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VOL #2

G29

"SULFITING AGENTS"

Appendix A Part I

Full text copies of all articles used in preparation of the monograph for

Department of Health, Education and Welfare
Public Health Service

Food and Drug Administration

Rockville, Maryland

under contract FDA-72-101

by

The Franklin Institute Research Laboratories

FIRL Project No. 80G-C3378-01

ANALYTICAL BIOCHEMISTRY 31, 484–492 (1969)

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Reaction of Dithionite and Bisulfite with Pyridoxal and Pyridoxal Enzymes

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Received May 12, 1969

In a recent study of alanine racemase from *Pseudomonas* (1), it was found that the homogeneous enzyme contains tightly bound pyridoxal phosphate. Because a flavin coenzyme has been proposed as a component of the alanine racemase of *Bacillus subtilis* (2), this possibility was investigated with the *Pseudomonas* alanine racemase. No flavin could be identified in the purified *Pseudomonas* enzyme by fluorometry nor was there any absorption band typical of enzyme-bound flavin (1). A more sensitive spectral test, the examination of difference spectra after dithionite treatment of the enzyme, was also employed. In order to interpret such difference spectra, it seemed prudent first to check possible spectral effects of dithionite on pyridoxal phosphate and on known pyridoxal enzymes, such as transaminases. It immediately appeared that dithionite does react both with pyridoxal phosphate alone and with several pyridoxal enzymes to produce prominent spectral changes. It was possible, however, to distinguish between the dithionite difference spectra obtained with a flavoenzyme and with a pyridoxal-phosphate enzyme.

This report describes the spectral changes produced by dithionite and also by bisulfite. Although the product of dithionite action on free or enzyme-bound pyridoxal phosphate has not been determined, evidence is presented that it is not an addition product with the bisulfite formed from dithionite autooxidation.

Spectral [illegible] on pyridoxal enzymes by dithionite, or bisulfite, seem not to [illegible] studied in any systematic manner. The presence of flavin [illegible] pyridine nucleotides, and cytochromes, all of which are reducible [illegible] dithionite, is commonly investigated by the use of dif[illegible] with this reagent. The frequent use of dithionite with [illegible] crude enzyme preparations makes it perti-

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neat to recognize the possible contribution of pyridoxal enzymes to such absorbance differences in the 300 and low 400 mμ range.

MATERIALS AND METHODS

Pyridoxal phosphate and pyridoxine hydrochloride were obtained from Sigma; pyridoxal hydrochloride was a sample obtained from Merck. Approximately 1 mM stock solutions of each were made in 0.05 M potassium phosphate buffer, pH 7.4; concentrations were determined more accurately by absorbance using literature values (3) for molar absorptivities. Sodium dithionite ("sodium hydrosulfite") was sampled from a recently purchased bottle ("purified," Fisher). It was not assayed, and concentrations given are nominal ones based on weighed samples. Solutions were made in buffer (usually 0.05 M potassium phosphate, pH 7.4) which had been gassed for at least 15 min with nitrogen. After preparation, solutions (usually 10 mg/ml) were further bubbled with nitrogen and kept in an ice bath in narrow screw-capped tubes. Such solutions were used only within several hours of preparation and were sampled from the bottom of the tube. Until it appeared that anaerobic precautions were irrelevant, spectra with sodium dithionite were also obtained using only nitrogen-gassed buffer both to make up solutions of pyridoxal compounds and to bring the cuvet contents to final volume.

Alanine racemase was the homogeneous enzyme recently described (1). Glutamic-aspartic transaminase, purified (Catalog No. 15335 EGAK), and a relatively crude preparation of glutamic-alanine transaminase (Catalog No. 15341 EGAL), were purchased from Boehringer-Mannheim Corp. D-Amino acid oxidase was the purified Worthington product ("DAOFS"). Commercially obtained enzymes were used without further treatment, except for glutamic-alanine transaminase, which was dialyzed overnight at 4° against 1000 vol. Tris, 0.05 M, pH 7.4, to remove ammonium sulfate and permit soluble stock solutions of greater concentration to be made. All measurements were made at room temperature with a Cary 14 recording spectrophotometer. Matched 1 ml cuvets (1 cm light path) were used for all the spectra shown, except as indicated. Using buffer-filled cuvets, the baseline was first balanced throughout the wavelength range scanned. Addition of reagents (sodium dithionite or sodium bisulfite) was always made identically to both a blank cuvet containing buffer and to a cuvet containing the same buffer together with pyridoxal compounds in the same total volume as the blank. To minimize dilution effects on spectra, the volume of

dithionite or bisulfite added never exceeded 0.05 that of the solution of pyridoxal compound or enzyme.

RESULTS

The change produced by excess sodium dithionite in the spectrum of pyridoxal phosphate at pH 7.4 is shown in Figure 1. At a relatively low molar excess of dithionite over pyridoxal phosphate (7:1), the dithionite-produced spectrum changed with time. At a high molar excess of dithionite, a spectrum with an unchanging peak at 360 mμ appeared immediately. Solutions made from stock bottles of sodium dithionite several months old or solutions which had been vigorously aerated before use gave spectral changes similar to those of Figure 1. At low concentrations of the added reagent, however, older stocks produced less spectral conversion than did the fresh reagent. This suggested that dithionite itself, rather than a decomposition or oxidation product, is responsible for the spectral change.

Since bisulfite is an autoxidation product of dithionite and since sulfite has been reported to change the spectrum of pyridoxal phosphate (4), it was of interest to examine bisulfite effects on pyridoxal phosphate. As shown in Figure 2, bisulfite addition to pyridoxal phosphate produces a spectrum different from that with dithionite; although both reagents abolish the absorbance peak of pyridoxal phosphate centering at about 390 mμ, the new absorption peak with dithionite is about 360 mμ, while that with bisulfite is at 350 mμ.

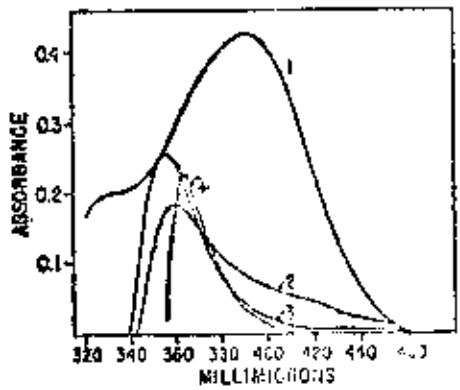

FIG. 1. Effect of sodium dithionite in excess on spectrum of pyridoxal phosphate: (1) pyridoxal phosphate (about 0.1 mM) in potassium phosphate, 0.05 M, pH 7.4; (2) immediately after addition of 0.7 mM sodium dithionite; (3) 15 min after 2; (4) immediately after addition of large excess of dithionite (5.8 mM) to 1.

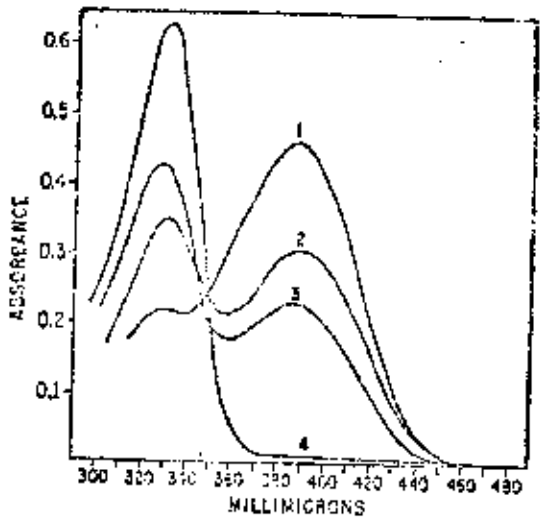

FIG. 2. Effect of increasing concentration of sodium bisulfite on spectrum of pyridoxal phosphate: (1) pyridoxal phosphate, approximately 0.0 mM, in potassium phosphate buffer, 0.05 M, pH 7.4; (2) immediately after adding 0.2 mM bisulfite; (3) immediately after adding further 0.2 mM bisulfite; (4) immediately after adding further 5.8 mM bisulfite.

The latter spectrum is similar to that produced by the addition of cysteine to pyridoxal phosphate (5).

The bisulfite effect on pyridoxal phosphate was also distinct from that of dithionite in that acetone interfered with the spectral change produced by bisulfite but not that produced by dithionite. At a final dilution of 10%, acetone largely abolished the effect of 9.6 mM bisulfite on the spectrum of pyridoxal phophate. In contrast, 10% acetone had little or no effect on the reactivity of 5.8 mM dithionite with pyridoxal phosphate as judged by the characteristic spectral change. The acetone effect in preventing the spectral change in pyridoxal phosphate with bisulfite is easily explained by the bisulfite-binding properties of the acetone carbonyl, which decreases the concentration of free bisulfite.

The effects of bisulfite or of dithionite on the spectrum of free pyridoxal are shown in Figure 3. Both compounds produced qualitatively different spectral changes, dithionite resulting in a spectrum similar to that seen after dithionite addition to pyridoxal phosphate. It is notable that bisulfite failed to produce the spectral change produced by lower concentrations of dithionite eliminating the possibility that the dithionite reaction results only from contaminating bisulfite.

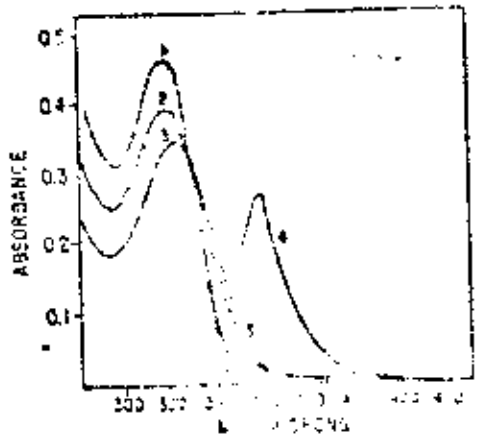

FIG. 3. Effect of bisulfite and di[illegible] spectrum of pyridoxal: (1) pyridoxal hydrochloride (0.0[illegible] mM) in [illegible] 0.05 M, pH 7.4; (2) immediately after addition of 2.9 mM [illegible], (3) immediately after addition of further 4.8 mM sodium [illegible] (4) immediately after addition of 2.9 mM sodium dithionite to a [illegible]

In contrast to the effect of dithionite on pyridoxal or pyridoxal phosphate, no change was observed in the spectrum of pyridoxine (over the range 310 to 500 mμ) after addition of 2.3 mM sodium dithionite.

Glutamic aspartic transaminase was also sensitive with relatively low concentrations of sodium dithionite, showing a progressive loss

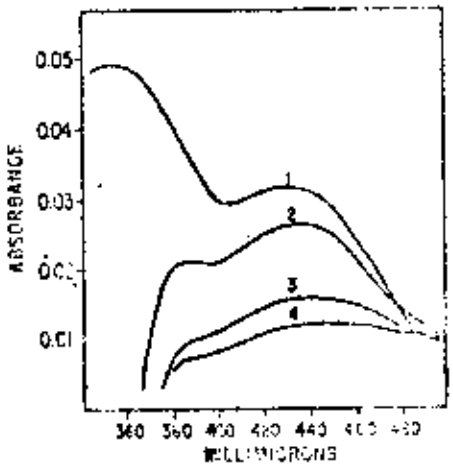

FIG. 4. Effect of [illegible] on the spectrum of [illegible] aspartic [illegible] nase. (1) enzyme, [illegible] ml, in [illegible] M [illegible] pH [illegible] (2) immediately after [illegible] 2.3 mM sodium dithionite, [illegible] (3) 20 minutes after 2.

of the 430 mμ peak with time (Fig. 4). In contrast, concentrations of bisulfite which were effective in all [illegible] the spectrum of pyridoxal phosphate at pH 7.4 had little or [illegible] on the spectrum of glutamic aspartic transaminase or of glu[illegible] and the transaminase at this pH. Lowering the pH, however, [illegible] a reaction between bisulfite and glutamic aspartic tra[illegible] a time-dependent spectral change (Fig. 5). [illegible] at pH 5.8 resembles that of the presumptive [illegible] with pyridoxal phosphate (Fig. 2). The [illegible] enzyme is sluggish compared with free pyr[illegible] phosphate and it apparently reaches equilibrium short of a [illegible] reaction when with a large excess of bisulfite (Fig. 5).

Dithionite may be useful in selectively reducing the absorption peak in the low 400 mμ range which is characteristic of several pyridoxal phosphate enzymes. This is illustrated for [illegible] in Figure 6 and for glutamic-alanine transaminase in Figure 7. It is notable that in each case the direct spectrum [illegible] tions would not have provided unambiguous indication of the characteristic peak attributed to the pyridoxal phosphate [illegible] thine complex, whereas in the difference spectra the peaks are unequivocal.

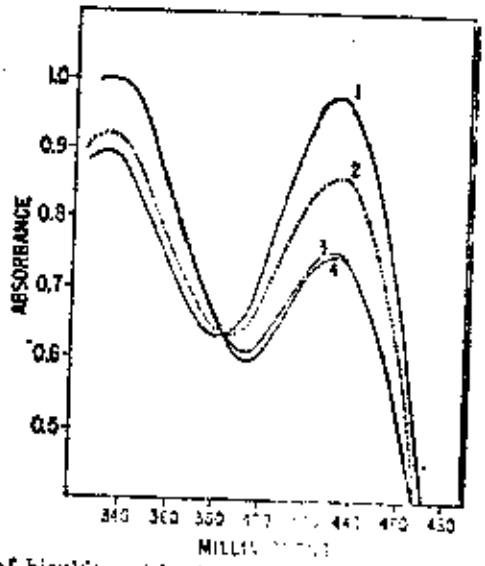

FIG. 5. Reaction of bisulfite with glutamic [illegible] transaminase at pH 5.8; (1) enzyme, approximately 4 mg/ml in 0.2 M [illegible] acetate, pH 5.8; (2) immediately after addition of 4.8 mM sodium bisulfite; (3) 10 min after (2); (4) 20 min after 2.

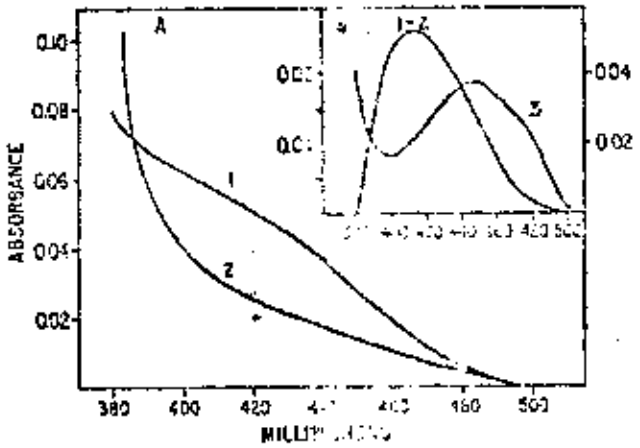

FIG. 6. Spectrum of alanine racemase before and after dithionite: (A) 1, alanine racemase, 0.017 mg in 0.1 ml [illegible] M potassium phosphate, pH 7.4, in self-masking 0.05 ml cuvet (Beckman, [illegible]); 2, immediately after adding sodium dithionite, 0.05 mg/0.005 ml. ([illegible] showing difference spectra: 1–2, alanine racemase (*before* minus *after* dithionite, from data of A); 3, difference spectrum (*before* minus *after* dithionite) for D-amino acid oxidase; conditions exactly as for alanine racemase except that enzyme was 0.5 mg/ml. Absorbance scale on left of inset refers to curve 1–2, that on right to curve 3.

The difference spectrum obtained on reducing the flavin of D-amino acid oxidase by dithionite is also shown in the inset of Figure 6. It can be distinguished from the difference spectrum attributed to the pyridoxal phosphate of alanine racemase (Fig. 6) not only by its peak and shape but also by its obliteration with hydrogen peroxide. The latter reagent produced only a modest drop in the dif-

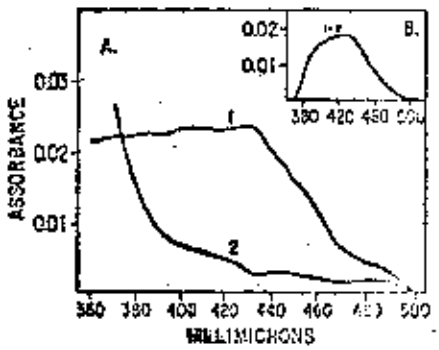

FIG. 7. Spectrum of glutamic alanine transaminase before and after dithionite: (A) 1, enzyme (approximately 1.5 mg/ml) in 0.2 M potassium acetate, pH 5.8; 2, immediately after adding 2.0 mM sodium dithionite. (B) Inset showing difference spectrum (1 minus 2, from A).

ference spectrum of alanine racemase after dithionite treatment, without qualitatively altering the shape of the difference peak.

DISCUSSION

The reaction products of dithionite with pyridoxal phosphate, pyridoxal, and enzyme-bound pyridoxal phosphate, remain to be determined. Because the reaction appears to involve dithionite itself, and is clearly not a reaction with bisulfite, a reductive reaction seems likely. Dithionite is capable of reducing a number of organic compounds including such nitrogen heterocycles as the pyridinium ring of pyridine nucleotides and the isoalloxazine ring system of flavin nucleotides. It is therefore conceivable that the pyridine ring of pyridoxal or its phosphate is also reduced, perhaps to the quinonoid stage. However, the apparent failure to obtain spectral conversion of pyridoxine by dithionite may implicate the aldehyde group as a requirement for this reaction. The simplest possibility, that the dithionite reaction involves only reduction of the aldehyde group of pyridoxal to an alcohol, can be rejected since the converted spectrum is not that of pyridoxine.

The presumed addition product of pyridoxal phosphate with bisulfite resembles spectrally the reaction product of pyridoxal phosphate with cysteine. It is notable that enzyme-bound pyridoxal phosphate is much less reactive with bisulfite than is free pyridoxal phosphate, a finding easily reconcilable with the belief that, in the enzymes tested, the aldehyde group of pyridoxal phosphate is not free but is present in rather stable azomethine linkage with an enzyme lysine.

Apart from the questions of chemical interest raised by the dithionite reaction with pyridoxal phosphate, recognition of its possibility may be pertinent in interpreting flavin difference spectra in protein mixtures. Although single enzymes containing both a flavin and pyridoxal prosthetic group have not been reliably described, multienzyme aggregates of physiological significance may conceivably involve both coenzymes. While the possibility of confusion exists, the spectral characteristics of the dithionite-pyridoxal product appear to make its difference spectrum distinguishable from that caused by the reduction of flavins.

SUMMARY

Both bisulfite and dithionite produce marked changes in the spectra of pyridoxal, pyridoxal phosphate, and pyridoxal phosphate enzymes. The spectrum of pyridoxal phosphate in bisulfite solutions

resembles that of the addition product of pyridoxal phosphate with cysteine. The spectral changes produced by dithionite are distinctly different from those produced by bisulfite and, unlike the latter, are not prevented by acetone. As judged by spectral change, pyridoxal phosphate enzymes appear less reactive with bisulfite than with dithionite, relative to the reactivity of the free coenzyme. The nature of the dithionite reaction and of its products has not been determined. However, the difference spectrum attributable to dithionite reaction with enzyme-bound pyridoxal phosphate, while distinguishable from that of a flavin difference spectrum must be kept in mind in interpreting presumptive flavin difference spectra. In addition, dithionite may be a useful reagent for delineating, by difference spectra, the characteristic low 400 mμ peak of pyridoxal phosphate enzymes.

ACKNOWLEDGMENT

This work was supported by Grant GM 11105 from the U. S. Public Health Service.

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Biochim. Biophys. Acta, 42 (1960) 206–211

FURTHER STUDIES ON CYSTATHIONINE SYNTHETASE-SERINE DEAMINASE OF RAT LIVER

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(Received January 4th, 1960)

SUMMARY

1. The purity of rat liver cystathionine synthetase-serine deaminase was more than doubled over that previously reported through the use of zone electrophoresis. The ratio of the two enzymic activities of the more purified preparation remained unchanged.

2. Enzymic activity was slightly inhibited by certain pyridoxine derivatives and strongly inhibited by carbonyl reagents, sulfhydryl reagents, various metal chelating agents, and by a number of heavy metal ions. Mg^{++} and Mn^{++} slightly stimulated the serine deaminase activity.

3. Study of the substrate specificity of the enzyme showed that only L-serine and L-threonine were deaminated and only homocysteine was utilized for synthesis of a sulfur-containing diamino acid with L-serine. L-cysteine strongly inhibited cystathionine synthesis.

INTRODUCTION

A procedure for the partial purification of cystathionine synthetase-serine deaminase from rat liver and some of the properties of this enzyme were reported earlier[1]. The

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present communication deals with the further purification of the enzyme by column electrophoresis, studies on the substrate specificity of its dual enzyme activities, and inhibition and activation by a variety of reagents.

EXPERIMENTAL MATERIALS AND METHODS

Materials

Geon (type 426) resin for use in column electrophoresis was generously supplied by the B. F. Goodrich Chemical Co., Avon Lake, Ohio. It was washed before use and the column was packed in the manner described by DERAIN[2].

The chemical compounds employed in this work and their sources are the same as previously reported[1].

Enzyme preparation

Preliminary purification of the enzyme for column electrophoresis was carried out by the published method[1]. This preparation was stored with ammonium sulfate as a frozen thick paste with almost no loss of activity. The serine deaminase specific activity of the preparations varied between 150 and 250.

Analytical methods

Protein concentrations were determined by the method of MONRASCH AND McGILVERY[3], based on the biuret reaction.

The enzyme assay methods previously reported[1] were employed, with the exception that mercaptoethanol was substituted for dimercaptopropanol because the former is more stable and more soluble in water, and, also, because at the concentration employed ($1.3 \cdot 10^{-4}$ *M*) mercaptoethanol slightly activated while the dimercaptopropanol somewhat inhibited the serine deaminase reaction.

In the assays, 0.25 mmoles of DL-homocysteine or 0.2 mmoles of L-serine 3 ml of incubation medium was employed as the substrates. The reaction medium contained between 0.35 to 0.45 mg of enzyme protein; 0.1 *M* potassium borate buffer, pH 8.3, and $5 \cdot 10^{-5}$ *M* pyridoxal phosphate with other components as indicated in the respective tables. The incubations were run for 30 min at 37° in a Dubnoff shaking incubator.

For column electrophoresis, the packed column (2 × 55 cm) was washed and equilibrated with 0.05 *M* potassium phosphate buffer, pH 7.5 for 24 h. The frozen enzyme preparation was dissolved in 6 volumes of 0.05 *M* potassium phosphate buffer, pH 7.5, containing $5 \cdot 10^{-4}$ *M* EDTA (ethylenediaminetetraacetic acid), and then dialyzed for 12 h against the same buffer. The small amount of protein precipitate that formed was removed by centrifugation.

The clear, yellowish solution (4 ml containing about 40 mg of protein) was slowly pipetted into the column. It was further allowed to travel, as observed by the movement of the yellow color, to a point about 17 cm from the top of the column by the gradual addition of the phosphate buffer. After setting the column into the electrophoresis apparatus, the two limbs of the assembly were dipped into the electrode vessels containing the same buffer. The power supply was adjusted to 750 V, allowing approximately 50 mA of current to flow for 10 h. At the end of the run, the column was removed, a stopcock was attached and it was placed over a fraction collector

and the contents were eluted with the same phosphate buffer at a flow rate of approx. 18 to 20 ml/h. 25 fractions were collected of about 3 ml each. The whole operation was carried out at about 6°.

The protein content and serine deaminase activity of the fractions were then determined, and the results plotted (Fig. 1). It was observed that the highest enzymic activity was always found in the fractions which were the deepest yellow in color, which aided in their location. This color was found to travel together with the enzyme protein. The L-serine deaminase purified in this manner attained a specific activity of 346 units/mg of protein.

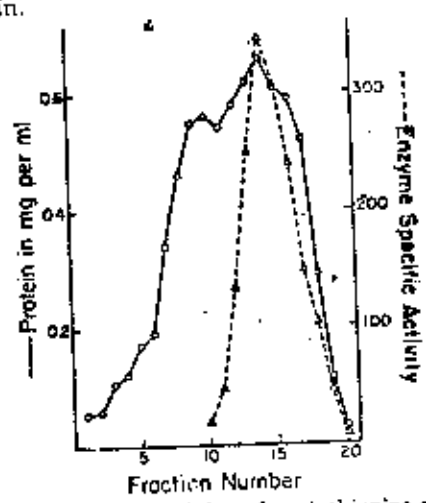

Fig. 1. Partition of protein and enzyme activity of cystathionine synthetase-serine deaminase on column electrophoresis.

In a second run performed in exactly the same way, a specific activity of 334 serine deaminase units/mg of protein was obtained. The specific activity of cystathionine synthetase, determined on the fraction with the highest serine deaminase activity, was 121. This gives a ratio for serine deaminase to cystathionine synthetase activities of 2.76, which is in good agreement with the previous findings[1].

For the additional studies reported here, batches of 100 to 120 mg of enzyme protein were fractionated by electrophoresis and those of highest enzyme activity collected. The combined fractions were brought to 50% saturation with ammonium sulfate by the slow addition of solid salt while stirring the mixture mechanically and periodically adjusting the pH to 7.2 with ammonium hydroxide. After standing for 6 h at 0°, the mixture was centrifuged at 8,000 × *g* and the resulting precipitate stored at −20°.

Effect of various inhibitors on the serine deaminase activity of the enzyme

Effect of pyridoxine derivatives: Addition of pyridoxal phosphate to the incubation media appreciably increased the enzyme activity[1]. It was of interest, therefore to determine the effect of pyridoxine derivatives on the enzyme activity. Tests were made with 4-deoxypyridoxine-5-phosphate, 4-deoxypyridoxine hydrochloride, and pyridoxine hydrochloride at a concentration of $4 \cdot 10^{-3}$ *M* on serine deaminase activity in the presence of $5 \cdot 10^{-5}$ *M* pyridoxal phosphate. The inhibitory effect found was small, amounting to only 10 to 15 %.

Inhibition by carbonyl reagents: The effect of a number of carbonyl reagents on serine deaminase activity are summarized in Table I. In this experiment, a second control was also run in the same manner as for the enzyme assay, except that the carbonyl reagent was added after the incubation, followed by the addition of trichloroacetic acid. A correction for any action of the carbonyl reagent on the development of the color of the pyruvyl-2,4-dinitrophenylhydrazone was thus determined and applied to the enzyme results. All carbonyl reagents inhibited the enzyme activity, decreasing in effectiveness in the order; hydroxylamine > cyanide > isonicotinic acid hydrazide > sodium bisulfite.

TABLE I

INHIBITION OF SERINE DEAMINASE BY CARBONYL REAGENTS

Components not listed in text were enzyme, 0.45 mg, EDTA, $5\cdot10^{-4}$ *M*; mercaptoethanol, $1.3\cdot10^{-4}$ *M*. Concentration of inhibitors, $6.7\cdot10^{-3}$ *M*.

Inhibitor	*Enzyme activity pmoles serine deaminated*	*Inhibition per cent*
None	65	
NaCN	5.6	91
Hydroxylamine hydrochloride	4	94
Isonicotinic acid hydrazide	44	32
$NaHSO_3$	52.2	20

TABLE II

EFFECTS OF METAL IONS ON SERINE DEAMINASE

Enzyme protein 0.36 mg. Concentration of each metal ion, $3.3\cdot10^{-4}$ *M*.

Metal ions added	*Enzyme activity pmoles serine deaminated*	*Per cent change*
None	52.3	
Cu^{++}	4.2	−92
Hg^{++}	1	−98
Cd^{++}	1.6	−97
Fe^{++}	47.7	−13
Co^{++}	52	− 7
Ni^{++}	48.8	−22
Mn^{++}	54.6	+ 4
Zn^{++}	53	+ 1
Mg^{++}	57	+ 9

Action of sulfhydryl reagents: Determination of the effect of *p*-chloromercuribenzoate, N-ethylmaleimide, *o*-iodosobenzoate and iodoacetate on serine deaminase activity at approximately 10^{-3} *M*, yielded the following result. The first three strongly inhibited the enzymic activity (80–100%); iodoacetate inhibited only moderately (37%).

Inhibition by metal ions: Table II summarizes the effect of various metal ions on serine deaminase activity. Controls which differed from the enzyme assay only in that the enzyme preparation was added after incubation were run with each assay. With the exception of Mg^{++}, Mn^{++} and Zn^{++}, all of the metal ions tested inhibited the enzymic activity. The most effective inhibitors were Hg^{++}, Cd^{++} and Cu^{++} which

produced almost complete inhibition at the concentration employed. Mg^{++} and Mn^{++} caused a small but significant activation of the serine deamination reaction.

Effect of metal chelating and related compounds. It was of interest to determine the effect of varying concentrations of EDTA, mercaptoethanol and dimercaptopropanol on serine deaminase activity in the presence of pyridoxal phosphate because of their use as components of the incubation media. Both EDTA and mercaptoethanol stimulated the enzymic activity slightly at the molar concentrations of $5 \cdot 10^{-4}$ and $1.3 \cdot 10^{-4}$ respectively. At higher concentrations both substances become inhibitory. The inhibition increases gradually with increasing concentration of the reagent, and becomes of considerable magnitude at a concentration of 10^{-2} M (−18.5% for EDTA and −11.5% for mercaptoethanol).

Dimercaptopropanol, on the other hand, was found to inhibit the enzymic activity even at the lowest concentration tested ($1.3 \cdot 10^{-4}$ M), and the degree of inhibition increased greatly at higher concentrations.

The effects of a number of heterocyclic aromatic compounds on serine deaminase activity are shown in Table III. Of the reagents tested, 8-hydroxyquinoline and *o*-phenanthroline showed a low degree of inhibition at the concentration of $4 \cdot 10^{-2}$ M. The most effective inhibitor in this group was 8-hydroxyquinoline-5-sulfonic acid, (30% inhibition). On the other hand, 2-hydroxyquinoline produced no inhibition possibly because this reagent does not form metal chelates[1].

Unlike the other metal chelating agents tested, 2,2'-bipyridine appreciably stimulated the enzymic reaction both in the absence and presence of EDTA. This may be attributed to chelation by this reagent of harmful metal ions such as those of iron or copper, thereby inactivating them.

TABLE III

EFFECT OF CHELATING AGENTS ON SERINE DEAMINASE ACTIVITY

Enzyme protein 0.37 mg; EDTA, $5 \cdot 10^{-4}$. Concentration of chelating agents, $4 \cdot 10^{-2}$ M.

Compound added	*Enzyme activity (μmoles amino deaminated)*	*Per cent change*
None	53	
8-hydroxyquinoline	46.2	−13
o-phenanthroline	50	−6
2-hydroxyquinoline	53	0
8-hydroxyquinoline-5-sulfonic acid	37.2	−30
2,2'-bipyridine	62.2	+17

Substrate specificity of serine deaminase-cystathionine synthetase

The relative activities of the enzyme on a number of hydroxy and S-containing amino acids and alanine are summarized in Table IV. The enzyme action on the amino acids was determined by the formation of α-keto acid. Lithium pyruvate was used as the color standard, regardless of the nature of the α-keto acid expected to be produced from a given amino acid.

Of the hydroxyamino acids tested, L-threonine was deaminated at about 20% of the rate of L-serine. Possibly this activity may reflect contamination of the enzyme with L-threonine deaminase, also present in rat liver, but this cannot be accepted

TABLE IV

ACTIVITY OF SERINE DEAMINASE ON HYDROXY AND SULFUR CONTAINING AMINO ACIDS

Enzyme protein, 0.36 mg; EDTA, $5 \cdot 10^{-4}$ M; mercaptoethanol, $1.3 \cdot 10^{-1}$ M.

Amino acid	*Amount added pmoles*	*Enzyme activity pmoles serine decomposed*
L-serine	100	57
L-threonine	200	11.4
L-homoserine	200	0.6
β-hydroxy-DL-aspartic acid*	100	0
D-glucosamine hydrochloride	200	1.1
DL-homocysteine	250	0.5
L-cysteine	200	0
DL (+ allo)-cystathionine	50	0
Lanthionine (L and meso)	50	0.6
S-isopropyl-DL-homocysteine	50	0
S-methyl-L-cysteine	100	0.2
S-carboxylmethyl-L-cysteine	50	0.6
DL-ethionine	100	0.4
L-methionine	100	1.0

* A synthetic preparation kindly furnished by Dr. E. Kun. Probably composed of mixture of the several possible configurations.

with certainty. The other hydroxyamino acids, and also the sulfur-containing amino acids tested were found to be essentially inert as substrates for the enzyme.

Activity of enzyme on L-serine in presence of certain sulfur-containing amino acids.

The effect of certain sulfur-containing amino acids on the serine deaminase and apparent cystathionine synthetase activity of the enzyme is recorded in Table V. The synthetase activity of the enzyme was evaluated by determining the amount of L-serine which reacted but was not converted to pyruvic acid, as described in the previous communication[1].

The results obtained with homocysteine and cysteine parallel those already published. Both of these amino acids virtually eliminate serine deaminase activity, but only homocysteine induces the synthetase reaction.

Substitution of the sulfhydryl group of these amino acids greatly reduced the

TABLE V

ACTIVITY OF SERINE DEAMINASE AND CYSTATHIONINE SYNTHETASE ON SULFUR CONTAINING AMINO ACIDS IN PRESENCE OF L-SERINE

Enzyme protein, 0.36 mg; mercaptoethanol, $1.3 \cdot 10^{-1}$; EDTA, $5 \cdot 10^{-4}$ M; 200 μmoles of L-serine and the sulfur amino acid in the amounts shown below.

Sulfur amino acid	*Amount added pmoles*	*Serine deaminase activity pmoles serine utilized*	*Cystathionine synthetase activity pmoles serine utilized*
None		58	
DL-homocysteine	250	1.3	21.5
L-cysteine	200	1.1	0
S-isopropyl-DL-homocysteine	100	45.7	3.3
S-methyl-L-cysteine	100	35	2.1

degree of inhibition of serine deaminase activity. Both *S*-isopropyl-DL-homocysteine and *S*-methyl-L-cysteine caused some apparent synthetase activity. For the former, this might be explained by some contamination or decomposition resulting in the occurrence of homocysteine. The explanation of the result indicating a synthetic effect of *S*-methyl-L-cysteine is not readily apparent.

Activity of the enzyme on hydroxyamino acids in the presence of homocysteine

The relative synthetase activity of the enzyme was tested on a number of hydroxyamino acids, in the presence of DL-homocysteine (Table VI). Synthetic activity was estimated by the disappearance of homocysteine from the reaction mixtures by means of the nitroprusside method of GRUNERT AND PHILIP[5]. Two controls were run simultaneously with the enzyme assay; in one, the enzyme preparation was added at the end of the incubation; in the second the hydroxyamino acid was added after incubation.

TABLE VI

ACTIVITY OF CYSTATHIONINE SYNTHETASE ON HYDROXYAMINO ACIDS IN PRESENCE OF HOMOCYSTEINE

Enzyme protein, 0.36 mg; EDTA, $5 \cdot 10^{-4}$ *M*; mercaptoethanol, $1.3 \cdot 10^{-4}$ *M*; 250 μmoles DL-homocysteine.

Hydroxyamino acid added	*Amount added μmoles*	*Cystathionine synthetase activity μmoles homocysteine utilized*
L-serine	200	21
L-threonine	200	0.45
Hydroxy-DL-aspartic acid	100	0.3
L-homoserine	200	0.7

The results indicate that, with the exception of L-serine which is a highly potent substrate for synthetic activity, all of the other amino acids were not utilized by the enzyme.

The results reported in this communication support the previous conclusion[1] that dehydrative deamination of serine and synthesis of cystathionine are functions of a single enzyme protein. Additional evidence has been obtained that pyridoxal phosphate is the coenzyme for the enzyme activities and that the enzyme requires free sulfhydryl groups for activity.

ACKNOWLEDGEMENT

This investigation was aided by a research grant from the National Heart Institute (H-3074), National Institutes of Health.

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3

Long-Term Continuous Exposure to Sulfur Dioxide in Cynomolgus Monkeys

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Cynomolgus monkeys were exposed to sulfur dioxide while a control group was exposed to filtered air. The exposure was for 24 hours a day, seven days a week, for 78 weeks. Frequent measurements were made to evaluate mechanical properties of the lung, distribution of pulmonary ventilation, diffusing capacity of the lung, and arterial blood oxygen tension. Hematological and clinical biochemical determinations were conducted. Microscopic evaluation of organs and tissues was conducted at termination of the exposure of the animals. No deleterious effect could be attributed to SO_2 exposure at concentrations of 0.14 to 1.23 ppm. Following 30 weeks of exposure to 4.69 ppm, an overexposure occurred in this group. This was followed by deterioration in pulmonary function which persisted during the following 48 weeks of observation, and alterations in pulmonary tissues were observed upon microscopic examination.

SULFUR DIOXIDE, a common pollutant of urban air, has received wide attention in scientific literature; two reviews of the work done to delineate its effect on vegetation, animals, and man have been published.[1,2] From these reviews, it becomes obvious that the research undertaken had been mainly concerned with short-term exposures of animals[3-10] or man[11-18] and usually at higher concentrations than those encountered in urban atmospheres, with the exception of a study on guinea pigs exposed to 0.16 ppm.[19] The few studies conducted to elucidate long-term effects in animals[20-23] also involved high concentrations. More recently, however, comprehensive pulmonary function tests have been used in long-term exposure studies to determine the effects of sulfur oxides at low concentrations in dogs[24-25] and guinea pigs.[26]

In the present study, cynomolgus monkeys (*Macaca irus*) were exposed for a period of 18 months at concentrations ranging from 0.1 to 5.0 ppm. This range was selected because it includes those concentrations encountered in urban atmospheres and extends to the current threshold limit value of 5.0 ppm established by the American Conference of Governmental Industrial Hygienists.[27]

Materials and Methods

Animals and Exposure Conditions.—Five groups of animals were used in this study; each group consisted of nine animals composed of five males and four females or vice versa. One group was maintained as the control group and was exposed to filtered air, whereas the other four groups were exposed to SO_2 at the four approximate nominal concentrations of 0.1, 0.5, 1.0, and 5.0 ppm.

Young cynomolgus monkeys (*M irus*) weighing 1.6 to 3.0 kg (3.5 to 6.6 lb) were used in this study. They were individually caged and held in a quarantine area. The animals were tested twice for tuberculosis during an eight-week period and then transported from the quarantine area to laboratory-animal holding rooms where they were observed for a period of two weeks. During the following 8 to 12 weeks, the animals were retested for tuberculosis, had a chest x-ray film taken, and passed through a screening procedure which consisted of evaluation of pulmonary function, electrocardiographic findings, and hematologic and clinical chemistry studies. Animals having values

Submitted for publication April 12, 1971; accepted July 29.

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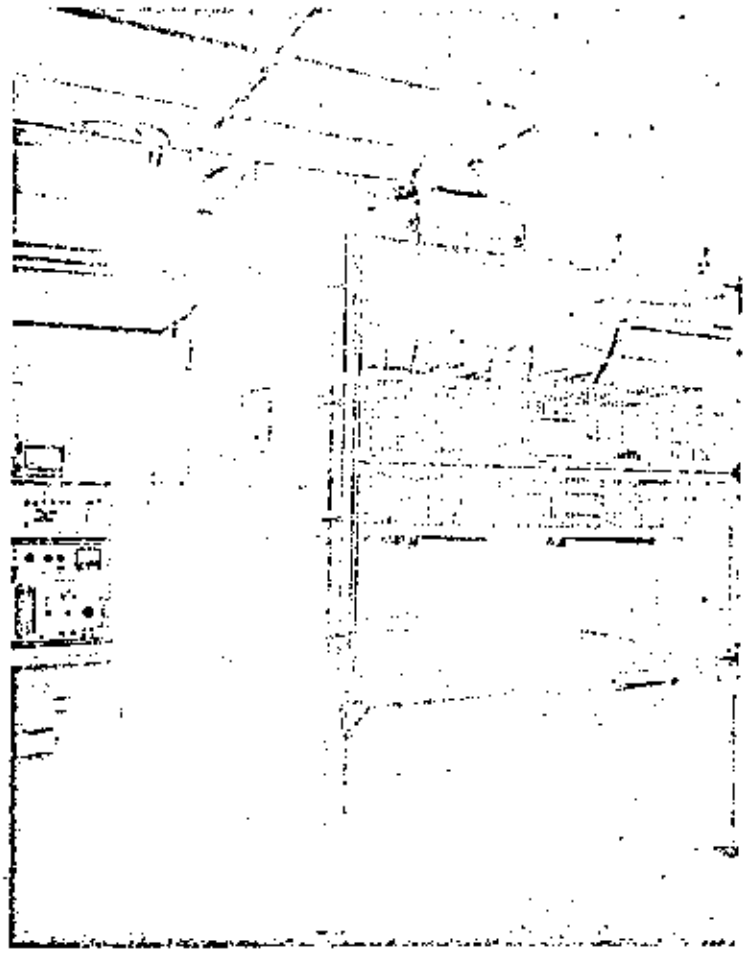

Fig 1.—Exposure chamber housing nine cynomolgus monkeys. Delivery and monitoring apparatus for SO_2 is also shown.

within normal limits were accepted. During this period, animals were also placed in a restraining chair with a face mask to adapt them to the pulmonary function testing procedures.

Animals accepted for studies were then transferred to individual exposure cages and placed in stainless steel and glass chambers. One of the exposure chambers used in this experiment is shown in Fig 1; the volume of each is approximately 6.5 cu m.

The air supplied to each exposure chamber passed through a charcoal bed and an absolute filter. The temperature was maintained at 72 ± 2 F and the relative humidity at 50% ± 5%. The airflow through each chamber was slightly in excess of 1,000 liter/min.

After placing the animals in the exposure chambers, a period of two weeks was allowed for adaptation. During the following eight weeks, all groups were exposed to filtered air only and physiological measurements were made to establish baseline values and normal variations present from week to week. Then the exposure to SO_2 was initiated and continued for a period of 78 weeks.

Each day the exposure was interrupted twice for a period of ten minutes for feeding the animals and cleaning the chambers. Exposures were also interrupted for the duration of the particular test on days when physiological measurements were made.

Samples of each chamber's atmosphere were taken once daily with an impinger and analyzed according to the method of West and Gaeke.[28] Also SO_2 analyzers (Melpar SR-1100-1) were used for semicontinuous analysis of chamber concentration of SO_2 and calibrated against the wet chemical method.[25] One analyzer was shared between the 0.1- and 0.5-ppm SO_2 exposure chambers and another was shared between the 1.0- and 5.0-ppm SO_2 chambers.

Measurements Made on Animals.—Body weights were recorded weekly during the eight-week preexposure period and the 78 weeks of exposure. Observation for survival and gross signs of behavioral abnormality were made daily. Pulmonary function tests included the measurements listed below. All were performed without anesthesia after 10 to 20 minutes of adaptation with the animal in the restraining chair in a sitting position with the face mask attachment.

Mechanical Properties of the Lung.—Measurements of total respiratory system flow resistance during inspiration (Rrs[i]) and during expiration (Rrs[e]) were made every week during the preexposure control period, every week during the first nine weeks of exposure, and every four weeks thereafter, employing a previously published method.[29] Using previously published techniques,[30] measurements of tidal volume (V_T), respiratory rate (RR), minute volume (MV), dynamic compliance of the lung (Cdyn[l]), pulmonary flow resistance (Rl), and work of breathing during inspiration (W[i]) and during expiration (W[e]) were made five times during the pre-

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...posure control period, every two weeks during the first ten weeks of the exposure period, and every four weeks thereafter.

Distribution of Ventilation.—The distribution of pulmonary ventilation was evaluated by the nitrogen washout technique as previously described.[31] These measurements were made on six occasions during the preexposure control period, every two weeks during the first four weeks of the exposure period, and every four weeks thereafter.

Diffusing Capacity of the Lung.—The carbon monoxide uptake and the diffusing capacity of the lung were measured by a rebreathing technique based on the same principles as those described by Bates,[32] Lewis et al,[33] Kruhoffer,[34] and Long and Hatch[35] after modification for use in monkeys. In doing so, the face mask and chair, which have been previously described,[29,31] were used. These measurements were made according to the same schedule as the distribution of ventilation measurements.

Arterial Blood Gas.—Arterial blood was obtained from the femoral artery for measurements of oxygen tension (Pa_{O_2}), carbon dioxide tension (Pa_{CO_2}), and pH. Measurements were made at 37.5 C utilizing oxygen (Po_2) and carbon dioxide (Pco_2) tension and pH electrodes on a radiometer, as previously described.[30] Each sample of blood was obtained 10 to 20 minutes after injection of phencyclidine hydrochloride for tranquilization of the animal. Measurements were made once during the preexposure period, every eight weeks during the first 24 weeks of the exposure period, and every 12 weeks thereafter.

Hematologic and Clinical Chemistry Studies.—Blood obtained from the femoral artery was used for the determination of the following values: hematocrit, hemoglobin, total erythrocyte and leukocyte counts, lymphocytes, segmented neutrophils, blood urea nitrogen (BUN), total bilirubin, serum total protein, serum albumin, serum electrolytes, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), serum lactic acid dehydrogenase, and serum alkaline phosphatase. We made the determinations, once during the preexposure control period and at weeks 11, 16, 25, 49, and 77 of the exposure period.

Statistical Analysis.—All data obtained were stored on digital computer system (IBM 1800) and statistical analyses were performed by this system. For each preexposure interval, data were analyzed to delineate baseline values, frequency distribution characteristics, mean values, standard deviation, and coefficient of variation of each individual group, of the five groups combined, and of differences between males and females. Regression analyses were used on the measured parameters when sufficient data points (greater than eight) were available. The proper polynomial regression function was chosen on the basis of the best fit for the control or one of the treatment groups. In these analyses, individual data points were used for each time interval. Comparisons of the regression coefficients between groups were made by Student's *t*-test, and confidence intervals were calculated as previously described.[30,31] In some instances a cumulative sum technique (CUSUM)[37-39] was used, via extension, to establish the time of occurrence of significant differences between the preexposure control period and the exposure period for a particular group or between the control group and any of the exposed groups. In performing statistical comparisons, $P < .05$ was chosen in all cases for accepting or rejecting significant differences between groups.

Killing Procedures.—Following the 78 weeks of exposure, the nine animals were killed with an overdose of pentobarbital sodium followed by exsanguination. Complete necropsies were performed on each animal, and specimens of the following tissues were fixed in 4% neutral buffered formaldehyde solution: trachea, peribronchial lymph node, heart, liver, and kidney. After fixation, 6μ hematoxylin-eosin-stained slides were prepared. In an inflated state, the lungs were removed from the animals and were perfused intratracheally with a volume of neutral buffered formaldehyde solution equal to the V_T of the animal. Slides were prepared from each of the seven lobes of the lungs.

Results

Sulfur Dioxide Concentrations.—The chamber concentrations of SO_2 as determined are presented in the Table. The arithmetic means of the monthly averages for each of the chambers and the standard deviations were 0.14 ± 0.05, 0.64 ± 0.25, 1.28 ± 0.42, and 4.69 ± 0.34 ppm. An accidental overexposure to SO_2 occurred after 30 weeks of exposure in the chamber in which animals were exposed to 4.69 ppm. The concentration during the overexposure was estimated to be not higher than 1,000 ppm and not lower than 200 ppm. The animals remained in that atmosphere for a period of one hour. Following this accident, the animals were not reexposed to SO_2 but were kept in the chamber

with filtered air for the following 48 weeks and physiological parameters were measured as originally scheduled until termination of exposure.

Measurements of Biological Parameters: Body Weight and Survival.—Analysis of the growth curve for each group revealed no significant differences between the control group and the exposed groups during the exposure to SO_2, although the group exposed to 0.14 ppm of SO_2 had a slightly higher body weight than the control group at the beginning of the experiment. The body weight growth curves for the control group and the group exposed to 4.69 ppm of SO_2 are presented in Fig 2. One death occurred in the control group and one in the group exposed to 0.64 ppm of SO_2. The death in the group exposed to 0.64 ppm of SO_2 was caused by intestinal infections accompanied by diarrhea and dehydration, whereas the death occurring in the control group was caused by gastric distention. No deaths occurred in the group exposed to 4.69 ppm of SO_2, which was accidentally overexposed, or in the groups exposed to 0.14 or 1.28 ppm of SO_2.

Mechanical Properties of the Lungs.—The V_T values obtained during the preexposure period and the 78-week exposure period for the control group and the groups exposed to SO_2 were compared. The groups exposed to 0.14, 1.28, and 4.69 ppm of SO_2 had increases in V_T comparable to the increase observed in the control group, but the group exposed to 0.64 ppm of SO_2 had a slight but significantly lower rate of increase in V_T than the control group. The RR values for the groups exposed to 0.14, 0.64, 1.28, and 4.69 ppm were comparable to the RR value of the control group. Comparable fluctuations were observed in RR in all groups, as were previously observed. The Rrs(i) and Rrs(e) values obtained during the preexposure period and the 78 weeks of exposure for the control group and the group exposed to 4.69 ppm of SO_2 are presented in Fig 3 and 4, respectively. There was a general trend toward decreasing values of both Rrs(i) and Rrs(e) in all groups, and no significantly higher values in Rrs(i) or Rrs(e) were observed in the groups exposed to SO_2 than in the control group. For the group exposed to 4.69 ppm of SO_2 there was, however, more variation in Rrs(i) values following the overexposure accident than in the Rrs(i) values of the control group. Values for RI followed the same trend, and no significant differences were observed between the control group and the groups exposed to SO_2. At the beginning of the exposure, Cdyn(i) values were comparable in all groups and were as follows: 7.86 ml/cm H_2O for the control group 8.00 mg/cm H_2O, 7.91 mg/cm H_2O, and 7.02 mg/cm H_2O, and 7.15 mg/cm H_2O for the groups exposed to 0.14, 0.64, 1.28, and 4.69 ppm of SO_2, respectively. The values for Cdyn(i) remained within a narrow range during the entire exposure period, and no significant trends were noted with exposure to SO_2. Similarly, values for W(i)/ml V_T and W(e)/ml V_T exhibited no significant changes due to SO_2 exposure.

Distribution of Ventilation, Diffusion, and Arterial Blood.—Values for the number of breaths between the beginning and end of the test it took to reach an end-expiratory N_2 concentration of 1% (N[1% N_2]) for nitrogen washout measured during the preexposure period and the 78-week exposure period are presented in Fig 5. The data were analyzed and a third-degree polynomial curve fitting is presented for the control group and the group exposed to 4.69 ppm of SO_2. There were no significant differences due to SO_2 in the groups exposed to 0.14, 0.64, and 1.28 ppm of SO_2. For the group exposed to 4.69 ppm of SO_2, a definite increase in N(1% N_2) values occurred following the overexposure accident. To further delineate this increase in N(1% N_2) values, the data were analyzed with the CUSUM technique[37-39] and the results are presented in Fig 6. In Fig 6, *top*, the CUSUM of the control group indicated that no change took place during the preexposure or exposure period, whereas the CUSUM of the group exposed to 4.69 ppm of SO_2 indicated a significant increase in N(1% N_2) values starting around week 33. Figure 6, *bottom*, also shows that a significant change occurred around week 33. Also measured during the nitrogen washout measurements were the time from the beginning of the test to reach an end-expiratory N_2 concentration of 1% (t[1% N_2]) and the cumulative V_T from the beginning to the end of the test (CV_T[1% N_2]), and in both instances anal-

values following the over- than in the $R_{rs}(i)$ val- l group. Values for R_1 trend, and no significant bserved between the con- groups exposed to SO_2 f the exposure, $C_{dyn}(1)$ rable in all groups and .86 ml/cm H_2O for the ng/cm H_2O, 7.91 mg/cm cm H_2O, and 7.15 mg/cm s exposed to 0.14, 0.64, of SO_2, respectively. The remained within a nar- e entire exposure period. trends were noted with milarly, values for $W(i)$/ ml V_T exhibited no sig- to SO_2 exposure.

ntilation, Diffusion, and lues for the number of beginning and end of each an end-expiratory 1% (N[1% N_2]) for measured during the and the 78-week expo- nted in Fig 5. The data hird-degree polynomi- sented for the control exposed to 4.69 ppm of significant differences roups exposed to 0.14, of SO_2. For the group of SO_2, a definite in- values occurred follow- accident. To further in N(1% N_2) values, ed with the CUSUM results are presented , the CUSUM of the d that no change took exposure or exposure USUM of the group of SO_2 indicated a N(1% N_2) values 33. Figure 6, *bottom*, icant change occurred measured during the surements were the g of the test to reach concentration of 1% cumulative V_T from end of the test both instances anal-

Analytical Concentrations of SO_2 in Exposure Chambers

Date	No.	First Level of Exposure, $\bar{X} \pm (SD)$	Second Level of Exposure, $\bar{X} \pm (SD)$	Third Level of Exposure, $\bar{X} \pm (SD)$	Fourth Level of Exposure, $\bar{X} \pm (SD)$
7/17/67-7/31/67	15	...	...	0.97 ± (0.25)	4.68 ± (0.35)
7/26/67-7/31/67	6	...	0.49 ± (0.06)	...	...
8/1/67-8/31/67	31	...	0.50 ± (0.03)	1.03 ± (0.07)	4.89 ± (0.24)
8/8/67-8/31/67	24	0.12 ± (0.05)	...	...	...
9/1/67-9/30/67	30	0.10 ± (0.01)	0.50 ± (0.03)	1.02 ± (0.13)	5.03 ± (0.19)
10/1/67-10/31/67	31	0.12 ± (0.03)	0.51 ± (0.13)	1.04 ± (0.22)	5.28 ± (1.62)
11/1/67-11/30/67	30	0.10 ± (0.01)	0.51 ± (0.04)	1.06 ± (0.14)	4.45 ± (0.53)
12/1/67-12/31/67	31	0.11 ± (0.02)	0.45 ± (0.07)	1.13 ± (0.24)	4.32 ± (0.52)
1/1/68-1/31/68	31	0.16 ± (0.26)	0.54 ± (0.23)	1.18 ± (0.10)	4.53 ± (0.61)
2/1/68-2/11/68	11	...	...	...	4.38* ± (0.17)
2/1/68-2/29/68	29	0.11 ± (0.02)	0.49 ± (0.08)	1.09 ± (0.22)	...
3/1/68-3/31/68	31	0.12 ± (0.05)	0.51 ± (0.03)	0.94 ± (0.13)	...
4/1/68-4/30/68	30	0.10 ± (0.02)	0.47 ± (0.06)	1.01 ± (0.13)	...
5/1/68-5/31/68	31	0.14 ± (0.03)	0.81 ± (0.04)	1.72 ± (0.16)	...
6/1/68-6/30/68	30	0.13 ± (0.03)	0.70 ± (0.03)	1.51 ± (0.20)	...
7/1/68-7/31/68	31	0.18 ± (0.05)	0.75 ± (0.05)	1.53 ± (0.11)	...
8/1/68-8/31/68	31	0.24 ± (0.03)	1.22 ± (0.06)	2.21 ± (0.11)	...
9/1/68-9/30/68	30	0.25 ± (0.02)	1.25 ± (0.04)	2.06 ± (0.14)	...
10/1/68-10/31/68	31	0.23 ± (0.02)	0.99 ± (0.04)	2.00 ± (0.09)	...
11/1/68-11/30/68	30	0.11 ± (0.01)	0.49 ± (0.05)	0.93 ± (0.09)	...
12/1/68-12/31/68	31	0.11 ± (0.03)	0.48 ± (0.05)	0.98 ± (0.10)	...
1/1/69-1/10/69	10	...	...	0.98 ± (0.09)	...
1/1/69-1/17/69	17	...	0.46 ± (0.05)	...	...
1/1/69-1/30/69	30	0.11 ± (0.03)	...	...	...
$\bar{X}\bar{X} \pm (SD)$		0.14 ± (0.05)	0.64 ± (0.25)	1.28 ± (0.42)	4.69 ± (0.34)

* Overexposure accident occurred on Feb 11, 1968, after 30 weeks of exposure. In this incident, the concentration was estimated to be as high as 1,000 ppm and not lower than 200 ppm for a period of one hour. Following this accident, the animals remained in the chamber for the following 48 weeks until Jan 9, 1969, when they were killed; they were not further exposed to SO_2 during that period.

ysis of the data similar to N(1% N_2) revealed a definite increase in those values in the group exposed to 4.69 ppm of SO_2 after the overexposure accident. All groups had comparable diffusing capacities of the lung for carbon monoxide prior to exposure and no significant trends were observed during the exposure to SO_2. There was a significant decrease in Pa_{O_2} values in the group exposed to the 4.69 ppm of SO_2 during the exposure period. A small decrease occurred in the group exposed to 1.28 ppm of SO_2 at the beginning of exposure, but in this group the values for Pa_{O_2} were comparable to the control group during the remainder of the exposure period. No significant changes occurred in the groups exposed to 0.14 or 0.64 ppm of SO_2. No changes were observed in Pa_{CO_2} values in the groups exposed to SO_2, and arterial pH values remained essentially constant with the exception of a low pH value of 7.32 observed at week 77 in the group exposed to 4.69 ppm of SO_2.

Hematological and Clinical Biochemical Determinations.—The values obtained for the hematocrit reading, hemoglobin, and erythrocyte count were within normal limits in all groups during the exposure to SO_2. High mean values for leukocytes of 11,000 to 15,000 were observed in all groups during the preexposure period. These values decreased during the exposure and reached 8,700 to 9,700 at week 77. A high value of 13,000 was observed at week 34 for the group exposed to 4.69 ppm of SO_2. This was three weeks after the overexposure accident which occurred in this group's chamber. Values for lymphocytes and neutrophils were within normal limits in all groups. Blood urea nitrogen and total bilirubin determina-

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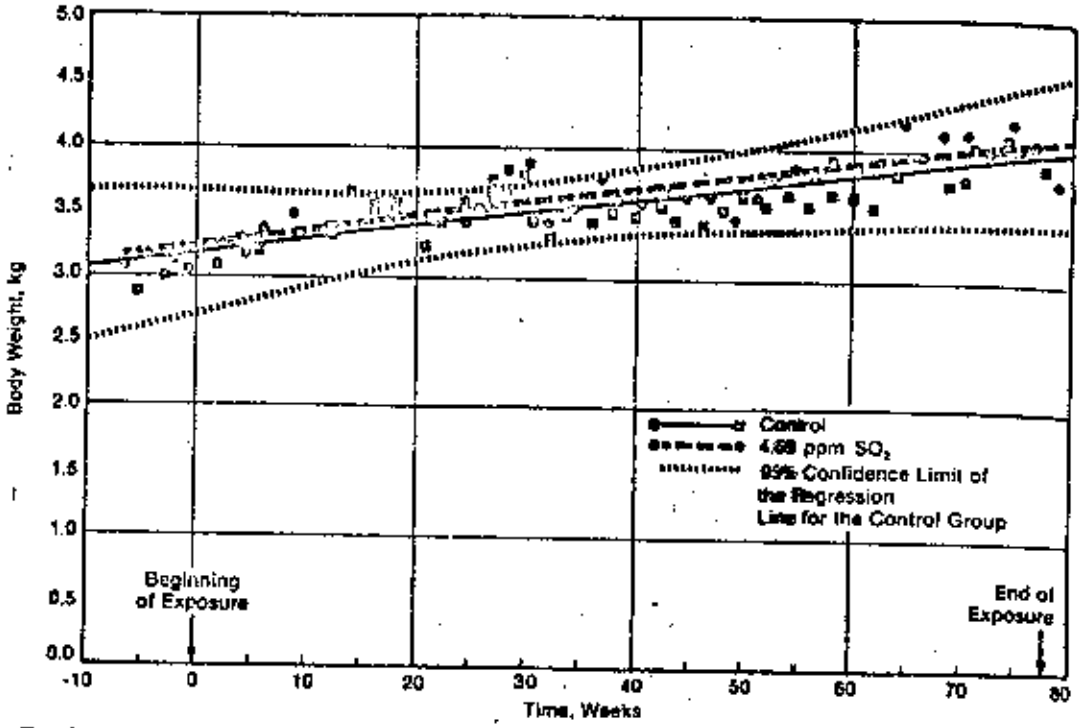
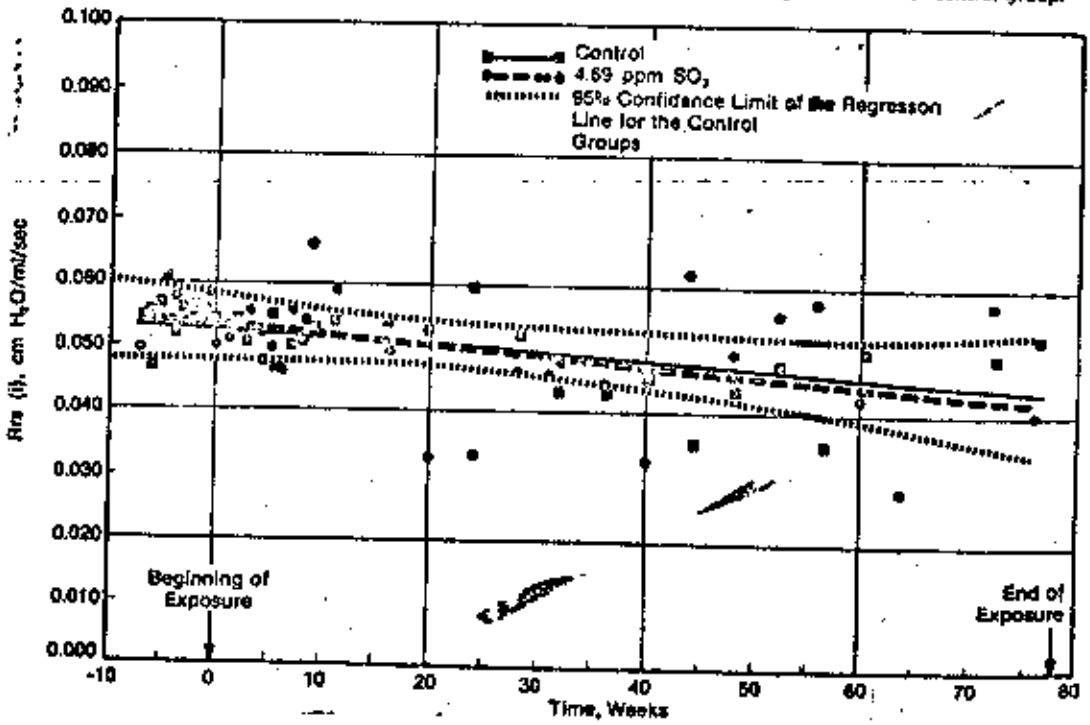
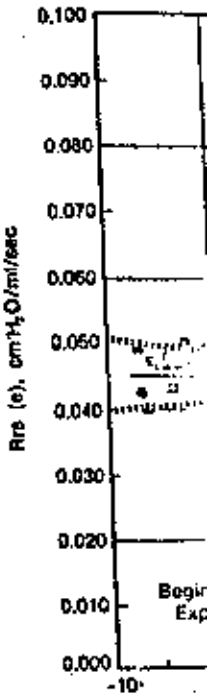


Fig 2.—Body weight data prior to and during exposure to SO_2. Each point represents mean of nine animals, and curves were fitted with first-degree polynomial. Regression lines for groups exposed to 0.14, 0.64, and 1.28 ppm of SO_2 are not presented as they fell between 95% confidence limits of regression line for control group.

Fig 3.—Rrs(i) data prior to and during exposure to SO_2. Each point represents mean of nine animals, and curves were fitted with first-degree polynomial. Curves for groups exposed to 0.14, 0.64, and 1.28 ppm of SO_2 are not presented since they lay between 95% confidence limits of regression line for control group.





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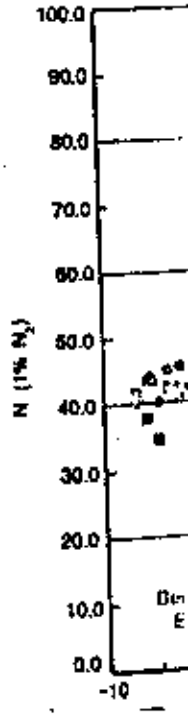

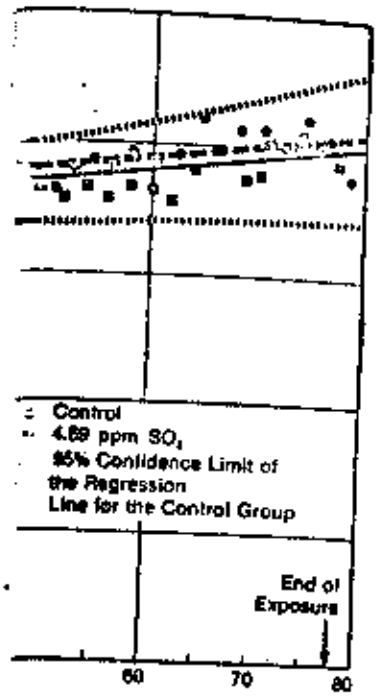

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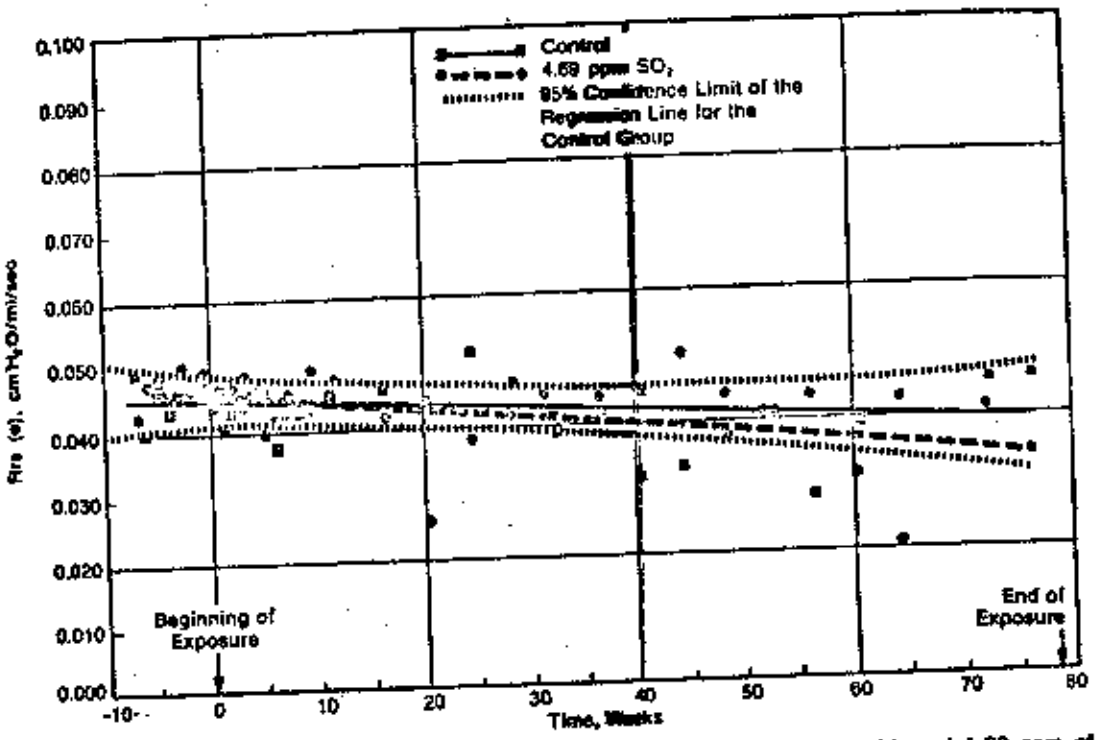
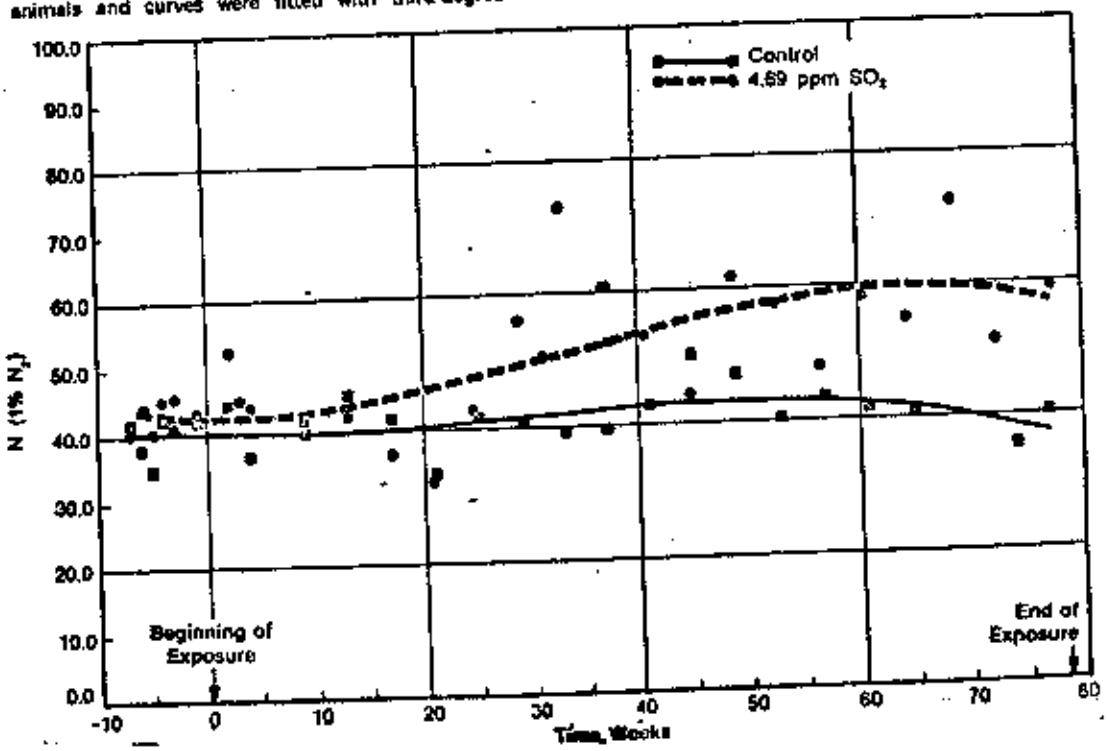


Fig 4.—Rrs(s) data prior to and during exposure to SO_2. Each point represents mean of nine animals, and curves were fitted with first-degree polynomial. Curves for groups exposed to 0.14, 0.64, and 1.28 ppm of SO_2 are not presented since they lay between 95% confidence limits of regression line for control group.

