролика при концентрации SO_2 2 мг/м³.

Как и следовало ожидать, изменения при концентрации сернистого газа 2 мг/м³ были менее резкие, чем при 5 и 20 мг/м³. Однако и при этой небольшой концентрации с момента подачи газа наступало увеличение глубины дыхания с преобладанием выдоха в начальный момент. Число дыханий почти не изменилось: до подключения газа было 23, после подключения — 25 дыханий в 1 минуту.

Очевидно, сернистый газ вызывает раздражение рецепторов слизистых оболочек верхних дыхательных путей, приводящее к рефлекторному изменению дыхания.

Влияние сернистого газа на вес животных

Опыты по изучению влияния сернистого газа на вес подопытных животных проводились на 12 кроликах, которые периодически взвешивались до и после затравки, одновременно с контрольной группой.

При концентрации сернистого газа 20 мг/м³ у всех трех кроликов этой серии в течение пяти недель со дня затравки

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наблюдалось либо значительное падение веса, либо он оставался стабильным, в то время как у контрольных кроликов в течение всего времени вес повышался. В то же время у всех опытных кроликов после затравки отмечалась значительная жажда и потери аппетита. Меньшие концентрации сернистого газа (2,5 и 5 мг/м³) не оказывали заметного влияния на вес животных.

Изучение влияния небольших концентраций сернистого газа на кровь, как и гистологические исследования трахеи и легких, не дали надежных результатов.

Наша работа представляет собою попытку физиологической проверки токсичности малых концентраций сернистого газа и является материалом для обоснования предложенных норм загрязнения атмосферного воздуха данным газом. Безусловно, должна быть соблюдена известная осторожность при переносе полученных результатов на людей.

Выводы

1. Результаты наших исследований показали, что сернистый газ в концентрациях, лежащих ниже порога раздражающего действия (20 мг/м³) оказывает хроническое токсическое действие на животный организм, вызывая стойкие нарушения в центральной нервной системе.

2. Физиологические методы, применявшиеся нами для исследования влияния сернистого газа на организм, в особенности методика изучения дыхательной функции и метод условных рефлексов, оказались наиболее чувствительными по сравнению с другими физиологическими и патогистологическими методами.

3. Изменения со стороны дыхательной функции при воздействии сернистого газа являются самым чувствительным тестом.

4. Наименьшая концентрация сернистого газа, вызывающая нарушение условнорефлекторной деятельности подопытных крыс, находится в пределах 3—7 мг/м³.

5. Сернистый газ в концентрации 20 мг/м³ вызывает серьезные нарушения условнорефлекторной деятельности, которые наступают уже в первый день затравки и выражаются: а) в стойком снижении корковой возбудимости вплоть до исчезновения условных рефлексов; б) в увеличении латентного периода условного рефлекса (в отдельных опытах в 3—3,5 раза); средняя величина латентного периода при этом увеличивалась в два с лишним раза; в) в частичном нарушении дифференцировок.

6. Изменения со стороны дыхательной функции в виде увеличения глубины дыхания с явным преобладанием выдоха в начале опыта наблюдались начиная с 2 мг/м³ SO_2.

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7. Опыты показали, что концентрация сернистого газа 20 мг/м³, установленная ГОСТ как предельно допустимая для воздуха цехов, не может считаться безразличной. Эксперименты на животных показали, что эта концентрация вызывает нарушение условнорефлекторной деятельности, изменение дыхания, потерю аппетита и веса животных. Результаты исследований дают основание рекомендовать пересмотр существующей нормы в направлении значительного ее снижения.

8. Сернистый газ в концентрации 5 мг/м³ при хроническом действии вызывает следующие явления: а) начальные изменения со стороны положительных условных рефлексов наступают на 5—6-й день затравки и носят волнообразный характер; б) латентный период условного рефлекса в отдельных опытах увеличивается в 2—2,5 раза; средняя величина латентного периода условного рефлекса увеличивается почти в 2 раза; в) помимо увеличения латентного периода, наблюдается частичное выпадение условных рефлексов; г) нарушений со стороны дифференцировки не наблюдается.

9. Сернистый газ в концентрации 2,5 мг/м³ при хроническом действии не оказывает влияния на условнорефлекторную деятельность, латентный период в результате затравки не изменяется, нарушения со стороны дифференцировок отсутствуют.

10. Изучение влияния сернистого газа на кровь, как и гистологические исследования трахеи и легких, не дали надежных результатов.

11. Результаты нашей экспериментальной работы являются материалом для физиологического обоснования предложенных норм предельно допустимых концентраций сернистого газа в атмосферном воздухе промышленных городов.

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Automated Analysis Applied to Soft Drinks Production and Quality Control

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V. T. WALKLEY and J. A. BUCHANAN

This paper reports the first stages of an investigation carried out by our laboratory into a system of automated analysis for the determination of certain constituents in fruit juices and soft drinks.

The analysis of soft drinks in terms of those analyses required for quality control purposes is a time-consuming laboratory process. This is particularly true where a number of bottling lines are in operation. In order to ensure that a full coverage of chemical parameters is maintained it was evident to us that some system of automated analysis would have to be used.

Our perusal of various techniques available indicated that the Technicon AutoAnalyzer offered certain advantages, and to this end an investigation into the use of this equipment was carried out.

Sulfur dioxide is widely used as a preservative in the soft drinks industry, and its concentration in the finished product is controlled by Statutory Order.

The literature relating to the analytical determination of sulfur dioxide is extensive. As early as 1882, Hass described a gravimetric technique for the determination of sulfur dioxide in wines, and Ripper in 1892 utilised a direct titration technique.

The method proposed by Monier-Williams (18) is probably the most widely used, although the simplified Jensen method (11) is frequently used for routine control testing, and Bennett and Donovan's (2) reducing substances technique can be incorporated.

Lloyd and Cowle (13) described a method for the determination of free sulfur dioxide in soft drinks using a desorption technique, followed by trapping in alkaline glycerol or sodium tetrachloromercurate reagent followed by an iodometric or colorimetric procedure. Burroughs and Sparks (4) recorded their findings obtained by desorption of sulfur dioxide (in vacuum) with an air stream, and titration of the sulfuric acid formed after absorption into hydrogen peroxide, using Tashiros indicator and standard alkali.

Each of these methods, and many others which appear in literature, have much to commend them in their own particular sphere. They are, however, time consuming when a large number of samples have to be screened at one time.

Bearing in mind the fact that sulfur dioxide forms a complex with carbonyl compounds to form hydroxysulfonates; or, in the case where alcohol is present, to form acetaldehyde bisulfate; this complexing of the sulfur dioxide molecule causes errors when any attempt is made to determine the total sulfur dioxide in soft drinks by simple titration procedure. It was evident, quite early in our methods trials with the AutoAnalyzer, that direct reaction techniques were not possible.

Scharoun and Saletan (28) developed a method for the automated determination of sulfur dioxide in beer using the AutoAnalyzer. This method consisted of automatically mixing the sample of beer with acidified mercuric chloride, diluting, adding weak sodium hydroxide to the sample stream and finally reacting with pararosanaline and formaldehyde. A time delay coil provided suitable reaction time, and the colour development, measured at 540 mμ, was compared to beers with known sulfite additions. Rate of sampling was twenty per hour.

We found two major objections to this method in the light of our own requirements for testing soft drinks. Firstly it is often necessary to examine a range of differently coloured soft drinks. The colour differences caused problems associated with the end point colour measurements. Secondly the presence of fruit pulp or cellular material gave rise to excessive noise on the recorder. A dual differential colorimeter provided suitable colour compensation and dialysis removed the pulp fruit particles. However, to obtain a total sulfur dioxide estimation, it was evident that a distillation system would have to be used.

In the light of these findings, an automated distillation technique was developed in conjunction with the Technicon Instruments Company Ltd., and the methodology is as outlined later in this paper.

A further report on this system is also to be found by reference to an article by Buchanan and Walkley (3).

A second estimation which we have effectively automated is that of Acetoin (A.M.C.) and diacetyl. A general review of this procedure has been reported by Walkley and Buchanan (29).

Certain microbial spoilage organisms develop,

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as an endproduct of metabolism, either or both, A.M.C. and diacetyl, the latter in particular producing a "buttery" off-flavour in citrus juices. This has been reported by numerous workers (6, 7, 9, 10, 19, 21, 22). A high quantity of either of these products in fruit juice indicates poor sanitary conditions. Estimation of A.M.C. and diacetyl by means of the Voges-Proskauer test (V.P. test) is now employed by a large number of citrus juice producers as a quality index since its initial introduction as a test procedure by Hill and Wenzel (9) in 1954.

The V.P. test is based on a colour reaction when diacetyl (or A.M.C. oxidized to diacetyl in the presence of strong alkali and oxygen) is reacted with alpha-naphthol and alkali. Many variations of this technique have been utilized but the method we have described was found to have advantages from the quality control point of view. It enabled both the diacetyl and A.M.C. estimation, as well as the ethyl alcohol estimation, to be made on the same distillate sample.

Diacetyl ($CH_3COCOCH_3$) is found in certain essential oils (clove, caraway, etc.), and is present in butter, where it is formed as a metabolic by-product of microbial proliferation. It is soluble in 4 parts of water and miscible with alcohol and ether.

Acetoin ($C_4H_8O_2$) is miscible with water and alcohol but sparingly soluble in petroleum ether and ether. Like diacetyl, A.M.C. is also a product of microbial metabolism.

The demonstration of these products in buffered glucose peptone water serves to distinguish certain types of intestinal bacilli, and the test used is known as the Voges Proskauer (VP) reaction and is described in numerous bacteriological handbooks.

Fields (7) proposed certain standards for A.M.C. and diacetyl concentrations in fruit juices, and indicated the close relationship between poor sanitary conditions in the manufacturing plant and high values of these components. We have found the system outlined to be of value in laboratory and control studies of both cultures and fruit juices. Marshall and Walkley (14, 15, 16, 17) studied various rot-producing fungi on fruit and from an examination of related organisms by Fields (7) much useful information as to the "quality" of juices may be derived from the estimation of these by-products of microbial metabolism. Patrick and Hill (24) noted the relationship between increased microbial growth in chilled orange juice and increased diacetyl values, and other workers have found diacetyl and A.M.C. values to be of assistance in evaluation of "Quality Indexes" in fruit juice processing.

The estimation of ethyl alcohol in soft drinks production is becoming an essential part of quality control operation, and the determination of alcohol content of ginger beer, low-gravity beer, soft drink blends, and related products necessitates many routine examinations.

In addition, the determination of alcohol production in yeast cell cultures and as a check on the undesirable formation of alcohol in fruit juices, has placed greater emphasis on this particular determination.

Holck and Fields (10) have suggested that the quantity of ethyl alcohol in apple juice may serve as an index to its microbial quality, and we have found similar determinations to be of value in the examination of fruit juices.

The standard laboratory procedure for the estimation of ethyl alcohol depends upon the well-known ebulliometric method, but this is time consuming, and as noted by Sawyer and Dickson (27) the need for a rapid screening method covering this type of examination is essential when large numbers of samples have to be examined. These authors (27) described a procedure for the automatic determination of both reducing sugars and alcohol in beer.

Alternative analytical procedures, for example the enzymic (yeast-alcohol dehydrogenase) method for ethyl alcohol estimation (8) and the employment of gas chromatography (5) were studied, but it was felt that the method outlined by Sawyer and Dickson (27) would provide a working basis for the development of an automated procedure suitable for the scope of our laboratory using the AutoAnalyzer.

The chromic oxidation of ethyl alcohol as a means of alcohol determination is well known, and Barini-Branchi (1) outlined a method for the estimation of alcohol in wines. The Cavett method has been modified by Nickolls (23) for the determination of alcohol in body fluids, and numerous other methods, based on chromic oxidation, exist.

Acidity determination forms an extremely important feature of quality control in soft drinks production. We are not immediately concerned with citric acid estimation, but rather with a total acidity-figure which would be equally suitable for study of such drinks as lemonades and products containing mixed fruit acids.

The Technicon Methodology

Citric Acid I, based on oxidation and bromination was found unsuitable for our particular purpose. Various modifications of a colorimetric method based on the conversion to pentabromacetone, followed by extraction into inert solvent and sodium sulfide colour producing reaction were unsuitable. Colour developing agents used as a

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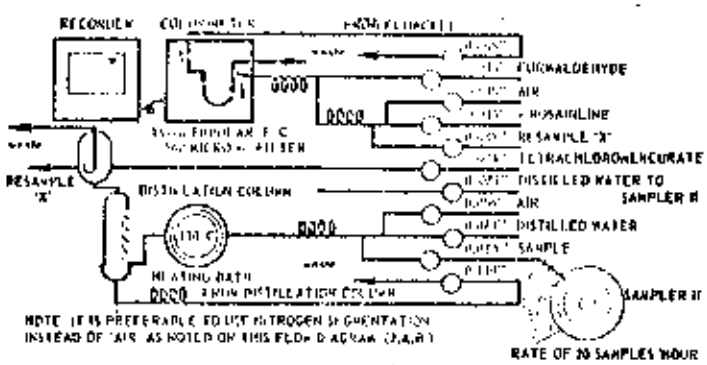

Figure 1

pentabromacetone reactant, such as iodine and alkaline resorcinol, were considered too elaborate for the variety of beverages to be studied.

Sulfur Dioxide

The apparatus for this method is the standard Technicon AutoAnalyzer with a Technicon distillation column fitted to the outlet of the heating bath.

Reagents

Formaldehyde:

1. Formaldehyde (40%)	5.0 ml
2. Distilled water, q.s.	1000 ml
3. Ethomeen	5 g

Preparation: Dilute the Formaldehyde to 1 litre with distilled water and add ethomeen. Prepare weekly. To prepare working solution, take 100 ml of the above solution and dilute to 1 litre with distilled water. Ethomeen is supplied by Armour Hess Chemicals Ltd.

Pararosaniline hydrochloride:

1. Pararosaniline hydrochloride	0.4 g
2. Concentrated hydrochloric acid	150 ml
3. Distilled water, q.s.	1000 ml

Preparation: Dissolve the pararosaniline hydrochloride in 500 ml of water. Slowly add the hydrochloric acid. Make up the solution to 1 litre in a 1 litre volumetric flask. This solution is stable for three months if stored in a cool dark place. To prepare working solution, dilute 100 ml of the above solution to 1 litre with distilled water.

Absorbing Reagent:

1. Mercury II chloride	6.8 g
2. Sodium chloride	2.9 g
3. Distilled water, q.s.	1000 ml

Preparation: Dissolve the mercuric chloride and the sodium chloride in water in a 1 litre volumetric flask. Dilute to volume with water. Keep tightly capped and refrigerated if it is to be stored for more than 24 hours.

Procedure

The sample is aspirated, diluted with distilled water, segmented with nitrogen and mixed in a single mixing coil. The solution is heated at 110°C. The vapour is then passed through the distillation column and absorbed in a continuous stream of absorbing reagent. The absorbed sulfur dioxide solution is resampled into a stream of nitrogen segmented pararosaniline hydrochloride and mixed in a single mixing coil. Formaldehyde is then introduced and the solution is mixed in a double mixing coil followed by mixing in a single mixing coil. The resulting colour is measured in a 15 mm flowcell using 560 mμ filters. Sample rate is 20 samples per hour with a 1:2 ratio of sample to wash. The heating bath must be lagged with glass fibre. We found that MS200 silicone fluid was satisfactory when used in the heating bath. This procedure follows the flow diagram shown in Fig. 1.

Discussion

This procedure provides a good rapid screening method for the evaluation and control of sulfur dioxide in soft drinks, and conserves time in laboratory operation. The method gives good reproducibility and at a sample rate of twenty per hour the cycle time is twelve minutes.

Sample standards if required are prepared from a solution of sodium metabisulfite added to a simulated soft drink. These prepared standards are included with each thirty samples tested. The range of standards used are adjusted according to the expected level of sulfur dioxide in the sample. We have found, for instance, that for a level of 70 ppm sulfur dioxide, standards of 60, 70 and 80 ppm are adequate, or samples themselves can be used as standards. Fig. 3 shows a recording of standards used in a test run.

Fig. 4 shows the relationship between percent transmission at 560 mμ and sulfur dioxide in parts

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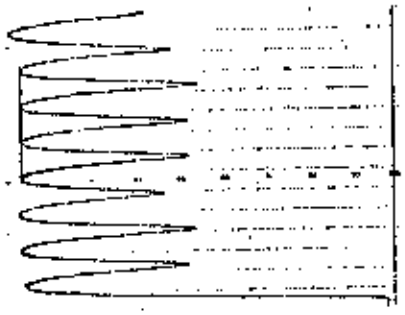

Figure 2

per million as set up for a particular test run. Fig. 2 reproduces a recorder trace showing the reproducibility obtained with four samples with identical sulfur dioxide concentration in a series of results taken from a typical test run. It is possible, also, by using a modification of the technique described in this paper, to determine the "free" sulfur dioxide in samples of soft drinks and fruit juice products using the AutoAnalyzer, and following a modification of Technicon's methodology S-11. If this is undertaken, the distillation column is removed, and the sample stream subjected to dialysis to remove fruit pulp and cellular material. The figure for "combined" sulfur dioxide can then be determined by deducting the "free" figure from the "total" figure. This "combined" sulfur dioxide figure can be of great value when studying the effective preservative value of sulfur dioxide when the latter exhibits varying degrees of complexing with carbonyl compounds.

Acetoin and Diacetyl

The apparatus for this estimation is the basic Technicon AutoAnalyzer pump, sampler II, colorimeter and recorder.

Reagents

Potassium hydroxide/creatine solution:

1.	Potassium hydroxide (AR)	30 g
2.	Creatine (AR)	0.2 g
3.	Distilled water, q.s.	1000 ml

Preparation: Dissolve the potassium hydroxide in a 1 litre flask, in distilled water. Make up to 1 litre mark, then add the creatine.

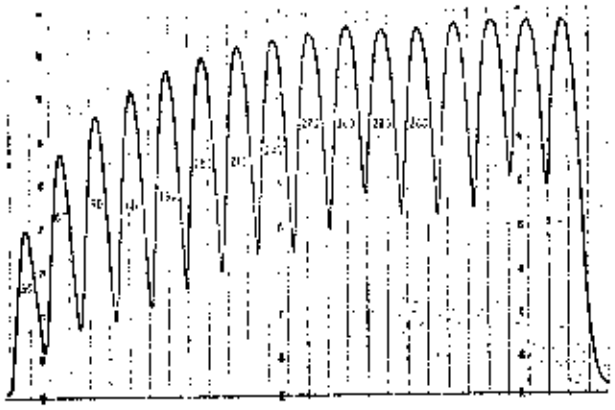

Figure 3

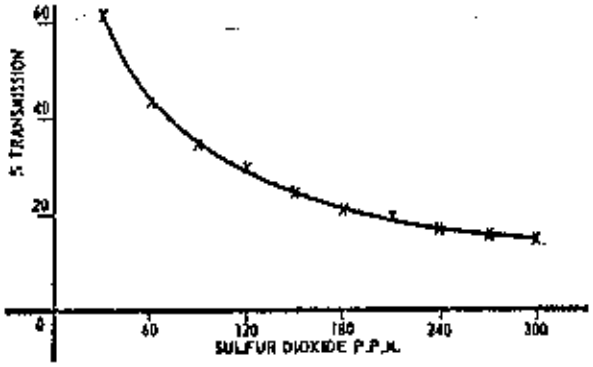

Figure 4

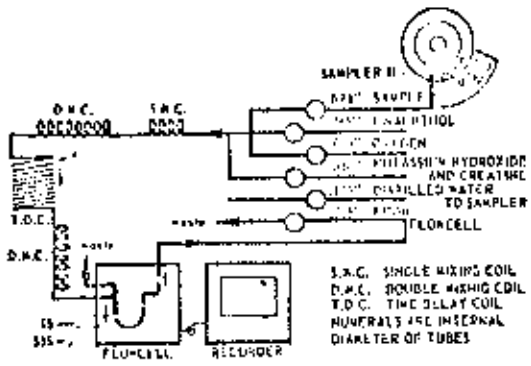

Figure 5

Alpha-naphthol Solution:

1.	Alpha-naphthol (AR)	2.5 g
2.	Isopropyl alcohol 99%	100 ml

Preparation: Dissolve the alpha-naphthol in the isopropyl alcohol in a 100 ml volumetric flask. Make up to the 100 ml mark.

The above reagents may be kept in amber bottles in a refrigerator for one week.

Oxygen:

Cylinder with reducing valve, flowmeter and pressure balance jar.

Standards:

Standards of Diacetyl and A.M.C. (and mixtures of both in varying proportions) were prepared daily in distilled water and used as control indicators in the AutoAnalyzer. Oxygen is drawn into the system via a reducing valve and atmospheric pressure balance jar.

Procedure

The sample is aspirated into a stream of oxygen segmented alpha-naphthol and mixed in a single mixing coil with potassium hydroxide/creatine solution. The solution is then mixed in a double mixing coil and passed through a time delay coil. The colour change is measured in a 15 mm flow-cell with 535 mμ filters. We found that a sample rate of 30 per hour with 1:1 ratio sample to wash give the most satisfactory results. This procedure follows the flow diagram as shown in Fig. 5.

Discussion

Cultures of fruit juice samples (concentrated fruit juices reconstituted to 12° Brix) were distilled in an apparatus fitted with a Parnas Wagner automatic discharge arrangement. 40 ml samples (with 10 ml distilled water) were distilled, the rate of distillation being such that each single distillation unit dealt with six samples per hour. 10 ml of the sample were distilled over.

Very good agreement is obtained between tests made on standard solution samples at varying concentrations and/or mixtures of diacetyl and A.M.C., and a factor of 0.9773 was used for conversion (oxidation) of A.M.C. to diacetyl. The method was found to be very specific and is of considerable value in examination of cultures or fruit juices (fruit juice concentrates reconstituted to 12° Brix before distillation). Fig. 6 shows pen recordings of standard solutions of A.M.C. and diacetyl and also results obtained from different mixtures of A.M.C. and diacetyl. Standard curves are shown in Figs. 7 and 8.

For extremely small amounts of A.M.C. or diacetyl, large samples may be distilled (i.e. 300 ml distilled and 25 ml distillate collected) and where applicable the concentration of diacetyl to A.M.C. may be determined by fractional distillation. The method is similar to that described by Fields (7) and Langlykke and Peterson (12).

The use of a time delay coil in the methodology outlined is necessary as there is a relationship between concentration of diacetyl and A.M.C. and the time taken for colour development. Murdock and Dennis (20) found that the addition of A.M.C. to a solution containing diacetyl retarded the rate of colour development, and as the colour production developed the rate of colour develop-

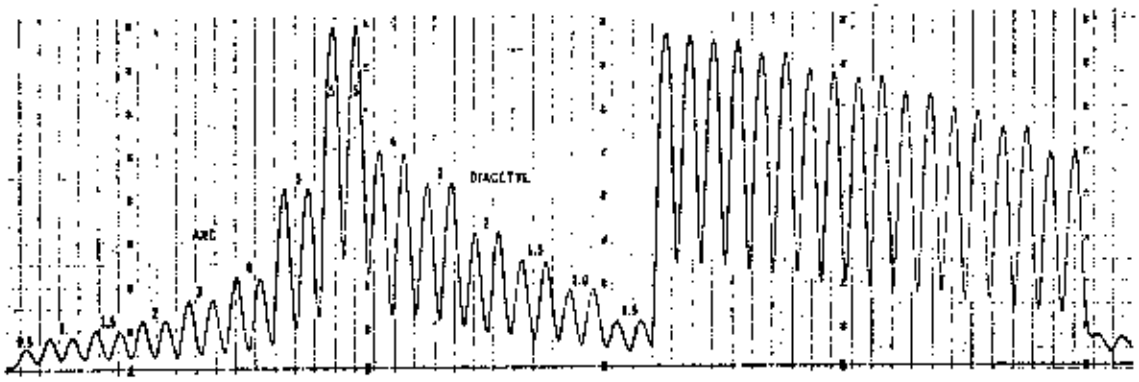

Figure 6

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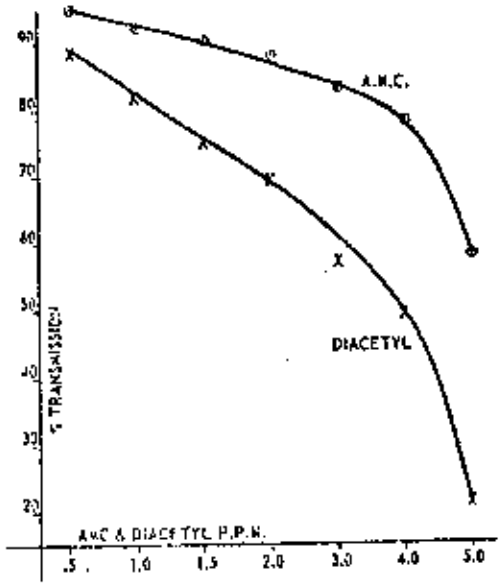

Figure 7

ment then increased until it exceeded that of solutions containing only A.M.C. or diacetyl. The time delay coil employed was such that a ten-minute holding time was effected. This can be varied, however, and although our laboratory has standardised the time to a ten-minute delay, no difficulty is experienced in either shortening or lengthening the time delay in order to examine particular features as outlined by Murdock and Dennis (20). The use of pure oxygen for segmentation was found to be an advantage and accelerated the A.M.C. oxidation.

Ethyl Alcohol

The apparatus used in this estimation is the basic Technicon AutoAnalyzer.

Reagents

Saline Solution:

1. Sodium chloride (AR)	9.0 g
2. Distilled water, q.s.	1000 ml

Preparation: Dissolve the sodium chloride in distilled water and make up to 1 litre in a volumetric flask.

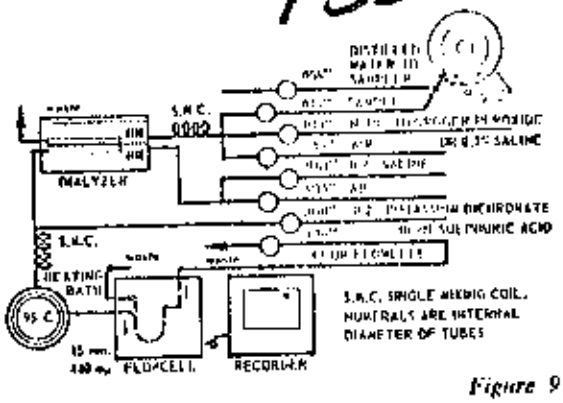

Figure 9

Potassium dichromate in sulfuric acid:

1. Potassium dichromate (AR)	2.0 g
2. 8N sulfuric acid	1000 ml

Preparation: Dissolve the potassium dichromate in the sulfuric acid. Make up to 1 litre in a volumetric flask.

N/100 Hydrogen peroxide:

Procedure

The methodology follows the outline shown in Fig. 9.

The sample is aspirated into an air-segmented stream of either distilled water or hydrogen peroxide and mixed in a single mixing coil. The solution is then dialysed into saline in the dialyser. Acidified potassium dichromate is introduced to the dialysate on emergence from the dialyser. The solution is then heated at 95°C in the heating bath. The colour change is measured at 440 mμ in the colorimeter using a 15 mm flowcell. Sample rate is 40 per hour with 2:1 ratio sample to wash.

Discussion

The methodology outlined for treatment of the distillate relies on the colour development produced after reacting the dialysed sample with a solution of potassium dichromate in sulfuric acid.

Ethanol standards in distilled water are prepared weekly and stored in the refrigerator in glass stoppered bottles. Hydrogen peroxide solution is

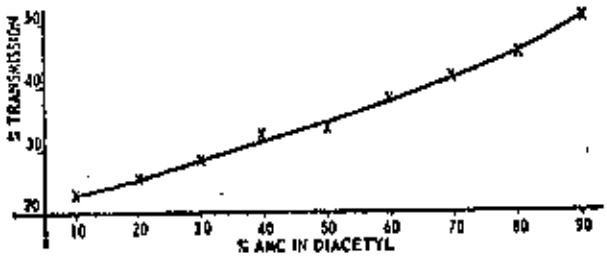

Figure 8

standardised by potassium iodide-thiosulfate titration and is prepared daily.

The methodology using the AutoAnalyzer follows that outlined in the Technicon Publication "Ethyl Alcohol IVb," but dichromate concentration to 0.2% and the primary neutralisation of sulfur dioxide (where required) by use of hydrogen peroxide is of new application.

It should be noted that although a dialyzer is shown in Fig. 9 this can be disregarded when using the predistillation technique described above.

Potassium dichromate oxidation of ethyl alcohol in the presence of sulfuric acid provides a primary oxidation of the molecule and yields acetic acid. The green colour in the completed reaction is due to chromic sulfate, and the colour is measured to give a quantitative value.

In the method described, samples were distilled using equipment fitted with a Parnas-Wagner automatic discharge arrangement, and this proved suitable for rapid routine preparation of samples both from low-gravity beer and soft drink products, fermentation cultures and other material containing ethyl alcohol. The range of alcohol studied was not in excess of 2% v/v.

Recovery rates of ethyl alcohol using the distillation technique, followed by estimation in the AutoAnalyzer, gave extremely good figures, and the methodology is shown later. When sulfur dioxide is present in the original sample it is necessary to effect neutralisation. This can be done by hydrogen peroxide reaction in the AutoAnalyzer. It was found convenient to neutralise in the distillation flask, but where neutralisation is carried out in the AutoAnalyzer, hydrogen peroxide was satisfactory. The use of iodine, ferrous salts or sodium hydroxide is unsuitable.

If neutralisation is carried out by primary reaction in the AutoAnalyzer, the hydrogen peroxide must not be in excess, and the concentration (N/100) is sufficient for sulfur dioxide to the order encountered in practical work (70 ppm). If neutralisation is carried out in the distillation flask the N/100 hydrogen peroxide in the AutoAnalyzer methodology should be replaced by 0.9% saline solution.

Production of hydrogen sulfide by yeasts has been reported by Rankine (25) and in a later paper (26) he studied this in more detail. Various strains of yeasts differ considerably in their hydrogen sulfide production, but when present in cultures, hydrogen sulfide causes side reactions in the methodology outlined. The presence, or absence, of hydrogen sulfide in cultures was established by using tubes of lead acetate supported on cellulose powder (as outlined by Rankine). Precipitation with bismuth nitrate in the presence of hydrochloric acid is found to be adequate for the removal of hydrogen sulfide when this step is indicated.

It would be meaningless in the context of this paper to record individual findings of ethyl alcohol concentration in the various cultures examined, or for that matter to make individual records of the results of analysis of low-gravity beer and soft drink products.

Reproducibility of results was of an extremely high order with concentrations up to 2.0 percent weight per volume ethyl alcohol and results were to an accuracy of ± 0.02% v/v in this range.

It was found that it is necessary to distil samples before analysing in the AutoAnalyzer, since the sugar present in either beverages or cultures interferes with this method and gives a false result.

Total Acidity

The apparatus is the basic Technicon AutoAnalyzer consisting of sampler II, pump, dialyser, heating bath (for carbonated beverages), colorimeter and recorder.

Reagents

Sodium chloride solution:

1. Sodium chloride	25 g
2. Distilled water, q.s.	1000 ml

Preparation: Dissolve the sodium chloride in distilled water in a 1 litre volumetric flask. Make up to the 1 litre mark with distilled water.

Stock Bromocresol green solution:

1. Bromocresol green	1 tube of B.D.H. water soluble
2. Distilled water q.s.	1000 ml

Preparation: Dissolve contents of one tube of Bromocresol green in distilled water in a 1 litre volumetric flask. Make up to the 1 litre mark with distilled water. To prepare working solution dilute 400 ml of stock solution to 1 litre with distilled water.

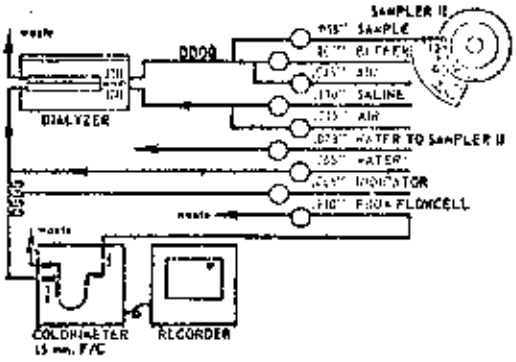

Figure 10

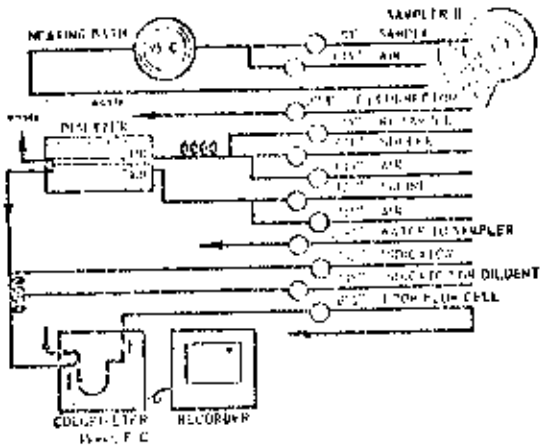

Figure 11

Stock Buffer Solution;

1. Citric acid monohydrate	4.2 g
2. Anhydrous dibasic sodium phosphate (Na_2HPO_4)	17.8 g
3. Distilled water	500 ml

Preparation: Dissolve the citric acid and anhydrous dibasic sodium phosphate in distilled water. Make up to the 500 ml mark in a 500 ml volumetric flask, with distilled water. To prepare working solution for carbonated and low acidity beverages, dilute 10 ml of the stock buffer solution to 1 litre with distilled water. To prepare working solution for squashes, cordial and high-acidity juices dilute 100 ml to 1,000 ml with distilled water.

Procedure

For non-carbonated drinks Fig. 10 shows the flow diagram for total acidity.

The sample is aspirated into a stream of buffer solution segmented with air and mixed in single mixing coil. The stream is then dialysed into a saline solution. The dialysate is diluted with distilled water and then mixed with bromocresol green in a single mixing coil. The colour reduction is measured in a 15 mm flowcell using 610 mμ filters. Sample rate is 50 per hour using a 2:1 ratio sample to wash.

Fig. 11 shows the flow diagram for total acidity estimation in carbonated beverages.

The sample, after de-gassing, is aspirated, segmented with air and mixed with buffer solution in a single mixing coil. The mixture is dialysed into a solution of saline in the dialyser. The dialysate is mixed with indicator in a double mixing coil and the colour reduction measured in a 15 mm flowcell using 610 mμ filters. Sample rate is 30 per hour with a 1:2 ratio sample to wash.

Discussion

It was found essential to remove dissolved carbon dioxide from the sample. Evolution of carbon dioxide caused sample size variation, and primary decarbonation had to be done either manually or with ultrasonic vibration. The dissolved carbon dioxide was eliminated by heating at 95°C for five minutes in a heating bath. After de-carbonating the stream is re-sampled through a C3 de-bubbler and mixed in a single mixing coil with buffer solution. The sample is then dialysed into saline solution in order to remove fruit pulp and cellular material, and the dialysate mixed with bromocresol green.

Fig. 12 shows the relationship between % transmission and % weight per volume of total acidity (citric monohydrate). Fig. 13 shows a pen recording of results from standards and reproducibility of this method.

The method employed for squashes, cordials and juices is similar to that employed for carbonated beverages, but since there is no need to eliminate dissolved carbon dioxide the heating bath has not been used.

We found that it was better to use water-soluble bromocresol green, since bromocresol green dissolved in alcohol causes noise on the recorder chart.

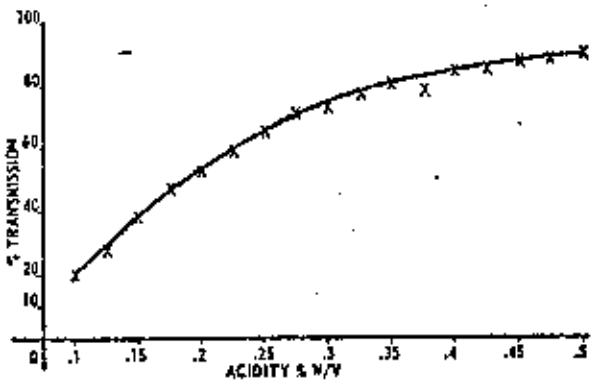

Figure 12

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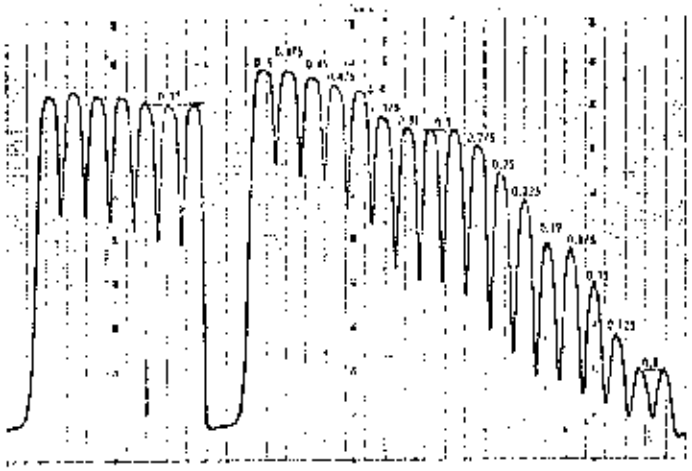

Figure 13

General Discussion

It must be pointed out that for purpose of routine control testing of soft drinks it is not always necessary to run through separate control samples with the tests themselves. We have found that the time consumed in the preparation of standards is wasteful and we use a small proportion of the actual samples themselves for control standards. This is achieved by taking a ratio of test samples which show either high or low chart traces and to carry out a separate titration for acidity determination, or a distillation test in the case of sulfur dioxide estimation. These results are then used to establish concentration figures upon which the remainder of the test samples can be evaluated. This method of short-circuiting the time for preparation of separate standards has been found to be of great value and is a departure from the more traditional methods.

The parameters (or tolerances) which are generally agreed by each laboratory or quality control centre must also be considered in relation to the interpretation of the tests, and we find it is quite adequate to "write into" the chart reader these values.

To quote an example, the level of sulfur dioxide in any particular product may be set at a level of 60 parts per million. The tolerance allowed by the quality control centre may be 58-62 parts per million. The number of samples to be examined may be to a total of one hundred during the day's operation on any one production line. The production line samples are run through the AutoAnalyzer, and during the course of the run, which takes a total time of five hours, a number of replicate samples are taken, generally those showing low and high results, and these are checked by distillation technique. The figures determined for sulfur dioxide concentration by the distillation technique are then plotted onto the chart reader and this enables the whole series of test results to be read off. To check for machine errors it is of course simple to put through a set of known standard values per each fifty tests. This same method of control can be used when determining total acidity and other parameters in soft drinks.

This approach to the problem of "control" standards has been found to be quite adequate. In particular, when dealing with volatile components such as sulfur dioxide in acid solution, considerable time is saved, as otherwise standard solutions have to be prepared frequently and just as frequently checked by traditional tests methods.

Such method of standardisation appears very different to the conventional approach, but is, nevertheless, perfectly accurate and satisfactory in every way.

The subject of tolerances was mentioned earlier, and it has been found that by using the AutoAnalyzer technique these tolerances can be reduced in actual terms of experimental error against production error.

Multi-channel techniques are possible with the AutoAnalyzer which enable the quality control laboratory to monitor a number of constituents simultaneously. This again represents a considerable step forward in time saving and in supplying quality information to operation staff.

It is also evident that the results of each test, in addition to being printed as a trace on a recorder, can also be given numerical values on a printer. With this point in mind, the automation of a number of quality control tests is being put in hand, as future plans may well encompass the storage of such information and the use of computer techniques to evaluate and process information covering a series of control tests.

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Summary

A technique for automating analytical procedure for sulfur dioxide and total acidity in soft drinks applicable both to carbonated and non-carbonated products was developed. Similarly, a technique for determination of acetoin, diacetyl and ethyl alcohol (following distillation in a Parnas-Wagner Still) was utilized for control of process in soft drinks manufacture. These techniques adapt certain existing manual procedures to automated methods and with suitable electronics can be adapted to instream analysis and quality control systems.

Résumé

Les auteurs ont orienté l'activité de leur laboratoire vers la mise au point d'une technique automatique applicable à l'industrie des boissons non alcoolisées et des jus de fruits. Ils ont développé une méthode entièrement automatique utilisant l'AutoAnalyzer Technicon pour le dosage du SO_2 "total", ainsi que du SO_2 "libre". Les auteurs ont également automatisé une méthode pour la détermination de l'éthanol ainsi que pour le dosage le l'acétylméthylcarbinol et du diacétyl. Ces deux derniers points présentent un intérêt certain en tant que "contrôle sanitaire" dans le fonctionnement d'une usine.

Zusammenfassung

Unter Verwendung des Technicon AutoAnalyzer wurde eine vollautomatische Methode zur Bestimmung des Gesamt-Schwefeldioxids entwickelt; ferner wird auch eine Methode zur Bestimmung des freien Schwefeldioxids verwendet.

Ebenso wird auch die Gesamtacidität von Getränken automatisch bestimmt.

Auch eine revidierte Methode zur Bestimmung des Äthylalkohols und eine Kontrollmethode zur Bestimmung von Acetylmethylcarbinol und Diacetyl ist zur Anwendungsreife entwickelt worden. Diese letztgenannten Punkte sind als Kenngrößen für die Betriebssauberkeit von Bedeutung.

Clin. Chem. 15 (1969)

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Measurement of Bisulfite in Biologic Solutions

James W. Wilkins, Jr., and James N. Rivecca*

In order to evaluate the toxicity of sodium bisulfite ($NaHSO_3$) used in peritoneal dialysis solutions it was necessary to measure bisulfite in simple solutions and serum. A microdiffusion technic is described for quantification of bisulfite. This involves evolution of SO_2 gas from bisulfite by acidification, trapping it as H_2SO_3, oxidation of the H_2SO_3 to H_2SO_4 by hydrogen peroxide, and titration of the hydrogen ion with dilute base. The method is easily performed and is quantitative in the range of 50–500 μg bisulfite per milliliter in simple solutions. Studies in serum show incomplete yet quantitatively reproducible recovery.

In assessing the toxicity of sodium bisulfite ($NaHSO_3$) which was being used in peritoneal dialysis solutions, it was necessary to measure low concentrations of bisulfite in dialysis fluid and serum. Existing methods used to determine bisulfite fall into three groups. All involve evolution of SO_2 by acidification, trapping of the SO_2 in a neutral solution, and analysis of the H_2SO_3 thus formed. In the first group bisulfite is oxidized to sulfate which is usually quantified gravimetrically following precipitation (*1*). In the second, bisulfite is determined as a reducing compound, such as by iodine titration (*2, 3*); and in the third, the H_2SO_3 is oxidized by H_2O_2 to H_2SO_4 and the resulting hydrogen ion titrated with alkali (*1, 4*). Such macroanalyses have been employed for many years, most notably in the food and leather industries. A modification of the Monier-Williams method (*1*) enables bisulfite to be quantified in biologic solutions by a microdiffusion technic.

Methods

Principle

Solutions containing bisulfite, when acidified, liberate SO_2 gas which may be trapped as H_2SO_3 in solution. The H_2SO_3 can easily be oxidized by hydrogen peroxide to nonvolatile H_2SO_4. The resulting solution

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can be titrated with weak base to a neutral endpoint. The amount of base used is proportional to the amount of hydrogen ion formed and, therefore, to the quantity of bisulfite liberated from the original solution. With acidification, carbon dioxide is also liberated from any bicarbonate in solution. Carbon dioxide may be trapped in the hydrogen peroxide solution as H_2CO_3, thereby increasing the titratable hydrogen ion content. The error introduced by the presence of bicarbonate can be eliminated by mild acidification of the peroxide solution and allowing the volatile CO_2 to escape.

Materials

The gas diffusion is carried out in Conway-type microdiffusion units (*5*) of glazed porcelain: 68 mm (OD) × 13 mm high with an inside depth of 10 mm and a central chamber of 35 mm (ID) × 5 mm deep. The Conway units are sealed airtight during determinations by covering them with plate glass lids (76 × 76 mm) coated with viscous soap or petrolatum. For titration a microburet is used.

Reagents

1. *Outer chamber solution* 25% (v/v) H_2SO_4 (Anal reagent, ACS).
2. *Central chamber solution* Dilute 5 ml of 3% (v/v) hydrogen peroxide and 1 ml of 1% (w/v) phenolphthalein in 95% ethanol to 100 ml by the addition of 0.0002 N HCl. This solution is filtered using Whatman No. 1 filter paper, and stored in a brown bottle.
3. *Bisulfite standards* $NaHSO_3$ (Reagent)—50 μg/ml, 100 μg/ml, 250 μg/ml, and 500 μg/ml in normal saline, which was prepared from CO_2 free distilled water.
4. *Titration base* 0.005 N sodium hydroxide.

Procedure

Pipet 1 ml of the central chamber solution into the central chamber of each Conway diffusion unit, and add 1 ml of the 25% H_2SO_4 to one side of each outer chamber. Next, pipet 1 ml of either a standard, unknown, or a saline blank into its respective chamber opposite the H_2SO_4 so that no mixing with the H_2SO_4 occurs. The unit is then sealed airtight with a plate glass lid coated with viscous soap, and the solutions in the outer well mixed by gentle rotation. After 1 hr the plate glass cover is removed and the units are exposed to room air for 10 min thus allowing any CO_2 trapped as H_2SO_3 in the central chamber solution to escape. The central chamber solution is titrated with 0.005 N NaOH to a neutral endpoint using phenolphthalein as an indicator.

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The volume of alkali used is recorded in μliters. As long as the relationship between the amount of bisulfite and the volume of base is linear the following ratio may be applied:

$$\frac{\mu g/ml \text{ unknown}}{\mu l \text{ unknown} - \mu l \text{ blank}} = \frac{\mu g/ml \text{ standard}}{\mu l \text{ standard} - \mu l \text{ blank}}$$

In all determinations the unknowns, standards, or blanks were run in triplicate and calculations were based on the mean.

Experimental

In order to determine linearity of the above method, standard solutions containing from 50 to 500 μg $NaHSO_3$ per milliliter were analyzed. To evaluate variation in this method replicate determinations were made on saline solutions containing 50, 100, 250, and 500 μg $NaHSO_3$ per milliliter. Recovery and reproducibility of the method for measuring bisulfite in serum were also assessed by adding bisulfite to serum in concentrations of 100, 250, and 500 μg $NaHSO_3$ per milliliter. All saline and serum replicate determinations were performed in triplicate using six separate specimens for each concentration.

Results

Using the method for bisulfite measurement described above, acceptable linearity was found in the range of 50–500 μg $NaHSO_3$ per milliliter (Fig 1). The six replicate determinations of $NaHSO_3$ in saline performed in triplicate for each specimen gave mean values ($\pm$2 SD) of 500 $\pm$ 30, 250 $\pm$ 10, 100 $\pm$ 12, and 50 $\pm$ 1.8 μg/ml. Replicate determinations in serum demonstrated incomplete recovery of added bisulfite when compared with the bisulfite containing saline standard. However, the reproducibility of the method in serum was not significantly impaired (Table 1).

Discussion

The method presented is easily performed and is reliable and accurate for measurement of bisulfite in simple solutions in the range of 50 μg–500 μg $NaHSO_3$ per milliliter. Reproducibility of bisulfite measurement in serum and saline was good. Recovery of bisulfite added to serum was incomplete at all three levels studied (100, 250, and 500 μg per milliliter). The incomplete recovery could be due to several factors. Some of the bisulfite could have been oxidized to sulfate, which would not be assessed by this method. Also the mixture of serum and 25% H_2SO_4 forms a gelatinous mass which may interfere with SO_2 evolu-

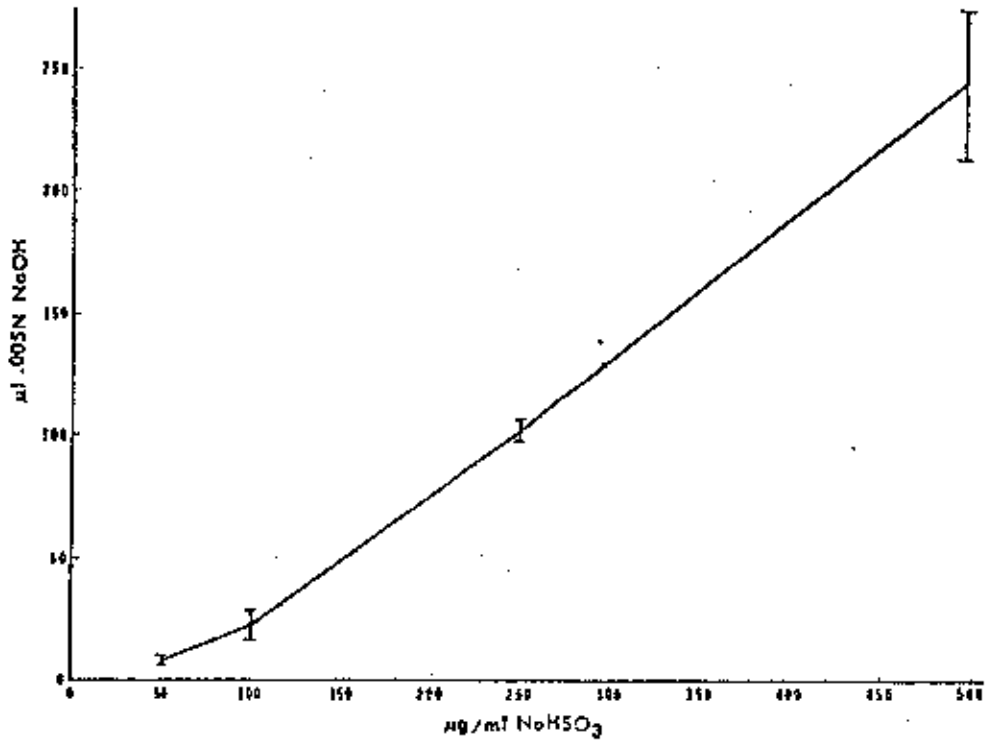

Fig 1. The standard curve obtained for bisulfite in saline. Net µliters of NaOH (standard-blank) plotted against concentration of $NaHSO_3$ (N = 18 [6 individual specimens; analysis performed in triplicate]). The vertical bars represent ±2 SD.

Table 1. Recovery of Bisulfite Added to Serum

Amount $NaHSO_3$ added (µg/ml)	Mean amount $NaHSO_3$ recovered* (µg/ml)	SD (µg/ml)	Recovery in serum (%)
100	82	5.4	82
250	214	23	86
500	382	28	76

* Mean of 6 triplicate determinations, N = 18.

tion. A similar difficulty has been reported with the alkalinization of whole blood in microdiffusion analysis of ammonia (*6*). It should be stressed, however, that this method is reliable in discerning quantitatively differing serum concentrations. The method has been employed for measuring levels of bisulfite in serum during continuous infusion in dogs and in peritoneal dialysis solutions before and after autoclaving (*7*).

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Toxicity of intraperitoneal bisulfite

Studies were carried out in animals to investigate the toxicity of intraperitoneal bisulfite. The LD_{50} (dose lethal to 50 per cent of the animals) for a single intraperitoneal injection of $NaHSO_3$ was calculated in the mouse, rat, rabbit, and dog. Studies indicated that the LD_{50} dose was increased by dilution of the bisulfite. When sodium bisulfite was infused at a rate of 80 mg. of $NaHSO_3$ per kilogram per hour in the portal vein of dogs having bilateral ureteral ligations, bisulfite did not accumulate in the serum. Autoclaving dialysis fluid substantially reduced its bisulfite concentration. These studies suggest that $NaHSO_3$ in amounts usually added to dialysis solutions is unlikely to cause toxicity in patients undergoing peritoneal dialysis.

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Sodium bisulfite ($NaHSO_3$) is added to parenteral solutions as an antioxidant. The peritoneal dialysis solutions used in our hospital contained 0.01 per cent $NaHSO_3$ in the 1.5 per cent glucose solution and 0.05 per cent $NaHSO_3$ in the 7 per cent glucose solution. A patient given 48 L. of dialysate daily, 12 of which are the 7 per cent glucose solution, would be exposed to 9,600 mg. of $NaHSO_3$. The possibility of excessive administration of $NaHSO_3$ by this route warranted further investigation. The following studies in animals were carried out to determine the toxicity of peritoneal dialysis solutions containing $NaHSO_3$.

Materials and methods

Experiment I—Single-dose intraperitoneal LD_{50} values of $NaHSO_3$. The animals used were male Swiss-Webster white mice weighing 19 to 25 grams; male Sprague-Dawley white rats weighing 235 to 310 grams; male New Zealand white rabbits weighing 1.1 to 1.9 kilograms; and male and female mongrel dogs weighing 7.8 to 15.8 kilograms. Animals were fasted 24 hours prior to and during the day of injection. Four animals of each species were tested at each of four dosage levels. Mice were given 300 mg. per kilogram of body weight* of a 1.25 per cent $NaHSO_3$ solution which was prepared immediately prior to injection in normal saline from which dissolved gases had been evacuated. Subsequent dose levels were each increased by 50 per cent. The solutions given to the rats, rabbits, and dogs contained 25 per cent $NaHSO_3$ similarly prepared. Rats were given the same dose as mice. Rabbits were given 133 mg. of $NaHSO_3$ per kilogram and this dose level was increased 50 per cent each time. Dogs were given

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*All doses throughout the rest of this paper are expressed in relation to body weight.

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174 mg. of $NaHSO_3$ per kilogram with each subsequent dose increased 33 per cent. Animals were observed for 72 hours following injection. LD_{50} (dose lethal to 50 per cent of the animals) values were calculated using the methods of Weil.[7]

Experiment II—Single-dose intraperitoneal LD_{50} values and the effect of the $NaHSO_3$ concentration. To investigate the effect of the $NaHSO_3$ concentration on the single-dose intraperitoneal LD_{50}, male Sprague-Dawley rats weighing 350 to 400 grams were used. Two concentrations of $NaHSO_3$ solution (5 and 1.25 per cent) were used to compare with the similarly prepared 25 per cent solution in Experiment I. Four animals were injected with 500 mg. of $NaHSO_3$ per kilogram and this dose level was increased by 33 per cent each of three additional doses. Animals were observed for 72 hours and LD_{50} values were calculated.

Experiment III—Measurement of bisulfite in biological fluids. A method was devised to measure bisulfite in various biological solutions.[8] The solution containing bisulfite was placed in a Conway microdiffusion unit and acidified. The liberated SO_2 gas was trapped as H_2SO_3 in solution, oxidized by hydrogen peroxide to nonvolatile H_2SO_4, then titrated with weak NaOH. This method was used in following serum bisulfite levels in Experiment IV and in determining bisulfite concentrations in dialysis solutions following autoclaving of six different bottles of 7 per cent glucose dialysis fluid and six different bottles of 1.5 per cent glucose dialysis fluid. All determinations were run in triplicate.

Experiment IV—Serum levels of bisulfite during continuous intravenous administration of solutions containing $NaHSO_3$. Three mongrel dogs weighing 15 to 22 kilograms were anesthetized with 30 mg. of pentobarbital per kilogram intravenously, and their ureters were ligated by an abdominal approach. The splenic vein was cut free, a polyethylene catheter inserted, and freshly prepared solutions of $NaHSO_3$ in normal saline were infused at a rate of 160 mg. per kilogram per hour for three hours. Immediately following this an infusion of 80 mg. of $NaHSO_3$ per kilogram per hour was carried out for an additional three hours. Blood samples for bisulfite analysis were drawn hourly from a catheter in the femoral artery.

Results

Experiment I. The single intraperitoneal dose LD_{50} values were determined to be 675, 498, 300, and 244 mg. of $NaHSO_3$ per kilogram for the mouse, rat, rabbit, and dog, respectively (Table I). In all cases of death due to bisulfite, animals died within 45 minutes of injection. The manner of death was best observed in the dog. The animals were extremely irritable and had clonic convulsions followed by respiratory instability with periods of apnea and cyanosis. Terminally, there was evidence of cardiovascular collapse associated with prolonged apnea.

Experiment II. The LD_{50} in the rat for a single intraperitoneal dose was found to be dependent upon the concentration of $NaHSO_3$ in the injected solution. The LD_{50} values were 498, 650, and 740 mg. of $NaHSO_3$ per kilogram with the 25, 5, and 1.25 per cent $NaHSO_3$ solutions, respectively (Table I).

Experiment III. Dialysis fluid bisulfite concentrations determined following autoclaving revealed a marked loss in the quantity of bisulfite in solution. The 7 per cent glucose solutions to which 500 mg. of $NaHSO_3$ per liter were added prior to autoclaving were found to contain 47 mg. of $NaHSO_3$ per liter (± S.E.M. of 5 mg. per liter) following autoclaving. The solutions of 1.5 per cent glucose to which 100 mg. of $NaHSO_3$ per liter had been added contained less than 25 mg. of $NaHSO_3$ per liter following autoclaving. (Reliability of this method for determining concentrations of $NaHSO_3$ less than 25 mg. per liter was not evaluated.)

Experiment IV. The serum concentration of $NaHSO_3$ in the dog was determined during continuous splenic vein infusion of

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CHIMIE BIOLOGIQUE. — *Inhibition des oxydases de l'*Agaricus campestris *par l'association de substances vitaminiques à l'anhydride sulfureux.* Note de MM. Igor Voinovitch, Henri Chretel, Jacques Durocher et Ernest Kahane, présentée par M. Maurice Javillier.

Au cours de l'étude du système enzymatique responsable du noircissement de certains champignons, nous avons été amenés à examiner l'action inhibitrice qu'exercent différentes substances sur ce phénomène. Il est classique d'utiliser l'anhydride sulfureux et les sulfites pour cette inhibition, mais on connaît les inconvénients d'ordre pratique de ces réactifs. Nous avons étudié l'action de faibles doses de trois substances à activité vitaminique.

Ces recherches étaient entreprises lorsque nous avons eu connaissance du travail de Titaiev (¹) sur l'action antioxydante, équivalente à celle de l'acide ascorbique, exercée par la thiamine dans les réactions d'oxydo-réduction. De notre côté, nous avions non seulement observé cette action, mais encore déterminé qu'elle est plus intense que celle de l'acide ascorbique sur l'oxydation enzymatique de la tyrosine, du pyrogallol et du substrat naturel préparé à partir de l'*Agaricus campestris*.

L'adjonction à la thiamine de faibles quantités de métabisulfite de potassium entrave la formation de thiochrome. En outre, diverses substances vitaminiques associées à l'anhydride sulfureux, soit libre, soit sous forme de métabisulfite, présentent une action synergique remarquable. On obtient une véritable exaltation de l'activité propre de chacun des composés utilisés, dont les doses nécessaires sont réduites très considérablement, et qui assurent une protection bien plus durable que chacun des constituants pris séparément.

Diverses associations dont l'acide ascorbique + SO_2, la thiamine + SO_2 et l'acide nicotinique + SO_2 ont été essayées. L'action antioxydante de la thiamine + SO_2 et de l'acide nicotinique + SO_2 paraît très supérieure à celle de tous les systèmes actuellement connus.

Les seuils d'activité dans les conditions choisies (20^{mg} de *tyrosine* et 1^{cm^3} d'un certain extrait oxydasique d'agaric pour 200^{cm^3} d'eau) sont les suivants :

SO_2	3^{mg}
Acide ascorbique	2^{mg}
Acide ascorbique + SO_2	$1^{mg} + 0^{mg},5$
Thiamine	$0^{mg},02$
Thiamine + SO_2	$0^{mg},01 + 0^{mg},005$

Sur 100^{mg} de *pyrogallol* et 1^{cm^3} d'extrait oxydasique d'agaric pour 200^{cm^3} d'eau, on obtient :

(¹) *Biochimia U. R. S. S.*, 13, 1948, p. 177 à 206.

SO_2	20^{mg} : arrêt total pendant 48 heures
Acide nicotinique	40-50^{mg} : arrêt partiel pendant 4 à 5 heures
Acide nicotinique + SO_2	4^{mg} + 4^{mg} : arrêt total pendant 15 jours
Thiamine	30 à 50^{mg} : arrêt partiel pendant 5 à 6 heures
Thiamine + SO_2	12^{mg} + 5^{mg} : arrêt total pendant 15 jours

Seule l'association de la thiamine ou de l'acide nicotinique avec l'anhydride sulfureux permet l'arrêt total de l'oxydation du pyrogallol et ceci pendant un laps de temps prolongé.

L'amide nicotinique ne présente aucune activité à cet égard.

Nous avons examiné l'action de quelques produits d'origine biologique riches en vitamines : macérés de levure de boulangerie et de brasserie, jus de citron et d'orange. La levure de boulangerie s'avère de loin la plus intéressante des quatre substances naturelles étudiées.

Nous n'avons observé dans aucun de nos essais avec la thiamine synthétique ou naturelle, et quel que fût le substrat, de développement de moisissures, contrairement à ce qui a été constaté avec l'acide ascorbique naturel ou non. Il semble donc que la thiamine soit douée de propriétés antiseptiques.

Nous avons étudié, à côté de l'action de la thiamine, celle des deux fractions de cette molécule. La thiamine agit par l'un ou l'autre de ses constituants suivant le substrat. Ainsi, le méthyl-4 hydroxyéthyl-5 thiazol protège la tyrosine, tandis que la méthyl-2 amino-4 pyrimidine protège le pyrogallol. Leur action est exaltée par l'anhydride sulfureux.

BIOLOGIE. — *Application de la méthode des dilutions isotopiques à l'étude de la spécificité des protéines.* Note (*) de MM. Georges Schapira, Jean-Claude Dreyfus et Jacques Kruh, transmise par M. Léon Binet.

L'emploi en Biologie des isotopes lourds et radioactifs permet de suivre le métabolisme d'une substance donnée. Il permet également, grâce à la méthode de dilution isotopique inaugurée par Rittenberg et Foster (¹), de doser dans un mélange un composé défini dont la séparation quantitative serait difficile. Cette dernière technique postule la connaissance préalable exacte de la nature chimique de la substance étudiée.

Nous avons tenté de l'appliquer à un problème voisin : étant donné un composé, isolé d'un milieu biologique, et présentant des caractères analogues à ceux d'une substance déjà connue, vérifier l'identité de ces deux composés. Il s'agit donc d'une étude qualitative de la spécificité d'un composé par rapport à une substance de référence identique ou voisine.

Le principe de la méthode que nous proposons est le suivant : on met en présence les deux substances étudiées. On soumet ensuite le mélange à un fractionnement par une méthode chimique ou physique et l'on compare

(*) Séance du 30 mai 1949.

(¹) *J. Biol. Chem.*, 133, 1940, p. 737.

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Table I. *Single-dose intraperitoneal LD_{50} studies of sodium bisulfite*

	Mouse		Rat						Rabbit		Dog	
	1.25 Gm. $NaHSO_3$ per 100 ml. solution		25 Gm. $NaHSO_3$ per 100 ml. solution		5 Gm. $NaHSO_3$ per 100 ml. solution		1.25 Gm. $NaHSO_3$ per 100 ml. solution		25 Gm. $NaHSO_3$ per 100 ml. solution		25 Gm. $NaHSO_3$ per 100 ml. solution	
	Dose (mg./Kg.)	Deaths	Dose (mg./Kg.)	Deaths	Dose (mg./Kg.)	Deaths	Dose (mg./Kg.)	Deaths	Dose (mg./Kg.)	Deaths	Dose (mg./Kg.)	Deaths
	300	0/4	300	0/4	500	0/4	500	0/4	133	0/4	174	0/4
	450	0/4	450	1/4	651	4/4	654	0/4	200	2/4	230	1/4
	675	2/4	675	4/4	852	2/4	852	3/4	300	1/4	300	4/4
	1,012	4/4	1,012	4/4	1,125	4/4	1,125	4/4	450	3/4	393	4/4
LD_{50}: (mg./Kg.)	675		498		650		740		300		244	
95% confidence range of LD_{50} (mg./Kg.)	534 to 853		406 to 610		558 to 756		650 to 845		183 to 491		213 to 279	

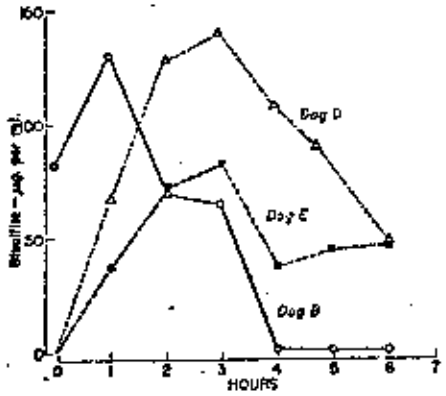

Fig. 1. The serum level of bisulfite (micrograms per milliliter) of three dogs during splenic vein infusion of $NaHSO_3$ is plotted against the hours of infusion. The infusion rate was 160 mg. of $NaHSO_3$ per kilogram per hour for the first three hours, then was reduced to 80 mg. of $NaHSO_3$ per kilogram per hour for the subsequent three hours. Dog B received a loading infusion of 240 mg. of $NaHSO_3$ per kilogram per hour for two hours prior to the determination of the serum level at time zero.

$NaHSO_3$ solutions. Following three hours of infusion at a rate of 100 mg. per kilogram per hour, levels of 50 to 140 µg $NaHSO_3$ per milliliter of serum were recorded. In all three animals, a prompt fall in the serum concentration of bisulfite followed reduction in the infusion rate from 160 to 80 mg. of $NaHSO_3$ per kilogram per hour. In one animal the serum bisulfite level after one hour fell to less than 25 µg per milliliter and in the other two to between 25 and 50 µg per milliliter (Fig. 1).

Discussion

Little is known about the acute toxicity of $NaHSO_3$, and even less about chronic toxicity. Hoppe and Goble[4] found the single-dose intravenous LD_{50} of $NaHSO_3$ to be 130 mg. per kilogram for the mouse, 115 mg. per kilogram for the rat, 95 mg. per kilogram for the hamster, and 65 mg. per kilogram for the rabbit. The LD_{50} per kilogram decreases as animals increase in size. The toxicity of long-term exposure to bisulfite also was studied by Hoppe and Goble[4] in the rabbit. The intravenous administration of two thirds of the intravenous LD_{50} dose, given every eight hours, five days a week, for three weeks, caused no deaths or pathophysiologic changes.