Fig 5.—N(1% N_2) data prior to and during exposure to SO_2. Each point represents mean of nine animals and curves were fitted with third-degree polynomial. Regression lines for groups exposed to 0.14, 0.64, and 1.28 ppm of SO_2 are not presented as they were comparable to control group.



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tions did not indicate any particular trend and a considerable variation was present. Serum total protein and albumin values were comparable in all groups and remained essentially constant during the exposure period. Similarly, serum electrolytes, including Na+, K+, Cl−, and Ca++, were comparable in all groups and remained within narrow limits during the exposure period. Serum glutamic oxaloacetic transaminase determinations remained within normal limits and considerable variation was present. For SGPT, no value higher than that of a control was observed but all groups exhibited low values at various time intervals. The results from measurements of serum lactic acid dehydrogenase and serum alkaline phosphatase indicated that all groups were comparable at the beginning of the exposure, and a definite trend toward lower values was present for all groups as the exposure progressed.

Terminal Body Weight, Organ Weight, and Organ-Body Weight Ratios.—Analysis of variance of terminal body weight, organ weight, and organ-body weight ratios obtained separately for males and females did not reveal any significant differences between the control and exposed groups.

Histopathological Examination.—*Control Group.*—Microscopic examinations of the lungs from the nine monkeys in the control chamber did not reveal any meaningful alterations. A slight to moderate degree of perivascular and peribronchiolar pigmentation was present in some of the animals in this group. This pigment was crystalline in form and generally brownish to black in color. The majority of this pigment had been phagocytized by tissue macrophages. A similar pigment was present in the peribronchial lymph nodes of these animals. The lungs from these animals were within the limits of normal histological variation and no evidence of occult disease was present.

Three instances of submucosal histiocytic infiltration and submucosal lymphoid hyperplasia were seen in the trachea. Here again, these microscopic alterations were slight in severity and are frequently seen in this species of primate. The livers were within the limits of normal histological variations for this species of monkeys. A few instances of slight hepatocyte pigmentation and Kupffer's cell pigmentation were seen. The kidneys from these animals were within the limits of histological variation and only a few incidental microscopic alterations were present. A minimal to moderate degree of interstitial nephritis was present in two of the males in this group.

One animal from this group died of gastric distention during the 77th week of exposure.

Group Exposed to 0.14 ppm of Sulfur Dioxide.—The lungs from all the animals in this group were comparable to those from the controls. Peribronchial and perivascular pigmentation, similar to that seen in the controls, was present from a slight to moderate degree in all nine animals. A parasitic granuloma involving some alveoli was present in one animal.

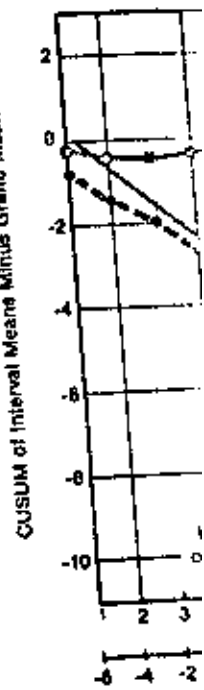
Crystalline pigment was present from a minimal to moderately severe degree in the peribronchial lymph nodes in six of the nine animals in this group. No meaningful alterations were seen in the tracheae, livers, or hearts.

Group Exposed to 0.64 ppm of Sulfur Dioxide.—The lungs from all the surviving animals within this group were within the limits of histological variation and compared well with the controls. In one animal, which died from gastroenteritis, a moderate pulmonary edema was present. The pulmonary edema seen in this animal was probably terminal and resulted from protracted clinical gastroenteritis.

With the exception of a moderate degree of submucosal lymphoid hyperplasia in one animal, the tracheae from the animals in this group were not remarkable. Minimal to moderate amounts of crystalline pigment were seen in the peribronchial lymph nodes in eight of nine animals in this group. The hearts and livers from the animals in this group were comparable to the controls.

Group Exposed to 1.28 ppm of Sulfur Dioxide.—Peribronchial and perivascular pigment, similar to that seen in the controls, was present in the lungs from the majority of the animals in this group. No other meaningful alterations were present in the lungs.

A minimal to slight degree of submucosal lymphoid hyperplasia was present in the tracheae of two animals in this group. Crystalline pigment was present in the peribron-



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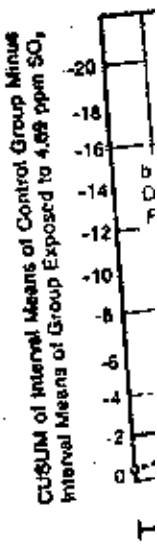

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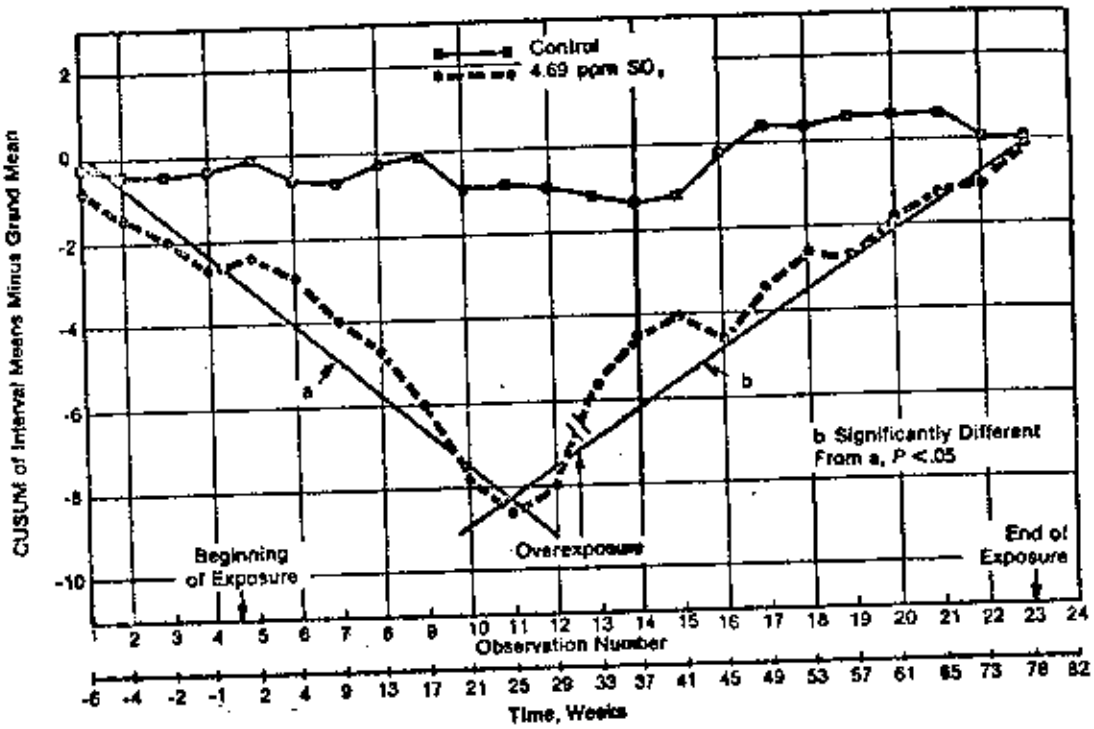
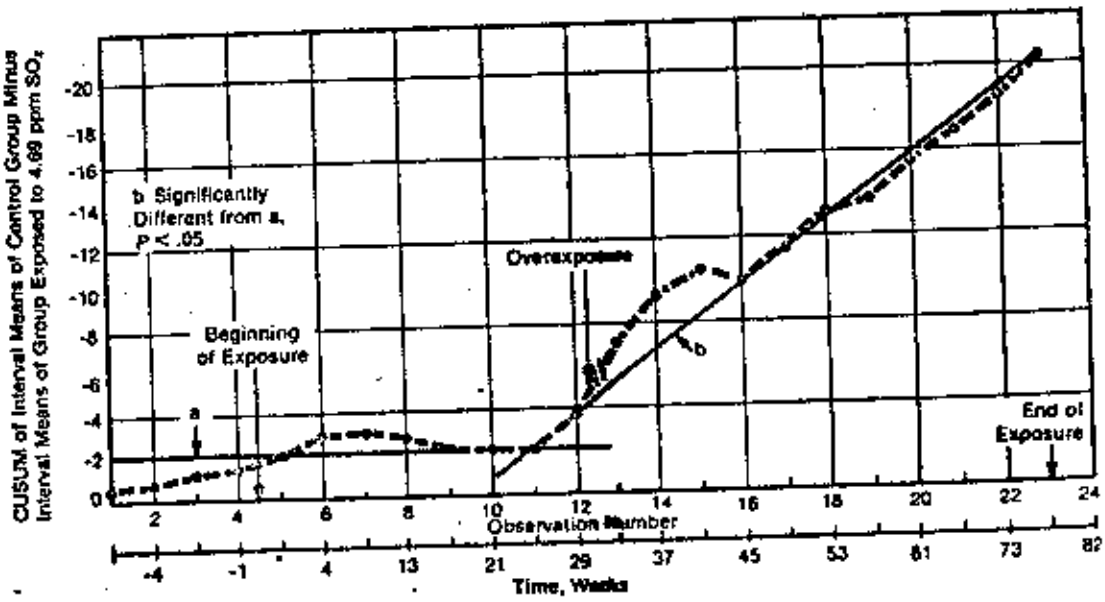


Fig 6.—Top, CUSUM transformation of data presented in Fig 5 for control group and group exposed to 4.69 ppm of SO_2. Data for control group remained essentially constant. Two distinct segments are present for group exposed to 4.69 ppm indicating a difference between data prior to and following overexposure. Bottom, CUSUM transformation of data presented in Fig 5 based on time-paired differences between control group and group exposed to 4.69 ppm of SO_2. Two distinct segments are present indicating that control group and exposed group were comparable before overexposure but significantly different following overexposure.



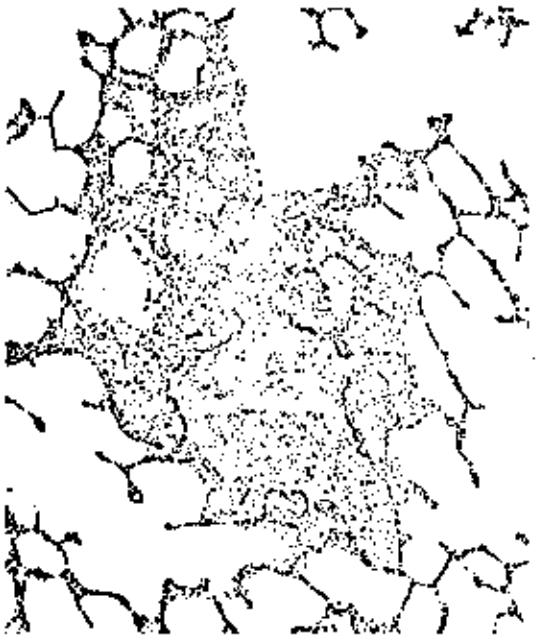


Fig 7.—Lung of animal overexposed to SO_2 showing alveolitis and bronchiolitis with pneumocyte hyperplasia (hematoxylin-eosin reduced from ×35).

chial lymph nodes in five of nine animals in this group. Two animals were affected with a minimal focal interstitial nephritis. The hearts and livers from all of the nine animals in this group did not show any meaningful alterations and were comparable to the controls.

Group Exposed to 4.69 ppm of Sulfur Dioxide and Overexposure Accident.—Histopathological alterations were seen in the lungs from all of these animals. Microscopic alterations were present in the respiratory bronchioles, alveolar ducts, and alveolar sacs. Focally distributed throughout all lobes of the lungs, several groups of alveoli contained moderate amounts of proteinaceous material and numerous alveolar macrophages. These alveoli were generally those opening directly off of the respiratory bronchioles. The alveolar walls were moderately thickened and infiltrated with histiocytes. Multinucleated giant cells were present in some alveolar lumina. The walls of the respiratory bronchioles associated with these foci were moderately infiltrated with histiocytes and leukocytes. Moderate hyperplasia of the bronchiolar epithelium was also present. Frequently the lumina of the respiratory bronchioles were plugged with proteinaceous material, macrophages, and leukocytes.

In eight of the nine animals, bronchiectasis and bronchiolectasis were present to a moderate degree. The walls of many of the affected bronchioles were moderately thickened.

Even though the overexposure had occurred 48 weeks before the termination of exposure of these animals, there was little evidence of fibrosis. These pulmonary lesions appeared active and would be classified as subacute. The alterations described above are demonstrated by Fig 7.

Microscopic alterations apparently related to the exposure to SO_2 were also seen in the liver. These changes were characterized by diffuse and focal hepatocyte vacuolation varying in severity from moderate to severe. This hepatocyte vacuolation was seen in all nine animals in this group and was not seen in any of the control animals.

Moderate amounts of crystalline pigment, similar to that seen in controls, were present in the peribronchial lymph nodes. In the kidneys, a focal, minimal to slight interstitial nephritis was seen in two animals. The tracheae and hearts from all the animals in the group were within the limits of histological variation.

Comment

We reported previously[26] that no detrimental changes to the pulmonary system in guinea pigs could be detected after one year of exposure to concentrations ranging from 0.13 to 5.72 ppm of SO_2. The results presented in this report indicate that no detrimental changes in monkeys can be attributable to continuous exposure to SO_2 at 0.14, 0.61, or 1.28 ppm for a period of 78 weeks. The group exposed to 4.69 ppm for a period of 30 weeks did not seem to be adversely affected. The overexposure accident was followed by a definite deterioration in pulmonary function which was demonstrated by the changes in the distribution of pulmonary ventilation as measured by the nitrogen washout technique. The microscopic findings in this group indicated that not only the lungs had been adversely affected, but also that alterations in the liver, similar to those seen in guinea pigs exposed to 5.72 ppm,[26] were present. Another group of animals is now being exposed to 5.0 ppm for 78 weeks.

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There are only a few reports involving chronic exposure of normal animals to sulfur oxides in which pulmonary function tests were included to delineate the effects on the pulmonary system. In a study by Goldring et al,[26] hamsters exposed chronically but not continuously to a very high concentration of SO_2 had only mild bronchitic lesions and relatively minor changes in the mechanical properties of the lung. These authors concluded that even at high concentrations, SO_2 failed to produce an important effect on the experimental production of obstructive lung disease in this species. Lewis et al[25] compared the effects of 5.1 ppm of SO_2, 5.1 ppm of SO_2 plus 835μg/cu m of H_2SO_4, or 0.755μg/cu m of H_2SO_4 in dogs impaired by prior exposure to 26 ppm of nitrogen dioxide with dogs not impaired. They did not report a statistically significant change in pulmonary function between the impaired and unimpaired control groups and any one of the exposed groups that received SO_2, H_2SO_4, or a mixture of both. They reported some differences between the various groups and suggested that the oxides of sulfur appeared to affect the unimpaired dogs more adversely than the impaired animals.[25] A study by Vaughan et al[24] involved dogs exposed 16 hours a day for a period of 18 months to a combination of 0.5 ppm of SO_2 and 100μg/cu m of H_2SO_4 mist. These authors concluded that after 18 months of exposure there were no detectable changes in the mechanical properties of the lung or in the diffusing capacity of the lung in the exposed animals. These results are similar to those obtained previously in guinea pigs[26] and those presented in this article. Vaughan et al[24] cautioned that their failure to detect impairment of pulmonary function might have been due to several factors, including (*a*) actual lack of effect due to exposure, (*b*) inability of the methods to detect subtle abnormalities, (*c*) quickly reversible effects due to removal of the animals from the contaminated atmosphere for measurements of pulmonary function, and (*d*) a large range of normal values in the control population necessitating large differences to validate the changes statistically. Also, in the studies by Vaughan et al[24] and Lewis et al,[25] measurements of pulmonary function were made during or following anesthesia, which, in itself may influence the results greatly. Thus, each of these points mentioned above must be evaluated before it can be concluded with any degree of certainty that the first, that is, actual lack of effects from exposure to SO_2, is the most probable choice. In such an attempt, the ability of the pulmonary function tests used in this study to detect subtle abnormalities must be considered first. Measurements of the mechanical properties of the lung, including V_T, RR, respiratory system flow resistance, RI, Cdyn(1), W(i), and W(e), have been made by numerous investigators in order to evaluate the effects of acute or subacute exposure to various pulmonary irritants, bronchoconstrictors,[3,40-43] and inert dusts[44,45] or to evaluate patients with obstructive respiratory diseases.[46-50] There is no doubt that these parameters are sensitive indices of respiratory impairment, particularly in acute exposure in which direct or reflex bronchoconstriction may occur[3]; but, when subtle changes have occurred, principally in the peripheral airways, parameters such as airway resistance can fail to indicate obstruction, as recently reported by Hogg et al.[50] As recent literature attests, there is also an obvious lack of knowledge about the correlation between subtle microscopic alterations in the lung and concomitant changes in these physiological parameters. In a review of the various techniques to assess the distribution of ventilation or distribution of inspired air in the lung, Bouhuys[51] has pointed out the usefulness of the nitrogen washout technique in detecting subtle changes due to small airway obstruction which would have otherwise gone undetected by measurements of airway resistance.[50] Bouhuys states that this measurement has proven a valuable tool in assessing the pathogenesis of bronchial asthma, emphysema, and worker's byssinosis.[51-54] Significant distribution changes could be demonstrated with inhaled histamine when subjective symptoms in humans were absent.[53] The technique has also been used in dogs exposed to phosgene and has revealed deterioration in pulmonary function.[43] This technique, adapted for the cynomolgus monkey, has been successful in detecting the effect of the overexposure of SO_2 sustained in the group exposed to 4.69 ppm.

The measurement of the diffusing capacity of the lung is probably the most difficult to evaluate. Forster[59,61] indicated the various factors governing the lung's diffusing capacity for carbon monoxide, the discrepancies between the methods used for evaluating this parameter, and the factors to be considered in the interpretation of the results. On the other hand, Long and Hatch[35] and Rinehart and Hatch[58] have used CO uptake measurements and have concluded that, following exposure to low levels of pulmonary irritants, a decrease could be observed without observable microscopic alterations in the lung. In addition, exposure to ozone has been reported to result in significant decreases in the diffusing capacity of the lung,[59,60] and reduction in CO uptake has been reported in cases of emphysema.[32] In a previously published article,[26] we reported a higher diffusing index in the group of guinea pigs exposed to 5.72 ppm of SO_2. This finding correlated well with the microscopic examination of the lung which revealed a much lower incidence of spontaneous pulmonary disease at this exposure level than in the control group.

While it is true that transient effects could not be detected by pulmonary function tests performed on animals removed from the exposure atmosphere, our study was directed toward the elucidation of effects resulting from chronic exposure. Thus, our experimental design purposely precluded the detection of immediate and possibly reversible effects of SO_2 reflex bronchoconstriction.[7,10] Moreover, on the basis of published data from short-term human exposures,[11-13,17] one would not expect consistent changes in the mechanical properties of the lung at concentrations up to 5 ppm. In guinea pigs, probably the most sensitive species studied in acute exposure to SO_2, an increase of only 10% in R1 is observed at 0.16 ppm of SO_2[19] and 25% at 26 ppm,[3] with a rapid return toward control values upon termination of the exposure.

Lastly, Vaughan et al[21] and Lewis et al[25] reported large variations, and large changes were needed to detect a statistically significant change. In our study, the five groups of animals had mean values which were very close for all parameters before the beginning of exposure. In addition, a sufficiently long preexposure observation period assured the establishment of baseline control values in each group, and measurements were made frequently during the entire 78 weeks of exposure to insure a higher chance of detecting any change.

In view of the above, we believe that when we have stated that there were no deleterious changes in the pulmonary system, there was indeed a genuine lack of effect ascribable to SO_2. This is also substantiated by the fact that histopathological examination of the lungs confirmed our observations with pulmonary function tests.

Finally, we must point out that negative results obtained with SO_2 on R1 and Rrs in monkeys exposed for a period of 18 months to SO_2 in no way contradict the findings in guinea pigs by Amdur and Mead[3] and Amdur[19] and in man by several authors[11-13,17] who found an increase, although relatively small, in R1 with short-term exposure up to 5 ppm SO_2. Indeed, in these studies, a slight bronchoconstriction was apparent during exposure.[7] In studies on long-term chronic exposure, increases in R1 or Rrs are more likely to be caused by tissue deterioration than by sustained reflex bronchoconstriction, and it is important to distinguish the two phenomena. Furthermore, the use of R1 or Rrs may not be quite appropriate to detect deterioration in pulmonary function if the effects are at the periphery, since a large increase, up to 100%, in the peripheral resistance would contribute only a small fractional increase in R1 or Rrs.[50] As pointed out by Hogg et al,[30] measurement of distribution of ventilation would be more appropriate to detect pulmonary damage in these instances. This was also found by Rossing[43] when exposing dogs to phosgene for periods of 13 weeks and also reported here in cynomolgus monkeys exposed to 4.60 ppm of SO_2, following the overexposure accident, and in a previous report[31] in cynomolgus monkeys exposed to NO_2.

The present findings in cynomolgus monkeys and the results reported earlier for the guinea pig[26] indicate that SO_2 in concentrations up to 1 ppm does not cause alterations in pulmonary function, nor are detrimental changes seen on microscopic examination of pulmonary tissues. In this connection, it should be remembered that Vaughan et al[24]

ration period assured the baseline control values in measurements were made the entire 78 weeks of a higher chance of detect-

above, we believe that ted that there were no in the pulmonary sys- need a genuine lack of SO_2. This is also substan- that histopathological ex- ngs confirmed our obser- ary function tests.

point out that negative SO_2 on R1 and Rrs in a period of 18 months ontradict the findings in ur and Mead[2] and Am- y several authors[11-13,17] ase, although relatively short-term exposure up eed, in these studies, a ction was apparent dur- ies on long-term chron- in R1 or Rrs are more by tissue deterioration ex bronchoconstriction, to distinguish the two more, the use of R1 or appropriate to detect monary function if the riphery, since a large in the peripheral re- ute only a small frac- or Rrs.[50] As pointed measurement of distri- would be more appro- nary damage in these so found by Rossing[13] phosgene for periods reported here in cy- posed to 4.69 ppm of verexposure accident, port[31] in cynomolgus O_2.

in cynomolgus mon- sported earlier for the at SO_2 in concentra- not cause alterations nor are detrimental scopic examination of this connection, it that Vaughan et al[34] also obtained normal results in dogs chronically exposed to low concentrations of SO_2; these authors have not presented histopathological findings as yet. Thus, the results of studies in a rodent, the dog, and a primate have all been normal.

In urban atmospheric pollution, populations are exposed to SO_2 in combination with other gaseous and particulate pollutants, and the possibility of synergistic effects under these circumstances must be considered. In our studies, exposure of two groups of cynomolgus monkeys to fly ash at 0.16 and 0.46 mg per cubic meter for a period of 18 months did not produce significant changes in pulmonary function in these animals[31]; other groups are now undergoing exposure to combinations of SO_2 and fly ash. It should be possible, therefore, to elucidate the possible synergistic action of these two pollutants in studies on chronic exposure. In short-term studies, no synergistic action was reported between SO_2 and fly ash, while certain metallic salts when added to SO_2 were shown to increase the response of guinea pigs.[12,51]

Dr. Alarie is under special fellowship 1-F03-ES10198-01 from the National Institute of Environmental Health Sciences.

The data reported in this article have resulted from the Electric Research Council's Air Pollution Research Program financed by the Edison Electric Institute with assistance from the Tennessee Valley Authority, the American Public Power Association, and Bituminous Coal Research, Inc.

Nonproprietary and Trade Names of Drug

Phencyclidine hydrochloride—*Sernylan*.

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Long-Term Continuous Exposure of Guinea Pigs to Sulfur Dioxide

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Three groups of guinea pigs were exposed to 0.13, 1.01, and 5.72 ppm for 12 months. Pulmonary function measurements including tidal volume, respiratory rate, minute volume, dynamic compliance, pulmonary flow resistance, and carbon monoxide uptake indicated that no detrimental changes could be attributed to sulfur dioxide. Hematology and clinical chemistry measurements were normal, and body weight, growth, and survival were not adversely affected. Microscopic examination of the lung and trachea revealed that the group exposed to 5.72 ppm had a lower incidence and severity of spontaneous disease normally present in those animals after a period of 52 weeks. Microscopic examination of the liver revealed alterations in the animals exposed to 5.72 ppm which were characterized by an increase in the size of the hepatocytes accompanied by cytoplasmic vacuolation.

SULFUR DIOXIDE, a common pollutant of urban air, has received wide attention in the scientific literature, and several studies in animals[1-6] and in man[9-16] have been conducted to delineate its effects on the respiratory system. However, these studies relate to short exposures and usually at concentrations higher than those encountered in the urban atmosphere, with the exception of guinea pigs exposed to 0.16 and 0.5 ppm SO_2 which produced an increase in pulmonary flow resistance of 10% and 13%, respectively.[7] Few studies have been conducted to elucidate the biological effects of chronic inhalation exposure in animals[17-19] but again the exposure levels were much higher than normally encountered in urban atmosphere. It is only recently that comprehensive pulmonary function tests have been used in chronic studies to determine the effects of low levels of sulfur oxides and reports on the effects of sulfur oxides in dogs have been published.[20,21]

We have undertaken studies to provide a basis for evaluating chronic effects of SO_2, and the part of our investigations reported herein was designed to determine the responses of guinea pigs to continuous inhalation of SO_2 at concentrations ranging from 0.1 to 5.0 ppm for a period of 12 months. These levels were selected because they represent those encountered in the urban atmosphere and the current threshold limit value (TLV) of 5 ppm established by the American Conference of Governmental Industrial Hygienists.[22]

Materials and Methods

Animal Groups.—Four groups of 120 Hartly

Submitted for publication Feb 20, 1970; accepted April 3.

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strain albino guinea pigs, weighing 180 ± 50 gm were used in this study. One group served as control while the other three were exposed to SO_2. Equal numbers of each sex were included. In each group, 40 animals were killed after three months of exposure.

Exposure Conditions.—The exposure to SO_2 was initiated after one week of adaptation and observation of the animals in the exposure chamber and one week of preexposure for physiological measurements on the animals. The duration of exposure was approximately 22 hours per day due to opening the chamber for cleaning purposes or for taking the animals out of the chamber for biological measurements. The animals were exposed seven days a week for 52 continuous weeks.

Exposure chambers of stainless steel and glass, having dimensions of 6 × 6 × 6 feet with pyramidal tops and bottoms, were used as shown in Fig 1. Air supplied to each chamber passed through a charcoal filter and an absolute filter before entering the chamber. Air left the chamber at the bottom and was exhausted through a charcoal filter by a positive displacement pump. Airflow through the chamber was maintained at approximately 1,000 liters per minute. The temperature was maintained at 72 ± 2 F. The relative humidity was maintained at 50% ± 10%.

Generation of the desired concentration of SO_2 was from cylinders of 1% or 5% SO_2 in nitrogen. Known amounts of these gas mixtures were metered into the incoming air and the concentration was frequently monitored by a sulfur dioxide analyzer. One instrument monitored both the 0.1- and 1.0-ppm chambers and another monitored the 5.0-ppm chamber. The instruments were calibrated against the wet chemical method of West and Gaeke.[23] Samples of chamber air were taken daily with an impinger and analyzed by this same method as a calibration check.

Biological Measurements.—Biological measurements included body weight, pulmonary function tests, hematology, and clinical biochemical determinations. Ten males and ten females were taken from each group before the beginning of the exposure, after three months of exposure, and after 52 weeks of exposure. Measurements were made of tidal volume (V_T), respiratory rate (RR), minute volume (MV), dynamic compliance [Cdyn (l)], pulmonary flow resistance (Rl), and work of breathing (W). These measurements were made according to techniques previously developed.[24-29] After these measurements, blood samples were taken and the following determinations were performed: hematocrit, hemoglobin, erythrocytes (RBC), leukocytes (WBC), blood urea nitrogen (BUN), serum glutamic oxaloacetic transaminase (SGOT), and serum glutamic pyruvic transaminase (SGPT). Thereafter, these animals were killed.

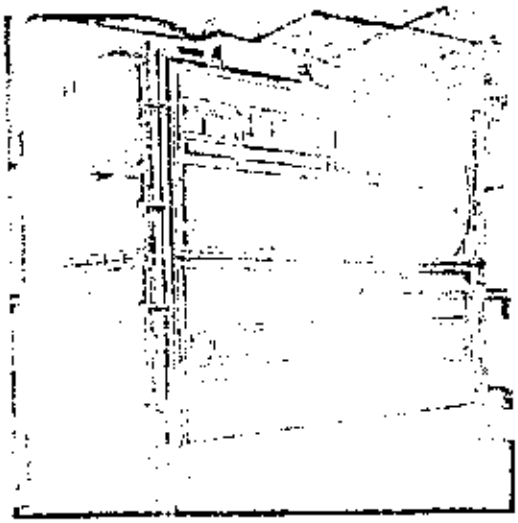

Fig 1.—Chamber holding guinea pigs for exposure to sulfur dioxide.

Measurements of respiratory system flow resistance (Rrs) and carbon monoxide uptake ($\dot{V}_{co}$) were made according to techniques pre-

Table 1.—*Monthly Mean Concentration ± SD of Sulfur Dioxide in Exposure Chambers During 12 Months*

Months	Low Level	Intermediate Level	High Level
1*	0.08 (0.025)	0.69 (0.23)	3.69 (0.87)
2	0.11 (0.084)	0.77 (0.35)	4.97 (0.67)
3	0.11 (0.020)	0.96 (0.24)	5.27 (0.85)
4	0.10 (0.019)	1.10 (0.34)	5.10 (0.64)
5	0.10 (0.022)	1.14 (0.22)	4.75 (0.59)
6	0.12 (0.052)	1.08 (0.15)	4.61 (0.40)
7	0.14 (0.128)	1.13 (0.62)	5.10 (0.38)
8	0.12 (0.040)	1.01 (0.08)	7.40 (0.40)
9	0.17 (0.022)	1.60 (0.16)	7.00 (0.32)
10	0.17 (0.028)	1.54 (0.12)	7.18 (0.36)
11	0.17 (0.023)	1.63 (0.25)	6.80 (0.45)
12	0.17 (0.021)	1.66 (0.31)	6.77 (0.51)
13*	0.12 (0.051)	1.34 (0.30)	... ...
Mean	0.13	1.01	5.72
SD	0.029	0.14	1.23

* Fraction.

Table 2.—Percent Survival in Guinea Pigs Exposed to Sulfur Dioxide After a Period of 52 Weeks

	Sulfur Dioxide Concentrations			
	Control	0.13 ppm	1.01 ppm	5.72 ppm
Males	65.0	53.8	56.4	79.1*
Females	65.8	75.5	48.8	85.4*
Combines	60.4	64.7	52.6	82.3*

* Significantly higher than control, $P < 0.05$.

viously developed.[30,31] These measurements, made on ten males and ten females as a grab sample from each group, were performed twice before the beginning of the exposure, every week during the first four weeks of exposure, every three weeks until the 28th week of exposure, then every four weeks until the end of the exposure.

Necropsies were performed on a representative number from each group of animals at the following intervals: before exposure, after three months of exposure, and at termination of exposure. Microscopic examinations were made of the lungs, trachea, bronchial lymph nodes, heart, liver, and kidneys.

Results

Sulfur Dioxide Concentration.—The chamber atmosphere concentrations as determined analytically are presented in Table 1.

Body Weight and Survival.—Weekly body weight results were analyzed by Rao's technique.[32] The growth rate was similar in all groups with the exception of the group exposed to 0.13 ppm in which a lower growth rate was observed during the first 14 weeks for the males and over the entire 52 weeks for the females ($P < 0.05$). Survival over the 52 weeks of exposure was analyzed by the life-table technique.[33] The results, as presented in Table 2, indicated a significantly higher survival in both males and females exposed to 5.72 ppm as compared to the control group.

Pulmonary Function Measurements.—A 3 × 4 factorial analysis of variance with time including preexposure, 12 weeks, and 52 weeks of exposure and four SO_2 levels, viz, 0.0, 0.13, 1.01, and 5.72 ppm, was performed for the following parameters: V_T, RR, MV, Cdyn(I), RI, and W. The results indicated that no dose-related changes could be attributed to SO_2, and all of the above parameters for all groups increased or decreased significantly ($P < 0.05$) with time. The data for Rrs during inspiration [Rrs(i)] and during expiration [Rrs(e)] are presented in Fig 2 and 3. A third-degree polynomial was fitted to the observed data for Rrs (i) for each group and the regression coefficients were compared by the Student's *t*-test. No significant differences were found among the groups. In addition, an exponential function was fitted to that portion of the data from 7 to 52 weeks. This time span represented the more stable portions of the curves. No significant differences were found among the groups with this analysis. However, it can be observed in Fig 2 that an earlier stabilization and less variation around the estimated curve were present for the group exposed to 5.72 ppm.

For Rrs(e), a first-degree polynomial was fitted to the observed data for each group and the regression coefficients were compared by the Student's *t*-test. No significant differences were found among the groups.

Table 3.—Regression Analysis of V_{rs} (ml/min × 10^3) in Guinea Pigs During 52 Weeks of Exposure to Sulfur Dioxide*

SO_2 Concentration, ppm	Time Interval, Weeks	a	b	Sy.x	Sb	r	Peak	Time-to-Peak, Weeks
Control	0-25	0.339	0.0193	0.038	0.0013	0.9774	6.49	24.5
	25-52	0.965	−0.0058	0.050	0.0019	−0.7659	...	...
0.13	0-22	0.355	0.0216	0.042	0.0017	0.9725	6.25	20.4
	22-52	0.883	−0.0042	0.093	0.0028	−0.4910	...	...
1.01	0-22	0.330	0.0244	0.053	0.0021	0.9663	6.80	20.6
	22-48	0.968	−0.0066	0.042	0.0016	−0.8480	...	...
5.72	0-19	0.332	0.0303†	0.036	0.0018	0.9860	7.38†	17.71
	19-48	0.952	−0.0047	0.045	0.0015	−0.7521	...	...

* Prepeak and postpeak segments of data presented in Fig 3 (log y=a+bx).
† Significantly higher than control and 0.13 ppm, $P < 0.05$.
‡ Significantly less than control, $P < 0.05$.

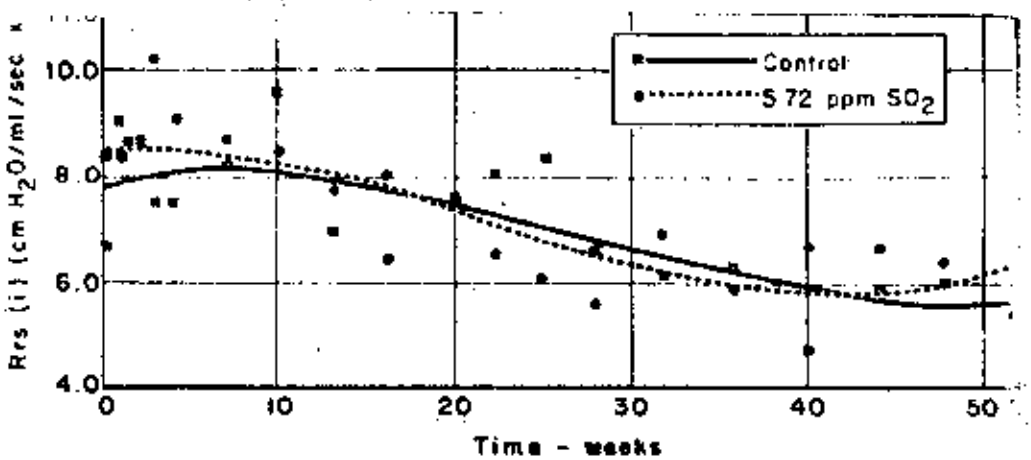

Fig 2.—Rrs (i) data in guinea pigs during exposure for 52 weeks. Each point represents mean of 20 animals. Curves were fitted with third-degree polynomial. Data points and curves for the groups exposed to 0.13 and 1.01 ppm are not presented as they overlap the control and 5 ppm SO_2.

Occasionally, higher than normal[13] values of Rrs(i) and Rrs(e) were encountered. These occurred randomly at various time intervals (Fig 2 and 3). They were particularly noted in the early phase of the experiment in all groups and were not dose-related.

The data obtained for $\dot{V}_{co}$ are presented in Fig 4. A fourth-degree polynomial was fitted, and the regression coefficients obtained for each group were compared by the Student's *t*-test. No significant differences were detected among the groups. However, it can be observed that for the group exposed to 5.72 ppm SO_2, the curve reached a higher plateau than the control group. This was observed for both the males and the females. To further delineate this observation, the prepeak and the postpeak segments of the untransformed curves for all the groups were examined by regression analysis. The equations were of the type log $y = a + bx$. The peak and time to peak curve intersections were calculated and results are presented in Table 3. The peak values for the group exposed to 5.72 ppm were significantly higher ($P < 0.05$) and the time to peak was significantly shorter ($P < 0.05$) than for the control group.

Hematological and Clinical Biochemistry Determinations.—A factorial analysis of variance was performed on the data obtained for those parameters. No dose-related effects could be detected in hematocrit, hemoglobin, RBC, WBC, SGOT, SGPT, or BUN.

Histopathological Examination.—The histopathological examination of tissues from animals from each group killed before exposure revealed no meaningful difference in incidence or severity of spontaneous diseases. The observations made on the lungs of animals exposed to 5.72 ppm and killed after 13 weeks of exposure revealed an apparent reduction of incidence and severity of spontaneous pulmonary disease. This was manifested primarily by a lack of histiocytic infiltration into the alveolar walls. Tracheitis was present in the control group and in the groups exposed to 0.13 and 1.01 ppm but was not seen in the group exposed to 5.72 ppm. Hepatocyte vacuolation was observed in a few animals from the control group, but was far less in degree and severity than for animals in the group exposed to 5.72 ppm.

The observations made on the lungs of animals killed after 52 weeks of exposure indicate a definite reduction in the incidence and severity of spontaneous pulmonary disease in the group exposed to 5.72 ppm. Representative lung sections are presented in Fig 5. In the control group and the groups exposed to 0.13 and 1.01 ppm, there was a relatively high incidence of spontaneous pulmonary disease characterized primarily by the presence of focal nodular lymphoid hyperplasia surrounding blood vessels, bronchi, and bronchioles. Histiocytic infiltration into the alveolar walls was prevalent in all groups except the group exposed to 5.72 ppm.

In the trachea, the histopathological alterations observed in the control group were characterized by submucosal infiltration of histiocytes and polymorphonuclear granulocytes. These were not prevalent in the ani-

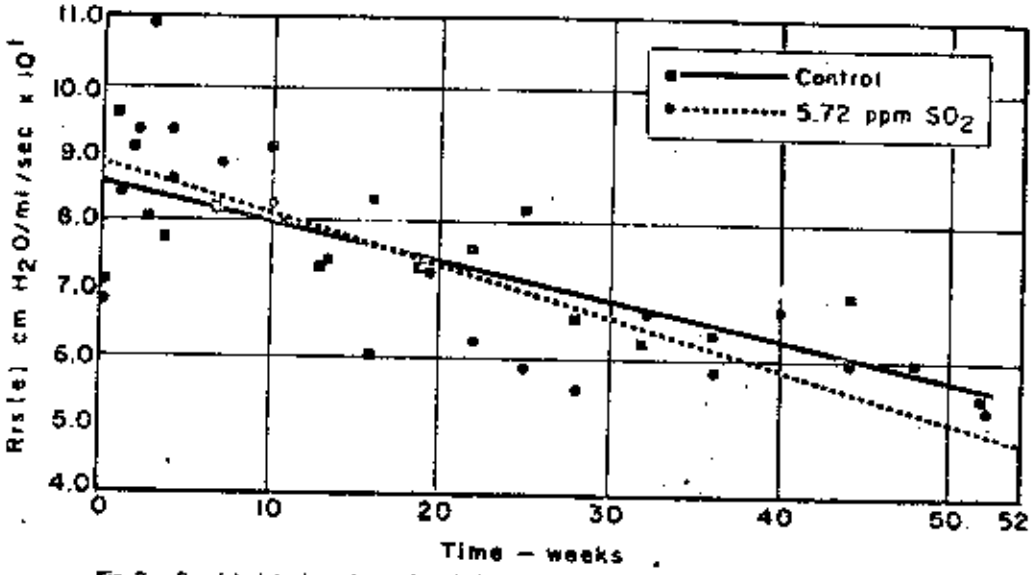

Fig 3.—Rrs (e) data in guinea pigs during exposure for 52 weeks. Each point represents mean of 20 animals. Curves were fitted with first-degree polynomial. Data points and curves for the groups exposed to 0.13 and 1.01 ppm are not presented as they overlap the control and 5 ppm SO_2.

mals exposed to 5.72 ppm. A low incidence of cytoplasmic vacuolation of the hepatocytes was present in the control group and the groups exposed to 0.13 and 1.01 ppm; however, the incidence and severity was much higher in the group exposed to 5.72 ppm as illustrated in Fig 6.

The hearts from all of the animals were within normal histological variations. Scattered instances of pericarditis and focal myocarditis were seen in a few of the exposed and control animals.

In control animals and those exposed to 0.13 and 1.01 ppm of SO_2 there was a relatively high incidence of nephritis and nephrosis, primarily in the males. This was greatly reduced in the animals exposed to 5.72 ppm of SO_2.

Comment

There are only a few reports on long-term exposure of animals to sulfur oxides in which pulmonary function measurements are featured.

Lewis et al[21] compared the effects of 225 days of exposure to 5.1 ppm of SO_2, 5.1 ppm of SO_2 plus 835μg/cu m of sulfuric acid, or 835μg/cu m of H_2SO_4 on dogs impaired by prior exposure to 26 ppm NO_2 for 191 days with dogs not thus impaired. Their analysis of the data revealed that: (a) dogs exposed to H_2SO_4 had a lower diffusing capacity than dogs in four other treated groups, but not exposed to H_2SO_4; (b) dogs exposed to SO_2 had a lower compliance and a higher resistance than dogs in four other treated groups, but not exposed to SO_2; and (c) unimpaired dogs exposed to SO_2 plus H_2SO_4 had a smaller residual volume than dogs in seven other treated groups. There was no indication that dogs exposed to SO_2, SO_2 plus H_2SO_4, or H_2SO_4 had significantly different values for diffusion capacity, compliance, resistance, or residual volume from control dogs.

A study, reported by Vaughan et al,[20] involved dogs exposed 16 hours a day for a period of 18 months to a combination of 0.5 ppm of SO_2 and 100μg/cu m of H_2SO_4. These authors concluded that after 18 months of exposure they could not detect any impairment in the pulmonary function of the animals. Their results are similar to ours obtained in guinea pigs in which no detrimental changes in pulmonary function could be detected. In fact, the group that we exposed to 5.72 ppm exhibited much less

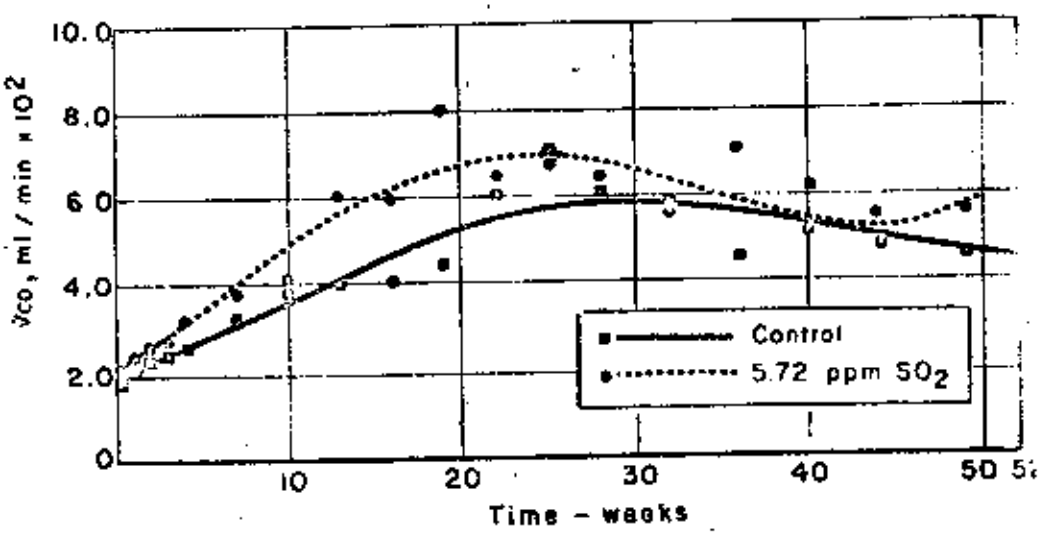

Fig 4.—$\dot{V}_{CO}$ data in guinea pigs during exposure for 52 weeks. Each point represents mean of 20 animals. Curves were fitted with fourth-degree polynomial. Data points and curves for the groups exposed to 0.13 and 1.01 ppm are omitted as they fell between the control group and the group exposed to 5.72 ppm SO_2.

variation in Rrs(i) than the control group and had a better diffusion index, as the $\dot{V}_{CO}$ values indicate. Our histopathological evaluation of the lung on animals exposed to 5.72 ppm revealed a decrease in the incidence and severity of spontaneous pulmonary disease normally present in 1-year-old control guinea pigs. This finding correlates well with the better diffusion index and the low variation in Rrs(i) of this group. In other studies[17-19] where microscopic examination was the criterion to evaluate the effects of SO_2 on mammals, there is no indication that levels below 5 ppm would induce alterations in the lung.

Our examination of the liver revealed hepatocyte vacuolation in the guinea pigs we exposed to 5.72 ppm.

This change did not influence the health of the guinea pigs as evidenced by the survival and growth curves. As we did not conduct a special investigation into the nature of the liver change, we can only speculate about its rate of development or its reversibility.

Our study, along with those of Lewis et al[21] and Vaughan et al,[20] is among the first designed to evaluate the chronic effects of SO_2, alone or in combination with other pollutants. They stand in contrast to the several[1-16] short-term animal and human experiments at concentrations higher than those encountered in the urban atmosphere man breathes, with the exception of guinea pigs exposed to 0.16 and 0.5 ppm of SO_2 which produced an increase in pulmonary flow resistance of 10% and 13%, respectively.[7] In our studies and those by Vaughan et al,[20] both with normal animals exposed to sulfur oxides for a long period of time, there was no indication of an increase in pulmonary resistance induced by the exposures. These findings are in contrast with increases in pulmonary flow resistance observed in guinea pigs and humans during short-term exposure.[1-16] Amdur has emphasized the applicability of short-term exposures to the general problem of air pollution as being relevant only in connection with short-term peak exposure and not with chronic effects.[34] Several epidemiological investigations have also contributed to our understanding of the biological effects of air pollution and these have been well reviewed.[35,36] However, these lack the precision of controlled animal experiments. Consequently, both epidemiological studies and long-term studies in animals are requisite to the proper evaluation of environmental quality. In view of the few long-term animal studies to date, much remains to be done.

Fig 5.—Lung sections from guinea pigs killed at 52 weeks (reduced from × 4). Top left, From animal in control group. Moderate to severe diffuse pneumonia is present in cardiac and diaphragmatic lobes. Dark areas are regions of diffuse histiocytic and lymphocytic infiltration. Top right, From animal in group exposed to 0.13 ppm SO_2. Moderate to severe focal nodular lymphoid hyperplasia is present throughout both lobes. Dark areas are regions of lymphoid hyperplasia around bronchioles and blood vessels. Bottom left, From animal in group exposed to 1.01 ppm SO_2. Moderate focal nodular lymphoid hyperplasia is present throughout both lobes. Dark spots are areas of focal perivascular and peribronchiolar lymphoid hyperplasia. Bottom right, From animal in group exposed to 5.72 ppm SO_2. Relatively free of lymphoid hyperplasia and diffuse pneumonia.

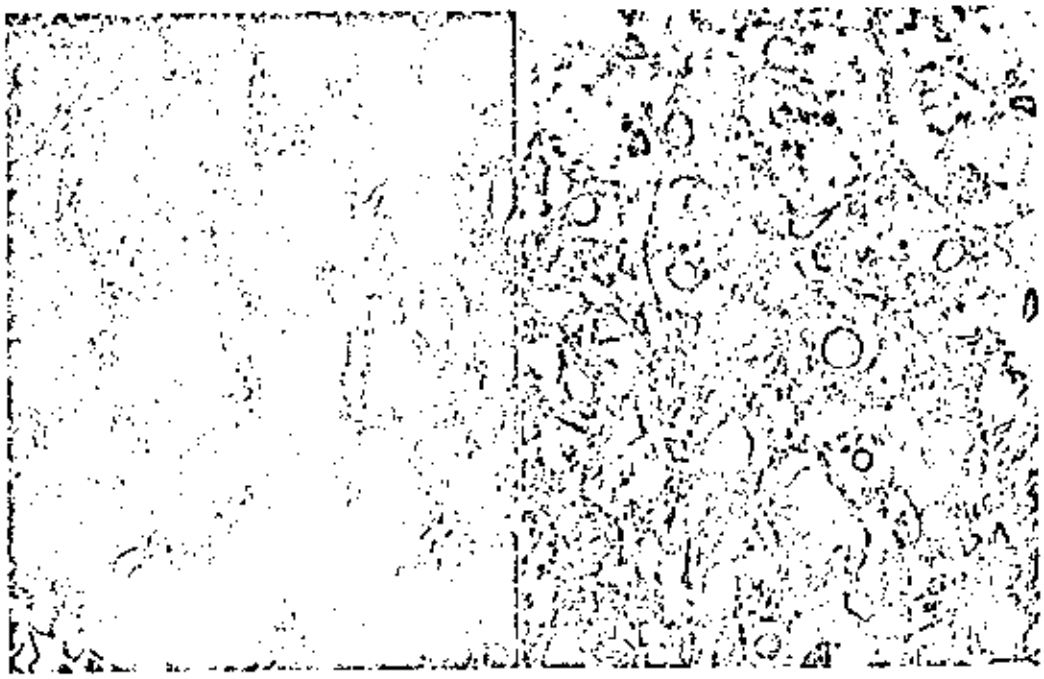

Fig 6.—Liver sections from guinea pigs killed at 52 weeks (× 250). Left, Normal hepatocytes surrounding central vein, from animal in control group. Right, Moderate swelling and vacuolation of hepatocytes with a few intracytoplasmic hyalin droplets, also present, from animal in group exposed to 5.72 ppm SO_2.

The data reported in this communication have resulted from the Electric Research Council's Air Pollution Research Program financed by the Edison Electric Institute with assistance from the Tennessee Valley Authority, American Public Power Association, and Bituminous Coal Research, Inc.

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CANCER FIGHT PROGRESS

Progress against cancer should be measured by several criteria: increased knowledge of the fundamental nature of malignant disease; identification and control of factors involved in cause and prevention; improvements in detection, diagnosis, and treatment leading to increased survival for the patient. The ultimate criterion, of course, is the control of cancer in man; and judged by this, treatment with drugs continued to provide encouraging evidence of progress during the past year.

Complete remissions of disease for extended periods have been induced with drugs in patients with widespread, rapidly progressive cancers, such as acute leukemia, Hodgkin's disease, childhood solid tumors, and a few other types. Some of these results are being produced by intermittent administration of combinations of drugs in high doses according to carefully planned schedules based on results of extensive laboratory studies of growth characteristics of cells. Such studies have shown that destructive effects of drugs against cancer cells can be increased without serious additional harm to normal cells. Intense exploration of the best methods of prolonging remissions in such patients is in progress. At the same time research is under way to extend the principles learned with these types of cancer to the more slowly growing tumors, such as those of the lung, breast, and colon-rectum, which make up the bulk of the cancers that afflict man.—"Cancer Research and Control," Progress Against Cancer 1969, a report of the National Advisory Cancer Council. US Dept of Health, Education, and Welfare, Public Health Service, National Institutes of Health, National Cancer Institute, part 1, p 2.

Proc. SOS/70, Int. Cong. Food Sci. Tech. (1970)

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Effects of Sulphur Dioxide in Animals and Man

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Introduction

Sulphur dioxide is used widely in foods as an antimicrobial preservative and to prevent both enzymic (Mapson, 1962) and non-enzymic (Burton *et al.*, 1963; McWeeney and Burton, 1963) discolouration. In foods it conserves carotene and also vitamin C content, a property which was of great nutritional importance during the 1939-45 wartime vegetable dehydration programme in the United Kingdom (Ministry of Food, 1946; Allen, Barker and Mapson, 1943, 1944). Sulphur dioxide, a gas obtained from the burning of sulphur, can be compressed to a liquid or prepared as a solution in water. The free gas is assumed to yield sulphurous acid as the active agent, when it dissolves in the water present in food. Sulphurous acid is often introduced to food as one of the sulphites of sodium, namely sodium metabisulphite, sodium sulphite, and sodium hydrogen sulphite. Also used are the sulphites of potassium and, less commonly, of calcium. For regulatory purposes, limits are prescribed in terms of sulphur dioxide, and this paper discusses the effects of sulphur dioxide added to food in any of the forms mentioned. Schroeter (1966) reviewed its use in foods, beverages, and pharmaceuticals.

The antimicrobial activity of sulphur dioxide is pH-sensitive. The quantity of sulphur dioxide needed to prevent fermentation is only 1/10 as much at pH 4 as at pH 5. Above pH 7 it is without effect on yeasts and moulds. The undissociated sulphurous acid is many times more active microbially than the bisulfite or sulfite ions, and the relative proportion of acid, bisulfite and sulfite is pH-dependent. The relationship between pH and antimicrobial activity has been studied extensively by Schellhorn (1951).

Sulphur dioxide can form complex additive compounds with other substances present in foods, for example with acetaldehyde, glucose, acetone, and formaldehyde. It reacts with ketones, including aldehydic sugars, in a reversible reaction, the equilibrium being influenced by temperature and pH. It also reacts reversibly with disulphide linkages in proteins, the disulphide being split into one part with a thiol group and another part with a thiosulphate group (Swan 1957). In foods, therefore, sulphur dioxide is present in free and bound forms, and the greater part of sulphur dioxide in treated food is in the bound form, especially bound to aldehydes or ketone groups. Fig. 1 illustrates the conversion of sulphur dioxide from the free to the bound form in a concentrated fruit drink.

Franz (1904) suggested that the sulphur dioxide additive compounds in food provoked toxicity symptoms similar to those induced by free sulphur dioxide, the speed of the response being determined by the rate at which they released free sulphur dioxide. Combined with acetaldehyde, for example, as CH_3 CHOH SO_3H, it displays one-fifth or less of the acute toxicity of free sulphurous acid (Leuch 1895).

Sulphur dioxide has been used in food for centuries. Today it is used internationally in a wide variety of foods, for example in fruit juices and pulps, dehydrated fruits and vegetables, fresh fruit such as grapes, sugar, wine (a use dating from Roman times), fruit syrups, soft drinks, beer, sausages (Pyett and Shelley 1962), and preserves. It is also used in some pharmaceutical preparations. All authorities, including for example, the Association of Food and Drug Officials of the United States (1957), accept sulphur dioxide as safe at the intended levels of use. Indeed, over 45 years ago a British Government Departmental Committee classified the sulphites as among the least harmful of preservatives (Report of the Departmental Committee on Preservatives and Colouring Matters in Food, 1924), presumably because it was known then that, after absorption, sulphurous acid and sulphites are readily oxidised by sulphite oxidase in the liver and excreted in the urine as sulphates. The Federal Food and Drug Authority classifies it as GRAS but does not permit its use in meat or foods recognised as a source of thiamine. Kidney (1968) has argued that, on balance, the use of sulphites in comminuted meat products, as practised in the United Kingdom, is beneficial to the public, and he has challenged, as incorrect, the widely

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held view that sulphites can restore the redness of dull-coloured meat, thereby masking initial putrefaction.

Sulphur dioxide imparts to food a characteristic unpleasant metallic flavour note. Consequently, food manufacturers have an incentive to use the minimum necessary to achieve the technological objective. Because of its adverse flavour effect, even in the absence of legal restrictions sulphur dioxide has the advantage over most food additives of being self-limiting in the concentrations at which it can be added to most foods. It can also be readily removed from foods by evaporation.

The greater the technological development of a community, the greater becomes its affluence, the better its diet, the greater its consumption of processed foods, and the higher its intake of sulphur dioxide. It is therefore appropriate that we should now look closely at this long-established food additive in the light of modern knowledge and standards and consider especially its toxicity in man.

FAO/WHO Assessment

Eight years ago, the acceptable intake for man, calculated as sulphur dioxide, was estimated (Joint FAO/WHO Expert Committee on Food Additives 1962) as follows:

Unconditional acceptance, 0–0.35 mg/Kg body weight

Conditional acceptance, 0.35–1.5 mg/Kg body weight.

During these eight years, there has been no reason to doubt the safety in use of sulphur dioxide at daily intakes up to these limits. Presumably because of long and widespread experience of the safety in use of sulphur dioxide, these limits embody a safety factor of only 10 to 23, compared with the more usual one of 100 in calculating a limit for man from the maximum no-effect-level in animals.

In the United Kingdom, total intake of sulphur dioxide was calculated to be unlikely to exceed 20 mg/day, a level within the conditional acceptance zone (Ministry of Agriculture, Fisheries and Food, 1959). For some possible consumptions, however, the FAO/WHO limits are relatively low. For example, the consumption of one third of a litre of wine containing 300 ppm of sulphur dioxide could readily provide the full acceptable daily intake for a 70-kilogram man.

More work on the toxicity of sulphur dioxide is now necessary to find whether the acceptable daily intake for man can be increased or, if it cannot, to speed the adoption of other preservatives in order to make possible a reduction in the total intake of sulphur dioxide. This paper assesses the acute and chronic toxicity risk of sulphur dioxide in man at concentrations likely to be encountered in food. Necessarily, our consideration is often based on evidence obtained in animal experiments. Little work with modern methods has been done in man.

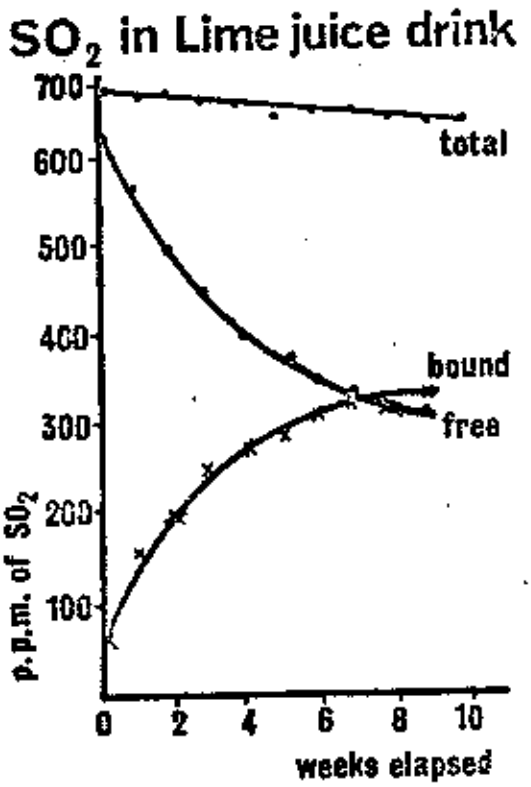

Fig. 1. Conversion of sulphur dioxide from the free to bound form in a concentrated fruit drink.

The Joint FAO/WHO Expert Committee (1962) considered that work was desirable: 1) to collect more data on the acute effects of sulphites in man; and 2) to confirm and/or elucidate in a second species the connexion between the toxic action of sulphites and the destruction of thiamine.

Acute Toxicity

The acute oral LD_{50} of sulphur dioxide was found to be 600–700 mg/Kg body weight in rabbits (Rost and Franz, 1913), and 400–450 mg/Kg for the cat (Jacobj and Wahlbaum, 1906). Recently, intravenous injection of sodium bisulphite solution in distilled water to determine acute LD_{50} in various species of animals indicates that, applying a tenfold safety rate, high doses can be tolerated (Hoppe and

Goble, 1961) in relation to the acceptable daily intake set for man of 1.5 mg/Kg (conditional level).

Acute intravenous LD_{50} in animals, mg/Kg as SO_2

	Mouse	Rat	Hamster	Rabbit
LD_{50} mg/Kg as SO_2	80	71	58	40

The acute intraperitoneal LD_{50} values of sodium bisulphite determined by Wilkins *et al.* (1968) were even higher than those obtained by the intravenous route.

Acute intraperitoneal LD_{50} in animals, mg/Kg.

	Mouse	Rat	Rabbit	Dog
Sodium bisulphite (1.25g/100ml)	675 (25g/100ml)	306	185	151
SO_2 equivalent mg/Kg	415	498	300	244

The early human oral toxicity studies have been briefly summarised by Sollman (1947). Greenwald (1954) reviewed the effects of inhaling sulphur dioxide on man and other animals.

Sulphur dioxide appears to be well tolerated by man until large doses are given. Over sixty years ago, Rost and Franz (1913) found that a single oral dose of 1 g sodium sulphite, equivalent to 0.25 g sulphur dioxide, slightly decreased the utilisation of proteins and fats but produced few gastro-intestinal symptoms, the effects mainly one of slight distention of the stomach. When the dose was increased to 4 g sodium sulphite (= 1 g sulphur dioxide), abdominal pains, vomiting, cold sweats, and diarrhoea were experienced by 6 of 7 treated. This high acute dose may perhaps be in excess of the body's capacity to oxidize sulphite to sulphate.

Earlier, Jacobj and Wahlbaum (1906) had reported that when humans were given much lower doses, 10–50 mg free sulphur dioxide dissolved in water, they experienced headaches, sore throat, intestinal pain, and some diarrhoea. Some of these symptoms are of a type not likely to be recorded in experimental animals.

Irritation of the mucous membranes and gastro-intestinal tract is the principal acute effect of sulphur dioxide in man (Grollmann and Slaughter, 1947), but the data available do not indicate the lowest dose which produces acute effects in man or the highest tolerable dose without harmful effect. However, oral administration of sulphurous acid and its salts as gastro-antiseptics was once practised, at doses of up to 2 g of sodium sulphite (Potter 1913). Since doses of sulfite in excess of 3.5 mg/Kg induce gastro-intestinal irritation leading to vomiting in man, acute poisoning is unlikely (Lafontaine and Goblet 1955).

Chronic Effects

Large doses of sodium sulphite, 50–200 mg sulphur dioxide equivalent, given daily by stomach tube over a long period (43–419 days) to dogs produced no adverse symptoms. The same large doses given to rabbits produced gastric haemorrhages and slight fissures in the mucous membranes (Rost and Franz, 1913). Sodium bisulphite in the diet of rats at 0.1% (equivalent to 30 mg sulphur dioxide/Kg body weight) over a long period, 1–2 years, in a series of experiments by Fitzhugh *et al.* (1946), inhibited growth and produced intestinal mucosal lesions, whereas lower doses of 0.05% (15 mg/SO_2/Kg/day) and below had no effect.

Thiamine Destruction in Sulphur-Dioxide-Treated Food

The ability of sulphur dioxide to convert thiamine into another substance has been known for over 30 years (Morgan, 1935). The destruction of thiamine by sulphur dioxide during drying of cabbages and potatoes is well established (Allen *et al.*, 1944; Pavcek, 1946). The destruction of a vitamin in food during processing is not unusual. For example, ascorbic acid destruction by heat during food processing is well known and may be corrected by fortification with added ascorbic acid.

Fitzhugh *et al.* (1946) attributed the greater part of the chronic toxicity of sulphur dioxide given as sulphites, which they observed in rats, to the decomposition of thiamine in the diet by the sulphur dioxide. In a series of experiments, they found that concentrations in the diet of less than 615 ppm sulphur dioxide had no significant effect on growth, whereas higher doses of 1538 ppm were associated with growth retardation, clinical polyneuritis, focal myocardial necrosis and fibrosis, spectacle eyes, bleached incisor teeth, brown uteri, atrophy of various viscera, calcified renal tubular casts, and atrophy of bone marrow and bones. The growth retardation on the sulphited diets was not prevented by twice-weekly injections of 100 μg of thiamine, but addition of sulphite to diets whose

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thiamine had been removed produced additional deleterious effects on the test animals. Their experiments were conducted for periods of one to three years, and the highest concentration of sulphur dioxide used (1538 ppm) reduced the average survival time. The lowest level in the diet which produced histopathological changes was 0.1% (615 ppm as SO_2).

Hötzel *et al.* (1966) showed that 300 mg/SO_2/Kg/day—given as glucose-sodium bisulphite complex, acetaldehyde-sodium bisulphite complex, or sodium metabisulphite—did not significantly affect rats when their diets were supplemented with all B-vitamins or with thiamine without the other B-vitamins. However, when no B-vitamins were present, as little as 50 mg/SO_2/Kg reduced growth and feed conversion, and increased mortality over that of controls not fed sulphur dioxide.

Fitzhugh *et al.* (1946) do not give sufficient information in their paper for us to calculate the intakes of sulphur dioxide per unit body weight, but the Joint FAO/WHO Expert Committee (1962) recorded that 0.05% of sodium bisulphite, equal to 307 ppm as SO_2, was equivalent to 15 mg sulphur dioxide/Kg body weight per day, this being the level they acknowledged to have no significant toxicological effect in rats.

Cremer and Hötzel (1966) showed that the weight gain of rats receiving a full supply of nutrients was not affected by the administration through stomach tube of up to 400 mg/SO_2/Kg daily over a three-month period, whereas in thiamine-deficient animals one-fifth of this amount depressed weight gain and increased mortality. These changes were also associated with a fall in liver thiamine content to a dangerously low level.

Thiamine Destruction in the Rest of the Diet

Thiamine supplementation of sulphur-dioxide-treated foods which are normally sources of thiamine cannot completely eliminate the risk of toxicity arising from thiamine deficiency. It is also necessary to assess the risk (Hötzel, 1962) that consumption of sulphur dioxide in foods not themselves sources of thiamine (beverages, for example) may effectively reduce the level of thiamine in the rest of the diet.

Lockett (1957) had found that fruit juice containing 350 ppm sulphur dioxide when given to thiamine-deficient rats at the rate of 0.5 ml/150 g body weight/day for 8 weeks, did not increase the severity of their deficiency symptoms. The experimental design was such that it might be argued that, since the rats received the sulphur-dioxide-containing fruit juice before they began to eat their solid diet, the results were of doubtful relevance to dietary thiamine destruction. Nevertheless, the experiment is useful as evidence of lack of chronic toxicity.

Lockett and Natoff (1960) gave 750 ppm sulphur dioxide to rats, in drinking water, for three years, covering three consecutive generations. Compared with control animals receiving drinking water without sulphur dioxide, no adverse effects were observed on fertility, growth rate, organ histology, or tumour development. Cluzan *et al.* (1965) gave 40 male and 40 female rats 75 mg/Kg/body weight sulphur dioxide daily for 20 months, as a solution of potassium metabisulphite in water. Compared with control animals given distilled water, there was no significant effect from the sulphur dioxide except for some increase in leucocyte number in the males, and a spleen weight increase. Reproduction studies over two generations revealed no untoward effect, although the sulphite groups had fewer young per litter and fewer male offspring.

As to wine, Lanteaume *et al.* (1965) fed groups of rats daily by stomach tube at 3 ml/100 g body weight with wine containing 450 ppm sulphur dioxide over 4 generations. No difference was seen between test and control animals in litter size, organ size, macro- and micro-pathological appearance of major organs, or protein utilization efficiency. There was some irregular reduction in the respiration of liver samples of animals from sulphur-dioxide-receiving groups, and a 7% reduction in weight gain of the second-generation males.

Most present evidence, therefore, is consistent with the view that sulphur dioxide ingested directly in beverages is unlikely to reduce thiamine levels in the rest of the diet. However, workers in Denmark have found *in vitro*, that fruit juice containing 350 ppm sulphur dioxide markedly reduced the thiamine present in food in conditions similar to those of the gastro-intestinal tract of man, and claimed that amounts of sulphur dioxide greater than 100 ppm in fruit juices cause destruction of thiamine on a scale which could lead to human deficiency (Hoff-Jorgensen, cited by Charley, 1970).

Work *in vivo* in man is clearly necessary, and such a study is now being undertaken by the British Industrial Biological Research Association. Preliminary results support the view that sulphur dioxide in beverages has no antithiamine effect in man (Sharratt, 1970). Fortification with thiamine of military rations containing sulphited dehydrated foods was studied

by Thomas and Berryman (1949), who concluded that thiamine destruction was not of nutritional significance to man on normal diets.