Reiss and Gerstl[5] investigated the intraperitoneal toxicity of $NaHSO_3$ in mice and reported that 0.18 mmole (18.54 mg.) or more given intraperitoneally caused death

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within a few minutes. Two of five mice died after injection of 0.12 mmole (12.36 mg.). Reiss and Gerstl found that bisulfite in toxic doses had an immediate effect on the central nervous system as indicated by clonic convulsions, and on the cardiovascular center with rapid fall of blood pressure.

Bhaghat and Lockett[1] administered $NaHSO_3$ intraperitoneally to rats and found that a single injection of an isotonic solution of bisulfite equal to 3 per cent of body weight (555 mg. per kilogram) killed half of the animals. Toxic doses caused restlessness, cyanosis, prostration, and finally cardiovascular collapse with death within 35 minutes. Eighty to 90 per cent of a sublethal dose of $NaHSO_3$ could be accounted for as sulfate in the urine within four hours.

The toxicity of peritoneal dialysis solutions containing $NaHSO_3$ has been investigated by Halaby and Mattocks.[2] They administered 7 per cent glucose dialysis solution containing S^{35}-labeled bisulfite in single or repeated intraperitoneal doses to rabbits and demonstrated accumulation of S^{35} in the plasma. Following death of several animals they concluded "that the use of 500 mg. per liter of $NaHSO_3$ in peritoneal dialysis fluids is dangerous and should be discontinued." To implicate $NaHSO_3$ toxicity in these deaths is unwarranted since: (1) The total dose that the rabbits received varied from 16 to 48 mg. per kilogram, which is less than the intravenous LD_{50} (65 mg.) reported by Hoppe and Goble[1] and considerably less than the intraperitoneal LD_{50} (300 mg.) reported here. (2) In the studies of Halaby and Mattocks[2] animals died from 337 minutes to 30 hours following injection, much longer than the period of time for bisulfite to cause death reported by others and confirmed in this study.[1, 3, 4] (3) The S_{35}-labeled material that Halaby and Mattocks[2] found accumulating in the serum of rabbits was not necessarily bisulfite. They did not distinguish that radioactivity due to bisulfite from that due to sulfate or other metabolites. Indeed, if one attempts to measure bisulfite in protein-free supernatants prepared with 15 per cent trichloracetic acid, there is poor and inconsistent bisulfite recovery.[5] Much of the S_{35} found in serum is probably in the form of sulfate, as there is rapid and relatively unlimited ability to metabolize bisulfite to sulfate.[1, 3]

As shown in Experiment II, when more dilute solutions are used, the bisulfite LD_{50} rises in rats. This indicates that with dilution a smaller amount of bisulfite moves across the peritoneum per unit time, therefore a greater total amount is required. The bisulfite concentrations used in these rats are several times greater than that present in the peritoneal dialysis fluid used in patients.

Previous investigators have reported that bisulfite given intravenously in sublethal doses over long periods of time is nontoxic.[3] There is rapid conversion of bisulfite to nontoxic substances.[1] Peritoneal dialysis is a chronic intermittent type of treatment. In Experiment IV, bisulfite was infused directly into the splenic vein to eliminate uncertainty regarding the rate of absorption of bisulfite from the peritoneal cavity. The ureters were ligated so that renal excretion would play a small role in protecting from bisulfite toxicity, since peritoneal dialysis is used in patients with greatly reduced renal function. Infusion of 160 mg. of sodium bisulfite per kilogram per hour resulted in a constant, albeit small, increase in bisulfite concentration in the serum. This rising level of bisulfite was reversed by decreasing the infusion rate to 80 mg. per kilogram per hour, indicating that in the dog the metabolism of sodium bisulfite to sulfate occurs at a rate of at least 80 mg. per kilogram of body weight per hour, at least four times the maximal conceivable exposure in a human undergoing chronic intermittent peritoneal dialysis (i.e., in a 50 kilogram patient receiving 2 L. of the 7 per cent glucose solution per hour, or 20 mg. of $NaHSO_3$ per

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kilogram). Moreover, the rate of absorption and, therefore, the likelihood of toxicity in a man would be minimized by the more dilute bisulfite solutions used in peritoneal dialysis.

Peritoneal dialysis solutions are usually sterilized by autoclaving. The amount of bisulfite remaining following autoclaving of the dialysis fluid is even less than 25 per cent of the formulated amount. On the basis of these studies, it seems unlikely, therefore, that death due to bisulfite can result in man from chronic intermittent peritoneal dialysis.

Conclusions

Studies in animals on the toxicity of intraperitoneally administered bisulfite and determination of the rate of metabolism of the bisulfite given intravenously, coupled with the finding that the amount of bisulfite in peritoneal dialysis solutions is greatly reduced by autoclaving, suggest that sodium bisulfite added to peritoneal dialysis solutions as an antioxidant is not likely to result in toxicity in patients.

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Die Abhängigkeit der SO_2-Toxizität von der Thiaminversorgung

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Von den bisher bekannten Reaktionen zwischen dem in der Lebensmittelbe- und verarbeitung vielseitig verwendeten technischen Hilfsstoff schweflige Säure und Nahrungsbestandteilen ist die von Williams und Mitarbeitern [7] und die von Morgan et al. [6] beschriebene Aufspaltung des Thiaminmolekül durch SO_2 für den Ernährungsphysiologen besonders interessant, da nach Ernährungserhebungen nicht alle Bevölkerungsgruppen genug Thiamin mit der Nahrung aufnehmen.

Fitzhugh und Mitarbeiter [3] konnten in Versuchen an Ratten jedoch keinen deutlichen Zusammenhang zwischen dem Grad der Thiaminversorgung und der Toxizität von SO_2 feststellen. Weder eine Verminderung des B_1-Angebots durch Erhitzen des Hefeanteils der Kost noch B_1-Injektionen bei einem Futter mit ausreichendem Thiamingehalt veränderten die Verträglichkeit für SO_2.

Untersuchungen von Hötzel [4] ergaben dagegen, daß Ratten bei B_1-Mangel deutlich weniger schweflige Säure vertragen als bei ausreichendem Thiamingehalt im Futter. In weiteren Arbeiten [5] wurde dieser Befund bestätigt und festgestellt, daß auch an Inhaltsstoffe von Nahrungsmitteln gebundene schweflige Säure in gleicher Weise wie SO_2 aus anorganischen Verbindungen bei Thiaminmangelratten deutlich toxischer wirkt.

Mit den Untersuchungen, über die hier berichtet wird, sollte erstens geklärt werden, ob ein über den normalen Bedarf hinausgehender B_1-Gehalt in der Kost die Verträglichkeit für SO_2 verbessern kann und zweitens, ob eine Abhängigkeit der SO_2-Toxizität von der B_1-Versorgung auch dann vorhanden ist, wenn Thiamin anstatt im Futter parenteral verabreicht wird.

Versuchstechnik

Die Versuche wurden an männlichen Ratten, die zu Beginn der 8 — 12 Wochen dauernden Versuche 21 Tage alt waren, durchgeführt. Die Tiere wurden in vollklimatisierten Räumen, in Einzelkäfigen und bei ad libitum-Fütterung gehalten.

Die schweflige Säure wurde in Form von Natriumdisulfit in einer Mischung mit Casein und Glucose täglich außer sonntags vom Futter getrennt verabreicht. Die SO_2-Dosis wurde wöchentlich nach dem jeweiligen Gewicht des Einzeltieres berechnet. Thiamin wurde als Thiaminchloridhydrochlorid einer praktisch thiaminfreien halbsynthetischen Kost in den angegebenen Mengen beigemischt oder in wässriger Lösung subkutan injiziert.

Für die Bestimmungen des Hämoglobingehaltes wurde durch Stutzen des Schwanzes gewonnenes Blut verwendet.

Die Versuche wurden so angelegt, daß innerhalb von Versuchsreihen mit gleich hoher Thiaminversorgung einzelne Tiergruppen unterschiedliche SO_2-Mengen erhielten. Die Ergebnisse sind als Dosiswirkungsfunktionen graphisch dargestellt. Gruppen mit gleicher Thiaminversorgung sind in den Diagrammen miteinander verbunden. Die eingetragenen Kreuzchen geben an, ab welcher SO_2-Dosis ein signifikanter Unterschied gegenüber der Kontrollgruppe ohne SO_2 auftritt, bezw. wann die toxische Grenzdosis erreicht ist.

Ergebnisse und Diskussion

Die Dosiswirkungsfunktion von schwefliger Säure in Abhängigkeit von der oralen Thiaminzufuhr für das Kriterium Gewichtszunahme ist aus Darstellung 1 zu ersehen. Bei 0,2 mg B_1 pro 100 g Futter sind bei 1600 mg SO_2 pro kg Körpergewicht alle Tiere gestorben. Bei derselben SO_2-Dosis aber 0,5 mg B_1 pro 100 g Futter überleben die Tiere zwar, aber sie haben eine signifikant niedrigere Gewichtszunahme als die Gruppen mit 1,0 oder 2,0 mg B_1 pro 100 g Futter, zwischen denen kein Unterschied besteht. Die Toxizität von SO_2 läßt sich somit durch eine über den Bedarf hinausgehende Thiaminzufuhr verringern. Eine Steigerung der B_1-Gabe über das fünffache des normalen Bedarfs hinaus kann die toxische Grenzdosis von SO_2 nicht weiter erhöhen. Anders liegen die Ergebnisse, wenn Thiamin anstatt mit dem Futter parenteral verabreicht wird (Abb. 2). Die toxische Grenzkonzentration von SO_2 wird dann durch die Höhe der B_1-Zufuhr *nicht* beeinflußt. Weder sind B_1-Mangeltiere empfindlicher für SO_2 noch sind die Tiere, die die fünffache der normalen Thiaminmenge bekommen haben, widerstandsfähiger als mit Thiamin gerade ausreichend versorgte Tiere.

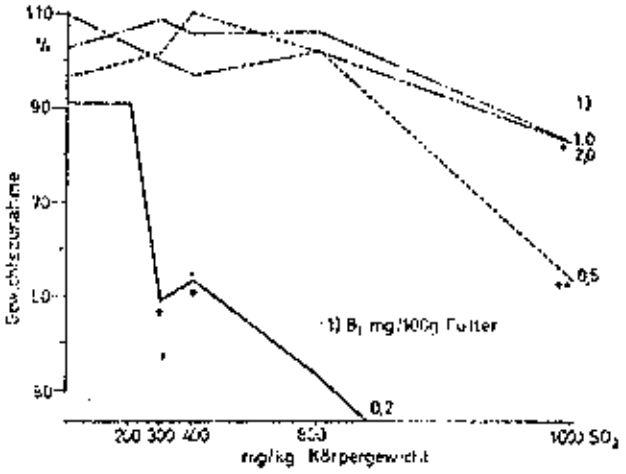

Abb. 1
SO_2-Toxizität bei unterschiedlich hoher peroraler Thiaminzufuhr.

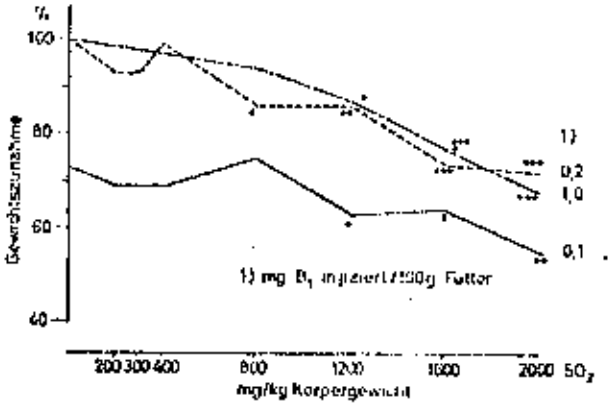

Abb. 2
SO_2-Toxizität bei unterschiedlich hoher parenteraler Thiamingabe.

Da manche Ratten bei hoher SO_2-Aufnahme sichtbar anämisch waren, wurden zur Objektivierung des Befundes Hämoglobinbestimmungen durchgeführt. Das Ergebnis ist wiederum als Dosiswirkungsfunktion dargestellt (Abb. 3). Ab 800 mg SO_2 pro kg Körpergewicht ist bei allen Thiaminversorgungsstufen ein statistisch sehr hoch gesicherter Unterschied gegenüber den Kontrollgruppen ohne SO_2 vorhanden.

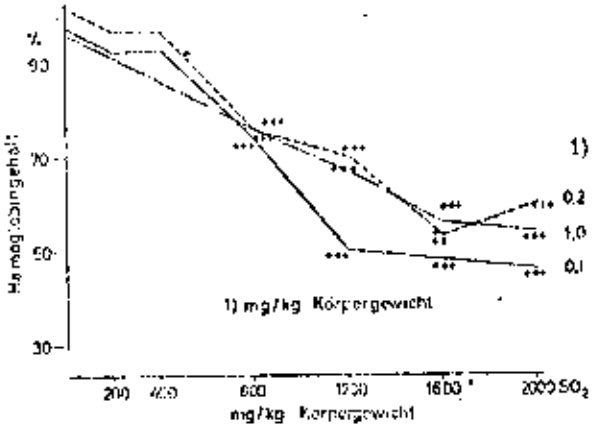

Abb. 3
Geringe Beeinflussung der durch SO_2 erzeugten Anämie durch Thiamininjektionen.

Aufgrund des Ergebnisses, daß eine unterschiedlich hohe Thiaminzufuhr per os die Toxizität der schwefligen Säure beeinflußt, nicht aber unterschiedlich hohe Thiaminmengen, wenn sie parenteral verabreicht wurden, kann man annehmen, daß eine nennenswerte Wechselwirkung zwischen Thiamin und schwefliger Säure nur im Verdauungskanal stattfindet. Offenbar hat, trotz getrennter Verabreichung von Teststoff und thiaminenthaltendem Futter, doch während der Verdauung eine Reaktion zwischen Thiamin und schwefliger Säure stattgefunden, durch die B_1 aufgespalten oder inaktiviert wurde. Obwohl im Speichel, Magensaft und Duodenalinhalt schweflige Säure oxydiert und damit entgiftet werden kann [2], ist nach Stoffwechseluntersuchungen von Baghat und Lockett [1] damit zu rechnen, daß noch ein erheblicher Anteil als SO_2 resorbiert wird. Man weiß bisher wenig darüber, wo das resorbierte SO_2 oxydiert wird, schweflige Säure selbst wird nur in geringer Menge im Urin ausgeschieden [1]. Da parenteral verabreichtes Thiamin nicht inaktiviert wird, kann man vermuten, daß die schweflige Säure während oder unmittelbar nach der Resorption oxydiert oder in eine andere mit Thiamin nicht reagierende Form überführt wird. Auch analytisch wurden jedenfalls bislang keine meßbaren Sulfitmengen in Organen festgestellt.

Ob die Senkung der Hämoglobinwerte durch SO_2 direkt oder über eine Wechselwirkung von SO_2 mit einem weiteren Nährstoff zustandekommt, konnte bisher noch nicht geklärt werden.

Zusammenfassung

In 10wöchigen Wachstumsversuchen an männlichen Ratten konnte bestätigt werden, daß die Toxizität von SO_2 bei Thiaminmangel erhöht ist. Eine bedarfsüberschreitende Thiaminzufuhr verbesserte die Verträglichkeit für SO_2. Wurde aber Thiamin anstatt oral parenteral verabreicht, so hatte der Grad der Thiaminversorgung keinen Einfluß auf die toxische Wirkung von SO_2. Gaben von mehr als 800 mg SO_2 pro kg Körpergewicht und Tag führten unabhängig von der Höhe der Thiamingabe zu niedrigerem Hämoglobingehalt.

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Isolation of a Crystalline Antithiamine Factor from Fern

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According to our previous investigations fern contains a thermostable water-soluble antithiamine agent with acidic character. A fairly good purification of this substance has been achieved, however it could not be isolated in the pure state. By changing the purification procedure a substance with a great antithiamine activity could be separated. The leaves of the dried fern were extracted with water, the extract concentrated, shaken with ethyl acetate and finally separated on a polyamide column.

In the course of the elution a fraction was obtained containing a substance with a high antithiamine activity. This compound could be isolated in the crystalline form. Its antithiamine activity is approximately 250 γ/mg, about 200 — 250 times more active than the raw extract and about ten times more active than the previously described antithiamine factor (hydrolysate II). The crystalline substance is—as assumed—a small molecule (molecular weight 200) and possesses a melting point of 190 — 192 °C. An aqueous solution of the crystals is thermostable. The crystals are soluble in ethanol, methanol, acetone and ethyl acetate and insoluble in chloroform and ether. Its composition is the following: C 60.83 %, H 4.57 %, O 33.80 %. The I. R., U. V. and N.M.R. spectra and other characteristic properties of this antithiamine agent are subsequently discussed. The complete elucidation of the chemical structure of the substance is in progress.

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Enzymatic Reactions Involving Sulfate, Sulfite, Selenate, and Molybdate*†

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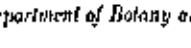
The pioneering work of Bernstein and McGilvery (1) and of De Meio *et al.* (2) established the participation of adenosine triphosphate in the enzymatic activation of sulfate. Further studies by Hilz and Lipmann (3) and by Segal (4) indicated that PP^i is eliminated from ATP during the activation reaction, whereas Robbins and Lipmann (5, 6) identified "active sulfate" as PAPS. Previous studies from this laboratory (7, 8, 9) and by Robbins and Lipmann (10) indicated a two-step reaction mechanism catalyzed by ATP-sulfurylase and APS-kinase, respectively.



$$ATP + SO_4^{=} \rightleftharpoons APS + PP \quad (1)$$

$$APS \xrightarrow{ATP} PAPS \quad (2)$$

In the present report we wish to present data on the purification of the sulfurylase protein, to adduce evidence for the net synthesis of APS, and to examine the reactivity of the sulfurylase with ATP and Group VI anions other than sulfate. It will be shown that sulfurylase transfers adenylic acid from ATP to sulfate or to selenate, forming the mixed anhydride (1), but that free adenylic acid is formed from ATP in the presence of sulfite, chromate, tungstate or molybdate.

MATERIALS AND METHODS

Sulfurylase Assay—The activity of ATP-sulfurylase was determined by following the enzymatic liberation of inorganic phosphate from ATP in the presence of molybdate and inorganic pyrophosphatase. The reaction mixture contained in μmoles: ATP, 5; Na_2MoO_4, 5; $MgCl_2$, 1; Tris-HCl buffer, pH 7.5, 20; EDTA, 0.3; purified pyrophosphatase, 1.8 μg., and the sulfurylase in a total volume of 0.50 ml. A unit of enzyme was defined as that amount which produced 1 μmole of inorganic phosphate in 60 minutes at 30°. The reaction proceeded linearly with time until 90 per cent of the ATP was hydrolyzed and was a linear function of enzyme concentration (Fig. 1). Enzymatic activity was halted by boiling, by alcohol, or by dilution. Trichloroacetic acid was not used owing to the nonenzymatic hydrolysis of ATP in the presence of molybdate at acid pH (12). Precipitated protein was removed by centrifugation when necessary. Inorganic phosphate was determined by the procedure of Fiske and SubbaRow (13). Controls with boiled enzyme or in which enzyme was omitted were used to correct for ATP hydrolysis during the phosphate determination; with crude sulfurylase preparations chloride was substituted for molybdate to measure ATPase activity.

Special Reagents—AMP and crystalline Na_2H_2ATP were obtained from the Sigma Chemical Company and ADP, UTP, CTP and GTP from Pabst Laboratories. The radioisotopes (S^{35} as $H_2S^{35}O_4$, P^{32} as $H_3P^{32}O_4$ and Se^{75} as $H_2Se^{75}O_4$) were supplied by the Oak Ridge National Laboratory. Labeled sulfate was used without further purification, usually after sufficient storage to permit contaminating P^{32} to decay. Labeled sulfite was prepared from labeled sulfate by the method of Chapoville and Fromageot (14). Unlabeled sulfite was prepared by decomposing commercial Na_2SO_3 with acid in a stream of oxygen-free N_2. The liberated SO_2 was purified by passage through sulfuric acid and an ice-jacketed condenser and trapped with a stoichiometric amount of NaOH in 0.002 M EDTA. Labeled and unlabeled $K_2S_2O_7$ were prepared from SO_3 by the procedure of Trelease and Beath (15). Inorganic phosphate labeled with P^{32} was purified before use by column chromatography (16). Inorganic pyrophosphate labeled with P^{32} was prepared by heating $Na_2HP^{32}O_4 \cdot 12\ H_2O$ at 300° for 5 hours (17) and purified by elution from Dowex 1 with 0.02 M NaCl-0.01 M HCl. Synthetic APS was prepared as the ammonium salt by the method of Baddiley *et al.* (18) and analyzed as 1.03 adenine to 1.00 organic phosphorus to 1.00 pentose to 0.86 sulfur. Adenine was determined by its absorbency at 259 mμ; organic phosphorus by the difference between P_i and total phosphorus following wet ashing with perchloric acid; pentose by the method of Mejbaum (19) and sulfur, after reduction to H_2S, as methylene blue (20). Purified yeast pyrophosphatase (step 3) free of detectable ATPase and 3′-nucleotidase activity was prepared according to the method of Hepple and Hilmoe (21). Adenylic acid deaminase was prepared by the method of Kalckar (22). Protein was determined by the trichloroacetic acid turbidimetric method (23) with bovine serum albumin as a standard.

Sulfurylase Preparation—Starchless bakers' yeast was emulsified, dried at room temperature, and stored at 0°. 3 gm. portions were mixed with 5 gm. of quartz sand and 15 ml. of Tris-EDTA buffer (0.005 M Tris-HCl and 0.001 M EDTA, both at pH 7.5) and ground at room temperature in an electrically driven mortar and pestle for 60 minutes. 15 ml. of buffer were added and the

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† This work constitutes departmental contribution No. 58 7.

[1] The abbreviations used are: APS, adenosine-5′-phosphosulfate; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; APSe, adenosine-5′-phosphoselenate; PAPSe, 3′-phosphoadenosine-5′-phosphoselenate; PP, inorganic pyrophosphate; P_i, inorganic orthophosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; PPase, inorganic pyrophosphatase.

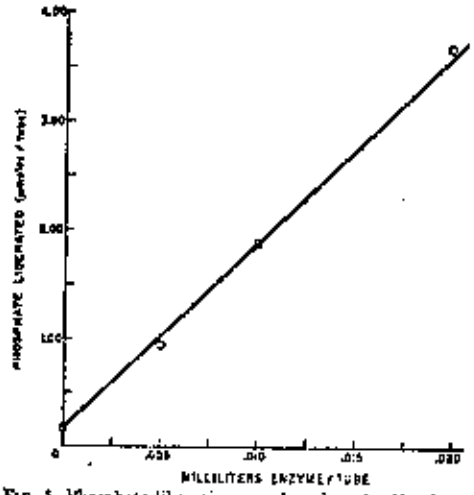

FIG. 1. Phosphate liberation as a function of sulfurylase concentration. Reaction conditions as described under sulfurylase assay with incubation for 2 hours. The enzyme solution contained 170 μg. of protein per ml.

autolytic extraction was continued for 30 minutes. Subsequent steps were carried out at approximately 1°. The suspension was centrifuged 15 minutes at 28,000 × *g*, filtered through cheesecloth, and frozen (Stage I). Eight portions of this fraction (120 ml.) were thawed and dialyzed for 12 hours against three changes of 1 l. portions of Tris-EDTA buffer. The dialyzed material was centrifuged for 15 minutes at 28,000 × *g*; the supernatant solution (dialyzed extract) was frozen, thawed, and centrifuged for 1 hour at 78,000 × *g*. The clear supernatant fluid, approximately 75 per cent of the total, was carefully removed and refrozen. This high speed supernatant fluid constituted the "crude enzyme" (Stage III) and contained the complete sulfate-activating system.

Protamine fractionation at pH 6.0 resulted in a separation of ATP-sulfurylase and APS-kinase. Aliquots of the crude enzyme were adjusted to pH 6.0 with dilute acetic acid and fractionated with 0.24 or 0.30 mg. of protamine per ml. of enzyme. After 10 minutes the precipitate was removed by centrifugation (20,000 × *g*, 5 minutes) and discarded. The supernatant fluid was then fractionated with successive 0.12 mg. portions of protamine and recentrifuged each time until no further precipitation occurred; *i.e.* until 0.48 to 0.60 mg. of protamine per ml. of enzyme had been added. The final supernatant fluid, designated as crude APS-kinase, was adjusted to pH 7.5 and frozen. The successive protamine precipitate fractions were each suspended in one-tenth of the original volume of Tris-EDTA buffer and assayed for sulfurylase activity with molybdate (Fig. 3). Usually 80 per cent of the total activity was recovered in a single precipitate fraction.

Further purification of 1 ml. aliquots of the protamine-purified enzyme (Stage IV) was obtained by electrophoresis on an 8 × 20 × 0.5 cm. block composed of 80 gm. dry weight of HCl-EDTA washed potato starch suspended in 0.05 M Tris-HCl buffer (0.001 M EDTA), pH 8.5. The enzyme moved toward the anode (6×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}) and was located by assay of small aliquots of the supernatant fluid of 8 × 2 × 0.5 cm. blocks suspended in 5 ml. of water. The starch block suspension containing most of the activity was then filtered and washed twice with additional 5 ml. portions of water. Bovine serum albumin (3 mg.) and glutathione (12 μmoles, pH 7.5) were added to the filtrate and the mixture was lyophilized. Concentrated stock solutions of electrophoretically purified sulfurylase were prepared for use by dissolving the lyophilized material in 1 ml. of distilled water. Such preparations were stable at −20° but lost activity upon repeated freezing and thawing. Aliquots of these stock solutions (Stage V) were used in the following experiments unless otherwise indicated.

TABLE I

Purification and recovery of ATP-sulfurylase from yeast

Stage of purification	Volume	Units	Specific activity	Recovery
	ml.	μmoles P_i	units/mg. protein	%
I. Crude extract	110	6350	3.4	100
II. Dialyzed extract	120	7920	4.4	100
III. Crude enzyme (78,000 × *g*)	77	4620	5.9	72
IV. Protamine ppt. fraction	7.7	2657	65	42
V. Electrophoresis fraction	7.7	909	347	14

TABLE II

Phosphate liberation with selected Group VI anions

Anion added	Total P_i	P_i due to Group VI anion
	μmoles/tube	μmoles/tube
NaCl (10 μmoles)	0.17	0.00*
Na_2SO_4 (5 μmoles)	0.22	0.05
Na_2SO_3 (5 μmoles)	0.56	0.39
K_2SeO_4 (5 μmoles)	0.63	0.46
Na_2MoO_4 (5 μmoles)	5.33	5.16

* Other experiments have shown that NaCl controls are identical with boiled enzyme controls. The reaction conditions are, except for anion added, as described under sulfurylase assay and sulfurylase, 0.8 μg., and incubation for 2 hours.

RESULTS

Enzyme Purification.—A 100-fold purification with 14 per cent recovery of ATP-sulfurylase activity was usually obtained (Table I). The increased activity at Stage II was due to removal by dialysis of sulfate, a competitive inhibitor of the molybdate reaction, and to loss of molybdate-inhibitable ATPase activity. Suspended protamine precipitates (Stage IV) were free of the ADP-sulfurylase of Robbins and Lipmann (19) and of ATPase and APS-kinase, and the purified sulfurylase (Stage V) was, in addition, free of inorganic pyrophosphatase and adenylate kinase.

Activity with Different Anions.—In the presence of sulfate as compared with blank controls, sulfurylase and pyrophosphatase liberated a small but reproducible amount of phosphate from ATP (Table II). This represented the equilibrium amount of phosphate formed in the presence of an excess of pyrophos-

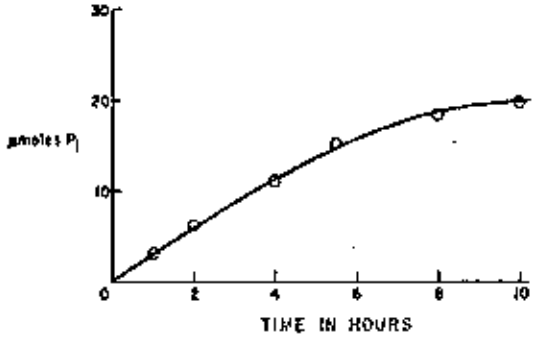

FIG. 2. Rate of phosphate liberation in the presence of sulfite. Each tube contained in μmoles: ATP, 10; $MgCl_2$, 2; Na_2SO_3, 50; Tris-HCl buffer, pH 7.5, 50; EDTA, 0.6; pyrophosphatase, 3.6 μg.; sulfurylase (stage IV), 560 μg. in a total volume of 1.00 ml. Incubation was in N_2 atmosphere at 30°, and aliquots of 0.1 ml. were removed at intervals for assay.

phatase since the amount was not increased by prolonged incubation or by additional sulfurylase (Equation 3).

$$ATP + SO_4^{=} \xrightarrow{\text{sulfurylase}} APS + PP \qquad (3)$$
$$PP \xrightarrow{PPase} 2P_i$$

With sulfite and molybdate, as contrasted with sulfate, sulfurylase liberated comparatively large amounts of phosphate (Table II). Earlier studies (8) showed that chromate and tungstate were also active in this respect. Phosphate liberation with sulfite, chromate, tungstate, and molybdate (shown for sulfite in Fig. 2) proceeded until all ATP was cleaved to AMP and PP (Equation 4).

$$ATP \xrightarrow{\text{anion}} AMP + PP \qquad (4)$$

Evidence cited below indicates that, with these 4 anions, a stable adenylic acid and a nucleotide was not formed.

With selenate as the substrate, phosphate was also liberated to a greater extent than with sulfate (Table II). The reaction with selenate, however, appeared to be a special case intermediate between that with sulfate and with molybdate since trace amounts of APSe could be recovered (see below). Nitrate, bicarbonate, and thiosulfate, like chloride, did not induce phosphorus liberation from ATP.

Several lines of evidence indicated that the reactivity of the Group VI anions was dependent upon the presence of a single enzyme in the various yeast preparations. Thus, most of the activity was obtained in a single protamine precipitate fraction (Fig. 3) regardless of which anion was used in assaying these fractions. Moreover, phosphate formation in the presence of sulfite and molybdate was inhibited by the addition of sulfate. In one experiment the addition of 20 μmoles of Na_2SO_4 reduced phosphate formation in the presence of 3 μmoles of Na_2MoO_4 by 67 per cent. In another, using a less purified preparation, the inhibition of the sulfite reaction by sulfate was shown to be competitive (Fig. 4). Finally, although the molybdate anion was used for enzyme assay during purification, the final preparation retained the ability to synthesize APS and APSe and to form PAPS upon the addition of APS-kinase (Table VI).

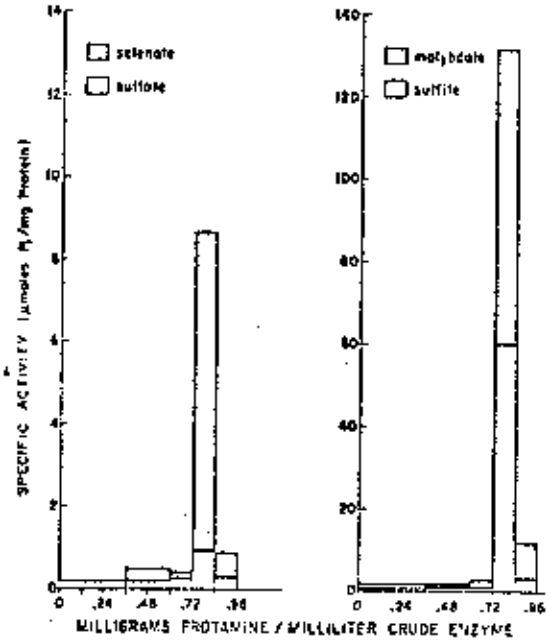

FIG. 3. Sulfurylase activity of successive protamine precipitate fractions as determined with four Group VI anions. Aliquots of each of the five fractions assayed in regular sulfurylase assays in the presence of K_2SO_4 (3 μmoles), K_2SeO_4 (3 μmoles), Na_2SO_3 (25 μmoles), or Na_2MoO_4 (3 μmoles).

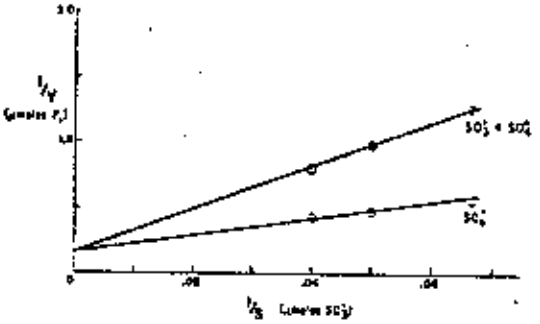

FIG. 4. Competitive inhibition of the sulfite reaction by sulfate. Each tube contained in μmoles: ATP, 5; $MgCl_2$, 1; Na_2SO_3 as indicated above; Na_2SO_4, 5, when present; Tris-HCl buffer, pH 7.5, 50; EDTA, 0.1; pyrophosphatase, 1.8 μg.; sulfurylase (Stage IV), 80 μg. in a total volume of 0.50 ml. with incubation for 2 hours at 30°.

Activity of Different Nucleotides—Experiments with ATP, ADP, UTP, CTP and GTP demonstrated that ATP was the nucleotide substrate for the sulfurylase reaction (Table III).

Proof of Pyrophosphate Formation—The fact that inorganic phosphate was liberated only in the presence of pyrophosphatase (Tables III and VI) showed that inorganic pyrophosphate, PP, was a product of the reaction. This was supported by studies

TABLE III

Nucleotide specificity of the sulfurylase reaction

Experiment I. Each tube contained in micromoles: nucleotide, 0.5; $MgCl_2$, 0.5; $NaMoO_4$, 0.5; Tris·HCl, pH 7.5, 10; EDTA, 0.1; glutathione, 2.5; pyrophosphatase, 1.8 μg; sulfurylase, 0.8 μg, in a total volume of 0.50 ml. and incubated 1 hour at 30°.

Experiment II. This was the same as above except: nucleotide, 1.0; $MgCl_2$, 1; sulfurylase, 1.7 μg, and incubation for 2 hours. The reaction was halted by boiling, and the aliquots were removed and incubated 1 additional hour with or without PPase.

Experiment	Reaction conditions	P_i	PP
		μmole/ml.	μmole/ml.
I.	ATP, PPase present	0.05	
	UTP, PPase present	0	
	CTP, PPase present	0.03	
	GTP, PPase present	0	
II.	ATP, PPase absent	0.01	0.01
	ADP, PPase absent	0.00	0.02

with protamine-purified enzyme (Stage IV) which demonstrated that 2 equivalents of P_i were liberated from ATP upon prolonged incubation with sulfurylase, pyrophosphatase and sulfate (Fig. 2) or with any other active anion. Owing to the unfavorable equilibrium, proof of PP formation with sulfate as anion rested upon the demonstration of P^{32}-PP exchange into ATP.

Formation of APS—The formation of APS as a product of the sulfurylase reaction was demonstrated with high specific activity, S^{35}-labeled sulfate. Aliquots of reaction mixtures containing the enzymatically synthesized, putative APS^{35} were chromatographed and subjected to electrophoresis together with authentic, synthetic APS (Fig. 3). S^{35}-radioactivity occurred only in the APS area, inorganic sulfate having moved into the electrode compartment. The exact coincidence of this radioactivity as determined by radiomicrography, and of the ultraviolet-absorbing APS region, as determined by visual and photographic comparisons, proved that the enzymatically synthesized, S^{35}-labeled compound was identical to the ultraviolet-absorbing nucleotide synthesized by the method of Baddiley *et al.* (18).

Estimates of the amount of S^{35}-labeled APS formed in the presence and absence of pyrophosphatase were made by eluting the APS area of an electrophoretogram (Table IV). A total of 0.02 μmole of APS was formed from 5 μmoles of ATP and 10 μmoles of sulfate in the presence of pyrophosphatase, whereas an average of only 0.0013 μmole was formed in the absence of pyrophosphatase. No attempts were made to insure that equilibrium had been reached; however, based on the molybdate assay more than sufficient sulfurylase was present; nor were experiments conducted to approach the equilibrium of the reaction in the reverse direction. Further, in these experiments only the amount of APS formed was measured and the amount of pyrophosphate liberated was assumed to be stoichiometrically equivalent. Until more definitive experiments can be made the apparent equilibrium constants at pH 7.5 and 30° are calculated as 4×10^{-7} for hydrolysis, and 6×10^{-7} for synthesis.

An independent estimate of the amount of APS formed in the presence of pyrophosphatase was made (Table V) by measuring the adsorption of molybdate-labeled sulfate to charcoal (24). This estimate, an average based on experiments with different batches of purified sulfurylase, is in agreement with the APS estimate obtained by eluting an electrophoretogram.

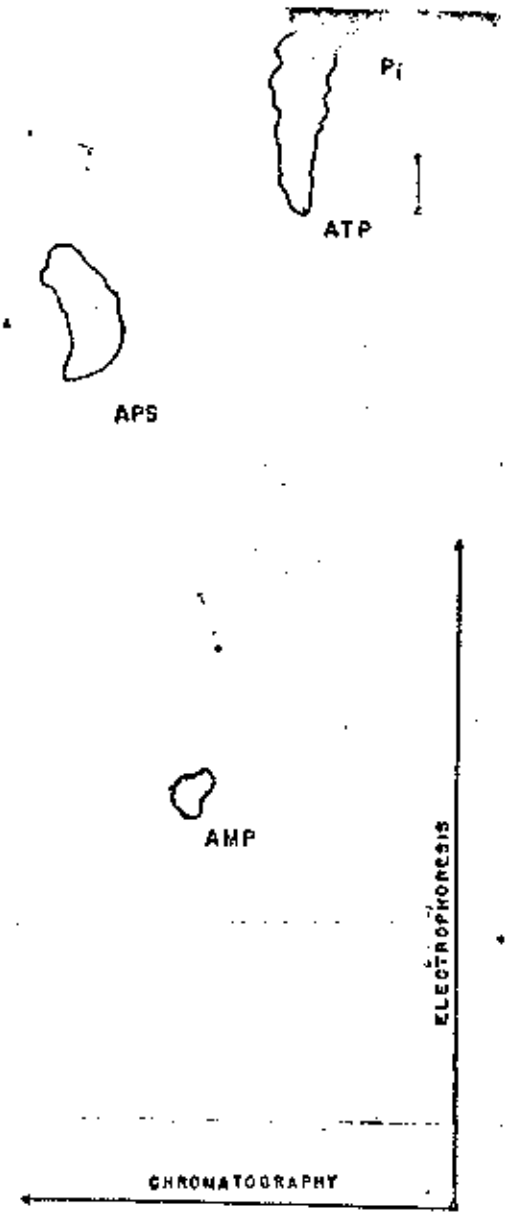

FIG. 3. A radioautograph of an electrophoretogram chromatogram showing coincidence of enzymatically synthesized APS^{35} and synthetic APS. The radiolabeled P represents P^{32} contamination of the radiosulfate. The synthetic APS spot shown in outline was located photographically by exposing other paper to ultraviolet radiation. Microcuries transmitted through the superimposed chromatograms are shown.

The incubation mixture contained in μmoles: ATP, 5; NaN_3, 50; [illegible] μmoles; [illegible]; Tris·HCl buffer (pH 7.5), 250; EDTA, 0.3; pyrophosphatase, 1.8 μg; sulfurylase, 0.8 μg, in a total volume of 0.50 ml. and incubated for 2 hours at 30°. The reaction was halted by boiling. The aliquots for electrophoresis consisted of 20 μl. of reaction mixture combined with 5 μl. 0.1 μmole of synthetic APS. Electrophoresis on Whatman No. 3MM HCl EDTA

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An attempt was also made to estimate the small amount of phosphate liberated. In these experiments the ratio of phosphate liberated to nucleotide-bound sulfate was sometimes as low as 2.5.

Formation of APSe—Radioactive selenate was used to demonstrate the formation of APSe, the selenate analogue of APS. Small but consistent amounts of nucleotide-bound selenate were detected by the charcoal adsorption technique and electrophoretic analysis of reaction mixtures revealed the presence of a Se^{75}-labeled compound with approximately the same mobility as APS. In marked contrast to the results obtained with sulfate, phosphate liberation from ATP was much greater than nucleotide-bound selenate accumulation (Table V). AMP was the major nucleotide product of the sulfurylase reaction in the presence of selenate (see below—Formation of AMP).

The consistent recovery of small amounts of APSe as compared with the amount of phosphorus liberated suggested that this nucleotide was unstable under the conditions of incubation or assay. Accordingly, experiments were carried out which demonstrated that, after stopping the reaction with alcohol the nucleotide-bound anion was stable for as long as 90 minutes, pH 7.5, and 30° (conditions of incubation). The compound was also stable at pH 4.5 and 1° for at least 45 minutes (conditions of assay). At pH 4.5 and 30°, however, the half-life of APSe was approximately 30 minutes. The comparative stability of this compound under the physical conditions of incubation and assay suggested that APSe was unstable in the presence of sulfurylase.

Coupling of Sulfurylase with APS-Kinase—When sulfate or selenate were present as substrates, the addition of APS-kinase to incubation mixtures containing sulfurylase resulted in increases both in liberation of phosphate and in synthesis of nucleotide-bound sulfate or selenate (Table V). In the case of sulfate the nucleotide-bound anion has been identified as PAPS (8, 10) and, as shown in Table V, the amount of PAPS formed was in fair agreement (Equations 1 and 2) with the amount of inorganic phosphate liberated. In the case of selenate, the increase in nucleotide-bound anion was much less than would be predicted from the increase in phosphate liberation. In some experiments phosphate liberation was doubled by the addition of APS-kinase to sulfurylase and selenate. Electrophoretic analysis showed that neither PAPSe (the selenate analogue of PAPS) nor 3′,5′-diphosphoadenosine (the expected decomposition product) had accumulated. The available data are not sufficient to explain these observations nor to establish the existence of PAPSe. They do, however, point to metabolic differences between the adenyl sulfates and selenates which are not accounted for by chemical instability of the selenate anhydride.

Addition of APS-kinase to incubation mixtures containing sulfite, chromate, tungstate, or molybdate, and sulfurylase did not increase phosphate liberation from ATP. This failure to obtain coupling between sulfurylase and APS-kinase and the absence of P^{32}-PP exchange into ATP in the case of these anions (see below and reference (8)) is evidence against the formation of stable adenylic acid-anion anhydrides of sulfite, chromate, tungstate, or molybdate.

Formation of AMP—Electrophoretic studies and phosphorus analyses indicated that AMP and PP were the products of the sulfurylase reaction when sulfite, chromate, tungstate, or molybdate were present as the active anion. AMP formation in the molybdate reaction was followed quantitatively by means of adenylic acid deaminase (Table VI). The analytical methods

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washed paper at 1° and 300 volts for 11 hours in 0.1 N acetate buffer at pH 5.1. Mobility of APS = 5.0 × 10^{-4} cm.² sec.$^{-1}$ volt^{-1}. The paper was dried for 1 hour, turned 90° as indicated, and subjected to ascending chromatography (18) for 21 hours at 1° in n-propanol-ammonia-water (6:3:1). R_F of APS = 0.34.

Table IV

Formation of S^{35}-labeled APS

Incubation and electrophoresis were the same as for Fig. 5 except as indicated below. Disks of equal size containing the S^{35}-labeled APS (located by scanning and by ultraviolet absorption of synthetic APS) were cut out, eluted, and dried in planchets for counting.

Addition	Incubation	Radioactivity/aliquot	APS formed
	hrs.	*c.p.m.*	*μmole/tube*
Sulfurylase + PPase	2	3390	0.020
Sulfurylase − PPase	2	395	0.002
Sulfurylase − PPase	4	236	0.001
Boiled sulfurylase − PPase	2	58	

Table V

Formation of phosphate and of nucleotide-bound sulfate and selenate by sulfurylase and by sulfurylase plus APS-kinase

Each tube contained in μmoles: ATP, 5; $MgCl_2$, 1; EDTA, 0.3; Tris-HCl, pH 7.5, 50; $Na_2S^{35}O_4$ (0.1 μc./μmole) or $K_2Se^{75}O_4$ (0.2 μc./μmole), 5; sulfurylase, 3.4 μg.; pyrophosphatase, 1.8 μg.; crude APS-kinase where indicated, 780 μg. In a total volume of 0.50 ml. with incubation for 2 hours at 30°.

Addition	Phosphate liberated	Nucleotide-bound anion	Ratio of phosphate liberated to nucleotide-bound anion
	μmoles	*μmoles*	
Sulfate	0.055	0.016	3.5
Selenate	0.223	0.002	111
Sulfate + kinase	0.250	0.107	2.5
Selenate + kinase	0.285	0.005	57

Table VI

Stoichiometry of the sulfurylase reaction

Each tube contained in μmoles: ATP, 2; Na_2MoO_4, 5; $MgCl_2$, 1; Tris-HCl buffer, pH 7.5, 50; EDTA, 0.3; and sulfurylase, 9.2 μg. The reaction was halted by boiling. PP was determined by additional incubation of aliquots with pyrophosphatase. AMP was determined by decrease in absorbancy at 265 mμ during additional incubation with adenylic acid deaminase (25).

Reaction conditions	Colorimeter reading		PP/tube	AMP/tube
	Aliquot before PPase	Aliquot after PPase		
			μmoles	*μmoles*
Complete, 2 hrs. incubation	8	251	1.72	1.59
Boiled enzyme, 2 hrs. incubation	20	14	−0.05	0.01
Boiled enzyme, zero time control	9	15	0.01	0

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mentioned would not have detected extremely small amounts of an AMP-anion anhydride had such a compound been formed. The principle evidence that an adenylic acid-anion anhydride was not formed in the case of sulfite, chromate, tungstate, and molybdate consisted of, (*a*) the failure to observe P^{32}-PP exchange into ATP in the presence of these anions, (*b*) with sulfite as the added anion, there was no detectable loss of sulfite during the course of the experiment as measured by the fuchsin reagent (20), and (*c*) in experiments with high specific activity, S^{35}-labeled sulfite, electrophoretic analysis failed to disclose any S^{35}-labeled nucleotides.

Incorporation of P^{32}-PP into ATP—Sulfate, selenate, sulfite, chromate, molybdate, and tungstate were tested for their ability to serve as substrates for the exchange of P^{32}-labeled PP into ATP. Sulfate-dependent exchange has been reported previously (4, 8). Charcoal adsorption (24) or a combination of paper chromatography (27) and paper electrophoresis were found to be suitable methods for the recovery of ATP from reaction mixtures containing $SO_4^{=}$, $SeO_4^{=}$, or $SO_3^{=}$. Complex formation by chromate, molybdate, and tungstate with nucleotides and with PP and P_i necessitated special precautions in the isolation of ATP for radioactivity determinations when these anions were present. Of the methods tested only column chromatography by the procedure of Cohn and Carter (16) proved suitable.

Following incubation, the reaction was stopped by boiling and the PP was hydrolyzed to P_i by additional incubation with PPase. The sample was made 1 M with respect to NH_4OH and applied to a 0.5 gm. Dowex 1 anion exchange resin column previously treated with 20 ml. of 1 M NH_4OH and washed until neutral. The column was eluted as follows: (*a*) 150 ml. of 0.003 M HCl; (*b*) 200 ml. of 0.01 M HCl-0.02 M NaCl; (*c*) gradient elution using 140 ml. of solution (*b*) in a mixing flask and 200 ml. of 0.01 M HCl-0.2 M NaCl in a reservoir. Fractions of 9 ml. were collected at a rate of approximately 3 ml. per minute. AMP and P_i were eluted with (*a*), ADP with (*b*), and ATP with (*c*). The overall recovery of radioactivity was 91 to 103 per cent. The overall recovery of nucleotide was low in the presence of molybdate.

With sulfate and selenate there was an exact correlation between the fractions containing ATP and those containing radioactivity. A 20 per cent exchange of ATP-PP and P^{32}-PP occurred during the 1 hour incubation with sulfate and an 11 per cent exchange occurred in the presence of selenate. In the case of sulfite, chromate, molybdate, tungstate, and the "no anion" control, significant amounts of radioactivity were not observed in the fractions containing ATP. Our earlier report (8) of exchange in the presence of sulfite was probably due to traces of sulfate in reagent grade sodium sulfite.

Discussion

Evidence is presented in this paper for three types of reactions catalyzed by ATP-sulfurylase in the presence of Group VI anions. The first reaction type (Equation 1) was encountered with sulfate as anion and involves formation of small amounts of adenosine phosphosulfate and of PP in equilibrium with ATP and sulfate. Adenosine phosphosulfate was recovered from reaction mixtures in amounts approximating those predicted by PP formation. The reaction equilibrium was shifted by the addition of inorganic pyrophosphatase, and P^{32}-PP exchange into ATP was observed.

The second reaction type was encountered with selenate as anion. P^{32}-PP exchange into ATP was observed and adenosine phosphoselenate was demonstrated, but in amounts much less than the PP produced. The reaction proceeded slowly to completion, at which time only AMP, PP, and selenate could be recovered. These results imply hydrolysis of the adenosine phosphoselenate permitting displacement of the reaction toward ATP cleavage (Equation 5).

$$\text{ATP} + \text{SeO}_4^{=} \rightleftharpoons \text{APSe} + \text{PP}, \quad \text{APSe} \xrightarrow{\text{HOH}} \text{AMP} + \text{SeO}_4^{=} \qquad (5)$$

The third reaction type was observed with sulfite, chromate, molybdate, and tungstate as anions and involves irreversible cleavage of ATP to AMP and PP. P^{32}-PP exchange into ATP was not observed and an AMP-anion anhydride could not be demonstrated. Possible mechanisms for this reaction include a catalytic cleavage of PP from ATP without intermediate anhydride formation (Equation 4) or formation of an unstable, AMP-anion anhydride (Equation 6) which is of insufficient half-life to permit PP exchange.

$$\text{ATP} + \text{MoO}_4^{=} \longrightarrow [\text{APMo}] + \text{PP}, \quad [\text{APMo}] \xrightarrow{\text{HOH}} \text{AMP} + \text{MoO}_4^{=} \qquad (6)$$

Available data do not permit distinction between these alternatives; this problem may ultimately be resolved by O^{18}-exchange studies. The molybdate type reaction bears a formal analogy to the arsenolysis of phosphate esters. In the arsenolysis reaction the formation of an acyl arsenate which decomposes spontaneously has been postulated (28) but never demonstrated.

The sulfurylase reaction with sulfate, although analogous to the acetate and amino acid "activation" reactions (29), appears to be unique in that demonstrable quantities of this AMP-anion anhydride are formed. Accumulation of small amounts of the nucleotide-sulfate anhydride has also been demonstrated by Robbins and Lipmann (10). The activity of PAPS in various biological sulfurylation reactions has now been established (10, 30-32). The reactivity of selenate in the sulfurylase reaction suggests that similar metabolic routes may exist for selenate in organisms in which selenium compounds occur (15, 33-37). Whether nucleotide-sulfate and nucleotide-selenate anhydrides have metabolic roles other than in sulfate or selenate esterification reactions remains uncertain.

Summary

1. The partial purification of adenosine triphosphate-sulfurylase from yeast is described. This enzyme in the presence of magnesium ions, adenosine triphosphate and sulfate catalyzes the formation of adenosine-5′-phosphosulfate and pyrophosphate.

2. Selenate, sulfite, chromate, molybdate, and tungstate also serve as substrates for sulfurylase, although stable adenylic acid-anion anhydrides are formed only with sulfate and selenate.

3. The catalytic cleavage of adenosine triphosphate to adenylic acid and pyrophosphate in the presence of sulfurylase and molybdate was utilized as a convenient assay procedure.

4. Adenosine diphosphate and uridine, cytosine, and guanosine triphosphates did not substitute for adenosine triphosphate.

5. Mechanisms are proposed for the reactivity of the various anions in the sulfurylase reaction.

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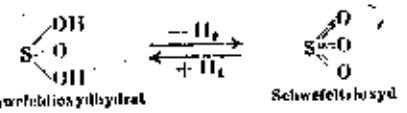
Karzinomatische Thiorespiration.

Wie ich experimentell in vitro nachweisen konnte, läßt sich unter dem Einfluß „atrophischer" Anoxybiose normales Gewebe in kanzerisierendes modifizieren. Diese anoxybiotisch induzierte Mutation wurde durch aerobe Glykolyse und Reimplantation der kanzerisierten Zellen testiert.

Sowohl organ-karzinomatöses als auch mutativ kanzerisiertes Gewebe vollzieht autonom, wie meine weiteren Untersuchungen ergeben haben, in überragendem Maße einen thiorespiratorischen Energiestoffwechsel, was ich durch den Nachweis eines neuen hochpotenzierten Redox-Systems:



experimentell deduzieren konnte.

Mit der hier erstmalig festgestellten energetischen Prävalenz der Zellproteine gewinnen diese, was bisher noch von keiner Seite erkannt wurde, neben den Kohlenhydraten, Fetten und Nukleinsäuren (energiepotenzierende Phosphorylierung) eine hervorragende Bedeutung in der Bio-Energetik des Zellstoffwechsels.

Berlin, Institut für Gärungsforschung.

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Besprechungen.

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Die nach der Entdeckung der Kristallinterferenzen mit Röntgenstrahlen einsetzende intensive Entwicklung unserer Kenntnisse vom festen Körper hat bekanntlich sehr bald gezeigt, daß die Annahme des periodischen Aufbaues der kristallinen Materie, die schon über 100 Jahre früher als Hypothese von einigen Kristallographen eingeführt worden war, nicht exakt erfüllt ist. Die Abweichung der realen Kristalle vom Idealbild der streng periodischen Struktur wird unter dem Begriff der „Sekundärstruktur" der raumgruppenmäßigen „Primärstruktur" eines Kristalls entgegengestellt. Während die Lehre von der Primärstruktur zur Stereochemie gehört, ist diejenige von der Sekundärstruktur eine physikalische Disziplin von besonderem technischen Interesse, da die meisten zur Zeit untersuchten, technisch wichtigen Effekte, wie Festigkeit, Plastizität, Aushärtung, Hubbleitung und Phosphorescenz, Sekundärstruktureffekte sind. Bedauerlicherweise besitzt der Sekundärstrukturforscher kein so durchschlagendes direktes Hilfsmittel, wie sie die Röntgenstrahlen für die Primärstrukturforschung darstellen, da einerseits die langwelligen Röntgenstrahlen in der Materie stark absorbiert werden und andererseits die Sekundärstruktur notwendig statistisch, d. h. sozusagen verwaschener als die Primärstruktur ist. Die Sekundärstrukturforschung ist also auf indirekte Methoden angewiesen, wie sie der Chemie vor Ausbildung der atomphysikalischen Untersuchungsverfahren allein zur Verfügung standen und die darin bestehen, daß aus einem umfassenden Erfahrungsmaterial Hypothesen begründet werden, deren Zutreffen an weiterem Erfahrungsmaterial immer wieder geprüft werden muß. Aus diesem Grunde ist es notwendig, bei Problemen, die in das Gebiet der Sekundärstruktur fallen, die Ergebnisse von oft recht weit auseinanderliegenden physikalischen und sogar technischen Sonderdisziplinen vor Augen zu behalten, um die richtigen und mit allen Einzelerkenntnissen verträglichen Hypothesen zu finden. Eine solche Sonderdisziplin bildet nun die Erforschung der Sekundärstruktur mit Röntgenstrahlen auf Grund der wellenkinematischen Interferenztheorie, und zwar handelt es sich um die Deutung von Feinheiten im Röntgendiagramm, wie Linienbreite, Linienform und diffuse Gebiete.

Der britische Physiker A. J. C. Wilson, der sich seit etwa 10 Jahren mit diesem Problem beschäftigt, gibt in vorliegendem Büchlein eine dankenswerte Zusammenfassung der bisher erreichten, zum größten Teil aus den angelsächsischen Ländern stammenden Ergebnisse. Diese Zusammenfassung eines noch im Fluß befindlichen Forschungsgebiets ist nicht in einer nur dem Eingeweihten verständlichen Form geschrieben, sondern genau dem Ziel der Sammlung, die etwa der deutschen Sammlung Göschen entspricht, so gehalten, daß auch ein Leser durchschnittlicher wissenschaftlicher Ausbildung sich über den neuzeitlichen Kenntnisstand in dem behandelten Gebiet unterrichten kann.

Nach einer Darstellung der Grundlagen der wellenkinematischen Interferenztheorie wird der Begriff des reziproken Gitters, das für die einfache Darstellung der späteren Überlegungen von grundlegender Bedeutung ist, entwickelt und veranschaulicht. Die Verbreiterung der Punkte des reziproken Gitters vermöge Teilchenkleinheit und die Auswirkung dieser Verbreiterung auf die im Debyeverfahren erhaltene Pulveraufnahme werden sodann behandelt, und im Anschluß daran werden in drei den Kern des Buches bildenden Kapiteln die Baufehler in Schichtstrukturen bzw. dreidimensionale Fehlordnungen und ihre Auswirkung auf den Charakter der Pulveraufnahme untersucht. Ein weiteres Kapitel ist den verzerrten Kristallen und den Auswirkungen der Wärmebewegung gewidmet. Jedem Kapitel sind ausführliche Hinweise auf die Originalliteratur beigegeben.

Der Reiz dieses Büchleins wird vergrößert dadurch, daß der Verfasser bestrebt ist, möglichst anschauliche Überlegungen zu benutzen — beispielsweise wird die komplexe Rechnung erst im 7. Kapitel eingeführt —, wodurch zwar die Übersichtlichkeit manchmal etwas leidet, aber die Eindringlichkeit zweifellos gewinnt. In dem Bestreben, zu einer elementaren Darstellung zu gelangen, mußte bedauerlicherweise auf die Reproduktion einiger Ergebnisse, wie z. B. die Arbeit von Patterson über die Linienformen in Debyeaufnahmen, verzichtet werden, was vielleicht in einer späteren Auflage nachgeholt werden kann. Die vereinfachte Rechnung, die zum Ersatz geboten wird, erscheint nicht sehr überzeugend. Ebenso stößt der Leser hin und wieder auf unwesentliche Unebenheiten, die sich in einer späteren Auflage beseitigen ließen, z. B. liegt es nach Ansicht des Ref. im Sinne der Deutlichkeit, die winkelabhängigen Intensitätsfaktoren symbolisiert durch einen Buchstaben in den Gleichungen mitzuführen.

Deutsche Arbeiten während des Krieges haben noch keine Berücksichtigung gefunden.

Das Buch entspricht einem wirklichen Bedürfnis, und besonders deutsche Leser werden es angesichts der bekannten Schwierigkeiten in der Literaturbeschaffung dankbar begrüßen und mit Gewinn studieren.

K. Schubert.

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Die physikalische und chemische Literatur enthält eine nicht geringe Zahl zum Teil ausgezeichneter Darstellungen der Thermodynamik einschließlich ihrer Anwendungen auf die chemischen Umwandlungen, von den Lehrbüchern der physikalischen Chemie und

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Disproportionation of Vitamin B_{12r} under Various Mild Conditions*

Ryo-hei Yamada, Shoichi Shimizu, and Saburo Fukui†

ABSTRACT: When vitamin B_{12r} dissolved in 1 M NaCl aqueous solution was allowed to stand in the presence of methyl iodide under anaerobic conditions in the dark overnight at room temperature, B_{12r} was converted into an almost equimolar mixture of methylcobalamin and aquocobalamin (vitamin B_{12a}). Under the experimental conditions mentioned above, the change did not occur in the absence of the salt. Other electrolytes, such as KCl, Na_2SO_4, $NaNO_3$, and $MgCl_2$, showed a similar effect. In the case of KCN or Na_2SO_3, cyanocobalamin or cobalamin sulfonate was formed instead of B_{12a}, respectively. Other alkylating agents, such as dimethyl sulfate, also yielded methylcobalamin. An equimolar mixture of B_{12r} and cobinamide$_2$, more easily reducible than B_{12r}, gave methylcobalamin and methylcobinamide in the yields corresponding to 16 and 80% of the initial amounts of B_{12r} and cobinamide$_2$, respectively. A comparative study on the effects of NaCl, NaI, and CsCl showed that the order of their effectiveness accorded with Hofmeister's lyotropic series. When a very high concentration of B_{12r} solution, where a portion of solid B_{12r} remained undissolved, was allowed to react with methyl iodide for 2 days in nitrogen atmosphere, methylcobalamin was obtained in about 20% yield without the help of electrolytes. In the visible absorption spectrum of B_{12r} in 1 M NaCl solution, no change was observed which demonstrated the occurrence of B_{12a} and B_{12s}. A comparative study on the alkylating actions of various monohaloacetates with different reactivity toward B_{12r} in 1 M NaCl revealed that the reactivity of alkylating agent had very marked influence on the reaction. These results would be explained as follows: a very small portion of B_{12r} disproportionates to B_{12a} and B_{12s}; B_{12s} thus formed is not only a very small amount but is also short lived; the formation of alkylcobalamin depends on whether an alkylating agent can trap this short-lived B_{12s} or not. When these reactions of B_{12r} were carried out in hydrogen atmosphere, the yield of alkylcobalamin was higher than that in nitrogen atmosphere.

The important role of reduced forms of vitamin B_{12} in biological systems has been increasingly suggested. Of the two reduced forms of the vitamin, B_{12r}, a two-electron reduction product, is highly reactive and yields alkylcobalamin by reacting with alkyl halide. This fact has led to the supposition that B_{12s} is an active species in several biochemical reactions, such as methane formation (Blaylock and Stadtman, 1964; Wood and Wolfe, 1966), methionine biosynthesis (Weissbach *et al.*, 1965), and conversion of cobalamin into its coenzyme form (Vitols *et al.*, 1964; Weissbach *et al.*, 1966). However, the appearance of B_{12s} has not been detected spectrophotometrically during the enzyme reactions. On the other hand, the absorption spectrum of B_{12s}, a one-elec-

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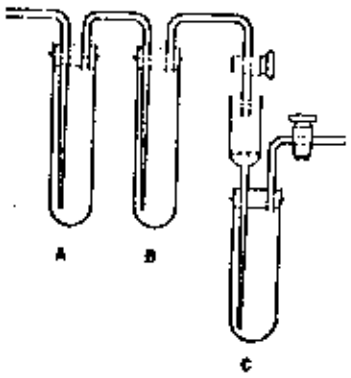

FIGURE 1: Apparatus used for preparation of B_{12r} by catalytic reduction of B_{12s} and subsequent reaction of B_{12r} with alkylating agents. The procedure is described in the text.

tion reduction product, or of something having analogous spectroscopic property, has been often observed during the course of some enzymatic reactions, such as biosynthesis of methionine (Taylor and Weisshach, 1967) and of 5′-deoxyadenosylcobalamin (Weisshach *et al.*, 1966).

A question also remains unsolved in the reduction of aquocobalamin (vitamin B_{12s}) with thiol compounds. It has been observed that the reaction product showed a B_{12r}-like absorption spectrum and an electron spin resonance signal like B_{12r} (Hill *et al.*, 1962, 1965). Nevertheless the product had a reactivity with alkyl halide yielding alkylcobalamin in a similar way to B_{12r} (Dolphin and Johnson, 1965).

This paper deals with the reaction of B_{12r} with appropriate alkylating agents *via* a disproportionation of B_{12r} to B_{12s} and B_{12r} under mild conditions. The results obtained would provide some information on the phenomena mentioned above.

Experimental Section

Materials. Cyanocobalamin was purchased from Roussel-Uclaf Co., France. Aquocobalamin, cyanodehydrocobalamin, and cyanocobinamide were prepared from cyanocobalamin according to the methods of previous workers (Bernhauer and Wagner, 1963; Bonnett *et al.*, 1957; Friedrich and Bernhauer, 1956). Alkylcobalamin, alkyldehydrocobalamin, and alkylcobinamide were prepared by the method of Smith and Mervyn (1963) from their corresponding cyano forms. Methyl iodide, *n*-propyl bromide, sodium monochloroacetate, and inorganic reagents were obtained from commercial sources. Sodium monobromoacetate and sodium monoiodoacetate were prepared from corresponding free acids.

Nitrogen used in this study was purified by passing through six wash bottles, each containing 100 ml of Fieser's solution (an alkaline sodium hydrosulfite solution containing sodium *β*-anthraquinone sulfonate) (Fieser, 1955). In an atmosphere of the nitrogen thus purified a solution of methylcobalamin (2.1×10^{-4} M) was irradiated with a 100-W tungsten lamp at a distance of 12 cm for 3 hr. A considerable amount of B_{12s} (*ca.* 35% of the initial amount of methylcobalamin) was detected by paper electrophoresis in the irradiated mixture. Dolphin *et al.* (1964) have observed that methylcobalamin was stable in the absence of oxygen (10^{-4} mM) in light of moderate intensity. On the other hand, Pratt (1964) has demonstrated that a trace of oxygen ($\lesssim 1.3 \times 10^{-4}$ M) markedly accelerated the rate of photolysis of methylcobalamin (5.1×10^{-4} M) in nitrogen atmosphere. These facts indicate that nitrogen used in our experiments still contained a trace of oxygen. By brisk bubbling of the nitrogen for 10 min through B_{12r} solution, B_{12r} oxidized to B_{12s}. But, when an aqueous solution (1×10^{-4} M) of B_{12r} was allowed to stand for 24 hr under a positive nitrogen pressure, no detectable spectral change, showing the formation of B_{12s}, was observed.