Adverse Effect of Food Stored in Contact with Sulphur Dioxide

The relevance to man of one other observation made on rats remains unclear, and research is necessary to assess its significance. Bhagat and Lockett (1964) concluded that a substance, or substances, toxic to rats may be formed slowly when a sulphur-dioxide-containing diet is stored for some time before use. They found that the storage of sulphited diets at room temperature led to the development of a toxicity to rats that was not apparent at 31 days but readily detectable at 75 days. Signs were observed, similar to thiamine deficiency, accompanied by diarrhoea and reduced weight gain, but these chronic adverse effects were not readily corrected by thiamine supplementation. Fitzhugh *et al.* (1946) had noted similar effects: stored sulphited diets reduced growth rate during the first 12 weeks more than did new diets that were otherwise the same. They also noticed that the aged diet increased the incidence of lesions of the teeth and uteri.

Work at B.I.B.R.A. has confirmed the adverse effect on rats of sulphur-dioxide-treated diets which have been stored. Experiments are in progress to determine which component, or components, of the diet react with the sulphur dioxide and, if possible, to identify the compound formed.

The literature has been refreshingly free from any suggestion that sulphur dioxide presents any carcinogenic or mutagenic hazard to humans or, indeed, to experimental animals. Since preparing the abstract of our paper, however, it has been suggested that sodium bisulphite and sulphur dioxide 'may constitute a genetic hazard to living organisms' (Shapiro *et al.*, 1970). This statement was made following an *in-vitro* study of chemical reactions. In these reactions, uracil and cytosine derivatives were converted to saturated adducts by the agency of sodium bisulphite. Sodium bisulphite also catalysed the deamination of cytosine to uracil. Shapiro *et al.* point out that the initial steps of the reaction of the potent mutagen hydroxylamine with cytosine derivatives (Phillips and Brown, 1967) is analogous to their reaction with sulphur dioxide.

The observations of Shapiro *et al.* (1970) should lead to further investigation, but are inadequate evidence of a genetic hazard to living organisms whose nucleic acids are present within living cells. It is of interest that mouse fibroblast and liver cells and human cervix epithelial cells in tissue culture in serum withstand relatively high concentrations of sulphur dioxide. In all three cell lines, growth continued, although inhibited, at 2000 ppm of sulphur dioxide. With 500 ppm of sulphur dioxide, cell growth continued at a rate comparable with that in control cultures (Thomson and Pace, 1962). The resistance of these mammalian cells in culture to cytotoxic effects from sulphur dioxide lends support to the view that sulphur dioxide in the diet poses a low order of health risk to man, although Thomson and Pace obtained some evidence supporting a view that humans are more sensitive than other mammals to sulphur dioxide.

Also in this connection, Golberg (1967) has pointed out that induction of mutation in yeast and bacteria cells by artificial colouring materials, even at concentrations of the same order of magnitude as those used in foodstuffs, are of questionable significance from the standpoint of mammalian toxicity.

It can be postulated that the sulphate formed metabolically by oxidation of sulphite might increase calcium loss from the body. At high levels of potassium metabisulphite intake by young rats, equivalent to 2885 and 5770 ppm of sulphur dioxide in the drinking water, increased calcium excretion was observed in the urine but not in the faeces when the diet supplied the normal amount of 0.5% calcium as carbonate. When the calcium content of the diet was higher, at 1.0%, calcium excretion in the urine was not increased but there was a significant increase of excretion in the faeces (Hugot *et al.*, 1965).

No effect on weight increase or on liver vitamin-A content was established when weanling Wistar rats were given drinking water containing 1200 ppm potassium metabisulphite (equivalent to 691 ppm of SO_2) (Causeret *et al.*, 1965).

Rate of Metabolism in Relation to Toxicity

It is of interest to consider the rate at which sulphite is oxidised to sulphate in the body, because knowledge of this should be a useful guide to the doses which can be safely tolerated. The little evidence available encourages one to be optimistic about the dose which man can tolerate. Wilkes *et al.* (1968), by infusing sodium bisulphite solution in the portal veins of three dogs whose ureters had been ligated to prevent renal excretion, demonstrated that not less than 80 mg/Kg/hr bisulphite was being

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metabolised to sulphate. This represents 49 mg/Kg/hr of sulphur dioxide. It would now be useful to establish the rate at which man converts sulphite to sulphate, but the animal evidence suggests that his capacity to do this is likely to exceed his possible exposure to sulphite.

General Aspects

Sulphur dioxide is a food additive used internationally in a wide variety of foods. Further study of its toxicity along the lines suggested by the Joint FAO/WHO Report (1962) might reasonably be regarded as a world public-health measure of some importance. There is no procedure to ensure that work of the kind required will be promptly undertaken. Individual organisations which do such work are contributing results of value throughout the world. It seems to us desirable to establish some international machinery to initiate and finance work on widely used food additives. We do not envisage new international laboratories but, rather, a body which can give grants to existing laboratories undertaking work of such wide international application as that being done on sulphur dioxide.

Summary and Conclusions

Foods containing sulphur dioxide to preserve their wholesomeness have been consumed by man for centuries. Investigations on man during the early part of this century record his tolerance of relatively large doses. Since technological development is likely to increase man's intake of sulphur dioxide, through greater use of processed foods, it is appropriate to review sulphur dioxide toxicity in the light of modern methods of assessment. The FAO/WHO Joint Expert Committee recommend an acceptable daily intake for man which is relatively low when related to possible consumption in some countries. Their finding placed much weight on relatively recent chronic-toxicity tests in rats, in which thiamine destruction in the diet by sulphur dioxide probably occurred. There is evidence that this can be corrected. The formation in stored sulphur-dioxide-treated diets of a substance or substances toxic to rats needs further study, but present animal evidence may prove to be irrelevant to the situation in man. Metabolism of sulphite to sulphate proceeds rapidly in animals, and a study of sulphur dioxide metabolism in man will probably provide reassurance of its safety in use at levels higher than those currently recommended by FAO/WHO. There would be value in organising and financing internationally the collection of essential toxicity data on widely used additives such as sulphur dioxide. At present there is no assurance that further work asked for by FAO/WHO Joint Expert Committee will be done.

Acknowledgments

We gratefully acknowledge help and advice in preparing this paper received from Dr. R. Crampton and Dr. Sharratt, of the British Industrial Biological Research Association; Miss Milliidge, of the British Food Manufacturers Research Association; Dr. V. C. S. Charley, and several of our colleagues in The Beecham Group.

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A Preliminary Report

6

Effect of a Combination of SO_2 and H_2SO_4 On Guinea Pigs

By MARY O. AMDUR, Ph.D.

RESEARCH of the past 4 years has shown that low concentrations of either sulfuric acid mist alone (*1*) or sulfur dioxide alone (*2*) cause a reflex production of shallow, rapid respiration in normal humans. Since both these substances can, and as a matter of fact often do, occur together, the obvious next step was to test the effect of a combination of the two. This was best done first on animals in which lung pathology as well as respiratory effects could be studied. This paper reports the results of these very brief preliminary studies on guinea pigs.

In the experiments using humans, the concentrations of sulfur dioxide or sulfuric acid mist varied in order of magnitude from those occurring in the atmosphere during such disasters as the London fog to those considered as safe levels for routine exposures in industry. In the case of sulfur dioxide, the average concentration for the day at the height of the London fog was 1.3 p.p.m. (see *Public Health Reports*, May 1953, p. 474). This is about 10 times the amount usually found in the London atmosphere and about one-tenth of the maximum allowable concentration for industry (10 p.p.m.). In the case of sulfuric acid mist, atmospheric concentrations of 0.25–0.4 mg. per cu.m. have been reported and the industrial level is 1 mg. per cu.m.

In the experiments using guinea pigs, concentrations much higher than these are used since the guinea pig is considerably more resistant to these substances than are humans. Levels 8 to 9 times the allowable industrial concentrations were used in the work reported here. These levels were chosen so that neither substance present alone would cause appreciable damage to the animals for the period of the exposure used.

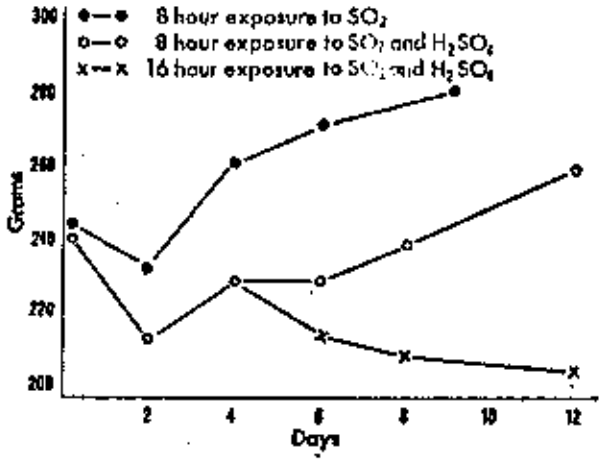
Dr. Amdur, research associate in the department of industrial hygiene, School of Public Health of Harvard University, who has been engaged in a series of studies on the effect on man and animals of sulfur dioxide and of sulfuric acid mist, presented this preliminary report on the effect of a combination of the two on guinea pigs, at the meeting in Boston of the American Association for the Advancement of Science, December 29, 1953.

Experimental Procedure

The apparatus was similar to that used in previous work on sulfuric acid mist alone (*1*, *3*). The mist was generated by warming concentrated sulfuric acid in a three-necked flask. Sulfur dioxide from a tank was metered into one neck of the flask while compressed air entering the second neck swept the vapors out of the flask through the third neck which was connected to the main air stream entering a 2-cu. ft. exposure chamber, made of corrosion-resistant materials (A).

For sampling the air in the chamber for sulfuric acid mist and sulfur dioxide, a membrane filter (B) and a sintered glass bubbler were connected in series. The sulfuric acid mist particles, which had a mean diameter of 1 micron, were caught by the filter. This filter was then washed with water and the conductivity of the resulting solution was measured. Concentration was calculated from a curve prepared from

Figure 1. Growth of guinea pigs following exposure to 89 p.p.m. of sulfur dioxide alone and to 89 p.p.m. of sulfur dioxide with the addition of 8 mg. per cu. m. of sulfuric acid mist.



standard sulfuric acid solutions. A sensitive fuchsin-formaldehyde colorimetric reagent (*4*) failed to indicate the presence of sulfur dioxide on the aerosol as collected. The sulfur dioxide, which passed the membrane filter (B), was trapped in the hydrogen peroxide-sulfuric acid reagent of Thomas and Abersold (*6*) and determined by conductivity. On several samples, the sulfur dioxide was determined by the iodine method. This checked the concentration found by conductivity showing that no appreciable quantity of the sulfur gas was present as sulfur trioxide.

In these experiments, 8 guinea pigs were exposed for 8 hours to a combination of 8 mg. per cu.m. of sulfuric acid mist and 89 p.p.m. of sulfur dioxide. Two of these animals were re-exposed 4 days later to the same concentrations for another 8 hours. Control data on the effects of exposure to sulfuric acid mist alone were readily available since in the course of previous work done in the same laboratory (*3*) a total of 68 guinea pigs from the same source had been exposed for 8 hours to 8 mg. sulfuric acid mist per cu. m. and 30 animals had been exposed to this concentration for 72 hours.

Due to various limiting factors, only 4 guinea pigs were exposed to 89 p.p.m. of sulfur dioxide uncontaminated by sulfuric acid mist. The response of the guinea pig to this gas has been, however, well documented by Weedon, Hartzell, and Setterstrom from the Boyce Thompson Institute (*6*). The response of the 4 animals exposed was as expected from the data reported.

Results

As criteria of damage, growth curves, respiratory response, and lung pathology were used. These combined criteria show that the effect of the combination was much more marked than the effect of either sulfuric acid mist or sulfur dioxide alone at the same concentrations.

Figure 1 shows the growth curves for animals exposed to sulfur dioxide alone and to the combination. In all cases the weight had fallen off the day following exposure. In the case of sulfur dioxide alone, growth was quickly resumed. When both sulfuric acid mist and sulfur dioxide were present, growth was slow to resume. The two animals that had been re-exposed ceased to grow entirely during the period of observation following re-exposure. The effect of the combination on growth is definitely greater than the effect of sulfuric acid mist alone, since in previous experiments even the survivors of LD_{50} concentrations (18 mg. per cu. m., a dose lethal to 50 percent of the animals exposed) or of 72-hour exposures to 8 mg. per cu. m. resumed growth following exposure. Figure 2 shows the growth curve of 12 animals exposed to 8 mg. sulfuric acid mist per cu. m.

Figure 2. Growth of guinea pigs following exposure to 8 mg. per cu. m. of sulfuric acid mist alone for 72 hours.

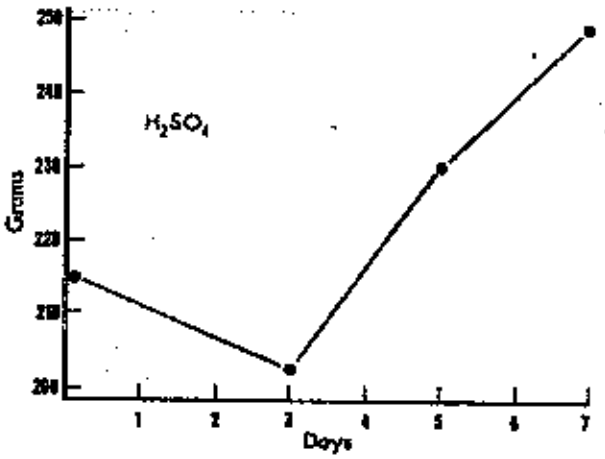


Figure 3. Lungs of three guinea pigs exposed for 8 hours to 89 p.p.m. of sulfur dioxide.

for 72 hours. The weight lost during exposure was quickly regained.

The lung pathology produced by the combination was much more extensive than that resulting from the same exposure to sulfuric acid mist alone or sulfur dioxide alone at the same dose levels. The lungs of animals exposed for 8 hours to 8 mg. per cu. m. of sulfuric acid mist alone (*3*) showed some damage with occasional areas of consolidation mostly limited to the hilar regions. Lungs of animals exposed to 89 p.p.m. of sulfur dioxide alone showed some damage, including pinpoint hemorrhages and occasional small areas of consolidation. Figure 3 shows the lungs of three of these animals. In neither case, however, was the lung damage severe.

Figure 4 shows the lungs of three animals exposed to the combination of sulfuric acid mist and sulfur dioxide for 8 hours. The lungs in all cases showed large areas of complete consolidation and hepatization involving entire lobes. Figure 5 shows the lung of one of the animals that was re-exposed for a second 8-hour period 4 days after the initial exposure. In this case hemorrhage and consolidation are widespread throughout the whole lung. The general ill health of these animals was very likely related to the presence of this extensive lung damage.

When guinea pigs are exposed to 5 mg. per cu. m. of sulfuric acid mist alone, there are no noticeable respiratory effects. This is true for exposures up to 72 hours. Grossly, the animals breathe and behave in a normal manner. At 89 p.p.m. of sulfur dioxide, the animals appear restless and annoyed when the gas is first turned on, but this passes in 5 to 10 minutes and they appear to become accustomed to it. Weedon and co-workers (*6*) report that at 150 p.p.m. of

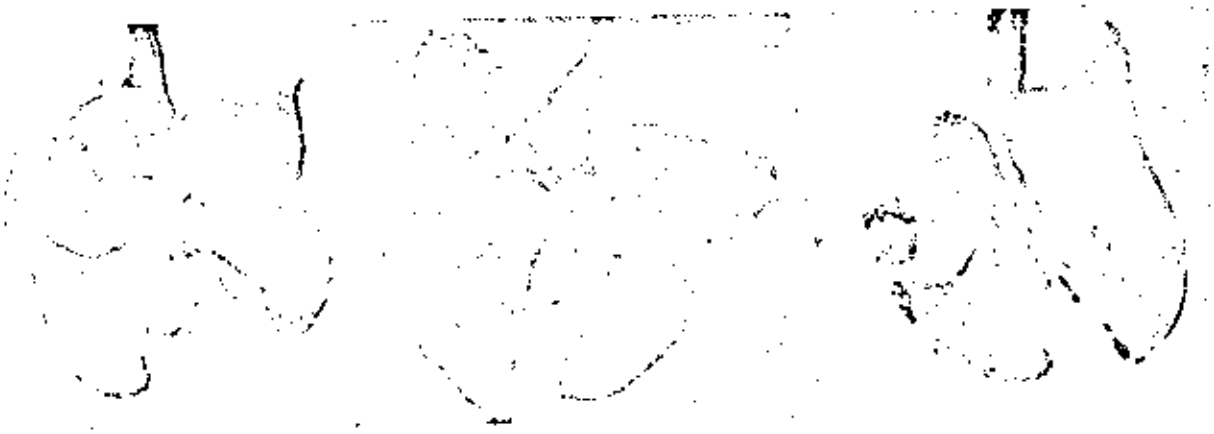


Figure 4. Lungs of three guinea pigs exposed for 8 hours to 89 p.p.m. of sulfur dioxide and 8 mg. per cu. m. of sulfuric acid mist.

Figure 5. Lung of guinea pig after 16-hour exposure to 8 mg. per cu. m. H_2SO_4 and 89 p.p.m. SO_2

sulfur dioxide severe respiratory distress does not set in until the animals have been exposed for 48 hours, and that even at 296 p.p.m. evidence of dyspnea only became apparent during the second day of exposure.

When these two substances are given together at the concentrations used, signs of respiratory distress become apparent within the first hour of exposure and become progressively worse as the exposure continues. Frothy fluid appears in the nose and mouth and dyspnea is very pronounced. Even more surprising is the fact that this labored breathing continues for 24 to 48 hours after the animals have been removed from the exposure chamber. At concentrations of sulfuric acid mist alone capable of killing 50 percent or more of the animals in 8 hours, those that survived were breathing normally within 2 hours after the exposure ended except in a few rare instances.

Discussion

None of the animals in these experiments died spontaneously either during exposure or in the short period of observation following exposure. Both the depressed growth curves and the severe lung pathology, however, indicated that considerable damage had occurred. Both these factors were markedly aggravated by the repeated exposure.

If a quantity of sulfuric acid mist equivalent to 89 p.p.m. of sulfur dioxide were given, guinea pigs would be dead within an hour or less of laryngeal spasm. With three times this concentration of sulfur dioxide alone, however, breathing is not affected until the second day of exposure. From this it appears that the small amount of sulfuric acid mist, which is ineffective alone, when combined with a concentration of sulfur dioxide, which is also ineffective alone, produces a very marked respiratory response.

Since only one concentration of sulfuric acid mist and sulfur dioxide was used in these experiments, the results must be regarded as purely preliminary. Further studies using different concentration combinations are needed before an explanation of the nature of the joint toxic action of these two compounds can be postulated.

Summary

It has been shown that for 8 mg. per cu. m. of sulfuric acid mist and 89 p.p.m. of sulfur dioxide given in combination to guinea pigs the effect on growth, lung pathology, and respiration are much more marked than would be predicted from the use of either alone.

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(A) Lucite. Rohm and Haas, Philadelphia.

(B) Filter, Millipore. Type HA, plain, 47 mm. discs in packages of 100. Lovell Chemical Co., Watertown, Mass.

Compt. Rend. 186 (1928)

7

EMBRYOLOGIE EXPÉRIMENTALE. — *Action de divers gaz sur l'œuf de poule. Assimilation de l'oxyde de carbone à un gaz inerte.* Note de Mlle SUZANNE ANGEL, présentée par M. Caullery.

Depuis les travaux de Gréhant (1), Haldane (2), Nicloux (3), l'oxyde de carbone est considéré comme un poison agissant spécialement sur le sang. « L'oxyde de carbone n'est toxique, dit Nicloux, que parce qu'il agit de telle sorte que l'animal n'est plus assuré du minimum d'oxygène nécessaire à la vie... il n'est un poison qu'indirectement. »

Cependant divers auteurs ont soutenu une opinion différente. C'est ainsi que Limossier (4), expérimentant sur l'escargot, considérait l'oxyde de carbone comme plus dangereux qu'un gaz inerte et que d'autres auteurs, dans des expériences que Balthazard (5) a d'ailleurs discutées, admettaient une action toxique de l'oxyde de carbone sur le tissu rénal, sur le muscle, ou sur le système nerveux. En outre il n'est pas démontré que l'oxyde de carbone ne soit pas un toxique de la cellule, car l'intoxication oxycarbonée chronique n'est pas encore expliquée et les auteurs ne sont pas d'accord pour rapporter à l'anoxémie les troubles si divers constatés dans l'intoxication aiguë.

J'ai recherché si l'on pouvait mettre en évidence une toxicité cellulaire de l'oxyde de carbone en comparant l'action de ce gaz à celle de gaz inertes et de gaz toxiques sur le germe de l'œuf de poule, constitué de cellules indifférenciées.

La méthode suivie consistait à placer des œufs, aussitôt après la ponte, dans des tubes ou des bocaux hermétiquement clos et à les maintenir pendant un temps donné dans une atmosphère ne renfermant que le gaz à étudier. Après séjour dans le milieu gazeux, les œufs sont exposés à l'air libre pendant 24 heures pour les débarrasser de la plus grande partie du

(1) N. GRÉHANT, *C. R. Soc. Biol.*, 39, 1887, p. 198.

(2) J. HALDANE, *Journ. of Physiol.*, 18, 1895, p. 201-217.

(3) M. NICLOUX, *L'oxyde de carbone et l'intoxication oxycarbonique (Étude chimico-biologique)*, 1 vol. 254 pages, 35 figures in-8°. Paris, Masson, 1924. Voir Chapitre V, p. 118 et suiv.

(4) G. LIMOSSIER, *C. R. et Mémoires Soc. Biol.*, 9e série, 1, 1889, p. 1-8.

(5) V. BALTHAZARD, *Intoxication par l'oxyde de carbone*, in *Nouveau Traité de Médecine*, par G.-H. ROGER, F. WIDAL, P.-J. TEISSIER, fasc. VI (*Intoxications*), Paris, Masson, 1926; voir p. 181.

gaz ayant agi, puis soumis à l'incubation pendant 48 heures. Ce temps écoulé, on observe le développement du germe.

Les œufs qui ont été maintenus pendant 8 jours dans des gaz inertes comme l'azote, l'hydrogène, puis mis à la couveuse pendant 48 heures, ont évolué normalement (¹). Par contre, des œufs placés dans des atmosphères de gaz toxiques se sont développés anormalement. Suivant la durée du séjour dans les gaz étudiés, l'évolution du germe s'est trouvée, ou retardée, ou annihilée totalement, ou encore elle s'est effectuée de telle sorte que seul, le blastoderme évoluait quelque peu, alors que l'embryon ne se formait pas, ou n'était représenté que par des masses cellulaires informes. J'ai obtenu ce développement du blastoderme *sans celui de l'embryon* après un séjour de 3 minutes dans l'hydrogène sulfuré, 3 minutes dans le gaz ammoniac, 2 heures dans le gaz sulfureux, 2 heures dans l'acide chlorhydrique, 5 heures dans le chlore, 2 jours dans l'acétylène commercial, 3 jours dans le gaz carbonique, 6 jours dans le gaz d'éclairage. Ainsi des œufs maintenus pendant 8 jours dans un gaz inerte comme l'azote ou l'hydrogène se développent normalement lorsqu'on les met en incubation, tandis que l'évolution du germe devient impossible quand l'œuf a préalablement séjourné dans un gaz toxique.

Ces données acquises, il devenait possible, grâce à la technique que je viens de préciser, de savoir si l'oxyde de carbone se comportait comme un gaz toxique ou comme un gaz inerte. A cet effet, j'ai placé des œufs pendant 8 jours dans une atmosphère d'oxyde de carbone pur et, après ce temps, les ai mis en incubation; ils se sont toujours développés normalement.

La conclusion s'impose donc : l'oxyde de carbone se comporte vis-à-vis de la cellule, non comme un gaz toxique, mais comme un gaz inerte. Ces faits, rapprochés de ceux déjà apportés par Nicloux (²), corroborent l'opinion qu'il soutient, que l'oxyde de carbone n'est toxique pour des organismes hautement différenciés, que parce qu'il les prive de l'oxygène absolument nécessaire à leur fonctionnement.

(¹) L'oxygène a été expérimenté; son effet a été analogue à celui des gaz inertes.

(²) M. Nicloux, *loc. cit.*

Brit. J. Industr. Med., 1950, 7, 82.

8

POSSIBLE LONG TERM EFFECTS OF EXPOSURE TO SULPHUR DIOXIDE

BY

A. ANDERSON

From the Anglo-Iranian Oil Co., Ltd., Abadan, South Persia

(RECEIVED FOR PUBLICATION JANUARY 3, 1950)

Sulphur dioxide is one of the oldest products of industry to which man has been exposed and its use under modern conditions is widespread. The effect of exposure to sulphur dioxide has attracted little attention, however, beyond studies of the immediate effects, and I have found only one controlled investigation into the long term effects (Kehoe, Willard, Kitzmiller, and Leblanc, 1932).

The immediate consequences of exposure to heavy concentrations are no concern of this paper, although at Abadan we are aware of the effects on the eye, the skin, and the lungs. They can be severe, but there has been no record of death due to exposure to sulphur dioxide in Abadan over the past five years.

The present investigation was stimulated by statements by labourers, supervisors, and others that in their view continued exposure to the fumes of sulphur dioxide must be a contributory cause, if not the cause, of tuberculosis, chronic bronchitis, and other chronic chest disease; and the confusion in the medical literature with regard to the immediate effects of exposure to heavy concentrations and the long term effects of ordinary industrial exposure. For instance, Kehoe and others (1932), Johnstone (1948), and Sappington (1943) maintain that chronic chest disease from ordinary industrial exposure will not occur. On the other hand, Romanoff (1939) and Dowling (1937) have reported cases of asthma following the inhalation of sulphur dioxide. Admittedly the asthma occurred in patients with a manifest allergic diathesis and after fairly severe exposure, although accurate assessment of the exposure was sometimes difficult to obtain. Enquiries as to long term effects have been made of authorities in the United Kingdom, but as a rule the answers have been expressions of opinion, and it is admitted that there is little direct evidence. Many people are influenced by the obvious immediate results of exposure to heavy concentrations and feel that chronic effects must inevitably occur.

Conditions at Abadan

Oil refineries are large users of sulphur dioxide, and at Abadan there are two important processes in which it is used (1) the refining plant (R.P. Area), and (2) the special products plant (S.P. Area).

These plants are housed in steel frame buildings, open on all sides and roofed over for protection from sun and rain so that ventilation is unusually good and escape from any major catastrophe unusually easy. This form of construction is also desirable in a country where the shade temperature may rise to 123° F. in summer and seldom falls below about 40° F. in winter.

Theoretically, the sulphur dioxide at each plant is in a closed circuit, but no mechanical pump yet devised is completely leak-proof in operation, and other minor sources of leak exist. Recent estimations of the concentration of sulphur dioxide in the atmosphere at these plants have shown that figures between 0 and 25 p.p.m. are usual. Estimations taken during the years 1938–45, when maintenance of the plant was relatively low, sometimes showed figures varying between 60 and 100 p.p.m., and one sample, which was deliberately taken near a pump gland temporarily out of commission, showed 200 p.p.m. The commonly accepted maximum allowable concentration is 10 p.p.m.

Method of Investigation

In each area (R.P. and S.P.) two groups of workers were chosen. The contact groups consisted of men who were exposed to sulphur dioxide, and the control groups of men engaged in the same areas, but with no recorded exposure to sulphur dioxide. The climatic conditions were the same for each group, and they had the added statistical advantages of being all Iranian nationals and all subject to similar conditions as regards working hours, clothing, housing, wage scales, and nutritional status. The choice was left to the supervisor

TABLE I

COMPARISON OF GROUPS BY AGE

Age Group (Years)	R.P. Area		S.P. Area	
	Contacts	Controls	Contacts	Controls
15	3	10	3	1
20	5	7	2	8
25	21	8	9	6
30	11	7	7	6
35	15	16	7	3
40	19	12	5	3
45	11	12	3	3
50	8	17	—	2
55	3	9	—	—
60	1	2	—	2
65	—	—	—	—
70	—	—	—	1
Total	97	100	36	35
Mean	37·60	39·95	33·05	35·50
S.D.	10·10	11·85	7·57	13·20
Difference	2·35		2·45	
S.E. of difference	1·56		2·56	

of each area and in fact almost all the exposed personnel were examined. The control groups were chosen by the supervisors from non-exposed personnel working in the areas concerned, and the working record of each man was consulted before the choice was made in order to eliminate the possibility of previous contact. These individual records are kept by the central labour office and are accurate.

A standard card was printed on which was recorded the results of clinical examination. In addition, the vital capacity was estimated and a radiograph of the chest obtained in each case. These results were recorded on the same card. All clinical examinations were carried out by one medical officer, except for the first 40 or so out of a total of 278 examinations successfully completed and recorded. All the vital capacities were estimated or supervised by one laboratory technician.

All the radiographs were examined and recorded by one person on the following standard system: (1) Shape of chest; (2) apices; (3) heart shadow; (4) hilar areas; (5) general lung fields. By these means it was hoped to eliminate as far as possible differences arising from personal bias. In the radiographs and the vital capacity estimations it was not known whether the particular result being assessed was from an exposed or a non-exposed worker. In the clinical examinations, it was impossible to eliminate this bias. It was hoped to show whether the contact groups showed any deviation in health compared with the control groups.

Analyses of Tabulated Results

In each area (Table 1) the means differ by approximately the standard error of the difference between them. This established a further similarity between the groups. The recording of age in an illiterate population is notoriously dangerous, but the risk almost certainly applied equally throughout.

In each area (Table 2) the means differ by approximately the standard error of the difference between them, a further similarity between the groups.

The means (Table 3) are very close together, and must be accepted as a further point of similarity between the groups.

The vital capacity (Table 4) is as good a measure of respiratory function as any at our command. Maxwell (1948) states that "vital capacity is an index of respiratory function", and Davidson (1948) that it has "frequently been adopted as a test of physical fitness". Best and Taylor (1945) state that the vital capacity is reduced in many diseased conditions, especially those involving the respiratory and vascular systems. They quote emphysema as an example. The estimation of vital capacity has also come to be almost a standard procedure when assessing the disability of the silicotic. As the groups used in this examination were comparable by weight and age, the correction for stature which

TABLE 2

COMPARISON OF GROUPS BY WEIGHT

Weight Group (lb.)	R.P. Area		S.P. Area	
	Contacts	Controls	Contacts	Controls
90	3	3	2	2
100	13	15	1	7
110	18	20	12	8
120	32	30	8	8
130	23	18	7	6
140	7	7	5	5
150	3	6	—	—
160	3	—	2	1
170	2	1	—	—
Total	104	100	37	37
Mean	126·44	124·60	126·35	122·84
S.D.	16·13	15·22	15·79	15·99
Difference	1·84		3·51	
S.E. of difference	2·19		3·71	

is sometimes used was not thought to be necessary. It will be seen that the mean vital capacity is better in each contact group, and that the means in the S.P. Area differ approximately by as much as the standard error of difference between them. In the R.P. Area, however, the difference between the means is 3·89 times the standard error of the difference, a figure usually accepted as statistically significant. The reasons for this are unknown. It can be conjectured that deeper breathing occurs on exposure to an irritant gas, but this is offset by the fact that no significant difference was found in the S.P. Area. It can only be stated that the contact group in the R.P. Area showed a significantly better mean vital capacity than the control group and that there is no evidence on this observation that their health was suffering as a result of exposure to sulphur dioxide. Considerable importance is attached to this finding, as it is the only time the vital capacity estimation has been used in this connexion, so far as is known, and it is felt that its simplicity of application and susceptibility to accurate measurement make it a valuable piece of evidence.

TABLE 3

COMPARISON OF GROUPS BY BLOOD PRESSURE

B.P. Group (Mm. Hg)	R.P. Area		S.P. Area	
	Contacts	Controls	Contacts	Controls
100	—	3	—	—
110	7	7	4	7
120	38	31	20	15
130	27	29	7	11
140	17	11	4	3
150	8	8	—	—
160	4	4	—	—
170	1	2	—	—
180	1	1	1	1
Total	103	96	36	37
Mean	135·19	134·79	129·72	129·32
S.D.	13·65	15·20	12·36	12·63
Difference	0·40		0·40	
S.E. of difference	2·05		2·92	

TABLE 4

COMPARISON OF GROUPS BY VITAL CAPACITY

Vital Capacity in Litres	R.P. Area		S.P. Area	
	Contacts	Controls	Contacts	Controls
1·8	—	1	—	1
2·0	—	2	1	—
2·2	6	5	1	3
2·4	3	11	2	4
2·6	8	9	2	5
2·8	8	13	6	5
3·0	10	20	3	2
3·2	11	14	4	7
3·4	17	11	7	4
3·6	11	2	6	1
3·8	5	4	3	2
4·0	4	—	—	2
4·2	1	—	—	—
4·4	1	—	—	—
4·6	—	—	1	—
4·8	1	—	—	—
5·0	—	—	—	1
Total	86	92	36	37
Mean	3·293	3·009	3·272	3·105
S.D.	·532	·434	·530	·623
Difference	0·284		0·167	
S.E. of difference	0·073		0·135	

Having been aware of the likelihood of inconsistency in the interpretation of radiographs for some time, I was particularly interested in the findings of Birkelo, Chamberlain, Phelps, Schools, Zacks, and Yerushalmy (1947) that the opinions of different observers on the same film were often at variance and that even the same observer would give different opinions on the same radiograph on different occasions. This phenomenon has been further examined by Fletcher and Oldham (1949) with particular reference to coal miner's pneumoconiosis. Therefore, I read all the *x*-ray films myself, recording the results in the manner noted above. By this means the differences between observers were eliminated, and I attempted to curb the effects of my own mood and differing outlook over a period of 18 months by reading the films in batches of 30 to 40.

No cases of active tuberculosis were found. This was surprising, as the incidence in Abadan is roughly twice that of the United Kingdom. The cases with quiescent Assmann's foci were subjected to full investigation in hospital, and the 20 cases designated "full investigation negative" were admitted because of other suspicious areas, usually in the region of the apex of the lower lobe. It will also be noted that the abnormal findings were distributed fairly evenly throughout the four groups.

TABLE 5

COMPARISON OF GROUPS BY RADIOGRAPHIC FINDINGS

Abnormality	R.P. Area		S.P. Area	
	Contacts	Controls	Contacts	Controls
Calcified hilar areas	12	15	4	3
Assmann's focus (quiescent) ..	4	2	—	—
Calcified extra hilar areas ..	8	10	—	4
Full investigation negative ..	11	2	3	4
Old pleurisy	2	4	1	1
Total	37	33	8	12

Odds and ends came to light of course. For example, several cases of syphilitic aortitis were discovered and treated, and one or two cases of amoebic hepatitis.

Length of Exposure to Sulphur Dioxide

In 101 subjects in the R.P. Area the exposure time was ascertained. Forty-five had been exposed from 1 to 4 years; 35 from 5 to 14 years; 21 from 16 to 19 years. The mean exposure time was 8·15 years. The S.P. Group, 37 in all, had a mean exposure time of 2·91 years due to the fact that the plant had been commissioned as recently as 1943.

Acclimatization Time

As a matter of interest each man in the contact groups was asked how long he thought he had taken to become accustomed to the effects of sulphur dioxide in his working atmosphere. The replies were expressions of opinion only, but are nevertheless interesting.

TABLE 6

STATED ACCLIMATIZATION TIME

	R.P. Area	S.P. Area
1–6 months ..	67%	92%
6–12 months ..	16%	8%
12–24 months ..	15%	—
Over 24 months ..	2%	—

"Acclimatization" is a word used frequently in Abadan with reference to hot weather conditions, and it was used as freely in Services medical circles during the recent war. The assessment of acclimatization is difficult, and not susceptible to measurement, but for some reason the S.P. workers felt that they became accustomed much more rapidly than did the R.P. workers. (It should be remembered that each clinical examination was an individual incident, so that collusion is unlikely.)

Summary

Workers exposed to daily concentrations sulphur dioxide for periods of time varying fr 1 to 19 years were examined. Special attenti was paid to age, weight, blood pressure, vi capacity, and radiography of the chest. The d concentration of sulphur dioxide varied between and 25 p.p.m. Occasionally, figures of 100 p.p. were recorded.

Workers not exposed to sulphur dioxide b otherwise subject to similar working conditions a to similar living conditions were also examine special attention being paid to the same details examination.

No evidence of adverse effects on health could found.

I wish to thank A. C. Turner, Esq., O.B.E., F.R.C. Chief Medical Officer to the Anglo-Iranian Oil C Ltd., in Iran, for permission to publish this pap Many members of the works management, too numer to mention, rendered invaluable assistance. I wi specifically to thank Dr. M. A. Khorram, who examin most of the cases clinically, Mr. J. Q. Gray, who pe formed or supervised the vital capacity estimations, a Mrs. T. L. Stephens, who did the laborious recordin Finally, I am indebted to 278 Persian workmen witho whose cooperation the investigation would have be impossible.

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Rom. Med. Rev. (1967)

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IMMUNOLOGICAL CHANGES IN ANIMALS EXPOSED TO LOW SULPHUR DIOXIDE CONCENTRATIONS *

I. ARDELEAN, M. CUCU, ELENA ANDRONACHE and SELMA BODURIAN

(From „Fiziologia normală și patologică", Vol. XII, No. 4, 1966)

Intensive development of industry led to a marked increase and diversity of air pollution in populated centres. Many recent epidemiological and statistical investigations have confirmed the increased incidence of respiratory diseases, a consequence of this new factor of environmental insalubrity.

Explanation of the physiopathological mechanisms that stand at the basis of acute morbid respiratory processes, is very difficult, owing to the fact that in the polluted atmosphere there are many different polluting substances in the form of gases, aerosols, dust, a.s.o., which are acting as a rule in low concentrations and whose effect is manifest after long time action.

Sulphur dioxide represents the most widespread polluting product in towns, being permanently present in the air due to the combustion of solid and fluid fuels. From the toxicological point of view, sulphur dioxide is classified in the category of irritating gases, with a sufficiently great water solubility coefficient to induce, when it exceeds the irritation threshold which is above 20 mg/m^3, the typical tracheobronchopulmonary syndromes determined by this group of toxic gases. However, such concentrations were not found in the urban atmosphere, not even in the case of acute accidents produced by air pollution. Notwithstanding, evident statistical correlations were established between acute and chronic respiratory diseases and the content of the atmosphere in sulphur oxidation products.

Some authors sustain that sulphur dioxide produces changes in the function of the respiratory tract and especially the alteration of pulmonary resistance, even in concentrations below 5 mg/m^3. In these investigations evidence was found of bronchic spasm, a phenomenon considered to be due to a reflex vegetative mechanism and to the direct action of the toxic substance on the smooth bronchic musculature.

Another aspect of the investigation concerns the experimental study of the action of atmospheric polluting products on infectious processes of the respi-

* Research carried out by the Department of General and Communal Hygiene of the Medico-Pharmaceutical Institute, Bucharest, and the Section of Communal Hygiene of the Institute of Hygiene, Bucharest.

ratory tract. Baetjer and Vintiner showed that prolonged exposure to smoke containing high proportions of CO and SO_2 did not lower the resistance of rats experimentally infected with type 1 pneumococcus. Similarly, neither did we find an increased mortality rate in mice experimentally inferted with type 21 pneumococcus and exposed to low SO_2 concentrations, but on the other hand we observed that SO_2 produced protracted forms of pneumococcal pulmonary condensations.

Considering of interest the perspectives opened, from the physiopathological viewpoint, by the study of the immunobiological changes that take place in the organism under the action of physical, chemical and biological factors, we investigated the action of polluting products in the atmosphere on infectious processes and immunity. The present paper deals with the action of low sulphur dioxide concentrations on the formation of agglutinins and on alexin titers.

Material and method

The investigations were carried out on 16 male rabbits separated into two equal groups: the one was exposed to sulphur dioxide and the other served as control.

The experimental group was kept in a gas chamber of the Pravdin type, for 7 hours daily, the average SO_2 concentration being 20 mg/m³. The experiment lasted 113 days. Concomitant to exposure, the animals were vaccinated with a formaldehyde pneumococcal antigen. Pneumococcus of type XIX was used and the antigen prepared from 10 hour cultures in fluid medium was administered by intravenous route in 0.5 ml amounts per animal, in both the experimental and the control group. The injections were carried out 3 days running, followed by a 5 days interval, in 6 series, the vaccination period lasting 48 days. Sixty days after the interruption of vaccination a booster dose was administered, consisting in an intravenous administration of a single 0.5 ml dose of antigen to each animal.

Blood was collected before each antigen administration for the estimation of the antipneumococcal agglutinin and alexin titers. Blood was likewise collected for these titrations 5 days after the end of vaccination and 5 days after the booster dose. Determination of the agglutinin and of the alexin titers was done according to current techniques.

For comparison of the titers obtained, we used the two-fold logarithm of the reciprocal values, starting from the known biomathematical finding that the relation between the amount of antigen and the titer that evidences it has an exponential and not a proportional character.

Results

a) *Agglutinins*

At the beginning of the experiment, no evidence was found of the presence of pneumococcal agglutinins. On day 8 and 16 from the beginning of vaccination, the animals under experiment presented high antibody titers, the difference against the control group being statistically significant. Thus, after eight days the t test (Student) was equal to 3.23, corresponding to a probability of $p < 0.01$, and after 16 days t was equal to 2.28 and $p < 0.05$ (table 1).

In the control group, the titer increased progressively from one series of vaccination to another, while the increase of the titer was much slower in the experimental group. On day 24 and 32 after the beginning of vaccination the differences were no longer stastistically significant ($p > 0.05$), but on day 40 and 48 the titers found in the control group were significantly higher than those of the group exposed to SO_2 ($t = 2.34$, $p < 0.05$ and respectively $t = 2.74$, $p \approx 0.02$). Increase in the titer after administration of the booster dose was not significant for the group exposed to SO_2 ($t = 0.79$, $p > 0.05$), while in the control group there was a significant increase in the agglutinin titer ($t = 2.44$, $p < 0.05$).

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Table I

Antipneumococcal agglutinin titer in immunized rabbits exposed to intoxication with SO_2 (ml)

Days		1	8	16	24	32	40	48	108	112
Intoxicated group	Average titer	0	1/50	1/76	1/91	1/153	1/327	1/503	1/48	1/75
	Average of log. 2 of reciprocal titer values	1	5.5	6.0	6.3	7.1	7.7	8.4	5.3	5.6
Control group	Average titer	0	1/18	1/31	1/242	1/329	1/1313	1/1949	1/60	1/256
	Average of log. 2 of reciprocal titer values	1	3.7	5.1	7.3	7.9	9.7	10.5	5.5	7.3
	Significance of log. 2 difference	t = 3.23 p < 0.01	t = 2.28 p < 0.05	t = 1.31 p > 0.05	t = 1.31 p > 0.05	t = 2.34 p < 0.05 = 0.02	t = 2.74 p < 0.05	t = 0.21 p > 0.05	t = 1.96 p < 0.05	

b) *Alexin*

The alexin titer increases slightly in the intoxicated animals (from 0.36 to 0.54 on day 24, but towards the end of immunization it is practically unchanged) (table II). In the control group the increase is constant, the difference at

Table II

Alexin titer in immunized rabbits exposed to intoxication with SO_2 (ml)

Days	1	8	16	24	32	40	48	108	112
Intoxicated group	0.86	0.85	0.55	0.54	0.70	0.73	0.86	1.10	1.00
Control group	0.90	0.86	0.93	0.00	0.53	0.55	0.60	1.00	0.70

the end of immunization, from 0.86 to 0.60 being statistically highly significant ($t = 5.5$, $p < 0.01$). The same phenomenon is evident after administration of the booster dose, the control group marking a significant increase of the titer from 1.00 to 0.70, whereas in the experimental group the increase was minimal from 0.10 to 1.00, that is statistically unsignificant ($p > 0.05$).

Discussion

A number of authors have investigated the effect of toxic gases on immunity processes. V. K. Navrotzki found inhibition of the formation of antityphus agglutination antibodies after the administration of T.A.B. vaccine to rabbits

exposed to low SO_2 concentrations, without noting however any significant changes in the proteinogram. I. G. Fridland considers that the toxic substances in the air lower the immunological resistance of the organism and increase the receptivity to infection, the most important factors being alteration of the phagocytic potential of the leukocytes, decrease of the serum bactericide potential and inhibition of antibody formation. Stoianovski and Rasskazova likewise observed a decrease in the phagocytic potential of leukocytes following exposure to SO_2, whereas Mitina reports a comparatively reduced effect of SO_2 on the phagocytic potential of leukocytes, the effect being more accentuated following exposure to nitrogen oxides or to a nitrogen oxide + SO_2 mixture.

L. Erhan investigated the production of precipitating antibodies to a foreign protein in animals subjected to the chronic action of low SO_2 concentrations. This author also studied the changes in the serum protein spectrum after prolonged exposure of the experimental animals to low SO_2 concentrations and found an increase of globulins, especially of beta-globulins, a phenomenon which he attributed to a stereotypical response of the organism provoked by primary nocuous factors rather than to the direct action of SO_2.

As regards the formation of agglutinating antibodies, the results we obtained are in agreement with those reported by Navrotzki, indicating inhibition of antibody formation under the action of low SO_2 concentrations. This phenomenon develops in stages, being preceeded by a transition period of stimulation of the globulin-forming system; inhibition of this system is still stronger towards the end of the period of exposure, when the intoxicated animals factually no longer respond to administration of the booster dose. The evolution in stages of the physiopathological processes has likewise been observed with other aggressions upon the organism, for instance with very large SiO_2 doses.

Similarly, the alexin titer has proved to be an immunological index sensitive tot the action of low SO_2 concentrations, suggesting moreover the evolution in stages of the intoxication process.

Changes in the immunobiological response of the animal after prolonged exposure to low SO_2 concentrations also suggest a general action of this toxic substance, associated tot the local action upon the respiratory tract. The increased sensitivity to microbial aggression in subjects exposed for a long time to low SO_2 concentrations is accounted for by bronchopulmonary irritation and by stasis of the bronchic secretion due to inhibition of the motility of the vibratile bronchic epithelium cili. However, this gas also penetrates into the general circulation, disturbing various functions, among which the immunity function which is of particular interest for the appraisal of the changes in resistance to infections. The possible existence of a general action of SO_2 is also sustained by the high solubility of this toxic substance in water and humoral fluids and its retention in high proportion in the respiratory tract during respiration, attaining approximately 84 per cent (both in the respiratory tract and the alveoles).

Conclusion

1. Low SO_2 concentrations inhibit the formation of agglutinins and alter the alexin titer, being therefore a toxic gas which, besides its local irratitive effect also exercises a general action on the mechanisms of immunogenesis.

2. The action of SO_2 on immunity processes takes place in stages, beginning with a transitory stimulation stage and continuing with inhibition of the immunological processes that become more accentuated as the intoxication progresses.

J. Inst. Brewing 75 (1969)

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THE INSTITUTE OF BREWING ANALYSIS COMMITTEE
DETERMINATION OF SULPHUR DIOXIDE IN BEER

Submitted by R. G. AULT for the Analysis Committee

Received 7th March, 1969

The method recommended for the determination of sulphur dioxide in beer is a modification of the well-known Monier Williams distillation procedure. For routine purposes a rapid technique combining simultaneous distillation and titration is also described.

THE Analysis Committee appointed the following Sub-Committee to recommend a method for the determination of sulphur dioxide in beer:—

R. G. Ault (Chairman), P. F. Bayles, L. R. Bishop, D. Goodwin, J. R. Hudson, M. O. Jones, C. Macfarlane, P. A. Martin, R. Parsons, A. Sinclair, K. Stowell and L. Taylor.

The findings of the Sub-Committee are given in this paper.

The method recommended by the Chemical Analysis Sub-Committee for determining sulphur dioxide in beer is a modification of the procedure submitted by the Institute of Brewing Analysis Committee in 1926[1] and is based on the well-known Monier Williams method.[2] Some latitude is considered justified in respect of the size of the sample and the distillation apparatus, the type of carrier gas and the end-point determination. The size of the apparatus determines to a large degree the size of the sample and also the time taken for distillation. For beer, practical tests have indicated that a satisfactory degree of accuracy can be obtained with a 50-ml. sample of beer used in combination with a 250-ml. capacity distillation flask and a distillation time of 15 min. (Procedure A).

Alternatively, a more rapid technique is available based on a simultaneous distillation and titration procedure. This method is especially suitable for routine purposes and gives an adequate degree of accuracy (Procedure B).

Since in Britain there is a legal requirement that beer shall not contain more than 70 p.p.m. SO_2, the Monier Williams method as described in the Analyst in 1927 must be considered as the official reference method and should be used in all cases involving arbitration.

PROCEDURE A

Reagents

(i) Hydrochloric acid, 5% (v/v) or phosphoric acid 5% (v/v).

(ii) Hydrogen peroxide, 10 vol. (titrated to bromophenol blue neutrality before use.)

(iii) Sodium hydroxide solution, N/50.

(iv) Bromophenol blue, 0·2% solution in 20% ethanol.

(v) Carrier gas, carbon dioxide, nitrogen or steam.

Apparatus

(i) Distillation flask. A three-necked, round bottom flask, capacity 250 ml. A suitable type is manufactured by Quickfit & Quartz (FR.250/35/22P). Alternatively, a single-necked flask, *e.g.*, FR.250/55, with a 3-way adaptor, *e.g.*, MA 4/25, can be used. The flask/adaptor carries respectively a tap funnel, a reflux condenser and a CO_2-delivery tube leading to the bottom. Ground-glass joints have been found satisfactory.

(ii) Reflux condenser. Double-surface type, *e.g.*, Quickfit & Quartz C5/22. The top end of the condenser is fitted with a splash head, *e.g.*, Q. & Q. SH7/02, connected to a glass delivery tube leading to the bottom of the receiver.

(iii) Receiver. Conical flask, 100 ml., containing hydrogen peroxide solution (10 vol.) into which the lower end of the delivery tube dips.

Method. 50 ml. hydrochloric acid (5%) is introduced into the distillation flask and boiled in a current of pure carbon dioxide

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until all air is removed from the apparatus (2–3 min.). The beer sample (50 ml.) is quickly added through the tap funnel and the mixture boiled in a slow current of carbon dioxide for 15 min. The flow of water in the reflux condenser is then stopped and any residual sulphur dioxide driven over into the receiver where it is absorbed into the hydrogen peroxide (10 ml. of 10 vol. hydrogen peroxide).

As soon as the vertical delivery tube becomes hot to the touch at its upper end, it is disconnected and washed down with distilled water into the receiver. The sulphuric acid produced by the oxidation of the sulphur dioxide is titrated at room temperature with N/50 sodium hydroxide solution using bromophenol blue as indicator. For relatively small amounts of sulphur dioxide as in beer, a guard tube has not been found necessary providing the carrier gas is bubbled slowly through the liquid in the receiver.

1 ml. N/50 NaOH = 12·8 p.p.m. SO_2.

If required, the sulphuric acid can be determined as barium sulphate by precipitation in the cold with barium chloride solution. In this case it will be necessary to take a larger sample of beer (*e.g.*, 250 ml.) in order that there may be a sufficient amount of precipitate to filter. The precipitate is washed carefully by decantation with hot water before filtration, dried and weighed.

Procedure B

Apparatus

(i) 1 litre round-bottomed flask (Quickfit FR 1L/3UM).

(ii) Multiple adaptor (Quickfit MA 1/3).

(iii) Vertical Splash head (Quickfit SH7/12).

(iv) Tap funnel (Quickfit D3/12).

(v) Condenser (Quickfit C1/12).

(vi) Delivery adaptor (Quickfit RA1/02).

Reagents

(i) Standard iodine solution 0·1 N.

(ii) Hydrochloric acid, analytical grade, s.g. 1·18.

(iii) Starch indicator solution. Mix 5 g. soluble starch into a paste with a little cold water. Pour the paste into 50 ml. boiling water and boil for 1 min. Cool, and dilute to 100 ml.

Method. Measure 250 ml. of beer sample into the boiling flask followed by 10 ml. hydrochloric acid. Bring rapidly to the boil and pass the distillate into about 15 ml. of water containing a little starch indicator solution and a few drops of 0·1-N iodine solution from a 10 ml. burette. As the distillation proceeds, run in iodine solution from the burette so that the blue colour is just maintained. The end-point is taken when the blue colour persists for 1 min.

SO_2 p.p.m. = ml. 0·1-N iodine × 12·8.

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1. Chaston Chapman, A., *Standard Methods of Analysis*, this *Journal*, 1926, 170.
2. Monier Williams, G. W., *Analyst*, 1927, 52.

THE INSTITUTE OF BREWING

ANALYSIS COMMITTEE

Method for Determination of Alpha-amylase Activity*

The method described utilizes a dextrinization procedure. One α-amylase unit is defined as the quantity of enzyme which will dextrinize a specified soluble starch in the presence of excess β-amylase at the rate of 1g. per hr. at 20°C.

Apparatus

(*a*) Constant temperature bath, 20° C. ± 0·05° C.

(*b*) *Comparator.* Either the standard Hellige comparator (Catalogue No. 607) or the pocket comparator (Catalogue No. 605) with prism attachment (Catalogue No. 605a). Alternatively, the standard Lovibond comparator may be used in conjunction with the Lovibond comparator discs 4/29. In use, illuminate the comparator with a 100-watt frosted lamp placed 6 in. from the rear opal glass of the comparator. Direct rays from

* This is a "Method which has become available" rather than a "Recommended Method."

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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

L. Balabajeva

Výzkumný ústav hygieny — NIHG, Sofija

Выводы: Эксперименты проводились над 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° C. 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 cysteïn 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.[2] 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 naším 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 dvouhlavých (v každé skupině 5 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 dokaždé 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 přími Varburgovou metodou podle výpočtů popsaných Umbrejtem[3].

Redukovaný glutathion byl určován v krvi a orgánech enzymovou glyoksalázovou 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, 0,4 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 ± 3,47	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 ± 2,50	25,40 ± 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 závislost 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 ± 4,09
I.	70,9 ± 9,64	73,7 ± 13,93	50,2 ± 8,24	137,6 ± 35,67	203,0 ± 32,25	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-

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 kyslíku 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

Komentář 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 zaměřeno 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 a ostatní prováděcí 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

VLIV KYSLIČNÍKU SIŘIČITÉHO NA TVORBU S-SULFOGLUTATHIONU A S-SULFOCYSTEINU „IN VIVO" U MORČAT

Образование сульфоглютатиона и сульфоцистеина «in vivo» при действии раствора сернистого газа на морских свинок

The Effects of Sulphur Dioxide upon the Creation of S. sulphoglutathione and S. sulphocysteine in Quinea-Pigs

L. Balabaeva, I. Gančev
Výzkumný ústav hygieny, Sofia

Выводы: Авторы инъектировали интраперитонеально 3 группы морских свинок изотоническим раствором, содержащим 2,5 мг сернистого ангидрида в 1 мл, однократно, двукратно и трикратно так, что животные получали от 5—60 мг сернистого ангидрида суммарно. Перед опытом и после декапитации животных определяли содержание общего и редуцированного глютатиона в крови. Из органов (печень, почки и тонкие кишки) и крови изолировали безбелковые нингидрин-положительные осадки, идентифицированные путем электрофореза на бумаге, в пиридин-уксуснокислом буфере с pH 4. Наряду с цистеином и глютатионом, на электрофореграммах устанавливаются и их сульфопроизводные — S-сульфоцистеин и S-сульфоглютатион. Содержание глютатиона в крови при однократном введении сернистого йона не изменяется, а при двукратном и трикратном введении уровень глютатиона повышается.

Čs. Hyg., 12, 1967, 7 : 376—380.

Summary: Three groups of guinea pigs were given intraperitoneal injections of isotonic solution containing 2.5 mgm of sulphur dioxide in 1 ml once, twice and three times, so that the animals obtained 5 to 60 mgm of sulphure dioxide on the whole. The whole and reduced glutathione, respectively, in blood were determined before the experiments as well as after decapitation of the animals. The ninhydrine — positive substances without proteins were obtained from different organs (liver, kidney, intestine) and blood and identified by means of electrophoresis on paper in pyridine-acetate buffer at pH 4. At the same time with cysteine and glutathione also their sulpho-derivatives (sulphocysteine and sulphoglutathione) were determined on the electrophoregram. The content of glutathione in blood after single administration of sulphite ion proved to remain at the same level while after two or three repeated injections the glutathione level increased.

Čs. Hyg., 12, 1967, 7 : 376—380.

Naše dřívější pokusy, prováděné v roce 1964, ukázaly, že S-sulfoglutathion se tvoří v hovězích červených krvinkách působením izotonického roztoku kysličníku siřičitého, dodaného v koncentraci 10krát silnější, než by ekvimolárně odpovídalo krevnímu glutathionu. Kinetiku reakcí mezi glutathionem, cysteinem a siřičitanem sodným sledovali Cecil a McPhee v čistých kulturách a dokázali, že při pH vyšším než 9 je tato reakce prostě bimolekulární a při pH nižším než 9 je mechanismus této reakce složitý. Stanovili, že disulfidové vaz-

by reagují s SO_3 ionty. Waley při sledování kyselých peptidů v očních čočkách stanovil přítomnost S-sulfoglutathionu. Tato sloučenina se lehce získává syntetickou cestou, je poměrně stabilní, ale reaguje rychle při pokojové teplotě s cysteinem a tvoří se vší pravděpodobností smíšené bisulfity. Waley předpokládá, že systém sulfit-sulfoglutathion-sulfát je zvratný, díky přítomnosti glutathion-reduktázové reakce. Robinson stanovil, že tato sloučenina se tvoří v tenkém střevě krys při zavedení radioaktivního (značeného) síranu. V následující práci tentýž autor stanovil, že ve větší míře se v těchto podmínkách tvoří ve střevní sliznici S-sulfocystein, ale přidáním okysličeného glutathionu k uvedené směsi se získá pouze S-sulfoglutathion. Zatím je těžko říci, probíhá-li

Tabulka 1

Skupina	Počet zvířat	Redukovaný glutathion v mg %		Celkový glutathion v mg %	
		před injekcí	po injekci	před injekcí	po injekci
jednou 5 mg SO_2	10	29.1 ± 1.39	29.4 ± 1.23	32.4 ± 1.47	32.2 ± 0.73
dvakrát 5 mg SO_2	10	23.7 ± 1.89	31.3 ± 2.76	25.8 ± 1.61	39.7 ± 2.88
třikrát 10 mg SO_2	6	29.0 ± 2.57	31.5 ± 1.38	30.4 ± 2.01	34.5 ± 1.87

tento proces v organismu enzymaticky, o čemž uvažují Arrigoni a Rossi, kteří zjistili v rostlinné tkáni enzymatický systém, rozkládající sulfoglutathion.

Úkolem této práce je prověřit, tvoří-li se S-sulfoglutathion a S-sulfocystein v organismu morčat vlivem intraperitoneálního zavedení izotonického roztoku hydrosulfitového iontu a má-li to vliv na úroveň krevního glutathionu.

Materiály a metody

K pokusu bylo použito 30 morčat o váze 160—200 g, rozdělených do 3 skupin. Všem zvířatům byla před započetím pokusu odebrána krev ze srdce punkcí, ve které byl stanoven obsah celkového a redukovaného glutathionu podle metody Woodwarda a Frye. Po skončení pokusu a dekapitaci zvířat byl znovu stanoven celkový a redukovaný glutathion v krvi. Pokusným zvířatům byl intraperitoneálně injikován izotonický roztok siřičitanového iontu (252 mg SO_3 . $7H_2O$ ve 12,2 ml destilované vody a 0,98 ml 2 n. kyseliny solné). V tomto roztoku tvoří chloridové a sodné ionty s krevním sérem izotonickou koncentraci, takže se celistvost krevních buněk neporušuje; toto bylo zjištěno v dřívějších pokusech „in vitro".

První skupině z desíti pokusných morčat byly injikovány jednorázově 2 ml izotonického roztoku, obsahujícího 5 mg SO_2. Druhé skupině zvířat byly vstřiknuty 2krát v průběhu dvou dnů 2 ml roztoku obsahujícího 5 mg kysličníku siřičitého. Třetí skupině morčat byly injikovány třikrát v průběhu pěti dnů 4 ml roztoku, obsahujícího 10 mg kysličníku siřičitého. Jednu hodinu po injekci byla zvířata usmrcena dekapitací a v heparinové krvi byl znovu stanoven obsah glutathionu. Desíti zvířatům z druhé skupiny byly vyjmuty ledviny, játra a tenké střevo a homogenizovány křemenným pískem v třecí misce, přidán 10 % roztok kyseliny trichlóroctové. Po filtraci se za studena přidal k bezbílkovinnému filtrátu 25 % roztok octanu barnatého (podle metody Waleyho), dokud se tvořila sedlina. Druhý den byla sedlina odstraněna filtrací a k čistému filtrátu byl přidáván 95 % alkohol, dokud se neobjevila lehká sraženina. Následujícího dne byly získané sedliny opět odfiltrovány, překrystalovány přidáním minimálního množství destilované vody do získání roztoku a opět sráženy alkoholem. Získané sedimenty z krve, jater, ledvin a tenkého střeva byly používány při elektroforetickém stanovení. Elektroforéza byla prováděna na Whatmannově papíře 3; v pyridinacetátovém pufru pH 4, při 200 Voltech a 12 mA, na proužcích 2,7/25 cm po dobu 2 hodin. Startovací čára byla 5 cm od katodového okraje. Standardní S-sulfoglutathion a S-sulfocystein byly získány syntézou podle metody po-

by reagují s SO_3 ionty. Waley při sledování kyselých peptidů v očních čočkách stanovil přítomnost S-sulfoglutathionu. Tato sloučenina se lehce získává syntetickou cestou, je poměrně stabilní, ale reaguje rychle při pokojové teplotě s cysteinem a tvoří se vší pravděpodobností smíšené bisulfity. Waley předpokládá, že systém sulfit-sulfoglutathion-sulfát je zvratný, díky přítomnosti glutathion-reduktázové reakce. Robinson stanovil, že tato sloučenina se tvoří v tenkém střevě krys při zavedení radioaktivního (značeného) síranu. V následující práci tentýž autor stanovil, že ve větší míře se v těchto podmínkách tvoří ve střevní sliznici S-sulfocystein, ale přidáním okysličeného glutathionu k uvedené směsi se získá pouze S-sulfoglutathion. Zatím je těžko říci, probíhá-li

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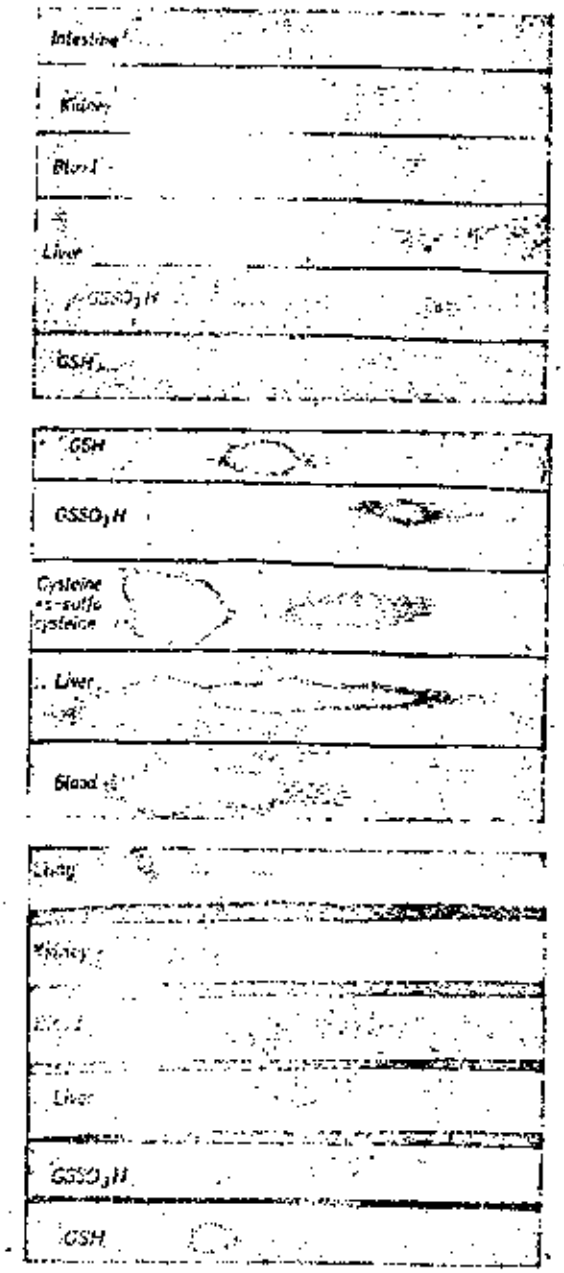
Materiály a metody

K pokusu bylo použito 30 morčat o váze 160—200 g. rozdělených do 3 skupin. Všem zvířatům byla před započetím pokusu odebrána krev ze srdce punkcí, ve které byl stanoven obsah celkového a redukovaného glutathionu podle metody Woodwarda a Frye. Po skončení pokusu a dekapitaci zvířat byl znovu stanoven celkový a redukovaný glutathion v krvi. Pokusným zvířatům byl intraperitoneálně injikován izotonický roztok siřičitanového iontu (252 mg $_2SO_3 \cdot 7H_2O$ ve 12,2 ml destilované vody a 0,98 ml 2 n. kyseliny solné). V tomto roztoku tvoří chloridové a sodné ionty s krevním sérem izotonickou koncentraci, takže se celistvost krevních buněk neporušuje; toto bylo zjištěno v dřívějších pokusech „in vitro".

První skupině z desíti pokusných morčat byly injikovány jednorázově 2 ml izotonického roztoku, obsahujícího 5 mg SO_2. Druhé skupině zvířat byly vstřiknuty 2krát v průběhu dvou dnů 2 ml roztoku obsahujícího 5 mg kysličníku siřičitého. Třetí skupině morčat byly injikovány třikrát v průběhu pěti dnů 4 ml roztoku, obsahujícího 10 mg kysličníku siřičitého. Jednu hodinu po injekci byla zvířata usmrcena dekapitací a v heparinové krvi byl znovu stanoven obsah glutathionu. Desíti zvířatům z druhé skupiny byly vyjmuty ledviny, játra a tenké střevo a homogenizovány křemenným pískem v třecí misce, přidán 10 % roztok kyseliny trichlóroctové. Po filtraci se za studena přidal k bezbílkovinnému filtrátu 25 % roztok octanu barnatého (podle metody Waleyho), dokud se tvořila sedlina. Druhý den byla sedlina odstraněna filtrací a k čistému filtrátu byl přidáván 95 % alkohol, dokud se neobjevila lehká sraženina. Následujícího dne byly získané sedliny opět odfiltrovány, překrystalovány přidáním minimálního množství destilované vody do získání roztoku a opět sráženy alkoholem. Získané sedimenty z krve, jater, ledvin a tenkého střeva byly používány při elektroforetickém stanovení. Elektroforéza byla prováděna na Whatmannově papíře 3; v pyridinacetátovém pufru pH 4, při 200 Voltech a 12 mA, na proužcích 2,7/25 cm po dobu 2 hodin. Startovací čára byla 5 cm od katodového okraje. Standardní S-sulfoglutathion a S-sulfocystein byly získány syntézou podle metody po-

psané Walcym a de Marcem. Abychom si mohli prověřit identitu standardu, požádali jsme prof. Waleyho o zaslání malého množství baryté soli S-sulfoglutathionu. Měla stejnou elektroforetickou pohyblivost, jako námi získaná sůl. Pro kontrolu jsme použili krve a orgánů morčat, která nedostala injekci SO_2.



Obr. 1.

Výsledky pokusu

Na tabulce 1 jsou uvedeny hodnoty obsahu celkového a redukovaného glutathionu v krvi morčat před a po injekci izotonického roztoku siřičitanového iontu, v mg%.

Na obr. 1 jsou elektroforetogramy standardu — glutathion (GSH) a S-sulfoglutathion ($GS\text{-}SO_3H$), společně s izolovanými sedlinami z jater [3],

krve (4), ledvin (5) a tenkého střeva (6) morčat, kterým byl podán injekčně roztok siřičitanového iontu (dvakrát 5 mg).

Obr. 2 ukazuje elektroforetické standardy glutathionu (GSH), S-sulfoglutathionu ($GS\text{-}SO_3H$), cysteinu a S-sulfocysteinu (3) současně s izolovanými sedlinami z jater (4) a krve (5) morčat, dvakrát injikovaných roztokem siřičitanového iontu.

Na obr. 3 jsou (zezdola nahoru) elektroforeogramy glutathionu-standardu (GSH), S-sulfoglutathionu ($GS\text{-}SO_3H$), sedlina jater (3), krve (4), ledvin (5) a plic (6) morčat kontrolních.

Diskuse výsledků

Jestliže jsme vzali v úvahu údaje, získané dřívějšími studiemi, jakož i údaje Waleyho a Robinsona, očekávali jsme snížení hladiny glutathionu v krvi morčat, injikovaných roztokem siřičitanového iontu. Jak je vidět z tabulky 1, v první skupině se hladina glutathionu celkem neměnila, rozdíl před a po injekci je v rámci chyby metody. V třetí a zvláště ve druhé skupině bylo objeveno značné, statisticky významné zvýšení hladiny glutathionu v krvi po injekci roztoky siřičitanového iontu. Tento jev může být vysvětlen mobilizací glutathionu ať jeho přesunutím z orgánů, nebo zvýšenou syntézou v přítomnosti toxického činidla. Hlavním úkolem této práce bylo elektroforeticky izolovat z nebílkovinných ninhydrin-pozitivních sedlin komponenty sloučenin glutathionu a cysteinu — S-sulfoglutathion a S-sulfocystein — a identifikovat pomocí elektroforetických standardů. Jak je vidět z přiložených fotografií, u zvířat, injikovaných siřičitanovým iontem, jsou pozorovány jak v orgánech, tak i v krvi 4 frakce, ze kterých první dvě jsou v blízkosti startovací čáry, cystein a glutathion a dvě následující s velkou elektroforetickou pohyblivostí — S-sulfocystein a S-sulfoglutathion. Na obr. 3 je vidět, že S-sulfoglutathion se neobjevuje v orgánech a krvi kontrolních zvířat a S-sulfocystein je v značně nižší koncentraci. Celkem je možno říci, že S-sulfoglutathion u pokusných zvířat se objevuje v mnohem nižší koncentraci než S-sulfocystein, především v ledvinách a v játrech. V krvi, kde koncentrace glutathionu značně převyšuje koncentraci cysteinu, nebyly pozorovány tak významné rozdíly.

Závěr

1. Po opakované intraperitoneální injekci izotonického roztoku siřičitanového iontu se v krvi a v orgánech morčat tvoří sulfoderiváty typu $RS\text{-}SO_3H$, které vznikají přímým působením siřičitanového iontu na glutathion a cystein „in vivo".

2. Při elektroforetické identifikaci nebílkovinných sedimentů, získaných z orgánů a krve, je pozorován značně vyšší obsah S-sulfocysteinu v porovnání s tvořícím se S-sulfoglutathionem.

3. Významná množství S-sulfocysteinu se tvoří též u kontrolních zvířat, takže S-sulfocystein je možno považovat za normální produkt metabolismu.

4. Obsah celkového a redukovaného glutathionu v krvi pokusných zvířat se po intraperitoneální injekci roztoku siřičitanového iontu při jednorázovém zavedení nemění, ale po dvakrát až třikrát opakované injekci se zvyšuje.

Autorka děkuje dr. Hájkovi z ÚH Praha za laskavou pomoc při úpravě práce.

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Souhrn

Autoři dali třem skupinám morčat intraperitoneální injekce izotonického roztoku obsahujícího 2,5 mg kysličníku siřičitého v 1 ml jednou, dvakrát a třikrát tak, že zvířata dostala od 5—30 mg kysličníku siřičitého celkem. Před pokusem a po dekapitaci zvířat byl stanoven obsah celkového a redukovaného glutathionu v krvi. Z orgánů (játra, ledviny, střeva) a krve byly získány ninhydrinpozitivní substance bez bílkovin, identifikované elektroforézou na papíře v pyridinacetátovém pufru o pH 4. Současně s cysteinem a glutathionem se na elektroforeogramu stanovily též jejich sulfodesiváty — S-sulfocystein a S-sulfoglutathion. Obsah glutathionu v krvi při jednorázovém zavedení siřičitanového iontu se nemění, ale po dvakrát až třikrát opakované injekci se hladina glutathionu zvyšuje.

Přeložila dr. Dadourková, Ústav hygieny, Praha 10, Šrobárova 48

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Došlo 10. 10. 1966.

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AMA. Arch. Ind. Health, 21 1960

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The Dynamics of Sulfur Dioxide Inhalation

Absorption, Distribution, and Retention

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The combustion of coal and fuel oil has resulted in a considerable contamination of the atmosphere by SO_2, amounting to as much as 2,000 tons per day in a large city such as New York.[1] The effects of the chronic inhalation of low concentrations of this substance are of particular importance, as urban atmospheric concentrations usually do not exceed 0.5 to 1 ppm. This aspect of SO_2 toxicity has been admirably reviewed by Greenwald.[2] The Committee on Threshold Limits of the American Conference of Governmental Industrial Hygienists in April, 1958, recommended that the threshold value for sulfur dioxide be limited to 5 ppm (13 mg. per cubic meter).[3] This is of significance, since the physiological effects of the inhalation of prolonged low-level concentrations of SO_2 in the human are essentially unknown.

Many studies[4-5] have dealt with the effects of relatively short exposures and high concentrations of SO_2, with observation of the symptoms and signs of the accompanying irritation of the respiratory tract. Recently the physiologic effects of exposure to SO_2 have come under investigation.[6-8] In addition, the rapidity of the absorption, distribution, and the length of time sulfur dioxide resides in plants[9] and animals[10] have been investigated.

Both phases of this problem have been of interest to us. A study is in progress in which Sprague-Dawley rats are being exposed to 0, 1, 2, 4, 8, 16, and 32 ppm of SO_2 throughout their lifetimes and for 24 hours each day. Observations include the length of life-span and the resulting histopathological changes. In addition, studies have been completed and are in publication regarding acute exposures of dogs to various concentrations of $S^{35}O_2$, inhalation being via a tracheostomy[11,12] and by nose and mouth breathing.[13] It has been demonstrated in the former study that SO_2 tagged with $S^{35}O_2$ is rapidly absorbed from the trachea and lungs and is distributed to all tissues examined, including the brain. A relatively high per cent retention of the inhaled gas by the trachea, lungs, hilar lymph nodes, liver, spleen, and kidneys occurred. The latter study indicated that when $S^{35}O_2$ is inhaled through the nose and mouth in concentrations of from 1 to 141 ppm a large proportion is deposited in the upper airways, a greatly reduced amount reaching the trachea, lungs, and other viscera. Even so, the smaller quantity of $S^{35}O_2$ reaching the lungs is absorbed and distributed to all tissues, including brain and bone marrow.

The present report is concerned with three additional aspects of interest in regard

Received for publication July 8, 1959.

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Supported in part by Public Health Service Contract SAph 70210, Air Pollution Medical Program, Experimental. Some of the facilities were available through grants-in-aid-of-research from the Public Health Service National Institute of Arthritis and Metabolic Diseases, Graduate Training Grant 2A5129; Army Medical Research and Development Command, DA-49-007-MD-905; The American College of Chest Physicians, and the United States Steel Research Foundation, Inc.

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TABLE 1.—*Pulmonary Function in Dogs Exposed to $S^{35}O_2$ only Through a Tracheal Segment in Vivo*

Dog No., Wt.	Length of Tracheal Seg. Exposed, In.	Inhaled Conc. SO_2, ppm	Exhaled Conc. SO_2, ppm	Decrease in Compliance of Lungs & Thorax *		Increase in Pulmonary Resistance †	
				At End of Exp. Period, %	1 Hr. After Exp., %	At End of Exp. Period, %	1 Hr. After Exp., %
1 33 lb.	4½	45	17	19	18	17	20
2 ‡ 28 lb.	3	91	66	2	3	4.5	0

* In millimeters per centimeter H_2O pressure.
† In centimeters H_2O pressure per liter per second.

to SO_2 transfer. These are (1) the extent of absorption of SO_2 from the trachea when it alone is exposed to SO_2, (2) the length of time that SO_2 can remain in the tissues, and (3) the distribution of $Na_2S^{35}O_4$ when injected into the blood stream, in contrast to the distribution after $S^{35}O_2$ inhalation.

Methods and Results

SO_2 Absorption from Trachea.—Two dogs were prepared so that an isolated segment of the trachea could be perfused with an $S^{35}O_2$ air-gas mixture, without the latter entering the main-stem bronchi or lungs. This was done by opening the chest in the midline through a sternum-splitting procedure and transecting the trachea about 1 in. above its bifurcation. A right-angle metal cannula was inserted into the distal trachea, and the lungs were ventilated only with air. A similar metal cannula was also inserted into the proximal part of the trachea, that is, the end just above the point of transection. This cannula served as the port of entry of $S^{35}O_2$. The chest then was tightly sutured around the metal cannulae and the air remaining in the pleural space aspirated. A tracheal tube with a rubber cuff was inserted through the mouth, and its tip situated just below the larynx. $S^{35}O_2$ gas could thus be pumped from a Mylar bag into the trachea and collected in a second Mylar bag as it exited through the upper tracheal tube. The whole animal was placed in a body plethysmograph so that the compliance of the lungs and thorax and the pulmonary resistance could be measured.[8,13] Pulmonary function measurements were determined serially during a 30-minute control period while the animal breathed air, during an exposure period when $S^{35}O_2$ was pumped through the isolated tracheal segment at a constant rate, and finally during a one-hour postexposure period when the tracheal segment was again perfused with air. The lungs were ventilated with air at all times by a respiration pump.

There was an appreciable decrease in compliance in the first dog and less decrease in the second animal (Table 1). Both demonstrated an increase in pulmonary resistance. These changes in compliance are of the same order of magnitude as seen in animals having their trachea and lungs directly exposed to SO_2.[11,12] The increases in pulmonary resistance are significant in degree and never have been observed to occur spontaneously. They may be com-

TABLE 2.—*Per Cent Retention by Organs After Passage of $S^{35}O_2$ Through an Isolated Segment of Dog Trachea in Vivo*

Dog No.	Vol. $S^{35}O_2$, L.	Exp. Period, Min.	Total Activity S^{35}, μc.	Visceral % Retention of S^{35}				
				Trachea	Lungs	Liver	Spleen	Kidneys
1 *	100	10	72	2.2	1	2.2	.3	.6
2 †	300	85	150	.6	.3	.5	.02	.1

* Length of trachea exposed, 4½ in.
† Length of trachea exposed, 2 in.

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TABLE 3.—*Concentrations of S^{35} in Tissues After Isolated Tracheal Segment Perfusion by $S^{35}O_2$ in Vivo* *

	Dog 1	Dog 2
Trachea	200	170
Lungs	5.2	3.0
Hilar lymph nodes	6.2	4.0
Liver	2.7	2.7
Spleen	3.9	2.0
Kidneys	4.0	3.0
Esophagus	5.0	8.4
Stomach	3.8	2.0
Duodenum	2.6	2.0
Jejunum	2.8	2.6
End-exposure, venous blood	2.0	3.9
End-exposure, arterial blood	8.4	4.7

* In millimicrocuries per gram.

pared to an average increase in pulmonary resistance of 230% as seen in animals whose lungs are directly exposed to SO_2[11,12] and 47% in animals breathing SO_2 through the nose and mouth.[13]

$S^{35}O_2$ was readily absorbed from these limited lengths of trachea (Table 2), and an appreciable amount of S^{35} was detected in the lungs, liver, spleen, and kidneys. Tissue S^{35} (Table 3) determinations indicated that concentrations were greater in the lungs, hilar lymph nodes, and midesophagus than in the remaining tissues assayed. The tracheal concentration was, of course, many times that in any other tissue.

Length of Time S^{35} Remains in Tissues.

Two dogs were exposed to $S^{35}O_2$ in the body plethysmograph via a tracheal tube inserted through the mouth.[12] Definite decreases in compliance (Table 4) and increases in pulmonary resistance occurred. They were then returned to their cages and autopsied two and seven days after exposure, respectively, and their tissues analyzed for S^{35} (Table 5). Ninety per cent or more of the $S^{35}O_2$ per unit of gas-air mixture inhaled was retained (Table 4). Even at the end of seven days appreciable concentrations of S^{35} were present in the tissues of the respiratory tract when compared to two animals acutely exposed to similar total amounts of S^{35} via a tracheostomy (Dogs 8 and 12, Table 5). The S^{35} concentration in the other viscera and tissues was either very low or nil. Therefore, the trachea and lungs retain S^{35} for as long as seven days after acute exposures to moderate concentrations of $S^{35}O_2$.

No S^{35} was detected in the feces at any time, but the S^{35} concentrations in the urine were 11.7 mμc. per milliliter in Dog 3 two days after exposure and 0.7 mμc. per milliliter in Dog 4 seven days after exposure.

Distribution of Intra-Arterially Injected $Na_2S^{35}O_4$.—One thousand microcuries of S^{35} in the form of $Na_2S^{35}O_4$ was injected into the femoral artery of a dog. Blood samples were taken for 100 minutes, and the dog was then autopsied. This time corresponds to that used in dogs which have inhaled $S^{35}O_2$ (40-minute exposure period plus a 60-minute postexposure follow-up period).[11-13] The concentrations in the tissues and per cent retentions by the organs were very low. Only 0.06% was retained by the trachea and 0.2% by the lungs. The

TABLE 4.—*Lung Function Tests During Exposure to $S^{35}O_2$-Tagged SO_2* *

Dog No., Wt.	SO_2 Inhaled, ppm μg/100 Cc.	SO_2 Exhaled, ppm μg/100 Cc.	SO_2 Retained, μg/μg/lb.	Total Exp. Time, Min.	Compliance of Lungs & Thorax † Control	Lowest	Final	40 Min. After Exp.	Pulmonary Resistance ‡ Control	Lowest	Final	60 Min. After Exp.	Autopsy
3 27 lb.	15 / 3.93	1.2 / 0.37	3024.1 / 113	43.5	10.4	19.6	18.6	18.8 −3.1%	51.0	66.5 +30%	66.5	54.5	2 days after SO_2 exp.
4 27 lb.	33 / 8.61	1.3 / 0.35	6015.4 / 245	40.8	22.6	22.0	21.8	21.6 −4.4%	27.4	55.6 +101%	48.6	26.7	7 days after SO_2 exp.

* Per Cent figures are increases and decreases, compared to control values.
† In millimeters per centimeter H_2O pressure.
‡ In centimeters H_2O pressure per liter per second.

TABLE 5.—S^{35} *Retained in Tissues and Organs*

	Dog 3, Autopsied After 2 Days		Dog 4, Autopsied After 7 Days		Acute Tracheal Exposure % S^{35} Retained *	
	S^{35}, Mμc/Gm.	% S^{35} Retained	S^{35}, Mμc/Gm.	% S^{35} Retained	Dog 12	Dog 8
Weight, lb.	27		27		43	[illegible]
Exp. time, min.	42.5		40		20	[illegible]
ppm	15		33		11.2	[illegible]
Total S^{35} retained, μc.	21.3		33.7		14.5	[illegible]
Larynx	—	—	1.4	0.07	—	—
Trachea	15	0.6	1.8	0.9	7.2	[illegible]
Lungs	0.4	0.23	0.42	0.11	6.9	2.4
Hilar lymph nodes	0	0	0	0	0.2	[illegible]
Abdom. lymph nodes	0	0	3.5	0.06	—	—
Right vent. muscle	0	0	0	0	0.6	—
Left vent. muscle	0	0	0	0	2.3	[illegible]
Liver	0	0	0	0	27.4	[illegible]
Spleen	0	0	0	0	5.4	[illegible]
Bile	0.7	—	0	0	—	—
Kidneys	0	0	7.8	0.02	2.6	[illegible]
Urin. bladder	0	0	0	0	—	—
Adrenals	0	0	0	0	—	—
Pancreas	0.2	0.02	0	0	—	—
Testes	2.4	0.15	1	0.01	—	—
Thyroid	0	0	0	0	—	—
Submax. gland	4.6	0.9	—	—	—	—
Esophagus	0.6	—	1.5	—	—	—
Fundus stomach	0.5	—	0.7	—	—	—
Jejunum	—	—	0	0	—	—
Brain	0	0	0	0	0.6	—
Skin	0	0	0	0	—	—
Muscle	0	0	0	0	—	—
Eye	0.03	—	—	—	—	—
Tongue	0.36	—	0.1	—	—	—

* Two dogs exposed via tracheostomy and autopsied 40 minutes after end of inhalation period. Figures given are in per cent of S^{35} which was retained by animal which is present in organ or tissue.

liver, spleen, kidneys, and brain retained 4.6%, 0.26%, 0.3%, and 0.6%, respectively, of the administered radioactive sulfate. The concentrations in the trachea and lungs were much lower than those in animals inhaling $S^{35}O_2$ in amounts which were only 1/10 of the activity which had been given intra-arterially. The remaining tissue concentrations were similar to those found in dogs receiving $S^{35}O_2$ by inhalation.[12,13] Therefore, S^{35} reaching the blood stream either after inhalation of $S^{35}O_2$ or by injection as $Na_2S^{35}O_4$ is distributed and becomes localized in a similar pattern in tissues other than those of the respiratory tract. No localization in the tissues of the respiratory tract follows intravenous administration of radioactive sulfate.

Comment

When inhaled through the nose and mouth in concentrations as low as 1 ppm, SO_2 is absorbed and reaches all tissues and viscera, including the brain.[11-13] The majority of the SO_2 (90% or more) is removed from an inhaled gas-air mixture and becomes localized mainly in the pharynx, trachea, lungs, and hilar lymph nodes. It is taken up from these sites of deposit, and a significant portion is found in the liver, spleen, and kidneys.

The SO_2 in the trachea and lungs is only slowly removed from these sites and, according to our experiments, can remain in these tissues for at least one week. According to Bystrova,[10] radioactive sulfur may be found in the tissues of mice, especially in the lungs, for as long as three weeks after exposure. It was detected in the protein portions of the tissues.

S^{35} is readily excreted in the urine, but none was found in the feces. It was stated to be excreted in both feces and urine

by Bystrova,[10] but in these experiments high concentrations of SO_2 were used and also a "priming dose" technique was used.

It was demonstrated by our tracheal transpiration experiments that the tracheal mucosa, when alone exposed, can absorb SO_2 in sufficient amounts to affect pulmonary function. Similar tests were not carried out to test uptake by the mucosa of the nose and mouth. However, $S^{35}O_2$ becomes deposited in these absorptive sites in high concentrations after nose and mouth breathing of this gas, and uptake into the blood most likely follows.[15]

The chemical nature of SO_2 as it exists in the tissues and blood after absorption has been little studied. Bystrova[10] has stated that SO_2 is attached in some manner to the proteins of the plasma and tissues. The mechanism by which pulmonary resistance and compliance are affected by SO_2 absorbed into the blood after tracheal exposure alone is unknown. Reflex bronchoconstriction after stimulation of nerve endings in the bronchi is possible, but a direct effect upon smooth muscle also has to be considered. In our preparation the nerve supply to the isolated tracheal segment as well as the bronchi and lungs was not disturbed.

S^{35}, given as $Na_2S^{35}O_4$ intra-arterially, did not become preferentially localized in the trachea and lungs and apparently became uniformly distributed throughout the body water.

Summary

SO_2 is readily absorbed from the upper and lower respiratory tract even when inhaled in low concentrations (1 ppm). It can be absorbed by the tracheal mucosa when the latter alone is exposed to a degree sufficient detrimentally to influence pulmonary function. After absorption from the sites of deposition in the trachea, bronchi, and lungs, it is distributed to all tissues, including the brain. It can still be detected in the trachea and lungs one week after exposure. The physiologic and metabolic effects of the prolonged inhalations of low-level concentrations of SO_2 are unknown, and experiments are now in progress in an attempt to obtain further data on this aspect of the effects of the inhalation of low concentrations of sulfur dioxide.

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J. Sci. Fd Agric. 1972, 23, 207–213

The Enhancement of Orange Oil Oxidation by Sulphur Dioxide

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Reactions at 35 °C of orange oil in contact with air and water were investigated by gas–liquid chromatography (g.l.c.) and by oxygen absorption measurements. Degradation of the orange oil was reduced in the presence of citric acid, but addition of sulphur dioxide caused a drastic increase, the extent and the rate of which were proportional to the initial concentration of the preservative. Several degradation products were identified.

1. Introduction

As part of a programme of research[b] concerning the storage stability of citrus oils and associated materials, numerous model systems have been investigated. Initially the investigation concerned the autoxidation of orange oil, and it was later extended to include variables, such as the presence of water, citric acid and preservatives.

The autoxidation reactions of orange oil[1,2] and limonene[3] have been studied previously. Baines *et al.*[4] have recently studied in detail the acid catalysed rearrangements of terpenes. Our studies have been concerned mainly with the effects of adding preservatives to the citrus systems.

Sulphur dioxide and benzoic acid are the two substances most widely used as preservatives for citrus products,[5] and it is generally recognised that sulphur dioxide also has the advantage of possessing antioxidant properties.[6–8]

Interactions of terpenes and sulphur dioxide have been studied previously.[9–11] The formation of oxidation products has only been reported for reactions carried out under very vigorous conditions, i.e. autoclaving at 145 °C for 3 h.[10,11] However, our initial experiments showed that the presence of sulphur dioxide was enhancing the oxidation of orange oil, even under relatively mild conditions, i.e. storage at 35 °C. Thus in these systems sulphur dioxide was behaving as a pro-oxidant and not as an antioxidant. Further experiments were therefore carried out in an attempt to confirm this observation and these are described in this paper.

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[b] This programme of research is being carried out in conjunction with Dr R. A. Jones and his associates, School of Chemical Sciences, University of East Anglia (see, for example, ref. 4).

2. Experimental

The orange oil used in this work was a Palestine sweet orange oil (Shamouti) from Naarden (London) Ltd. Sulphur dioxide was added as sodium metabisulphite. The experiments were of two types: one type involved the use of g.l.c. and the other Warburg manometers.

2.1. Gas–liquid chromatography experiments

This series (see Table 1) involved the interaction of orange oil with different combinations of water–citric acid–preservatives. Mixtures of the orange oil (1.8 ml) and the aqueous solutions (215 ml) were stored at 35 °C in glass bottles (capacity = 230 ml) fitted with screw-caps. The oil was not dispersed in the aqueous phase because a simple homogenisation would not have given rise to a stable emulsion, and the addition of an emulsion stabiliser may have complicated the interaction.

The reaction mixtures were examined visually after 4 weeks and 9 weeks, and their oils were analysed by direct injection of 1 μl samples into a Beckman GC-2A gas chromatograph under the following conditions.

Column oven temperature = 100 °C (isothermal). Column: (a) 2% LAC 2R446 on Chromosorb G (2 m × 6 mm stainless steel), (b) 30% Carbowax 1540 on Firebrick C22 (2 m × 5 mm stainless steel). Carrier gas = nitrogen (flow rate = 0.50 ml/s). Flame ionisation detector.

2.2. Warburg manometer experiments

This series of experiments (see Figures 1 and 2) measured the oxygen uptake of orange oil in the presence of different combinations of water–citric acid–sulphur dioxide. Similar experiments in which the orange oil was replaced by various monoterpenes were studied in order to aid interpretation of the orange oil results.

Oxygen uptake was measured in Warburg manometers[12] (B. Braun, Melsungen, West Germany), containing Braun's solution as the barometric fluid and thermostatted at 35.00 ± 0.02 °C.

All reaction mixtures consisted of 2 ml of aqueous solution (citric acid concentration = 2% w/v) and 0.25 ml of orange oil or monoterpene. Pressure changes were measured at constant volume and allowance was made for fluctuations in atmospheric pressure by use of control manometers containing distilled water.

3. Results and discussion

After storage at 35 °C for only 4 weeks, all the reaction mixtures containing sulphur dioxide showed marked changes in appearance and odour. The extent of the changes increased with the sulphur dioxide concentration. The mixtures containing high concentrations of sulphur dioxide had strong odours (similar to those normally associated with oxidised orange oils), the oils had become brown (some had a brown ring of semi-solid material above the oil), the aqueous phase had become cloudy, and there was a deposit of brown sludge at the bottom of the bottles. The brown ring and the sludge were, presumably, polymeric reaction products from the oil.

The only reaction mixture not containing sulphur dioxide which showed any obvious changes was the mixture of oil and water. This mixture had a faint odour indicative of an oxidised oil and both the oil and aqueous phases were slightly cloudy. The mixture containing oil and citric acid and the mixtures containing oil, citric acid and benzoic acid showed no obvious changes.

After storage for 9 weeks, no further major changes had taken place apart from the loss of natural orange oil colour in all the remaining samples.

The results of the gas chromatographic analyses confirmed those of the visual examinations and are briefly summarised in Table 1 (together with a description of the reaction mixtures). Retention times are relative to limonene = 10 and peak heights relative to limonene = 10 000. Although the limonene is probably taking part in the reaction, the relative changes in its concentration are small when compared to those of other reactants and products. Limonene can therefore be used as a "pseudo" internal standard for detecting large changes in the concentrations of the other components.

The results of the g.l.c. analyses show that the magnitude of the changes induced in the orange oil increased in the following order:

$$\begin{matrix}\text{Citric acid}\\ 180\ \text{B}\\ 1800\ \text{B}\\ 3600\ \text{B}\end{matrix} < \text{Water} < 150\ \text{S} < \begin{matrix}1200\ \text{S} + 500\ \text{B}\\ 1500\ \text{S}\end{matrix} < 3000\ \text{S}.$$

Thus, the presence of citric acid reduced the extent of the oil–water reaction, and the further addition of benzoic acid had no obvious effect. However, the addition of sulphur dioxide to the oil–water–citric acid system drastically increased the extent of the reaction.

The results of the gas chromatographic analyses of the reacted oils were compared with those of the unreacted orange oil. The main components which were used up in the reaction (relative to limonene) were tentatively identified as: α-pinene, β-pinene, myrcene, octanal, decanal and linalol. It is well known that many compounds (e.g. alkene[13] and carbonyl[14] compounds) will react with bisulphite, forming stable compounds such as bisulphite addition complexes and sulphonic acids. However, the gas chromatographic analyses also showed the presence of reaction products which were tentatively identified as: *p*-cymene, *p*,α-dimethylstyrene,[4] carvone, carveol and α-terpineol. These are oxidation products of orange oil (*N.B.* It must be assumed that the concentrations of these products would also have been reduced by formation of bisulphite addition compounds.)[13, 14] There were numerous other reaction products, many of which were low boiling compounds, which were not identified. The reaction mixtures containing high concentrations of sulphur dioxide also gave rise to a polymeric material (presumably formed, at least in part, from the limonene), which was shown by infrared analysis to be a mixture containing a high proportion of hydroxyl groups and a lower proportion of carbonyl groups.

Thus some of the compounds formed during the reaction (see Table 1) were the normal autoxidation products of orange oil[1,2] and obviously include those of limonene[3] (its major constituent). Other major components of the reacted oils were also tentatively identified by g.l.c. retention data as products of oxidative reactions. Work is in progress to identify these products more positively and to determine the mechanism of their

TABLE 1. Summary of gas–liquid chromatographic analyses of samples stored at 35 °C for 9 weeks

Relative retention time	Tentative identity	Relative peak heights									
		U.O.O.	Water	Citric acid	150 S	1500 S	3000 S	180 B	1800 B	3000 B	1200 S 500 B
4	α-pinene	118	115	106	128	79	45	110	112	112	112
6	β-pinene	106	105	92	9	5	5	91	93	59	5
8	myrcene	227	186	225	128	9	—	213	217	193	28
10	limonene	10 000	10 000	10 000	10 000	10 000	10 000	10 000	10 000	10 000	10 000
15	p-cymene	—	1	—	12	36	286	—	—	—	100
16	octanal	16	3	13	2	—	—	12	11	12	—
22	unidentified	—	—	—	—	42	169	—	—	—	111
36	p,α-dimethylstyrene	—	—	—	1	31	81	—	—	—	49
48	decanal	6	—	6	1	—	—	6	6	6	—
53	linalol	9	6	—	1	—	—	—	1	2	—
98	unidentified	—	4	—	2	8	16	—	—	—	3
125	α-terpineol	1	—	2	2	4	8	2	2	1	11
166	carvone	—	19	—	3	21	26	—	—	—	7
218	carveol	—	6	—	1	7	8	—	—	—	3

U.O.O., unreacted orange oil; Water, orange oil and water; Citric acid, orange oil and 2% w/v citric acid in water. In the remainder of the samples sulphur dioxide (S) or benzoic acid (B) was present, at the levels (p.p.m.) indicated, in the citric acid.

formation. It is probable that the mechanism includes acid catalysed rearrangements.[4] Nonetheless, since terpene oxidation products were formed during the reaction it can be concluded that the presence of sulphur dioxide actually enhanced the oxidation of the orange oil.

This conclusion was confirmed by the results of the Warburg experiments, which showed (Figure 1) that the rate of oxygen absorption by the orange oil–sulphur dioxide mixtures increased almost linearly with the added sulphur dioxide concentration.

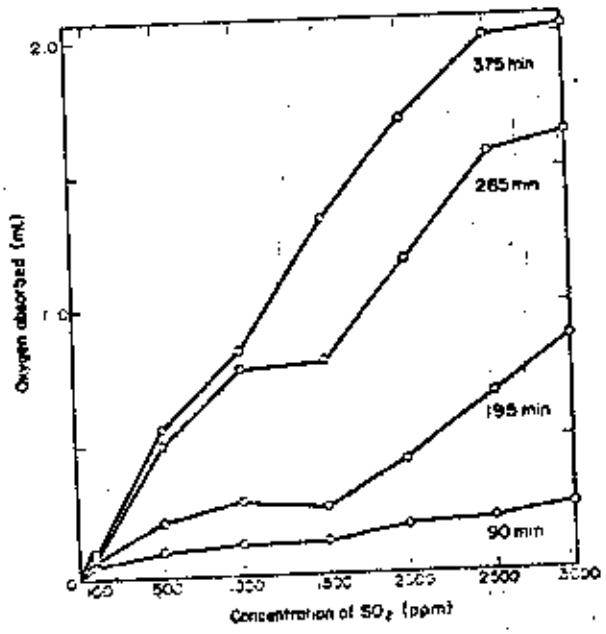

Figure 1. The variation of oxygen absorption with sulphur dioxide concentration. The reaction mixtures consisted of orange oil and aqueous solutions of various concentrations of sulphur dioxide (100, 500, 1000, 1500, 2000, 2500 and 3000 p.p.m.). The volumes of oxygen absorbed by the solutions were measured at the following times: 90, 195, 285 and 375 min.

Furthermore, the reaction slowed down considerably when the molar quantity of oxygen absorbed approached the molar quantity of sulphur dioxide present initially. This implied that sulphur dioxide was being used up in the reaction, and in addition that each molecule of sulphur dioxide was responsible for absorption of one molecule of oxygen (i.e. twice the quantity required to convert sulphite to sulphate). The decrease in rate as the sulphur dioxide was used up also confirmed that the rate of oxygen absorption was dependent on the sulphur dioxide concentration. However, in the absence of orange oil, less than one twentieth of its molar equivalent of oxygen was absorbed by the sulphur dioxide within the same time and the rate of oxygen absorption was therefore much slower. The excess of oxygen absorbed by the orange oil–sulphur dioxide mixtures must have been utilised for oxidising reactants other than the sulphur dioxide, i.e. components of the orange oil, and this, therefore, provides further confirmation that the presence of sulphur dioxide enhances the oxidation of orange oil.

The presence of citric acid slowed the rate of oxygen absorption by both orange oil and by sulphur dioxide solutions, but its presence accelerated the rate of oxygen absorption by mixtures of orange oil and sulphur dioxide solutions (Figure 2). This indicated that the reaction is pH dependent.

The reaction mixtures of sulphur dioxide solutions with individual monoterpenes (i.e. limonene, myrcene, citral and geraniol) also absorbed oxygen rapidly, which again indicates mutual acceleration of oxidation. It has been shown[15] that the oxidation of

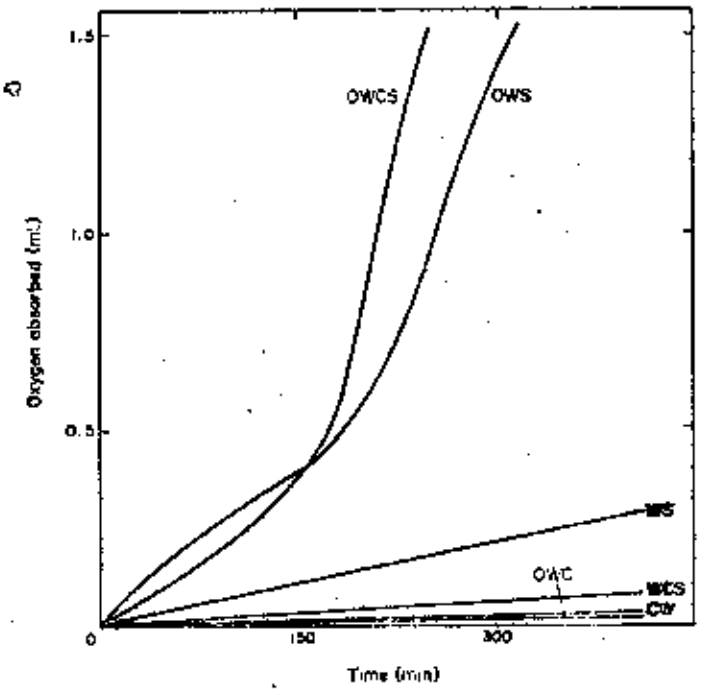

Figure 2. The rate of oxygen absorption by various combinations of orange oil–water–citric acid–sulphur dioxide.

The reaction mixtures consisted of various combinations of: O = orange oil (0.25 ml), W = water (2.00 ml), C = citric acid (2% w/v), S = sulphur dioxide (1500 p.p.m.).

aqueous solutions of sulphur dioxide in the presence of air proceeds by a free radical chain mechanism. A wide variety of organic compounds are known to inhibit sulphite oxidation by chain termination.[16] Thus, the behaviour of orange oil and the monoterpenes, which appear to enhance the oxidation of the sulphur dioxide (and *vice versa*), is unusual.

The mutual acceleration of the oxidation of sulphur dioxide and orange oil could be related to the fact that both reactions proceed via a free radical chain mechanism.[15, 17] Further evidence for this has been obtained by our recent experiments which have shown that the addition of an antioxidant, such as butylated hydroxyanisole, slows the oxidation rate of orange oil–sulphur dioxide mixtures. Details of these experiments will be published later.

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J. Agr. Food Chem.

Colorimetric Determination of Sulfur Dioxide from Malt and Beer by Complexing with Sodium Tetrachloromercurate(II)

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A rapid, sensitive method is presented for the determination of sulfur dioxide in malt and beer which embodies a distillation procedure in conjunction with a pararosaniline hydrochloride—formaldehyde colorimetric determination. A solution of sodium tetrachloromercurate(II) is used as the absorbing medium for sulfur dioxide. This results in the formation of the stable disulfitomercurate (II) ion which does not require an immediate analysis for sulfur dioxide. The method is specific for sulfur dioxide, and no interferences have been found. When the method was applied to the determination of sulfur dioxide in the range of 1 to 75 p.p.m., a maximum error of ±5% was realized.

Extensive investigations involving a colorimetric analysis of sulfur dioxide in low concentrations have been carried out by Grant (*1*), Steigman (*4*), and Joslyn (*2*), who successfully used a basic fuchsin–formaldehyde complex as the color reagent. West and Gacke (*6*) have reported increased stability and sensitivity of the color reagent by substitution of hydrochloric acid–bleached pararosaniline for the previously used sulfuric acid–bleached fuchsin.

In the present study, preliminary application of a colorimetric procedure to malt and beer was based on the work of Steigman in conjunction with a modified Monier-Williams method (*3*). Evolved sulfur dioxide was absorbed in a solution of 5% glycerol in 0.1 *N* sodium hydroxide as suggested by Urone and Boggs (*5*). West and Gacke have improved upon the absorbing medium through introduction of a sodium tetrachloromercurate(II) solution. Use of this solution as an absorbing medium for sulfur dioxide results in formation of the stable disulfitomercurate(II) ion. The method presented here for determination of sulfur dioxide in malt and beer embodies a distillation procedure together with an adaptation of the colorimetric method for sulfur dioxide developed by West and Gacke.

Reagents and Apparatus

Pararosaniline Hydrochloride Solution. Dissolve 0.1 gram of pararosaniline hydrochloride (Certified Dye, National Aniline Division, Allied Chemical and Dye Corp.) in distilled water and dilute to 100 ml. Allow it to stand for 1 day to assure complete solution.

Bleached Pararosaniline Hydrochloride Solution. Pipet 20 ml. of pararosaniline hydrochloride solution into approximately 100 ml. of distilled water contained in a 500-ml. volumetric flask. To this add 30 ml. of reagent grade concentrated hydrochloric acid; allow the dye to bleach to a pale yellow-green color and dilute to 500 ml. with distilled water.

Sodium Tetrachloromercurate(II) Solution. Dissolve 11.7 grams of reagent grade sodium chloride and 27.2 grams of reagent grade mercuric chloride in 1 liter of distilled water.

Formaldehyde Solution. Dilute 5.5 ml. of 36% reagent grade formaldehyde to 100 ml. with distilled water.

Standard Sulfite Solution. Accurately weigh a quantity (approximately 0.2 gram) of reagent grade sodium bisulfite (iodometric assay 58.5% sulfur dioxide) and dissolve in 100 ml. of sodium tetrachloromercurate(II) solution. Dilute to 500 ml. with distilled water. Transfer a 10-ml. aliquot to a 100-ml. volumetric flask and dilute to volume with distilled water.

Alkaline Pyrogallol Solution. Dissolve 5 grams of pyrogallic acid in 25 ml. of hot distilled water. Cool this solution and mix with 100 ml. of an 80% potassium hydroxide solution.

Spectrophotometer. A Beckman Model DU spectrophotometer with 1-cm. borosilicate glass cells.

Distillation Apparatus. The assembled distillation apparatus is illustrated in Figure 1.

Analytical Procedure

Preparation of Standard Curve. Aliquots of 1 to 10 ml. of the standard sulfite solution were pipetted into 100-ml. volumetric flasks containing 25 ml. of bleached pararosaniline hydrochloride solution and 2 ml. of formaldehyde solution. The color was developed for 7 minutes at room temperature, followed by dilution to volume. The absorbance was immediately determined at a wave length of 560 mμ using matched 1-cm. borosilicate glass cells. The color was stable for 5 minutes and then faded slowly. Absorbance readings can be expected to decrease by about 2% over a period of 60 minutes. The standard curve was linear in the concentration range of 0 to 0.2 mg. of sulfur dioxide per 100 ml.—effective for malt and beer.

Experimental. Three hundred grams of beer were weighed into the 1-liter distillation flask and degassed for 5 minutes with nitrogen, which had been purged of oxygen by passage through the alkaline pyrogallol trap. Twenty milliliters of reagent grade concentrated hydrochloric acid were added via the aspirator bottle and connecting tube. In this manner, air was not introduced into the distillation system.

The distillation flask was heated for 45 minutes with continuous nitrogen flushing at the rate of about 75 ml. of nitrogen per minute. Sulfur dioxide was distilled into 100 ml. of sodium tetrachloromercurate(II) solution contained in a 125-ml. gas absorption tower, the gas dispersion tube of which was fitted with a medium porosity fritted disk. After completion of the distillation, a 5-ml. aliquot was withdrawn from the gas absorption tower and transferred to a 100-ml. volumetric flask containing 25 ml. of bleached pararosaniline hydrochloride solution and 2 ml. of formaldehyde solution. The color was developed

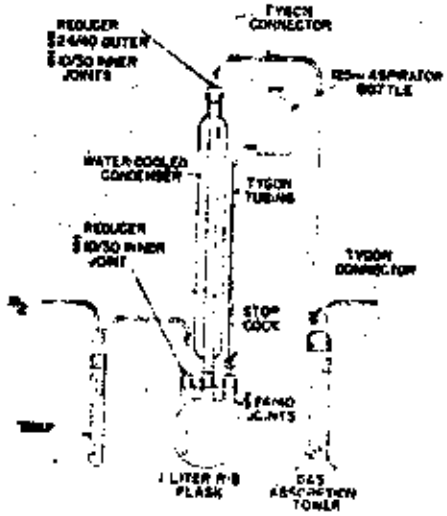

Figure 1. Assembled distillation apparatus

Table I. Recovery of Sulfur Dioxide from Synthetic Solutions

Added, Mg.	Recovered, Mg.	Recovery, %
1.40	1.42	101.6
3.50	3.43	98.0
7.66	7.55	98.6
11.71	11.85	100.5
14.00	14.30	102.1
18.90	19.10	101.6
23.70	24.60	103.6

Table II. Precision of Method on a Commercial Beer Sample

Sample	Sulfur Dioxide, P.P.M.	Sample	Sulfur Dioxide, P.P.M.
1	9.20	7	8.83
2	8.80	8	9.20
3	8.73	9	9.17
4	9.33	10	9.26
5	9.40	11	9.20
6	9.38	12	9.23
		Mean	9.14
		S.D.	0.23

Table III. Comparative Analysis of Commercial Beers

Beer	Sulfur Dioxide, P.P.M. Colorimetric	Sulfur Dioxide, P.P.M. Gravimetric
A	0.81	0.90
B	3.00	2.93
C	3.23	3.33
D	4.83	4.77
E	8.16	8.87
F	9.83	9.33

Table IV. Comparison of Colorimetric and Gravimetric Determinations of Sulfur Dioxide in Malt

Malt	Sulfur Dioxide, P.P.M. Colorimetric	Sulfur Dioxide, P.P.M. Gravimetric
A	19.0	19.2
	17.2	17.6
	18.4	22.5
	18.6	18.1
B	39.0	43.3
	39.6	43.9
	39.6	38.4
	38.4	42.1
C	70.0	71.9
	69.9	73.0
	70.0	65.5
	70.0	72.5

as for preparation of the standard curve. The sulfur dioxide concentration was obtained from the standard curve.

For the determination of sulfur dioxide in malt, experimental conditions outlined for beer were adhered to with the exception of the following modifications: Three hundred milliliters of distilled water were added to the distillation flask, and the sample consisted of 50 grams of well-mixed whole malt.

Results and Discussion

Efficiency of the distillation apparatus was established by recovery and distillations, as outlined for the experimental procedure, with known amounts of sodium hydrogen sulfite. Results of these experiments are tabulated in Table I. The data indicate that the distillation apparatus, time of distillation, and a single absorption tower give satisfactory recoveries. A number of initial experiments were conducted using two absorption towers; in every case, no detectable quantities of sulfur dioxide were found in the second absorption tower. These observations corroborate the findings of West and Gacke relative to the efficiency of sodium tetrachloromercurate(II) as a sulfur dioxide absorption medium when used with a single absorption tower.

Precision of the method was ascertained on a commercial beer sample (Table II). The data indicate that reproducibility is within the desired range of ±5% maximum error.

A comparative analysis of commercial beers and malts was conducted utilizing both the currently accepted Monier-Williams gravimetric and the colorimetric procedure presented in this investigation. Results of these analyses are presented in Tables III and IV, respectively. According to the data of both tables, satisfactory agreement exists between results of the two methods. In general, gravimetric results for malt are slightly higher than similar colorimetric results.

Attempts to apply the 1.0% pararosaniline hydrochloride concentration proposed by West and Gacke to the present study were prohibitive, as it would have necessitated a reduction of the malt sample size and subsequently introduce sampling errors. A 0.1% pararosaniline hydrochloride solution was, therefore, substituted for the 1.0% solution of pararosaniline hydrochloride used by West and Gacke. This change yielded the degree of desired sensitivity for the method.

Although, as previously indicated, the standard curve showed conformity to Beer's law to a concentration of 0.2 mg. of sulfur dioxide per 100 ml., the curve continued to be linear to a concentration of 0.3 mg. of sulfur dioxide per 100 ml. Concentrations of sulfur dioxide are limited in application to a precision spectrophotometer such as the Beckman DU. Attempts to adapt the method to a filter instrument were successful to a limited degree. Instrument response was such that the standard curve obtained conformed to Beer's law only at low concentrations of sulfur dioxide. Therefore, if usage of a filter instrument is contemplated, the limiting concentration range corresponding to sensitivity of the particular instrument must be ascertained.

During malt analyses, an occasional sample was encountered containing as much as 200 p.p.m. of sulfur dioxide. A 5-ml. aliquot of the sodium tetrachloromercurate(II) trapping solution resulted in high absorbance readings. This condition was satisfactorily overcome by using a smaller (2- or 3-ml.) aliquot without loss of sensitivity.

In the course of the present investigation, attention was given to stability of the reagents. Of reagents used, bleached pararosaniline hydrochloride exhibited a minor stability problem. After standing for 1 week, the bleached pararosaniline hydrochloride yielded results which were slightly higher than those realized for a freshly prepared solution; therefore, bleached pararosaniline hydrochloride solution should be used for a maximum of 3 days.

In addition to the possible interferences studied by West and Gacke, distillable substances from malt and beer were investigated. Color development was not affected by volatile substances produced in reflux distillation.

Conclusions

The Monier-Williams method was modified to the extent that sodium tetrachloromercurate(II) was used as the absorbing medium for sulfur dioxide in malt and beer. This solution has the advantage over the usual alkali solutions generally employed as sulfur dioxide absorbing media in that very stable disulfitomercurate(II) ion is formed. Determination of sulfur dioxide was based on the color produced when pararosaniline hydrochloride and formaldehyde were added to the absorbing medium. The method proved to be sensitive and much less time-consuming than the usual gravimetric procedure.

The ability to use 50 grams of dry material eliminates many sampling difficulties. This suggests possible application of the analysis to other food products containing low levels of sulfur dioxide.

In the malting industry, sulfur dioxide is at times applied to malt during the kilning cycle just prior to drying. Recent application of the colorimetric procedure to analysis of kiln air containing high levels of sulfur dioxide has presented very encouraging results, although the study is incomplete at this time.

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Received for review January 5, 1957. Accepted April 11, 1957.

Industr. alim. agr. 1961
V=L. 78

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Possibilités d'application des "osmopiles", piles galvaniques spéciales, à la détection d'arômes alimentaires et à la mesure de leur intensité (*)

par Alain BERTON
Maître de Recherches au C. N. R. S.

LA spécialisation de l'auteur en toxicologie industrielle lui avait fait rechercher des détecteurs simples de traces de gaz et vapeurs toxiques.

A la suite d'essais par variation de conductibilité électrique de liquides ayant dissous des gaz ou des vapeurs, il réussit à mettre au point des piles galvaniques spéciales, dont une des électrodes, généralement sous la forme d'un tamis, se trouve en contact, à la fois avec l'électrolyte et avec l'atmosphère analysée, l'autre électrode étant complètement immergée (fig. 1).

Le courant gazeux projeté sur le tamis en contact, par sa face inférieure, avec l'électrolyte, produit par action électrochimique, ou même électrophysique, une variation de courant galvanique entre les deux électrodes, qui peuvent être de même nature. On se trouve de la sorte en présence de récepteurs sensibles à des gaz et des vapeurs en très minimes quantités, ce qui lui a fait appeler ces récepteurs des piles qui sentent « des osmopiles » (du grec ôsmein = sentir).

Ainsi, avec un tamis de platine à la surface d'eau distillée, l'autre électrode électrode étant en platine également ou en plomb, on peut déceler des traces très faibles (moins de quelques parties par million en volume de gaz ou vapeurs ionisables dans l'eau) d'acides et de bases.

LES OSMOPILES

Le courant galvanique, de quelques microampères, sous quelques microvolts à quelques millivolts, circule dans un sens, pour les acides et leurs anhydrides (sulfureux, formique, acétique, butyrique, etc.), sauf pour l'acide sulfhydrique ou hydrogène sulfuré et l'acide cyanhydrique possédant un pouvoir supérieur à celui de l'eau distillée employée, qui est généralement de pH 5 environ. Le courant circule en contre de sens contraire, pour les vapeurs basiques (ammoniac, aniline, etc.).

Ainsi ainsi que les fromages, à pâte crue fermentée, présentent des vapeurs basiques, et cela d'autant plus qu'ils sont plus faits (camemberts, Pont-l'Evêque, Munster), alors que les fromages à pâte cuite, type gruyère, dégagent des vapeurs acides. Les Port-Salut, les Hollande sont neutres.

Grâce à ces piles, que nous appelons *LES OSMOPILES « A »*, on pourra contrôler la fraîcheur de certains aliments, par exemple : poissons, viandes, graisses alimentaires, qui peuvent être plus ou moins rancies.

Dans le cas de ces dernières, suivant le type d'oxydation auquel on a affaire, on utilisera des détecteurs différents. L'oxydation acidifiante sera décelée par LES OSMOPILES précédemment dé-

(*) *Communication faite à l'Association des Chimistes, le 20 Octobre 1960).*

crites. En présence d'une oxydation moins brutale, avec formation d'aldéhydes, d'alcools, de cétones

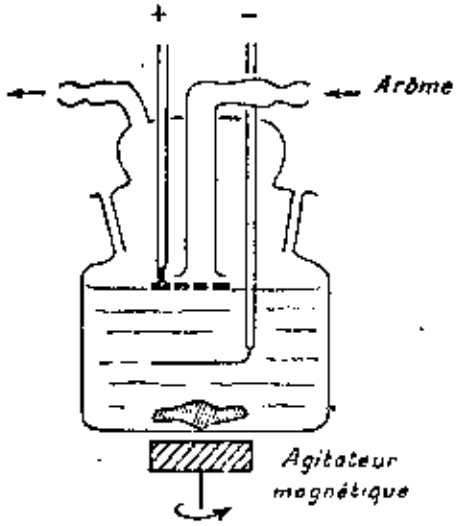

Fig. 1
Osmopile Breton
Brevetée S.G.D.G.

principalement, on fera appel *aux PILES « C »*.

Ces dernières comportant un électrolyte oxydant, se révèlent comme très sensibles à toute substance facilement oxydable : aldéhydes, alcools, esters. Elles détecteront donc également les vapeurs odoriférantes provenant des fruits, des condiments. C'est ainsi que la *PILE « C »* réagit très fortement à l'odeur du melon, à l'huile essentielle de zeste d'orange ou de citron, à l'arôme des clous de girofle, de poivre, de cannelle, de persil, d'estragon, d'oignon, d'ail, de truffes, de champignons.

Il existe une autre *PILE appelée « I »*, particulièrement sensible aux composés aromatiques à noyaux benzéniques, ou à liaisons non saturées par exemple les terpènes.

Nous avons mis au point, d'autre part, des piles sensibles aux cétones volatiles, aux dérivés nitrés organiques (nitrobenzène ou essence de mirbane), aux dérivés soufrés (sulfures d'allyle), au gaz carbonique, à l'oxyde de carbone, à la vapeur d'eau au fourneau, etc.

SPECIFICITE

Malheureusement, ces détecteurs, en général, ne sont spécifiques que de groupes de familles chimiques (acides, bases, produits facilement oxydables), et non d'une famille seule ou d'un produit chimique déterminé. Cela n'empêche pas qu'ils puissent être utilisés pour doser des gaz et des vapeurs dont on connaît par avance la nature, ces cas sont les plus fréquents dans le contrôle industriel. La spécificité absolue est atteinte en isolant chaque composé par une petite colonne de chromatographie gazeuse très simple, à l'extrémité de laquelle on dispose une OSMOPILE. On observe alors avec le temps des maxima de courant galvanique dont la position, pour des conditions expérimentales définies, est caractéristique d'un composé et dont l'intensité est fonction de la concentration de ce dernier dans le mélange analysé. (Fig. 2).

En chromatographie gazeuse classique, les récepteurs utilisés détectent indifféremment tous les composés présents et on peut assister à des superpositions gênantes des maxima ou « pics ». Avec les OSMOPILES, on ne décèlera, par contre, que les composés soufrés, ou les aromatiques, ou les alcools, ou les aldéhydes, ou les acides. C'est une chromatographie gazeuse sélective.

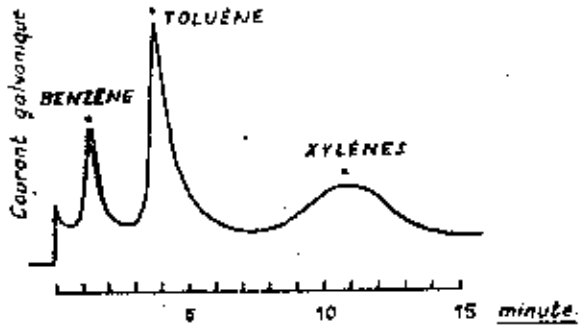

Fig. 2
Chromatographie sélective des vapeurs d'un solvant complexe base d'essence minérale, contenant des hydrocarbures aromatiq

FONCTIONNEMENT

Quel est le mécanisme de fonctionnement des OSMOPILES ?

Dans tous les cas où les courants observés sont relativement importants, c'est-à-dire de l'ordre de quelques microampères, sous une tension de quelques millivolts, la vapeur décelée doit réagir avec l'électrolyte : ionisation intense de l'eau distillée dans le cas des PILES « A », oxydation par les piles à électrolyte oxydant, réduction dans le cas des piles réductrices, déplacement d'un acide plus ionisant.

Au niveau de l'électrode active, on assiste à des variations de la concentration électronique ou polarisation, ce qui a pour effet de produire une augmentation, ou une diminution du courant galvanique.

Les phénomènes ne sont pas aussi simples, comment expliquer, dans le cas des piles à eau pure, qu'un air sec tombant sur le tamis-électrode diminue le courant ? Les molécules d'eau superficielles deviendraient moins ionisées par dessiccation comme si, suivant la loi générale de l'e

elles contractaient leurs orbites électroniques, pour s'opposer à leur évaporation.

Nous n'avons pas eu le temps d'étudier théoriquement les piles que nous avons mises au point, c'est ce que nous nous proposons de faire dans le cadre de nos recherches futures.

Indiquons néanmoins ici que si la réponse à l'action d'une vapeur est pratiquement instantanée, le retour aux conditions initiales pour procéder à une nouvelle mesure, est plus lent et peut durer plusieurs minutes, après la suppression de l'agent actif (fig. 3). Ce temps est raccourci par agitation de l'électrolyte. Notons enfin qu'après chaque essai, le pouvoir réactif de ce dernier est très peu changé, la minime quantité de vapeur étant considérablement plus petite que le volume du liquide galvanique.

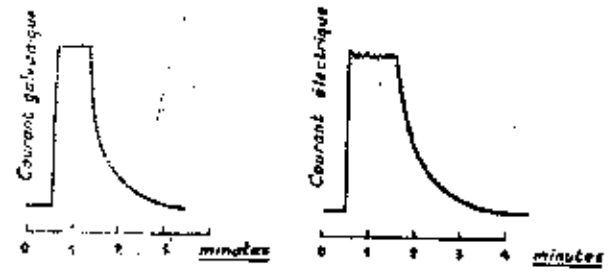

Fig. 3

Réponse d'une Osmopile à l'action d'une petite quantité de vapeur active

Réponse intégrée de nerfs olfactifs de lapin à l'action d'une petite quantité de vapeur odorante

APPAREILLAGE

Pratiquement, comment se présentent les OSMOPILES ?

Celles-ci sont enfermées dans un petit récipient en verre avec deux tubulures dont l'une est reliée à une petite pompe à membrane et l'autre dirige sur l'électrode-tamis l'air aspiré (fig. 1). Les variations de courant galvanique sont mesurées directement par un microampèremètre à aiguille, ou un galvanomètre à spot plus sensible. Si on veut les enregistrer, on utilise un suiveur de spot SEFRAM ou un appareil de notre conception qui emploie un papier enregistreur photographique spécial, noircissant instantanément, sans révélation intérieure, sous l'impact du spot du galvanomètre, constitué par une lumière ultraviolette intense.

Pour des mesures qualitatives, il suffit de présenter la vapeur considérée devant l'ajutage aspirant de l'OSMOPILE. Des mesures quantitatives nécessitent des échantillons d'un volume déterminé, qui l'on peut injecter, au moyen d'une seringue, dans le circuit d'air purifié par du charbon actif alimentant la pile.

Mentionnons que nous mettons présentement au point des piles sèches, portatives, à électrolyte inversable, retenu dans un support poreux.

MESURES

Spécialisés en toxicologie, l'étude des arômes alimentaires nous a, jusqu'à présent, peu occupé. Nous ne pouvons donc donner comme exemples l'application des OSMOPILES, que des méthodes assez éloignées de celles qui pourraient résoudre les problèmes intéressant les chimistes analystes dans les industries de l'alimentation. Toutefois les principes de base restent valables.

Nous avons mis au point les méthodes de dosage suivantes :

— alcool dans l'air des ateliers, dans l'air expiré, et dans le sang ;

— styrène, anhydride sulfureux dans l'air ;

— analyses chromatographiques d'hydrocarbures benzéniques dans des essences de pétrole et les solvants complexes ;

— même analyses pour les dérivés chlorés ou soufrés.

Ces méthodes devant faire l'objet d'autres publications, nous n'en donnerons ici que le principe : un volume déterminé, par exemple de 10 ml de l'échantillon gazeux, est injecté, au moyen d'une seringue, dans le circuit d'air pur alimentant la pile. On observe alors un certain courant galvanique que l'on compare aux courants galvaniques obtenus dans les mêmes conditions expérimentales avec des atmosphères-étalons.

Dans le cas des liquides, on en évaporera quelques microlitres dans une enceinte de volume déterminé, et on en prendra, à l'aide d'une seringue une part aliquote que l'on traitera comme précédemment. Les mesures sont reproductibles à plus ou moins 2 %.

ANALYSES CHROMATOGRAPHIQUES

Dans le cas des analyses chromatographiques, quelques millilitres des vapeurs provenant de l'évaporation de 10 microlitres du liquide dans un volume de 20 ml, sont injectés dans le circuit d'air par traversant la colonne. Celle-ci est constituée par un tube de verre de 6 mm de diamètre intérieur rempli de kieselguhr pur, imbibée uniformément de 10 % de phtalate de butyle, qui joue le rôle de

la phase stationnaire. Avec un débit d'air de 1 litre à la minute, il suffit de longueurs de colonnes de quelques dizaines de centimètres pour séparer les hydrocarbures benzéniques légers, les alcools et les solvants chlorés couramment utilisés.

Qualitativement, l'intérêt de la méthode ressort de l'examen des graphiques. En quelques minutes, on peut distinguer les différents composants d'un mélange, décelables par les OSMOPILES utilisées (fig. 2).

Quantitativement, on compare les intensités des pics obtenus avec celles de mélanges étalons.

A propos de ces méthodes, nous mentionnerons que la chromatographie gazeuse classique a permis d'analyser la plupart des constituants de certains arômes alimentaires. Nous citerons les cherches de Harold JACKSON sur le fromage cl dar et celles de Joseph CORSE et Keene DIMICK le parfum des fraises. Les parties volatiles au préalable extraites par entraînement de va d'eau ou par distillation dans le vide, puis pas dans des colonnes chromatographiques chau pour accélérer l'évaporation et la séparation composés les plus lourds.

Il est hors de doute que la chromatographie lective par OSMOPILES devra apporter une contribution dans ces études, étant donné leur sibilités extrême pour certains composés, et primant le chauffage des colonnes, procédé qu à éviter dans le cas de produits peu stables.

PRINCIPAUX AROMES ALIMENTAIRES

Pour se faire une idée des possibilités d'emploi des OSMOPILES, rappelons ici succinctement la composition connue des principaux arômes alimentaires.

Ceux-ci, pour *LES METS CUISINES* ont été assez peu étudiés, mais il semble que les composés soufrés y jouent un grand rôle. Ils pourront être décelés, sélectivement après pyrolyse, par les OSMOPILES sensibles à l'anhydride sulfureux.

LES COMPOSES SOUFRES se trouvent, par exemple, dans les choux sous forme d'isothiocyanates, de mercaptans, de sulfoxydes, de sulfure de méthyle ; on en trouve également dans les radis, les oignons, l'ail, la moutarde (sévenols). La B-méthylmercaptopropionaldéhyde serait caractéristique du fromage Cheddar, alors que les cétones 2-heptanone et 2-pentanone caractériseraient les fromages bleus. On rencontre naturellement de l'ammoniac dans la plupart des variétés crues fermentées.

Le composé principal de *L'AROME DU BEURRE* est comme on le sait le diacéthyle que l'on trouve également dans *LE PAIN* à côté de l'acétylméthylcarbinol, produits que l'on décèlera à l'aide des PILES « A ». Nous avons personnellement caractérisé l'alcool éthylique dans le pain frais. Mentionnons aussi que *L'AROME DU LAIT* est dû particulièrement à des traces de méthional et d'autres dérivés soufrés.

Dans *LES VIANDES ET LES POISSONS* peu frais on décèlera à l'aide de ces mêmes récepteurs les amines et l'ammoniac.

Dans *LES FRUITS*, les principes parfumés sont généralement des esters. On les utilise dans les bonbons à l'état de mélanges artificiels. C'est ainsi que pour rappeler le parfum de la pomme, on emploiera le valérianate d'amyle (apple oil) ; pour rappeler celui de la poire, le butyrate, et celui de la banane, l'acétate d'isoamyle ; le butyrate d'éthyle rappelera l'ananas ; l'e-nodecalactone, la pêche ; le méthylphényl glucidate d'éthyle, la fraise ; mais ces produits industriels ne possèdent pas la fraîcheur des parfums naturels, qui sont des mélanges très complexes, comportant des dizai de constituants, parmi lesquels ce sont des tr d'alcools, d'aldéhydes, d'acides, qui donnent « note de fraîcheur fruitée ».

Dans la *FLEUR DU HOUBLON*, nécessair la fabrication de la bière, on trouve principalem du linalol et de l'isononate de linalyle et sa leur marchande est appréciée par olfaction, op tion qui pourra s'effectuer à l'aide d'OSMOPIL oxydantes.

Dans l'huile essentielle de *ZESTE DE CITR OU D'ORANGE*, le constituant principal est le limonène à côté d'autres terpènes qui seront dé lés par les OSMOPILES oxydantes « I » et « C On y trouve également de petites quantités d cools et d'acides supérieurs, des esters, etc.

La présence presque générale et importante composés terpéniques dans la plupart des parfu de fruits et de fleurs fait que c'est surtout par composés qu'on observera une réaction galvaniq Pour déceler et doser les principes contribuan plus à l'individualisation des parfums, il fau donc procéder à une séparation par chromatog phie gazeuse. Néanmoins, un essai direct aux O MOPILES permettra de se faire une idée, d'ap les teneurs en terpènes, de la concentration du p fum considéré.

LES LEGUMES sont également odorants, m moins que les fruits : la carotte doit son arô particulier au carotol, alcool terpénique, le conce bre au nonadeniol et au nonadenial.

On peut rapprocher des fruits *LES CON MENTS* qui, par leur arôme puissant, jouent rôle si important en gastronomie. Dans ce domai le poivre doit ses propriétés à un alcaloïde, la périne. On retrouve un autre alcaloïde « poivré la capsicine, dans les piments.

L'apiol, éther-oxyde de tétraphénols propé lés, se rencontre dans le persil ; l'anéthol, métho 1-isoallyl-4 benzène dans l'anis ainsi que la f chone, une cétone, qui se retrouve dans le feno

Ces composés pourront être également « senti par les OSMOPILES oxydantes, ainsi que la

illine, qui est une aldéhyde-phénol ; l'eugénol ans les clous de girofle.

On sait que les dérivés soufrés, cités plus haut ans l'oignon, dans l'ail, dans la moutarde, constituent les principes actifs et lacrymogènes de ces ondiments. On pourra les doser soit directement, râce aux piles oxydantes, ou après pyrolyse, au oyen de l'OSMOPILE sensible aux vapeurs acies.

Nous terminerons ce résumé succinct par les *OISSONS ALCOOLISEES*. On sait que le bouquet des vins est dû à la présence de traces, principalement d'esters, d'aldéhydes et d'alcools supérieurs.

L'aldéhyde benzylique joue un rôle important dans la composition du Kirsch, comme des dérivés furfuryliques dans celle de vins doux, comme des traces de phénols dans celle du whisky.

Tous ces composés pourront être décelés et dosés par nos OSMOPILES, mais après extraction et isolement par chromatographie gazeuse.

L'OLFACTION

Avant de terminer cet exposé, nous voudrions oucher un mot concernant l'olfaction, ce phénoène qui, avec le sens de la gustation, joue un rôle i important dans l'appréciation d'un mets.

Il n'est pas connu avec précision. Néanmoins, il st hors de doute que les traces de vapeurs, resonsables de l'odeur, viennent au contact de la artie de la muqueuse nasale possédant une sensibilité particulière, et qu'on a nommée tache olfaive, par le fait qu'elle est pigmentée en jaune oncé. Cette zone, de quelques centimètres carrés, st tapissée de cellules possédant des fins prolongements ou cils olfactifs qui baignent dans la couche aqueuse secrétée à sa surface. Ceux-ci doivent ouer un rôle important ; malheureusement le mécanisme intime de l'action des molécules odoriférantes, à leur niveau, n'est pas connu avec précision. On peut faire les deux hypothèses suivantes :

1° Adsorption physique des molécules avec ransformation de la structure superficielle du milieu aqueux, ce qui a pour effet de changer spécifiquement le régime vibratoire des cils ou leur polarisation électrique.

2° Action plus profonde des molécules étrangères, telles celles d'ammoniac ou d'acides, qui jouent eur rôle habituel d'agents chimiques puissants.

Si cette deuxième hypothèse peut être retenue ans ces cas, comment expliquer, si ce n'est par la remière, la sensibilité extrême du nez pour des omposés, comme le scatol et les mercaptans, relativement peu réactifs chimiquement, aux températures ordinaires.

Le nez arrive, en effet, à déceler moins de quelques milliardièmes de milligramme de ces produits par litre d'air ! Dans le cas de l'ammoniac, e seuil est de l'ordre de 1/100° de milligramme alement. Il est vrai qu'on pourrait songer à l'action des enzymes dans les cellules facilitant les associations biochimiques, mais celles-ci sont généralement lentes.

La première hypothèse rendrait compte, en outre de la différence d'odeur d'isomères optiques ui, différents dans leur configuration spatiale, sont identiques du point de vue réactivité chimique. Indiquons que des travaux récents ont attiré l'attention sur le rôle des granules pigmentés en jaune ncé, et qui envahissent les cellules.

En tout cas, quel que soit le mécanisme proposé, la sensation olfactive est un phénomène subjectif. Les influx nerveux provenant de millions de cellules olfactives sont transmis au cerveau par un réseau extrêmement complexe de conducteurs nerveux, avec relais, faisant songer aux fils d'un réseau téléphonique. Certains de ces relais, par suite d'associations antérieures avec d'autres sensations, ou même de traumatismes plus ou moins importants, doivent contribuer à la personnalisation de la sensation.

Rappelons, en outre, que certains individus ne sentent pas certaines odeurs. Dans le cas de ces anosmiques, il faudrait supposer qu'il existe des cellules plus sensibles à certains composés qu'à d'autres, comme d'ailleurs on l'observe pour les cellules gustatives.

Enfin, l'étude, « *in vivo* », sur une cellule olfactive unique, des variations observées de courant électrique, a montré que l'influx, constitué par un train d'ondes, est complexe, et que ses caractéristiques sont liées à la nature de la vapeur odoriférante, et à sa concentration.

On se trouve donc en présence d'une « symphonie » de sensations et non d'une sensation unique.

Dans ces conditions, il ne semble pas possible de rapprocher le mécanisme de nos OSMOPILES de celui d'une cellule olfactive. La constitution de cette dernière présente une toute autre complexité qu'une simple pile, et se trouve associée à tout l'ensemble biochimique de l'individu (relations, par exemple, avec l'état hormonal de ce dernier). Néanmoins, le comportement de nos récepteurs fait songer à un nez artificiel, et c'est pour cela que nous les avons nommés des OSMOPILES. En effet, si la différence entre leur structure intime est péremptoire, leur réponse, en présence des produits qu'ils sentent, est assez proche : rapidité comparable d'action des vapeurs odorantes, retour aux conditions initiales un peu plus lent, comme cela se reflète sur les variations de courant en fonction du temps (graphiques, fig. 2), après la suppression de l'admission des vapeurs.

N'oublions pas, d'autre part, qu'on se trouve en présence de phénomènes ayant lieu à la surface de liquides aqueux, dans les deux cas.

EN CONCLUSION

Les OSMOPILES, détecteurs d'une très grande simplicité, peuvent présenter une utilité certaine pour mesurer l'intensité de certains arômes alimentaires, dans un souci de contrôle de qualité. C'est ainsi qu'on pourrait déterminer le degré de rancissement d'huiles, de matières grasses, le degré de fraîcheur de viandes, le degré de mûrissement de fromages, le degré de maturation de fruits...

On pourra également suivre les processus de fermentation par dosage galvanique, du gaz carbonique, de l'éthylène, des alcools, contrôler la conservation de matières alimentaires, doser l'anhydride sulfureux dans les jus de fruits, les vins, etc.

En ce qui concerne l'analyse qualitative de ces arômes, il est nécessaire d'effectuer, au préalable, une chromatographie gazeuse, afin de déceler par des OSMOPILES les différents constituants, ainsi séparés.

Suivant la sensibilité de ces dernières à certaines familles chimiques, on ne décèlera de la sorte que certains composés d'une manière sélective, ce qui présente de l'intérêt dans le cas des mélanges complexes, constituant généralement les arômes.

(Laboratoire de Physique de la Faculté de Pharmacie de Paris.)