Preparation of Vitamin B_{12r} and Its Application to Reactions. METHOD A. B_{12r} was prepared by the catalytic hydrogenation of B_{12s} (Diehl and Murie, 1952). After mixing with an aqueous solution of an electrolyte and removal of hydrogen by bubbling nitrogen through the solution, the catalyst, $PtO_2 \cdot 2H_2O$, was filtered and the filtrate was led to a reaction vessel. In a representative experiment using a reaction apparatus as shown in Figure 1, hydrogen was bubbled for 20 min through 8 ml of aqueous solution of B_{12s} (4.3×10^{-4} M) containing 1 mg of $PtO_2 \cdot 2H_2O$ in vessel A and 8 ml of 2 M NaCl solution in vessel B. B_{12s} was almost completely converted into B_{12r} within 10 min. Nitrogen was then bubbled for 10 min and the B_{12r} solution was transferred into vessel B. After further 10-min bubbling of nitrogen, during which the catalyst was precipitated, the solution in vessel B was transferred into the reaction vessel C through a glass filter with pore diameter of 100–200 mμ.

METHOD B. B_{12r} was prepared by anaerobic photolysis of *n*-propylcobalamin and was applied to reactions with a similar apparatus to that used in method A except that the reaction vessel C was not furnished with the glass filter. In a typical experiment 5 ml of aqueous solution of *n*-propylcobalamin in vessel A and 5 ml of 2 M NaCl solution in vessel B were deoxygenated by 20-min bubbling of nitrogen. Then *n*-propylcobalamin solution was exposed to a 300-W tungsten lamp at a distance of 10 cm under vigorous bubbling of nitrogen. Photolysis was almost complete in 5 min.

In order to remove B_{12s} which would be formed (Yamada *et al.*, 1966b), nitrogen containing a trace of oxygen was briskly bubbled through the solution for further 10 min. By this treatment B_{12r} reoxidized to more stable B_{12s}. Then the B_{12r} solution was transferred into vessel B and next the mixed solution was poured into the reaction vessel C.

METHOD C. Solid B_{12r} prepared as previously described (Yamada *et al.*, 1966c) was used. (1) The apparatus used was similar to that of method A except that vessel A was omitted. Solid B_{12r} was placed on the glass filter (pore diameter, 20–30 μ) attached to the reaction vessel C and water or an aqueous solution of electrolyte was placed in vessel B. Nitrogen was made to pass through the filter *via* the solution in vessel B for 20 min. Then the solution was transferred into the vessel C

through the filter. By the treatment solid B_{12}, placed on the filter was dissolved. (2) When a large amount of B_{12} was used, a two-necked flask was employed as a reaction vessel, and a glass filter (pore diameter, 20–30 μ) was attached to one neck. The solid B_{12} was placed inside of the filter, and the flask was inclined so that the powder of B_{12} might not drop into water in the bulb of the flask. Nitrogen, flushed into the flask through the other neck, was bubbled through the water and passed out through the filter for 20 min. Then the flask was made to stand vertically and was shaken to transfer the powder of B_{12} into the water. Cobinamide, and dehydrovitamin B_{12} were prepared and applied to subsequent reactions according to method B or C.

An appropriate alkylating agent was placed and deoxygenated in the reaction vessel (vessel C in method A, B, and C-1) prior to the addition of B_{12} solution. In the case of methyl iodide, 0.5 ml in the reaction vessel was mildly bubbled with cooled nitrogen for 2–3 min before the addition of B_{12} under cooling in ice water. In a particular case (see method C-2), methyl iodide was deoxygenated in another vessel and poured into the reaction vessel. In the cases of sodium monohaloacetates, these were dissolved in water and deoxygenated by 20-min bubbling of nitrogen before the addition of B_{12} solution.

Separation of Reaction Products. Paper electrophoresis was performed using the apparatus, type II-C of Toyo Roshi Co., and Toyo filter paper no. 50 in 0.5 M acetic acid (pH 2.7) and in 0.1 N phosphate buffer (pH 7.0), respectively. Paper chromatography was carried out using Toyo filter paper no. 50 by an ascending method. Solvent systems used were as follows: (1) water-saturated *sec*-butyl alcohol, (2) water-saturated *sec*-butyl alcohol–acetic acid (100:1, v/v), (3) *sec*-butyl alcohol–water–aqueous ammonia (28%) (100:36:14, v/v), (4) 0.1% aqueous KCN-saturated *sec*-butyl alcohol, and (5) *sec*-butyl alcohol–0.2 N aqueous KCN–aqueous ammonia (1000:10:2, v/v).

When the reaction mixture contained only cobalamins, products were well separated by paper electrophoresis in 0.5 M acetic acid and were extracted with water from the paper. Then they were determined, after converted into dicyanocobalamin. Recoveries of methylcobalamin and B_{12} in these procedures were 87.0 ± 0.62 (standard deviation) and 88.4 ± 1.58% (standard deviation), respectively. In a case where the reaction mixture contained methylcobalamin, aquocobalamin (B_{12}), methylcobinamide, and aquocobinamide, products were separated by a two-dimensional method which consists of a successive use of paper electrophoresis and paper chromatography. On paper electrophoresis in 0.5 M acetic acid the products were separated into three spots, the first, somewhat tailing, was that of aquocobinamide, the second was an overlapped spot of aquocobalamin and methylcobinamide, and the last was that of methylcobalamin. After the electrophoresis, the paper was exposed to light in order to convert the methylcorrinoids into aquocorrinoids. The paper chromatography was carried out at right angles to the paper electrophoresis with the solvent system 4 containing cyanide. The four products were separated in the forms of cyanocobalamin and dicyanocobinamide. Their recoveries were 86.5 ± 0.82 and 87.2 ± 0.76%, respectively.

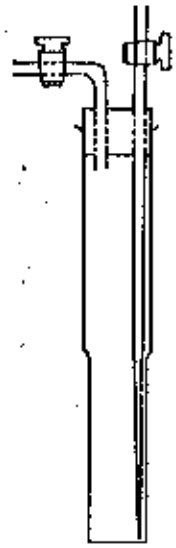

FIGURE 2: Modified quartz cell for absorption spectrum measurement under anaerobic conditions.

Absorption Spectra Measurement. A Hitachi EPS-II self-recording spectrophotometer was used. The measurement under anaerobic conditions was performed using a modified quartz cell fitted with a capillary in a manner as shown in Figure 2.

Results

Reaction of Vitamin B_{12} with Methyl Iodide in 1 M NaCl. It has been generally accepted that B_{12} is not reactive with methyl iodide. Under our experimental conditions, there was in fact no reaction observed when methyl iodide was added to a dilute aqueous solution of B_{12}. In the concomitant presence of an electrolyte, *e.g.*, NaCl, Na_2SO_4, $NaNO_3$, NaOH, KCl, KCN, or $MgCl_2$, however, a considerable amount of methylcobalamin was found to be produced. An isolated product other than methylcobalamin was cyanocobalamin in the presence of KCN, cobalamin sulfonate in the presence of Na_2SO_4, and B_{12} in the presence of the other electrolytes mentioned above. For subsequent experiments we mostly used NaCl, a typical, simple, and neutral electrolyte.

When 1 M NaCl solution containing B_{12} prepared by method A and methyl iodide was vigorously shaken and was allowed to stand in the dark overnight under nitrogen, the brown color of B_{12} turned to red, which was attributed to methylcobalamin and B_{12}. After destilling with phenol extraction, methylcobalamin and B_{12} were separated by paper electrophoresis in 0.5 M acetic acid (pH 2.7). Methylcobalamin was identified with the authentic sample by paper chromatography using the solvent systems 1–3. As shown in Table I the yield of methylcobalamin was slightly less than 50%. When the

TABLE I: Yield of Products from the Reaction of Vitamin B_{12r} with Methyl Iodide in 1 M NaCl.

Expt	Product (%)	
	Methylcobalamin	Aquocobalamin
1[a]	46	54
2[a]	44	56
3[b] (without NaCl)	0	100[c]

[a] In expt 1 and 2, 16 ml of 2.1×10^{-4} M and 16 ml of 3.2×10^{-4} M B_{12r} prepared by method A were, respectively, allowed to react with methyl iodide. [b] B_{12r} prepared by method C was used in comparable amounts with that in expt 1 and 2. [c] Oxidation product of remaining B_{12r} by exposure to air.

TABLE II: Yield of Products of All the Corrinoids from the Reaction of an Equimolar Mixture of Vitamin B_{12r} and Cobinamide, with Methyl Iodide in 1 M NaCl.[a]

	Methylcorrinoid (%)	Aquocorrinoid (%)
Cobalamin	8	42
Cobinamide	40	10
Total	48	52

[a] Reaction mixture (12 ml) contained 2.1×10^{-4} M B_{12r} and 2.1×10^{-4} M cobinamide, prepared by method B.

reaction of B_{12r} with methyl iodide was attempted in the absence of NaCl, there was no formation of methylcobalamin as mentioned above, and only B_{12s} was obtained, which was considered to be derived from B_{12r} unreacted, by exposure to air. These results suggest that the yield of methylcobalamin would be 50% in ideal conditions in the presence of NaCl, and the slightly lower yield is owing to the difficulty of complete removal of oxygen in methyl iodide and nitrogen used.

Therefore, a following reaction route was supposed. Two molecules of B_{12r} disproportionated into one molecule of B_{12s} and one molecule of B_{12r}, the former of which produced methylcobalamin by reacting with methyl iodide. Dimethyl sulfate also served as alkylating agent, but the activity was markedly lower than that of methyl iodide under our experimental conditions.

Reaction of a Mixture of Vitamin B_{12r} and Cobinamide, with Methyl Iodide in 1 M NaCl. If B_{12r} disproportionated as described above, the presence of another corrinoid, which is more or less easily reducible than B_{12r}, would affect the disproportionation process and consequently the ratio of methylcobalamin to B_{12s} produced by the reaction with methyl iodide would not become 1:1.

In order to investigate this point, an equimolar mixture of B_{12r} and cobinamide, which were prepared by method B, was allowed to react with methyl iodide in 1 M NaCl. After standing overnight under slightly positive nitrogen pressure, products were separated by a successive use of paper electrophoresis in 0.5 M acetic acid and paper chromatography in the solvent system 4. As shown in Table II nearly 50% of all the corrinoids was methylated. However, the yield of methylcobalamin was 8% of all the corrinoids, namely 16% of the initial amount of B_{12r}. On the other hand, methylcobinamide was obtained in the yield of 40% of all the corrinoids, corresponding to 80% of cobinamide, used.

In this reaction system, it appears probable that some of the following routes would take place as a primary reaction: (1) disproportionation of B_{12r}, (2) disproportionation of cobinamide, and (3) crossed disproportionation between B_{12r} and cobinamide. The result in Table II strongly suggests that cobinamide, which would be more easily reducible than B_{12r}, was preferentially converted into cobinamide, by the crossed disproportionation and reacted with methyl iodide. The presumption that cobinamide, was more easily reduced than B_{12r} was supported by the following observation; the conversion rate of aquocobinamide into cobinamide, was higher than that of B_{12s} to B_{12r} in reduction with $NaBH_4$. The presumption was also supported by polarographic investigation (unpublished result).

Absorption Spectrum of Vitamin B_{12r} in 1 M NaCl. As shown in Figure 3, the visible absorption spectrum of B_{12r} in 1 M NaCl, was identical with that in water. There was nothing found that demonstrated the occurrence of B_{12s} or B_{12a}. This suggests that a very small portion of B_{12r}, if any, disproportionated in 1 M NaCl under the conditions.

Reaction of Vitamin B_{12r} with Monohaloacetates in 1 M NaCl. To obtain information on the property of B_{12s} formed by the disproportionation of B_{12r}, alkyl halides of different reactivity were allowed to react with B_{12r} in 1 M NaCl. For this purpose sodium monochloroacetate, sodium monobromoacetate, and sodium monoiodoacetate were used as alkylating agent and the degree of formation of carboxymethylcobalamin was compared.

In nitrogen atmosphere a mixture of B_{12r} prepared by method C and monohaloacetate in 1 M NaCl was allowed to stand overnight in the dark. This reaction gave by-products which were more acidic than carboxymethylcobalamin. When the reaction time was limited (335 min) for the comparison of the reactivities among the monohaloacetates, only one by-product was obtained in addition to carboxymethylcobalamin. A similarly behaving compound was obtained from carboxymethylcobalamin and monoiodoacetate irrespective of the presence of oxygen and NaCl. B_{12s}, cyanocobalamin, and methylcobalamin did not give such a compound in the presence of monoiodoacetate. Therefore the by-product is regarded as a secondary reaction product formed from carboxymethylcobalamin.

As given in Table III, when monoiodoacetate was

TABLE III: Yield of Products from the Reaction of Vitamin B_{12r} with Monohaloacetates in 1 M NaCl.[a]

Monohaloacetate Used	Product (%)		
	Aquocobalamin	Carboxymethylcobalamin	By-Product
ICH_2COONa	64	28	8
$BrCH_2COONa$	89	8	3
$ClCH_2COONa$	100	0	0

[a] Reaction mixture (10 ml) contained 2.7×10^{-4} M B_{12r} prepared by method C and 5×10^{-1} M monohaloacetate. Reaction was stopped before it was complete (335 min).

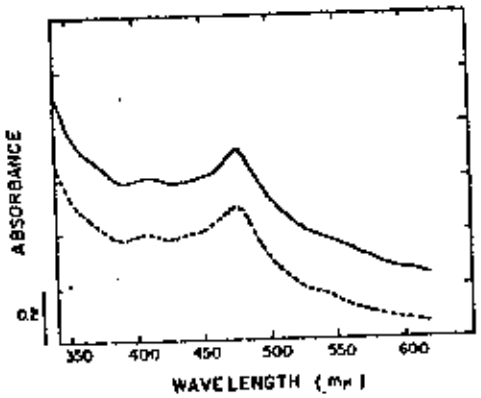

FIGURE 3: Absorption spectra of vitamin B_{12r} (5.1×10^{-5} M) in water and 1 M NaCl, respectively. (———) Vitamin B_{12r} in water and (– – – –) vitamin B_{12r} in 1 M NaCl.

used the yield of carboxymethylcobalamin was 28% and that of the single by-product was 8%. This means that carboxymethylation of B_{12r} occurred in the yield of 36%. Similarly, when monobromoacetate was used, 8% plus 3%, that is, 11% was carboxymethylated, while carboxymethylated products were negligible when monochloroacetate was used. Evidently the reactivity of alkylating agent had very marked influence on the reaction. These results would be explained as follows. B_{12r} formed from B_{12r} in 1 M NaCl is not only in a small amount, but also short lived, and the occurence of the alkylation reaction depends on whether an alkylating agent can trap this short-lived B_{12r} or not. Thus B_{12r} is considered to be indeed in an equilibrium with B_{12r} and B_{12s} and this equilibrium probably lies far to the direction of B_{12r}.

Comparison of the Effect of NaCl, NaI, and CsCl on the Reaction of Vitamin B_{12r} with Methyl Iodide. In order to investigate the role of electrolytes on the reaction of B_{12r} with methyl iodide, the effects of NaCl, NaI, and CsCl were compared. In 1 M solution of these salts, B_{12r} was allowed to react with methyl iodide in a limited time (140 min). As shown in Table IV, NaCl was most effective and yielded 19% of methylcobalamin, while the yield of methylcobalamin was markedly low, only 6 or 7%, respectively, when NaI or CsCl was employed. This order of the effectiveness of the salts was in accord with the Hofmeister's lyotropic series. Therefore it may be probable that the electrolytes remove water molecules around B_{12r} molecules and render them easier to interact with each other. This is in accord with an observation that solid B_{12r} is much less easily dissolved in 1 M NaCl than in water.

TABLE IV: Comparison of Effect of NaCl, NaI, and CsCl on the Reaction of Vitamin B_{12r} with Methyl Iodide.[a]

Electrolyte Added	Product (%)	
	Methylcobalamin	Aquocobalamin
NaCl	19	81
NaI	6	94
CsCl	7	93

[a] Reaction mixture (10 ml) contained 1 M electrolyte and 1.4×10^{-4} M B_{12r}. Reaction was stopped before it was complete (140 min).

Reaction of Vitamin B_{12r} with Methyl Iodide in the Absence of Electrolytes. If the role of electrolytes is to stimulate the interaction of B_{12r} molecules as described above, it is possible that the disproportionation would occur in a very high concentration of B_{12r} solution without electrolytes. To test this presumption, 11 μmoles of solid B_{12r} prepared by method C was mixed with 0.1 ml of water, where a portion of solid B_{12r} remained undissolved. After adding methyl iodide the mixture was kept for 2 days in nitrogen atmosphere. Then methylcobalamin was obtained in the yield of 23%. Thus the disproportionation was proved to occur without the help of electrolytes under such conditions.

Effect of Hydrogen on the Reaction of Vitamin B_{12r} with Methyl Iodide. The reaction of B_{12r} with methyl iodide was compared in nitrogen and hydrogen atmosphere to test the possibility that molecular hydrogen serves as reducing agent to B_{12r}. When B_{12r} prepared by method C was allowed to react with methyl iodide overnight in 1 M NaCl, methylcobalamin was obtained in the yield of 43% under nitrogen, while 59% under hydrogen (Table V). When the reaction of B_{12r} or dehydrovitamin B_{12r} was performed in 0.1 N NaOH, the yield of the methylation product was also higher in hydrogen than in nitrogen. These results indicate that molecular hydrogen can act as a reducing agent to corrinoid. Tackett *et al.* (1963) have demonstrated the reduction of H_2O by B_{12r}, which is possibly a reverse of the reaction described here.

It was surprising that the reaction in 0.1 N NaOH gave methylcobalamin in the yield of more than 50% even in nitrogen atmosphere. Heating of cyanocobalamin or aquocobalamin in 0.1 N NaOH is known to lead to the dehydrogenation on the ring B of corrin nu-

TABLE V: Effect of Hydrogen on the Reaction of Vitamin B_{12r} and Dehydrovitamin B_{12r} with Methyl Iodide.[a]

Expt	Substance	Atmosphere	Electrolyte Added	Product (%) Methylcorrinoid	Product (%) Aquocorrinoid
1	B_{12r}	N_2	1 M NaCl	43	57
2	B_{12r}	H_2	1 N NaCl	59	41
3	B_{12r}	N_2	0.1 N NaOH	61	39
4	B_{12r}	H_2	0.1 N NaOH	83	17
5	Dehydro-B_{12r}	N_2	0.1 N NaOH	52	48
6	Dehydro-B_{12r}	H_2	0.1 N NaOH	58	42
7	Dehydro-B_{12r}	N_2	0.1 N NaOH	56	44

[a] Volume of reaction mixture and concentration of B_{12r} or dehydrovitamin B_{12r} was as follows: expt 1 and 2, 7 ml and 2.0×10^{-4} M; expt 3, 8.6 ml and 4.0×10^{-4} M; expt 4, 6.3 ml and 6.2×10^{-4} M; expt 5 and 6, 7 ml and 2.2×10^{-4} M; expt 7, 20 ml and 6.5×10^{-4} M; Dehydrovitamin B_{12r} used in expt 7 was prepared by method B and the others were prepared by method C.

cleus with simultaneous reduction of the cobalt atom. To test whether such a reaction occurred or not, methylcobalamin and B_{12s} produced from B_{12r} were converted into their dicyano forms and examined by visible absorption spectrum and by paper chromatography in solvent system 5 (Yamada *et al.*, 1966a). There was no dehydrocobalamin detected. Reaction of dehydrovitamin B_{12r}, which was already dehydrogenated on ring B, with methyl iodide in 0.1 N NaOH also gave its methylation product in the yield over 50%. Therefore another additional reduction mechanism should be sought in this case.

Discussion

Although a trace oxygen contaminating in our reaction systems might have some influences on the reaction, such as its equilibrium, reaction rate, or yields of alkylated products, the results obtained above strongly suggest the mechanism of the change of B_{12r} to B_{12s} and alkylcobalamin in eq 1.

$$\underset{B_{12r}}{2Co^{II}} \rightleftharpoons \underset{B_{12s}}{Co^{I}} + \underset{B_{12a}}{Co^{III}} \qquad (1)$$

$$\downarrow \text{alkylating agent}$$

$$\text{alkyl } B_{12}$$

In aqueous solutions B_{12r} disproportionates to B_{12s} and B_{12a} until an equilibrium is attained. This equilibrium lies so far to the direction of B_{12r} that B_{12s} or B_{12a} cannot be detected spectrophotometrically. An electrolyte added to this system promotes the disproportionation. When alkylating agents trap B_{12s} as alkylcobalamin to take it out of the equilibrium system, the equilibrium moves toward the right in eq 1 and finally an equimolar mixture of alkylcobalamin and B_{12a} is produced. Although it is difficult to define a conclusive mechanism of the action of electrolytes, a possible explanation might be offered by assuming that the electrolytes facilitate the interaction of B_{12r} molecules by removing the water molecules surrounding them. This view would be supported by the facts that the order of the effectiveness of the electrolytes, so far tested, was in accord with the lyotropic series and a very high concentration of B_{12r} solution gave alkylcobalamin even in the absence of electrolytes.

Schrauzer and Windgassen (1966) reported the occurrence of characteristic absorption peaks of B_{12s} when they added KOH at 1 N concentration to a methanolic solution of B_{12r}. This fact indicates that under specially vigorous conditions a sufficient amount of B_{12s} is formed as detected spectrophotometrically although the possibility of dehydrogenation on ring B or action of other reduction mechanisms cannot be ruled out. These phenomena show that B_{12s} produced by the same mechanisms can be spectrophotometrically detected in one case where vigorous condition is utilized, and cannot be detected in another case where the reaction was mildly carried out in neutral aqueous solution analogous to biochemical reactions.

For example, a reduction product of cyanocobalamin with ferredoxine can serve as substrate in the enzymatic synthesis of 5′-deoxyadenosylcobalamin and can also be methylated with methyl iodide, although it has an identical absorption spectrum with that of B_{12r} (Weissbach *et al.*, 1966). Considering the results of Vitols *et al.* (1964) that B_{12s} produced by $NaBH_4$ reduction of B_{12r} serves as substrate in the deoxyadenosylating enzyme system, a possible explanation for the phenomena mentioned above would involve a transient formation of B_{12s}. Similarly, in the terminal reaction of methionine biosynthesis, the spectrum of B_{12s} has not been observed in spite of the property of the B_{12} enzyme to be propylated with propyl iodide. In this case the spectrum of the enzyme is similar to that of B_{12s} (Brot and Weissbach, 1965), although the absence of electron spin resonance signal leaves a problem to be solved.

Reduction of cobalamins with several thiols gives a

compound having a similar absorption spectrum to that of B_{12} (Hill *et al.*, 1962) as well as a characteristic electron spin resonance spectrum of B_{12} (Hill *et al.*, 1965). This compound reacts with methyl iodide and 5′-iododeoxyadenosine to give methylcobalamin and 5′-deoxyadenosylcobalamin (Murakami *et al.*, 1966), respectively, while it hardly reacts with 2′,3′-isopropylidene-5′-tosyladenosine (Dolphin and Johnson, 1965; Morley and Blakley, 1967). These facts can be also understood as that the reactant is a small amount of B_{12} which has only a short life. The difference of the behaviors between 5′-iododeoxyadenosine and 2′,3′-isopropylidene-5′-tosyladenosine is explained by the marked dependence of transient B_{12} on the reactivity of alkylating agent, as is shown when B_{12} was allowed to react with different monohaloacetates. When 2′,3′-isopropylidene-5′-tosyladenosine is employed, the rapid reaction will not be seen unless a considerable amount of B_{12} is maintained with a strong reducing agent. An analogous interpretation may be offered for our findings that a B_{12}-like compound formed by anaerobic heating of cyanocobalamin in 0.1 N NaOH reacts with methyl iodide or *n*-butyl bromide (Yamada *et al.*, 1964, 1966a).

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Distribution of ^{35}S in the Blood and its Excretion in Urine of Dogs Exposed to $^{35}SO_2$

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Blood ^{35}S levels in anesthetized dogs rose progressively when the isolated upper airways were exposed to $^{35}SO_2$ for 30 to 60 minutes, and decreased slightly in the hours following exposure. The plasma contained more ^{35}S than the red blood cells (RBC); more than half of the plasma-^{35}S was dialyzable, the greatest radioactivity among the nondialyzable fraction of the plasma being associated with α-globulin proteins. Two thirds of the RBC-^{35}S appeared to be intracellular. RBC-^{35}S/Plasma-^{35}S was higher following in vivo exposure of animals than when plasma-^{35}S was mixed with nonradioactive RBC. Most of the urinary ^{35}S was in the form of inorganic sulfate.

EXPOSURE to sulfur dioxide (SO_2) may be associated with changes in the concentration of plasma proteins,[1,2] hemoglobin concentration,[3,4] and red blood cells (RBC).[5,6] The mechanism for these changes is not certain, nor is it clear whether impairment of nutrition, water or electrolyte balance, or both were in part responsible for their occurrence. The ^{35}S is known to enter the blood within minutes of the onset of exposure to both $^{35}SO_2$[7,8] and sulfuric acid mist.[9] Little is known of its subsequent distribution and exchange among the components of the blood, knowledge that might clarify the hematologic changes attending exposure to the gas. The primary purpose of our study was to extend preliminary observations that had been made on the interactions of ^{35}S and blood[10] as well as to identify the forms in which ^{35}S is excreted in the urine. Our results are based on the exposure of both the isolated upper airways of dogs and in vitro samples of blood to $^{35}SO_2$.

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Methods

In Vivo Experiments.—The surgically isolated upper airways of nine mongrel dogs were exposed to $^{35}SO_2$. In five animals the concentration of $^{35}SO_2$ was 22 ± 2 ppm[10] the level of radioactivity being 3.7×10^4 picocuries/ml for the first experiment (dog 1). The other four animals were exposed to 50 ppm of $^{35}SO_2$[10] the level of radioactivity was 2.1×10^4 picocuries/ml for the first animal (dog 7) in this

group. The details of the surgical preparation have been described separately.[10] The animals were anesthetized with intravenously administered pentobarbital sodium (initial dose, 30 mg/kg; maintenance dose, 30 to 60 mg, as needed), paralyzed with succinylcholine chloride (initial dose, 3 mg/kg; maintenance dose, 0.02% solution in 5% dextrose in saline infused continuously at the approximate rate of 1 ml/min), and ventilated on ambient air by a displacement pump. Arterial blood and urine samples were collected during and following the exposure; the postexposure period lasted up to three hours. The blood was sampled through indwelling polyethylene tubing that had been passed through one of the femoral arteries into the abdominal aorta. The sampling syringes were moistened with heparin sodium. Samples were drawn at 5 to 10 minute intervals during the exposure, less often following completion of the exposure. The blood, within minutes of collection, was separated into its plasma and cellular components by centrifugation. The urine was collected through a catheter that was implanted in the bladder through an incision in the lower anterior abdominal wall. At least 1.5 ml of urine were obtained with each sample. Urinary sampling was less frequent and less regular than that of blood, the interval depending on the rate of urinary flow. All samples were refrigerated at about 5 C until analyzed. One-milliliter samples of the plasma and cellular components of the blood and urine were counted with a thin window flow-type counter (Geiger-Müller). We calculated the whole blood activity usually by summing the radioactivity of the plasma and cellular components and correcting for the hematocrit value. To correct radioactivity of the RBC, we assumed that trapped plasma represented 5% of the sample volume.[12] All values for radioactivity were corrected for self-absorption.[10]

In Vitro Experiments.—The arterial blood of unexposed dogs and the venous blood of healthy volunteers were exposed to $^{35}SO_2$ in a slowly rotated tonometer at room temperature.

Distribution of ^{35}S in Plasma.—We determined the fraction of the plasma radioactivity that was bound to protein by the method of dialysis[13]: The cellophane tubing (Visking 9/16 inches inflated diameter) containing the radioactive plasma was placed in a 25% solution of polyvinylpyrrolidone (PVP) and refrigerated at about 5 C. The PVP was stirred continuously. Counts were made on 1-ml samples of the plasma before dialysis, and on the PVP after dialysis. All data reported for this technique were obtained after at least 24 hours of dialysis at which time the transfer was virtually complete. The percentage of dialyzable ^{35}S was calculated as follows:

$$\% \text{ dialyzable } ^{35}\text{S} = \frac{\text{PVP activity after dialysis} \times \text{PVP vol}}{\text{Plasma activity before dialysis} \times \text{Plasma vol}} \times 100.$$

The nondialyzable radioactive plasma was subjected overnight to electrophoresis on 36- × 15-cm strips of filter paper in a buffer (Veronal) of pH 7.3. Following electrophoresis, the strips were dried at 100 C and then were developed in bromphenol blue solution. The dried and stained strips were cut into six segments: albumin, α-, β-, and γ-globulins, and two unstained portions of the paper beyond albumin and globulin. The segments were taped to planchets and were counted (Geiger-Müller). Because of the low ^{35}S radioactivity present on each segment of paper, we counted both the background and sample for more than half an hour.

Distribution of ^{35}S in RBC.—We determined the relative distribution of ^{35}S between the surface and the interior of the RBC by comparing the radioactivity of the cells before and after they were washed. To wash the cells, three parts of normal saline (NaCl) or isotonic phosphate buffer were added to one part of the radioactive cells, the mixture was rotated gently and centrifuged for 15 minutes; the supernatant fluid was then discarded. The procedure was repeated three times.

To test if any ^{35}S was fixed to the cell membrane, we counted the radioactivity of RBC ghosts that were prepared by the method of Dodge et al[14]: The ghost sample was prepared from 1 ml of the washed cells suspended in an equal volume of isotonic phosphate buffer, and then resuspended in 1 ml of 0.1N potassium hydroxide.

In Vitro Exchange of ^{35}S Between Plasma and RBC.—We combined equal volumes of radioactive plasma and nonradioactive RBC in a syringe and rotated the contents in a water bath at 37 C for at least 15 minutes. (In initial tests, we found no difference in the magnitude of the transfer of ^{35}S to RBC for periods ranging from 15 to 60 minutes). The mixture was then separated into plasma and RBC components for counting. We measured the concentrations of ^{35}S in the plasma before and after mixing with the RBC, and in the RBC component following the mixing. The radioactivity of the RBC was corrected for trapped plasma.

Urinary Sulfate.—We used the method of Folin[15] to analyze the urine for ^{35}S. Inorganic sulfate was precipitated by the addition of 5%

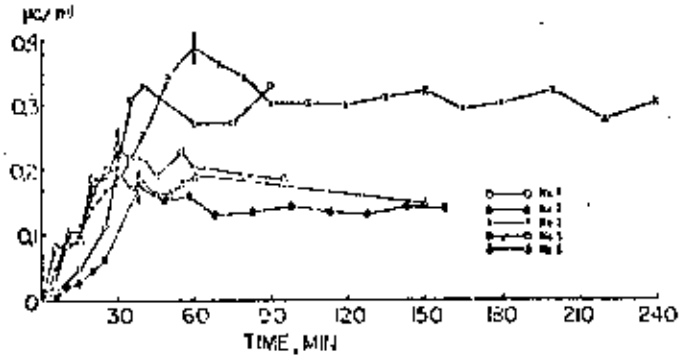

Fig 1.—Changes in level of ^{35}S in whole blood of five dogs. The $^{35}SO_4$ = 22 ± 2 μc/m. Decay constant of ^{35}S was used to correct all levels of radioactivity to the day at which the first dog was exposed. Vertical bars indicate end of exposure.

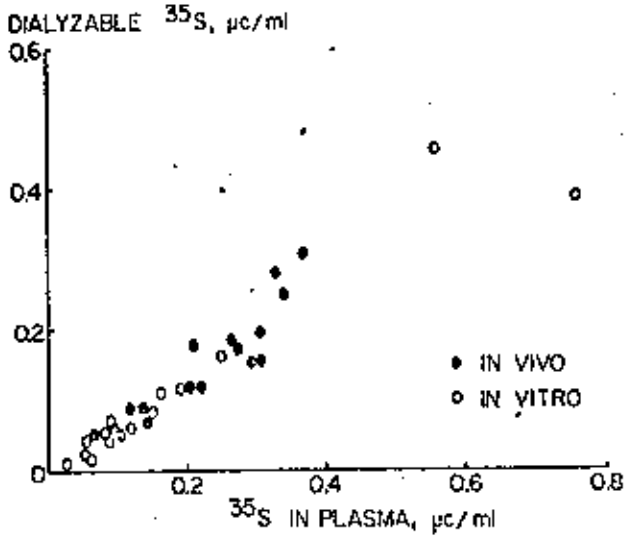

Fig 2.—Relation between total plasma ^{35}S and dialyzable ^{35}S following dialysis with 25% PVP for 24 hours.

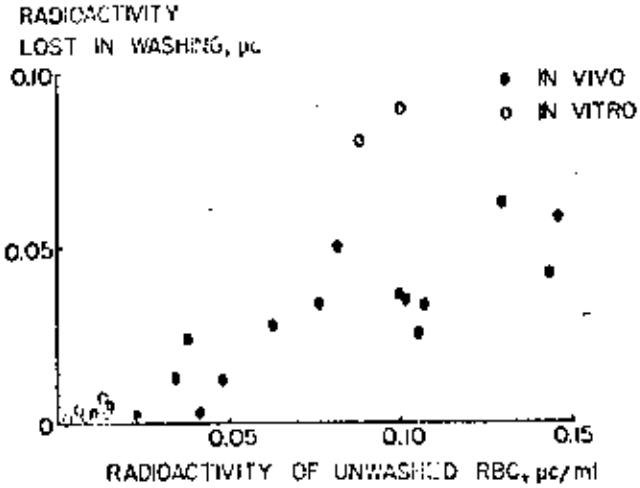

Fig 3.—Relation between the ^{35}S activity of unwashed RBC and activity lost from these cells by washing with normal saline or isotonic phosphate buffer.