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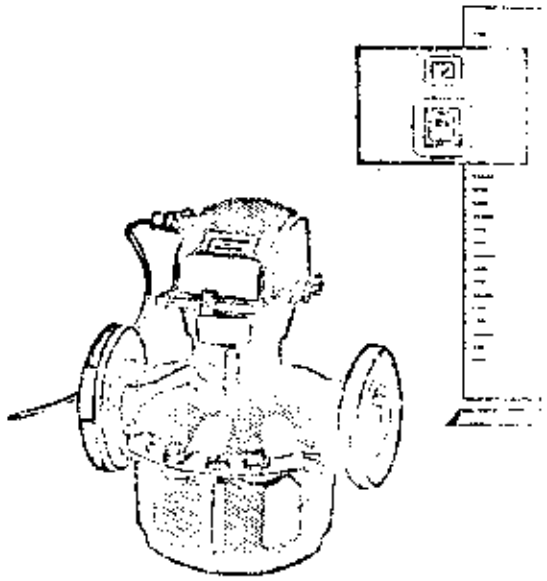

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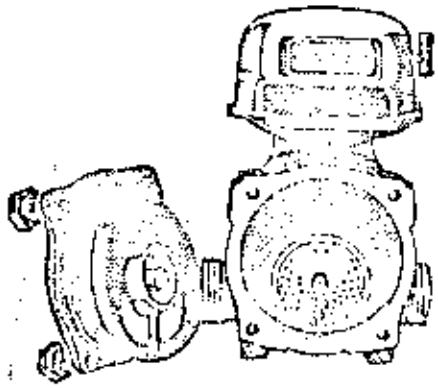

APPAREILS ÉTANCHES, INOXYDABLES, DÉSIGN

Fd Cosmet. Toxicol. Vol. 2, pp. 1–13. Pergamon Press 1964. Printed in Great Britain

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Research Section

The Effect of Sulphite in Solid Diets on the Growth of Rats

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(*Received* 15 *November* 1963)

Abstract—The toxicity of diets mixed with metabisulphite to 0·6%, at room temperature and stored at room temperature was found to pass through two phases. The early form of toxicity, characteristic of diets used within 2 months of their preparation, proved to be a thiamine deficiency complicated by the presence of weak antithiamine activity. The later form of toxicity, which developed in diets stored for 3–4 months, was only partly reversed by large amounts of thiamine and was accompanied by diarrhoea.

INTRODUCTION

Lockett & Natoff (1960) found growth, general health and reproduction unaffected by the addition of sodium metabisulphite (1,500 ppm as SO_2) to all drinking water supplied to 3 consecutive generations of rats. By contrast, Fitzhugh, Kaudsen & Nelson (1946) found evidence of toxicity when concentrations of 'sodium bisulphite' above 0·1% were present in a solid diet. Rats fed on this diet mixed with 0·25% sodium bisulphite grew less rapidly than did controls, and the reduction in weight was not prevented by twice-weekly intramuscular injections of 100 μg thiamine. These injections were given because of the well-known destruction of thiamine by SO_2 (Morgan, Kimmel, Field & Nichols, 1935). Unfortunately, all rats that received diets containing 0·25% sodium bisulphite or more developed severe diarrhoea, which may have occasioned a multiple avitaminosis responsible for the stunted growth, clinical polyneuritis, spectacle eyes, bleached incisor teeth, browned uteri, visceral atrophy and focal myocarditis found in these animals. Since prolonged storage of such diets increased the incidence of browned uteri and tooth lesions, but not of polyneuritis, at least two factors were operative in the production of these changes. One of these was almost certainly deficiency of thiamine, and Fitzhugh *et al.* (1946) were inclined to attribute all the deleterious effects of their more heavily sulphited diets to this cause and considered their view supported by demonstration that thiamine-deficient diets were rendered more toxic by the addition of sulphite.

The object of our work has been to confirm and extend the observations of these previous authors. We have therefore compared the effects of autoclaving with those of sulphiting on the growth-maintaining properties of each ingredient of the diet used by Fitzhugh *et al.* (1946), and have sought correlation between the initial and final thiamine contents of such diets. We have thereby demonstrated the formation of antithiamine activity in yeast in diets treated with sulphite.

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METHODS

Animals. Newly weaned female rats from a single colony of Wistar origin were used throughout. Animals to be used in an experiment were divided into groups of 5. Each group occupied a single Boot's type standard cage and was allotted a treatment at random. All cages were stored under similar conditions of lighting and humidity in a single room maintained within the range of 21·5–34·5°C by thermostats. Water was supplied *ad lib.* in bottles and fresh paste diet was provided *ad lib.* in hoppers attached to the inner walls of cages.

Diets. The standard diet used was that of Fitzhugh *et al.* (1946) and had the following percentage composition: corn starch, 60; casein, 18; corn oil, 6; powdered desiccated whole liver, 5; brewers yeast, 5 (dried yeast was substituted); U.S.P. (XII) salt mixture No. 2, 4; the 2% codliver oil was omitted (see vitamin supplements below). All dietary constituents were obtained from Hopkins & Williams Ltd., London, with the exception of desiccated whole liver which was obtained from Oxo Laboratories Ltd., London. The moisture content of each ingredient was determined by drying it to constant weight over P_2O_5 at room temperature, and the thiochrome method (Aneurine Panel of the Sub-committee on Vitamin Estimations, 1951) was used to assay any thiamine present. These values are shown in Table 1.

Table 1. *Moisture and thiamine contents of dietary constituents*

Constituent	Water (%)	Thiamine (μg/g)
Corn starch	10·8	ND
Corn oil	0·07	ND
Casein	2·4	ND
Desiccated whole liver	2·8	7·0
Yeast (dried)		
Sample I	4·1	16·5
Sample II	—	29·3
Sample III	—	20·5

ND = None detected

Diet II. This diet was low in thiamine and had the following percentage composition: rice starch, 60; casein, 23; corn oil, 6; powdered desiccated whole liver, 5; U.S.P. (XII) salt mixture No. 2, 4. An oral vitamin supplement was always used with this diet (see below).

Fresh diets. These were prepared in bulk sufficient for the whole experiment 1–3 days before the experiment began. They were kept in tightly closed polythene containers at room temperature.

Stored diets. These were prepared and were then kept, sealed in polythene containers, at room temperature until used. Ingredients were always weighed without correction for the water content.

Vitamin supplements. Every rat received 0·5 ml cod-liver oil weekly, orally, by pipette. The oral supplement of mixed vitamins was added daily to the food, when used, and consisted, per rat per day, of: riboflavine, 0·3 mg; calcium pantothenate, 0·03 mg; *p*-aminobenzoic acid, 2·0 mg; pyridoxine hydrochloride, 0·45 mg. Thiamine hydrochloride and cocarboxylase (thiamine pyrophosphate) were administered only as stated in the text.

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The sulphiting of diets. Sulphited diets were prepared by mechanical mixing of fresh standard diet with 0·6% (w/w) of solid sodium metabisulphite. The resulting diet contained not less than 3,400 ppm sulphite as SO_2. Curves showing the rates of disappearance of both sulphite and thiamine from such diets, kept under sealed conditions at room temperature, are shown in Fig. 1. Sulphite was determined as described (*British Pharmacopœia* 1953, p. 771).

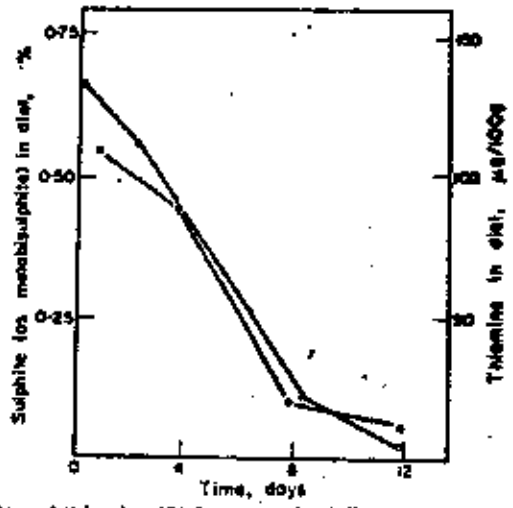

FIG. 1. Loss of sulphite (●) and thiamine (O) from standard diet. The diet, which initially contained 0·6% sodium metabisulphite and 137·5µg thiamine/100 g (yeast sample III was used), was stored under sealed conditions at room temperature.

Autoclaving. When stated in the text, constituents of the diets were autoclaved, sealed, at 15 lb pressure/in^2 for 4 hr.

Biological measurements. Body weight, the daily intake of water and food and the wet weights of faeces were measured as previously described (Lockett & Natoff, 1960). Electrocardiographic records were taken in some experiments.

RESULTS

In the initial experiments, one sample of each ingredient was separately autoclaved,

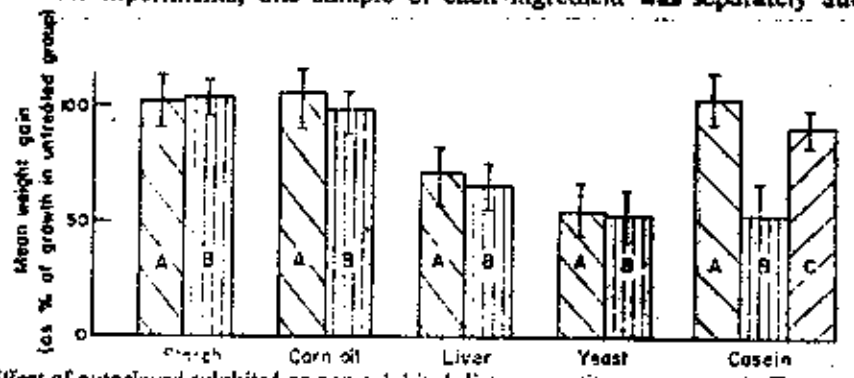

FIG. 2. Effect of autoclaved sulphited or non-sulphited dietary constituents on growth. Treatment of constituents is indicated as follows: A, autoclaved; B, sulphited and autoclaved; C, kept at room temperature under sealed conditions for 8 days after admixture with 0·6% sodium metabisulphite. The resultant standard diets, differing only in the pretreatment of a single constituent, were fed to young rats for 5 weeks. Standard error of the mean is indicated in each case.

Table 2: *Growth and other measurements in rats on a diet containing metabisulphite*

Diet	Observation	Growth and other measurements at week					
		0	1	2	3	4	5
Without sulphite	Body weight (g)	36·4±0·39(70)	63·6±0·82(70)	77·1±1·26(70)	98·6±1·29(70)	104·6±1·52(70)	133·5±1·53(70)
	Food intake (g/100g body weight)	—	13·6±0·24(98)	12·2±0·11(98)	9·9±0·09(98)	8·6±0·09(98)	8·6±0·07(98)
	Fluid intake (ml/100g body weight)	—	29·2±0·56(98)	27·8±0·31(98)	24·4±0·29(98)	20·7±0·25(98)	19·3±0·48(98)
	Faeces, wet weight (as % of food intake)	—	6·2±0·33(14)	6·8±0·59(14)	8·7±0·93(14)	7·5±0·59(14)	7·2±0·12(14)
With sulphite	Body weight (g)	35·5±0·45(70)	60·9±0·63(70)	73·4±0·78(70)	82·0±1·18(70)	89·4±1·39(70)	95·0±1·66(70)
	Food intake (g/100g body weight)	—	14·1±0·23(98)	11·7±0·15(98)	9·0±0·14(98)	7·3±0·09(98)	6·9±0·15(98)
	Fluid intake (ml/100g body weight)	—	30·7±0·52(98)	29·9±0·38(98)	25·8±0·29(98)	21·4±0·39(98)	19·4±0·34(98)
	Faeces, wet weight (as % of food intake)	—	5·6±0·28(14)	6·9±0·83(14)	8·2±0·52(14)	9·8±1·52(14)	9·4±1·08(14)

0·6% Sodium metabisulphite in freshly-prepared solid diet stored at room temperature was fed to groups of 70 female rats. Numbers in parentheses represent the total number of measurements made. Results represent mean ± standard error of mean.

sealed at 15 lb/in² for 4 hr, and another sample was similarly treated after mixing to 0·6% (w/w) with solid sodium metabisulphite. Next day, a series of standard diets were prepared differing in the pretreatment of single ingredients. The weight gains amongst groups of young rats fed on these diets for 5 weeks were compared. Fig. 2 shows the result of these experiments; mean weight gains of rats fed on each treated diet have been expressed as a percentage of the mean weight gain of rats fed on untreated diet. The growth-sustaining properties of yeast and liver were equally reduced by the two treatments, whereas neither process altered the food value of starch or corn oil. The autoclaving of casein in the presence of sulphite did, however, result in toxicity unattributable to heat alone. By contrast casein, that had been allowed to stand under sealed conditions at room temperature for 8 days after mixing with 0·6% (w/w) sodium metabisulphite before inclusion in a standard diet, had developed no toxicity.

A. *The toxicity of freshly prepared diets mixed with 0·6% (w/w) sodium metabisulphite at room temperature and stored at room temperature throughout experiments lasting 5–7 weeks.* Fourteen experiments were made in which the growth, food and water intakes and the wet weights of the faeces of young rats fed on untreated standard diet and on the same diet mixed with 0·6% (w/w) sodium metabisulphite at room temperature were compared. The combined results of these experiments are shown in Table 2. The appetite and growth of those animals receiving the sulphited diet fell off progressively and in parallel from week 3. Weakness, apathy, bradycardia and clinical polyneuritis were evident in week 4 or 5. The water intake of these animals remained normal, and the moisture content of the faeces rose significantly in only 4 experiments. The fact that these animals showed no tendency to eat their faeces, despite obvious thiamine deficiency, suggested that antithiamine activity had been formed by interaction of the sulphite with constituents of the diet, or that the bacterial flora of the intestine had become grossly modified.

The next two experiments demonstrated that the toxicity of the freshly sulphited standard diet was mainly attributable to loss of thiamine. The diets used for these experiments were prepared by the daily addition of selected, untreated ingredients to appropriate sulphited stocks of the residual diet, so completing the standard formula for each. Table 3 shows that the weight gains of young rats fed on these diets for a period of 5 weeks were related to their thiamine content.

Table 3. *Growth of rats on various diets*

Untreated constituents added to sulphited diet	Thiamine in final diet (μg/100g)	Mean body weight	
		Week 0	Week 5
Sulphited standard diet	trace	36±0·5	70±4·9
Casein	trace	37±1·3	69±4·7
Yeast	147·5	38±0·6	90±5·2
Yeast+casein	147·5	37±0·9	89±4·6
Liver+yeast	182·5	38±0·6	109±5·5
Casein+liver+yeast	182·5	40±0·9	119±5·2

The diets used were prepared by the daily addition of selected, untreated constituents to appropriate sulphited stocks of the residual standard diet. The various diets were fed to groups of 10 young rats for 5 weeks. Results represent mean ± standard error of mean.

This point was more closely investigated, since a possible indication of the presence of antithiamine activity in the sulphited standard diet had been afforded by the behaviour of the rats (see above). Search for evidence supporting or disproving this hypothesis was therefore made by comparing the extent of toxicity developing from the addition of sulphite to standard diets containing 117·5 and 182·5 μg thiamine/100 g (yeast samples I and II respectively) and to diet II containing 35 μg thiamine/100 g (no yeast, oral vitamin supplement). The growth curves of rats fed on these diets, untreated and sulphited, are shown in Fig. 3.

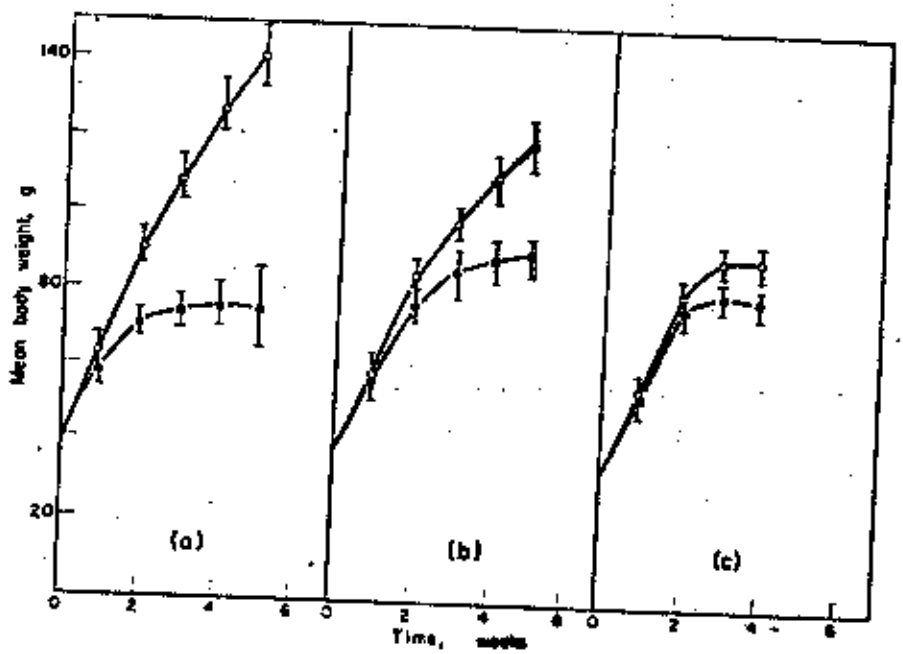

FIG. 3. Effect of dietary thiamine on growth of rats on untreated (O) or sulphited (●) fresh diets. The results are grouped as follows:

(a)—growth on standard diet containing yeast sample I; thiamine content 182·5 μg/100 g diet
(b)—growth on standard diet containing yeast sample II; thiamine content 117·5 μg/100 g diet
(c)—growth on diet II: thiamine content 35 μg/100 g diet.
Standard error of the mean is indicated in each case.

Control growth rates were related to the thiamine content of the untreated diets but so was the extent of the toxicity which resulted from the interaction of these diets with metabisulphite. Moreover, the quantities of thiamine needed to prevent the development of thiamine deficiency in rats fed on freshly sulphited diets bore relationship to the amount of altered thiamine in those diets. The results summarized in Table 4 show that 0·1 mg thiamine hydrochloride per rat, applied intramuscularly twice weekly, was needed to restore growth completely when the diet contained 137 μg altered thiamine/100 g diet. In contrast weekly intramuscular doses of 0·02 mg thiamine hydrochloride per rat were more than adequate to antagonize the effect of 35 μg altered thiamine/100 g diet.

Correspondingly, if the deficiency of thiamine caused by fresh sulphited diets was allowed to develop for 5 weeks before restorative treatment was begun, diets containing the greater amount of altered thiamine proved the more refractory to treatment (Table 5). Evidence of a

Table 4. *Effect of supplementary thiamine on growth of rats given sulphited diets*

Diet	Altered thiamine (μg/100g)	Supplementary thiamine (mg by intramuscular injection)	No. of rats	Final body weight (g)
Standard	0	—	10	123±6·1
Standard, sulphited	137	—	10	66±7·6
	137	0·1 thiamine (twice weekly)	5	124±7·5
	137	0·02 thiamine (twice weekly)	10	81±6·1
	137	0·01 cocarboxylase (twice weekly)	5	64±5·1 (4 weeks)
Diet II	0	—	5	92±3·0
Diet II, sulphited	35	—	5	81±3·3
	35	0·02 thiamine (weekly)	10	104±3·4

Untreated and sulphited diets, which were freshly prepared, were fed to rats (initial body weight 28·3±0·2) for 5 weeks. Results represent mean ± standard error of means.

pure thiamine deficiency was also afforded by these experiments, since the restoration of growth caused by the daily inclusion of 1·0 mg thiamine hydrochloride in the diet was not augmented by an oral mixed vitamin supplement that contained riboflavine, pantothenate *p*-aminobenzoic acid and pyridoxine hydrochloride (see Methods).

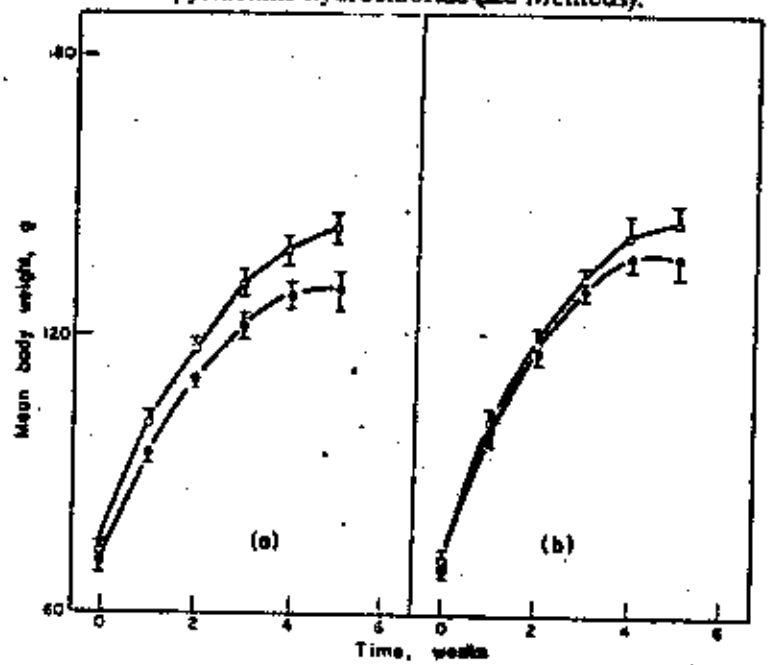

FIG. 4. Effect of pyrimidine and thiazole moieties of thiamine on growth of rats given non-sulphited (O) or sulphited (C) freshly-prepared standard diet containing 137 μg thiamine/100 g diet (yeast sample III was used) for 5 weeks.

(a)—results with untreated rats

(b)—rats injected intramuscularly bi-weekly with 350 μg of the pyrimidine and 240 μg of the thiazole moieties resulting from the cleavage of thiamine by sulphite.

Table 5. *Restoration of growth by administration of thiamine to rats given sulphited diets*

Diet	Altered thiamine (µg/100g diet)	No. of rats	Body weight (g)				Curative treatment	
			Before thiamine		After thiamine			
			Week 4	Week 5	Week 6	Week 7	Thiamine/rat (mg)	Mixed vitamin supplement
Standard	0	35	109±2·1	115±2·3	123±3·2	134±2·9	—	—
		10	105±3·7	114±4·1	120±4·6	131±4·8	0·2 orally daily	—
Standard, sulphited	150–180	30	78±2·5	81±2·8	111±4·1	126±4·0	1·0 injected 1·0 orally daily	—
		10	82±3·1	84±3·7	87±5·3	104±4·8	1·0 orally daily	—
		10	83±5·2	84±7·2	90±5·6	102±5·1	1·0 orally daily	orally daily
		5	75±5·0	79±5·5	76±5·5	90±2·0	0·2 orally daily	—
		5	77±6·6	73±8·8	93±7·6	115±7·5	0·1 cocarboxylase twice weekly intramuscularly	
			Week 3	Week 4	Week 5	Week 6		
Diet II	0	10	91±3·7	92±3·0	107±4·3	128±3·7	0·05 intramuscularly weekly	orally daily
Diet II, sulphited	35	10	83±3·4	81±3·3	90±5·5	105±7·6	0·05 intramuscularly weekly	orally daily

Freshly-prepared diets were fed to groups of animals for 7 weeks. Supplementary thiamine was given to animals on the untreated and sulphited standard diets at the beginning of week 5 and to those on untreated and sulphited diet II one week earlier.

The next step was to identify the dietary component that reacted with metabisulphite at room temperature to yield antithiamine activity

This investigation began with an examination for antithiamine action *in vivo* of the thiazole and pyrimidine moieties (Williams, 1938) resulting from the cleavage of thiamine by sulphite. This was done because these moieties have been shown to act as antithiamines *in vitro*. However, it is clear from the results recorded in Fig. 4 that these compounds do not act as antithiamines *in vivo*, but have instead a thiamine-sparing effect (Fig. 5).

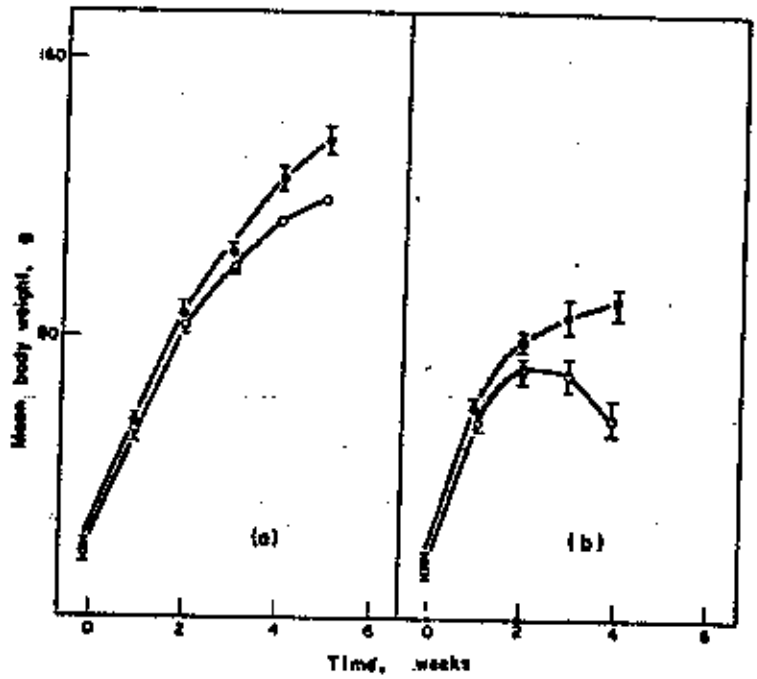

FIG. 5. Effect of pyrimidine and thiazole moieties of thiamine on growth of rats given diet II. Two samples of diet II, (a) and (b), containing 40 and 28 μg thiamine/100 g diet respectively were given to rats for 5 weeks. Animals were either untreated (O) or received bi-weekly intramuscular injections of pyrimidine and thiazole moieties (●) as detailed in legend to Fig. 4.

Finally, it was conclusively demonstrated that the only component of the standard diet that yielded a significant amount of antithiamine activity when sulphited was the yeast. First, the combined results of two experiments, shown in Fig. 6, demonstrate that no antithiamine activity is formed when the standard diet lacking yeast (diet II) is sulphited. Rats fed on untreated diet II grew at the same rate as those fed on the same diet II in which all components except the liver had been sulphited together and to which fresh liver powder had been added daily to complete the formula (sulphited base, fresh liver powder). Rats fed on sulphited whole diet II grew at the same rate as those fed on the same non-sulphited prepared diet without the liver and to which sulphited liver powder was added daily to complete the formula. The low thiamine content of all these diets (35 μg/100 g) which was provided almost solely by the liver powder led to the development of deficiency. This lack of thiamine was effectively treated by the weekly intramuscular administration of only 0·05 mg thiamine hydrochloride.

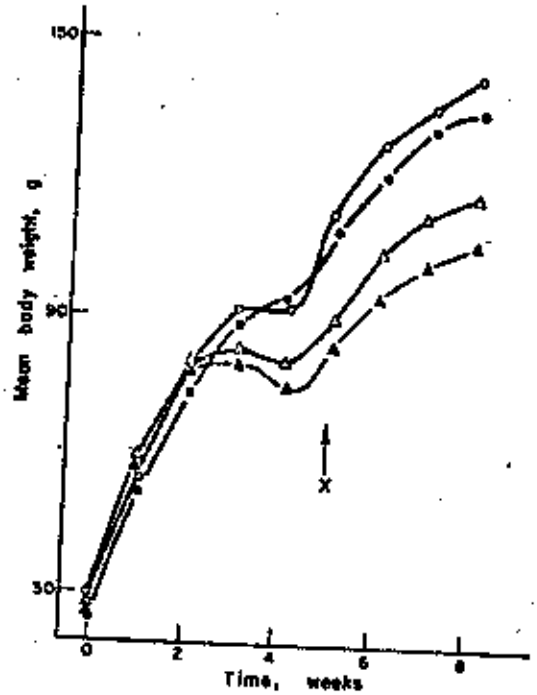

FIG. 6. Effect of different freshly-prepared sulphited diets on growth of groups of 10 rats given diet II: O, non-sulphited diet; O, sulphited diet (liver component of diet non-sulphited); ▲, liver component sulphited, remainder of diet non-sulphited; △, whole diet sulphited. X—Start of weekly intramuscular injections of 0·05 mg thiamine HCl (for injection schedule see Table 5).

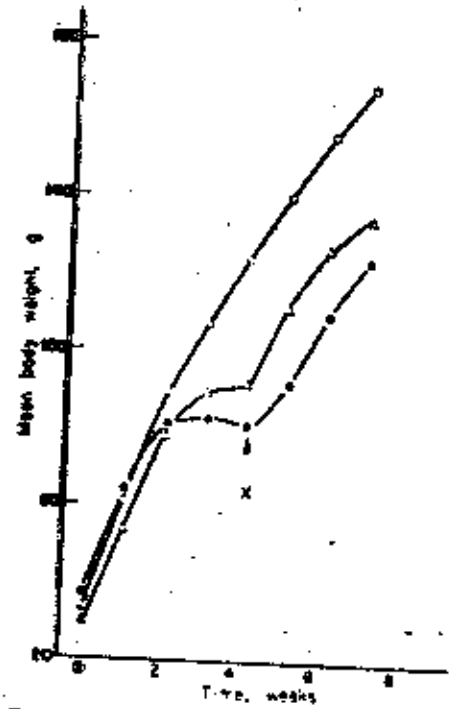

FIG. 7. Growth of groups of 10 rats on different freshly-prepared diets: O, standard diet containing yeast; △, diet II omitting yeast; O, sulphited standard diet (liver component of diet non-sulphited). X—Start of intramuscular injections of thiamine HCl, administered to groups indicated by △ and O (for injection schedules see Table 5).

By contrast, the standard diet, which contains yeast, does develop antithiamine activity when sulphited. This fact is clearly demonstrated by the results of the next group of experiments, which are combined in Fig. 7. Here comparison has been made of the growth rates of young rats fed on the standard diet containing yeast (182·5 μg thiamine/100 g), this same diet sulphited in the absence of the liver fraction and to which fresh liver powder was added daily to complete the formula (35 μg thiamine/100 g) and diet II, which contained no yeast (35 μg thiamine/100 g). Deficiency of thiamine developed rapidly in those animals fed on the low thiamine diets. Whereas this deficiency was effectively corrected by weekly intramuscular injection of 0·05 mg thiamine hydrochloride in rats receiving diet II, this treatment was ineffective for the recovery of rats fed on the low thiamine diet containing sulphited yeast. These animals responded only to the daily intramuscular injection of 1 mg thiamine hydrochloride per rat. The large doses of thiamine needed both to combat the development of thiamine deficiency (Table 4) and to cure it (Table 5) when produced by sulphited diets containing yeast has been repeatedly confirmed.

B. *The toxicity of sulphited diets is increased by storage at room temperature.* Sulphited standard diets that had been stored for 75 or 91 days before use in an experiment were less able to maintain the growth of young rats than was a fresh sulphited diet or one stored for 31 days, despite the fact that the initial thiamine contents of these diets did not differ

(Fig. 8). Moreover, the diets stored for 75 days caused diarrhoea and an increase in fluid consumption (Table 6), whereas the freshly sulphited diets did not (Table 2). In addition,

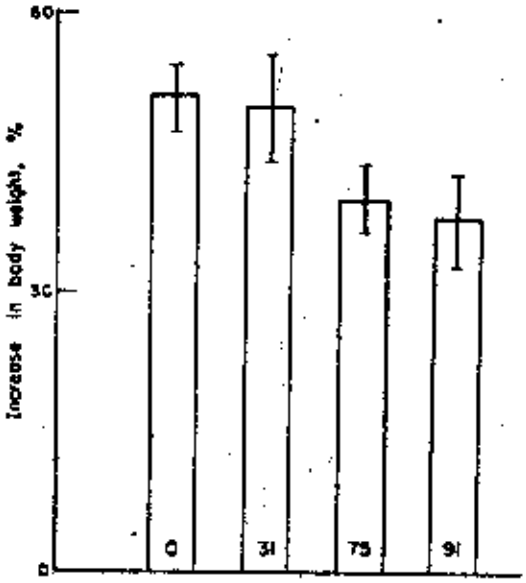

FIG. 8. Effect of storage of sulphited standard diets (thiamine content 137 μg/100 g diet) on toxicity to rats. Duration of storage, in days, is indicated in histogram.

the depression of growth resulting from the provision of sulphited standard diet after storage for 75 days proved even more resistant to preventive treatment with thiamine than did that occasioned by the freshly sulphited diets (Table 7).

Table 6. *Effect of stored sulphited diet on food and fluid intakes, and weight of faeces of rats*

Sulphited standard diet	Observation	Measurements at week 1	2	3	4	5
Fresh	Food intake (g/100 g body weight)	14·7±1·00	12·2±0·45	9·8±0·40	8·4±0·40	7·8±0·25
	Fluid intake (ml/100 g body weight)	34·1±1·65	29·5±1·21	25·6±1·32	22·6±1·15	19·8±0·95
	Faeces, wet weight (as % of food intake)	7·7±0·65	10·8±1·65	9·4±1·93	13·1±1·05	7·7±0·49
Stored for 75 days	Food intake (g/100 g body weight)	9·7±0·88	9·6±0·45	8·7±0·43	8·6±0·38	8·3±0·37
	Fluid intake (ml/100 g body weight)	29·7±2·02	32·1±1·50	28·3±1·30	24·2±1·17	25·8±1·19
	Faeces, wet weight (as % of food intake)	15·1±1·01	15·4±0·95	14·5±1·09	12·7±0·89	12·7±0·82

Groups of 20 rats received fresh sulphited standard diet for 5 weeks. Sulphited standard diet that was stored for 75 days was given to groups of 80 animals for 5 weeks. Each measurement was carried out at weekly intervals. Results represent mean ± standard error of mean.

Table 7. *Failure of thiamine supplementation to reverse growth retardation of rats brought about by stored sulphited diet*

Standard diet	Altered (thiamine µg/100 g)	Supplementary thiamine: Intramuscular injection mg (weekly)	Supplementary thiamine: Oral dose (mg daily)	No. of rats	Mean body weight (g): Week 0	Mean body weight (g): Week 5
Non-sulphited, fresh (thiamine content 137 µg/100 g diet)	0	—	—	10	30±0·5	123±6·1
Sulphited, fresh	137	—	0·1 thiamine	5	25±0·8	112±8·6
		—	0·1 cocarboxylase	5	28±0·7	120±4·2
		—	0·3 thiamine	5	26±0·6	118±4·2
		—	0·3 cocarboxylase	5	28±0·6	124±3·7
		—	—	10	29±0·7	66±7·6
Non-sulphited, stored 31 days (thiamine content 137 µg/100 g diet)	0	—	—	10	27±1·6	122±5·4
Sulphited, stored 75 days	137	—	—	10	28±1·4	53±6·2
		0·03 thiamine	—	10	30±0·8	72±2·7
		—	0·03 thiamine	10	30±1·0	74±5·8
		0·3 thiamine	—	10	30±0·7	72±4·0
		—	0·3 thiamine	10	30±0·8	72±6·1
		0·3 cocarboxylase	—	10	31±0·3	74±5·9

Four types of diet were used during the 5-week period: non-sulphited and fresh, sulphited and fresh, non-sulphited and stored, sulphited and stored. Supplementary thiamine was administered as indicated in the Table. Initial and final body weights represent mean ± standard error of mean.

DISCUSSION

The evidence demonstrates that the reduction in the growth of young rats that results from the use of diets within 7 weeks of their admixture with sodium metabisulphite to give 0·6% (w/w) at room temperature is attributable to lack of thiamine. The basis of this claim may be briefly summarized. First, thiamine and sulphite disappear rapidly and in parallel from the diet in the first 8 days after sulphiting (Fig. 1). Secondly, the toxicity of a fresh sulphited diet is related to the loss of thiamine it has sustained (Table 3). Thirdly, the toxic effects of these sulphited diets can be prevented or cured (Tables 4 and 5) solely by administration of thiamine or cocarboxylase. Whereas no antithiamine activity is formed when metabisulphite reacts with casein, liver or muscle (unpublished results), starch or corn oil (Fig. 6), antithiamine activity is formed by interaction of sulphite with dried yeast (Fig. 7, Tables 4 and 5), and this antithiamine activity necessitates the use of large doses of thiamine to prevent or cure the toxic effects resulting from the use of diets containing sulphited yeast (Tables 4 and 5, Fig. 7).

The storage of sulphited diets at room temperature led to slow development of an additional toxicity, which was not apparent at 31 days, but was readily detectable at 75 days (Fig. 8). The signs of this late toxicity resemble those described by Fitzhugh *et al.* (1946); signs of thiamine deficiency were accompanied by diarrhoea (Table 6), and the stunting of

growth could not be reversed by administration of thiamine (Table 7). The cause of this late toxicity is still under examination. No explanation can yet be offered for the toxic interaction of sulphite and casein on autoclaving: no such toxicity has resulted from the similar treatment of tyrosine or cystine (unpublished observation).

Acknowledgements—The expenses of this work were defrayed by Unilever Ltd., who also made a grant to B. Bhagat for personal maintenance during training in research.

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Les effets sur la croissance des rats d'un régime solide additionné de sulfite

Résumé—Dans le cas de régimes auxquels avait été mélangé 0,6% de métabisulfite à une température normale de 20°C et conservés à cette même température, il a été démontré que la toxicité qui caractérise les régimes utilisés dans les deux mois qui suivent la préparation, s'est présentée sous forme de carence de thiamine, compliquée par la présence d'une action anti-thiamine faible. La forme tardive de toxicité qui a suivi l'emploi de régimes conservés pendant 3 à 4 mois n'a pu être que partiellement contrôlée par de fortes doses de thiamine, et était accompagnée de diarrhée.

Die Wirkung von Sulfit in der festen Nahrung auf das Wachstum von Ratten

Zusammenfassung—Es wurde gefunden, dass die Toxizität eines Futters, das bei Raumtemperatur mit 0,6% Metabisulfit versetzt und bei derselben Temperatur gelagert wurde, zwei Phasen durchläuft. Die erste Form der Giftwirkung ist charakteristisch für derartiges Futter mit einer Lagerzeit von weniger als 2 Monaten und zeigt sich in B_1-Mangelerscheinungen kompliziert durch das Vorliegen einer leichten Antithiamin-Wirkung. Die spätere Form der Giftwirkung von solchem Futter mit 3–4 Monaten Lagerung konnte nur teilweise durch hohe B_1-Gaben aufgehoben werden und war von Diarrhöe begleitet.

J. Pharm. Pharmacol. 12 (1960)

THE ABSORPTION AND ELIMINATION OF METABISULPHITE AND THIOSULPHATE BY RATS

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Received May 30, 1960

Rats eliminated 55 per cent of the sulphur of metabisulphite and 23 per cent of that of thiosulphate into the urine in the 4 hours after the oral administration of these compounds. This sulphur was excreted almost entirely as inorganic sulphate.

A search of the literature yielded much information about the formation of sulphite and its detoxification in the animal body, but little evidence about the absorption of sulphite from the gastrointestinal tract and the manner of its excretion. Strong indication exists that orally administered metabisulphite solution is absorbed by rats, for Lockett and Natoff[1] found that the ingestion of 11·1 to 25·5 mg. of sodium metabisulphite per 100 g. weight per day did not increase the wet weight of the faeces excreted. Such metabisulphite would have required 0·57 to 1·3 ml. of water for its isotonic solution. Less than a third of this volume of water would have caused a significant increase in the wet weight of the faeces if added to them, for the average wet faeces excreted was 2·31 ± 0·01 g. per 100 g. weight per day. The object of the present work has been to make some estimate of the speed and degree of this absorption and discover in what form ingested metabisulphite is excreted by this species.

Experimental

Female Wistar rats were fed diet 41 b of Stein and were housed in the laboratory for at least 1 week before use.

Experiments of Group I

Eight rats weighing 147·8 ± 1·8 g. were divided into pairs matching in weight. Two pairs drank only sodium metabisulphite (2,000 p.p.m. as SO_2) and two pairs water, for 3 days: all were deprived of food for the last 24 hours of this period. Then each animal received a volume of its drinking fluid equivalent to 5 per cent of weight by stomach tube. Immediately afterwards the bladders were emptied by gentle suprapubic pressure, each pair was put into a separate metabolism cage, and the urine was collected for 4 hours. A cross-over test was made 4 days later in which those pairs which had previously drunk the metabisulphite solution drank the water, and *vice versa*. The whole procedure was repeated.

Experiments of Group II

Twelve rats, weighing 260·5 ± 1·6 g., were assigned to four groups for tests in which oral treatments were allocated to groups according to

a 4 × 4 Latin Square design and were administered by stomach tube at intervals of 3 days. On each occasion an hydrating volume of water equivalent to 2·5 per cent body weight was given after a fast of 18 hours and was followed, 1 hour later, by a volume of test solution equal to 5 per cent of body weight. Urine was collected from each group separately throughout the next 4 hours (see Experiments Group I). The test solutions used were NaCl, 0·74 per cent; $Na_2S_2O_3$, 5 H_2O, 4·51 per cent; Na_2SO_4,10 H_2O, 5·86 per cent; $Na_2S_2O_5$, 3·46 per cent; all w/v in water.

Experiments of Group III

These differed from those of Group II in that the rats used weighed 267·5 ± 2·4 g., and were assigned to six groups. Treatments were given by intraperitoneal injection, in volume equivalent to 3 per cent of body weight, and consisted of 0·9 per cent NaCl w/v; 13·4 ml. 1·46 per cent anhydrous Na_2SO_4, 8·0 ml. 1·95 per cent $Na_2S_2O_3$ and 8·0 ml. 2·54 per cent $Na_2S_2O_5$,5 H_2O all w/v in water, each diluted to 100 ml. with 0·9 per cent NaCl w/v, administered to each group in turn in a four-part cross over test according to a random block design.

TABLE I

THE EFFECT OF THE INGESTION OF 5 PER CENT WEIGHT OF A SOLUTION OF SODIUM METABISULPHITE ON THE URINARY EXCRETION OF SULPHUR BY RATS IN THE SUCCEEDING 4-HOUR PERIOD

	Water load	Metabisulphite load	t calc. n = 14
Urine volume, ml.	8·1 ± 0·68 (16)	9·3 ± 0·64 (16)	1·36
Inorganic sulphur as SO_4^{--} in mg.	10·7 ± 2·13 (16)	33·2 ± 3·38 (16)	5·56
Reducing power as SO_4^{--} in mg.	1·3 ± 0·27 (16)	2·0 ± 0·29 (16)	2·53
Organic sulphur as SO_4^{--}	0·3 ± 0·03 (16)	0·4 ± 0·12 (16)	0·76

The values shown are means for pairs of rats ± S.E. of the mean (number of observations).

Chemical Methods

The total and inorganic sulphur of urine was estimated as sulphate by the method of Bray, Humphris, Thorpe, White and Wood[2], modified only by substitution of a Gallenkamp photoelectric colorimeter N.3615 for a Spekker photoelectric absorptiometer, and replacement of the Chance Neutral Filter H 508 by one with a neutral density of 1·15. Since sulphite, thiosulphate and thiocyanate reduce iodine, they were collectively estimated in urine as an increase in the reduction of iodine above control levels. 1 ml. urine was slowly added to 5 ml. of 0·1N aqueous iodine. After 2 minutes 0·2 ml. HCl (B.P.) was added and the excess iodine was titrated with 0·01N thiosulphate using mucilage of starch as indicator. Blank tests were made throughout.

RESULTS

Changes Induced in the Urinary Excretion of Sulphur by the Oral Administration of Sodium Metabisulphite in Rats

Comparison was made of the sulphur-containing compounds excreted in the urine of rats in the 4-hour period immediately following the

administration by stomach tube of either water or a solution of sodium metabisulphite in Experiments of Group I. The results of the eight tests made are summarised in Table I. The sulphate equivalent of the mean oral dose of metabisulphite administered per rat was 44·4 mg. Of this, 53 per cent was excreted in the urine in the first 4 hours after its administration. Much the larger part of this sulphur was in inorganic form, and was sulphate. There was also a small but significant increase in the reducing power of the urine attributable to the presence of sulphite, thiosulphate or thiocyanate.

Comparison of the Changes Induced in the Urinary Excretion of Sulphur by the Oral Administration of Sulphate, Thiosulphate and Metabisulphate to Rats

Experiments of Group II were used to make comparison of the sulphur containing compounds excreted in the urine in the 4 hours immediately after the administration by stomach tube of solutions of sodium chloride,

TABLE II

THE URINARY EXCRETION OF WATER AND SULPHUR BY GROUPS OF THREE RATS IN THE 4 HOURS AFTER THE ORAL ADMINISTRATION OF AQUEOUS SOLUTIONS OF SODIUM CHLORIDE, SULPHATE, METABISULPHITE AND THIOSULPHATE IN A VOLUME EQUIVALENT TO 5 PER CENT WEIGHT

Salt in loading fluid	NaCl	Na_2SO_4	$Na_2S_2O_5$	$Na_2S_2O_3$
Water load excreted per cent	46·5 ± 9·32 (4)	6·9 ± 0·62 (4)	65·5 ± 4·40 (4)	45·2 ± 11·61 (4)
Inorganic sulphur as SO_4^{--} in mg.	8·9 ± 0·62 (4)	22·2 ± 3·94 (4)	197·4 ± 6·47 (4)	79·2 ± 11·11 (4)
Organic sulphur as SO_4^{--} in mg.	1·5 ± 0·21 (4)	3·4 ± 0·37 (4)	2·1 ± 1·61 (4)	4·0 ± 1·42 (4)
Sulphur load excreted per cent	—	7·1 ± 1·15 (4)	55·1 ± 0·24 (4)	23·1 ± 3·11 (4)

The values shown are means ± S.E. (number of groups).

metabisulphite, thiosulphate and sulphate in a volume equivalent to 5 per cent of body weight. Equivalent amounts of sodium were present in all these solutions and equal amounts of sulphur in the last three. The results of these experiments are summarised in Table II. The differences between the proportions of the sulphur load excreted in the 4 hours immediately after the ingestion of the three sulphur compounds were highly significant ($P = 0·01$ by t test). Approximately 55 per cent of the sulphur load of metabisulphite, 23 per cent that of thiosulphate and 7 per cent that of sulphate were eliminated. The sulphur load was excreted solely in inorganic form in each case.

Comparison of the Changes Induced in the Urinary Excretion of Sulphur by the Intraperitoneal Injection of Isotonic Solutions of metabisulphite, thiosulphate, and sulphate

Intraperitoneal injections of isotonic aqueous sodium metabisulphite in volume equivalent to 3 per cent of body weight caused an immediate restlessness rapidly followed, in each of 12 rats, by cyanosis, prostration and cardiovascular collapse. Half the animals died at this stage, within 35 minutes of the injection. The remainder had begun to recover at 40 minutes and appeared normal half an hour later. No such toxic

effects resulted from similar intraperitoneal injections of sodium thiosulphate or sulphate. The toxic symptoms of intraperitoneal metabisulphite were not seen when the dose was reduced to one-quarter by dilution of the original isotonic solution 1 part in 4 with isotonic sodium chloride. After trial, one-twelfth the LD50 concentration of metabisulphite was selected for use in comparison of the effect of intraperitoneal injections of isotonic solutions of metabisulphite, thiosulphate and sulphate in volume equivalent to 3 per cent of weight on the urinary excretion of sulphur. The results of one such experiment, typical of three, are shown in Table III. In these Group III experiments the total sulphur injected, expressed as mg. of sulphate per 100 g. weight, amounted to 4·81 when metabisulphite or thiosulphate, and 3·95 when sulphate solutions were used. Neither the form of excretion nor the extent of the excretion of this sulphur differed whether metabisulphite, thiosulphate or sulphate had been given by the intraperitoneal route. In the first 4 hours after injection

TABLE III

COMPARISON OF THE URINARY SULPHUR OUTPUTS IN THE 4 HOURS FOLLOWING THE INTRAPERITONEAL INJECTION OF SODIUM METABISULPHITE, THIOSULPHATE, SULPHATE AND CHLORIDE IN ISOTONIC SOLUTIONS, IN VOLUME EQUIVALENT TO 3 PER CENT WEIGHT

Loading solution	Sodium metabisulphite	Sodium thiosulphate	Sodium sulphate	Sodium chloride
Water load per cent	36·7 ± 5·23 (6)	44·3 ± 9·38 (6)	39·6 ± 4·42 (6)	37·2 ± 5·8 (6)
S.G.	1030 ± 3·43 (6)	1029 ± 3·59 (6)	1022 ± 2·94 (6)	—
Inorganic sulphur as SO_4^{--} in mg.	28·4 ± 1·57 (6)	27·6 ± 2·85 (6)	21·3 ± 6·73 (6)	6·50 ± 3·11 (6)
Total sulphur as SO_4^{--} in mg.	30·0 ± 1·35 (6)	29·2 ± 2·95 (6)	23·2 ± 1·84 (6)	2·70 ± 1·58 (6)
Reducing power as SO_4 in mg.	2·0 ± 0·63 (6)	2·4 ± 0·42 (6)	2·4 ± 0·33 (6)	—
Sulphur load excreted per cent	85·6 ± 3·29 (6)	84·9 ± 11·7 (6)	87·7 ± 14·15(6)	—

The values shown are means ± S.E. (numbers of groups).

80 to 90 per cent of the administered sulphur was eliminated as inorganic sulphate; this was accompanied by very small amounts of iodine reducing substances. The diureses evoked by these sulphur containing solutions matched that caused by nearly equivalent sodium loading (Table III).

DISCUSSION

The rates of the urinary elimination of sulphur, provided in the forms of metabisulphite, thiosulphate or sulphate, were indistinguishable when these compounds were given by intraperitoneal injection (Table III) but differed when the oral route was substituted (Table II). The form in which the sulphur appeared in the urine was the same whether metabisulphite, thiosulphate or sulphate was administered. Therefore it may be inferred that the rates of absorption of these compounds from the gastrointestinal tract differ. Rats absorb metabisulphite more rapidly than thiosulphate, and sulphate is, as expected, absorbed only to a very limited extent.

Administered thiosulphate and metabisulphite are not excreted as such by rats because the ingestion or injection of these two iodine reducing substances caused no greater increase in the iodine reducing power of the urine during their renal excretion than did that of sulphate (Tables I and

III) and that increase was very small (Table I). All three compounds were excreted in the urine almost solely in the form of sulphate. It must therefore be concluded that the only important detoxification system for metabisulphite and thiosulphate in the rat is an oxidative one (Table III). Fridovich and Handler[3,4], have shown that the oxidation of sulphite by rat livers does take place. An intermediate thiosulphonate is formed and hydrolysed to yield a suphydryl compound and a sulphate. These reactions were shown to involve the reversible participation of a flavoprotein and hypoxanthine proved an obligatory co-factor at pH values of and in excess of 7·6.

The small amount of iodine reducing substance in normal rat urine may be, by analogy, thiosulphate (Table I) for small amounts of thiosulphate are usually present in the urine of man[5] and in that of cats and dogs[6]. This thiosulphate of normal urine probably arises from the sulphur containing amino acids of the diet[7]. Since rat liver converts mercaptopyruvate rapidly and quantitatively to thiosulphate in the presence of sulphite[8] the slight increase in the iodine reducing power of rat urine during the elimination of the metabisulphite should probably be attributed to the excretion of slightly more than the usual trace of thiosulphate.

Acknowledgements.—The expenses of this work were defrayed from a grant made by Unilever & Co. Ltd., which also provided a personal grant to B. Bhaghat for assistance during training in research.

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Internat. Z. Vit. Forschung 39 (1969)
Eingegangen am 7. November 1969

G-6-PDH
LDH
NADH-Diaphorase
Sulfit
Thiamin im Blut

Thiamin im Harn
Thiaminmangel
Thiaminstatus
Transketolase

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Thiaminmangel und Unbedenklichkeit von Sulfit für den Menschen

2. Mitteilung:

Der Thiaminstatus und die Aktivität einiger Dehydrogenasen in den Erythrozyten

I. Bitsch, K. Schiele, E. Kunsemüller, I. Benthien,
unter Mitarbeit von D. Beckmann [1]

Zusammenfassung: Bei menschlichen Probanden wurde durch Gabe einer thiaminarmen Kost eine biochemisch nachweisbare, aber nicht zu klinischen Ausfallserscheinungen führende Thiaminunterversorgung erzeugt. In diesem Stadium des Thiaminmangels hatten orale Gaben von Sulfit (400 mg pro Vpn pro Tag) keinen Einfluss auf den Thiaminstatus, ermittelt durch Bestimmung der Transketolaseaktivität und des Thiamingehalts im Blut sowie der Thiaminausscheidung im Harn. Unter unseren Versuchsbedingungen erfolgte somit keine Zerstörung des Thiamins durch Sulfit in vivo. Die Aktivitäten der Erythrozytenenzyme LDH, G-6-PDH und NADH-Diaphorase sanken um 10–40 % bezogen auf den Ausgangswert unter dem Einfluss der thiaminarmen Kost. Es konnte keine zusätzliche Beeinflussung der genannten Enzyme durch Sulfit in vivo beobachtet werden.

Im Tierversuch konnte in einigen Fällen der experimentelle Beweis erbracht werden, dass Sulfit Thiamin in vivo zerstören kann. So fand Hötzel (1962) in Lebern von Ratten eine signifikante Verminderung des freien Thiamins bei chronischer, vom Futter getrennter Applikation von 40 oder 80 mg Sulfit/kg/Tag. Lhuissier (1966) sowie Causeret und Mitarbeiter (1965) beobachteten einen

[1] Dr. D. Beckmann, Psychosomatische Klinik, sei für Beratung bei den statistischen Berechnungen gedankt.

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signifikanten Abfall des Thiamins im Harn von Ratten, denen Sulfit getrennt vom Thiamin im Futter verabreicht worden war. Der Hauptanteil der Umsetzung scheint dabei im Magen erfolgt zu sein. Sidorenkov (1965) beschrieb ein Absinken der Thiaminausscheidung im Urin bei Vergiftung mit Sulfit. Um zu überprüfen, ob auch beim Menschen eine *in vivo*-Zerstörung des Thiamins durch Sulfit möglich ist, und zwar unter «praxisnahen» Bedingungen in bezug auf Thiamingehalt der Kost und Sulfitzufuhr, kontrollierten wir laufend den Thiaminstatus der Versuchspersonen durch Messung der Transketolaseaktivität und des Thiamins im Vollblut sowie der Thiaminausscheidung im Harn.

Von verschiedenen Dehydrogenasen ist bekannt, dass sie von Sulfit z. T. in erheblichem Mass gehemmt werden. So beobachteten Pfleiderer, Jeckel und Wieland (1956) eine starke Hemmung durch Sulfit bei Lactatdehydrogenase (LDH) und Malatdehydrogenase (MDH) aus Schweineherzmuskel. Eine synergistische Hemmung verschiedener Lactatdehydrogenasen durch Bisulfit und 3-Acetyl-pyridin-adenin-dinucleotid beschrieben Ciaccio und Keller (1962), Ciaccio (1966) und Ciaccio, Boxer, Devlin und Ford (1967). Die Hemmung von LDH durch sulfitabspaltende Verbindungen, insbesondere 1,3-Butandiol cycl. Sulfit, untersuchten Ciaccio, Keller, Kenderes, Boxer und Devlin (1966) sowie Mizumo und Humphrey (1966). In jüngster Zeit wurde beobachtet, dass auch durch Thiaminmangel die Aktivität von Dehydrogenasen vermindert werden kann. So fanden Deus, Blum und Holzer (1968) eine Erniedrigung der Aktivität der Isocitratdehydrogenase in Rattenlebern und Rattenerythrozyten bei Thiaminmangel, während die Aktivität der MDH nahezu konstant blieb. Bei chronischer Unterernährung sank die Aktivität der Isocitratdehydrogenase, während die Aktivität der MDH stark anstieg. Gubler (1968) beschrieb einen deutlichen Abfall der LDH-Aktivität bei thiaminfrei ernährten Ratten in der Leber, einen geringen Abfall im Herzen, während in Plasma, Niere und Gehirn keine Aktivitätsänderung beobachtet werden konnte. Colajacomo und Mitarbeiter (1963) fanden eine starke Verringerung der Glukose-6-phosphat-Dehydrogenase (G-6-PDH)-Aktivität bei der «Beriberi»-Ratte.

Uns erschien es interessant, gleichzeitig den Einfluss eines Thiaminmangels und den von Sulfit auf Dehydrogenasen zu untersuchen; wir kontrollierten daher während des Versuchsablaufs von Periode II bis Periode IV zweimal wöchentlich die Aktivitäten von LDH, G-6-PDH und NADH-Diaphorase in hämolysierten Erythrozyten der Versuchspersonen.

Methoden

Transketolase:

Bestimmung nach Dreyfus (1962) in der Modifikation von Schouten und Mitarbeitern (1964). Berechnung als U/l Blut (HK = 35 %). Die Aktivität der Transketolase wurde mit und ohne Zusatz von Thiaminpyrophosphat bestimmt und die Aktivierbarkeit, bezeichnet als «TPP-Effekt», in Prozent angegeben.

Thiamin im Harn:

Bestimmung des freien Thiamins nach Chromatographie über Permutit T (Stroheckeer u. Henning, 1963). Die Berechnung erfolgte als mcg Thiamin/Tag und als mcg Thiamin/g Kreatinin (Bestimmung des Kreatinins nach Popper, Mandel und Mayer, 1937).

Thiamin im Blut:

Bestimmung des Gesamtthiamins (Thiamin + Thiaminpyrophosphat) nach 12–15stündiger Inkubation mit Taka-Diastase (Serva-Entwicklungslabor, Heidelberg) und Chromatographie über Permutit T (Stroheckeer und Henning, 1963). Berechnung als mcg Thiamin/100 ml Blut.

Erythrozytenzahl:

Bestimmung durch automatisches Auszählen.

Hämolyse:

1,0 ml heparinisiertes Blut wurde dreimal mit 10 ml physiologischer Kochsalzlösung gewaschen, nach jeder Waschung wurde 10 min bei ca. 3000 U/min zentrifugiert. Die gewaschenen und zentrifugierten Erythrozyten wurden in 2,5 ml 0,02prozentiger Digitoninlösung suspendiert, 15 min bei ca. +4° C stehen gelassen und anschliessend erneut zentrifugiert. Der klare Überstand wurde für die Aktivitätsbestimmungen verwandt.

LDH:

Vorschrift nach Biochemica Informationen, Boehringer (1966). Das Hämolysat wurde 1 : 5 mit Phosphatpuffer verdünnt und von der Verdünnung 0,05 ml pro Bestimmung verwandt. Berechnung als U/10³ Erythrozyten.

G-6-PDH:

Vorschrift nach Biochemica Test Combination, Boehringer (1967). Pro Bestimmung wurde 0,10 ml Hämolysat verwandt. Berechnung als mU/10³ Erythrozyten.

NADH-Diaphorase:

Vorschrift nach Scott u. McGraw (1962). Pro Bestimmung wurden 0,10 ml Hämolysat verwandt. In Abänderung der Methode von Scott und McGraw wurde genau 30 sec. nach Zugabe des Hämolysats die Zeit gestoppt, während der eine Extinktionsänderung von 0,050 erfolgte. In gleicher Weise wurde ein Blindwert ohne NADH gemessen, da Hämoglobin selbst eine rasche Reduktion von Dichlorphenolindophenol bewirkt. Berechnung als mU/10³ Erythrozyten.

Berechnungen

Jeder Einzelwert wurde durch 4 Parallelbestimmungen ermittelt, für jede Versuchsperson wurde aus den Einzelwerten der Periodenmittelwert errechnet (x) und aus je 6 Periodenmittelwerten die Mittelwerte der Test- und der Kontrollgruppe bestimmt ($\bar{x} \pm s$). Es wurden somit 8 Perioden-Gruppenmittelwerte erhalten, die den folgenden mathematischen Berechnungen zugrunde liegen:

1. Der Vergleich der Periodenmittelwerte der Kontrollgruppe (K) mit den Periodenmittelwerten der Testgruppe (T) zur Prüfung, ob Abfall und Wiederanstieg der Perioden-Gruppenmittelwerte

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signifikant sind, erfolgte mit Hilfe des t-Tests für unabhängige Stichproben (Vergleich T_{II} mit $K_{IV(V)}$ und K_{IV} mit T_V).
In gleicher Weise wurden die Mittelwerte T_{III} und K_{III} verglichen, um einen evtl. zusätzlichen Einfluss von Sulfit auf die Aktivitäten zu erfassen.

2. Zum Vergleich der verschiedenen Periodenmittelwerte innerhalb der gleichen Gruppe auf Signifikanz von Abfall und Wiederanstieg wurden die Differenzen der Messwertpaare ermittelt und der theoretische Erwartungswert der mittleren Differenz gleich 0 gesetzt. Der t-Test bezieht sich auf den Vergleich der mittleren Differenz mit dem theoretischen Erwartungswert (Vergleich T_{II} mit $T_{IV(V)}$ und K_{II} mit $K_{IV(V)}$).

Ergebnisse

Die Ergebnisse sind in den Tabellen 1 bis 8 zusammengefasst. Die Aktivität der *Transketolase* sank von Periode II bis Periode IV unter dem Einfluss der thiaminarmen Kost um ca. 15 % ab und stieg nach Gabe der Normalkost mit hohen Thiamindosen wieder auf den Ausgangswert an. Der Abfall ist innerhalb der gleichen Gruppe nicht statistisch sicherbar. Beim Vergleich der unabhängigen Mittelwerte K_{II} mit T_{IV} und T_{II} mit K_{IV} auf Signifikanz, war nur der Abfall von K_{II} auf T_{IV}, nicht der von T_{II} auf K_{IV} gesichert. Das gleiche Bild ergibt sich bei Überprüfung des Wiederanstiegs: Der Unterschied zwischen K_{IV} und T_V ist nicht gesichert, wohl aber der von T_{IV} auf K_V.

Bei Betrachtung der Aktivierbarkeit der Transketolase durch Thiaminpyrophosphat *(TPP-Effekt)* zeigte sich erwartungsgemäss die gegenläufige Tendenz: Anstieg bei Gabe der thiaminarmen Kost und Abfall auf 0 nach Gabe von Normalkost und hohen Dosen Thiamin. Ein zusätzlicher Einfluss von Sulfit auf die Aktivität oder Aktivierbarkeit der Transketolase war nicht feststellbar.

Bei der Bestimmung von *Thiamin im Harn* wurde sowohl bei Berechnung der Gesamtausscheidung pro Tag als auch bei Berechnung des Thiamin-Kreatinin-Verhältnisses ein gesicherter Abfall und Wiederanstieg bei Vergleich der Periodengruppenmittelwerte T_I mit K_{IV} und T_{IV} mit K_V erhalten. Nicht signifikant waren wiederum Abfall und Wiederanstieg innerhalb der gleichen Gruppen. Ein Einfluss von Sulfit auf die Thiaminausscheidung im Harn war nicht nachweisbar.

Der *Thiamingehalt im Blut* sank im Durchschnitt um ca. 30 % von Periode I bis Periode III. Bemerkenswert dabei ist, dass der Thiamingehalt im Blut der weiblichen Vpn zu Beginn des Versuchs nur etwa halb so hoch war wie der der männlichen Vpn und im Laufe des Versuchs nur um ca. 10 % abnahm, während der Thiamingehalt im Blut der männlichen Vpn um mehr als 50 % absank. Wegen der durch die unterschiedliche Ausgangslage bedingten grossen Streubreite der Werte der Periode I liess sich der z. T. sehr gravierende Abfall des Thiamin-

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Tab. 1: Transketolaseaktivität in hämolysiertem Vollblut (U/l Blut, Hämatokrit = 35 %)

Periode	Datum	Testgruppe T						$\bar{x} \pm s$	Kontrollgruppe K						$\bar{x} \pm s$
		♂			♀				♂			♀			
		1	3	5	15	16	18		6	7	8	11	14	15	
II	4. Nov.	41.4	58.2	48.2	76.5	58.6	68.2		50.8	63.7	64.2	51.3	75.9	93.5	
	11. Nov.	60.8	57.8	44.7	64.0	51.3	51.2		51.8	53.5	57.2	52.5	59.1	56.0	
	x	51.1	58.0	46.5	70.3	55.5	59.7	56.8 ± 8.1	51.3	58.6	60.7	51.9	67.5	74.8	60.8 ± 9.1
III	18. Nov.	48.2	53.8	43.1	56.8	49.8	42.3		42.8	67.8	58.0	53.9	49.6	52.3	
	21. Nov.	51.7	52.8	48.0	58.2	52.3	51.9		44.1	51.8	49.0	48.3	67.8	57.5	
	25. Nov.	55.2	35.3	37.5	65.4	41.9	55.0		42.3	59.4	53.7	45.3	59.6	49.9	
	28. Nov.	50.8	52.7	51.4	60.3	68.7	56.8		39.1	68.8	69.8	48.3	68.7	71.0	
	2. Dez.	42.9	54.8	41.7	58.8	66.6	57.3		39.8	55.0	61.5	44.3	63.4	70.3	
	x	46.6	49.9	44.5	59.9	55.9	52.7	51.5 ± 5.8	41.6	60.6	58.4	48.0	61.4	60.2	55.0 ± 8.2
IV	9. Dez.	48.7	43.6	55.7	55.8	43.2	46.4		40.7	57.8	59.6	43.8	69.8	51.1	
	16. Dez.	52.1	48.8	38.1	59.1	46.4	40.8		40.8	60.8	55.7	40.4	48.7	53.3	
	x	50.4	45.9	47.2	57.5	44.3	43.4	48.1 ± 5.2	40.8	59.0	56.7	41.9	55.3	52.2	50.9 ± 7.7
V	17. Dez.	56.0	56.1	59.8	69.8	70.5	56.6		52.8	59.6	64.5	52.5	58.3	67.0	
	18. Dez.	55.7	46.4	54.7	57.5	53.9	50.1		53.0	61.8	53.5	58.8	57.4	69.5	
	x	55.9	51.3	57.3	63.7	62.2	53.4	57.3 ± 4.8	52.9	60.6	59.0	55.7	57.9	68.3	59.0 ± 5.2

$T_{II} : K_{IV}\ p > 0.05$ $K_{II} : T_{IV}\ p < 0.05$ $T_{IV} : K_{V}\ p < 0.02$ $K_{IV} : T_{V}\ p > 0.05$

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Tab. 2: TPP-Effekt, Anstieg der Transketolaseaktivität (%)

Periode	Datum	Testgruppe T ♂ 1	2	3	♀ 15	16	18	k ± s	Kontrollgruppe K ♂ 6	7	8	♀ 11	14	15	k ± s
II	4. Nov.	20	15	28	21	19	10		23	15	23	35	6	9	
	11. Nov.	14	28	26	29	26	23		22	25	23	24	18	27	
	x	19	22	27	25	23	17	22 ± 5	23	20	23	30	12	19	21 ± 5
III	18. Nov.	18	15	47	24	30	32		35	12	26	26	41	41	
	21. Nov.	21	20	28	35	31	14		31	32	33	32	15	20	
	25. Nov.	40	27	28	19	52	18		28	22	24	34	15	41	
	28. Nov.	26	21	35	20	17	35		19	21	5	24	24	31	
	2. Dez.	37	22	44	15	6	11		18	19	17	29	25	11	
	x	28	21	35	23	27	22	26 ± 5	25	21	21	29	23	29	24 ± 3
IV	9. Dez.	20	13	0	15	18	6		14	11	7	3	11	15	
	16. Dez.	34	24	29	15	16	30		25	30	23	26	30	21	
	x	27	19	15	15	12	19	17 ± 5	20	21	15	15	16	18	17 ± 2
V	17. Dez.	0	0	0	0	0	0		1	0	0	0	2	0	
	18. Dez.	0	0	0	0	0	0		0	18	0	0	0	0	
	x	0	3	0	0	0	0	0 ± 1	1	9	0	0	1	0	1 ± 3

$T_{II} : K_{III(IV)}\ p > 0.05$ $K_{II} : T_{III(IV)}\ p > 0.05$ $T_{IV} : K_V\ p < 0.02$ $K_{IV} : T_V\ p < 0.01$

Tab. 3: Thiamin im Harn (mcg/24 h)

Periode	Datum	Testgruppe T ♂ 1	3	5	♀ 13	16	18	x̄ ± s	Kontrollgruppe K ♂ 6	7	8	♀ 11	14	15	x̄ ± s
I	21. Okt.	56.1	94.0	70.5	58.7	6.3	19.1		91.6	122.7	79.0	50.6	205.1	158.1	
	28. Okt.	49.5	93.8	25.2	41.8	11.0	25.7		93.6	92.7	52.0	17.7	95.7	76.9	
	x	54.9	93.9	47.8	50.3	9.7	22.4	46.4 ± 29.1	92.0	107.7	65.5	38.7	145.4	117.5	95.0 ± 38.8
II	4. Nov.	22.4	41.9	14.0	9.0	4.9	16.7		12.0	28.1	23.7	7.0	22.1	24.3	
	7. Nov.	19.4	34.2	8.0	15.8	8.5	14.8		7.7	12.9	26.2	15.1	36.1	28.5	
	11. Nov.	25.3	28.5	12.5	27.2	7.5	3.5		16.3	24.5	20.0	9.5	25.1	16.2	
	x	22.7	34.9	11.5	16.3	7.0	11.6	17.3 ± 10.1	12.0	21.6	25.3	9.9	27.8	23.0	19.9 ± 7.3
III	14. Nov.	18.1	36.4	17.2	25.2	31.1	12.5		13.4	19.9	22.1	16.9	31.2	12.1	
	18. Nov.	13.5	42.1	13.6	24.1	7.9	16.5		13.3	27.5	12.9	13.3	15.1	22.6	
	21. Nov.	20.5	34.6	12.7	14.5	8.7	6.2		9.4	12.9	19.1	11.6	10.7	14.5	
	25. Nov.	15.2	27.0	8.6	11.9	5.4	4.9		7.3	13.4	12.1	7.5	11.9	13.2	
	28. Nov.	14.2	23.1	10.9	11.6	7.7	11.1		10.3	23.5	15.2	8.4	11.0	19.3	
	2. Dez.	16.5	21.4	10.2	11.6	8.6	5.2		7.9	13.8	13.7	6.1	13.5	13.5	
	5. Dez.	13.9	31.5	9.1	10.3	11.1	5.3		5.0	13.7	15.6	8.4	12.0	12.4	
	x	17.5	30.9	11.6	15.7	11.1	8.8	15.9 ± 7.8	9.5	18.5	17.2	10.0	15.0	15.4	14.2 ± 3.7
IV	8. Dez.	9.6	14.8	8.5	8.6	8.8	5.3		4.8	9.6	18.9	6.0	12.7	10.3	
	12. Dez.	17.3	26.3	9.5	13.2	10.7	3.8		6.6	17.0	20.4	9.1	16.1	18.0	
	16. Dez.	15.5	27.1	10.5	11.5	8.7	5.4		11.1	22.8	19.8	7.1	19.3	19.1	
	x	14.0	23.6	9.5	10.0	7.7	5.5	11.8 ± 6.2	7.4	16.8	18.0	7.4	13.0	16.0	13.1 ± 4.7
V	17. Dez.	2.90×10^6	2.02×10^6	1.32×10^6	1.73×10^6	1.45×10^6	2.15×10^6		4.17×10^6	1.68×10^6	1.96×10^6	1.27×10^6	2.20×10^6	9.66×10^6	
	18. Dez.	3.12×10^6	2.65×10^6	2.62×10^6	1.41×10^6	1.19×10^6	3.00×10^6		3.54×10^6	1.89×10^6	2.39×10^6	2.06×10^6	3.05×10^6	2.12×10^6	
	x	3.06×10^6	2.31×10^6	2.22×10^6	1.57×10^6	1.32×10^6	2.58×10^6	$2.26 \times 10^6 \pm 0.88 \times 10^6$	3.86×10^6	1.83×10^6	2.18×10^6	1.67×10^6	2.63×10^6	2.90×10^6	$2.51 \times 10^6 \pm 0.81 \times 10^6$

$T_I : K_{IV}$ $p < 0.05$ $T_{IV} : K_V$ $p < 0.001$

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Tab. 4: Thiamin-Kreatinin-Verhältnis im Harn (mcg/g)

Periode	Datum	Testgruppe T ♂ 1	3	5	♀ 13	16	18	x̄ ± s	Kontrollgruppe K ♂ 6	7	8	♀ 11	14	15	x̄ ± s
I	21. Okt.	56.1	94.0	70.5	58.7	6.3	19.1		91.6	122.7	79.0	50.6	205.1	158.1	
	28. Okt.	49.5	93.8	25.2	41.8	11.0	25.7		93.6	92.7	52.0	17.7	95.7	76.9	
	x	54.9	93.9	47.8	50.3	9.7	22.4	46.4 ± 29.1	92.0	107.7	65.5	38.7	145.4	117.5	95.0 ± 38.8
II	4. Nov.	22.4	41.9	14.0	9.0	4.9	16.7		12.0	28.1	23.7	7.0	22.1	24.3	
	7. Nov.	19.4	34.2	8.0	15.8	8.5	14.8		7.7	12.9	26.2	15.1	36.1	28.5	
	11. Nov.	25.3	28.5	12.5	27.2	7.5	3.5		16.3	24.5	20.0	9.5	25.1	16.2	
	x	22.7	34.9	11.5	16.3	7.0	11.6	17.3 ± 10.1	12.0	21.6	25.3	9.9	27.8	23.0	19.9 ± 7.3
III	14. Nov.	18.1	36.4	17.2	25.2	31.1	12.5		13.4	19.9	22.1	16.9	31.2	12.1	
	18. Nov.	13.5	42.1	13.6	24.1	7.9	16.5		13.3	27.5	12.9	13.3	15.1	22.6	
	21. Nov.	20.5	34.6	12.7	14.5	8.7	6.2		9.4	12.9	19.1	11.6	10.7	14.5	
	25. Nov.	15.2	27.0	8.6	11.9	5.4	4.9		7.3	13.4	12.1	7.5	11.9	13.2	
	28. Nov.	14.2	23.1	10.9	11.6	7.7	11.1		10.3	23.5	15.2	8.4	11.0	19.3	
	2. Dez.	16.5	21.4	10.2	11.6	8.6	5.2		7.9	13.8	13.7	6.1	13.5	13.5	
	5. Dez.	13.9	31.5	9.1	10.3	11.1	5.3		5.0	13.7	15.6	8.4	12.0	12.4	
	x	17.5	30.9	11.6	15.7	11.1	8.8	15.9 ± 7.8	9.5	18.5	17.2	10.0	15.0	15.4	14.2 ± 3.7
IV	8. Dez.	9.6	14.8	8.5	8.6	8.8	5.3		4.8	9.6	18.9	6.0	12.7	10.3	
	12. Dez.	17.3	26.3	9.5	13.2	10.7	3.8		6.6	17.0	20.4	9.1	16.1	18.0	
	16. Dez.	15.5	27.1	10.5	11.5	8.7	5.4		11.1	22.8	19.8	7.1	19.3	19.1	
	x	14.0	23.6	9.5	10.0	7.7	5.5	11.8 ± 6.2	7.4	16.8	18.0	7.4	13.0	16.0	13.1 ± 4.7
V	17. Dez.	2.90×10^6	2.02×10^6	1.32×10^6	1.73×10^6	1.45×10^6	2.15×10^6		4.17×10^6	1.68×10^6	1.96×10^6	1.27×10^6	2.20×10^6	9.66×10^6	
	18. Dez.	3.12×10^6	2.65×10^6	2.62×10^6	1.41×10^6	1.19×10^6	3.00×10^6		3.54×10^6	1.89×10^6	2.39×10^6	2.06×10^6	3.05×10^6	2.12×10^6	
	x	3.06×10^6	2.31×10^6	2.22×10^6	1.57×10^6	1.32×10^6	2.58×10^6	$2.26 \times 10^6 \pm 0.88 \times 10^6$	3.86×10^6	1.83×10^6	2.18×10^6	1.67×10^6	2.63×10^6	2.90×10^6	$2.51 \times 10^6 \pm 0.81 \times 10^6$

IV	12. Dez.	11.2	16.0	8.3	13.2	10.7	8.8		8.0	17.8	20.4	9.1	16.1	18.9	
	16. Dez.	15.8	27.1	10.5	11.3	—	8.4		11.2	22.6	12.6	7.1	10.9	7.1	
	x	14.0	22.6	9.5	10.8		8.5	11.3 ± 0.2	7.4	16.0	10.9	7.4	13.0	13.0	13.1 ± 4.7
V	17. Dez.	2.99×10^5	2.92×10^5	1.82×10^5	1.73×10^5	1.45×10^5	2.15×10^5		4.17×10^5	1.66×10^5	1.96×10^5	1.27×10^5	2.20×10^5	3.68×10^5	
	18. Dez.	3.12×10^5	2.69×10^5	2.62×10^5	1.41×10^5	1.19×10^5	3.06×10^5		3.54×10^5	1.99×10^5	2.30×10^5	2.06×10^5	3.05×10^5	2.12×10^5	
	x	3.06×10^5	2.81×10^5	2.22×10^5	1.57×10^5	1.32×10^5	2.58×10^5	$2.26 \times 10^5 \pm 0.68 \times 10^5$	3.86×10^5	1.83×10^5	2.18×10^5	1.07×10^5	2.63×10^5	2.90×10^5	$2.51 \times 10^5 \pm 0.81 \times 10^5$

$T_I : K_{IV}$ $p < 0.05$ $T_{IV} : K_V$ $p < 0.001$

Tab. 4: Thiamin-Kreatinin-Verhältnis im Harn (mcg/g)

Periode	Datum	Testgruppe T						x ± s	Kontrollgruppe K						x ± s
		♂			♀				♂			♀			
		1	2	3	12	13	16		6	7	8	11	14	15	
I	21. Okt.	26.5	47.2	28.4	35.5	7.3	13.0		57.5	85.8	39.0	41.4	149.4	153.5	
	28. Okt.	24.4	64.2	17.3	28.4	10.0	16.0		44.8	38.6	26.4	11.1	64.1	72.6	
	x	25.5	55.7	22.8	32.0	8.7	14.5	26.5 ± 16.5	51.1	61.8	32.9	26.2	106.7	113.6	65.2 ± 36.8
II	4. Nov.	16.7	29.5	7.5	6.1	4.6	11.2		6.4	23.0	14.2	6.9	20.6	22.3	
	7. Nov.	10.7	16.7	5.0	14.5	6.5	8.9		4.1	11.5	13.9	9.5	31.2	27.9	
	11. Nov.	11.2	16.7	7.2	14.5	5.5	8.0		5.6	13.8	13.8	6.7	17.1	14.9	
	x	10.9	19.6	6.6	11.6	5.5	7.7	10.5 ± 5.1	5.4	16.1	13.9	7.7	23.0	21.7	14.4 ± 6.6
III	14. Nov.	8.4	18.3	9.5	25.6	10.1	8.1		6.6	12.0	15.8	9.3	33.5	7.8	
	18. Nov.	7.4	20.8	6.9	15.8	5.7	9.3		7.6	14.2	8.5	7.5	18.7	16.7	
	21. Nov.	9.6	18.9	6.8	9.6	6.4	9.1		4.3	8.4	10.3	7.0	8.0	12.0	
	25. Nov.	6.0	16.0	4.9	7.1	5.5	3.1		4.0	18.3	6.7	5.6	8.7	10.5	
	28. Nov.	7.4	13.3	6.4	6.8	5.8	7.8		6.4	13.6	10.3	6.8	10.0	17.2	
	2. Dez.	7.5	11.6	5.7	8.2	4.0	3.1		4.6	10.5	9.2	4.6	9.8	10.9	
	5. Dez.	9.5	10.4	5.1	7.8	7.4	3.4		2.5	7.3	7.7	4.2	9.0	9.8	
	x	7.9	16.0	6.3	11.2	7.4	5.4	8.1 ± 4.1	5.2	10.9	9.8	6.4	12.9	12.1	9.5 ± 3.0
IV	9. Dez.	4.9	8.1	4.9	3.1	2.7	3.6		2.5	6.3	8.5	3.3	9.8	9.4	
	12. Dez.	8.6	14.0	5.6	9.9	8.1	6.1		4.0	10.8	11.9	6.8	15.6	18.4	
	16. Dez.	9.7	15.8	6.4	8.3	7.2	8.7		6.6	13.4	12.1	5.0	5.3	18.5	
	x	7.7	12.9	5.6	7.1	6.0	4.4	7.2 ± 3.0	4.3	10.1	10.8	5.0	11.5	15.4	9.5 ± 4.2
V	17. Dez.	1.37×10^5	1.56×10^5	0.99×10^5	1.12×10^5	0.94×10^5	1.24×10^5		2.10×10^5	0.77×10^5	1.25×10^5	0.74×10^5	1.69×10^5	2.42×10^5	
	18. Dez.	1.67×10^5	1.53×10^5	1.50×10^5	0.85×10^5	0.75×10^5	1.79×10^5		1.99×10^5	1.09×10^5	1.25×10^5	1.18×10^5	1.83×10^5	1.66×10^5	
	x	1.52×10^5	1.54×10^5	1.25×10^5	0.99×10^5	0.84×10^5	1.51×10^5	$1.28 \times 10^5 \pm 0.26 \times 10^5$	2.04×10^5	0.93×10^5	1.24×10^5	0.96×10^5	1.76×10^5	2.04×10^5	$1.50 \times 10^5 \pm 0.54 \times 10^5$

$T_I : K_{IV}$ $p < 0.05$ $T_{IV} : K_V$ $p < 0.001$

Tab. 3: Thiamin im Blut (mcg/100 ml)

Periode	Datum	Testgruppe T ♂ 1	♂ 3	♂ 5	♀ 13	♀ 16	♀ 18	x ± s	Kontrollgruppe K ♂ 6	♂ 7	♂ 8	♀ 11	♀ 14	♀ 15	x ± s
I	28. Okt.	14.1	8.0	7.5	6.7	5.8	5.9		11.8	17.6	8.1	6.8	5.4	5.0	
	x	14.1	8.0	7.5	6.7	5.8	5.9	7.9±3.1	11.8	17.6	8.1	6.8	5.4	5.0	9.1±4.8
II	4. Nov.	8.0	5.1	7.0	6.7	4.7	2.9		10.1	9.0	9.8	5.9	6.7	7.0	
	11. Nov.	5.2	6.9	5.2	4.8	5.1	5.4		7.3	6.5	6.0	11.6	7.0	5.3	
	x	6.6	6.0	6.1	5.8	4.9	4.2	5.6±0.8	8.7	7.8	7.9	8.8	6.9	6.2	7.7±1.0
III	18. Nov.	6.8	4.5	5.2	7.2	4.1	5.6		5.1	6.6	5.0	4.5	6.0	4.9	
	21. Nov.	12.3	5.7	5.5	9.7	5.8	6.6		10.3	6.5	6.1	7.6	5.5	5.5	
	25. Nov.	4.7	4.5	8.4	5.4	5.0	3.9		3.1	5.9	6.9	3.7	7.0	4.9	
	28. Nov.	3.8	3.3	3.3	7.8	4.6	6.1		5.2	4.5	5.2	3.5	6.1	4.5	
	2. Dez.	3.8	4.2	4.3	4.0	5.4	4.3		4.1	4.0	4.7	5.0	6.0	6.0	
	x	6.8	4.4	4.8	5.9	5.0	5.3	5.0±0.5	5.9	5.8	5.8	4.7	6.5	5.2	5.2±0.8
IV	9. Dez.	5.9	5.7	5.8	6.9	5.0	4.8		6.4	6.5	6.4	4.9	6.0	5.5	
	16. Dez.	12.2	11.5	12.2	14.4	12.6	9.3		11.7	12.1	13.1	11.3	12.9	11.9	
	x	9.1	8.6	9.0	10.7	8.8	7.1	8.8±1.1	9.1	9.3	9.8	8.1	9.5	8.7	9.0±0.5
V	17. Dez.	18.2	20.7	20.5	22.6	23.2	24.5		25.4	22.2	20.6	23.0	21.5	22.7	
	18. Dez.	24.4	22.5	20.9	23.2	20.9	23.7		22.5	26.8	22.0	20.2	23.1	23.1	
	x	21.3	21.7	20.6	22.9	22.1	24.1	22.1±1.2	24.0	24.5	21.3	24.6	22.2	22.9	23.2±1.3

$T_{II} : K_{III}$ $p > 0.05$ $K_{II} : T_{III}$ $p > 0.05$ $T_{III} : K_{V}$ $p < 0.001$

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	x	9.1	8.6	9.0	10.7	8.0	7.1	8.8±1.1	9.1	9.0	9.3	8.1	9.8	8.7	9.7
V	17. Dez.	18.2	20.7	20.3	[illegible]	23.2	24.5		25.4	22.2	20.6	25.0	21.3	22.7	
	18. Dez.	24.4	22.5	20.9	23.2	20.9	23.7		22.5	26.8	22.0	26.2	23.1	23.1	
	x	21.3	21.7	20.6	22.9	22.1	24.1	22.1±1.2	24.0	24.5	21.3	24.6	22.2	22.9	23.2±1.5

$T_{II} : K_{III}\ p > 0.05$ $K_{II} : T_{III}\ p > 0.05$ $T_{III} : K_{V}\ p < 0.001$

Tab. 6: LDH-Aktivität in hämolysierten Erythrozyten (U/10⁹ Erythrozyten)

Periode	Datum	Testgruppe T						$\bar{x} \pm s$	Kontrollgruppe K						$\bar{x} \pm s$
		♂			♀				♂			♀			
		1	3	5	13	16	18		6	7	8	11	14	15	
II	4. Nov.	3.12	3.15	2.90	3.01	2.77	3.60		2.72	2.90	2.52	2.93	2.79	3.14	
	11. Nov.	2.05	2.03	1.58	2.39	2.90	3.14		1.50	1.94	1.92	2.47	2.50	1.99	
	x	2.58	2.59	2.24	2.70	2.83	3.37	2.71±0.36	2.11	2.42	2.22	2.70	2.64	2.56	2.44±0.22
III	18. Nov.	2.44	2.85	2.05	2.61	2.67	2.80		2.50	2.45	2.18	2.43	2.28	2.26	
	21. Nov.	2.62	2.78	2.14	2.42	2.70	3.14		2.46	1.85	1.96	2.64	2.71	2.27	
	25. Nov.	2.23	2.63	1.75	2.18	2.57	2.72		2.11	1.70	2.03	2.44	2.56	1.74	
	28. Nov.	2.48	1.96	1.48	2.14	2.30	2.44		1.84	1.53	2.10	2.27	1.96	1.74	
	2. Dez.	2.00	2.33	1.95	1.95	2.06	2.49		1.95	1.68	1.84	2.13	1.90	2.08	
	x	2.35	2.51	1.87	2.26	2.46	2.74	2.36±0.28	2.17	1.84	2.02	2.38	2.24	2.02	2.11±0.17
IV	9. Dez.	2.19	2.22	2.06	2.56	2.45	2.70		2.24	2.29	2.11	2.22	2.33	2.32	
	16. Dez.	2.41	2.15	1.96	2.41	2.57	2.60		1.98	2.04	2.53	2.42	2.60	1.94	
	x	2.30	2.18	1.99	2.53	2.51	2.68	2.36±0.24	2.11	2.16	2.32	2.32	2.47	2.13	2.25±0.10
V	17. Dez.	2.58	2.64	2.28	2.81	2.65	2.78		2.34	2.04	2.47	2.61	2.20	2.18	
	18. Dez.	2.56	2.02	2.33	2.60	2.89	2.92		1.61	2.27	2.46	2.70	2.34	2.27	
	x	2.57	2.32	2.30	2.70	2.77	2.85	2.58±0.22	1.97	2.15	2.46	2.65	2.27	2.22	2.28±0.22

$T_{II} : K_{IV}\ p < 0.02$ $K_{IV} : T_{V}\ p < 0.01$

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Tab. 7: G-6-PDH-Aktivität in hämolysierten Erythrocyten (mU/10⁹ Erythrocyten)

Periode	Datum	Testgruppe T						$\bar{x} \pm s$	Kontrollgruppe K						$\bar{x} \pm s$
		♂			♀				♂			♀			
		1	2	3	13	16	18		6	7	8	11	14	15	
II	4. Nov.	104.3	100.2	119.0	156.8	137.5	115.7		111.3	175.2	115.9	119.5	186.6	99.4	
	11. Nov.	94.4	100.3	82.5	130.6	157.1	167.1		76.0	126.0	124.0	143.3	165.5	122.4	
	x	99.3	100.3	100.7	143.6	147.3	141.4	122.1 ± 24.2	93.6	147.6	119.9	131.4	158.5	110.9	125.6 ± 21.9
III	18. Nov.	103.4	117.6	115.9	129.4	103.2	136.2		86.3	141.3	115.3	109.6	100.7	102.2	
	21. Nov.	109.7	109.9	115.9	121.7	107.6	113.1		102.2	108.7	92.5	97.0	132.8	110.6	
	25. Nov.	90.1	108.8	101.6	123.3	127.3	125.9		102.0	137.2	101.5	116.1	107.7	91.2	
	28. Nov.	109.4	93.1	100.4	126.5	124.5	110.4		80.7	131.3	95.0	111.1	141.9	110.4	
	2. Dez.	94.1	107.6	102.7	102.9	125.7	125.5		105.8	117.1	113.7	117.1	117.8	123.5	
	x	101.3	107.4	106.0	121.1	117.7	122.2	112.7 ± 8.6	95.4	127.1	103.6	110.1	114.8	107.5	109.7 ± 10.7
IV	9. Dez.	95.1	104.4	111.5	103.3	124.5	122.0		121.1	128.4	105.5	107.6	127.9	94.4	
	16. Dez.	100.4	98.9	113.1	116.1	123.3	103.1		83.7	114.3	114.2	99.8	126.5	95.1	
	x	97.7	101.6	112.3	109.7	123.9	112.5	109.6 ± 9.2	97.9	121.3	109.8	103.7	127.2	94.7	109.1 ± 15.0
V	17. Dez.	68.5	73.0	100.5	97.1	102.9	106.6		86.5	107.7	111.3	88.4	109.3	96.9	
	18. Dez.	66.4	84.7	112.8	112.6	125.3	126.3		74.8	135.8	111.0	93.4	113.0	100.1	
	x	67.4	78.8	106.6	104.8	114.1	117.4	102.1 ± 14.9	80.6	120.7	111.2	90.9	111.1	98.2	98.1 ± 20.3

$T_{II} : K_V \; p > 0.05$

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Tab. 8: NADH-Diaphorase-Aktivität in hämolysierten Erythrozyten (mU/10⁸ Erythrozyten)

Periode	Datum	Testgruppe T ♂ 1	3	5	♀ 13	16	18	$\bar{x} \pm s$	Kontrollgruppe K ♂ 6	7	8	♀ 11	14	16	$\bar{x} \pm s$
II	4. Nov.	117.1	109.1	78.3	100.6	80.6	93.7		105.6	85.6	71.0	91.0	75.3	126.0	
	11. Nov.	42.2	64.1	43.0	111.4	76.0	92.9		55.3	59.1	53.0	57.2	66.0	76.8	
	x	79.7	86.6	60.6	106.0	78.3	93.3	84.0 ± 15.3	80.5	72.4	62.0	74.1	70.7	101.4	76.8 ± 13.4
III	18. Nov.	61.8	82.6	79.9	105.2	93.3	72.2		77.2	73.3	76.0	77.2	58.8	90.2	
	21. Nov.	59.1	76.4	51.5	74.5	83.3	63.0		82.2	60.3	54.1	76.4	74.5	67.6	
	25. Nov.	47.6	53.0	60.2	78.7	56.8	61.1		86.0	61.4	49.5	54.1	69.1	83.3	
	28. Nov.	54.9	44.9	49.5	70.3	54.1	68.0		61.1	58.0	42.6	60.3	74.9	68.0	
	2. Dez.	40.7	35.7	40.0	55.3	60.3	40.0		48.8	66.8	55.3	47.6	64.9	71.8	
	x	52.8	58.5	60.2	76.8	69.6	62.1	63.3 ± 8.5	71.1	64.0	55.5	63.1	68.4	76.2	66.3 ± 7.1
IV	9. Dez.	30.0	31.7	26.0	61.1	57.6	59.9		66.0	51.9	53.0	46.0	48.5	44.4	
	16. Dez.	27.8	41.0	57.6	51.8	70.3	58.2		64.5	39.4	47.5	38.8	51.8	34.1	
	x	28.8	36.4	41.8	56.3	68.0	59.2	48.4 ± 15.0	66.3	55.7	50.5	53.8	50.2	39.3	52.6 ± 8.7
V	17. Dez.	46.7	73.8	62.3	60.0	57.0	51.3		79.5	76.7	62.3	55.3	53.3	66.8	
	18. Dez.	55.4	63.9	59.9	52.4	34.5	55.4		58.8	59.9	55.4	42.0	50.3	54.5	
	x	51.1	69.9	61.1	60.2	45.8	53.4	56.7 ± 8.2	69.2	68.3	58.9	49.0	51.8	50.7	57.9 ± 8.9

$T_{II} : K_{IV}\ p < 0.01$ $K_{IV} : T_V\ p > 0.05$ $T_{III} : K_{III}\ p > 0.05$ $T_{IV} : K_{IV}\ p > 0.05$

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gehalts im Blut beim Vergleich der Periodengruppenmittelwerte T_I mit K_{III} oder K_I mit T_{III} statistisch nicht sichern. Signifikant dagegen war der Wiederanstieg nach dem Thiaminstoss. Eine zusätzliche Beeinflussung des Thiamingehalts im Blut durch Sulfit konnte nicht beobachtet werden.

Bei allen drei Dehydrogenasen konnte während Gabe der thiaminarmen Versuchskost ein Aktivitätsabfall um 10 bis 40 % und z. T. ein Wiederanstieg nach Gabe der Normalkost mit hohen Thiamindosen beobachtet werden. Abfall und Wiederanstieg liessen sich innerhalb der gleichen Gruppen (= abhängige Stichproben) statistisch nicht sichern, wohl aber z. T. beim Vergleich von Periodenmittelwerten der Testgruppe mit Periodenmittelwerten der Kontrollgruppe (= unabhängige Stichproben). Signifikant war der Abfall der *NADH-Diaphorase* von Periode II auf Periode IV, ebenfalls der Abfall von *LDH*, während sich der Abfall der *G-6-PDH-Aktivität* um ca. 10–20 % wegen der grossen Streubreite statistisch nicht sichern liess. Der Wiederanstieg in der Nachperiode war nur bei LDH signifikant. NADH-Diaphorase wies als einziges der untersuchten Enzyme einen verstärkten Abfall in der Testgruppe auf, möglicherweise als Folge eines zusätzlichen Hemmeffekts durch Sulfit. Allerdings war der Unterschied gegenüber der Kontrollgruppe statistisch nicht zu sichern.

Diskussion

Die Überprüfung des Thiaminstatus ergab eine Verarmung der Versuchspersonen während des Zeitraums, in dem die thiaminarme Kost verabreicht wurde. Besonders eindruckvoll war der Rückgang der *Thiaminausscheidung im Harn* auf Werte, die nach Angaben des Council on Food and Nutrition (Pearson, 1962) als ungenügend angesehen werden müssen (< 40 mcg/Tag bzw. < 27 mcg/g Kreatinin). Weniger ausgeprägt waren die Veränderungen des *Thiamingehalts im Blut* und der *Aktivität der Transketolase*, die beide nur wenig abfielen. Nach der Definition von Brin (1964) hatten die Versuchspersonen das Stadium B der Thiaminunterversorgung erreicht, d. h. jenen Zustand, in dem zwar schon biochemische Veränderungen nachweisbar sind (nach Brin Absinken der Transketolaseaktivität, Anstieg des TPP-Effektes auf ca. 15 %, tägliche Thiaminausscheidung im Harn 25 mcg), in dem es aber noch nicht zu klinischen Ausfallerscheinungen kommt. Auch beim Thiamingehalt im Blut werden erst Werte unterhalb von 2–3 mcg/100 ml Blut als Zeichen ungenügender Versorgung angesehen (Lzevy u. Mitarb., 1965). Bei diesem Grad der Thiaminunterversorgung konnten wir keinen Einfluss von Sulfit auf den Thiaminstatus der Versuchspersonen nachweisen; d. h., dass eine *in vivo*-Zerstörung von Thiamin

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durch orale Gaben von Sulfit unter den genannten Versuchsbedingungen nicht erfolgt ist.

Das Absinken der *LDH-Aktivität* entspricht dem von Gubler (1968) bereits im Tierversuch nachgewiesenen Abfall unter dem Einfluss einer thiaminarmen Kost. Dass kein zusätzlich hemmender Effekt durch Sulfit beobachtet wurde, spricht dafür, dass bei unseren Versuchsbedingungen (Sulfitmangel) Sulfit bereits im Magen-Darmtrakt bzw. nach der Resorption völlig oxydiert worden ist und als solches im Blut nicht mehr vorlag, denn schon sehr geringe Sulfitmengen sind in der Lage, nach Untersuchungen von Pfleiderer, Jeckel und Wieland (1956), Giaccio, Keller, Kenderes, Boxer und Devlin (1966) sowie Mizumo und Humphrey (1966) verschiedene Lactatdehydrogenasen zu hemmen.

Auch der beobachtete Abfall der Aktivitäten von *G-6-PDH* und *NADH-Diaphorase* ist mit ziemlicher Sicherheit – da die übrigen Nährstoffe in ausreichender Menge gegeben wurden – auf die Verarmung der Versuchspersonen an Thiamin zurückzuführen. Beide Enzyme haben in Erythrozyten besondere Funktionen: *G-6-PDH* ist das Schlüsselenzym des oxydativen Glukoseabbaus über den Pentosephosphatzyklus (Aebi, 1967). Bei einem Mangel an G-6-PDH, der als angeborener Defekt auftreten kann, kommt es zu einem Mangel und/oder zu einer Instabilität von reduziertem Glutathion. Hierdurch sind die reduzierten Pyridinnukleotide und die für die Zellintegrität essentiellen SH-Gruppen der Membran und verschiedener Enzyme sowie das Hämoglobin nicht ausreichend gegen Oxydationsprozesse geschützt, so dass es unter dem Einfluss bestimmter Pharmaka und Extrakte aus *Vicia faba*, welche Glutathion oxydieren, zu hämolytischen Krisen kommen kann (Waller, 1968, Beutler, 1959, Walker und Bowman, 1960). Auch Sulfit soll nach Untersuchungen von Ezrielev (1968) hämolytisch wirken können. Über die Ursache macht der Autor keine Angaben. Denkbar wäre, dass Sulfit mit Glutathion zu S-Sulfoglutathion reagiert, welches Balabaeva und Gancer (1967) in Blut und Organen von Meerschweinchen nach i. p. Injektion von Sulfit nachweisen konnten. Durch Überführung in S-Sulfoglutathion büsst Glutathion seine physiologische Funktionsfähigkeit ein (Aufrechterhaltung von SH-Gruppen und des Hämoglobins in reduzierter Form). Möglicherweise lässt sich die im Tierversuch von Hötzel (s. Mitt. I) bewiesene erhöhte Toxizität von Sulfit bei Thiaminmangel wenigstens z. T. damit erklären, dass sowohl Thiaminmangel als auch Sulfit zu einer Verminderung von reduziertem Glutathion in Erythrozyten führen können. Voraussetzung ist allerdings, dass Sulfit unverändert in den Blutkreislauf gelangt, was nach den Untersuchungen von Pfleiderer, Stock, Otting und Diemair (1968) zumindest im Tierversuch möglich ist.

Im vorliegenden Versuch konnte kein Sulfit im Blut nachgewiesen werden (s. Mittl. 5). Es ergab sich auch kein Hinweis auf eine mögliche hämolytische Reaktion, da die Aktivitäten der Serumenzyme, insbesondere der LDH, während des Versuchszeitraums nicht anstiegen (s. Mittl. 1), was nach Gerlach, Losse und Schmidt (1961) bei Hämolysen durch Austritt von Erythrozytenenzymen zu erwarten ist. Auch führte der Thiaminmangel nur zu einer Minderung der G-6-PDH-Aktivität um ca. 20 %, so dass, selbst wenn Sulfit in geringen Mengen unverändert in den Blutkreislauf gelangt wäre und mit dem Glutathion reagiert hätte, es unter unseren Versuchsbedingungen sicher nicht zu einer drastischen Minderung des Glutathions in den Erythrozyten gekommen wäre.

NADH-Diaphorase wirkt in Erythrozyten als Methämoglobinreduktase (Bethke, Kleinhauer, Gärtner und Schiebe, 1964; Scott und Griffith, 1959; Scott, 1960, und Scott, 1962). Ein Mangel an NADH-Diaphorase – er tritt auf in den ersten Lebensmonaten (Bethke, Kleinhauer, Gärtner und Schiebe, 1964; Kübler und Simon, 1969), oder als angeborener Defekt (Linneweh, 1962; Löhr, 1964; Scott und Griffith, 1959; Scott, 1960) – führt zu einer besonderen Empfindlichkeit gegenüber Methämoglobinbildnern (z. B. Nitrit). Ob Sulfit in der Lage ist, *in vivo* Methämoglobin zu bilden, ist nicht mit Sicherheit bekannt. *In vitro* gelang es Engelhardt (1964) in Hämolysaten, Oxyhämoglobin durch Sulfit in Methämoglobin zu überführen. Auch die Befunde von Rost und Franz (1912), die in Selbstversuchen nach oraler Gabe von hohen Dosen Natriumsulfit Cyanose beobachteten und die Untersuchungen von Wilmes (1966), der nach chronischer Verabreichung sehr hoher Sulfitdosen bei Ratten eine Abnahme des Hämoglobingehaltes in den Erythrozyten fand, sprechen für die Annahme, dass Sulfit – genügend hohe Resorption vorausgesetzt – zur Methämoglobinbildung führen kann. Eine Abnahme des Hämoglobingehaltes bei chronischer Dosierung fanden auch Ezrielev (1968), Luedke, Bratzler und Dunne (1959), Sleisinger und Tusl (1968) sowie Simon (1964). Dagegen beobachteten Mitina (1968) sowie Lee und Danner (1966) einen Anstieg des Hämoglobins nach Sulfit-Applikation. Ezrielev (1968) deutet diese widersprüchlichen Angaben mit der Vermutung, dass nach einem anfänglichen Abfall eine Induktion der Hämoglobinsynthese erfolge.

Wir konnten während der Versuchszeit keine Veränderung des Hämoglobingehalts im Blut der Versuchspersonen feststellen. Der recht erhebliche Rückgang der NADH-Diaphorase-Aktivität um 30–40 % scheint also noch keinerlei Auswirkung auf die Beständigkeit des Hämoglobins ausgeübt zu haben (s. Mitt. 1).

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Summary: A thiamine deficient diet caused thiamine deficiency in human volunteers with biochemical effects but no clinical signs. On giving sulfite by oral application to these volunteers, there was no influence on thiamine status as shown by transketolase-activity and thiamine content in blood and thiamine excretion in urine, suggesting that there was no destruction of thiamine by sulfite in vivo. The enzyme activities of LDH, G-6-PDH and NADH-diaphorase decreased in red blood cells when giving the thiamine deficient diet, but oral application of sulfite did not influence these enzyme activities under the conditions of this experiment.

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LACTIC DEHYDROGENASE ISOZYMES IN THE NERVOUS TISSUE

I. THE REACTION OF ISOZYMES WITH DIPHOSPHOPYRIDINE NUCLEOTIDE ANALOGUES AND THEIR INHIBITION BY SODIUM METABISULFITE

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(Received September 4th, 1961)

SUMMARY

1. Brain L(+)lactic acid dehydrogenases from several species have been submitted to agar-gel electrophoresis. When the pH is 7.6, the beef-brain enzyme, which has been studied most extensively, may be resolved into three main components.

2. Some kinetic properties of three L(+)lactic acid dehydrogenase isozymes from beef brain have been investigated by using several DPN analogues and the determination of other kinetic parameters (K_m for sodium pyruvate, excess substrate inhibition, inhibition by sodium metabisulfite).

3. Sodium metabisulfite has been found to be a powerful competitive inhibitor of brain L(+)lactic acid dehydrogenase. Resolved isozymes are less sensitive to the inhibitor than the native enzyme. pH–activity curves in the presence of sodium metabisulfite show definite differences among the multiple forms of L(+)lactic acid dehydrogenase.

4. Experiments with the pyridine analogues of DPN and sodium metabisulfite, and also the analysis of the enzyme affinity for pyruvate, have shown that the electrophoretic migration on agar gel causes a conformational change of the single L(+)lactic acid dehydrogenases. The nature of this modification has been discussed.

INTRODUCTION

It is the purpose of the present paper to report on the use of DPN analogues as a simple means of discriminating between LDH isozymes of beef brain as they can be separated by agar gel electrophoresis. It will be shown, however, that the separation procedure itself causes a conformational change of LDH molecules.

KAPLAN *et al.*[1] used a catalytic technique (*i.e.* the use of coenzyme analogues) for discriminating between differences in proteins which serve the same function; they applied it to crude or purified LDH's from different sources, but it has not yet been extended to electrophoretically-separated isozymes.

Abbreviations: APDPN, 3-acetylpyridine-DPN; LDH, L(+)lactic acid dehydrogenase; Py3ALDPN, pyridine-3-aldehyde-DPN; TNDPN, thionicotinamide-DPN.

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While this work was in progress, FLEXNER *et al.*[2] published a paper on the lactic dehydrogenase of the developing cerebral cortex, after chromatography on DEAE and CM-cellulose. Kinetic differences between the isolated fractions were shown by comparing the oxidation rates of lactate in the presence of DPN, APDPN, and Py3ALDPN.

Recently PLAGEMANN *et al.*[3] also investigated the problem of discriminating between LDH isozymes of rabbit and human tissues by means of their catalytic activities. As a matter of fact, some time ago the problem of catalytic differences among isozymes had already attracted the interest of investigators, and WIELAND AND PFLEIDERER[4] pointed out the correlation between sulfite inhibition and the electrophoretic behaviour of rat LDH's.

While sodium sulfite has been described[5] as an inhibitor of LDH, inhibition by sodium metabisulfite has never been reported. This paper describes in some detail inhibition by sodium metabisulfite of native LDH and also the resolved isozymes.

The technique of agar gel electrophoresis has been used for the separation of LDH multiple forms, because, when starch gel is used, only a partial recovery of the enzyme[3] is possible. WIEME[6] has summarized the existing studies of agar gel electrophoresis. However, because agar is poorly defined chemically, it can be supposed that one of the pitfalls of agar gel electrophoresis is the possibility that it may be accompanied by modifications of the protein molecules submitted to migration. The choice of agar gel as a supporting medium has, in fact, shown that molecular modifications do take place in LDH isozymes submitted to electrophoretic migration.

EXPERIMENTAL

Chemicals

DPN and DPNH were purchased from Pabst Laboratories. APDPN, Py3ALDPN and TNDPN were a generous gift from Dr. N. O. KAPLAN (Brandeis University, Waltham, Mass. (U.S.A.)). Sodium pyruvate and L(+)lactic acid 40% (*puro*) were obtained from Fluka. Sodium metabisulfite was a product of Merck and Co. All other compounds were reagent grade.

Enzyme preparation

Preliminary experiments have shown that a partial purification of the brain extracts causes only minor modifications of the electrophoretic pattern on agar gel. Most experiments were therefore, carried out with a partially-purified preparation of the brain enzyme. The procedure consisted essentially in steps 1–3 of the purification technique proposed by NEILANDS[7] for the heart-muscle enzyme. Whole beef brains were homogenized in two volumes of cold distilled water and centrifuged at 30 000 × *g* for 30 min. Calcium phosphate gel[8] was then added to the supernatant (15%, v/v). After 30 min stirring at 4°, the enzyme solution was centrifuged again and the phosphate gel was then extracted twice with 0.2 *M* sodium phosphate buffer, pH 7.4. At this point, the total volume was 0.2 of the original volume. On the resulting solution a fractionation with ammonium sulphate was carried out by adding the solid salt up to about 0.6 saturation. The precipitate, collected by centrifugation, was dissolved in a small amount of sodium phosphate buffer (about 0.025 of the original volume), clarified by centrifugation, and dialysed with stirring for 24 h at 4° against

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sodium phosphate buffer, 0.05 M (pH 7.4), containing 10^{-4} M cysteine. DPNH and sodium pyruvate were added to the enzyme before the salt fractionation, in final concentrations of $2 \cdot 10^{-4}$ M and $1 \cdot 10^{-3}$ M, respectively.

This technique, when it was applied to the beef brain, resulted in a 12–14 times purification. Usually the final enzyme solution was frozen in 1-ml aliquots to be thawed just before the electrophoretic migration. If the enzyme is frozen only once, no alteration of the electrophoretic pattern is found. It is, however, to be noted that the isozymes are not equally stable, the less stable being the protein which migrates faster towards the cathode (see next section).

Enzyme activity assay

LDH activity was determined in the following manner. For the lactate oxidation, the reaction mixtures contained 2 or 0.7 μmoles of the oxidized dinucleotide and an amount of the enzyme capable of giving an absorbancy change at 340 mμ of 0.015 to 0.030 in 1 min. The final volume of the reaction mixtures was 3.0 ml, and a glycine–NaOH buffer, 0.1 M (pH 10), was used. The absorbancy change was recorded as a function of time at the absorption maxima reported[9] for each of the coenzyme analogs. All the reactions were initiated by the addition of the enzyme solution. For the pyruvate reduction, experimental conditions were, unless indicated otherwise, those suggested by KORNBERG[18].

The amount of the enzyme varied according to the coenzyme used (see Table I).

Agar gel electrophoresis

Zone electrophoresis with the use of Bacto-agar Difco was done by the method

TABLE I

RATIOS OF REACTION RATES MEASURED WITH NATIVE LDH AND THREE ISOZYMES FROM BEEF BRAIN, AFTER ADDITION OF SEVERAL OXIDIZED DINUCLEOTIDES AND TWO CONCENTRATIONS OF L(+)LACTATE

The data collected in this table have been obtained by assaying the LDH activity with two concentrations of L(+)lactate (0.074 and 0.0074 M, which are referred to as (H) and (L), respectively). Thus, according to KAPLAN *et al.*[1], DPN(L) symbolizes the rate of lactate dehydrogenation, when DPN is the coenzyme and lactate is 0.0074 M. Py3ALDPN(H) symbolizes the rate of reaction with 0.074 M lactate and Py3ALDPN as a coenzyme. All the data have been obtained in the presence of 0.7 μmole of dinucleotide. The rates with APDPN have been determined by using 8 times as much enzyme and the rates with Py3ALDPN by using 4 times as much enzyme.

Enzyme	DPN(L)/DPN(H)	APDPN(L)/APDPN(H)	Py3ALDPN(L)/Py3ALDPN(H)	TNDPN(L)/TNDPN(H)	DPN(L)/APDPN(L)	DPN(H)/APDPN(H)	DPN(L)/APDPN(H)
Native LDH	0.86	0.95	0.393	0.53	27.4	30.6	26.3
Isozyme A	0.54	0.83	0.60	0.33	9.6	14.7	8.0
Isozyme B	0.75	1.40	0.42	0.38	11.45	22.4	16.0
Isozyme C	0.90	1.50	0.8	0.5	26.0	20.0	17.3

Enzyme	DPN(L)/Py3ALDPN(L)	DPN(H)/Py3ALDPN(H)	DPN(L)/Py3ALDPN(H)	DPN(L)/TNDPN(L)	DPN(H)/TNDPN(H)	DPN(L)/TNDPN(H)
Native LDH	20.0	9.15	7.9	2.78	1.71	1.47
Isozyme A	14.5	11.20	9.6	1.25	0.84	0.42
Isozyme B	14.0	7.20	5.6	1.60	0.85	0.62
Isozyme C	9.0	7.60	7.2	1.80	1.00	0.90

of GRABAR AND WILLIAMS[11]. Most separations were made in gels 18 × 12 × 0.4 cm. Barbital buffer, 0.05 *M* (pH 7.6), was used. This pH value was chosen after preliminary experiments on the mobility of LDH isozymes from beef brain at neutral or alkaline pH values. Soon after the removal of the power supply (a voltage drop of 100 V was applied for 3.5 h) the agar gel was divided with a cutting apparatus into fragments 0.5 or 0.2 cm wide. The quantitative extraction of the LDH fractions from agar gel was obtained by using 0.40 *M* sodium phosphate buffer, pH 7.35. With this buffer the recovery ranged from 90 to 95%. It has been found that the extraction of the isozymes can be performed by two techniques, *i.e.* by allowing the enzyme to diffuse from the gel over a period of 12–16 h at 4°, or by thoroughly dissolving the agar in the buffer with a glass rod. The first procedure was followed in most of the experiments.

RESULTS

Electrophoretic pattern

Fig. 1 shows a typical electrophoretic pattern on agar gel of LDH from beef brain. The pattern is highly reproducible as far as the location of the isozymes and their amounts are concerned, although minor changes can be observed.

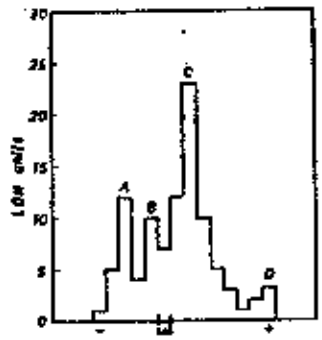

Fig. 1. The distribution of LDH isozymes from beef brain as separated by agar gel electrophoresis. The starting point is shown below the graph. One LDH unit is the amount of the enzyme which gives an absorbancy decrease of 0.001 in 1 min at 340 mμ.

The four peaks have been designated as A, B, C and D. This last fraction has been observed only in the most active purified preparations. At pH 7.6, A and B have a net positive charge, while C and D have a net negative charge. Owing to the low level of peak D, the kinetic analysis has been restricted to A, B and C.

Though another paper of this series will describe electrophoretic patterns of LDH from the brain of different zoological species, it is worth noting that great differences can be found. In our opinion, however, patterns obtained by submitting extracts of the whole brain to agar gel electrophoresis can represent a rough simplification. In other words, single structures of the central nervous system may have a peculiar isozymatic composition, which cannot be revealed by electrophoretic analysis of the whole organ. Preliminary experiments[12] support the hypothesis.

It is also to be questioned whether the LDH fractions isolated by agar gel electrophoresis of the whole brain are contaminated by minor components, which do not appear as peaks in the electrophoretic pattern.

Studies with the pyridine analogues of DPN

As previously noted, the kinetic study of the isozymes has been restricted to fractions A, B and C, owing to the fact that component D is present only in the purified preparations. Table I summarizes a group of experiments performed in order to investigate the behaviour of each isozyme with DPN and three of its pyridine analogues. It can be readily realized that the fractions separated by electrophoresis behave differently with the natural and the artificial coenzymes. If one considers the ratios calculated for DPN, APDPN and TNDPN, it can be noted that there is a gradual modification of the kinetic behaviour, as one goes from A to C. This is not so when Py3ALDPN is used as a coenzyme. However, Fig. 2 shows that, even with respect to Py3ALDPN, the three molecular forms of LDH behave differently and also how fraction B is most sensitive to changes in lactate concentration, while the reverse happens with A.

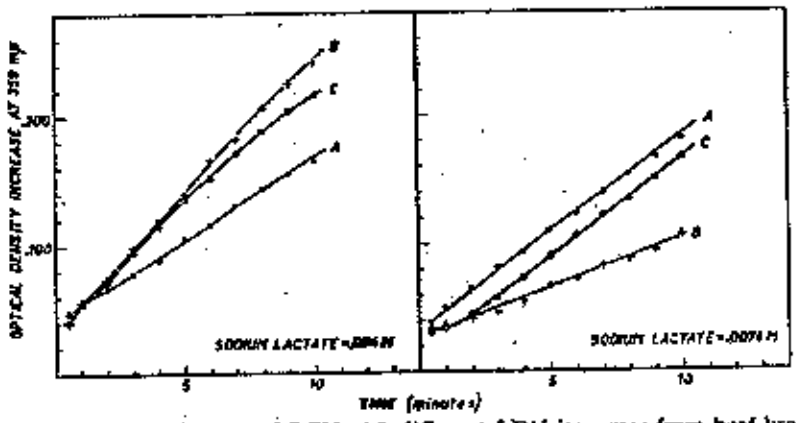

Fig. 2. Rate of reduction of Py3ALDPN with different LDH isozymes from beef brain. The final concentrations of L(+)lactate were as indicated in the figure. For details see EXPERIMENTAL.

The results of Table I are quite intriguing. The velocity ratios calculated on the reaction rates of the three isozymes do not account for the ratios determined with the native enzyme. This finding has stimulated the subsequent investigation in order to establish whether a real modification of LDH occurred during the electrophoretic migration.

Michaelis constants (K_m) for pyruvate of LDH isozymes

Fig. 3 shows curves of per cent activities as a function of pyruvate concentration. It can be seen that the shapes of the curves for B and C are quite similar, while the curve for A shows a different behaviour. Moreover, the curve obtained with the native enzyme does not correspond to the kinetics of an artificial mixture of the isozymes, and, as a finding of major interest, the LDH components isolated by electrophoresis show a decrease of the inhibition by excess substrate. Table II gives the K_m values for pyruvate of the three isozymes and of the native LDH. An artificial mixture of the three electrophoretic fractions showed a K_m for pyruvate corresponding closely to the calculated mean value ($K_m = 3.65 \cdot 10^{-5}$ M).

When the last steps of the purification procedure (ammonium sulphate fractionation, dialysis and agar gel electrophoresis) were not performed in the presence of

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DPNH and pyruvate, the K_m for pyruvate of the native enzyme increased up to $3.3 \cdot 10^{-4}$ M, and the electrophoresis caused a tremendous increase of the K_m values of the single isozymes. In this connection, two findings deserve to be mentioned: (a) the complete disappearance of the inhibition of isozymes by excess substrate (pyruvate up to $3.3 \cdot 10^{-2}$ M was used); and (b) the different extent of the changes in the K_m values of isozymes "not protected" for pyruvate. Fig. 4 illustrates the first point and Table II the second one.

On the basis of the data reported heretofore, there is no doubt that a molecular change occurred in the protein components during agar gel electrophoresis. Most likely, this modification was not represented only by oxidation of –SH groups, because the preincubation with 10^{-4} M cysteine of LDH submitted to electrophoresis did not significantly affect the K_m of the single isozymes*.

Experiments with sodium metabisulfite gave further information on this point.

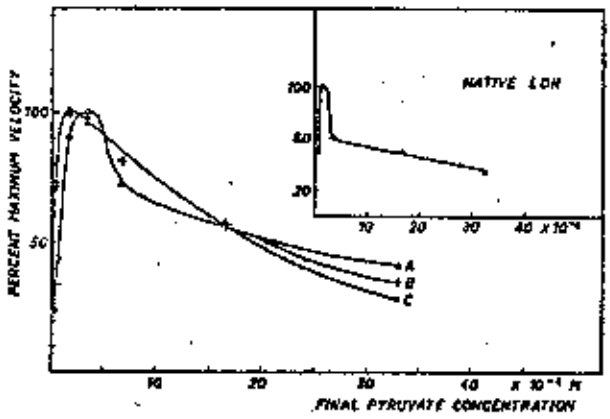

Fig. 3. Effect of pyruvate concentration on the reaction velocity with LDH isozymes and native LDH from beef brain.

TABLE II

MICHAELIS CONSTANTS FOR PYRUVATE

Enzyme	Protected (M)	Not protected* (M)
Native LDH	$1.31 \cdot 10^{-5}$	$3.3 \cdot 10^{-4}$
Isozyme A	$7.15 \cdot 10^{-5}$	$2.0 \cdot 10^{-4}$
Isozyme B	$3.15 \cdot 10^{-5}$	$4.4 \cdot 10^{-4}$
Isozyme C	$1.95 \cdot 10^{-5}$	$7.5 \cdot 10^{-4}$

* See EXPERIMENTAL.

Experiments with sodium metabisulfite

While the inhibition of LDH by sodium sulfite has already been described[4], the effect of sodium metabisulfite has not yet been reported. We have found that this last compound is a powerful competitive inhibitor of the LDH from beef brain. Fig. 5

* Although we lack definite evidence, preliminary findings may suggest that similar, but slighter, modifications occur during starch gel electrophoresis[12].

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shows a typical plot, from which a K_m for pyruvate equal to $1.31 \cdot 10^{-5}$ M and a K_s equal to $1.10 \cdot 10^{-4}$ M have been calculated.

As was previously found with sulfite, a differential inhibition of the isozymes can

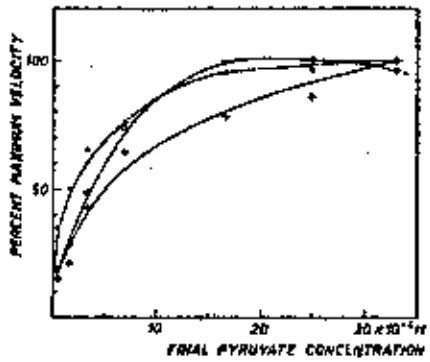

Fig. 4. Effect of pyruvate concentration on the reaction velocity with "not-protected" isozymes. See text.

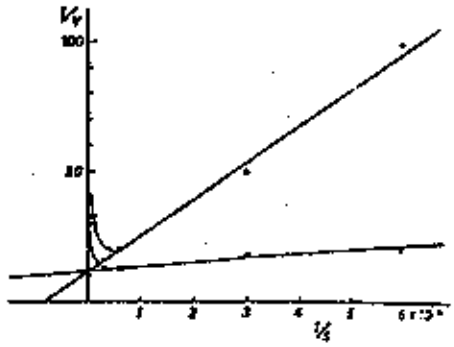

Fig. 5. Effect of sodium metabisulfite ($1.66 \cdot 10^{-4}$ M) on the LINEWEAVER-BURK plot for native LDH with sodium pyruvate.

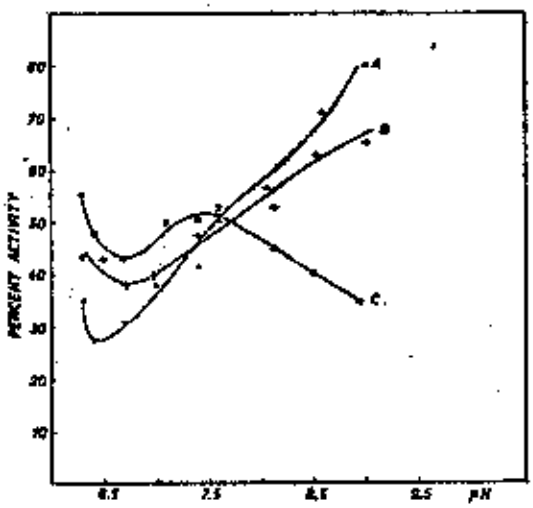

Fig. 6. pH-activity curves in the presence of sodium metabisulfite ($1.66 \cdot 10^{-4}$ M) with three isozymes from beef brain. For details, see EXPERIMENTAL.

TABLE III

LDH INHIBITION BY SODIUM METABISULFITE

Sodium metabisulfite concentration, $1.66 \cdot 10^{-4}$ M.

Enzyme	Per cent inhibition
Native LDH	84
Isozyme A	70
Isozyme B	56.8
Isozyme C	40
Artificial mixture of	
A + B + C, observed	53
calculated	56.2

be observed with sodium metabisulfite (Fig. 6 and Table III). Fig. 6 summarizes a typical experiment in which the LDH activity of each electrophoretic fraction was determined at different pH values. It can be seen that fractions A and B are substantially similar, while a different curve is obtained with peak C.

Table III gives the per cent inhibition of each isozyme, of the native enzyme and of the recomposed mixture. If one compares the per cent inhibition of the artificial mixture (A + B + C) with the theoretical mean values, a good agreement is found. From a kinetic standpoint, the recombination of the isozymes does not give any more the original enzyme.

Details on sodium metabisulfite inhibition will be published elsewhere[11]. It seems, however, worth mentioning that, at neutral pH and with $1.66 \cdot 10^{-4}$ M sodium metabisulfite, no spectrophotometric evidence of reaction between the compound and DPN or DPNH was obtained. On the contrary, if 1 μmole of DPN is added to 3 ml of a concentrated solution of sodium metabisulfite (0.35 M) at pH 7.4, the instantaneous formation of a complex with an absorption maximum at 320 mμ is observed. At variance with the formation of the DPN–bisulfite complex[23], the attack of sodium metabisulfite on DPN is scarcely sensitive to changes in the hydrogen ion concentration, a fact which shows that the $S_2O_5^{2-}$ ion exists as such in aqueous solutions[14]. Finally, it may also be mentioned that sodium metabisulfite strongly inhibits even the lactate dehydrogenation.

DISCUSSION

That the pyridine analogues of DPN are capable of discriminating LDH fractions separated by agar gel electrophoresis is most obvious from data collected in Table I. However, as previously noted, the data show a disagreement between the velocity ratios determined with the native enzyme and the theoretical ratios which can be calculated from data obtained with the three isozymes. It seems, therefore, that a well-defined conclusion can be drawn: a conformational change occurs in the LDH isozymes during the electrophoretic migration.

The most interesting question is, however, the nature of this modification. The relative affinity of LDH isozymes for the DPN analogues is higher than the affinity of the unresolved enzyme for them, and the major increase of this affinity is observed with the acetylpyridine analogue. The obvious conclusion is that the molecular change in the isozymes does facilitate the attack of a compound containing the electron-withdrawing acetyl group more than it facilitates the interaction with the carboxamide or the aldehyde group.

The findings with the DPN analogues parallel the results concerning the K_m values of the isozymes for pyruvate. In this connection, data reported in Table II are worthy of comment. It has been stressed that the lack of protection of LDH causes a tremendous increase of the K_m values of the resolved isozymes and that this increase is not the same for all the three forms of the enzyme. In fact, when LDH is not protected, the component which comes out with the higher K_m value is peak C, while the smaller change is observed with fraction A. PLAGEMANN *et al.*[9] have observed a decrease in the amount of excess pyruvate inhibition of rabbit isozymes, when the purified fractions were stored or treated with trypsin. It is important to note that the structural change which causes a decrease of the excess pyruvate concentration facilitates also the reaction of the isozymes with APDPN and TNDPN (Table I).

The inhibition by sodium metabisulfite deserves a few comments. Of particular interest is the finding that the native LDH is much more sensitive to the inhibitor than are the three electrophoretic fractions. Because sodium metabisulfite is a competitive inhibitor, one would assume that the conformational change occurring during the electrophoresis concerns the active site, or some neighbouring groups. Experiments with the pyridine analogues and experiments on pyruvate affinity support this view.

In relation to Fig. 6, it is tempting to correlate the kinetic behaviour of the isozymes in the presence of sodium metabisulfite with their electrophoretic mobility. Such an attempt seems, however, premature, for it must be remembered that the electrophoretic migration deeply modifies LDH isozymes. The curves in Fig. 6 may at any rate suggest differences in the dissociation constants of some molecular grouping on the isozymes. In the pH range investigated, sulfhydryl, α-amino, ε-amino and imidazol groups can all be taken into account, and it is important to note that imidazoles can react with the pyridine ring[5].

All this is only speculative, because the groups of the LDH proteins involved in the binding to the coenzymes are still unknown. However, the transfer of energy[15] from the aromatic amino acids of the enzyme to the dihydropyridine suggests that these amino acids can be near the binding site.

Finally, it is stressed that zone electrophoresis in a solid medium such as agar gel may alter the configuration of protein molecules. Such an alteration may help us to understand the mechanism of the formation of the enzyme–coenzyme–substrate complex, and also the nature of the active site; but it makes the kinetic analysis of the isozymes meaningless, when the analysis is aimed at determining the natural reactivity of the separated proteins.

ACKNOWLEDGEMENTS

This investigation has been made possible by grants from the National Institute of Neurological Diseases and Blindness (Grant B-2917) and the Consiglio Nazionale delle Ricerche (Rome).

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result from hydrolysis is negligible and would have little effect on the ratio (4), whereas the liberation of pantoate would have a considerable effect.

These observations do not detract from the possible usefulness of pantoyltaurine and other analogues of pantothenic acid against Group A streptococci and presumably other organisms which are incapable of utilizing, or cannot readily utilize, pantolactone or pantoic acid for pantothenic acid synthesis. As already indicated, reduction of analogue concentration (by hydrolysis) would be inconsequential. Furthermore, the inhibition of growth of Group A streptococci, for instance, with pantothenic acid analogues is an established fact.

Summary

Like pantothenic acid, pantolactone, and the pantoate ion, pantoyltaurine and other pantothenic acid analogues of this type antagonize the inhibitory effect of salicylate on *E. coli*.

It is suggested that the active antagonist in a solution of such an analogue is pantolactone or pantoate. These (particularly the former) could conceivably be contaminants of the analogues. However, the liberation of pantoate by hydrolysis, appears to be a more likely explanation for the antisalicylate effect.

The presence of pantolactone or pantoate in a solution of pantoyltaurine and similar analogues of pantothenic acid provides a possible explanation for the ineffectiveness of these drugs in inhibiting the growth of microorganisms which can utilize pantolactone or pantoate for the synthesis of pantothenic acid.

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Interference With the Antibacterial Action of Streptomycin by Reducing Agents[1]

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The authors have repeatedly observed that the antibacterial action of streptomycin is significantly reduced by incubation in an anaerobic jar. When filter-paper discs are saturated with streptomycin and placed on the surface of infusion blood agar plates inoculated with *E. coli*, the resulting zones of inhibition on plates incubated anaerobically are much smaller than those on the comparable aerobic plates. The reduced activity of streptomycin anaerobically is not due to better growth conditions, since *E. coli* produces its maximum growth when incubated aerobically. Similar results may be obtained with staphylococci and other species of bacteria whose growth is favored by aerobic incubation.

These observations seemed to us to be significant, and further studies were carried out, the results of which suggest that they may have some bearing on the antibacterial action of streptomycin.

Method and results. Duplicate sets of infusion agar plates containing 2-fold concentrations of streptomycin were inoculated with various species of bacteria. One set of plates was incubated aerobically, the other in the anaerobic jar by the palladium-hydrogen method as modified by Spaulding and Goode (8). Plates were read for evidence of growth inhibition at the end of 24 and 48 hours incubation at 37° C. Table 1 shows the results of these tests.

TABLE 1

Inhibition of Bacterial Growth by Streptomycin on Infusion Agar

Organism	Incubation	Growth* in presence of streptomycin; units/ml. of agar							
		None	1.0	2.0	4.0	8.0	16.0	32.0	64.0
E. coli-S	aerobic	4	4	1	–	–	–	–	–
	anaerobic	3	3	3	3	3	3	–	–
E. coli-M	aerobic	4	4	2	–	–	–	–	–
	anaerobic	3	3	3	2	–	–	–	–
S. aureus-SM	aerobic	4	4	1	1	1	–	–	–
	anaerobic	4	4	3	3	2	2	–	–
S. aureus #7159	aerobic	4	1	–	–	–	–	–	–
	anaerobic	3	3	3	3	2	1	–	–
S. paratyphi B	aerobic	4	4	4	3	–	–	–	–
	anaerobic	3	3	3	3	2	2	2	1
S. typhimurium	aerobic	4	4	4	3	2	–	–	–
	anaerobic	2	2	3	2	2	2	1	–
Pneumo. Type I	aerobic	4	4	4	4	4	1	–	–
	anaerobic	4	4	4	4	2	1	–	–
Str. Hemolyticus C-203	aerobic	4	4	4	4	4	3	–	–
	anaerobic	4	4	4	4	3	1	–	–

* Readings made at end of 24 hours incubation; 48-hour readings were essentially the same.

Under anaerobic conditions the amount of streptomycin necessary to produce bacteriostasis was from 2 to 16 times greater than the comparable aerobic concentration. With one exception (*S. aureus*-SM), these results were observed only with organisms which produced their maximum growth on aerobic plates.

A similar experiment was carried out with extract agar. Although there was some evidence that the antibacterial action of streptomycin was greater on the aerobic plates, the difference was not large. It appeared that various reducing agents in the infusion medium played a role in inhibiting streptomycin anaerobically. The greater reducing power of infusion agar was evident from the observation that methylene blue in a concentration of 1:100,000 in this medium

[1] Streptomycin used in this study was generously supplied through the courtesy of Dr. D. P. Robertson, Merck and Company, Inc., Rahway, New Jersey.

was reduced during anaerobic incubation, while methylene blue extract agar plates similarly incubated were not reduced. The presence or absence of streptomycin had no apparent effect.

The anti-*E. coli* action of streptomycin was subsequently determined in tryptone and infusion broth containing cysteine or sodium thioglycollate (Table 2).

TABLE 2

THE EFFECT OF CYSTEINE AND THIOGLYCOLLATE ON THE ANTIBACTERIAL ACTION OF STREPTOMYCIN AGAINST *E. coli*

Inhibiting concentration of streptomycin	
Medium	Units/ml.
Tryptone*	1.0
Tryptone plus 0.1 per cent thioglycollate	2.0
Tryptone plus 0.1 per cent cysteine	64.0
Infusion†	16.0
Infusion plus 0.1 per cent thioglycollate	64.0
Infusion plus 0.1 per cent cysteine	> 128.0

* Difco tryptone 1.0 per cent.
† Veal infusion, 1.0 per cent tryptose, 0.03 per cent glucose.

Aside from the added SH compounds, infusion broth itself contains interfering substances, since 16 times as much streptomycin was necessary for bacteriostasis in the infusion control as in the tryptone control. The presence of cysteine increased the concentration required for bacteriostasis 64-fold in tryptone and at least 8-fold in infusion broth; the increase with thioglycollate was only 2- to 4-fold.

TABLE 3

EFFECT OF INORGANIC REDUCING AGENTS ON THE ANTIBACTERIAL ACTION OF STREPTOMYCIN AGAINST *E. coli*

Inhibiting concentration of streptomycin	
Reducing agent*	Units/ml.
Tryptone control	2.0
0.5 per cent sodium formate	32.0
0.05 per cent stannous chloride	32.0
0.05 per cent sodium bisulfite	32.0
0.05 per cent sodium hydrosulfite	16.0
0.5 per cent sodium thiosulfate	32.0

* Final concentration in tryptone broth.

The effect of cysteine on streptomycin could be due either to a reducing action or to some specific chemical reaction, especially with the sulfhydryl groups. The inactivation of penicillin (*2*) and other antibiotic agents (*1*) by cysteine is well known. The action of cysteine on streptomycin appears, however, to be different from that on penicillin, as was demonstrated in the recent report of Denkelwater, Cook, and Tishler (*4*), published while this manuscript was being prepared. These workers state that the inactivation of streptomycin by cysteine is reversible. Similar interference by sulfhydryl compounds with the antibacterial properties of mercurials (*6*) and quinones (*3*) has been reported.

If interference with the activity of streptomycin by sulfhydryl compounds is due to reducing powers, other reducing agents should likewise interfere with its action. Consequently, various reducing agents have been tested. Each of the substances studied definitely antagonized the antibacterial action of streptomycin against *E. coli*, as shown in Table 3. It is evident that the antibacterial action of streptomycin is greatly diminished by a variety of reducing agents other than sulfhydryl compounds.

Discussion. Reducing agents definitely antagonize the antibacterial activity of streptomycin. Further work will be required, however, to determine the true nature of this interference. Whether it is due to a lowering of oxygen tension or to some specific chemical reaction or both acting simultaneously, is still unknown. The SH compounds and the inorganic reducing agents could function in either capacity. The reduction in activity of streptomycin in the presence of glucose, as reported by Waksman and co-workers (*9*), could be attributed to the reducing action of this carbohydrate.

It is entirely possible that this phenomenon is related to the mode of action of streptomycin. This antibiotic displays its greatest activity against bacteria which grow better aerobically than anaerobically, e. g. *E. coli*, *S. aureus*, and *Myco. tuberculosis*. None of the obligate anaerobes is susceptible (*7*). The antibacterial action of streptomycin may be due to its ability to block some enzyme system, oxidative in nature, which is essential only to the growth of susceptible aerobic bacteria, an enzyme system which anaerobes do not possess. If this is true, it is possible that the antagonism by the reducing agent is a specific effect upon this streptomycin-enzyme relationship rather than a direct effect on the streptomycin itself. In fact, addition of the SH compounds and the inorganic reducing agents to a concentrated solution of streptomycin (1,000 units/ml.) does not result in material loss of activity, although Denkelwater and co-workers (*4*) recently reported that this antibiotic in a lower concentration was inactivated by cysteine.

Certain other practical implications are worthy of note. It is conceivable that in certain parts of the body where organic reducing agents are present or where a low oxygen tension exists, larger concentrations of streptomycin may be necessary to inhibit the growth of bacteria. Elias and Darso (*5*) recently reported that typhoid bacilli were isolated from stools in spite of the presence of large concentrations of streptomycin. These investigators suggested the presence in the body of a substance inhibitory to streptomycin.

mycin. It remains to be seen whether these two phenomena are related.

It is evident that the choice of a medium is important in assaying streptomycin and in determining the susceptibility of bacteria to its antibacterial activity. Because of its antagonism for streptomycin, an infusion medium would be an unwise choice for this purpose. Eventually one of these reducing agents may prove helpful in testing the sterility of streptomycin solutions or in the culturing of fluids from patients under treatment. Cysteine may prove to be such an agent.

Summary. The antibacterial activity of streptomycin in infusion agar plate cultures of *E. coli* and other bacteria is diminished by anaerobic incubation. The bacteriostatic activity of this antibiotic for *E. coli* is reduced in the presence of cysteine, sodium thioglycollate, stannous chloride, sodium bisulfite, sodium hydrosulfite, sodium formate, and sodium thiosulfate. Cysteine was the most active of the agents tested. Further investigation is necessary to determine the nature of this interference. It is possible that this phenomenon is related to the mode of action of streptomycin.

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Water-level Regulator for a Series of High-temperature Water Baths[1]

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It was necessary in this laboratory to conduct a series of tests at 70° C., the nature of which required the use of water as a bath liquid. The tests were carried out in two water baths, and, as efficient covers for the baths were not feasible, the diagrammed system (Fig. 1), set up in less than an hour, served to maintain the levels in both baths to within ¼ in.

In operation, the reservoir bottle, located on a shelf above the water baths, is filled from a tap, J, by closing screw-clamps, I and I′, and opening clamp I″. After the reservoir bottle is filled, clamp I″ is closed and I and I′ are opened. Line D extends below the surface of bath B and serves as a delivery tube, while line E is the regulator and cuts off the supply of air to the reservoir, F, when it is reached by the water level in the bath. Siphon C keeps the level in bath A at the same level as that in B.

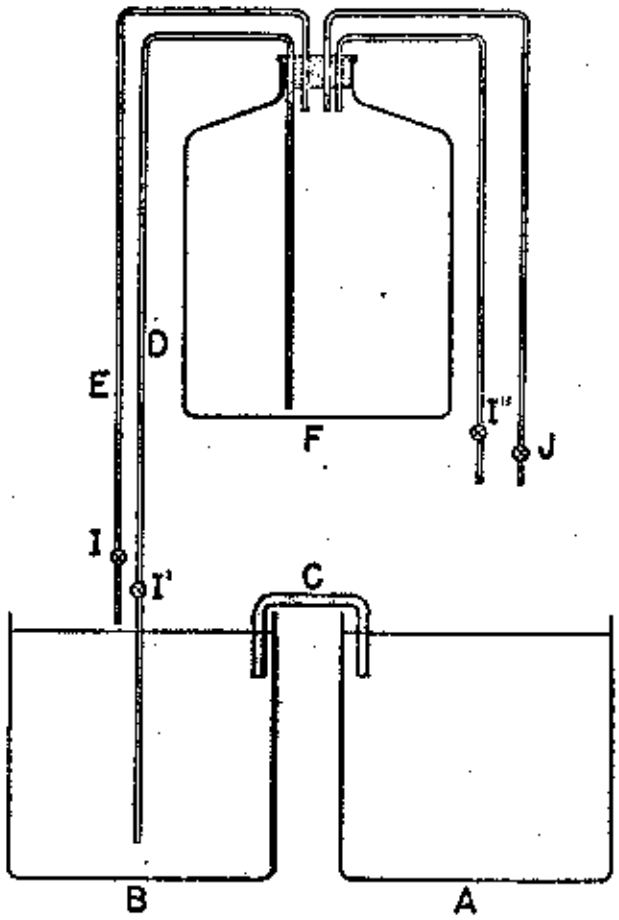

Fig. 1

The baths are of 6-gallon capacity, and the reservoir bottle holds 5 gallons. If the reservoir is filled in the late afternoon, this capacity is sufficient to make up for evaporation overnight. The tubes leading out of the reservoir are of glass, and the cork holding them is sealed in with DeKhotinsky cement. The siphon is of 15-mm. glass tubing to preclude stoppage by air bubbles.