Table 1.—Relative Counts per Minute of Paper Segments Following Electrophoresis of Non-dialyzable Portions of Radioactive Plasma

Dog No.	Blank	Albumin	α Globulin	β-Globulin	γ-Globulin	Blank
9	6.6 (13)*	9.6 (18)	21.1 (41)	4.0 (8)	6.6 (13)	3.6 (7)
15	2.3 (10)	4.6 (20)	8.8 (38)	2.9 (12)	2.8 (11)	2.0 (9)

* Numbers in parentheses are equal to % of total count.

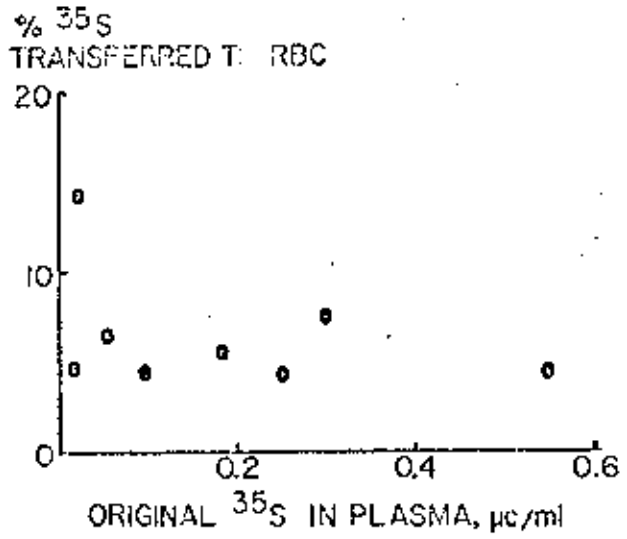

Fig 4.—Relation between plasma ^{35}S and percentage of ^{35}S transferred to RBC following mixing of plasma and RBC in a syringe at 37C for 15 minutes.

barium chloride to the diluted, acidified urine. To precipitate total sulfate, we heated the urine slowly to boiling before adding the barium chloride. The mixture was allowed to stand two to three hours, filtered through a funnel (Buchner), and the precipitate was washed with cold water. The radioactivity of the filtrate, including the washings, was determined with a counter (Geiger-Müller). The radioactivity of the sulfate was calculated from the difference in radioactivity between the urine and the filtrate.

Results

The ^{35}S in Whole Blood, Plasma, and RBC.—The absolute levels of radioactivity in whole blood, corrected to the day of the first experiment by application of the decay constant for ^{35}S,[16] are shown in Fig 1. In general, the whole blood levels rose steadily throughout the exposure (30 to 60 minutes). The lowest levels were found in dog 2 which was found to have pulmonary edema at autopsy. The edema is thought to be related to an excessive infusion that was given inadvertently. Four of the five animals showed a fall in ^{35}S blood levels once exposure was ended, though the fall was slight and was not necessarily progressive with time. The maximal postexposure period lasted three hours. The time course of the changes in

Table 2.—Relative Urinary Excretion of ^{35}S as Inorganic and Total Sulfate

Sample Number	% ^{35}S as Inorganic Sulfate	% ^{35}S as Total Sulfate
1 (Dog 7)	88.2	91.5
2 (Dog 8)	82.9	99.1
3 (Dog 8)	81.2	90.1
4 (Dog 9)	86.1	...
5 (Dog 9)	87.3	91.3
6 (Dog 9)	83.2	90.2
Mean	84.8	92.4

plasma and RBC levels of ^{35}S have been reported.[10] They may be summarized as follows: The ^{35}S rose progressively in both compartments during the exposure. In the postexposure period the plasma levels tended to fall whereas the RBC levels, following an initial fall, gradually rose. The ^{35}S concentrations were always higher in the plasma than in the RBC, although the difference between concentrations was variable.

The ^{35}S Distribution Within Plasma.—The sum of the ^{35}S in the dialyzable and nondialyzable fractions did not differ statistically from the ^{35}S measured independently in aliquots of the same whole plasma. The percentage of dialyzable ^{35}S was essentially constant over the entire range of radioactivity (Fig 2): 61.4% (SE ± 2.3%) in 31 measurements. The partitioning was similar for the in vivo and in vitro samples.

To test the results of dialysis, the radioactivity of eight samples of plasma was measured before and after precipitation of the proteins with trichloroacetic acid. The average percentage of ^{35}S remaining in the plasma filtrate was 74.7% (SE ± 8.8%), a value not significantly different from the percentage that was dialyzed.

Protein Fractions.—Only two samples had sufficient radioactivity for electrophoretic analysis. Since we did not correct for self-absorption or for differences in the paper size among the segments, the results are useful for describing only the relative distribution of ^{35}S. Table 1 lists the count number per minute for each segment of the paper. The highest count was found on the segment containing the α-globulin fraction, the next highest on the segment containing albumin.

RBC Radioactivity.—*Surface Versus Interior.*—Figure 3 shows the relation between the radioactivity lost from the cells through washing and the radioactivity of the unwashed cells for samples analyzed within one or two days of their exposure. The mean percentage of radioactivity lost from the RBC through washing was 35.1% (SE ± 3.6%) for the in vivo samples, and 63.8% (SE ± 10.3%) for the in vitro samples. The difference between the two groups was significant ($P < 0.01$).

Cell Membrane Radioactivity.—We found no radioactivity in the ghosts of six samples of RBC.

In Vitro Exchange of ^{35}S Between Plasma and RBC.—An average of only 8.0% (SE ± 1.5%) of the plasma ^{35}S underwent transfer to the RBC following the mixing of equal volumes of the two constituents (Fig 4). The ratio of RBC radioactivity per plasma radioactivity was significantly lower following this in vitro form of transfer than following exposure of the upper airway to $^{35}SO_2$ (in vivo).

Urinary Excretion of ^{35}S.—The time course of urinary excretion of ^{35}S and the relative concentrations of urinary-^{35}S and plasma-^{35}S were reported previously.[10]

An average of 84.4% of the urinary-^{35}S was in the form of inorganic sulfate; 92.4% was present as total sulfate (Table 2).

Comment

The level of ^{35}S in the blood should reflect the balance between the rate at which ^{35}S enters the circulation, principally from the mucosa of the upper airways, and the rates at which it is excreted from the body and deposited in peripheral tissues. There is no direct information about the magnitude of these functions, nor how they may change with the concentration of ^{35}S in different tissues and with time. In general, we found that the radioactivity of whole blood decreased relatively little during postexposure periods lasting up to three hours despite the continuous renal excretion of ^{35}S. The observation confirms Bystrova's experience with white rats.[7] It would appear that the rate of transfer of the bulk of the ^{35}S from the site of absorption to the circulation, though delayed, is sufficient to sustain whole blood levels for many hours after the exposure is ended. The time required to mobilize all ^{35}S from the adsorptive site is not known. Balchum et al[17] were able to detect ^{35}S in the airway tissues of a dog one week after the animal had been exposed to $^{35}SO_2$ through a tracheal cannula.

We found that ^{35}S was always more concentrated in the plasma than in the RBC. About one third of the radioactivity in plasma appeared to be in association with proteins, especially the α-globulins. The nature of the ^{35}S-protein bond was not determined. The α-globulins are considered to be hetero-

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serum—mixtures of proteins that are bound to carbohydrates, lipids, and other substances. They include most of the glycoprotein found in human plasma.[15] Smith et al[16] showed by means of paper electrophoresis that following the intravenous injection of $Na_2{}^{35}SO_4$ into rats, the greatest accumulation of radioactivity occurred on the strips containing the α_2-globulin. The authors assumed that the ^{35}S was principally contained in sulfonated carbohydrate-globulin complexes. In the light of this binding of ^{35}S to the α-globulins, it is interesting to note that both Erban[1] and Symon[2] reported elevated serum α-globulin in experimental rabbits that were exposed to an average concentration of 36 mg/cu m (12.6 ppm) of SO_2 for 80 days. Erban suggested that the change represented a nonspecific response to chronic stress rather than a consequence of the direct action of the gas; our finding suggests that a more specific mechanism may also operate. Alternatively, Navrotsky[24] reported no effects on the serum protein spectrum of rabbits after long-term exposure to SO_2.

In our experiments about two thirds of the ^{35}S associated with the RBC appeared to be intracellular. The remainder is assumed to have been adsorbed on the surface of the cell. We could not demonstrate any ^{35}S that was bound to the cell membrane. During the postexposure period the amount of ^{35}S in association with the RBC increased, while the plasma concentration of ^{35}S fell. The net gain in the RBC concentration of ^{35}S is difficult to explain, particularly if the radioactivity within the cell is assumed to reflect principally the sulfate ion. The latter is thought to be passively transported across the mammalian RBC membrane.[21] Consequently, the increase in the RBC concentration of ^{35}S relative to the plasma concentration might reflect either a change in membrane permeability or a nonionic binding of the ^{35}S inside the cell. On the other hand, the experiments showing that the transfer of ^{35}S from the plasma to the RBC was considerably less in vitro than in vivo are consistent with the possibility that an active transport mechanism may function during life.

Whether the inspired sulfur that enters the RBC may affect these cells is unclear. Lee[9] reported an increase in the hemoglobin concentration of guinea pigs exposed to 6 to 310 ppm of SO_2 for only one hour. The increase in hemoglobin concentration was roughly proportional to the concentration of the gas between 6 and 20 ppm but the proportionality ceased at concentrations above 20 ppm. The result of long-term exposure of animals to SO_2 have been inconsistent. Volkova[22] found no significant changes in the concentrations of hemoglobin and RBC in rabbits exposed to concentrations below 20 mg/cu m (7.0 ppm) of SO_2 for 3.5 hours/day over a period of 1.5 months. Recently, Battigelli et al[23] reported no detectable change in the hematocrit value of rats exposed to a mixture of SO_2 (1 ppm) and dust (graphite, 1 mg/cu m) for four months. However, Ball et al[4] reported an increase in hemoglobin concentration together with increases in the white blood cell count and the percentage of neutrophils in rats exposed to 1 to 32 ppm of SO_2 for periods up to 13 months. Hayashi[6] reported that an initial increase of RBC was followed by a gradual return to the pre-exposure level in rabbits that were exposed to 100 ppm of SO_2 for ten months.

Our previous data[10] showed that the kidney excreted ^{35}S at a rate approximately proportional to the level of ^{35}S in plasma and whole blood; in addition, the rate of ^{35}S excretion was not influenced by mannitol, an osmotic diuretic. These findings would be expected to the extent that ^{35}S is excreted principally as an inorganic sulfate and that the reabsorptive mechanism for the sulfate is readily saturated.[21] Generally, urinary sulfur is present in three forms: inorganic sulfate, ethereal sulfate, and neutral sulfur.[25] We found that 84.4% of the urinary ^{35}S was excreted as inorganic sulfate, 7.6% as ethereal sulfate, while the form of the remainder was not determined.

If it is assumed that all of the $^{35}SO_2$ entering the nasopharynx was taken up by the mucosa in these experiments, then 1% to 6% of the total amount that entered the body was excreted in the urine during the few hours of the study. As shown in the previous report,[10] the amount of ^{35}S present in the circulation, coincident with the peak concentration, ranged from 5% to 18% of the dose. If little or none of the ^{35}S was

excreted in the feces during this period, the remaining three fourths or more of the inspired dose must have been distributed between the site of absorption and the peripheral tissues of the body.

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Phillip Waithe performed surgery, and Mrs. Sally Stonehouse and Miss Dominica Paci provided technical assistance.

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Effect of Certain Compounds on *in vitro* Degradation of Serotonin by Ceruloplasmin.* (26176)

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It was recently demonstrated in this laboratory that *in vitro* degradation of serotonin by ceruloplasmin is strongly inhibited by both isoniazid and iproniazid(1). This finding may be of importance in connection with the psychopharmacologic and other clinical effects of these compounds. Moreover, it may have significance with regard to a concept which postulates that fibrosis may result from an imbalance between serotonin and serotonin degradation mechanisms at the tissue level(1,2,3). In view of these and other considerations, it became of interest to determine the *in vitro* effect of a wide range of other compounds on this pathway of serotonin oxidation.

Accordingly, tests were performed with selected reducing, sulfhydryl containing, and metal binding compounds; with hydrazine derivatives and amine oxidase inhibitors; and with a number of antihypertension and psychopharmacologic agents. The findings form the basis of this report.

Materials and methods. Initial titration tests with purified preparations of ceruloplasmin confirmed the activity of this enzyme over a wide pH range. Although optimal activity was at pH 6.0, it was elected to perform these *in vitro* tests at a more closely physiological pH, that is, 7.4. Accordingly, serial 2-fold dilutions of ceruloplasmin were made in physiological saline solution. The enzyme concentration which was found to metabolize between 65% and 80% of the substrate (300 μg serotonin-creatinine sulfate) was used in the final test system. This degree of enzyme activity was found to be present in a 1:8 dilution of the human ceruloplasmin employed. Dilutions of 3 different preparations of ceruloplasmin were used. These were designated DWZ 37, DWZ 42, and DWZ 44, and had been prepared by the Kabi Co., Stockholm, Sweden†

Procedure. To a 20 ml beaker were added 1.0 ml of ceruloplasmin solution; 1.0 ml of 0.5 M phosphate buffer, pH 7.4; and 0.5 ml of the solution containing the substance to be tested for inhibition of the reaction. The beakers were pre-incubated for 15 minutes on a Dubnoff metabolic shaker. Solutions of the test substances were freshly prepared and placed in the test system within 10 minutes of preparation. Final concentration in the incubation mixture was 4.9×10^{-4} M for serotonin. Incubation was continued for 1 hour, and determination for residual serotonin was made by the colorimetric method of Udenfriend, *et al.*(4). Controls included tests with boiled ceruloplasmin, and, for color interference, recovery of serotonin after incubation with the respective test substance in absence of enzyme.

The following substances were tested for reaction inhibition at a final concentration of 1×10^{-3} M: sodium metabisulfite, ascorbic acid, glutathione, British anti-lewisite (BAL), cysteine, methionine, D, L penicillamine, sodium cyanide, and ethylene diamine tetraacetic acid (EDTA). Among the hydrazine derivatives and among oxidase inhibitors tested at the same concentration were: amphetamine, procaine amide, iproniazid,‡ isoniazid,‡ isocarboxazid‡ (Marplan),§ hydralazine§ (Apresoline),§ Compound Ba-20533§ (an analog of hydralazine), thiocarbohydrazide, semicarbazine, and aminoguanidine. Hydrazine hydrate, phenylhydrazine, 2-4 dinitrophenylhydrazine, 1-acetyl-2-phenylhydrazine, and diphenylthiocarbazone were employed in a final dilution of 1×10^{-4} M be-

* Aided by a grant from Pennsylvania Trudeau Soc.

† The ceruloplasmin used in this study was generously made available by the Wyeth Laboratories, Radnor, Pa.

‡ Kindly provided by Hoffmann-LaRoche, Nutley, N. J.

§ Contributed by Ciba Pharmaceutical Products, Summit, N. J.

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...se of color interference at greater concentrations.

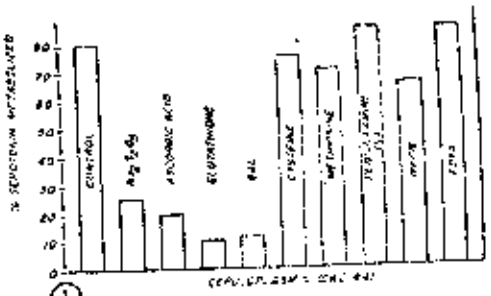
The antihypertensive and psychopharmacologic substances tested at a final concentration of 1 x 10^{-3} were Su-7057§ (a thiazide diuretic), Su-5879§ (Esidrix,® hydrochlorothiazide), Su-6355§ (an analog of hydroflumethiazide), reserpine, Su-3118§ (syrosingopine, Singoserp,® a synthetic analog of natural Rauwolfia alkaloids), Su-5171§ (an isomer of syrosingopine), chlorisodamine chloride§ (Esidrix),® hexamethonium bromide, tetraethylammonium chloride, methylphenidate hydrochloride§ (Ritalin),® and Ro-50600§ (Librium).® Su-5864§ (guanethidine, an antihypertensive agent), and Su-6157§ (a thiazide diuretic) were employed in a final concentration of 0.0265 mg/ml.

All of the reagents were chemically pure substances except for BAL which was prepared from a solution containing peanut oil and benzyl benzoate.

Results. The majority of test findings are presented in Fig. 1-3. At pH 7.4, an inhibitory effect on *in vitro* degradation of serotonin was exhibited by sodium metabisulfite, ascorbic acid, glutathione, BAL, isoniazid, iproniazid, nocarboxazid, hydralazine, Ro-50523, thiosemicarbazide, and hydrazine hydrate. An effect about equal to the latter was also noted with compounds Su-3118 (Singoserp) and its isomer, Su-5171. Lesser changes in activity were found in presence of reserpine, tetraethyl ammonium chloride, and Su-7057, as well as with phenylhydrazine, 2-4-dinitrophenylhydrazine, and 1-acetyl-2-phenylhydrazine.

None of the other substances appeared to alter the reaction rate under the conditions of testing.

Discussion. It is evident from the foregoing results that enzymic degradation of serotonin by ceruloplasmin is markedly inhibited at physiologic pH by a number of widely differing compounds. Reducing agents such as sodium metabisulfite, ascorbic acid, glutathione, and BAL are particularly active. In this connection, it may be noted that Holmberg and Laurell demonstrated reduction of ceruloplasmin to a colorless compound by sodium hydrosulfite, ascorbic acid, hydroxylamine, and thioglycolic acid(5), while ascorbic acid was further shown to diminish enzyme activity(6). This property of ascorbic acid has been utilized by R. B. Davis to block the destruction of serotonin present in specimens of clotted blood(7). Holmberg and Laurell also found that potassium cyanide inhibited oxidation of paraphenylene diamine, especially on the acid side of pH 7(6). This is in contrast to the minimal effect of



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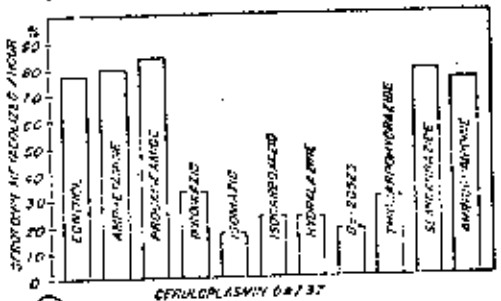

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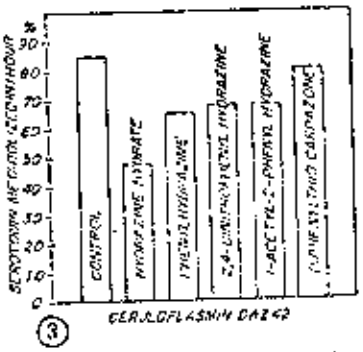

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FIG. 1. *In vitro* effect of certain reducing, sulfhydryl containing, and metal binding agents on serotonin degradation by ceruloplasmin.

FIG. 2. Effect of indicated compounds on *in vitro* degradation of serotonin by ceruloplasmin preparation DWZ 37.

FIG. 3. Effect of certain hydrazine derivatives on *in vitro* degradation of serotonin by ceruloplasmin preparation DWZ 42.

NaCN as observed in this study at pH 7.4.

It is of interest that EDTA and penicillamine did not inhibit the reaction. It appears, therefore, that the copper of ceruloplasmin was not chelated. R. P. Davis observed a similar failure to chelate enzyme metal in studies with carbonic anhydrase(8). He suggested that the lack of chelation of carbonic anhydrase zinc might reflect a more internal location of this metal in the carbonic anhydrase molecule than is the case for other zinc enzymes where chelation results in competitive inhibition and where a superficial location is necessary in the substrate binding function of these enzymes. It seems reasonable to consider that a similar relationship probably exists with regard to the copper of ceruloplasmin.

With regard to the hydrazine derivatives, Martin, *et al.*(9) demonstrated that oxidation of 5-hydroxytryptamine by serum from normal and pregnant humans was inhibited by .003 M iproniazid. These workers suggested that the enzyme involved was ceruloplasmin and further noted that this offered a potential degradation pathway for serotonin transformation other than the monoamine oxidase system. The present studies with purified ceruloplasmin revealed not only iproniazid, but also isoniazid, hydralazine, an analog of hydralazine (Ba-20523), isocarboxazid, and thiocarbohydrazide are inhibitory, as is hydrazine hydrate to a lesser extent. On the other hand, semicarbazide, aminoguanidine, and diphenylthiocarbazone were without effect. Thus, the presence of the basic hydrazine nucleus in a compound is not sufficient in itself to produce inhibition of this reaction.

Several of the test substances are classified by some as "monoamine oxidase inhibitors," while others are termed "diamine oxidase inhibitors." Among the former are amphetamine, procaine amide, iproniazid, and isocarboxazid. It can be seen from the results that some inhibited ceruloplasmin activity whereas others did not. Similarly, thiocarbohydrazide, a "diamine oxidase inhibitor," markedly depressed ceruloplasmin oxidation, while semicarbazide and aminoguanidine were ineffective.

Hydralazine, and its analog, Ba-20523, are both potent diamine oxidase inhibitors. Hydralazine also strongly inhibits monoamine oxidase, but its analog is about 30 times less active in this regard(10). Both substances were markedly inhibitory to ceruloplasmin activity. The findings, therefore, fail to show correlation with either monoamine or diamine oxidase inhibition.

Numerous reports have dealt with the relationship of serotonin and other amines, such as norepinephrine, to central nervous system activity. The psychopharmacologic effects of monoamine oxidase inhibitors, for example, are believed to be mediated through inhibition of this pathway of destruction of brain serotonin and/or norepinephrine(11). Recent observations by Sulser and Brodie(12) underscore the complexity of interaction of these mechanisms at the brain tissue level. Their findings suggest that the tranquilizer action of reserpine is not related to brain norepinephrine loss but rather to a change in level of brain serotonin.

Our findings indicate an even more complex relationship is probably involved. Thus, reserpine and 2 of its analogues (syrosingopine and its isomer, Su-5177) were found to have some inhibitory effect on degradation of serotonin by ceruloplasmin. Degree of inhibition was not as striking as that obtained with hydralazine, its analog (Ba-20523), isoniazid, and isocarboxazid. Nevertheless, it should be apparent that modifying effects on pathways of serotonin degradation other than monoamine oxidase must also be taken into account when interpreting the clinical actions of various compounds.

Of the remaining substances tested in this study, only TEAC and Su-7057 exhibited some inhibitory effect on the reaction test-system. That TEAC produced inhibition despite the presence of the chloride ion is of interest since Helmberg and Laurell(13) showed that certain monovalent anions, including Cl^- and Br^-, had an accelerating effect on this enzymic process. Hexamethonium bromide, however, was without evident effect. Su-7057, which showed slight inhibition, was the only thiazide derivative tested to do so. The reason for this finding is not known.

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Summary. In an *in vitro* test system at pH 7.4 it was found that degradation of serotonin by ceruloplasmin was markedly inhibited by sodium metabisulfite, ascorbic acid, glutathione, BAL, isoniazid, iproniazid, hydralazine, an analog of hydralazine (Ba-20523), isocarboxazid, thiocarbohydrazide, hydrazine hydrate, syrosingopine (Su-3118) and its isomer, Su-5171. Lesser effects were produced by reserpine, tetraethylammonium chloride, and a thiazide diuretic (Su-7075). A number of other sulfhydryl containing, chelating, anti-hypertension, and psychopharmacologic agents were without effect in the test system employed. It was not possible to correlate reaction inhibition with simple presence of sulfhydryl groups, the hydrazine nucleus, or with monoamine oxidase and diamine oxidase inhibition.

The authors wish to express their appreciation to Mrs. Claire Groff and Miss Faye Baldwin for technical assistance during these studies.

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Pyridoxal Phosphate in Plasma and Leukocytes in Patients with Leukemia and Other Diseases.* (26177)

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The concentration of pyridoxal phosphate, the coenzymatically active form of Vit. B_6, can be estimated in leukocytes and plasma using a modification of the manometric method of Umbreit *et al.*(1). Previously, this has been done in the blood of normal (2,3,4) and pregnant subjects(3,4) and in the cord blood of new born babies(3,4). In addition, load tests were performed in normal controls and in pregnant women with measurement of the B_6 content of plasma and leukocytes following oral administration of pyridoxine hydrochloride(4). The present communication deals with estimation of pyridoxal phosphate in the blood and plasma of patients with chronic and acute leukemia and various other diseases.

Method. For simultaneous estimation of pyridoxal phosphate in leukocytes and plasma, 5 ml of venous blood were collected in a tube containing about 0.002 g of sodium versenate. The assay method is based on coenzyme function of pyridoxal phosphate in a tyrosine decarboxylase system according to Boxer *et al.*(2) with minor changes as previously described(4). Streptococcus fecalis was obtained from the Dept. of Bacteriology, Rutgers Univ., New Brunswick, N. J. Leukocytes were isolated using freeze-dried phytohemagglutinin prepared from navy beans. The phytohemagglutinin does not contain any measurable B_6. The samples were usually

* Supported by Vitamin Fn.

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α-HYDROXYSULFONATES AS INHIBITORS OF THE ENZYMATIC OXIDATION OF GLYCOLIC AND LACTIC ACIDS

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The action of glycolic acid oxidase and glyoxylic acid reductase, two enzymes which have been isolated from green leaves in a highly purified form, has recently been investigated (1, 2). It was demonstrated that these two enzymes could function together in crude homogenates of leaves and in a model system to transfer electrons from DPNH[1] to oxygen by the following mechanism (1, 2):

(1) Substrate + DPN^+ → oxidized substrate + DPNH + H^+ (dehydrogenases)

(2) DPNH + $CHO.COO^-$ + H^+ → DPN^+ + $CH_2OH.COO^-$ (glyoxylic acid reductase)
Glyoxylate Glycolate

(3) Glycolate + $\frac{1}{2}O_2$ → glyoxylate + H_2O (glycolic acid oxidase, catalase)

Sum. Substrate + $\frac{1}{2}O_2$ $\xrightarrow{DPN^+ \text{ and glyoxylate or glycolate}}$ oxidized substrate + H_2O

In order to evaluate the role of such a system in the living leaf, a specific inhibitor of either Reaction 2 or 3 would be desirable. The relationship between sulfanilamide and the metabolism of *p*-aminobenzoic acid (4) suggested that α-hydroxysulfonic acids by analogy might be effective inhibitors of the action of the enzymes concerned with the metabolism of α-hydroxycarboxylic acids.[2] The bisulfite addition compounds of aldehydes and ketones have been shown to be salts of α-hydroxysulfonic acids (5, 6), and sodium hydroxymethanesulfonate ($CH_2(OH)-SO_3Na$), the bisulfite addition compound of formaldehyde, is a stable substance (7) which is analogous in structure to glycolic acid.

The present experiments show that this compound and several other α-hydroxysulfonates are effective inhibitors of the enzymatic oxidation of

[1] The following abbreviations are used: DPN^+ and DPNH for oxidized and reduced diphosphopyridine nucleotide, respectively; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride.

[2] The author is indebted to Dr. C. W. Partridge of Yale University for pointing out this possibility.

glycolate. The action of these compounds on the oxidation of L(+)-lactate, which is catalyzed by glycolic acid oxidase and by the DPN+-linked lactic dehydrogenase of rabbit muscle, has also been investigated, and the effect of these inhibitors upon glycolic acid oxidase has been compared in detail with that of sodium bisulfite. The specificity of the action of sodium hydroxymethanesulfonate as an inhibitor of glycolic acid re-

TABLE I

Comparison of α-Hydroxysulfonates As Inhibitors of Glycolic Acid Oxidase

	Inhibition of oxygen uptake					
	Glycolate as substrate at 5×10^{-4} M			L-Lactate as substrate at 1×10^{-3} M		
	Final concentration of inhibitor					
	1×10^{-4} M	2.5×10^{-4} M	5×10^{-4} M	5×10^{-4} M	7.5×10^{-4} M	1×10^{-3} M
	per cent	per cent	per cent	per cent	per cent	per cent
Sodium bisulfite	20	40	57			80
" hydroxymethanesulfonate	31	52	62	32	46	
" 1-hydroxy-1-ethanesulfonate	20		66	44	54	75
Disodium sulfoglycolate		42	67			

The Warburg flasks contained 200 μmoles of Tris-HCl, pH 8.3; riboflavin phosphate, 0.20 μmole; glycolic acid oxidase, about 2.5 γ. Potassium glycolate, 10 μmoles, or potassium L-lactate, 20 μmoles, was placed in the side arm together with the indicated inhibitor to give the final concentration shown. The volume was made to 2.0 ml. with water; potassium hydroxide in the center well; gas, air; temperature, 30°. After 10 minutes, the substrate and inhibitor were tipped in. The oxygen uptake was measured for two successive 10 minute intervals beginning 8 minutes after the additions. The uninhibited rate with glycolate was about 30 μl. per 10 minutes and that for L-lactate about 17 μl. per 10 minutes.

ductase, D-glyceric acid dehydrogenase, and malic dehydrogenase has also been studied.

Effect of α-Hydroxysulfonates and Sodium Bisulfite on Glycolic Acid Oxidase Reaction

The action of the bisulfite addition compounds of three aldehydes and of sodium bisulfite on the initial rate of oxygen uptake with glycolate or L-lactate as substrates is compared in Table I. With glycolate, it was found that sodium hydroxymethanesulfonate, sodium 1-hydroxy-1-ethanesulfonate (acetaldehyde-bisulfite addition compound), and disodium sulfoglycolate (sodium glyoxylate-bisulfite addition compound) were nearly

equally effective at concentrations about 1 per cent of that of the substrate. Sodium bisulfite was slightly less inhibitory than any of the three α-hydroxysulfonates tested. Formaldehyde, at the same concentration as the inhibitors, was without effect on the rate of oxygen uptake. With L-lactate, a substrate of glycolic acid oxidase which has a higher Michaelis constant than that of glycolate (1), the inhibitors tested were even more effective than with glycolate. It would also appear that the sulfonate analogue of lactic acid, sodium 1-hydroxy-1-ethanesulfonate (CH_3-$CH(OH)$-SO_3Na), more effectively inhibits the oxidation of lactate than does the sulfonate analogue of glycolic acid, sodium hydroxymethanesulfonate.

The competitive nature of the inhibition of the oxidation of glycolate by sodium hydroxymethane-sulfonate is shown in Fig. 1. From the form of the Lineweaver and Burk equation derived by Wilson (8) it is clear that

$$\frac{v}{v_i} = 1 + \frac{K_s}{K_i}\left(\frac{[I]}{K_s + [S]}\right)$$

straight lines should result with an intercept of 1 and with a slope dependent on the substrate concentration [S] if the inhibition is competitive. From the slopes of the regression lines in Fig. 1, the dissociation constant of the enzyme-inhibitor compound, K_i, can be calculated if the Michaelis constant, K_s, is known. The Michaelis constant for glycolate is 3.8×10^{-4} M (1), and K_i can be calculated to be 1.7×10^{-5} M from Curve A and 1.9×10^{-5} M from Curve B. The relationship between these constants indicates that sodium hydroxymethanesulfonate is indeed an effective inhibitor of the oxidation of glycolate by the glycolic acid oxidase of spinach leaves.

The effect of sodium hydroxymethanesulfonate upon the oxidation of glycolic acid is compared at two concentrations in Fig. 2 and is shown to be essentially constant. The inhibitor is as effective at pH 8.3, the pH optimum for the enzyme, as at pH 7.0. In other experiments, which were carried out at pH 6.4, inhibitions of similar magnitude were observed. With 5×10^{-3} M glycolate as substrate, the initial rate of oxygen uptake was inhibited about 95 per cent by 1×10^{-2} M inhibitor. In the control experiment, there was essentially no oxygen uptake without glycolate.

The competitive inhibition observed and the low dissociation constant of sodium hydroxymethanesulfonate suggested that the inhibition was not caused by free sodium bisulfite which dissociated from the inhibitor. Moreover, although sodium bisulfite appears to inhibit well, it was a less effective inhibitor of the oxidation of glycolate than sodium hydroxymethanesulfonate (Table I). This is inconsistent with the view that free sodium bisulfite was the real inhibitor. These observations suggested that sodium bisulfite inhibits the oxidation of glycolate by interacting with the

glyoxylate produced in the reaction to form disodium sulfoglycolate ($NaO_3C{-}CH(OH){-}SO_3Na$), which is the actual inhibitor. It proved possible to verify this explanation of the observed inhibition in two independent ways.

Under the experimental conditions described in Table II, the inhibition of glycolate oxidation is essentially the same with either sodium bisulfite

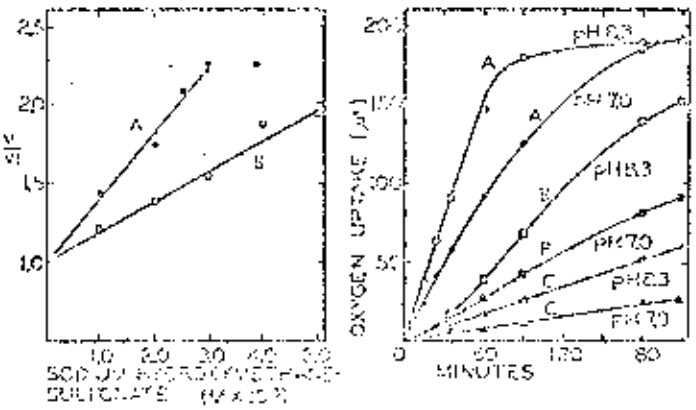

FIG. 1 FIG. 2

FIG. 1. Competitive inhibition of glycolate oxidation by sodium hydroxymethanesulfonate. Warburg vessels were prepared and oxygen uptake was measured as described in Table I. The initial rate of oxygen uptake per 10 minutes without inhibitor (v) divided by the rate in the presence of the final concentration of sodium hydroxymethanesulfonate indicated (v_i) is plotted against the final concentration of inhibitor. Curve A, glycolate concentration 5×10^{-3} M. Curve B, glycolate concentration 1×10^{-2} M.

FIG. 2. Effect of pH and time on inhibition of glycolate oxidation by sodium hydroxymethanesulfonate. The experimental conditions were essentially those described in Table I. Tris-HCl buffer at the pH indicated was used; glycolate added, 5×10^{-3} M. Curves A, no inhibitor added. Curves B, 1×10^{-4} M sodium hydroxymethanesulfonate. Curves C, 1×10^{-3} M sodium hydroxymethanesulfonate.

or disodium sulfoglycolate at an inhibitor concentration of 1×10^{-4} M. If the bisulfite concentration is lowered to 1×10^{-5} M, the inhibition diminished from 77 to only 20 per cent. When a large excess of glyoxylate, 1×10^{-2} M, is added, reaction with the free bisulfite to form sulfoglycolate occurs, but the inhibition remains the same, 81 per cent. However, if free sodium bisulfite were the inhibitor, since the addition of excess glyoxylate lowers its concentration, a marked lowering of the inhibition would be expected. Since there was no reduction in inhibition, sodium bisulfite itself cannot be the only inhibitor.

If sulfoglycolate, the product formed from glyoxylate and sodium bi-

sulfite, is the inhibitor rather than the free bisulfite which is added, it should be possible to trap the glyoxylate formed in the reaction and thus reduce the effectiveness of bisulfite. Such experiments are shown in

TABLE II

Inhibition of Oxidation of Glycolate by Sodium Bisulfite and by Disodium Sulfoglycolate in Presence of Excess Glyoxylate

	Inhibition of oxygen uptake	
	Inhibitors at 1×10^{-3} M	Inhibitors at 1×10^{-4} M
	per cent	*per cent*
Sodium bisulfite	77	20
Disodium sulfoglycolate	79	
Glyoxylate* + sodium bisulfite	81	16

* Glyoxylate was added so that its final concentration was 1×10^{-3} M. Glyoxylate added alone at this concentration has no effect on the rate of oxidation of glycolate. The Warburg vessels were prepared as in Table I, with glycolate at 5×10^{-3} M.

TABLE III

Inhibition of Oxidation of Glycolate by Sodium Bisulfite and by Disodium Sulfoglycolate in Presence of Cyanide

	Inhibition of oxygen uptake	
	Experiment 1	Experiment 2
	Inhibitors at 5×10^{-3} M; cyanide at 1×10^{-3} M	Inhibitors at 1×10^{-4} M; cyanide at 1×10^{-3} M
	per cent	*per cent*
Sodium bisulfite	45	67
Disodium sulfoglycolate	52	74
Sodium cyanide	8	1
" " + sodium bisulfite	8	33
" " + disodium sulfoglycolate	9	69

The Warburg flasks were prepared as in Table I, with a final concentration of 5×10^{-3} M glycolate. Cyanide was added to the center well of the flasks containing this component to maintain the concentration (9).

Table III. Cyanide has little effect on the glycolic acid oxidase reaction. When cyanide is added in large excess of the inhibitor (Table III, Experiment 1), both sodium bisulfite and disodium sulfoglycolate no longer inhibit the reaction. This result is consistent with the view that sulfoglycolate is the inhibitor and that it dissociates in the presence of an excess

of cyanide to form the glyoxylate cyanohydrin compound. When equal amounts of inhibitor and cyanide are added (Table III, Experiment 2), sodium bisulfite is much less effective than disodium sulfoglycolate. It seems clear that the cyanide, by reacting with the glyoxylate produced by the reaction, prevents the formation of sulfoglycolate, and the α-hydroxysulfonate must therefore be the active inhibitor of the reaction.

Effect of α-Hydroxysulfonates and Sodium Bisulfite on Reaction of Muscle Lactic Dehydrogenase

Since the α-hydroxysulfonates tested were markedly inhibitory to the oxidation of L-lactate by the glycolic acid oxidase of spinach leaves, it was of interest to determine their effectiveness on the reaction catalyzed by the DPN+-linked lactic dehydrogenase of muscle. This reaction can readily be assayed in either direction. The experiments under "Pyruvate as substrate," Table IV, show that sodium bisulfite almost completely inhibits the reduction of pyruvate, as would be expected, since it reacts with the substrate. Sodium hydroxymethanesulfonate is ineffective as an inhibitor, and sodium 1-hydroxy-1-ethanesulfonate exerts only a slight action. It is known that this last inhibitor dissociates more than sodium hydroxymethanesulfonate, which may account for its greater effectiveness. These data emphasize that little free bisulfite could be available from the dissociation of sodium hydroxyethanesulfonate.

The data under "L-Lactate as substrate," Table IV, suggest that sodium bisulfite and sodium 1-hydroxy-1-ethanesulfonate (the sulfonate analogue of lactate) competitively inhibit the oxidation of L-lactate by the DPN+-linked enzyme at low inhibitor concentrations. It should be pointed out that, at the concentrations of lactate employed, the enzyme was not saturated with substrate, and this could account in part for the greater effectiveness of these inhibitors against lactate oxidation than against pyruvate reduction. Although the mechanism of the inhibition has not been studied in detail for lactic dehydrogenase as it has for glycolic acid oxidase (Tables II and III), the inhibition of lactate oxidation by sodium bisulfite at these low concentrations of inhibitor may well arise from the formation of the sodium bisulfite addition compound of pyruvate.

Effect of Sodium Hydroxymethanesulfonate and Sodium Bisulfite on Enzymatic Reduction of Glyoxylate

In view of the observation that the oxidation of glycolate to glyoxylate is markedly inhibited by sodium hydroxymethanesulfonate, the effect of this inhibitor on the reverse reaction, the enzymatic reduction of glyoxylate to glycolate, was investigated. This reaction was studied with a crystalline preparation of glyoxylic acid reductase by measuring the rate of oxidation of DPNH at 340 mμ in the presence of glyoxylate (2). The Michaelis

constant for glyoxylate with this enzyme is 0.1×10^{-4} M. With glyoxylate at 0.01 M, essentially no inhibition of the reaction is observed with 0.04 M sodium hydroxymethanesulfonate. Under the same experimental conditions, sodium bisulfite at 0.01 M completely inhibits the reaction, and the inhibition is completely reversed when glyoxylate at 0.01 M is added after

TABLE IV

Inhibition of Lactic Dehydrogenase of Muscle by α-Hydroxysulfonates

	Per cent inhibition				Molar concentration of inhibitor to give 50 per cent inhibition	
					L-Lactate as substrate at	
	Pyruvate as substrate at 0.001 M				0.10 M	0.033 M
Final concentration of inhibitor	0.001 M	0.002 M	0.01 M	0.033 M		
Sodium hydroxymethanesulfonate	12	10	26	24	6.3×10^{-4}	
Sodium 1-hydroxy-1-ethanesulfonate	19	26	52	48	1.5×10^{-4}	7.0×10^{-5}
Sodium bisulfite	54				1.0×10^{-4}	5.0×10^{-5}

For assay with pyruvate as substrate, experimental cuvettes of 1 cm. light path contained 100 μmoles of potassium phosphate at pH 6.4, 3 μmoles of potassium pyruvate, about 4 γ of crystalline lactic dehydrogenase from rabbit muscle, inhibitor as indicated, and water to make the final volume 3.0 ml. DPNH, 0.15 μmole, was added at zero time, and readings were taken at wave length 340 mμ in the Beckman spectrophotometer for two 15 second intervals. 1 unit is defined as a decrease in optical density of 0.01 per minute at 25°. Under the conditions described, the uninhibited enzyme assayed 22.0 units; with 1 μmole of pyruvate, it assayed 29.8 units; with 6 μmoles of pyruvate, it assayed 24.4 units. For assay with L-lactate as substrate (bu), experimental cuvettes contained 100 μmoles of glycine-KOH buffer at pH 9.7, 30 μmoles or 10 μmoles of potassium L-lactate as shown, about 8 γ of lactic dehydrogenase from muscle, 1.5 μmoles of DPN+, inhibitor as indicated, and water to make the final volume 3.0 ml. The enzyme was added at zero time, and the increase in optical density at 340 mμ was measured as described above. The uninhibited enzyme assayed 12.1 units with 30 μmoles of L-lactate and 6.3 units with 10 μmoles of L-lactate.

the rate measurement is made. Analogous experiments on the inhibition of the reduction of glyoxylate by muscle lactic dehydrogenase (11) instead of glyoxylic acid reductase have given similar results.

Effect of Sodium Hydroxymethanesulfonate on Other Enzymatic Oxidations of α-Hydroxy Acids

In order to test further the specificity of sodium hydroxymethanesulfonate as an inhibitor, the effect of this compound on D-glyceric acid de-

hydrogenase (12) and malic dehydrogenase was examined. D-Glyceric acid dehydrogenase activity is associated with the crystalline glyoxylic acid reductase of tobacco leaves (2) and, accordingly, this preparation was used. Assays were carried out spectrophotometrically, by the method described in Table IV for lactic dehydrogenase, with sodium pyrophosphate buffer at pH 8.9, potassium D-glycerate, 0.9 μmole of DPN^+, and about 10 γ of glyoxylic acid reductase. At a final concentration of D-glycerate of 0.033 M, 11.2 units of enzyme were assayed, and, at a concentration of 0.017 M, 10.8 units were found. The concentration of sodium hydroxymethanesulfonate required to give 50 per cent inhibition was 9.0×10^{-4} M at 0.033 M D-glycerate and 5.7×10^{-4} M at 0.017 M D-glycerate. The sodium hydroxymethanesulfonate is considerably less effective as an inhibitor of the oxidation of D-glyceric acid than it is of the oxidation of glycolic and lactic acids. On the other hand, with sodium bisulfite, 5×10^{-4} M, and D-glycerate, 0.017 M, 84 per cent inhibition was observed. Again by analogy with the experiments cited in Tables II and III, the effect of bisulfite may be attributed to the α-hydroxysulfonate produced by the interaction with the hydroxypyruvate formed.

Malic dehydrogenase was assayed in the same manner as D-glyceric acid dehydrogenase with potassium L-malate and 0.45 μmole of DPN^+. Of the preparations of malic dehydrogenase examined, one was an ammonium sulfate fraction precipitated between 40 and 100 per cent of saturation from an extract of tobacco leaves. The other was a partially purified commercial preparation from pig heart. The results were similar with both preparations. At 0.033 M L-malate, no inhibition was observed with 0.01 M sodium hydroxymethanesulfonate. The effect of sodium bisulfite at the latter concentration is difficult to evaluate because of its non-enzymatic reaction with DPN^+ (13). With the partially purified malic dehydrogenase at 0.1 M malate, 0.01 M sodium bisulfite apparently inhibited the rate of the reaction by 77 per cent.

DISCUSSION

Because it reacts so readily with carbonyl compounds, bisulfite has frequently been used as a trapping agent in enzymatic reactions. Carbonyl-trapping reagents are required in systems that have been used for the assay of lactic dehydrogenase (14), but there is no record of the use of bisulfite for this purpose. In view of the experiments reported here, it is clear that bisulfite would have severely inhibited such systems. There are also reports that bisulfite can inhibit enzymatic reactions by interacting with the substrate (15). Inasmuch as bisulfite combines with DPN^+ under certain conditions (13), enzymatic inhibition might be observed because of this reaction.

The inhibition by bisulfite of the oxidation of glycolate by glycolic acid oxidase is brought about by the α-hydroxysulfonate formed from the glyoxylate produced. This observation suggests that the analogous inhibitions of the oxidation of L-lactate, L-malate, and D-glycerate, which were examined, arise from the same mechanism.

Sodium 1-hydroxy-1-ethanesulfonate (acetaldehyde-bisulfite) in particular is much more effective against muscle lactic dehydrogenase when the reaction is measured from the side of L-lactate oxidation under the conditions shown in Table IV. The smaller effect of the inhibitor, when measurement is made from the pyruvate side of the reaction, may arise in part from the lower Michaelis constant for pyruvate than for L-lactate. It is also possible that the enzymatic sites are altered by the different substrates or by the conditions of assay in the two opposite directions. These inhibitors might prove useful in studies of the mechanism of action of this enzyme.

Sodium hydroxymethanesulfonate (formaldehyde-bisulfite) is stable at boiling temperature, and a 10 % solution liberates hardly any formaldehyde (7) when refluxed with sodium carbonate. As an inhibitor, the substance seems to be fairly specific in its action. It strongly inhibits the oxidation of glycolate (Reaction 3) without affecting the reduction of glyoxylate (Reaction 2). If the terminal respiratory system which involves these two reactions operates to a significant extent *in vivo*, it should be possible to devise conditions under which the oxygen uptake of intact leaf tissue is inhibited and at the same time glycolic acid accumulates.

Methods and Preparations

Sodium hydroxymethanesulfonate monohydrate was obtained as large lustrous plates (7) from the reaction of formaldehyde with sodium bisulfite. It was crystallized three times from 60 per cent ethanol. Sodium, calculated 15.1; found 15.8 per cent. Sodium 1-hydroxy-1-ethanesulfonate was prepared in a similar manner from acetaldehyde. Disodium sulfoglycolate was prepared as described by Weinhouse and Friedmann (16). Sodium glyoxylate monohydrate was prepared by the method of Uadin and Metzler (17), and potassium pyruvate was made by the method of Kirkes *et al.* (18). DPNH was prepared with dithionite (10). A preparation of glycolic acid oxidase from spinach leaves of about 25 per cent purity obtained by a modified procedure (1) was a gift of N. A. Frigerio of Yale University. Crystalline lactic dehydrogenase of rabbit muscle (20) was provided by Dr. E. Racker.

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SUMMARY

Sodium hydroxymethanesulfonate (formaldehyde-bisulfite) competitively inhibits the oxidation of glycolate by glycolic acid oxidase of spinach leaves. The inhibition is effective at concentrations of inhibitor 1 per cent of that of the substrate. Other α-hydroxysulfonates such as sodium 1-hydroxy-1-ethanesulfonate (acetaldehyde-bisulfite) and disodium sulfoglycolate (glyoxylate-bisulfite), and sodium bisulfite itself, are about equal in effectiveness in the inhibition of the oxidation of glycolate. The inhibition brought about by sodium bisulfite arises from the α-hydroxysulfonate formed from the glyoxylate that is produced in the reaction.

The α-hydroxysulfonates studied effectively inhibit the enzymatic oxidation of L(+)-lactate by glycolic acid oxidase and by muscle lactic dehydrogenase, but are less effective in inhibiting the oxidation of D-glycerate and are ineffective against the oxidation of L-malate. Sodium bisulfite inhibits all of these reactions probably through the formation of α-hydroxysulfonates with the products. Sodium hydroxymethanesulfonate is not an effective inhibitor of the enzymatic reduction of glyoxylate by glyoxylic acid reductase of tobacco leaves or of the enzymatic reduction of pyruvate by muscle lactic dehydrogenase.

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poate determina pentru o arie găsită, cantitatea corespunzătoare de component. Curbele de etalonare sînt prezentate în figura 4.

Pentru a stabili eroarea metodei s-au preparat probe de concentrații cunoscute, conținînd trei componenți, care au fost cromatografiate. Rezultatele obținute au fost calculate prin interpolări pe curbele de etalonare.

Tabela 1 cuprinde cantitățile de componenți luate în probă, rezultatele obținute și eroarea procentuală relativă stabilită față de cantitatea inițială.

Tabela 1

Substanța	Mg introduse în probă	Mg determinate în probă	Eroare, %
Benzen	245	246	+0,40
Toluen	287	284	—1,04
Etilbenzen	102	100	—1,96
Stiren	165	168	—1,81
Izopropilbenzen	153	156	—1,93
α-Metilstiren	180	180	—

Autorii lucrării au verificat metoda la Combinatul de Cauciuc sintetic din Orașul Gheorghe Gheorghiu-Dej. Probele au fost recoltate în paralel prin linii de recoltare și prin soluții absorbante pentru metoda autorilor. Rezultatele comparative obținute, sînt redate în tabela 2. Figura 5 reprezintă cromatograma unei probe recoltate la instalația de stiren de la Combinatul de Cauciuc Sintetic Orașul Gheorghe Gheorghiu-Dej.

Tabela 2

Substanța determinată	Determinări prin cromatografiere, mg/m³ aer	Determinări prin metoda, mg/m³ aer
Benzen	129	115
Toluen	155	138
Etilbenzen	218	212
Stiren	174	170

Concluzii

Față de metodele existente descrise în literatura de specialitate [3, 4, 5, 6, 7, 8, 9, 10], metoda din această lucrare are următoarele avantaje:

— folosește un aparat existent în țară — cromatograf Pye Argon cu o anexă originală de adaptare a tubului de recoltare cu probă;

— este o metodă rapidă — recoltarea și determinarea durează circa 20 min;

— reproductibilitatea este bună;

— limitele de eroare se încadrează în limitele permise de aparat, 10% în valoare relativă;

— sensibilitatea metodei se situează în jurul a 0,1 mg la m³ aer;

— metoda permite determinarea unui amestec de mai mulți componenți existenți la un moment dat în atmosferă printr-o singură recoltare într-un tub;

— tuburile de recoltare pot fi folosite în mai multe recoltări, fără schimbarea umplutului;

— pentru obținerea unei diagrame cu vîrfuri suficient de mari, pentru a evita erorile, aparatul permite lucrul la sensibilități diferite, adică pentru concentrații foarte mici se poate lucra la o sensibilitate de 10 ori mai mare față de componenții normali.

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SCURTE COMUNICĂRI

P. Gh. Zugrăvescu și M. A. Zugrăvescu: Metodă rapidă pentru determinarea bioxidului de sulf

Stabilirea conținutului de bioxid de sulf într-o serie de produse conservate și semiconservate ca mărunțiș, sucuri, conserve, reprezintă o condiție esențială din punct de vedere calitativ al acestor produse. Datorită condițiilor de depozitare, a cantităților mari precum și a lipsei personalului calificat, dozarea bioxidului de sulf nu poate fi făcută prin metodele chimice titrimetrice cunoscute, care necesită pe lîngă calificare deosebită, și o trusă de sticlărie și reactivi care îngreunează lucrul «la teren», fără a mai lua în considerare timpul de lucru pentru o determinare, care depășește cu mult, în acest caz, cerințele unei metode rapide. Ținînd seama de aceste cerințe, metoda propusă constă în realizarea unor hîrtii indicatoare care, utilizate în mod similar cu hîrtiile indicatoare pentru determinarea pH-ului soluțiilor, sînt sensibile față de bioxidul de sulf, în prezența căruia se obține o colorație care permite determinarea lui semicantitativă.

Principiul metodei se bazează pe reacția de reducere a iodatului de către iodură, în prezența ionilor H^+

$$IO_3^- + 5I^- + 6H^+ \rightleftharpoons 3\,I_2 + 3H_2O \quad (1)$$

Pentru ca reacția să nu se producă decît în prezența H_2SO_4, amestecului iodat-iodură I se adaugă soluție de $Ba(OH)_2$ care menține pH-ul soluției în domeniul alcalin. Hîrtia de filtru impregnată cu această soluție în care s-a dizolvat și amidon, se introduce în soluția probei cu SO_2. Prezența ionilor H^+ datorită acidului sulfuros, permite declanșarea reacției (1), apoi are loc reacția:

$$SO_3^{2-} + I_2 + H_2O \rightleftharpoons SO_4^{2-} + 2\,HI \quad (2)$$

care determină colorația amidonului (albastru-brun-maron)

Modul de lucru. Se cîntărește 0,38 g $Ba(OH)_2 \cdot 8H_2O$ peste care se adaugă 72 cm³ apă distilată fără bioxid de carbon. Dacă se formează totuși carbonat de bariu, acesta se îndepărtează prin decantare. În 10 cm³ soluție obținută, se dizolvă 1 g amidon solubil, iar în restul soluției se dizolvă 1 g iodat de potasiu și 2 g iodură de potasiu. După dizolvare, se adaugă soluția de amidon, se agită bine și se lăsăzește cîteva minute.

Se lasă să se răcească la temperatura camerei și se adaugă 30 cm³ glicerină pură. Se agită pînă la omogenizare.

Se taie fîşii de hîrtie de filtru (calitativă, cu bandă neagră sau cu bandă albă, sau hîrtie de filtru pentru cromatografie Whatman nr. 1) de dimensiuni 1 cm × × 10 cm şi se impregnează capetele acestora lăsîndu-le scufundate în soluţie 30 s. După aceea se usucă pe hîrtie de filtru. Astfel impregnate, hîrtiile se pot utiliza aproximativ 7 zile.

Rezultate şi discuţii

Pentru aprecierea cantitativă a determinărilor, este necesară compararea intensităţii de colorare a hîrtiei indicator, cu un etalon de nuanţe care să exprime concentraţia în funcţie de intensitate. Cea mai mică concentraţie de SO_2 care poate colora hîrtia indicator, este de 5 p.p.m. Peste concentraţii mai mari de 1000 p.p.m. intensitatea de colorare este maximă şi nu se mai pot sesiza diferenţele.
Pentru acest interval de concentraţii se stabileşte etalonul de nuanţe cu ajutorul căruia se apreciază vizual concentraţia SO_2 în soluţie. Soluţiile concentrate se diluează corespunzător pentru a face posibilă determinarea. Se recomandă ca diluarea să se facă de un număr întreg de ori, de 2,3... ori, pentru ca rezultatul să se poată aprecia uşor.
Metoda se poate aplica şi la determinarea SO_2 în gaze. În acest caz, SO_2 dintr-un anumit volum se absoarbe într-o soluţie potrivită (sodă 10%; o parte K_2CO_3 + + o parte apă + două părţi glicerină; soluţie KCl acidulată s.a.) sau se trece direct peste hîrtia indicator, un volum cunoscut de gaz.
Pe lîngă rapiditatea maximă această metodă mai prezintă avantajul utilizării unor reactivi uzuali, uşor de păstrat şi manipulat, putînd fi folosită de personal fără calificare deosebită.

Ileana Weisemann, A. Butucelea şi R. Nistor*: **Determinarea spectrală a purităţii zincului**

Necesitatea unui control rapid al purităţii zincului utilizat în diferite ştiinţe a dus la experimentarea unei metode spectrografice. S-a măsurat raportul dintre intensitatea unei linii a elementului analizat (Cu, Fe, Sn, Pb sau Cd) şi intensitatea elementului de bază (Zn) şi, pe baza unor curbe de bază trasate în prealabil pentru fiecare din elementele de analizat, s-au determinat concentraţiile acestor elemente. Ca linii analitice au fost alese anumite linii sensibile care apar la excitarea probei în arc alternativ. S-a căutat un regim de lucru în care curentul de descărcare să nu depăşească 3 A, pentru a reduce la minimum interferenţele dintre elemente; de asemenea, un studiu al condiţiilor de excitare cele mai potrivite pentru a asigura concomitent stabilitatea, reproductibilitatea, sensibilitatea şi rapiditatea maxime, a dus la alegerea pentru generatorul de arc (de tip Durs) a unui regim de lucru de 1 500 cm capacitate şi 320 μH inductanţă.
Pentru analiză s-au folosit un spectrograf Jobin & Yvon tip Nouvelle Zélande (dispersie liniară 5 Å/mm în regiunea 2 500 Å), echipat de firma «Cameca» (Franţa) cu înregistrare cu fotomultiplicatori a intensităţilor liniilor, programare a condiţiilor analizei şi înscriere automată a raportului de intensităţi (Spectrolecteur SL-15). În acest montaj, un fotomultiplicator (I) rămîne în permanenţă fixat asupra unei linii a elementului major (Zn 4 680,11), iar celălalt (X) trece succesiv, după un program prestabilit, peste liniile analitice ale elementelor de determinat (tabela 1), oprindu-se asupra fiecăreia din ele timp de 17 s; din aceste 17 s, numai 13 s sînt utilizate efectiv pentru înregistrarea şi integrarea intensităţii liniei, 1 s este rezervată pentru eliminarea unor semnale parazite, iar 3 s sînt destinate înregistrării raportului dintre intensităţile integrate X/T. Înainte de începerea analizei este asigurat un timp de preardere de 5 s. Un raport al tensiunilor aplicate la diodele fotomultiplicatorilor X/T = 10/2, împreună cu sensibilităţile la amplificare indicate în penultima coloană a tabelei 1 pentru fiecare linie folosită, s-au dovedit adecvate pentru a cuprinde în scala potenţiometrului înregistrator domeniile de concentraţii cele mai probabile de a fi întîlnite în eşantioane, şi anume cele indicate în ultima coloană. Panta de intrare a spectrografului a fost fixată la 25 μ.
Atît eşantioanele utilizate la etalonare, cît şi celor care se analizează, li se execută la strung o faţă plană. Ca electrod se foloseşte un baston de grafit (Péchiney, 6 mm ⌀) tăiat conic (60° la vîrf). La începutul analizei, entroda a fost fixată la 2 mm.
Pentru etalonare s-a folosit un set de 5 eşantioane-etalon în formă de disc cu diametrul de 6 cm, provenind de la firma «Zinc et Alliages» (Franţa), al căror conţinut în Cu, Fe, Sn, Pb şi Cd acoperă întreg domeniul, acestea avînd certificate de analiză chimică provenite de la diferite laboratoare. Fiecare eşantion a fost înregistrat de 6—7 ori, în diferite regiuni, pentru a elimina eventuale efecte ale neomogenităţii. Curbele de etalonare obţinute sînt cele din figura 1, unde pe abscisă s-a înregistrat raportul (X/T) · 100 al intensităţii liniei elementului analizat către intensitatea liniei elementului de bază, iar pe ordonată — concentraţia elementului determinat, în p.p.m.
Se menţionează că abaterea pătratică medie relativă a înregistrărilor este de numai 1...3%, ea fiind, însă ceva mai ridicată pentru valori X/T extreme. Durata unei analize nu depăşeşte 10 min de la sosirea probei în laborator.

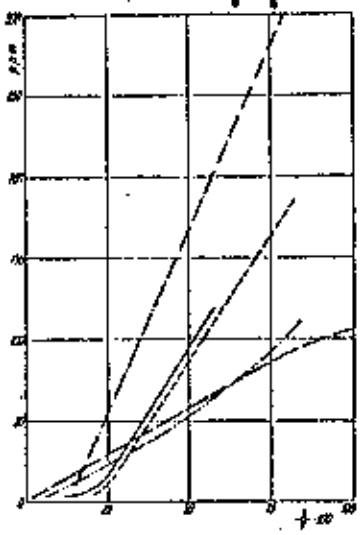

Fig. 1. Curbele de etalonare: — · — · Pb; — — — Fe; —— Cu; — · — · — · Cd; ———— Sn.

Tabela 1

LINIILE ANALITICE UTILIZATE, CONDIŢIILE DE AMPLIFICARE ŞI SENSIBILITATEA METODEI

Elementul	Linia analitică utilizată (Å)	Intensitatea relativă la arc	Energia de excitare a liniei (eV)	Sensibilitatea la amplificare	Domeniul de concentraţii de determinat (p.p.m.)
		ar (I)			
Zn	4 680,14	300	6,7	3	—
Cu	3 247,54	5 000	3,8	3	2...110
Fe	3 020,64	1 000	4,1	6	6...185
Sn	2 839,99	300	4,8	6	3...110
Pb	2 833,07	500	4,4	6	14...250
Cd	2 288,02	1 500	5,4	6	2...105

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0277A)

VLIV KYSLIČNÍKU SIŘIČITÉHO NA DEKARBOXYLACI V ERYTROCYTECH A NA OBSAH REDUKOVANÉHO GLUTATHIONU V ORGÁNECH A KRVI KRÁLÍKŮ

Влияние сернистого газа на декарбоксилирование в эритроцитах и на содержание восстановленного глютатиона в органах и в крови кроликов

Effect of Sulphur Dioxide upon Decarboxylation in Erythrocytes and upon the Content of Reduced Glutathione in the Organs and Blood of Rabbits

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Выводы: Эксперименты проводились на двумя группами кроликов — подопытной и контрольной. Подопытным животным вводилась внутривенно однократная доза раствора, содержащего 5 мг сернистого газа. Через 1 час животные были обезглавлены. Одна часть крови была использована для определения декарбоксилирования, расхода кислорода в эритроцитах манометрическим методом в присутствии 0,3 м цистеина в качестве стимулятора дыхания. Установлено, что сернистый газ снижает декарбоксилирование в эритроцитах приблизительно на 16 % и расход кислорода на 18 % при инкубации, продолжающейся 1 час при температуре 37° С. Вторая часть крови, печень, почки, сердце, селезенка и надпочечники были использованы для определения восстановленного глютатиона глиоксалевым методом. Установлено, что при однократном введении сернистый газ снижает содержание глютатиона в печени и в легких на 50 %, в почках на 40 %, в то время как в крови, в селезенке и в надпочечниках содержание глютатиона в общем не меняется.

Čs. Hyg., 13, 1968, 1: 23—26.

Summary: The experiments were done in two groups of rabbits — experimental and control. The experimental animals were given a single intravenous dose of a solution containing 5 mg sulphur dioxide. The animals were decapitated one hour later. One part of the blood was used to determine decarboxylation and, by the manometric method, oxygen consumption of erythrocytes in the presence of 0,3 M cystein as stimulator of respiration. It was found that sulphur dioxide decreased decarboxylation in erythrocytes by about 16% and oxygen consumption by 18% at one hour incubation at 37 °C. The second part of the blood and the liver, kidney, heart, spleen and adrenals were used to determine reduced glutathione by the glyoxal method. It was found that suplphur dioxide in the single dose decreases the glutathione content in the liver and lungs by 50%, in the kidneys by 40%, but does not influence glutathione content in blood, spleen and adrenals.

Čs. Hyg., 13, 1968, 1: 23—26.

Naše dřívější výzkumy o vlivu kysličníku siřičitého na dekarboxylaci hovězích erytrocytů „in vitro" ukázaly, že kysličník siřičitý, v závislosti na koncentraci, zákonitě potlačuje tento proces.[3] Bylo nutno zjistit, bude-li tato zákonitost pozorována také v živém organismu při jednorázovém zavedení kysličníku siřičitého. Poněvadž dekarboxylace v erytrocytech je spojena pouze s oxidačně re-

dukčními procesy oxidace glukózy v pentozovém cyklu[7], bylo našim úkolem zjistit, má-li kysličník siřičitý vliv na systém glutathion-NAD-P a na změny hladiny glutathionu v krvi a některých orgánech. Předtím jsme zjistili, že dlouhotrvající chronické působení kysličníku siřičitého na organismus pracujících, zaměstnaných v tavírně mědi v městě Pirdop, značně snižuje obsah celkového a redukovaného glutathionu v krvi[1].

Provedení pokusu

Pokusy byly prováděny na dvou skupinách králíků asi dvouklíbových (v každé skupině 4 králíků). Králíkům pokusné skupiny byla jednorázově intravenózně vpíchnuta injekce 2 ml fyziologického roztoku obsahujícího 5 mg kysličníku siřičitého. Za hodinu byla pokusná zvířata zabita dekapitací. Všechna krev byla odebírána do nádobky, ve které byl šťavelan draselný. Jedné části krve bylo použito pro stanovení spotřeby kyslíku a dekarboxylace erytrocytů, druhé části pro stanovení redukovaného glutathionu. Kromě toho se redukovaný glutathion stanovil také v játrech, plicích, ledvinách, slezině a nadledvinkách.

Spotřeba kyslíku a dekarboxylace byla studována na erytrocytech, získaných patnáctiminutovou centrifugací krve při 3000 ot./min. a následným odstraněním plazmy z povrchové vrstvy buněk obsahující leukocyty. Inkubace byla prováděna v izotonickém ústojném roztoku podle Koji (3), který měl toto složení: 0,154 M . NaCl — 3 díly, 0,154 M . KCl — 4 díly, 0,154 M . $MgSO_4$ — 1 díl a fosfátový pufr podle Sörensena, pH 7,4 — 2 díly.