With a sufficiently large reservoir, the system can be used to control a large number of baths. If the cost of the water is neglected, as it can be in most cases, this arrangement has a definite advantage over mineral oil baths in that cleaning of any glassware placed in the baths is much less troublesome.

[1] Contribution from the Industrial Fellowship on Optical Cements, Mellon Institute. Published by permission of the donor, Ordnance Department, U. S. Army.

Eur. J. Toxicol 4 (1971)

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Etude sur la fixation et la transformation du SO_2 dans le sang in vitro

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Résumé

L'étude des modes de fixation et l'évolution du SO2 in vitro du sang humain prélevé soit sur ACD, soit sur héparine apporte les résultats suivants :

— le sang total apparaît capable de transformer des quantités importantes de SO2, surtout au niveau des éléments figurés.

— il existe une inhibition partielle, de ce processus de transformation du SO2, observée surtout au niveau des éléments figurés lorsque le sang est saturé d'oxyde de carbone.

— l'hypothèse d'une action enzymatique ne peut être retenue dans les conditions de l'expérience alors que l'action catalytique oxydante par les ions cuivre a été confirmée.

— au niveau du plasma, le SO2 est presque totalement fixé sous forme de sulfite alors que la fraction restante transformée ne paraît pas nécessiter l'intervention des protéines plasmatiques.

Des auteurs [1] faisant inhaler du $S^{35}O_2$ à des animaux ont retrouvé l'élément marqué dans le sang après un délai relativement court de cinq minutes. Nous avons pensé qu'il serait intéressant d'étudier les modes de fixation et l'évolution du SO_2 in vitro du sang humain prélevé soit sur A. C. D., soit sur héparine. Au cours de cette expérimentation préliminaire, nous avons tenté d'évaluer, d'une part, le SO_2 dissous dans le sang par déplacement de ce dernier sous l'influence du vide et d'un balayage par un courant de N_2 et, d'autre part, le SO_2 fixé sous forme de SO_3^{2-} ou SO_3H^- par action de l'acide phosphorique. La différence entre le SO_2 total introduit et les quantités précédemment déterminées représente la fraction transformée par le sang. Voici la description de la technique utilisée.

Technique

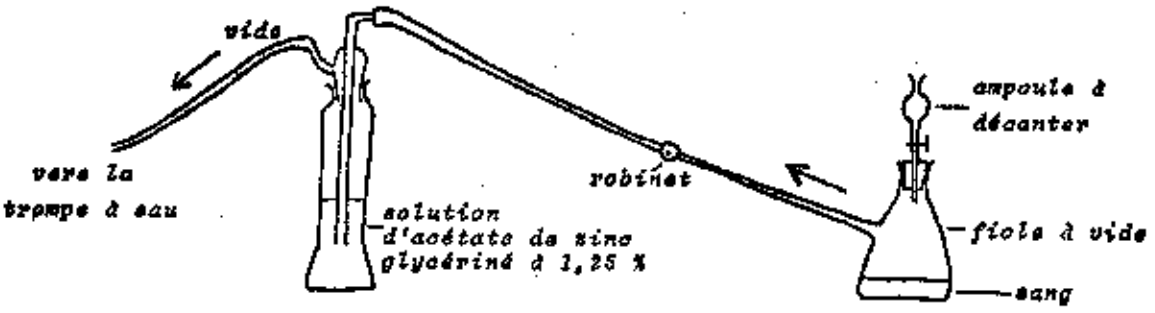
25 ml de sang sont introduits dans un flacon obturé par un bouchon de caoutchouc dans lequel passent deux tubes en verre permettant de brasser par un courant de N_2 l'atmosphère au-dessus du sang. La quantité de SO_2 désirée (de 1 à 10 ml), c'est-à-dire de 2,65 à 26,50 mg est introduite dans le flacon à l'aide d'une seringue après avoir obturé par une pince de Mohr les tubes en polyvinyle surmontant les deux tubes en verre. On agite ensuite le sang ainsi placé dans une atmosphère de SO_2 durant trente minutes, puis on fait passer un courant d'azote qui, à la sortie du flacon, traverse un barboteur contenant de l'acétate de zinc : l'éventuel SO_2 non dissous, fixé ou transformé sera ainsi capté. Le sang additionné de quelques gouttes d'alcool octylique est ensuite introduit dans une fiole à vide surmontée d'une ampoule à décanter permettant d'introduire l'acide phosphorique (50 ml). On relie à l'aide d'un tube en polyvinyle la fiole à vide à un barboteur à acétate de zinc lui-même branché sur une trompe à eau. On soumet ainsi le sang à l'action du vide et l'éventuel SO_2 déplacé pourra être capté par l'acétate de zinc. Le même dispositif (voir schéma) permet de soumettre le sang à l'action combinée de PO_4H_3 et du vide. On peut même adjoindre à ces deux facteurs (PO_4H_3 + vide), en utilisant le même appareillage, l'action de la chaleur en plongeant la fiole à vide dans un bain-marie.

L'évaluation du dioxyde de soufre s'effectue sur une partie aliquote de la liqueur d'acétate de zinc selon la méthode pyridine-nitroprussiate [2]. La quantité de SO_2 introduite dans l'atmosphère du flacon à réaction est évaluée par cette même technique au cours d'étalonnages préliminaires.

Résultats

Dans les conditions expérimentales décrites, nous avons, à la suite de nombreux essais, constaté que 25 ml de sang prélevés sur A. C. D. sont

Manuscrit reçu le 5 avril 1971.



susceptibles de transformer 3 ml de SO_2, soit 7,95 mg. En effet, pour des doses inférieures, il n'a jamais été possible de mettre en évidence du dioxyde de soufre soit par action du vide seul, soit par action conjuguée du vide et de l'acide phosphorique. Pour la dose de 7,95 mg de SO_2, l'action d'un acide fort et du vide permet de libérer 0,030 mg de SO_2.

Afin d'étudier la répartition de SO_2 entre le plasma et les éléments figurés, nous avons procédé à deux séries d'essais. La première série consiste à injecter 7,95 mg de SO_2 dans 25 ml de sang. Après agitation de trente minutes, on centrifuge et on dose le SO_2 récupéré par l'action du vide seul ou du vide avec, en plus, l'action d'acide phosphorique, d'une part, sur le plasma, et, de l'autre, sur les éléments figurés. Dans la deuxième série, on centrifuge 25 ml de sang et, après séparation du plasma et des éléments figurés, on injecte, d'une part, 2,65 mg de SO_2 dans le plasma, et, de l'autre, 5,30 mg de SO_2 dans les éléments figurés. On procède ensuite au dosage du SO_2 recueilli par les mêmes procédés que ceux précédemment décrits. Dans ces conditions, nous avons mis en évidence que :

1° La proportion de dioxyde de soufre transformée par les éléments figurés du sang est sensiblement le double de celle transformée par le plasma.

2° Pour un même échantillon de sang, nous avons constaté que le pourcentage de SO_2 transformé n'est pas identique si l'on compare les résultats obtenus sur le sang total à ceux obtenus séparément par le plasma d'une part et les éléments figurés d'autre part. Dans ce dernier cas, le pourcentage est diminué.

Il nous a paru intéressant, en fonction des constatations précédentes, de fixer chimiquement l'hémoglobine sous la forme très stable de carboxyhémoglobine. Nous avons ensuite étudié la fixation du SO_2 sur ce sang oxycarboné à 100 %, selon le protocole déjà décrit. Nous avons pu mettre en évidence que le sang total oxycarboné transformait une quantité de SO_2 nettement inférieure à celle observée pour le sang normal. Cette différence est due au fait que les éléments figurés du sang oxycarboné possèdent à un degré bien moindre la propriété de transformer le SO_2, alors qu'au niveau du plasma, aucune différence n'est enregistrée.

Ces résultats nous ont incités à effectuer le même protocole expérimental avec des échantillons de sang oxygéné à saturation, 75 % ; 50 % ; 25 % ; nous n'avons pu mettre en évidence aucune variation par rapport au sang normal, même en injectant des quantités plus importantes de SO_2 pour un volume identique de sang.

L'ensemble de ces résultats ont été confirmés en associant une élévation de température à l'action de PO_4H_3 et du vide. Nous pensons pouvoir en déduire que le SO_2 récupéré est bien sous forme de sulfite et qu'une fraction très importante a été transformée en dérivés soufrés non identifiables par la technique utilisée.

Il était normal de penser [3, 4] que le soufre du dioxyde de soufre pourrait se fixer au niveau des protéines plasmatiques ou du stroma des hématies. Nous avons donc dénaturé des protéines soit par la chaleur, soit par l'urée : nous n'avons constaté aucune variation dans nos résultats. On peut donc dire que la perte des structures secondaires, tertiaires et quaternaires des protéines n'a aucune incidence sur la fixation du SO_2.

Il nous paraît intéressant de mentionner que l'addition au sang d'inhibiteurs enzymatiques tels que bichlorure de mercure, versénate, n'a apporté aucune variation à nos résultats. Par contre, nous avons vérifié l'action catalytique oxydante des ions cuivre.

S'adressant ensuite à une technique utilisant le même principe, mais différente dans l'appareillage (note à paraître), nous avons étudié l'action du plasma totalement déprotéinisé par l'action de l'alcool sur le dioxyde de soufre. Les résultats obtenus semblent nous autoriser à confirmer que les protéines plasmatiques ne jouent aucun rôle important. De plus, sachant que les sulfites [5] réagissent avec les ponts di-sulfures des protéines selon la réaction réversible :

$$R - S - S - R + SO_3^{=} \rightleftharpoons R - S^{-} + S - SO_3^{-}$$

Nous avons étudié la réaction du SO_2 en présence de CU^{2+}, de Cu^{+} et Hg^{2+}, de Hg^{2+} et urée, de Hg^{2+} et guanidine, d'une part, sur un « plasma normal » et, de l'autre, sur un plasma « déprotéinisé ». Les différences d'action sur le SO_2 observées entre « un plasma normal » et ce même plasma additionné, soit de CU^{2+}, de CU^{+} et Hg^{2+}, de Hg^{2+} et urée, de Hg^{2+} et guanidine sont insignifiantes. Une expérimentation similaire sur le même plasma « déprotéinisé » donne des résultats identiques. Ceci semble donc bien confirmer le rôle passif des protéines dans la transformation de SO_2 par le plasma.

Conclusion

En fonction des résultats que nous avons obtenus, il nous paraît permis de dire :

1° Que le sang total est capable de transformer des quantités importantes de SO_2 et principalement au niveau des éléments figurés.

2° Qu'il existe une inhibition partielle observée surtout au niveau des éléments figurés de ce processus de transformation du SO_2 lorsque le sang est saturé d'oxyde de carbone.

3° Que l'hypothèse d'une action enzymatique ne peut être retenue dans les conditions où nous nous sommes placés, alors que l'action catalytique oxydante par les ions cuivre a été confirmée.

4° Qu'au niveau du plasma, le SO_2 est presque en totalité fixé sous forme de sulfite, alors que la fraction restante transformée ne paraît pas nécessiter l'intervention des protéines plasmatiques.

Summary

Study of the methods of fixation and the evolution of SO2 in human blood in vitro, taken from Dextrose Citric Acid or heparin emphazises the following results :

Whole blood and particularly blood cells appear able of transforming important quantities of SO2.

A partial inhibition in the process of transformation of SO2 exists when the blood is saturated by carbon monoxide and is particularly noticeable in the blood cells

The hypothesis of enzymatic action cannot be retained in experimental conditions as the catalytic action, oxidizing by copper ions, has been confirmed.

At the plasma level, SO2 is almost entirely fixed in the form of sulfite, and the remaining transformed fraction does not appear to need the intervention of plasmatic proteins.

Zusammenfassung

Die Bindung und die Entwicklung von SO2 in vitro im menschlichen Blut wird unter Verwendung von ACD und Heparin untersucht. Die Resultate sind folgende : Vollblut erscheint fähig, grössere Mengen von SO2 zu transformieren immer dann, wenn das Blut mit Kohlenoxyd gesättigt ist, besteht eine partielle Hemmung des SO2-Transformation. Die Hypothese einer Fermentwirkung konnte unter den Versuchsbedingungen nicht aufrecht erhalten werden, wohl aber die einer katalytisch oxydierenden Wirkung der Kupferionen. Im Plasma wird das SO2 fast vollständig als Sulfit gebunden, während die Restfraktion kein Eingreifen von Plasmaproteinen zu benötigen scheint.

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Amer. Soc. Brew. Chem., Proc.
(1955)

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Determination of Volatile Sulfur Compounds
V
Sulfur Dioxide

[illegible]

Introduction

Of the [illegible] has been [illegible] papers on the [illegible] the [illegible] of foods and beverages.

The literature on sulfur [illegible] Monier-Williams [illegible] man in a [illegible] up to date.

The majority of methods [illegible] either of two categories [illegible] solution. The direct [illegible] in the past, but they have [illegible] that the sample [illegible] and under [illegible] is oxidized by [illegible] Alternately, the sulfate [illegible] cipitated and weighed [illegible] time-consuming, but Monier-Williams and [illegible] of Official Agricultural Chemists [illegible]

The direct methods and the [illegible] Monier-Williams [illegible] theoretical and practical shortcomings. [illegible] sulfur dioxide [illegible] bromine, etc., or volatile [illegible] peroxide. Practically [illegible] very careful manipulation. For example, in the Monier-Williams [illegible] some hydrochloric acid vapor may be carried over into the [illegible] laboratories, the peroxide solution used in this procedure is always tested for chloride after completion of the [illegible] by adding a few drops of silver [illegible] Furthermore, these methods are [illegible] sulfur dioxide [illegible]

As long as sulfur dioxide [illegible] levels, [illegible] sensitivity is [illegible] sulfur dioxide [illegible] the [illegible] sulfur dioxide [illegible] sulfur dioxide [illegible]

For these [illegible] from methods for [illegible] of SO_2 in [illegible]

not necessary because of the thousandfold lower ... beer. In over a thousand samples of beer analyzed in our laboratory ... hydrogen sulfide, only one sample contained over one hundred parts of su... per billion, and this unusual level would give rise to an error of only ... tenths of a part per million of sulfur dioxide.

The method is convenient because it uses the same solutions and the calibration curve as do the determinations of hydrogen sulfide and merca... Thus three volatile sulfur compounds of beer may be determined by the colorimetric procedure, and the complete volatile sulfur analysis may, b... justing the volumes of beer taken for hydrogen sulfide and mercaptan an... be done on a single twelve-ounce sample of beer.

Methods

Reagents

Stannous Chloride: Dissolve 100 g. of stannous chloride dihydrate ... ml. of concentrated hydrochloric acid and dilute to 1 lite... distilled water. The stannous chloride must be of such ... that 5 g. of it will dissolve clearly in 100 ml. of conce... hydrochloric acid and must be free of metals whose sulfi... less soluble than SnS.

Buffer: Dissolve 69 g. of sodium dihydrogen phosphate monohy... 750 ml. of distilled water. Adjust to pH 7 with 20% ... hydroxide and dilute to 1 liter. (This is the same buffer ... the mercaptan determination[2]).

Zinc Acetate: Dissolve 20 g. of zinc acetate, c.p., in 1 liter of distill... Add five drops of glacial acetic acid to prevent hydrolysi...

p-Amino-Dimethylaniline Sulfate: Dissolve 150 mg. of p-amino-... aniline sulfate (Eastman) in 150 ml. of diluted sulf... (2 + 1), previously cooled to room temperature. Store ... frigeration.

Ferric Chloride: Dissolve 2.7 g. ferric chloride hexahydrate in 50 ... centrated hydrochloric acid and dilute to 100 ml. wit... water.

Note: The last three reagents are the same as previously descrit... water used in the preparation of solutions must be free of copper ... heavy metals. Water containing 0.1 p.p.m. of Cu, if used in the buff... may lead to an error of 1 p.p.m. of SO_2.

Apparatus

Three 250 ml. glass-jointed Drechsel-type gas washing bottles, ... ing, graduated cylinder and gas bubbler[3,4]. See Figure 1.

Procedure

The three gas washing bottles and the graduate cylinder are ... train, as illustrated. The first and second bottles contain 100 ml. ... stannous chloride solution, the third contains 5 ml. of buffer a... distilled water; and the graduate cylinder holds 40 ml. of zinc ace...

[3] The accessories are available from Labtician Products Company, Hollis 2, N. Y.

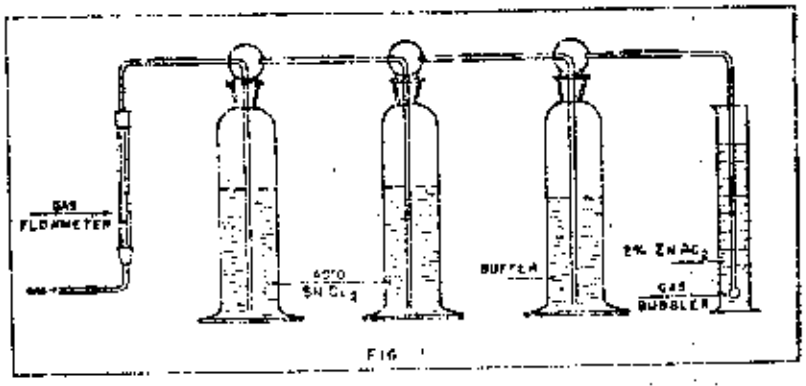


FIGURE 1. APPARATUS FOR SULFUR DIOXIDE DETERMINATION.

Add the sample of beer (or other material) containing between 5 and 100 micrograms of sulfur dioxide (5 ml. of beer, if it has between 1 and 20 p.p.m. SO_2) to the first bottle.

Pass a stream of nitrogen at about 600 ml./min. through the apparatus for one hour.

Cool the graduated cylinder containing the zinc acetate to 10° C and add 5 ml. of p-amino-dimethylaniline and 1 ml. of ferric chloride. Make up to 50 ml. with zinc acetate solution.

Mix well and let stand for one hour at room temperature.

Read in a spectrophotometer or colorimeter[7].

If greater sensitivity seems desirable, it may be obtained by reducing the volume of zinc acetate[8].

A blank determination, omitting the beer, is also run. The blanks are usually very low, and depend on the purity of the stannous chloride and hydrochloric acid used.

Typical Calculation

$$\frac{20 \text{ mcgm. } H_2S \times \frac{64}{34} \times \frac{1000}{1000}}{5 \text{ ml. sample}} = 7.5 \text{ mg.} SO_2/\text{liter.}$$

As many as five samples of beer may be run without changing the stannous chloride solution in the first bottle. The solution in the second bottle may be used for a week. The buffer is refreshed for each test by adding 5 ml. of fresh concentrate to the solution in the third gas washing bottle. This is necessary to absorb hydrochloric acid vapor which would otherwise render the zinc acetate ineffective.

Recovery of Sulfur Dioxide

Because of the rapid oxidation of sulfur dioxide in the dilute solutions needed for verifying this procedure, and because of the combined state in which at least part of the sulfur dioxide in beer exists, recoveries of sulfur dioxide were done on solutions of known amounts of acetone-sodium bisulfite, acetaldehyde-sodium bisulfite and di-n-propyl sulfite (all from Eastman) in water. The compounds were found to give stable dilute solutions, whose sulfur dioxide content could be checked by titration with iodine[9]. Recoveries were thus determined on "bound" sulfur dioxide, in three forms, as an aldehyde complex, as a ketone complex and as an ester. The data are shown in Table 1.

Sulfur Dioxide in Brewing Materials

The levels of sulfur dioxide in some brewing materials were determined (Table IV). The amounts are such as to contribute about 2-5 parts per million to wort. Much of this is lost during mashing and boiling, as will be seen later. The higher sulfur dioxide content of refined grits, as opposed to coarse grits, probably reflects the use of sulfur dioxide in refining.

TABLE IV
Analysis of Brewing Materials

	SO_2, mg./kg.
Malt	42; 37
	40; 42
	28; 28
	34
	47
	25
	36
Corn Grits, Coarse	[illegible]
	[illegible]
	41
Corn Grits, Refined	47; 48
	35
	41
Hops	[illegible]
	8.5

Sulfur Dioxide Levels During Processing

Samples at various stages of fermentation and storage were taken from a brewery and analyzed for sulfur dioxide. The results are shown in Table V.

The rather low values obtained on the samples of wort indicate partial losses of sulfur dioxide derived from the malt, adjuncts and hops. The highly significant increase in sulfur dioxide content during fermentation proves, for the first time, that brewers' yeast fermentation produces this compound. It also indicates that reducing conditions prevail during fermentation. The somewhat erratic but generally low levels during storage indicate possibilities of oxidation, complexing or utilization of the sulfur dioxide.

TABLE V
SO_2 Levels During Fermentation, P.P.M.

Days	Brew 1	2	3	4
0	1.0; 1.0			
1	2.0	1.1		
2	6.0	6.0		
3				11
4			11	
5				
6				
7	9.9	9.9		23
8			20	
9				

SO_2 Levels During Storage, P.P.M.

Days	Brew 1	2	3	4	5
10					
11				17	
12			19		1.8
13					5.3
16					3.6
21	6.6	1.2			
22					3.0
23				13	
27			7.8		
29					1.1

tions were made without changing reagents or removing beer residues from the first reductors. The data are typical of the replication which is obtainable and represent only one of several similar sets.

The precision warrants reporting data to two significant figures, when results are stated as parts per million (or as micrograms per milliliter).

TABLE VI

Precision of the Method on a Single Beer Sample

	Apparatus 1	Apparatus 2	Apparatus 3	Aver.
Run 1	10.3 ppm	9.9 ppm	11.0 ppm	10.1
2	9.9	9.9	9.5	9.8
3	10.2	9.8	10.2	10.1
4	10.0	10.1	10.1	10.1
Average	10.10	9.92	10.20	10.07

Minimum	9.5 ppm
Maximum	11.0 ppm
Average	10.07 ppm
Stand. Dev.	0.46 ppm

Sulfur Dioxide Levels in Commercial Beers

Analyses of twenty beers from large and small breweries, representing over 40% of domestic production, were conducted by the present procedure and by the volumetric Monier-Williams method[1], modified by having the condenser in a vertical position. (See Table VII).

TABLE VII

SO_2 Determinations on Twenty Beers

Present Method	Monier-Williams	Present Method	Monier-Williams
1.7 ppm	5	6.0 ppm	9
1.9	5	6.0	10
2.6	4	6.5	11*
3.4	8	6.8	13*
3.6	7	6.9	12*
3.7	7	7.4	15
3.8	10*	8.1	14
4.0	8	10.5	14*
5.0	6	11.5	17
5.0	9*	12.5	13*

* Not on same bottle.

The results given by the Monier-Williams method are invariably higher. This is clearly in accord with expectations. Several reasons for this discrepancy are immediately apparent. Monier-Williams[1] himself reported interference from other sulfur compounds. The volumetric method, almost universally employed in prac-

erence to the gravimetric procedure, actually determines substances oxidizable to acids, and is not specific for sulfur dioxide.

In view of these differences and the possible interferences in the Monier-Williams procedure, a review of the chemistry of sulfur dioxide seems warranted.

Discussion

Sulfur dioxide is the most common oxide of sulfur. It results from the burning of sulfur and most sulfur compounds. Depending on conditions, it is either an oxidizing or reducing agent. In beer, it may act as a reducing agent (or antioxidant), since it is known to protect ascorbic acid in fruit juices[18]. Conversely, it may be an oxidizing agent, as in its reaction with stannous chloride.

$$3SnCl_2 + SO_2 + 6HCl \longrightarrow H_2S + 2H_2O + 3SnCl_4$$

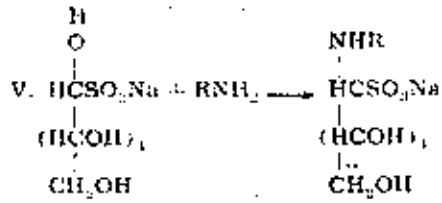
In water solutions, sulfur dioxide exists as sulfurous acid, a weak, dibasic acid. The reactivity of sulfurous acid stems from the tendency of sulfur to become hexacovalent. Bisulfite has long been known to react with aldehydes and ketones (I). It also reacts, almost as quickly, with unsaturated organic compounds (II). It combines with amines (III), and the amine bisulfites may further react with aldehydes and ketones (IV). Glucose sodium bisulfite also condenses with amines (V)[1].

I. $RCHO + NaHSO_3 \longrightarrow RCHOHSO_3Na$

II. $RCH = CHR' + NaHSO_3 \longrightarrow RCH_2CHSO_3NaR'$

III. $RNH_2 + H_2SO_3 \longrightarrow RNH_3SO_3H$

IV. $RCHO + R'NH_2SO_3H \longrightarrow RCHOHSO_3H_2NR'$

V.


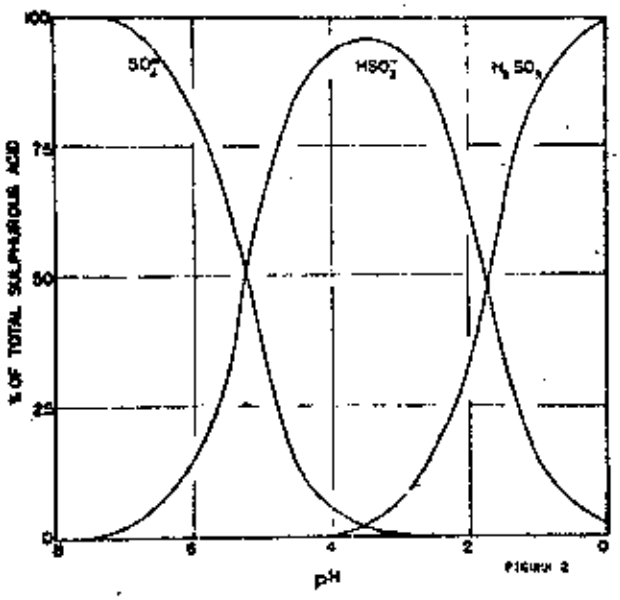


FIGURE 2. EFFECT OF pH ON H_2SO_3, HSO_3^-, $SO_3^=$ EQUILIBRIA

... the types of reaction ... above is possible ... properties of some of the products are worth examining.

The question of "free" and "bound" sulfur dioxide must first be considered. Countless attempts have been made to distinguish between these. The distinction between free and bound sulfur dioxide is analogous to the difference between pH, or free hydrogen ions, and total acidity, or total hydrogen ions. And similarly, any method of measurement must not disturb the equilibrium between sulfur dioxide adducts and the free compound. Indicators, or an electrode sensitive to free sulfur dioxide would be the ideal, but none has been found.

Monier-Williams' citing the work of Kerp gives the dissociation of aldehyde and ketone bisulfites (Table VIII). These dissociations are markedly affected by pH. The addition compound with acetaldehyde is of such stability that less than 1 part per 1000 ℔ oxidized when exposed to air for 14 hours, despite the known instability of equally dilute solutions of uncomplexed bisulfite.

TABLE VIII

Compound	Percentage Dissociated in N/30 Solution
Formaldehyde Sodium Bisulphite	0.155
Acetaldehyde Sodium Bisulphite	0.71
Benzaldehyde Sodium Bisulphite	1.90
Acetone Sodium Bisulphite	23.67
Glucose Sodium Bisulphite	81.89

To further illustrate the mobility of the equilibria involved, several experiments were performed.

First, beer was oxidized with hydrogen peroxide at several concentrations. The excess peroxide was destroyed (with catalase) and the residual SO_2 determined. Comparison with SO_2 content of the beer before oxidation gave the amount of SO_2 "free" enough to be oxidized by H_2O_2. The results in Table IX indicate that some 20-40% of the SO_2 is the free, and show that the equilibrium is somewhat labile to the oxidant.

Secondly, several beers were distilled, and portions of the distillate, residue and original beer were analyzed for SO_2. For convenience the distillation was conducted in accordance with the American Society of Brewing Chemists' procedure for alcohol. The results are shown in Table X. It has been reported that 90% of the SO_2 added to wine as aldehyde bisulfite is liberated on heating. The data obtained here indicate that about the same may hold true for beer. The equilibrium is displaced by heat and by the removal of the products as vapors.

As mentioned previously, other adducts of SO_2 are possible in beer. Of these

TABLE IX
"Free" and "Bound" SO_2 By Peroxide

Beer A	H_2O_2	"Free" SO_2, ppm	"Bound" SO_2, ppm	% "Free"
350 ml.	1 ml.	0.6	3.4	15
	3 ml.	1.4	2.9	27
	10 ml.	1.2	2.8	30
Beer B				
350 ml.	1 ml.	1.7	5.2	25
	3 ml.	2.2	4.7	32
	10 ml.	2.[illegible]	4.9	42

TABLE X
"Free" and "Bound" SO_2 By Distillation

Beer	SO_2 In Orig. Beer ppm.	SO_2 In Dist. ppm.	% of Total	SO_2 In Res. ppm.	% of Total
A	8.0	6.2	78	1.7	21
B	8.5	6.6	78	0.9	11
C	7.9	6.2	78	1.1	14

the combinations with unsaturates and tannins are very likely. Monier-Williams[16] ascribes great stability to such compounds, but with slow decomposition occurring in hot acid solutions.

The concept of "bound" SO_2 is thus seen to be rather complex — some SO_2 is bound with respect to oxidation by H_2O_2; some remains bound after distillation, some may not respond to reduction by $SnCl_2$, and some is bound by water.

At the pH of beer, Figure 2[17] shows that practically no H_2SO_3 is possible. Calculations from the equilibrium constants show that a sulfurous acid solution at pH 4 containing 20 p.p.m. of SO_2 should have only about 2 p.p.m. of H_2SO_3, which may then evolve some gaseous sulfur dioxide.

One view emerges clearly from the evidence relative to the binding or freedom of SO_2. It is highly probable that free sulfur dioxide can exist in beer only as a minor part of the total sulfite present.

The comparison of results on beer by the present procedure with those of the A.O.A.C. method demonstrates that the hot procedure (Monier-Williams) gives higher values. When the first reductor in the present method is heated to 80°-90° C., after a regular analysis has been completed, a significant increment of SO_2 is obtained.

Similarly, Nichols and Reed[18] reported that no definite end point was reached during distillation of SO_2 from dried fruits in the presence of hydrochloric acid, even after five hours of distillation (Figure 3). Earlier in this paper, mention was made of the slow decomposition of lignin sulfonates, sulfones, etc., in boiling

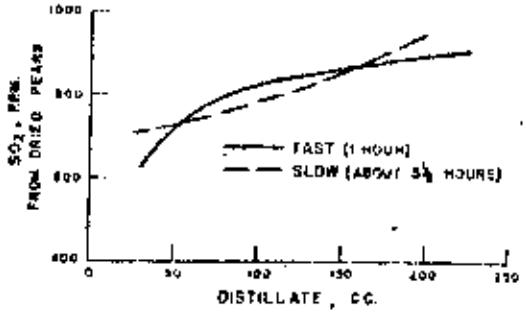


FIGURE 3. RELEASE OF SO_2 DURING DISTILLATION.

In continuing ... jected to analysis for sulfur dioxide ... gave a negative result, in keeping with the fact that all sulfur ... able substances had just been destroyed. However, the Monier-Williams pro cedure on portions of the same iodine-treated beers gave indications of over 5 p.p.m. of sulfur dioxide, when none should have been present.

Advancing knowledge during the past 25 years seems to cast doubt on the meaning of sulfur dioxide determinations by the Monier-Williams procedure Any method which may create additional quantities of the substance being measured should be suspect. A review of the collaborative work on this metho which preceded its adoption by the A.O.A.C.[1] confirms the need for suspicion The method was the best available at the time, and it has been useful throug the years. However, it was not based on a wholly adequate foundation (Josly and Braverman[2] state that any method which does not apply knowledge of th aldehyde and sugar sulfonic acids and does not use recovery of added pu sulfonates may not be considered accurate; recovery of added sulfurous acid bisulfite can not be accepted as sufficient proof of accuracy). Its lack of spec city and susceptibility to interference are well known. It suffers from lack precision on small samples and on low levels of SO_2, even when large samp are available.

The present procedure is free of these handicaps.

Influence of SO_2 on Odor and Taste of Beer

Sulfur dioxide, present in practically all beer and ale as a natural t constituent, has an odor which is familiar to most people. The sharp pung of a freshly lighted match arises from the combustion of sulfur to SO_2; chemists are certainly well acquainted with the characteristic odor arising a burning sulfur candle or an acid sulfite solution.

So it is not surprising that critical beer tasters for many years, knowing sulfur dioxide was present in beer and ale, should sometimes find products seemed to have a "sulfite" odor or taste. The authors have personal recoll of such observations.

With the striking example of the "chemical odor filters" applied to old testing for H_2S and mercaptans, it was very natural to seek to extend thi ciple to sulfur dioxide. The first step was to ascertain the levels of SO_2 ad to beer which a select panel of six experienced tasters could detect.

Since sulfur dioxide is known to exhaust or paralyze the olfactory participants were presented a series of bisulfite solutions in water, in as order, with appropriate cautions about dwelling over-long on any sampl one of the six panel members found any recognizable "sulfite odor" at a centration of SO_2 below 50 p.p.m., and it took 100 p.p.m. of SO_2 in de water to bring recognition to all six. (Cf. Berg, et al.[3]).

The threshold series was repeated using additions of sodium bisulfite soda (with over 4 volumes of CO_2). The results supported the first series

Further experiments revealed that sensitive tasters will usuall differences in odor or taste of beer samples with 20 p.p.m. or added SO

will sometimes distinguish differences of 10 p.p.m. of added SO_2. But recognition of characteristic SO_2 odor or taste did not occur until 50 p.p.m., or more, of sulfur dioxide was present.

These results seemed unreasonable because all six participants just knew that they had been able to recognize much less in the odor of beer or ale, backed up and fully confirmed by analyses which showed such "sulfitey" beers might contain somewhere between 10 and 30 p.p.m. of sulfur dioxide.

However, when some of these beers were examined further, it was found that so-called "sulfite" odors disappeared from beer on addition of copper sulfate, which would have destroyed the mercaptan and H_2S odors, but not that of sulfur dioxide. The inter-relationships between the various sulfur compounds described in the previous paper in this series[1] would seem to allow the possibility that a beer with 20 p.p.m. of SO_2 might have arrived at the beer examiner's nose with a more *sulfury* odor than another with lower sulfur dioxide, since only 0.01 or 0.02 p.p.m. of mercaptan might give a very pronounced odor, while 20 p.p.m. of SO_2 would not be recognizable.

Although SO_2 *per se* is not ordinarily significant in the odor or flavor of malt beverages, it may be, by reaction with carbonyl groups, double bonds, oxygen, etc., quite potent in its protective influence on the character and stability of such products. The exact mechanism of the protection is no better known for beer than it is for wine or other foods.

Acknowledgement

The authors wish to give thanks to Miss Lillian Pfautsch and Mr. R. T. Steinberg for their help in the preparation of these papers.

Summary

1. A method for determining from 5-100 megms. of sulfur dioxide has been presented. For most beers, this requires a 5 ml. sample. The three common volatile sulfur compounds in beer may now be determined — all terminating in the same colorimetric procedure.

2. The levels of SO_2 in a few brewing materials and some present-day beers are presented.

3. The amount of sulfur dioxide present during fermentation and storage is shown. The increases during fermentation revealed the formation of SO_2 during this process.

4. A discussion of sulfur dioxide and its chemical partners is given.

5. The concept of "free" and "bound" SO_2 is shown to be relative to the agent which measures it. To the nose, bisulfite is bound SO_2, to iodine it is free; to iodine, acetaldehyde bisulfite is bound SO_2, to stannous chloride, it is free.

6. The Monier-Williams method gives higher numbers for beers than the procedure described herein. Since the former technique is subject to positive errors of appreciable magnitude, its use should be reconsidered.

7. The specific influence of SO_2 on the odor of beer was found to be un[illegible] [illegible] less than 20 p.p.m.

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APPLICATION DE L'AMPEROMETRIE A LA DETERMINATION DE L'ANHYDRIDE SULFUREUX DANS LES VINS

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Introduction.

L'anhydride sulfureux introduit comme stabilisant dans les vins, soit sous forme de gaz sulfureux, soit sous forme de sulfite, se combine en peu de temps à diverses substances présentes dans le vin, acétaldéhyde, sucre, aux polyphénols et en particulier à la matière colorante. Finalement on ne trouve qu'une fraction de cet anhydride sous forme libre titrable par l'iode. Il est nécessaire de noter à cet égard que la fraction anhydride sulfureux libre SO_2 n'est pas rigoureusement sous forme d'anhydride SO_2 mais bien souvent à l'état de bisulfite acide, c'est-à-dire d'anion SO_3H, en équilibre avec une petite quantité d'anhydride sulfureux SO_2 qui seul possède l'odeur caractéristique et qui, seul, soit véritablement antiseptique. Notons que cette proportion d'anhydride sulfureux « vraiment libre » dans le vin est fonction du pH. Elle est d'autant plus abondante que le pH du vin est plus bas. RIBEREAU-GAYON et JAULMES, rapportent que cette fraction représente 10 % de l'anhydride sulfureux total lorsque le pH est de 2,8 et 1 % lorsque le pH est de 3,8.

Du point de vue législation, la plupart des pays producteurs de vins ont rigoureusement reglementé l'emploi de cet antiseptique dans le but, bien entendu, de préserver la santé du consommateur et de conserver au vin son caractère de produit naturel. En France la quantité totale d'anhydride sulfureux tolérée est de 450 mg./litre. La législation exigeait jusqu'en 1934 que la quantité d'anhydride sulfureux libre ne dépasse pas 100 mg./litre avec une marge de 10 % au maximum. Dans d'autres pays la législation est encore plus sévère. C'est ainsi que l'Italie tolère au maximum 150 mg. d'anhydride sulfureux total par litre de vin et 15 mg. d'anhydride sulfureux à l'état libre. Il en résulte donc que le dosage de l'anhydride sulfureux est d'une importance capitale.

Principe du dosage.

La méthode de dosage est basée sur l'oxydation de l'anhydride sulfureux par l'iode. L'anhydride sulfureux libre est dosé directement par l'iode en milieu acide. Par contre, l'anhydride sulfureux combiné n'est pas oxydable par l'iode à froid en milieu acide. Il est nécessaire de le déplacer par distillation en milieu acide et de le titrer alors par l'iode. En ce qui concerne les vins blancs, le dosage de l'anhydride sulfureux libre peut se faire directement par l'iode en présence d'empois d'amidon comme indicateur de virage. Par contre, avec un vin rouge, le titrage est beaucoup plus délicat d'autant plus que la coloration propre du vin est accentuée par acidification. C'est pourquoi nous avons pensé utiliser

pour le titrage de l'anhydride sulfureux libre une méthode électrochimique où la fin de réaction au lieu d'être signalée par un indicateur coloré serait indiquée par un système électrométrique. Nous avons repris le procédé utilisé par les Suisses Tanner et Rutschmann mettant en jeu des électrodes de platine polarisées, connu sous le nom de « Dead Stop End Point », et qui n'est finalement qu'un procédé ampérométrique simplifié.

L'ampérométrie est une méthode de dosage titrimétrique mettant en jeu :

1° Une réaction de dosage : la substance à titrer (A) est dosée au moyen d'une solution titrée d'un réactif (B). Une réaction chimique a lieu entre A et B. On cherche à mettre en évidence le point équivalent de cette réaction, c'est-à-dire le point où la substance A à doser a totalement réagi sur la substance B.

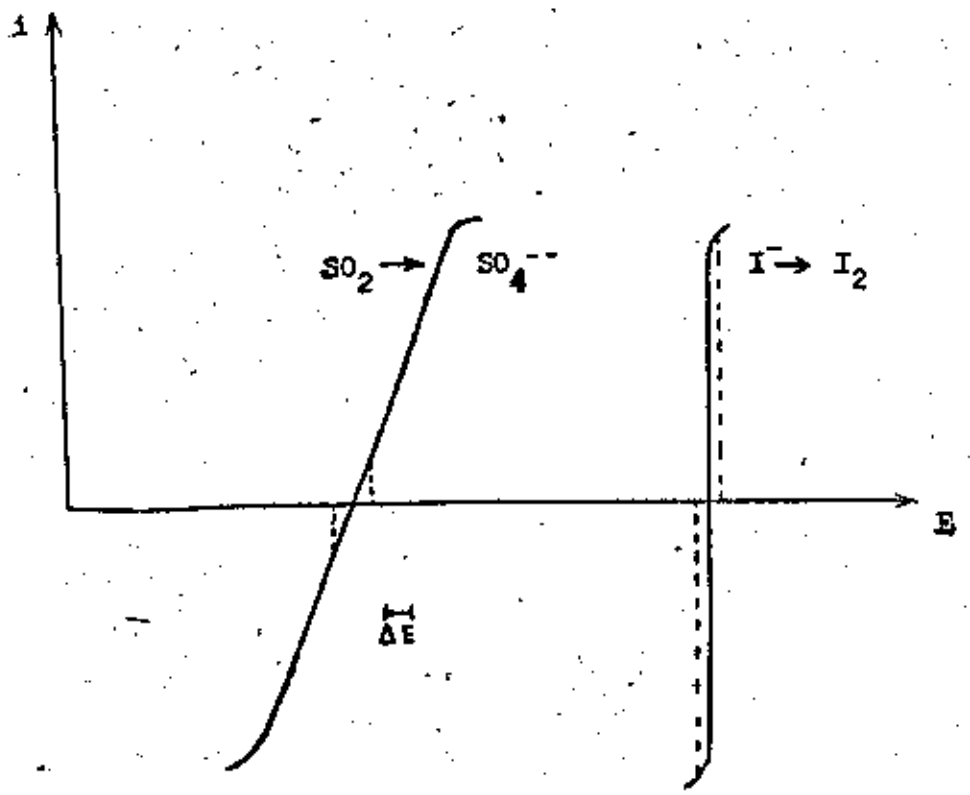
2° Une réaction indicatrice : en même temps que l'on effectue le dosage, on réalise une électrolyse dont le potentiel est fixé. L'intensité I du courant de l'électrolyse est fonction des concentrations des corps électrolysés. Cette électrolyse intéresse un des corps présents ou formés lors de la réaction. On suit les variations de l'intensité au cours du dosage, le point équivalent est indiqué par un point singulier de la courbe I = f (quantité de réactif ajouté).

Dans notre cas particulier, nous avons utilisé comme méthode indicatrice une ampérométrie avec deux électrodes de platine. Entre ces deux électrodes on applique une très faible différence de potentiel constante ΔE (10 à 30 millivolts).

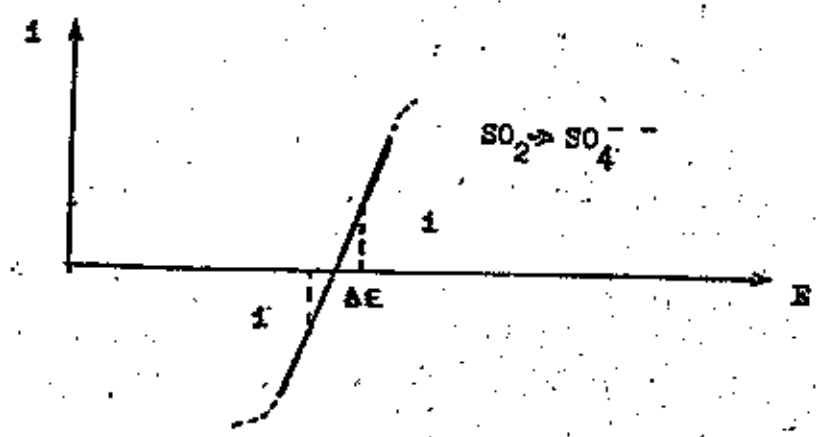
La réaction de dosage est la suivante :

$$SO_2 + I_2^{-} + 2\,H_2O \longrightarrow SO_4^{--} + 4\,H^+ + 2\,I^-$$

Si nous représentons les courbes intensité-potentiel des systèmes oxydoréducteurs en présence : $SO_2 \Longleftrightarrow SO_4^{--}$ et $I_2 \Longleftrightarrow I^-$, nous avons :



Au début du dosage nous avons seulement le système $SO_2 \rightarrow SO_4^{--}$ car l'iode ajouté est entièrement transformé à l'état I^-. Nous allons observer une intensité qui correspond à l'application de ΔE au système $SO_2 \rightarrow SO_4^{--}$.



Lorsque tout l'anhydride sulfureux aura disparu l'addition d'une nouvelle quantité d'iode nous mettra en présence du système $I^- \rightarrow I_2$. A ce moment là, l'intensité correspondra à l'application ΔE au système très rapide : $I^- \rightarrow I_2$.

Nous observerons donc une intensité beaucoup plus importante au point équivalent.

Appareillage.

Du point de vue pratique l'appareillage est très simple :

Nous avons utilisé une fiole titrimétrique cylindrique de 150 ml de capacité avec deux tubulures supérieures soudées. A ces tubulures s'adaptent, par des rodages coniques rigoureusement étanches, deux électrodes de platine. La fiole possède en plus un col pouvant recevoir la pointe de la burette. D'autre part comme source électrique nous avons utilisé un titrimètre électronique à œil magique de la maison Biolyon ; il se compose essentiellement d'un amplificateur électronique équipé d'une double triode, d'un œil magique, d'une valve et d'autre part, d'un circuit de polarisation des électrodes. L'alimentation s'effectue par le secteur alternatif 110 ou 220 v. L'appareil est indépendant des variations de tension jusqu'à plus ou moins 10 % ainsi que des variations de fréquence. Sa manipulation est simple et se réduit à la manœuvre de deux boutons dont l'un commande l'ouverture de l'œil magique et l'autre permet de régler la tension aux bornes des électrodes. Ce dernier réglage ne doit être retouché que lorsqu'on change d'électrode ou de récipient à titration.

L'agitation au cours du dosage est réalisée en plaçant la fiole titrimétrique sur un agitateur électro-magnétique.

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Dosage.

1° *Anhydride sulfureux libre.*

Le dosage est effectué selon la méthode directe de RIPPER. 50 ml de vin sont acidulés par 1,5 ml d'acide sulfurique au 1/5 en volume et on ajoute l'iode N/20 de façon à amener l'ouverture de l'œil magique.

Afin de régler rigoureusement l'appareil, on fait un essai avec du vin blanc auquel on a rajouté quelques gouttes d'empois d'amidon. L'ouverture de l'œil magique doit correspondre rigoureusement *au début* de virage de l'empois.

On règle ainsi le titrimètre électronique une fois pour toutes. La tension aux bornes des électrodes doit être de l'ordre de 15 à 20 mv., tension qui peut être mesurée à l'aide d'un galvanomètre de laboratoire suffisamment sensible. Nous avons utilisé pour cela un galvanomètre manispot Sefram Mavb. L'intensité du courant de passage doit être de l'ordre de 0,5 µA. Cette intensité peut être lue sur un microampèremètre ordinaire. Celui que nous avons utilisé présente une résistance interne de 2.300 ohms environ. Nous conseillons vivement de faire un réglage préalable à l'aide de vin blanc. De plus, l'adjonction d'iode devra cesser lorsque l'ouverture de l'œil sera stable durant 20 secondes au moins. Le dosage de l'anhydride sulfureux libre n'a présenté en lui-même aucune difficulté et a confirmé les observations de TANNER et RUNTSCHLER. La méthode nous a semblé beaucoup plus intéressante dans la mesure où elle pouvait s'appliquer au dosage de l'anhydride sulfureux total et par suite combiné.

2° *Anhydride sulfureux total.*

La méthode officielle française comporte un essai préliminaire selon le procédé de RIPPER. Cette méthode est basée sur la destruction à froid des combinaisons de l'anhydride sulfureux avec les aldéhydes du vin à l'aide d'une solution de potasse. L'anhydride sulfureux passe ainsi à l'état de sulfite de potasse, on réacidifie par l'acide sulfurique et on titre l'anhydride sulfureux par l'iode. RIBEREAU-GAYON applique cette méthode de la façon suivante :

25 cm³ de vin reçoivent 10 cm³ de solution normale de soude : on laisse en contact 5 minutes, on acidifie par 3 à 5 cm³ d'acide sulfurique au tiers et titre immédiatement par l'iode à 4 g par litre.

On considère que cette méthode est acceptable pour le travail courant de contrôle et au-dessous de 300 mg d'anhydride sulfureux par litre. Lorsque l'on désire une méthode précise, la législation et les conventions internationales conseillent une méthode de distillation, en particulier les méthodes de HAAS, FLANZY et DEHNER ou DEHNER et BENARD.

D'après RIBEREAU-GAYON, les causes d'erreurs de la méthode RIPPER sont les suivantes :

« 1° L'oxydation du sulfite durant l'alcalinisation, notamment en présence de polyphénols ; cette erreur est diminuée en ne laissant que 5 minutes en contact avec la soude ;

2° La recombinaison avec l'acétaldéhyde de l'anhydride sulfureux libéré au moment où on acidifie, qu'il est impossible d'éviter complètement dans les vins riches en acide sulfureux combiné, malgré toute la célérité que l'on peut apporter au titrage ;

3° La formation par l'alcalinisation des vins, de substances consommant de l'iode ».

La méthode fut améliorée par JAULMES et DIECZEIDE et voici comment RIBEREAU-GAYON la conduit :

Une prise de 25 cm³ de vin est alcalinisée par 10 cm³ de soude N ; on agite pour mélanger. Après 5 minutes, on ajoute de l'empois d'amidon et on verse rapidement en agitant 1,5 cm³ d'acide sulfurique au tiers. On titre immédiatement par l'iode à 4 g par litre. On alcalinise à nouveau par 40 cm³ de soude N ; on attend 5 minutes et on acidifie par 5 cm³ d'acide sulfurique au tiers. On titre l'acide sulfureux libéré une seconde fois. Le calcul porte sur le volume d'iode utilisé pour ces deux titrages successifs. De cette façon cette méthode, dénommée « RIFFER double », conduit à des résultats plus proches de la réalité.

Nous avons essayé de la combiner avec l'ampérométrie et de la modifier de telle façon que l'on puisse doser d'un même coup l'anhydride sulfureux libre et l'anhydride sulfureux combiné. Voici le mode opératoire que nous avons adopté :

1° 25 ml de vin sont acidifiés par 0,75 ml d'acide sulfurique au tiers.

On agite légèrement. Le pH, dans ces conditions, oscille entre 0,8 et 1 selon les vins, pH qui nous semble normal pour une bonne titration par l'iode. On titre par l'iode N/32 : soit n le nombre de ml d'iode N/32 versé.

2° On ajoute alors 15 ml de soude normale.

Le pH, dans ces conditions, est supérieur à 9, on ferme hermétiquement la fiole, on agite légèrement et on attend 5 minutes que la soude libère l'anhydride sulfureux de ses combinaisons aldéhydiques et autres. Au bout de 5 minutes, on réacidifie par 1,5 ml d'acide sulfurique au tiers (pH 0,8 à 1), on agite légèrement et on titre à nouveau par l'iode N/32 : soit n' le nombre de ml d'iode versé au cours de ce titrage.

3° On ajoute alors 25 ml de soude normal (pH > 9).

On bouche hermétiquement la fiole, on agite légèrement et on attend 5 minutes. Au bout de ce temps on acidifie par 2,5 ml d'acide sulfurique au tiers, on agite légèrement et titre à nouveau par l'iode N/32 : soit n'' le nombre de ml d'iode utilisé pour cette 3e titration. Finalement, les résultats sont donnés par les formules suivantes :

$$\text{Anhydride sulfureux libre en mg/litre} = (n \times 40)$$

$$\text{Anhydride sulfureux combiné en mg/litre} = [(n' + n'') \times 40]$$

Chaque adjonction de soude et d'acide doit, de préférence, se faire en plongeant la fiole titrimétrique dans un bain d'eau glacée. Durant les titrations, l'agitation dans la fiole se fait à l'aide de l'agitateur électromagnétique.

Discussion.

Afin de pouvoir connaître la précision de cette nouvelle méthode de dosage, nous avons parallèlement opéré des contrôles à l'aide de la méthode MARCILLE, modifiée par DEIBNER et BENARD. Comme toutes les méthodes distillatoires, elle est considérée comme très précise.

Méthode Marcille (Deibner et Benard).

1° *Principe.*

L'anhydride sulfureux est chassé du vin par ébullition en milieu acide et à l'abri de l'air. Le distillat est recueilli sur de la soude en excès. L'anhydride sulfureux distillé est titré par iodométrie en évitant l'erreur due à la présence de sa combinaison avec l'acétaldéhyde.

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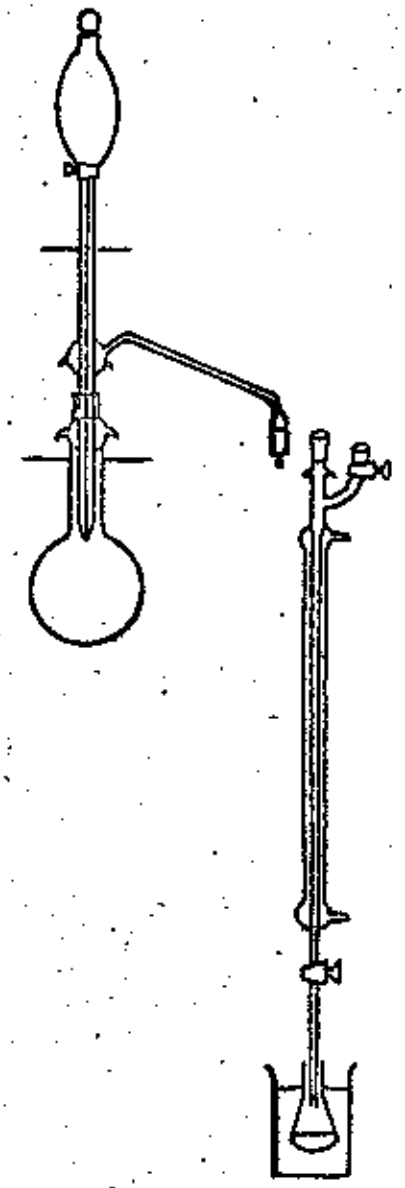

Appareil de Dehner et Marcille pour la distillation de l'acide sulfureux à l'abri de l'air.

2° *Appareil.*

L'appareil se compose d'un ballon de 500 cm³ muni d'une ampoule à robinet de 100 cm³ et relié à un réfrigérant de West vertical de 30 cm de longueur utile ; ce dernier est muni à sa partie supérieure d'un tube latéral fermé par un petit robinet et d'un robinet à sa partie inférieure.

La capacité interne de ce réfrigérant doit être de 50 à 55 cm³. Le robinet inférieur est suivi d'une tige effilée qui est introduite dans un erlenmeyer récepteur de 200 cm³. Le ballon est posé sur un disque de métal ou d'amiante percé d'une ouverture circulaire de 5 cm de diamètre. L'ampoule à robinet a une douille assez longue pour que son robinet se trouve à 25 cm au-dessus du bouchon du ballon et que sa pointe effilée arrive jusqu'au bas du col du ballon. Deux disques d'amiante sont placés entre le robinet et le ballon pour éviter tout échauffement du vin dans l'ampoule à robinet.

3° *Technique.*

Dans le réfrigérant de West, dont le robinet inférieur est fermé, on place 25 cm³ de soude 4 N et de l'eau jusqu'au niveau du tube latéral supérieur. Un erlenmeyer de 200 cm³ immergé dans un bain d'eau et de glace est placé au bas du réfrigérant pour recueillir le distillat.

Dans le ballon on place 1 cm³ d'acide chlorhydrique pur et 20 cm³ d'eau. On porte à l'ébullition ce liquide, en laissant ouvert le tube latéral supérieur du réfrigérant. L'air est ainsi chassé. On ferme le robinet du tube latéral tout en maintenant l'ébullition et aussitôt on ouvre le robinet placé à la partie inférieure du réfrigérant.

D'autre part, on a placé dans l'ampoule à robinet 100 cm³ de vin. On ouvre le robinet de l'ampoule progressivement, tout en maintenant un chauffage assez énergique sous le ballon pour que l'ébullition ne cesse pas. On continue à chauffer après avoir rincé l'ampoule à robinet avec deux fois 5 cm³ d'eau distillée bouillie. On arrête la distillation lorsque le volume contenu dans le ballon a été réduit à 30 cm³ environ.

Le distillat, alcalin et fortement refroidi, contenu dans l'erlenmeyer, est alors versé lentement dans un bécher de 400 cm³ constamment agité dans lequel on a placé préalablement 30 cm³ d'acide sulfurique au 1/5, 50 cm³ d'iode N/32, quelques morceaux de glace et 20 cm³ d'iodure de potassium à 10 %. On titre l'excès d'iode par la solution de thiosulfate de sodium N/32 ; soit n le volume de thiosulfate utilisé pour décolorer l'empois d'amidon.

On vérifie qu'aucune trace d'anhydride sulfureux n'existe, combinée à l'acétaldéhyde en ajoutant deux gouttes de phénolphtaléine et de la soude N jusqu'à virage au rose, on décolore par une goutte d'acide N et on ajoute 20 cm³ de solution saturée de borate de sodium. On ajoute de l'iode N/32 jusqu'à recoloration au bleu stable de l'empois d'amidon ; soit n' le volume d'iode employé. Ce dernier volume doit être diminué du petit volume n'' d'iode N/32 nécessaire pour amener le virage au bleu de l'empois dans un essai à blanc fait en mélangeant les mêmes réactifs, mais sans distillat, qui est remplacé par un volume égal d'eau distillée. Très généralement (n' - n'') est pratiquement nul, lorsqu'on a opéré d'une façon correcte.

La quantité d'anhydride sulfureux total est égale à (50 - n + n' - n'') 10, exprimée en mg d'anhydride sulfureux par litre.

Pour les vins contenant plus de 500 mg d'anhydride sulfureux par litre, il faut recommencer le dosage en utilisant 50 cm³ d'iode N/16. Le résultat exprimé en mg par litre est alors : (50 - n + n' - n'') 20.

Dans cette méthode nous avons pratiqué la correction recommandée par Denigès et Bexard et nous avons constaté que le terme n' - n'' est en effet pratiquement nul, lorsque le dosage est fait correctement. Toutefois, nous tenons à signaler qu'il est préférable de ne pas pousser la distillation jusqu'à ce que le volume contenu dans le ballon soit réduit à 30 ml

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ETUDE COMPARATIVE DE L'ANHYDRIDE SULFUREUX PAR DISTILLATION ET MÉTHODE AMPEROMETRIQUE

VINS Références	Méthode chimique SO_2 total en mg/l	Méthode Ampérométrique		
		libre en mg/l	combiné en mg/l	total en mg/l
Vin n° 1	103,68	22,27	82,71	104,98
Vin n° 2	270,08	10,24	267,51	277,75
Vin n° 3	28,44	6,40	22,40	28,80
Vin n° 4	250,24	14,08	238,08	252,16
Vin n° 5	131,84	26,88	101,12	128,00
Vin n° 6	101,12	34,56	64,64	99,20
Vin n° 7	53,76	19,20	37,12	56,32
Vin n° 8	80,00	7,68	72,96	80,64
Vin n° 9	119,68	19,20	98,56	117,76
Vin n° 10	162,56	32,00	128,00	160,00
Vin n° 11	224,00	17,92	200,96	218,88
Vin n° 12	90,84	19,20	74,24	93,44
Vin n° 13	115,20	19,20	95,70	114,90
Vin n° 14	156,16	20,88	131,00	151,88
Vin n° 15	128,[illegible]	19,20	106,24	125,44
Vin n° 16	222,72	38,40	177,92	216,32
Vin n° 17	144,64	23,04	119,40	142,08
Vin n° 18	119,68	19,20	98,56	117,76
Vin n° 19	156,80	32,00	122,88	154,88
Vin n° 20	111,36	19,20	90,88	110,08
Vin n° 21	48,00	12,00	34,00	46,00
Vin n° 22	135,50	26,00	112,00	138,00
Vin n° 23	197,00	26,00	174,00	200,00
Vin n° 24	127,00	20,00	108,00	128,00
Vin n° 25	339,00	70,00	270,00	340,00
Vin n° 26	250,00	10,00	234,00	244,00
Vin n° 27	130,42	22,00	105,60	127,60
Vin n° 28	137,30	16,80	119,20	136,00
Vin n° 29	113,70	24,00	86,70	110,70
Vin n° 30	127,00	20,00	103,60	123,60
Vin n° 31	38,00	16,00	24,20	40,20
Vin n° 32	67,50	20,00	50,00	70,00
Vin n° 33	162,00	24,00	134,00	158,00
Vin n° 34	157,00	22,00	140,00	162,00
Vin n° 35	43,00	10,00	36,00	46,00
Vin n° 36	151,00	54,00	102,00	156,00
Vin n° 37	220,00	46,00	176,00	222,00
Vin n° 38	129,50	44,00	88,00	132,00
Vin n° 39	122,50	32,00	91,00	126,00
Vin n° 40	109,00	34,00	76,00	110,00
Vin n° 41	105,50	38,00	70,00	108,00

environ, surtout pour les vins riches en sucre. Nous arrêtons généralement la distillation lorsque le volume contenu dans le ballon est de l'ordre de 45 à 50 ml, car on risque, en milieu acide, pour des vins riches en sucre, et en poussant trop loin la distillation, d'obtenir une dégradation des sucres avec formation de furfurol qui peut gêner par la suite le dosage de l'anhydride sulfureux.

Résultats et conclusions.

Nous avons pratiqué ainsi une cinquantaine de déterminations comparatives et nous avons constaté que notre méthode donnait des chiffres en anhydride sulfureux total très concordants avec la méthode de DEIBNER et BENARD.

Dans l'ensemble, l'écart maximum entre les deux techniques ne dépasse pas 4 %. Il semble donc que cette méthode puisse rendre certains services par sa rapidité d'abord, mais du fait aussi que dans les vins rouges elle élimine l'incertitude du virage de l'empois d'amidon. Elle permet avec une très bonne précision d'avoir dans une seule opération et l'anhydride sulfureux libre et l'anhyride sulfureux combiné. Nous insistons sur le fait que les proportions précédemment données doivent être rigoureusement respectées et qu'il est préférable que les adjonctions de soude et d'acide soient réalisées après avoir placé la fiole titrimétrique dans un bain d'eau glacée.

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The Determination of the Free Sulphur Dioxide Content of Ciders

By L. F. BURROUGHS and A. H. SPARKS
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The conditions necessary for determining free sulphur dioxide in cider were established by studying the rate of removal of sulphur dioxide from aqueous solutions by a gas stream. The cider is acidified and the free sulphur dioxide removed at room temperature in an air-stream under reduced pressure; it is then absorbed in neutralised hydrogen peroxide and titrated with standard alkali. Possible interferences are considered and allowed for by applying a correction for the blank value.

When sulphur dioxide is used as a preservative in ciders, wines and other fruit beverages, part of it combines with the carbonyl compounds present to form hydroxysulphonates ("bisulphite compounds") and part remains free in the inorganic state, mainly as sulphurous acid and bisulphite ions. It is widely recognised that only free sulphur dioxide is effective against micro-organisms, and it is therefore important to be able to determine this analytically.

Ripper's method,[1] of iodimetric titration in acid solution without previous treatment with alkali, is the one most commonly used for determining free sulphur dioxide in ciders and similar beverages. Unfortunately, it also records other substances oxidisable by iodine, and for this reason the end-point is often ill defined. The errors involved in this method and various attempts to overcome them were reviewed by Joslyn and Bravermann.[2]

The colorimetric method of Steigman,[3] involving the use of pararosaniline and formaldehyde, has been developed by Stone and Laschiver[4] for determining both free and total sulphur dioxide in beer. With ciders, however, interference by polyphenols greatly reduces its sensitivity for determining free sulphur dioxide and renders it useless for determining total sulphur dioxide.

Kielhöfer and Aumann[5] and Paul[6] removed free sulphur dioxide from acidified wine at room temperature by a stream of air, absorbed it in neutralised hydrogen peroxide and titrated with 0·01 N sodium hydroxide. This method is based on sound theoretical principles and appeared worthy of investigation. When the wine (10 or 20 ml) is acidified with 5 ml of 25 per cent. v/v orthophosphoric acid, the pH is lowered to between 1 and 1·5. This has the double purpose of rendering the free sulphur dioxide more volatile and of greatly reducing the rate of decomposition of the combined sulphur dioxide; furthermore, at this pH sulphur dioxide resists oxidation by the atmosphere. Kielhöfer and Aumann passed a stream of air for 30 minutes, whereas Paul, possibly using a more rapid rate of flow, found that 15 minutes was sufficient. However, as observed by Kielhöfer and Aumann, the precise stoicheiometric removal of free sulphur dioxide from a wine is not to be expected, since even at this low pH there is a slight tendency for combined sulphur dioxide to dissociate once the equilibrium is disturbed by removal of the free sulphur dioxide. Kielhöfer and Aumann found that the amount of sulphur dioxide liberated in this way was small, even if the wine was aerated for a long time, and they regard the value obtained after 30 minutes as being the best practicable estimate of free sulphur dioxide. The extent of the dissociation of bound sulphur dioxide will, however, depend on the nature and amount of the carbonyl substances present in the particular sample and cannot always be assumed to be negligible.

The method described in this paper is a modification of the methods of Paul and of Kielhöfer and Aumann; it was developed from a study of the conditions necessary to ensure complete removal of sulphur dioxide from solution in the absence of combined sulphur dioxide.

Rate of removal of sulphur dioxide from dilute acid solution

The problem of removing sulphur dioxide from solution at a given temperature and pH is a physical one, and depends on the flow-rate of carrier gas and on the partial pressure of the sulphur dioxide. The flow-rate of gas should be as fast as possible, consistent with efficient trapping of the entrained sulphur dioxide by the absorbing solution.

In a preliminary experiment nitrogen was bubbled through a solution of sodium hydrogen sulphite (0·85 M) in 5 per cent. v/v orthophosphoric acid. Portions (20 ml) were removed and titrated with 0·01 N iodine. The concentrations of sulphur dioxide remaining in solution after different times were—

Length of bubbling time, minutes ..	0	10	15	20	30	40
Sulphur dioxide remaining, p.p.m. ..	54	24	7	4	1	trace

It appears, therefore, that bubbling for at least 30 minutes is required for the complete removal of sulphur dioxide from solution under these conditions. This was attributed to the extremely low partial pressure of sulphur dioxide in the carrier gas when the concentration of sulphur dioxide in solution had been reduced to about 10 p.p.m. This difficulty could obviously be overcome by removing the sulphur dioxide under a partial vacuum.

A similar experiment was carried out, in which a stream of nitrogen at a pressure of 80 mm of mercury was drawn through the solution, by using a water-pump; the concentrations of sulphur dioxide remaining in solution after different times were—

Length of bubbling time, minutes ..	0	5	10	15
Sulphur dioxide remaining, p.p.m. ..	53	12	1	nil

Similar results were obtained in a further experiment in which a stream of air at 80 mm pressure was used. Removal of sulphur dioxide was therefore complete in 15 minutes, and this provided the basis for the method described below. The hydrogen peroxide solution used for absorbing the sulphur dioxide was shown to be completely stable to aeration under reduced pressure for 15 minutes.

Method

Apparatus—

The apparatus (see Fig. 1) is similar to that described for determining total sulphur dioxide,[7] but requires only two absorption tubes, A and B, and no condenser. The 250-ml round-bottomed flask, having a B24 neck joint and a side inlet, and the B14 air-leak and the tubes (200 × 25 mm) are standard pieces of glassware. The absorption heads are made from B24 cone joints with the internal delivery tubes drawn out to a jet. The second absorption tube, B, is connected to a mercury manometer and a water-pump.

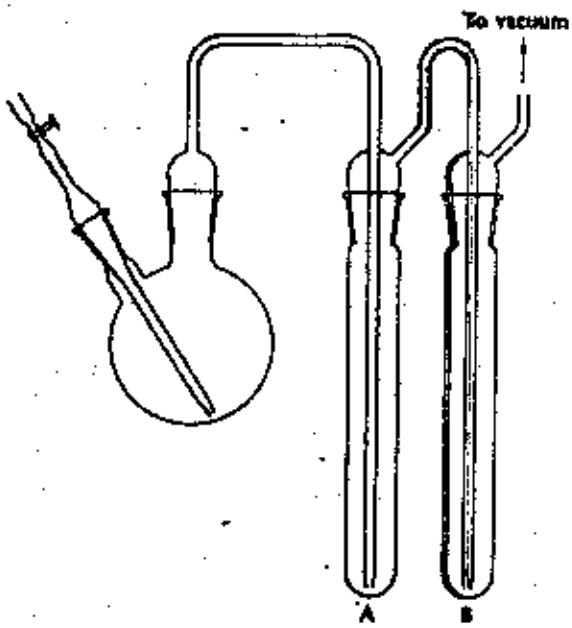

Fig. 1. Absorption apparatus

REAGENTS—

Hydrogen peroxide, 1 per cent. w/v—Dilute 3·3 ml of 100-volume analytical-reagent grade hydrogen peroxide to about 90 ml with distilled water. Add 1 ml of Tashiro indicator (2 volumes of 0·1 per cent. methyl red, *plus* 1 volume of 0·1 per cent. methylene blue, both in 95 per cent. ethanol), and neutralise with 0·01 N sodium hydroxide to the first appearance of a green colour. Dilute to 100 ml.