Jako stimulátoru dýchání se používalo 0,3 M roztoku cysteinu[4]. Údaje manometru se odečítaly na Varburgově přístroji při 37 °C každých 15 minut během jedné hodiny.

Pro stanovení mikrolitrů kysličníku uhličitého, izolovaného erytrocyty, se do každé Varburgovy nádobky dal: 1 ml erytrocytové suspenze, 0,1 ml 0,3 M glukózy (190 mg%), 0,2 ml 0,3 M cysteinu (do postranního ramene) a 1,7 ml inkubačního roztoku.

Pro stanovení mikrolitrů kyslíku, absorbovaného krví, se do každé Varburgovy nádobky dal: 1 ml erytrocytové suspenze, 0,1 ml 0,3 M glukózy, 0,2 ml 0,3 M cysteinu (do postranního ramene), 1,5 ml inkubační směsi a 0,2 ml 20% roztoku hydroxidu draselného pro absorpci kysličníku uhličitého.

Množství produkovaného kysličníku uhličitého a kyslíku absorbovaného erytrocyty se stanovilo přímou Varburgovou metodou podle výpočtů popsaných Umbrejtem[5].

Redukovaný glutathion byl určován v krvi a orgánech enzymovou glyoxalázovou metodou popsanou Pattersonem, Lazarowem[6]. Metylglyoxal byl získán z glycerinového aldehydu destilací z 20% kyseliny sírové. Jako enzymového materiálu bylo použito pekařských kvasnic, promývaných podle metody popsané Pattersonem a Lazarowem. Pro stanovení glutathionu z orgánů bylo odebíráno po 1 gramu s výjimkou nadledvinek, které byly použity celé, odstranila se pouze vrchní blána. Tkáně byly homogenizovány v přítomnosti 4% kyseliny sulfosalicylové. Roztok byl neutralizován 1 M roztokem uhličitanu sodného do pH 7 a filtrován přes filtr, promytý horkou vodou. Pro stanovení glutathionu bylo používáno po 0,5 ml filtrátu. Pro stanovení glutathionu v krvi byly odebírány 3 ml krve, která byla homogenizována 21 ml destilované vody, bílkoviny byly vysráženy 25% sulfosalicylovou kyselinou. Po filtraci se roztok neutralizoval 1 M roztokem uhličitanu sodného ($NaHCO_3$).

Do každé Varburgovy nádobky byl dán: 1 ml 20% kvasinkové suspenze, 0,1 ml roztok metylglyoxalu, 0,1 ml 0,2 M roztoku $NaHCO_3$ a 0,5 ml zkoumaného roztoku nebo standardního roztoku glutathionu do postranního ramene. Pro stanovení slepé zkoušky se do postranního ramene dalo 0,5 ml destilované vody. Inkubace začíná patnáctiminutovým temperováním, potom

Tabulka 1

Skupina zvířat	Počet sledování	Mikrolitry vzniklého kysličníku uhličitého			
		15 min.	30 min.	45 min.	60 min.
Kontrolní	10	56,82 ± 3,40	73,65 ± 3,17	93,16 ± 3,92	106,20 ± 4,80
Pokusná	10	52,60 ± 4,80	66,00 ± 7,16	82,40 ± 4,81	89,50 ± 4,30

se roztok přelévá z postranního ramene do nádobky hlavní, za 4 minuty se kohouty uzavřou a každých 5 minut se na manometru odečítají údaje tvorby kysličníku uhličitého, který odpovídá vzniklé kyselině mléčné v důsledku glyoxylové reakce.

Experimentální údaje

Na tabulce 1 a 2 jsou uvedeny hodnoty pro dekarboxylaci a spotřebu kyslíku erytrocyty u kontrolní skupiny a u králíků, jimž byl aplikován fyziologický roztok obsahující 5 mg kysličníku siřičitého.

Tabulka 2

Skupina zvířat	Počet sledování	Mikrolitry spotřebovaného kyslíku			
		15 min.	30 min.	45 min.	60 min.
Kontrolní	10	26,17 ± 2,96	50,70 ± 3,87	80,40 ± 7,21	103,50 ± 10,2
Pokusná	10	12,20 ± 2,56	25,60 ± 3,20	52,00 ± 3,52	85,70 ± 6,85

Diskuse výsledků

Jak je vidět z tabulky 1, je dekarboxylace v erytrocytech u pokusné skupiny králíků při jednorázovém zavedení kysličníku siřičitého značně snížena, což je patrné už po 30 minutách inkubace. Rozdíl v hodnotách mezi kontrolní a pokusnou skupinou vzrůstá během inkubace. Podobná zákonitost je pozorována také při absorpci kyslíku erytrocyty s tím rozdílem, že v začátku inkubace je potlačení spotřeby kyslíku výraznější a během doby se procentuálně snižuje (tab. 2). Protože kyslíková spotřeba erytrocytů je celkovým ukazatelem jejich oxidace, vztahujícím se nejenom k procesům oxidace glukózy v pentozovém cyklu, nebudeme se jí zabývat v závěrech.

Tab. 3. Hodnoty glutathionu v mg% v orgánech a krvi králíků: I. injikovaných roztokem obsahujícím 5 mg kysličníku siřičitého a II. kontrolní skupiny

Skupina zvířat	Játra	Ledviny	Plíce	Slezina	Nadledvinky	Krev
II.	133,1 ± 5,34	116,1 ± 18,11	111,3 ± 7,28	119,9 ± 18,14	183,7 ± 38,21	84,9 ± 4,69
I.	70,9 ± 9,64	73,7 ± 13,93	59,2 ± 8,24	137,6 ± 35,67	203,0 ± 32,23	74,5 ± 10,25

Je-li inhibiční účinek kysličníku siřičitého na dekarboxylaci v erytrocytech spojen s potlačením oxidačně redukční rovnováhy systému glutathion-NAD-P, má kysličník siřičitý vliv na hladinu redukovaného glutathionu v organismu. Naše pokusy vyjádřené v tabulce 3 ukazují, že jednorázové zavedení kysličníku siřičitého značně sníží hladinu redukovaného glutathionu v některých orgánech. V játrech a plicích pokusné skupiny se hladina glutathionu sníží o 50 %, v ledvinách asi o 40 % ve srovnání s kontrolní skupinou králíků. Ve slezině, nadledvinkách a krvi se hladina glutathionu celkem nemění, tj. získané rozdíly nejsou statisticky významné. Předpokládáme-li, že kysličník siřičitý se slučuje s glutathionem, přičemž tvoří S-sulfoglutathion, můžeme objasnit snížení gluta-

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thionu v játrech, plicích a ledvinách, orgánech, které hrají významnou detoxikační úlohu. Udržení určité hladiny glutathionu ve slezině, nadledvinkách a krvi může být spojeno s jeho mimořádnou úlohou v těchto orgánech a uskuteční se díky rychlé syntéze glutathionu nebo extrakcí z ostatních orgánů.

Souhrn

1. Při jednorázovém nitrožilním podání fyziologického roztoku, obsahujícího 5 mg kysličníku siřičitého králíkům bylo zjištěno snížení dekarboxylace v erytrocytech asi o 16 % a absorpce kysliku v erytrocytech asi o 18 % po jednohodinové inkubaci.

2. Při jednorázovém nitrožilním zavedení izotonického roztoku obsahujícího 5 mg kysličníku siřičitého bylo za hodinu u dekapitovaných králíků zjištěno snížení redukovaného glutathionu v játrech a plicích o 50 % a v ledvinách o 40 %, ale v krvi, slezině a nadledvinkách nebyly zjištěny statisticky významné změny v obsahu redukovaného glutathionu.

Přeložila B. Dadourková, Ústav hygieny, Praha 10, Šrobárova 48

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Léčebně preventivní péče

Komenář k vyhlášce 42/1966 Sb. a souvisejícím předpisům

MUDr. J. Boukal a kolektiv

Práce vychází ze základních myšlenek zákona o péči o zdraví a čerpá ze zkušeností a uplatňováním starého léčebného řádu. Je zaměřena na aktivní charakter léčebně preventivní péče a její preventivní zaměření, všímá si nejen nároků občanů na léčebně preventivní péči, ale podrobně rozvádí ustanovení o součinnosti občanů při prevenci a dispenzarizaci a ustanovení o jejich povinnostech pečovat o zdraví. Dále jsou vyloženy návaznosti s ostatními prováděcími předpisy k zákonu a také zcela nová ustanovení. Vhodným doplňkem publikace jsou 4 schémata, ukazující organizační strukturu zdravotnictví u nás.

Aktuality MZd, sv. 164 — stran 176, vyobrazení 4, kartonované 10 Kčs

Poultry Science, 30 1951 470A)

Chemistry of Shell Egg Deterioration: Effect of Reducing Agents

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INTRODUCTION

THIS report is concerned with storage deterioration of eggs induced by exposure of fresh eggs to chemical reducing agents. Apparently few investigators have employed this method for reaching conclusions as to possible mechanisms by which deteriorations occur. Almost 50 years ago, Pollacci (1904) in Pavia, Italy, during studies of discoloration of egg yolk and white, observed that hydrogen sulfide caused thinning of white. Our interest was stimulated by a doctoral thesis (Hoover, 1940), representing earlier work conducted in this Bureau, in which it was reported that cysteine and thioglycolic acid caused thick egg white to become thin. Hoover suggested that the thinning might be due to reduction of the S—S bonds of the egg white protein, ovomucin.

Hoover's observations have been confirmed and, in addition, we have shown that other reducing agents cause thinning of egg white and that all of these reducing agents cause the yolk membrane to weaken. Thus, the over-all effect of chemical reduction is a simulation of natural, nonmicrobial egg breakdown. Data are reported on broken-out whole eggs, egg yolk, egg white, crude preparations of ovomucin, and on shell eggs that were exposed to reducing agents in gaseous form.

[1] Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

MATERIALS AND METHODS

Fresh eggs were obtained from a local wholesale distributor. Homogeneous samples of egg white were prepared by mixing fresh whites in a blender as needed (MacDonnell *et al.*, 1950).

Relative viscosity measurements were made at 25°C. with an Ostwald pipette calibrated against water.

Crude ovomucin was prepared from egg white from which the major part of the lysozyme had been crystallized by the method of Alderton and Fevold (1946). The lysozyme-free filtrate was adjusted to pH 4.2–4.5 and diluted with 10–15 volumes of water to precipitate the ovomucin, which flocculates and rapidly settles. The precipitated ovomucin can be reconstituted to form a clear, very viscous solution at slightly alkaline pH values. As precipitated by this technique, it comprises about 2 percent of the total nitrogen of the egg white.

White and yolk indexes were measured according to Sharp (1929), Heiman and Carver (1936), and Gibbons (1947), and percentage of thick white was estimated according to Lorenz and Almquist (1934).

Nitrogen values were determined by the micro-Kjeldahl technique with a mercury catalyst.

RESULTS

Effect of Reducing Agents on a Smooth (Blended) Mixture of Thick and Thin Egg Whites: The addition of 156 parts per million of thioglycol causes the relative vis-

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Table 1.—*Effect of dilute thioglycol on the viscosity of blended egg white*

Thioglycol conc.[a]		Relative viscosity at various times[b]			
Molarity $\times 10^{-3}$	p.p.m.	0	10 min.	100 min.	19 hr.
0	0	4.52	—	—	4.35
4	3	(4.52)	4.21	3.61	4.41
20	16	(4.52)	3.79	3.24	3.33
100	78	(4.52)	3.72	2.85	2.85
200	156	(4.52)	3.06	2.65	2.63

[a] The thioglycol was added to 100 ml. of blended egg white and the samples allowed to stand at room temperature (22–25°C) for the time indicated.
[b] Viscosity determinations were made with Ostwald pipettes calibrated against water at 25°C.

cosity of blended egg white to decrease from 4.52 to 3.06 in 10 minutes (Table 1). After 19 hours the viscosity had decreased to 2.63. As little as 3 parts per million of thioglycol caused a significant decrease in viscosity, but in 19 hours it had decreased to only 3.49, whereas the control sample had changed from 4.52 to 4.35 (Table 1). These effects for another sample of egg white are shown graphically in Figure 1. Sodium bisulfite also markedly affects the viscosity of blended egg white, but it appears to require about twenty times as much sulfite as thioglycol to produce the same change in viscosity and the rate of decrease with sulfite is much lower. Other chemical reducing substances also cause viscosity decreases, although none was as effective as thioglycol. Sodium cyanide, sodium sulfite, and sodium hyposulfite were effective. The sodium hyposulfite, in addition to causing viscosity reduction, bleached the egg white, presumably by reduction of riboflavin or other pigments present therein. Sodium thiosulfate had no effect on egg white.

The Effect of Thioglycol on Isolated Ovomucin (Crude): As shown in Table 2, thioglycol markedly reduces the viscosity of isolated ovomucin which, of course, is reputedly the protein responsible for the gel-like structure of thick egg white (Almquist and Lorenz, 1932). As was the case with egg white, the viscosity decreased sharply on addition of reducing

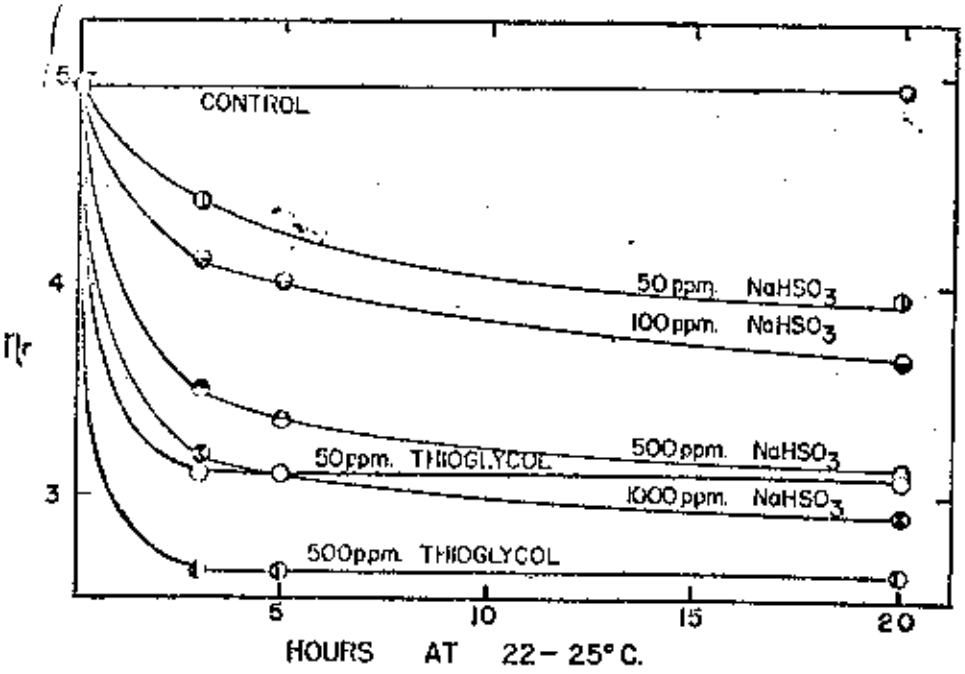

Fig. 1. Viscosity decreases of blended egg white caused by thioglycol and $NaHSO_3$.

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agent. The decrease in viscosity was somewhat greater with increase in amount of reducing agent. To facilitate the comparison, the concentration of ovomucin used was selected to be equivalent approximately to the concentration of ovomucin in egg white. Evidence that ovomucin was being affected by the reducing agent when whole egg white was treated was obtained from the observation that the character of the crude ovomucin precipitate differed when prepared from natural egg white and from thioglycol-treated egg white (Hoover, 1940). The crude ovomucin (a complex containing lysozyme and other constituents) precipitated by diluting the white with three volumes of water (Balls and Hoover, 1940) appeared as a rapidly settling, heavy, and fibrous precipitate in the case of untreated white, while that precipitated from chemically reduced egg white appeared as a fine precipitate, which could only be separated by long-standing or high-speed centrifugation. This changed precipitability was not unlike the change in precipitability caused by high shear homogenization of egg white (MacDonnell *et al.*, 1950).

Qualitative Observations on Egg Yolk and Unblended White: When the white of a broken-out egg was treated with dilute thioglycol, the thick white rapidly disappeared and resembled thin white, and the thin white already present decreased slightly in viscosity. Chalazae separated from the egg weakened in dilute alkaline thioglycol and largely disintegrated after several hours' exposure with only mild handling. The yolk membrane weakened when treated with thioglycol. A few drops of 0.5*M* alkaline (pH 8.8) thioglycol dropped on a separated yolk caused the yolk to rupture immediately. The surface of yolks suspended in slightly alkaline saline (pH 9–10) containing 50 ppm. thioglycol underwent a rapid and visible change. Within a few minutes, simply shaking the container gently caused the yolk to burst. Also, isolated, saline-washed yolk membranes partially dissolved almost immediately in alkaline thioglycol (0.5*M*, pH 8.8). A very sheer, web-like membrane, which was resistant to further change, remained. Immersion in the buffer alone at pH 8.8 did not cause this change. Under slightly acid conditions (pH 4–5) the yolk membranes were not visually altered by thioglycol; however, qualitatively, the membranes had less tensile strength as judged from their tendency to tear on handling.

TABLE 2.—*Viscosity decrease of solutions of crude ovomucin caused by thioglycol*[a]

Thioglycol conc.		Relative viscosity at various times		
Molarity $\times 10^{-4}$	p.p.m.	0	1 hr.	20 hr.
0	0	15.2	—	15.8
60	47	(15.2)	6.35	2.81
300	250	(15.2)	3.59	2.61
1,500	1,070	(15.2)	2.86	2.03
7,000	5,500	(15.2)	2.29	1.62

[a] Ovomucin concentration, 0.55 mg. N/ml.; pH of solutions, 9.5 ± 0.5.

Effect of Thioglycol on Whole, Broken-out Eggs: When small quantities of thioglycol were added to whole, broken-out eggs they rapidly simulated old, badly deteriorated storage eggs. The whites became thin and the yolks became flat. These experiments were performed by breaking whole eggs into 4-oz. wide-mouth jars containing dilute solutions of the thioglycol (1.0 ml. of dilute thioglycol) and allowing the bottles to stand at room temperature. After a few hours the eggs were poured on a glass plate and quality evaluations were made. Table 3 gives the yolk and white indexes and the percentage thick white for treated and control eggs. The changes in these qualities caused by a 4-hour exposure of whole egg to 30 and 300 ppm. thioglycol are

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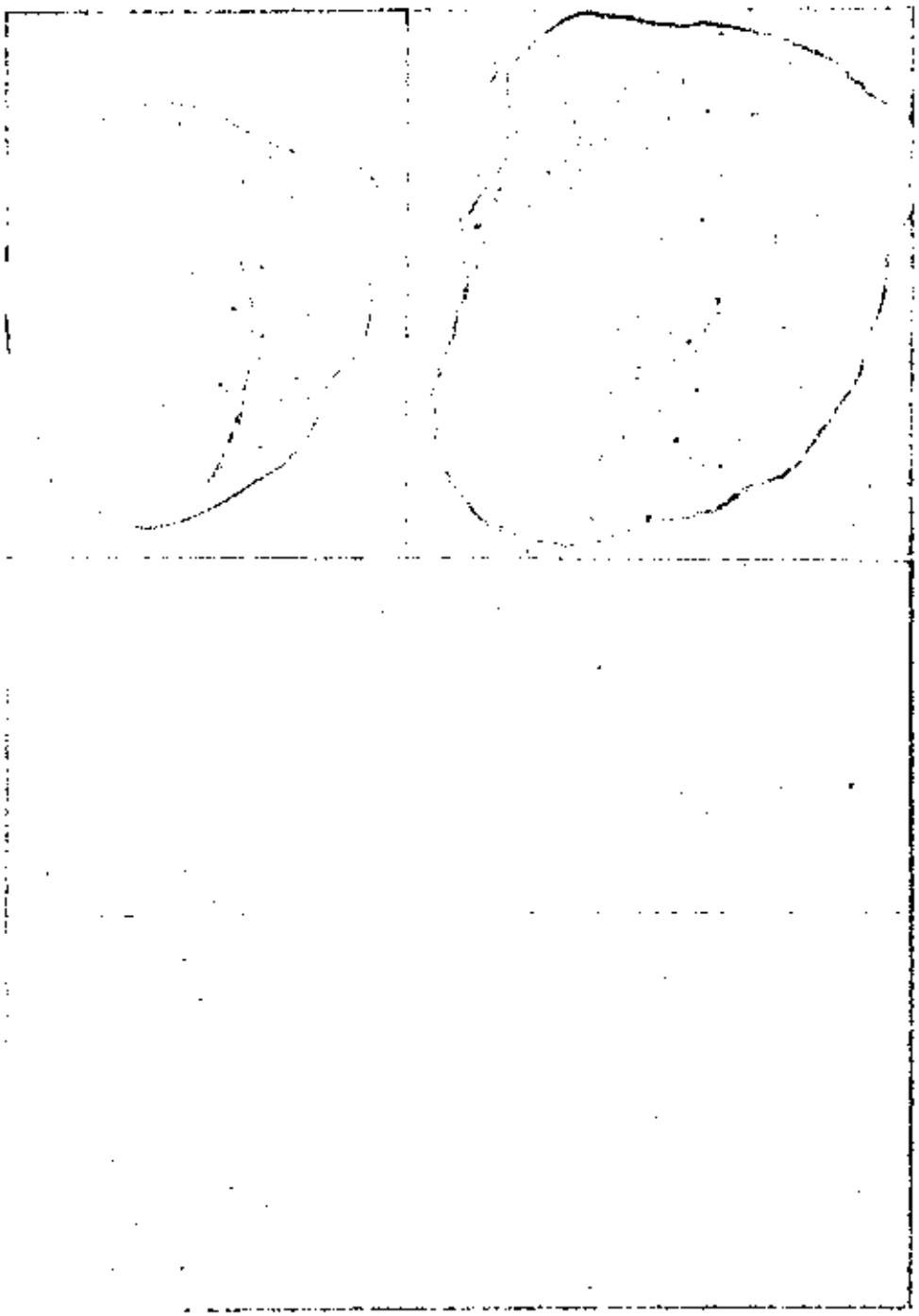

FIG. 2. Effect of adding dilute thioglycol to broken-out eggs (top view). A. Coated after 4 hours at 25°C. B. 300 p.p.m. thioglycol after 4 hours at 25°C. C. 300 p.p.m. thioglycol after 4 hours at 25°C. Essentially the same results were obtained by a 2-hour treatment with the same amounts of reducing agent.

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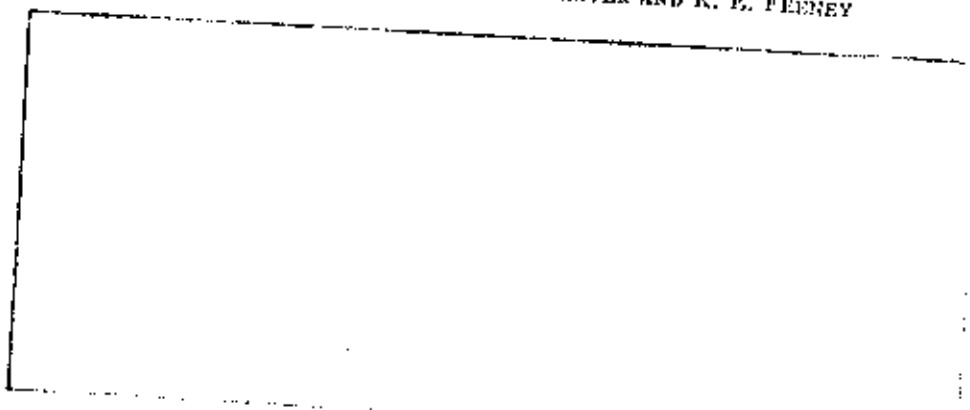

Fig. 3. Same as Figure 2 (side view).

similar for the two experiments reported. Figures 2 and 3 give top and side views of thioglycol-treated eggs.

Changes in Shell Eggs Caused by Reducing Agents Dispersed in the Air Surrounding the Eggs: Since reducing agents added to egg markedly affected their properties, it obviously became important to determine whether gaseous substances such as hydrogen sulfide can penetrate the egg shell and cause these changes when the reducing agent is present in the air in low concentration. Eggs held in desiccators which contained air plus various amounts of hydrogen sulfide or sulfur dioxide were examined for white index, percentage of thick white, and yolk index (Tables 4 and 5). Significant damage was caused when the gases were present to the extent of 0.1 percent or less and for short storage times (less than 17 days) at 2°C. (35.6°F.).

The effect of the gaseous reducing agent was largely prevented, as might be expected, when the eggs were oil-treated before they were placed in the desiccator.

Discussion

Hoover (1940) advanced the hypothesis that reducing agents thin egg white because they split the S—S bonds of mucin by reduction. The present results support this view, which it seems must be extended to include the effect of reducing agents on egg yolk, or preferably on the yolk membrane. The increased solubility and decreased viscosity accompanying reduction of the mucin is quite consistent with

Table 3.—*Effect of addition of thioglycol to broken-out eggs*[a]

Expt.	Thioglycol p.p.m.	No. eggs	Av. white index	Av. percent thick white	Av. yolk index
1	0	8	0.057	58	0.48
	50	3	<0.02	48	0.29
	300	3	<0.02	23	0.21
2	0	8	0.066	71	0.45
	50	3	0.034	49	0.31
	300	3	<0.02	27	0.26

[a] Eggs were less than 7 days old and, if refrigerated, were warmed to room temperature before breaking. Eggs were broken out into 4 oz. wide-mouthed jars containing the required amount of thioglycol. The thioglycol was in 0.1 M solution and p.p.m. were calculated on the basis of 45 ml. of egg with an assumed uniform distribution of thioglycol throughout the egg. This assumption, obviously, is not true at the start of the treatment. Samples were held at room temperature (22–25°C.) for 4 hours before evaluation.

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TABLE 4. *Deteriorations of shell eggs exposed to low concentrations of reducing gases*

Expt.	Gas exposure[a]	No. eggs	Av. white index	Av. percent thick white	Av. yolk index
1 13 days at 2°C.	Air	6	0.056	77	0.46
	H_2S, 0.02%	6	0.036[b]	65	0.43
	H_2S, 0.10%	6	<0.02[c]	35[c]	0.35[c]
2 10 days at 2°C.	Air	6	0.072	63	0.45
	H_2S, 0.05%	12	0.038[c]	56	0.37[c]
	SO_2, 0.05%	6	0.037[c]	60	0.41
	SO_2, 0.20%	6	0.031[c]	55	0.29[c]

[a] Eggs were placed in desiccators of 9.5 liters total volume. Gas was introduced with a syringe and the silicone-sealed desiccator was stored undisturbed at 2°C. for entire period of storage. 6 eggs were placed in each desiccator.

[b] Significantly different (5% level) from air sample.

[c] Highly significantly different (1% level) from air sample.

observations that reduction of S–S bonds of proteins frequently affect their solubility and/or viscosity. Thus reduction increases the solubility of highly insoluble keratin proteins such as feathers, wool and hair (Goddard and Michaelis, 1934); reduction decreases the solubility of insulin (Miller and Andersson, 1942) and it greatly decreases the viscosity of gluten suspensions (Olcott *et al.*, 1943).

Although the results obtained do not prove that chemical reduction is the natural mechanism of non-microbial deterioration of the white and yolk, the hypothesis is none the less an attractive one. The small amounts of reducing agent required to cause the deteriorations and the fact that the yolk, as well as the white, is affected by the same chemical agents support the hypothesis.[2] Thus chemical

[2] It is possible that the observed weakening of the yolk membrane is due to the solubilization of ovomucin, either in the membrane or in the thin layer of white surrounding it.

TABLE 5.—*Deteriorations of natural and oiled shell eggs exposed to low concentrations of hydrogen sulfide*[a]

Expt.	H_2S in gas phase % by volume	Oil treatment[b]	Av. white index	Av. percent thick white	Av. yolk index
1 10 days exposure at 2°C. 6 eggs per sample	0	None	0.047	66	0.39
	0.03	None	<0.03	43	0.34
	0.10	None	<0.02	20	0.32
	0	Oiled	0.055	58	0.40
	0.03	Oiled	0.012	53	0.38
	0.10	Oiled	0.038	51	0.35
2 17 days exposure at 2°C. 9 eggs per sample	0	None	0.051	66	0.45
	0.02	None	<0.02	41	0.36
	0.05	None	<0.02	14	0.36
	0	Oiled	0.016	66	0.40
	0.02	Oiled	0.052	64	0.43
	0.05	Oiled	0.047	62	0.40

[a] Eggs for experiment 1 were treated as described in Table 4. In experiment 2 the desiccators were aired and reopened after 4, 5, 7, 10 and 13 days storage. In both experiments 3 natural and 3 oiled eggs were held in each desiccator.

[b] Eggs were oiled by dipping for 10 sec. in a standard coating oil at 125°F. and allowed to drain overnight on pad flats.

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reduction of the ovomucin and yolk membrane could slowly occur by the action of minute amounts of reducing agents, if present in or if formed within the egg. While thick-white breakdown has been attributed to changes of solubility of ovomucin which might occur as a result of CO_2 loss and increase of the egg white pH, it is also true that the action of the sulfhydryl reducing agents is greatly accelerated by increase of the alkalinity of the reaction medium.

It is well known that ovalbumin of egg white contains sulfhydryl groups which, however, are not as reactive as the sulfhydryl groups of thioglycol. Because of the breaking of hydrogen bonds during denaturation, the SH groups in denatured ovalbumin are more reactive than in the native protein. Whether native or denatured ovalbumin formed during storage plays a part in the deterioration of eggs is for the present an untested possibility. However, if only 2% of the SH groups in ovalbumin were to become as active as the SH of thioglycol, marked deterioration of the egg would result. The observation that separated egg white becomes thin without the intervention of any outside agent (Feeney *et al.*, 1951) is, of course, consistent with the possibility that the thinning of egg white involves reduction but it is also consistent with the often mentioned possibility that thinning is due merely to the slow solution of mucin at the alkaline conditions of egg white (without the intervention of reduction).

The potential deterioration of shell eggs that might result during transportation and storage, if the eggs encounter reducing agents, should not be overlooked. It is imperative to determine whether reducing agents occur significantly in areas where eggs are handled and, if so, to determine the practical significance of their occurrence.

On the positive side, it may be possible to utilize chemical thinning advantageously in certain processing operations where thin egg white is desired.

CONCLUSIONS

Small amounts (less than 50 ppm.) of reducing agents such as thioglycol, hydrogen sulfide, and sulfur dioxide not only cause egg white to thin but also cause the yolk to simulate a deteriorated appearance. Small amounts of hydrogen sulfide and sulfur dioxide in the atmosphere surrounding eggs is absorbed through the shell and causes deterioration-like changes in the egg. Oiling greatly retards the absorption of these substances.

The possibility that the mechanism of the non-microbial deterioration of shell eggs involves reduction of the S—S bonds in mucin is discussed. The need for practical evaluation of these laboratory findings is indicated.

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Association Notes

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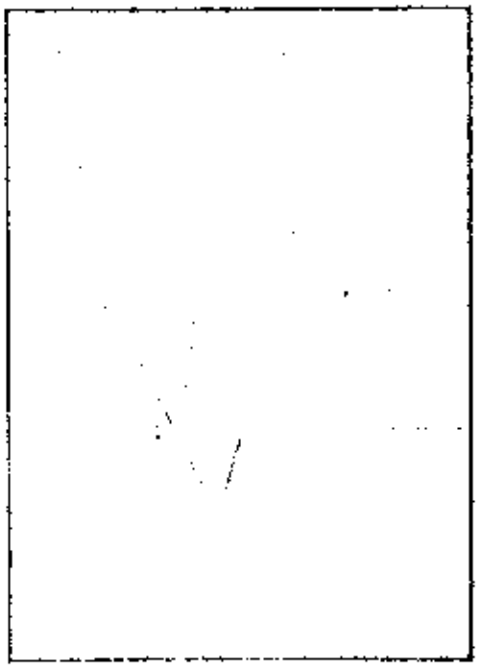
being Professor of Poultry Husbandry Extension.

Dossin is recognized as a leader in the field of producing and maintaining egg quality, having written a number of extension circulars, leaflets and news articles on the subject. At the time that egg auctions were being established in Pennsylvania, he made an analysis from which the Pennsylvania Quality Egg Program was formulated.

He early recognized the value of visual aids in extension work and has personally taken many pictures for this purpose. Dossin has also assumed aggressive leadership in educational work on production of hatching eggs, broiler production and disease control.

He is married and has one son. He is a member of the Poultry Science Association, World's Poultry Science Association, American Poultry Association, Pennsylvania Poultry Federation, Alpha Gamma Rho and Epsilon Sigma Phi.



H. L. KEMPSTER
Winner of the Poultry Science Teaching Prize

POULTRY SCIENCE TEACHING PRIZE

The Poultry Science Teaching Prize of one hundred dollars was presented to Professor Harry Laverne Kempster, Department of Poultry Husbandry, College of Agriculture, University of Missouri, Columbia.

Professor Kempster was born in Cold-

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