Sodium hydroxide, 0·01 N—Prepare in the usual way, store in an automatic 10-ml burette with soda-lime guard tubes and standardise against potassium hydrogen iodate. It is convenient to use the same solution of potassium hydrogen iodate, suitably diluted, to standardise 0·01 N sodium thiosulphate for determining total sulphur dioxide.[7]

Orthophosphoric acid, 25 per cent. v/v—Containing 250 ml of 85 per cent. w/v orthophosphoric acid per litre.

PROCEDURE—

By pipette, transfer 5 ml of 1 per cent. hydrogen peroxide into each absorption tube. Similarly transfer 10 or 20 ml of sample to the flask, and add 5 ml of 25 per cent. orthophosphoric acid.

Attach the flask to the apparatus, and evacuate to a pressure of 70 to 80 mm while admitting a steady stream of air, controlled by the clip on the inlet tube. After 15 minutes shut off the vacuum, and carefully open the screw-clip. Lower the first absorption tube, A, and expel the drop of solution in the tip of the delivery tube by warming the flask with the hand; rinse the delivery tube. Titrate the contents of tube A with 0·01 N sodium hydroxide to a grey end-point. Replace the tube on the apparatus to rinse the inside of the delivery tube; re-adjust the end-point, if necessary.

Immediately attach to the apparatus another absorption tube, A′, containing 5 ml of 1 per cent. hydrogen peroxide. Close the screw clip, apply the vacuum, and continue aeration for a second 15 minutes (including the time taken in titrating the contents of tube A, normally about 2 minutes). Finally remove tube A′, titrate the contents as described above, and subtract from the result of the titration of tube A, the blank value obtained. The reasons for this blank value are discussed below.

Calculate results as p.p.m. of sulphur dioxide.

1 ml of 0·01 N sodium hydroxide ≡ 0·32 mg of sulphur dioxide.

The absorption of sulphur dioxide in tube A is highly efficient, and the second tube, B, rarely yields any significant titration unless the hydrogen peroxide in tube A is completely reduced by sulphur dioxide. This occurs only with samples containing an extremely high concentration of free sulphur dioxide (much beyond the limits of normal cider), since 5 ml of 1 per cent. hydrogen peroxide will oxidise 100 mg of sulphur dioxide.

DISCUSSION OF METHOD

RECOVERY OF FREE SULPHUR DIOXIDE—

The method was tested by comparing the recovery of free sulphur dioxide (10-ml portions containing 200 p.p.m.) from a solution containing 0·157 g of sodium metabisulphite ($Na_2S_2O_5$) per 500 ml with that obtained simultaneously by titrating the same solution into 10-ml portions of 0·01 N iodine. To ensure that the two methods should be strictly comparable, 0·1 N hydrochloric acid was used as a common standard for both the 0·01 N sodium hydroxide and the 0·01 N iodine (via potassium iodate and 0·1 N sodium thiosulphate).

Results of three determinations showed 100·0, 100·2 and 100·2 per cent. recovery; in each determination the second absorption tube remained neutral, and no residual sulphur dioxide was detectable (by 0·01 N iodine and starch) in the contents of the flask.

It is, unfortunately, not possible to test the recovery of free sulphur dioxide in the presence of cider, because, when standard sodium hydrogen sulphite is added to cider, part of it combines with the carbonyl substances present. Nor is there any established method for the precise determination of free sulphur dioxide in cider, with which the present method could be compared. It is, however, reasonable to assume that, since the proposed method gives complete recovery of sulphur dioxide from sodium hydrogen sulphite solution, it will also give complete recovery from cider.

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Two factors may, however, lead to high results; these are the presence of volatile acids in the cider and the liberation of free sulphur dioxide by dissociation of "bisulphite compounds" during the determination.

EFFECT OF VOLATILE ACIDS—

The volatile acidity of cider is normally not more than about 0·1 per cent. as acetic acid. If any of this is carried over in the air stream it may be trapped in the absorption tube and so be recorded as sulphur dioxide. The probable size of the blank value due to volatile acid was investigated by determining the apparent free sulphur dioxide content of 0·2 per cent. acetic acid. Results correspond to 2·5 p.p.m. as sulphur dioxide on a 20-ml sample and were substantially unchanged in the blank solution, tube A′, collected during the second 15 minutes. In a normal cider, therefore, with a much lower volatile acidity, the error caused by volatile acids should be less than 2·5 p.p.m. and can be allowed for by subtracting the blank value.

DISSOCIATION OF COMBINED SULPHUR DIOXIDE—

The equilibrium between free and combined sulphur dioxide in a cider at a given temperature and pH depends on the concentrations of the carbonyl compounds present and on the equilibrium constants of their "bisulphite compounds". If the free sulphur dioxide is removed, as in the proposed procedure, each "bisulphite compound" dissociates into free sulphur dioxide and free carbonyl compound at a rate dependent on its concentration and on the velocity constant of its decomposition. These velocity constants are greatly reduced by lowering the pH, but even at pH 1·5 the dissociation is measurable and may interfere in the determination of free sulphur dioxide. It is therefore to be expected that part of the sulphur dioxide determined in the blank titration, tube A′, is caused by the dissociation of combined sulphur dioxide; this may be called the "dissociation blank value."

In ciders and similar alcoholic beverages much of the combined sulphur dioxide is present as acetaldehyde bisulphite, but other carbonyl compounds are also involved. Burroughs and Whiting[9] have calculated the sulphite-binding power of certain of the known constituents of cider, and Kielhöfer and Wurdig[9,10] have examined similar problems in wines.

The liberation of sulphur dioxide combined with acetaldehyde, pyruvic acid and glucose was investigated in model systems. A solution, containing 100 p.p.m. of sulphur dioxide as acetaldehyde bisulphite in dilute phosphate - citrate buffer at pH 3·5, yielded a blank value, tube A′, of only 0·3 p.p.m. in the determination of free sulphur dioxide; dissociation of acetaldehyde bisulphite is therefore negligible under these conditions. This was confirmed by an experiment in which a solution containing 200 p.p.m. of sulphur dioxide and 70 p.p.m. of acetaldehyde was allowed to come to equilibrium, thus giving approximately 100 p.p.m. of free and 100 p.p.m. of combined sulphur dioxide. The results for the determination of free sulphur dioxide by the proposed method (excluding the correction for the blank value) agreed within 1 p.p.m. with those by direct iodimetric titration of the original solution at pH 1·5. There was similarly close agreement between the bound sulphur dioxide content (determined by iodimetric titration at pH 8·14) of the original solution and of the contents of the flask after determination of free sulphur dioxide. The presence of acetaldehyde bisulphite in the sample, therefore, does not interfere in the determination of free sulphur dioxide by the proposed method, nor does it contribute appreciably to the dissociation blank value.

An experiment, similar to the iodimetric determination described above, was carried out on a solution containing 200 p.p.m. of total sulphur dioxide and 220 p.p.m. of sodium pyruvate, again corresponding to approximately 100 p.p.m. of free and 100 p.p.m. of combined sulphur dioxide. Here, the apparent free sulphur dioxide determined by vacuum aeration was 8 to 9 p.p.m. higher than by direct iodimetric titration, with a corresponding loss of bound sulphur dioxide in the contents of the flask. The amount of free sulphur dioxide liberated during the second 15 minutes aeration, tube A′, was 6 to 7 p.p.m., so that a close approximation to the true value could be obtained by subtracting this correction.

It can be calculated from the results obtained by Vas[12] for glucose, that the amount of glucose bisulphite likely to occur in a normal cider is small, corresponding to only 8 p.p.m. of combined sulphur dioxide at 1·8 per cent. of glucose and 50 p.p.m. of free sulphur dioxide. It can also be calculated that, at pH 1·5, only 0·5 per cent. of the glucose bisulphite present will dissociate in 15 minutes at 20° C. The effect of this on the determination of free sulphur

dioxide in ciders should therefore be negligible. This was confirmed by using a solution of glucose (1·8 g per 100 ml) and sodium metabisulphite, $Na_2S_2O_5$ (0·03 g per 100 ml), at pH 3·5. After equilibrium had been attained, this solution contained 181 p.p.m. of total sulphur dioxide,[7] 157 p.p.m. of free sulphur dioxide and therefore, by difference, 24 p.p.m. of combined sulphur dioxide; the dissociation blank value obtained during the second 15 minutes was only 0·3 p.p.m. With a similar solution containing ten times as much sodium metabisulphite, the dissociation blank value was 5 p.p.m. in the presence of 1500 p.p.m. of free and 218 p.p.m. of combined sulphur dioxide. The effect of the dissociation of glucose bisulphite on the determination of free sulphur dioxide in ciders and similar beverages can therefore be neglected, but it may be significant in products with a high concentration of glucose or of free sulphur dioxide.

It appears, therefore, that the blank value due to dissociation of combined sulphur dioxide in ciders is not, in the main, attributable to acetaldehyde or glucose. Pyruvic acid certainly yields a dissociation blank value, as probably do other carbonyl compounds, since ciders having a relatively low content of pyruvate can give blank values up to 8 p.p.m. of sulphur dioxide. Numerous determinations of free sulphur dioxide in different ciders have shown blank values ranging from 1 to 10 p.p.m., the higher values being obtained in the presence of relatively high concentrations of sulphur dioxide bound to unidentified carbonyl compounds.

For a given cider with incremental additions of sulphur dioxide, the size of the blank value increases with increasing concentration of free sulphur dioxide. Results obtained on a commercial cider were—

Apparent content of free sulphur dioxide (tube A), p.p.m.	3·6	11·6	30·4	54·6	90·0
Blank value (tube A′), p.p.m.	1·5	2·8	4·3	5·8	7·3
Corrected content of free sulphur dioxide (A − A′), p.p.m.	2·1	8·8	26·1	48·8	82·7

Although the blank value corrections may be small, they frequently represent more than 10 per cent. of the apparent free sulphur dioxide content, especially at low concentration.

EFFECT OF TEMPERATURE ON THE FREE SULPHUR DIOXIDE CONTENT OF CIDERS—

The equilibrium between free and bound sulphur dioxide in solution is influenced by temperature. If a cider is heated the "bisulphite compounds" tend to dissociate, liberating free sulphur dioxide; conversely, if it is cooled, the carbonyl compounds react further, lowering the concentration of free sulphur dioxide. The size of this effect may be considerable: for example, the free sulphur dioxide content of a cider was found to increase from 26 p.p.m. at 20° C to 42 p.p.m. at 40° C.

The rate at which these changes occur has not yet been studied, but it is obviously a wise precaution to keep the sample at the appropriate temperature until just before analysis. Thus, it would be wrong to analyse a sample that had been stored overnight in a refrigerator if the results are intended to relate to equilibrium conditions at some other temperature.

REPRODUCIBILITY—

Six replicate determinations of free sulphur dioxide were made on two ciders. In the first, results ranged from 13·4 to 13·0 p.p.m. with a coefficient of variation of 1·4 per cent. In the second, the range was 57·5 to 58·7 p.p.m. with a coefficient of variation of 0·8 per cent.

COMPARISON WITH A METHOD FOR SOFT DRINKS—

After this manuscript was submitted, Lloyd and Cowle[13] published a method for determining free sulphur dioxide in soft drinks that is similar to ours and to the earlier methods of Kielhöfer and Aumann[8] and Paul.[9] The essential differences between these methods are listed below.

Desorption of sulphur dioxide—In the methods of Lloyd and Cowle, Kielhöfer and Aumann and Paul the sulphur dioxide is desorbed in a fast stream of carrier gas at atmospheric pressure. Our method of desorption *in vacuo* is quicker and more efficient, and therefore facilitates the application of a correction for the dissociation blank value.

Absorption of sulphur dioxide—Lloyd and Cowle absorbed the sulphur dioxide in 0·1 N potassium hydroxide in 10 per cent. v/v glycerol, since they found that absorption in neutralised hydrogen peroxide and titration with sodium hydroxide gave poor end-points with screened methyl orange. This is contrary to our experience, and no such difficulty is mentioned by Kielhöfer and Aumann or by Paul. We obtained sharp end-points provided the solutions were made up in good quality distilled water; poor end-points can occur with some samples of water de-mineralised by ion exchange.

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The use of an alkaline absorbent (with subsequent acidification and titration with iodine) involves the risk of loss of sulphur dioxide in the absorption tube by oxidation. This is overcome by adding glycerol as an anti-oxidant and using oxygen-free nitrogen as the carrier gas; it is also necessary to de-aerate the apparatus by passing nitrogen for 5 minutes before adding the sample. In our proposed method, no such precautions are required since the sulphur dioxide is stable to oxidation in dilute orthophosphoric acid (pH 1 to 1·5) and is deliberately oxidised by hydrogen peroxide in the absorption tubes.

Dissociation of bound sulphur dioxide—Lloyd and Cowle state that after the desorption period of 15 to 20 minutes no subsequent dissociation of sulphur dioxide of any significance was observed. Thus, they make no correction for a dissociation blank value; this probably reflects the fact that glucose was the main binding agent for sulphur dioxide in their samples. With ciders a correction for the dissociation of bound sulphur dioxide is essential for accurate determinations.

Reproducibility—From an examination of the results of duplicate analyses shown in Table V of the paper, we judged that Lloyd and Cowle's procedure of desorption and trapping is less reproducible (coefficient of variation 2·85 per cent.) than our method.

Colorimetric modification—For samples having a low content of free sulphur dioxide, Lloyd and Cowle absorb the sulphur dioxide in sodium tetrachloromercurate and determine it colorimetrically. In our method it is possible to determine as little as 5 to 10 p.p.m. of free sulphur dioxide with sufficient accuracy for most purposes (±0·3 p.p.m.) by the normal procedure.

Conclusions

Although Lloyd and Cowle's procedure may be satisfactory for soft drinks with a relatively high content of free sulphur dioxide and low dissociation blank, it is not suitable for products for which the dissociation blank value is appreciable compared with the free sulphur dioxide content. On the other hand, our method would appear to be equally applicable to soft drinks and, if the dissociation blank value can be omitted for such products, is quicker as well as being more precise and easier to manipulate.

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Received *May 16th*, 1963

The Determination of the Total Sulphur Dioxide Content of Ciders

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The factors involved in determining total sulphur dioxide in alcoholic beverages are discussed, and an iodimetric method is described in which reflux distillation from dilute orthophosphoric acid and a carrier-stream of nitrogen are used. The reflux condenser effectively retains other iodine-reducing substances, and the iodimetric titration eliminates interference due to the presence of acetaldehyde.

In principle, the determination of sulphur dioxide in a foodstuff requires the distillation of the sample with acid and collection of the sulphur dioxide in some suitable solution in which it can be determined. The fact that so many methods and modifications of conditions of distillation and determination have been proposed[1,2,3] indicates that no entirely satisfactory general procedure has yet been devised. The extensive literature on the determination of sulphur dioxide is confused and sometimes contradictory, possibly owing to lack of appreciation of the errors involved in a particular method, especially if it is applied to a product for which it is not entirely suitable. In most methods there are several possible sources of error, some causing high results and some low, so that apparently correct results can be obtained fortuitously by combination of these effects. Unless, therefore, the separate errors are recognised and each minimised or evaluated, even the apparent complete recovery of added sulphur dioxide is no guarantee of the general reliability of a method. Further, different foodstuffs present different problems owing to the presence of interfering substances.

Any method for the accurate determination of sulphur dioxide in alcoholic beverages should take account of (*a*) the presence of volatile substances other than sulphur dioxide that will give appreciable blank values and (*b*) the presence of acetaldehyde, which can react with sulphur dioxide before it is oxidised (with hydrogen peroxide or iodine) in the receiver. This latter requirement is frequently overlooked; the error involved is usually small (1 to 2 per cent.), but cannot be neglected in an accurate determination.

The various methods for determining sulphur dioxide in wines have been fully reviewed by Deibner.[3] Apart from Ripper's method[4,5] of direct titration with iodine, which is well known to be subject to errors, the most commonly used methods all involve distillation and can be divided into two main groups.

Alkalimetric methods—The acidified sample is boiled under reflux and the sulphur dioxide carried over in a stream of air or inert gas into neutralised hydrogen peroxide, where it is oxidised to sulphuric acid and, finally, titrated with sodium hydroxide. The result can be checked gravimetrically by precipitation as barium sulphate. This is essentially Monier Williams' method.[6] The presence of volatile acids in the sample may lead to high results by titration (depending on the efficiency of the reflux condenser), but does not affect results determined gravimetrically.

Iodimetric methods—The sulphur dioxide distilled from the acidified sample is collected in a suitable receiving solution and subsequently titrated with iodine. Various receiving solutions have been proposed, *e.g.*, sodium hydroxide,[3] sulphuric acid,[7] and iodine.[8] The advantage of this method is the convenience and sensitivity of the iodimetric titration.

Two methods for determining total sulphur dioxide in wines have been described recently, one alkalimetric and the other iodimetric. In describing the first, Kielhöfer and Aumann[9] discussed possible sources of error in the iodimetric titration of sulphur dioxide and concluded that Paul's alkalimetric method is preferable; Paul later described his method in more detail.[10] The wine (10 or 20 ml) is boiled under reflux with 5 ml of 25 per cent. phosphoric acid for 15 minutes in a Lieb - Zacherl apparatus[11]; the liberated sulphur dioxide is carried over in an air stream into 3 ml of 0·3 per cent. hydrogen peroxide (previously neutralised to Tashiro

indicator) and titrated with 0·01 N sodium hydroxide. The essential feature of this apparatus is a highly efficient reflux condenser. Blank determinations of any volatile acids not retained by the condenser can be made by adding a trace of hydrogen peroxide to the sample before distillation. Paul, however, claims that blank determinations for normal wines are negligible, and this was confirmed by Kielhöfer and Aumann. Although this method is satisfactory from the point of view of the blank value, it cannot allow for the error due to acetaldehyde.

In the second method, described by Diemair, Koch and Hess,[12] the sample is distilled from dilute phosphoric acid, and the distillate is collected in buffer at pH 3·4 to prevent loss of sulphur dioxide by volatilisation. The distillate is titrated with 0·01 N iodine, first at pH 3·4 to oxidise the free sulphur dioxide and then after solid sodium hydrogen carbonate has been added to dissociate any acetaldehyde bisulphite. Although this method caters for the presence of acetaldehyde, it makes no allowance for the blank value of volatile iodine-reducing substances other than sulphur dioxide. With ciders this blank value has been found to represent about 5 p.p.m. of sulphur dioxide.

The proposed method combines the advantages of both the procedures described above, since it gives zero blank corrections with ciders and complete recovery of sulphur dioxide in the presence of acetaldehyde.

Method

General principles—

The development of this method was based on the considerations listed below:—

1. In order to be able to titrate acetaldehyde bisulphite it is essential to use an iodimetric method, since the adjustment of pH necessary to dissociate the bound sulphur dioxide is not permissible in the alkalimetric procedure.

2. In principle, Diemair, Koch and Hess's method[12] appeared to offer a rapid and convenient means of determining total sulphur dioxide, particularly if it could be done by steam-distillation in a Markham apparatus.[13] Recovery of sulphur dioxide (10 ml at 300 p.p.m.) from acetaldehyde bisulphite was 99·5 per cent. by this procedure. When, however, ciders that were shown to be completely free from sulphur dioxide (by acid distillation and colorimetric determination[14] by pararosaniline and formaldehyde) were analysed by this method, the cider blank value, due to volatile iodine-reducing substances, corresponded to 3 to 8 p.p.m. of sulphur dioxide. No means could be found of reducing the magnitude of this blank value. The alternative, of determining the blank value by adding a trace of hydrogen peroxide to the cider to oxidise the sulphur dioxide before distillation, was tested on a sulphur dioxide free cider. This was unsuccessful since the blank value was much greater (18 p.p.m. as sulphur dioxide) after treatment with hydrogen peroxide than without (3 p.p.m.); further, traces of hydrogen peroxide in the distillate reacted slowly with potassium iodide to liberate iodine. It was therefore impossible to differentiate chemically between sulphur dioxide and the other volatile iodine-reducing substances present in the distillate obtained by steam-distillation.

3. A physical separation of the volatile iodine-reducing substances, which produce the cider blank value, from sulphur dioxide was achieved by distillation under reflux, as in the alkalimetric procedures. The combination of iodimetric titration with distillation under reflux in a stream of nitrogen is made possible by the fact that the vapour pressure of iodine in potassium iodide solution is greatly reduced by increasing the concentration of potassium iodide. In the proposed method the small amount of iodine volatilised by passing nitrogen through 0·01 N iodine in 5 per cent. potassium iodide solution is completely absorbed in a second tube containing 5 per cent. potassium iodide. This second tube also acts as a second absorber for sulphur dioxide, for which purpose a trace of iodine is initially added to the contents. Since it is essential that no iodine should escape from the second tube, a third absorption tube is included, containing 5 per cent. potassium iodide solution and a drop of starch indicator. This tube normally remains colourless during several determinations.

4. During the distillation, a small amount of the sulphur dioxide re-combines (presumably in the vapour phase) with acetaldehyde, which also distils over. In order to determine this bound sulphur dioxide, the combined contents of tubes 1 and 2 are made weakly alkaline (pH 8·3) to allow the acetaldehyde bisulphite to dissociate. After 30 seconds the solution is acidified and the residual iodine titrated with 0·01 N sodium thiosulphate. Control experiments showed that acetaldehyde itself is not oxidised by iodine under these conditions.

5. In the alkalimetric methods described by Paul[18] and by Kielhöfer and Aumann[9] the sulphur dioxide is carried over in a stream of air. The good recoveries quoted by them are surprising (as recognised by Kielhöfer and Aumann), since most other workers have found it necessary to carry out the distillation in the presence of carbon dioxide or nitrogen. The necessity of avoiding oxidation in the method described below was established by comparing recoveries of sulphur dioxide from 10 ml of a solution containing 245 p.p.m. When the sulphur dioxide was carried over in a stream of nitrogen, the recovery was 245·5 p.p.m.; 223·5 p.p.m. were recovered when a stream of air was used.

APPARATUS—

The apparatus (see Fig. 1) is based on the Monier Williams principle of distillation under reflux in a stream of a carrier gas. It is similar to the Lieb - Zacherl apparatus used by Paul, except that the latter has only one absorption tube. The flask (round-bottomed, 250 ml, with side inlet), double-walled condenser (B24 joints) and tubes (200 mm × 25 mm) are standard pieces of glassware, obtainable from Messrs. Quickfit and Quartz, Ltd. The absorption heads are made from B24 cone-joints with the internal delivery tubes drawn out to a jet. The separate absorption units are joined together by small lengths of polyvinyl chloride tubing. Blood urea distillation tubes, obtainable from Messrs. Quickfit and Quartz, Ltd., can be used but are more difficult to rinse.

The flask is heated by a micro-bunsen burner, with a shield of asbestos with a hole (1½ inches in diameter) being used to prevent charring of the sample. The absorption tubes are shielded from the heat of the bunsen burner by a vertical sheet of asbestos.

The nitrogen supply is taken from a cylinder of oxygen-free nitrogen, reduced to a pressure of about 15 cm of water.

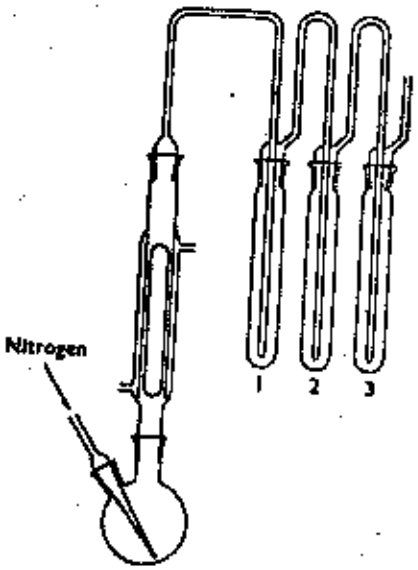

Fig. 1. Apparatus for determining total sulphur dioxide

REAGENTS—

All materials should be of analytical-reagent grade.

Iodine, 0·01 N in 5 per cent. w/v potassium iodide solution—This need not be freshly prepared and can be kept as a stock solution.

Potassium iodide, 5 per cent. w/v—Freshly prepare free from iodine.

Sodium thiosulphate, 0·1 N—Standardise accurately against potassium iodate. Dilute to 0·01 N as required for use.

Sodium phosphate, 0·2 M—A solution containing 72 g of disodium hydrogen orthophosphate, $Na_2HPO_4.12H_2O$, per litre.

...er and Aumann[9] the ... quoted by them are ...other workers have found it ... dioxide or nitrogen. The ...s established by comparing ...ing 245 p.p.m. When the recovery was 245·5 p.p.m.;

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Orthophosphoric acid, 25 per cent. v/v—A solution containing 250 ml of 85 per cent. orthophosphoric acid per litre.

Sulphuric acid, N.

Starch indicator—Prepare a fresh 1 per cent. w/v solution of soluble starch.

PROCEDURE—

By pipette transfer 5-ml portions of 5 per cent. potassium iodide solution to tubes 2 and 3, and add one drop of starch indicator to the contents of tube 3; transfer 10 ml of 0·01 N iodine to tube 1.

By pipette place the sample (10 or 20 ml, containing up to 3 mg of sulphur dioxide) in the 250-ml flask, and add 5 ml of 25 per cent. phosphoric acid. Attach the flask to the condenser. Adjust the flow of nitrogen to a moderate rate (2 to 3 bubbles per second). Heat the flask for 15 minutes with a small flame, and gently boil the contents.

Remove the flame, but keep the nitrogen flowing. Disconnect tube 2, and rinse the delivery tube. Similarly disconnect and rinse tube 1.

Pour and rinse the contents of tube 2 into tube 1, and add 5 ml of 0·2 M sodium phosphate solution. After 30 seconds add 2 ml of N sulphuric acid, and titrate the excess of iodine with 0·01 N sodium thiosulphate solution, adding 5 drops of starch indicator near the end-point. During the titration it is convenient to stir the solution in the tube with a magnetic stirrer having a small polythene-coated iron rod (1 cm long). Subtract the volume of titre from the volume of sodium thiosulphate equivalent to 10 ml of 0·01 N iodine *plus* 5 ml of 5 per cent. potassium iodide found by direct titration. One millilitre of 0·01 N sodium thiosulphate is equivalent to 0·32 mg of sulphur dioxide.

NOTES—

1. A blank titration, carried out by distilling 20 ml of distilled water instead of the sample, should agree with the direct titration of 10 ml of 0·01 N iodine to within ±0·02 ml of sodium thiosulphate solution. This shows that any iodine volatilised from tube 1 is completely retained in tube 2. This is confirmed by the fact that the contents of tube 3 normally remain colourless, and therefore do not need to be replaced during several determinations.

2. Duplicate determinations usually agree within ±0·02 ml of 0·01 N sodium thiosulphate, equivalent to 0·6 p.p.m. of sulphur dioxide in a 10-ml sample.

3. For samples containing more than 3 mg of sulphur dioxide, the volume of 0·01 N iodine in tube 1 can be suitably increased. Similarly, for extremely small amounts of sulphur dioxide the volume of iodine can be reduced, but 5 per cent. potassium iodide solution should then be added to keep a total volume of 10 ml.

4. Samples in which most of the sulphur dioxide is in the free state (*i.e.*, not combined as bisulphite compounds) rapidly evolve their sulphur dioxide in the early stages of boiling, and traces of sulphur dioxide may fail to be absorbed in tube 1. With such samples it is advisable to prolong the flow of nitrogen before starting to heat the solution, so that part of the free sulphur dioxide is carried over in the cold and sufficient iodine is volatilised into tube 2 to retain any sulphur dioxide escaping past tube 1. Alternatively, 2 or 3 drops of 0·01 N iodine may be placed in tube 2 from the pipette before the remainder is placed in tube 1.

TESTS OF RECOVERY OF SULPHUR DIOXIDE

The accuracy of the method was established by investigating (*a*) recovery of free sulphur dioxide from sodium bisulphite solution, (*b*) recovery of bound sulphur dioxide from acetaldehyde sodium bisulphite added to cider and (*c*) the magnitude of the cider blank value due to volatile iodine-reducing substances other than sulphur dioxide.

A solution of sodium bisulphite, calculated to contain approximately 250 p.p.m. of sulphur dioxide, was assayed by the distillation method and by direct iodine titration. In the latter procedure, 10 ml of solution was added by pipette, with stirring, to 10 ml of 0·01 N iodine and 5 ml of 5 per cent. potassium iodide solution; the excess of iodine was then titrated

as in the distillation method. Blank determinations on 10 ml of distilled water were made, both by distillation and by direct titration. The results expressed as millilitres of 0·01 N sodium thiosulphate solution were—

	Distillation	Direct titration
10 ml of distilled water	9·76, 9·74	9·75, 9·74
10 ml of sodium bisulphite	2·08, 2·10	2·00, 2·10

It can be seen that free sulphur dioxide is completely recovered by the distillation procedure, but, in order to ensure this, it is important to have the initial trace of iodine in tube 2 (see Note 4 above). The close agreement between the blank values obtained by distillation and by direct titration supports the observation in Note 1 above.

Recovery of bound sulphur dioxide from acetaldehyde bisulphite added to cider was tested on three ciders that were shown to be completely free from sulphur dioxide (by distillation from acid solution and determination of sulphur dioxide by the colorimetric pararosaniline - formaldehyde method). Results are shown in Table I.

TABLE I

RECOVERY OF BOUND SULPHUR DIOXIDE FROM ACETALDEHYDE BISULPHITE ADDED TO CIDER

Recovery expressed as a percentage of the results obtained by direct titration

Procedure (see text)	Cider A			Cider B			Cider C*		
	Thiosulphate titre, ml	Sulphur dioxide found, mg	Recovery, %	Thiosulphate titre, ml	Sulphur dioxide found, mg	Recovery, %	Thiosulphate titre, ml	Sulphur dioxide found, mg	Recovery, %
I	7·42	2·375	100·0	7·43	2·378	100·0	8·14	2·605	100·0
	7·44	2·380		7·42	2·375		8·12	2·599	
II	7·43	2·378	100·0	7·40	2·370	99·7	8·11	2·595	99·8
	7·42	2·375	99·9	7·41	2·372	99·8	8·12	2·599	99·9
III	7·34	2·350	98·8	7·30	2·336	98·3	8·02	2·565	98·7
	7·35	2·352	98·9	7·28	2·330	98·0	8·00	2·560	98·4
IV	0·01	0·003	—	0·02	0·006	—	0·02	0·006	—
	0·01	0·003	—	0·02	0·006	—	0·02	0·006	—
V	0·01	0·003	—	0·00	0·000	—	0·00	0·000	—
	0·01	0·003	—	0·00	0·000	—	0·00	0·000	—
VI	0·01	0·003	—	0·01	0·003	—	0·01	0·003	—
	0·01	0·003	—	0·02	0·006	—	0·01	0·003	—

* The acetaldehyde bisulphite solution used with cider C was different from that used with ciders A and B.

Portions (10 ml) of acetaldehyde bisulphite solution, prepared from sodium metabisulphite and redistilled acetaldehyde, were added by pipette to 10 ml of 0·01 N iodine and 5 ml of 5 per cent. potassium iodide solution and titrated as described in the distillation method (Procedure I). Further 10-ml portions were distilled in the presence of 10 ml of cider and 5 ml of 25 per cent. phosphoric acid, and the sulphur dioxide was determined (*a*) by the full procedure described above and (*b*) by omitting the treatment with 0·2 M sodium phosphate. The former (Procedure II) gave complete recovery of sulphur dioxide, but when sodium phosphate was omitted (Procedure III) recovery was slightly low, owing to recombination of sulphur dioxide with acetaldehyde. Blank distillations (Procedure V) were also made on 20-ml portions of the ciders to ensure that none of the sulphur dioxide recorded in the previous distillations was due to other iodine-reducing substances.

Although these three ciders showed zero blank values, it cannot be assumed that all ciders would behave similarly, and some means of measuring the blank value of a cider containing sulphur dioxide is needed. Paul[19] added a trace of hydrogen peroxide to the contents of the flask in order to oxidise the sulphur dioxide before distillation. In Procedure IV, therefore, 10 ml of acetaldehyde bisulphite solution, 10 ml of cider and 5 ml of 25 per cent. phosphoric acid (*i.e.*, as in Procedure II) were treated with 10 drops of 1 per cent. w/v hydrogen peroxide before distillation; the sulphur dioxide was completely oxidised, and the blank values were negligible. In Procedure VI, 20 ml of cider, without added acetaldehyde bisulphite, were oxidised with hydrogen peroxide before distillation; the blank

f distilled water were made, ass s millilitres of 0·01 N

Direct titration
9·75, 9·74
2·09, 2·10

recovered by the distillation the initial trace of iodine in blank values obtained by Note 1 above.

sulphite added to cider was m sulphur dioxide (by dis- e by the colorimetric para- e I.

SULPHITE ADDED TO CIDER
ed by direct titration

Cider C*		
Thio-sulphate titre, ml	Sulphur dioxide found, mg	Recovery, %
8·14	2·605	100·0
8·12	2·599	
8·11	2·595	99·8
8·12	2·599	99·9
8·02	2·565	98·7
8·00	2·560	98·4
0·02	0·006	—
0·02	0·006	—
	0·000	—
	0·000	—
0·01	0·003	—
0·01	0·003	—

ferent from that used with

ed from sodium metabisul- ml of 0·01 N iodine and 5 ml d in the distillation method e of 10 ml of cider and 5 ml determined (*a*) by the full th 0·2 N sodium phosphate. dioxide, but when sodium w, owing to recombination edure V) were also made on ide recorded in the previous

annot be assumed that all the blank value of a cider hydrogen peroxide to the efore distillation. In Pro- 10 ml of cider and 5 ml of with 10 drops of 1 per cent. e was completely oxidised. ml of cider, without added efore distillation; the blank

values were again negligible. Thus, although hydrogen peroxide was found to increase the amount of steam-volatile iodine-reducing compounds (see Note 2 under "General Principles," p. 305), these substances were effectively retained by the reflux condenser in the apparatus described.

For ordinary purposes and with normal ciders, there seems to be no need to carry out routine blank determinations. The above treatment with hydrogen peroxide would only be needed if other evidence suggested the presence of volatile iodine-reducing substances that might interfere with the determination of sulphur dioxide.

REPRODUCIBILITY—

The method gives highly reproducible results; seven replicate determinations on 20-ml portions of a cider, containing 130 p.p.m. of sulphur dioxide, gave results between 8·06 and 8·08 ml of 0·01 N sodium thiosulphate, with a coefficient of variation of only 0·10 per cent.

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Received *August 23rd*, 1962

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Inhibition of Prothrombin, Thrombin and Autoprothrombin C with Enzyme Inhibitors*

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As a means of determining active groups on a molecule organic chemists use reagents which specifically react with chemical groups. Biochemists have employed this same method to determine active sites on enzymes. Using these methods the inhibitory effect of various chemical modifications of three molecules important in blood coagulation, prothrombin, thrombin and autoprothrombin C, was explored in this investigation.

Bovine prothrombin, thrombin and autoprothrombin C have been isolated in this laboratory, and their amino acid composition determined. For easy reference to the amino acid residues of all three, a table has been published by Seegers, Cole, Harmison and Marcinink (21). All amino acids are present in each of these three molecules, and hence all of the polar groups – amino, imidazole, guanidyl, carboxyl, disulfide, sulfhydryl, phenol, hydroxyl, indole, amide, and thiomethyl – are present, and could function as an active site.

By specifically blocking groups and then measuring the activity of the molecule to see if this produced a loss, we expected to get information concerning molecular structures essential for activity. If activity was lost, one might assume that this particular group was essential for the molecule to display its proper activity. It is possible that more than one group is essential for the activity, but unfortunately the number of specific blocking reagents is very limited. So, in all cases, several reagents were used to determine the essential position of one particular group.

Nanninga (15) studied the effect of numerous chemicals on the activity of thrombin and concluded that thrombin has an essential amino group. He found neither sulfhydryl groups nor disulfide bonds essential for thrombin activity.

* This investigation was supported in part by a research grant HE 03424-08 from the National Heart Institute, National Institutes of Health, U.S. Public Health Service.

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Thromb. Diath. Haemorrh. v. 13 (3-4) 1965

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Carter and Warner (4, 5) found that reduction of disulfide bonds resulted in decreased activity in prothrombin but not thrombin. Sulfhydryl blocking agents did not result in lost activity of either prothrombin or thrombin. They concluded from these observations that prothrombin has an essential disulfide bonding, but thrombin does not, and that sulfhydryl groups are not essential for the activity of either.

Koppel, *et al.* (8, 9), found that sulfhydryl blocking agents decreased the prothrombin concentration of plasma as determined by the two-stage analysis. Through a series of substitution experiments which included the substitution of purified or concentrated clotting factors, they concluded that the essential sulfhydryl group was not in prothrombin. It was an accelerator of prothrombin activation, which they decided was Ac-globulin (factor V). Aoki, Harmison and Seegers (1) confirmed this observations. They found that their highly concentrated preparations of Ac-globulin (factor V) lost activity following incubation with sulfhydryl blocking agents.

Materials and Methods

Bovine prothrombin was prepared according to the method of Seegers (19, 24). Thrombin and autoprothrombin C were prepared by methods previously described (21). The chemicals used were added to distilled water immediately prior to use. The pH of each solution was neutralized so that it was between pH 6.5 and 7.5, and then made up to the desired molarity, which in most cases was 0.1 M. An aliquot of this solution was diluted so that two different concentrations of each chemical were used. Unless otherwise indicated, pH adjustments of acidic solutions were accomplished with NaOH, and of alkaline solutions with HCl.

Each chemical was tested against the three proteins, prothrombin, thrombin and autoprothrombin C. One part chemical and one part protein to be tested was mixed together in a plastic tube, making the final concentration of chemical in most cases 0.05 and 0.01 M. At zero time, immediately upon mixing protein and chemical, an aliquot was removed and its activity determined. This value was considered to be 100% activity, and also served as a control to determine if at this concentration the chemical reagent interfered with the assay procedure for the protein. The mixture of one part chemical reagent and one part protein was left in stoppered plastic tubes at room temperature. After two hours another aliquot was removed and the activity of the protein again measured. After 24 hours a third aliquot was taken and its activity determined. The protein activities after two and 24 hours incubation were reported as percentages of the activities at zero time.

Controls were set up which contained one part of the protein and one part distilled water. These controls measured any spontaneous deterioration of the protein during the 24-hour incubation period. The proteins were in concentrated solutions (15,000 U/ml prothrombin or thrombin and 1,000 U/ml autoprothrombin C) during incubation periods and could thus be greatly diluted before assaying their activities. The chemicals with which they had been incubated were, consequently, also diluted. This was perhaps sometimes important in preventing the chemical from interfering with the assay.

The prothrombin concentrations were determined by the modified two-stage assay of Ware and Seegers (28). Thrombin concentration was determined by its ability to clot fibrinogen as described by Seegers and Smith (26). The method described by Cole, Marciniak and Seegers (8) was employed to quantitate autoprothrombin C activity.

Experimental Results

Table 1 is designed as a summary of the results with numerous chemicals which were tested.

Reducing agents. Reducing agents act on unmodified proteins to convert disulfide bonds to sulfhydryl groups. The reducing agents which were employed in these experiments included several reagents having SH groups: cysteine, thioglycolic acid, 2, 3-dimercapto-1-propanol (BAL) and reduced glutathione. Other reducing agents, having no SH group, included: sodium sulfite, sodium bisulfite, sodium hydrosulfite, and ascorbic acid.

In this study all of the reducing agents altered the prothrombin and autoprothrombin C molecules so that they no longer displayed their typical activity. The thrombin molecule was unaffected by the reducing agents having SH groups. It was somewhat affected by sodium sulfite in two hours; however 24 hours were required to show a marked inhibitory effect of sodium sulfite, sodium bisulfite, and sodium hydrosulfite upon the ability of thrombin to clot fibrinogen.

Later we found that thrombin could after all be inactivated with reducing agents. Thioglycolic acid, first neutralized with sodium hydroxide, destroyed the activity at 0.2 M concentration. At 0.05 M some of the activity was first lost and then recovered spontaneously. Since much depends upon the relative concentration of thrombin and reducing agent we at first failed to observe a change.

Oxidizing agents. Oxidizing reagents containing the disulfide linkage specifically oxidize proteins having free sulfhydryl groups. Typical reagents which were used for studying this group were: cystine, and oxidized glutathione. As a strong oxidizing agent potassium permanganate was employed. The mild oxidizing agents employed were: hydrogen peroxide, potassium ferricyanide, 2,6-dichlorophenolindophenol sodium salt, and sodium selenite.

Oxidizing reagents having disulfide linkages had no inhibitory effect on prothrombin, thrombin or autoprothrombin C activity. A strong oxidizing agent such as potassium permanganate, even in low concentration, inhibited all three molecules within two hours incubation. With mild oxidizing agents there was decreased activity of all three after 24 hours, but varied in their inhibitory effect in two hours, generally showing a low degree of inactivation of prothrombin, thrombin and autoprothrombin C. Autoprothrombin C was the most sensitive of the three to hydrogen peroxide. Its activity was reduced to approxi-

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Table 1. Chemical Modification of Prothrombin, Thrombin and Autoprothrombin C and Effect of Their Activity.

Reagent	Prothrombin		Thrombin		Autoprothrombin C	
	2 hrs	24 hrs	2 hrs	24 hrs	2 hrs	24 hrs
Reducing agents:						
Ascorbic acid	++	++++	0	0	++++	++++
Thioglycolic acid	++	++	0	0	+++	+++
BAL	++++	++++	0	0	++++	++++
Reduced glutathione	++	+++	0	0	+++	++++
Cysteine	+++	++++	0	0	+++	++++
Sodium bisulfite	++++	++++	+	+++	++++	++++
Sodium hydrosulfite	++++	++++	0	+++	++++	++++
Sodium sulfite	++	++++	+	++++	++	++++
Alkylating agents:						
Iodoacetate 0.05 M	0	+++	+	+	+	+++
Iodoacetate 0.01 M	0	+	0	0	0	0
Oxidizing agents:						
Oxidized glutathione	0	0	0	0	0	0
Cystine	0	0	0	0	0	0
Hydrogen peroxide	0	++	0	+++	++	+++
Potassium permanganate[1])	++++	++++	++++	++++	++++	++++
Potassium ferricyanide	0	++	0	0	0	+
2,6-Dichlorophenolindophenol[2])	++	+++	0	++++	++	++++
Sodium selenite	0	0	0	0	0	0
Mercaptide forming agents:						
Parachloromercuribenzoate[3])	0	0	0	0	0	0
Mapharside	0	0	0	0	0	0
Merthiolate	0	0	0	0	0	0
Phenylmercuric Hydroxide	0	++	0	0	0	0
P-arsanilic acid	0	0	++	++	++	+++
Phenylmercuric nitrate	0	+	0	0	0	0
Carbonyl reagents:						
Phenylhydrazine	++	++++	+	++++	++	++++
Hydroxylamine	+	++++	0	0	++	++++
Sodium bisulfite	++	++++	+	++++	++	++++
Amino group reagents:						
Formaldehyde[4])	+++	++++	+	+	0	+
S-acetylmercaptosuccinic anhydride	++++	++++	++++	++++	0	++++

1) Final concentration 0.0005 M.
2) Final concentration 0.005 M.
3) Final concentration 0.005 M.
4) Final concentration 0.015 M.

Tab. 1: Continued

Reagent	Prothrombin		Thrombin		Autoprothrombin C	
	2 hrs	24 hrs	2 hrs	24 hrs	2 hrs	24 hrs
Miscellaneous:						
Phenol[5]	0	0	0	0	0	0
Guanidine hydrochloride	0	0	0	0	0	0
Lithium bromide	0	0	0	0	0	0
Potassium ferrocyanide	0	+	++	+++	0	+

5) Final concentration 1% solution.

The first column under each protein tabulates the effect of two hours incubation with the chemical, and the second column 24 hours incubation. If from 90 to 100% of the original activity was present at the end of the time period, it is designated in the table by zero. One plus indicates that between 70 and 90% of the original activity remained. Two plus indicates that between 50 and 75% of the original activity was retained. Three plus indicates that between 25 and 50% of the original activity was present, and four plus indicates that less than 24% of the original activity remained at the end of the period of incubation. Similar results were obtained whether the final concentration of the chemical was 0.05 or 0.01 M except in the case of iodoacetate. Therefore results for both concentrations of iodoacetate are recorded in the table. Other results in the table are for a final concentration of 0.05 M unless otherwise indicated. Solutions were neutralized to a pH range of pH 6.5 to 7.5. Incubation was carried out at room temperature. In the case of thrombin reducing agents such as thioglycolic acid are effective in higher concentrations.

mately 60% of its original activity within the first two hours whereas the others still retained their activity. All of the materials, however, had lost activity during 24 hours incubation with hydrogen peroxide. Potassium ferricyanide did not decrease the activities of any of the proteins during the first two hours. Prothrombin and autoprothrombin C lost some activity during 24 hours incubation, but thrombin remained fully active. The compound 2,6-dichlorophenolindophenol destroyed the activity of all three in 24 hours. It was most destructive of prothrombin and autoprothrombin C. Within two hours approximately 30% of the prothrombin was gone. Activity of all three molecules was greatly reduced during 24 hours incubation.

Mercaptide forming reagents. Heavy metals, particularly mercury, arsenicals, and selenites, combine with single sulfhydryl groups under mild conditions. The mercury containing compounds which were used in this study included: p-chloromercuribenzoate, merthiolate, phenylmercuric nitrate, and phenylmercuric hydroxide.

These reagents exerted no inhibitory effect during the first two hours incubation with prothrombin, thrombin or autoprothrombin C. After 24 hours incubation, the only inhibitory effects noted were a decrease of 45% and 25% of prothrombin activity by phenylmercuric hydroxide and phenylmercuric nitrate, respectively.

The arsenic containing compounds employed were both pentavalent arsenicals: mapharside, and p-arsanilic acid. Mapharside had no effect upon the proteins tested. The p-arsanilic acid had the most inhibitory effect against autoprothrombin C, which lost 25% of its activity within the first two hours and 60% in 24 hours. Thrombin decreased in activity approximately 25% in two hours and nearly 50% in 24 hours. Prothrombin retained its activity during the 24 hours incubation with p-arsanilic acid.

Alkylating agents. The only reagent of this groups we studied was iodoacetic acid. In the presence of a 0.01 M concentration, the proteins were unaffected, with the exception of prothrombin, which lost 28% of its activity during a 24-hour incubation period. However, when the proteins were incubated in a 0.05 M solution of neutral iodoacetic acid, thrombin activity decreased about 12% within the first two hours, then remained constant during a 24-hour period of incubation. Although prothrombin lost no activity during the first two hours, a 70% decrease occurred during the 24-hour period. Autoprothrombin C activity decreased 15% during the first two hours and about 55% during the 24-hour incubation period.

Amino group reagents. Representing this group of reagents were: formaldehyde solution, acetylmercaptosuccinic anhydride. Prothrombin activity proved to be quickly decreased in the presence of formaldehyde. Thrombin activity dropped nearly 25% in the first two hours and did not decrease further during the next 22 hours incubation. Autoprothrombin C was resistant to inhibition by formaldehyde during the first two hour incubation period and lost only about 15% during 24 hours incubation.

Prothrombin and thrombin activities were lost during two hours incubation with acetylmercaptosuccinic anhydride. Autoprothrombin C activity was unaffected following two hours incubation with this reagent, but nearly all activity disappeared following 24 hours incubation.

Carbonyl group reagents. Three reagents were employed to study the possibility of carbonyl groups being essential groups in prothrombin, thrombin and autoprothrombin C: phenylhydrazine hydrochloride, hydroxylamine sulfate, and sodium bisulfite. Activity of all three proteins decreased over 75% during 24 hours incubation with phenylhydrazine. Autoprothrombin C activity decreased approximately 45% within the first two hours, prothrombin approximately 30% and thrombin approximately 20%. Thrombin activity was stable during a 24 hour incubation period with hydroxylamine. Autoprothrombin C activity fell approximately 40% during two hours incubation and 80 to 85% during 24 hours. Prothrombin activity diminished 15 to 20% during two hours incubation and between 70 and 80% after a 24-hour incubation period with hydroxylamine.

The prothrombin and autoprothrombin C activity was rapidly lost in contact with sodium bisulfite. Thrombin was somewhat affected by sodium bisulfite in two hours; however 24 hours was required to show a marked inhibitory effect upon the ability of thrombin to clot fibrinogen.

Miscellaneous reagents. Four reagents were tested which do not nicely fall into any of the above categories: phenol, lithium bromide, guanidine hydrochloride, and potassium ferrocyanide.

Phenol, in a 1% solution, did not destroy the activity of prothrombin, thrombin or autoprothrombin C during a 24-hour incubation period. Lithium bromide and guanidine hydrochloride, in the low concentrations used, did not affect the activity. Potassium ferrocyanide was not very effective in decreasing the activity of prothrombin or autoprothrombin C. Activity was unaffected after two hours but, after 24 hours, up to 25% of the activity was lost. Nearly 50% of the thrombin activity disappeared after two hours incubation with this agent and nearly 75% after 24 hours.

Discussion

Disulfide Group

Reducing agents act on unmodified proteins to reduce disulfide bonds to sulfhydryl groups. Those reducing reagents containing sulfhydryl groups are very specific in their action on disulfide bonds; on the other hand, they activate enzymes requiring sulfhydryl groups (18). Sulfites and bisulfites add to disulfide bonds to give a sulfhydryl and thiosulfite (6).

In this series of investigations reducing the disulfide bond did not decrease thrombin activity in the regular concentrations we were using. By increasing the concentration to 0.2 M inactivation was observed. At 0.05 M the results were variable, and there was even spontaneous recovery of activity subsequent to inactivation. Both prothrombin and autoprothrombin C activity rapidly decreased following reduction of the disulfide bond, which indicates disulfide bondings are essential for their activity. This agrees with the statements of Carter and Warner (4, 5) concerning prothrombin and thrombin, with Koppel, *et al.* (8, 9) concerning prothrombin and Nanninga's (15) investigation of thrombin, with the exception of the observation that thrombin can be inactivated if the concentration of reducing agent is high enough.

Sulfhydryl Groups

Sulfhydryl groups oxidize readily to disulfides. However, the only reagents, which can be considered to specifically oxidize sulfhydryls, are disulfide reagents (3). The disulfide oxidizing reagents used in this investigation did not

decrease the activity of prothrombin, thrombin or autoprothrombin C. On the other hand, strong oxidizing agents markedly decreased activity of all three proteins, and since a low degree of inactivation was seen in all three following incubation with mild oxidizing agents, it must be assumed that this effect was due to oxidation of groups other than sulfhydryl. This does not exclude the essential position of sulfhydryl groups, however, since these groups must be freely reacting and be located close together in order for oxidation to disulfides to occur.

Mercaptide forming reagents are able not only to react with free sulfhydryl groups but are also able to react with sluggish ones. The heavy metals, mercury, arsenic and selenite combine with single sulfhydryl groups under mild conditions. Among this type of reagent, p-chloromercuribenzoate is one of the most widely used and probably most specific. It is able to combine with a single sulfhydryl group at low concentrations of reagent.

The trivalent arsenicals have a striking affinity for sulfhydryl groups. Mapharside has been used clinically in the past for treatment of trypanosomal and spirochetal infections. *In vivo* this pentavalent arsenical is converted to a trivalent form and then exerted its effect by blocking essential sulfhydryl containing enzymes (16); therefore, one would except that mapharside would block sulfhydryl groups only when used *in vivo*.

In the presence of sulfhydryl groups iodoacetic acid forms a thioether. At one time iodoacetic acid was thought to be specific for proteins which contained sulfhydryl groups. It is now realized that, during a longer incubation period, iodoacetate reacts with other groups, including amino, phenol and indole (14). During this investigation, the clotting proteins decreased in activity only in high concentrations and following long incubation, so most likely some group other than sulfhydryl was involved.

Considering the results of the three methods used here to study the essential position of sulfhydryl groups – that is, specific oxidizing agents which contain disulfide linkages, the mercaptide forming reagents, and rapid inactivation with iodoacetate – we have found that sulfhydryl groups are not essential for the clotting activities of prothrombin, thrombin, or autoprothrombin C.

Amino Group

Acetic anhydride is a valuable reagent for studying the essential nature of amino groups without causing denaturization of the protein, if the protein is kept cold and subjected to a short exposure period. More active or prolonged exposure will probably result in other than amino groups being involved. A method for acetylation of prothrombin and thrombin has been reported which does not denature either molecule but certainly changes their properties (11, 12).

Prothrombin, after being acetylated, is no longer able to give thrombin, which clots fibrinogen, upon activation with thromboplastin or concentrated sodium citrate. The prothrombin molecule, in contact with thromboplastin, however can be converted to acetylated thrombin. Acetylated thrombin no longer has the ability to clot fibrinogen but retains its ability to split synthetic substrates such as p-toluene sulfonyl-l-arginine methyl ester – that is, it has retained its esterolytic capacity. Previous publications of Seegers and Landburn (23) have pointed to the fact that esterolytic activity of thrombin is not always coexisting with clotting activity. Normally thrombin has both esterolytic and clotting activity.

The fact that acetylated prothrombin is activated to give acetylated thrombin indicates that the amino groups acetylated in prothrombin are probably those which are acetylated in thrombin. Acetylated thrombin which is obtained from activation of acetylated prothrombin has the same characteristics as acetylated thrombin which has been obtained by acetylating isolated thrombin.

Acetylmercaptosuccinic anhydride has been reported to have a strong preference for amino groups (10). Prothrombin and thrombin activities were completely lost following two hours incubation with acetylmercaptosuccinic anhydride. Autoprothrombin C activity was unaffected following two hours incubation with acetylmercaptosuccinic anhydride, but nearly all of its activity was lost following 24 hours incubation with this reagent.

Formaldehyde in neutral solution reacts with free amino groups in an equilibrium type reaction. The reaction of formaldehyde with proteins is complicated and, although it cannot be considered a specific reagent, it is of value when considered in conjunction with other reagents. Prothrombin activity was markedly decreased by formaldehyde, thrombin activity was decreased slightly and autoprothrombin C activity was not affected until after a long incubation period.

Markwardt (13) found that as thrombin activity disappeared in formaldehyde hirudin binding capacity was diminished and, with the return of thrombin activity, hirudin binding capacity was restored. He also found that hirudin did not combine with acetylated thrombin. This was interpreted to indicate that an amino group of thrombin must be available to combine with hirudin. In another study we found that hirudin does not combine with autoprothrombin C, and here we are reporting that autoprothrombin C activity is not lost when incubated with reagents which bind amino groups.

Nitrous acid reacts with amino groups of proteins, but it also reacts with other groups including sulfhydryl and phenol. Although it cannot be considered a specific reagent, it is felt that, if it inhibits rapidly, the reaction is with amino groups and, if slowly, it is with the phenol groups. Seegers reported in 1940 (19)

that the thrombin and prothrombin activity was rapidly destroyed by dilute solution of nitrous acid at pH 5.5, which indicates that their activities were dependent upon the integrity of alpha amino groups.

Compiling the results of this present report, and those previously published, free amino groups are required for normal prothrombin and thrombin activity, but autoprothrombin C does not appear to require amino groups for its activity.

Carbonyl Group

Hydrazine and hydroxylamine are known to react with aldehyde and ketone groups. Prothrombin and autoprothrombin C activities were lost following incubation with both phenylhydrazine and hydroxylamine. The results with thrombin were variable in that it retained full activity following incubation with hydroxylamine, but decreased somewhat with phenylhydrazine.

In addition to reducing disulfide bonds, sodium bisulfite in a saturated aqueous solution will react to form addition compounds with aldehydes and methyl ketones (17). Although the sodium bisulfite incubated with prothrombin, thrombin and autoprothrombin C was only 0.05 M, the decreased activity noted could possibly have been a result of some combination with aldehyde or methyl ketone groups. Prothrombin and autoprothrombin C activities had the greatest decrease and thrombin activity was somewhat decreased.

Olcott and Fraenkel-Conrat (18) state that no protein group is known to react with hydroxylamine or hydrazines in the concentrations used and they draw the conclusion that few enzymes which are markedly inhibited by these low concentrations should be ascribed to the presence of prosthetic groups or coenzymes containing ketone or aldehyde groups. On the other hand, the inhibiting effect has not been adequately explained in all cases. The results found here indicate that prothrombin and autoprothrombin C activity is decreased by inhibiting aldehyde or ketone groups, and loss of thrombin activity is inconclusive.

Miscellaneous Reagents

Phenol solution did not decrease the activity of the clotting factors investigated. This does not give any information about the active sites on the molecules, but allows the use of phenol as a preservative agent in the prevention of bacterial contamination if such is desired.

Progressive inhibition of thrombin activity by potassium ferrocyanide is in agreement with the report by Nanninga (15).

Comparative Abilities of Various Reagent Groups to Inhibit Clotting factors

Table 2 contains a summary of the effect of various reagent groups on activities of five purified clotting factors. Included in the table are the results of

Table 2. Comparative Abilities of Various Reagent Groups to Inhibit Clotting Factors.

	Prothrombin	Thrombin	Autoprothrombin C	Ac-globulin[1]	Autoprothrombin I[2]	Platelet Cofactor I[3]
Reducing agents	+	−	+	−	+	−
Sulfhydryl blocking agents	−	−	−	+	−	−
Amino group	+	+	−	−	?	+
Oxidizing agents						
a) Nonspecific	+	+	+	+	+	+
b) Disulfide	−	−	−	+	±	−
Carbonyl group	+	±	+	±	+	+

+ Indicates that reagents used caused inhibition of the protein activity.
− Indicates that reagents used did not cause inhibition of protein activity.
? Indicates that the reagents were not tested with clotting factor.
± Results inconclusive. In the case of Ac-globulin hydroxylamine tended to stabilize.
1) Reference (1).
2) Reference (22).
3) Reference (2).

prothrombin, thrombin and autoprothrombin C reported in this publication; Ac-globulin (factor V) published by Aoki, *et al.* (1); autoprothrombin I published by Seegers and Kagami (22); and platelet cofactor I (factor VIII) published by Baker and Seegers (2).

In the table a plus indicates that the representative reagents of the group caused inhibition of the activity of the clotting factor. A plus also indicates that the group is essential for the activity of that clotting factor. From the table it can be noted that disulfide bonds which are readily reduced are essential for activities of prothrombin, autoprothombin C and autoprothrombin I (factor VII). Sulfhydryl groups are essential only for the activity of Ac-globulin. Prothrombin, thrombin, and platelet cofactor I (factor VIII) contain essential amino groups; autoprothrombin C does not; and it has not been determined for Ac-globulin or autoprothrombin I. A carbonyl group is essential for activities of prothrombin, autoprothrombin C, autoprothrombin I, and platelet cofactor I. All of the clotting factors are readily inactivated by oxidizing agents, but not by those oxidizing agents containing disulfide groups, which means that groups other than sulfhydryl were oxidized. Ac-globulin (factor V) is the exception. Due to its essential sulfhydryl group Ac-globulin readily loses activity by oxidizing agents containing disulfide groups.

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Summary

Numerous chemical enzyme inhibitors were employed to determine the active polar groups in the prothrombin, thrombin and autoprothrombin C molecules. Prothrombin and autoprothrombin C activities were decreased by the reduction of disulfide bonds, but thrombin activity was affected only at higher concentrations of reducing agent. Amino groups were not essential for the activity of autoprothrombin C; however, the blocking of such groups decreased the activities of prothrombin and thrombin. The blocking of carbonyl groups decreased the activity of both prothrombin and autoprothrombin C, but thrombin was not inactivated by hydroxylamine. The activities of none of the three were decreased by sulfhydryl blocking agents. Nonspecific oxidizing agents reduced the activities of all three molecules; however, oxidizing agents which specifically oxidize sulfhydryl groups, were incapable of decreasing the activities of prothrombin, thrombin or autoprothrombin C. The sulfhydryl groups are not essential for the activities of prothrombin, thrombin or autoprothrombin C.

Résumé

On a utilisé de nombreux inhibiteurs d'enzyme afin de déterminer les groupes polaires actifs des molécules de prothrombine, thrombine et autoprothrombine C. L'activité de la prothrombine et celle de l'autoprothrombine C sont diminuées par la réduction des liaisons disulfures, l'activité de la thrombine n'est affectée qu'aux concentrations plus élevées de l'agent réducteur. Les groupes aminés ne sont pas essentiels pour l'activité de l'autoprothrombine C, par contre, le blocage de ces groupes diminue l'activité de la prothrombine et de la thrombine. Le blocage des groupes carbonyles fait diminuer l'activité de la prothrombine et de l'autoprothrombine C, mais la thrombine n'est pas inactivée par l'hydroxylamine. L'activité d'aucune des trois molécules n'est bloquée par les réactifs du groupe sulphydryle. Les agents oxydants non spécifiques réduisent l'activité des trois molécules, par contre les oxydants spécifiques des groupes sulphydryles sont incapables d'altérer l'activité de la prothrombine, de la thrombine et de l'autoprothrombine C. Les groupes sulphydryles ne sont pas essentiels pour l'activité de la prothrombine, de la thrombine et de l'autoprothrombine C.

Zusammenfassung

Zahlreiche chemische Fermenthemmstoffe wurden angewendet, um die aktiven polaren Gruppen in den Molekülen von Prothrombin, Thrombin und Autoprothrombin C zu bestimmen. Die Aktivität von Prothrombin und Autoprothrombin C wurde durch Reduktion der Disulfid-Bindungen vermindert, die

Aktivität von Thrombin wurde aber nur von wesentlich höheren Konzentrationen des reduzierenden Agens beeinflußt. Aminogruppen waren nicht von Wichtigkeit für die Aktivität von Autoprothrombin C; Blockierung solcher Gruppen hingegen verminderte die Aktivität von Prothrombin und Thrombin. Die Blockierung von Carbonylgruppen verminderte die Aktivität von Prothrombin und Autoprothrombin C, Thrombin hingegen wurde von Hydroxylamin nicht inaktiviert. Die Aktivität keiner der drei untersuchten Substanzen wurde durch sulfhydrylblockierende Agenzien vermindert. Unspezifisch oxydierende Agenzien reduzierten die Aktivität aller drei Moleküle; oxydierende Agenzien hingegen, welche in spezifischer Weise Sulfhydrylgruppen oxydieren, verminderten die Aktivität von Prothrombin, Thrombin oder Autoprothrombin C nicht. Sulfhydrylgruppen sind also nicht von Wichtigkeit für die Aktivität von Prothrombin, Thrombin und Autoprothrombin C.

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Int. Sugar. J. 67 (1965)

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DETERMINATION OF SULPHUR DIOXIDE IN WHITE SUGAR

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Introduction

Any method for the determination of sulphur dioxide in white sugar should have an effective range applicable to sulphur dioxide contents of from less than 1 p.p.m. to more than 20 p.p.m. At the midpoint of this range the concentration of sulphur dioxide is only about 0·3 micro-equivalents per gram of sugar and it is desirable to measure sulphur dioxide at less than one tenth of this concentration.

Most of the conventional procedures involve either direct titration of a solution of the sugar with iodine[1] or else modifications[2,3] of the Monier-Williams distillation[4], and, since the total amount of sulphur dioxide in the distillate is normally too low for the gravimetric variations, the distillation procedures are also completed by titration of the sulphur dioxide either as such or after oxidation to sulphuric acid. Even with 0·02N titrant, the quantitative determination of the sulphur dioxide from 50 g of sugar is only sufficient to yield a titre of 0·075 ml for each 1 p.p.m. of sulphur dioxide in the sugar so that the precision of the determination is low in the lower ranges of concentration; in distillation techniques, the physical size of the apparatus necessary to handle the large quantities of sugar can lead to excessive oxidation and physical losses, while direct titration of the bulky sugar solutions is subject to interference and to oxidation and end-point errors.

More sensitive and specific procedures for estimating low concentrations of sulphur dioxide were therefore investigated and the selected procedure has been developed from the acid-bleached fuchsin method of STEGMANN[5] and from the bleached para-rosaniline method of WEST & GAEKE[6].

The WEST & GAEKE method is somewhat misleading in that the bleached reagent is prepared from "a 1% aqueous solution of para-rosaniline hydrochloride". None of the rosaniline dye-stuffs examined in the present investigation have had a solubility approaching 1% and the probable explanation is that of PATT *et al.*[7], who suggested that the high solubilities reported for some samples were due to the presence of diluting amounts of some inert contaminant in the dyestuff. In consequence the bleached reagents reported here have invariably been prepared from a saturated solution of the dyestuff. The bleached dye prepared from the saturated solution was found to be stable for at least five weeks and identical standard curves were obtained on bleaching aliquots from a single sample of saturated dye solution over a period of 3 months.

Various authors have selected different rosaniline dyes for use in this type of reaction. As none of the dyes would be chemically pure it is probable that the stated preferences refer to selection between the particular commercial samples which were available to the authors and not necessarily to any fundamental superiority of one chemical compound. Whereas WEST and GAEKE preferred para-rosaniline to rosaniline, little difference was found in the sensitivity of the reagents available for the present investigation; although para-rosaniline hydrochloride (Hopkin and Williams 6496.6) was marginally the more sensitive, it did not appear to be as stable in the bleached form as rosaniline hydrochloride (British Drug House, Laboratory Reagent) and so the latter was adopted for use.

The amounts of bleached rosaniline and formaldehyde have been increase to twice those used by WEST and GAEKE because, with the increased amount of reagent, the absorption curve departs less from linearity at the higher concentrations of sulphite.

An additional stage has been introduced into the procedure; sugar solutions are adjusted to approximately pH 11 before addition of the chromogenic reagents.

Reagents

A. Saturated aqueous solution of rosaniline hydrochloride: Suspend excess (about 1 g per 100 ml) rosaniline hydrochloride in distilled water, heat to about 50°C and allow to cool with shaking, then stand for 48 hours with occasional shaking and filter.

B. Bleached rosaniline: To 4 ml of rosaniline solution A, add 6 ml of concentrated hydrochloric acid, mix and dilute to 100 ml. The decolorization is not instantaneous and the reagent should not be used for at least 1 hour after preparation.

C. Formaldehyde solution, 0·2%: Dilute 5 ml of 40% formaldehyde with water to 1 litre.

Method

Dissolve the sugar under test in distilled water with gentle mixing and add dilute sodium hydroxide to produce a solution containing 0·5 to 30 µg of sulphur dioxide per 10 ml and which is approximately 0·004N with respect to sodium hydroxide. For most sugars this concentration may be achieved in solutions containing 4 ml of 0·1N sodium hydroxide and either 10 g or 20 g of sucrose per 100 ml. If the general level of sulphur dioxide is less than 2 p.p.m.

[1] AMBLER *et al.*: *Ind. Eng. Chem., Anal. Ed.*, 1931, 3, 339.
[2] SAUSHKIN: *Proc. 12th Sessions I.C.U.M.S.A.*, 1959, 166.
[3] DUBOUR & BUSSAT: *Int. Abstr. Agric.*, 1959, 72, 508, 673.
[4] MONIER-WILLIAMS: *Ministry of Health, Public Health Rpt.*, 1927, 643.
[5] *J. Soc. Chem. Ind.*, 1942, 61, 18.
[6] *Anal. Chem.*, 1956, 28, 1816.
[7] *ibid.*, 1962, 34, 1660.

the sucrose concentration may be increased to 40 g per 100 ml.

Transfer 10 ml of the sugar solution to a clean dry test tube (alternatively 1 to 4 g of dry sugar may be weighed and dissolved directly in a test tube graduated at 10 ml and made up to volume after addition of 0·4 ml of 0·1N sodium hydroxide); add 2 ml of bleached rosaniline (B) followed by 2 ml of 0·2% formaldehyde (C). Mix and allow to stand at room temperature for 30 ± 5 minutes.

Measure the optical density of the solution in a 1 cm cell at approximately 560 mμ (a Kodak No. 5 filter, peak transmission 565 mμ is suitable) and determine the sulphur dioxide content by reference to a standard absorption curve produced under the same conditions.

Standard Absorption Curve

The absorption curve is prepared by use of standard solutions of known amounts of sodium sulphite added to sulphur dioxide free sucrose, e.g. B.D.H. "Analar" sucrose, dissolved in 0·004N sodium hydroxide. The sucrose is added to all the standard solutions with the prime objective of minimizing oxidation of the sulphite during preparation and handling of the standards. Provided that the solutions are made approximately 0·004N with respect to sodium hydroxide before addition of the acidic bleached rosaniline, the presence of sulphur dioxide-free sucrose in concentrations of up to 40 g per 100 ml does not sensibly affect the colour yield and it is unnecessary to match the sucrose concentration in the standards to that in the test solutions.

Prepare a solution containing approximately 0·5% $Na_2SO_3,7H_2O$ in 10% sucrose and determine accurately the concentration of sulphite by titration against standard iodine solution.

Dilute 5 ml of the sulphite solution to 100 ml with 10% sucrose to give a primary standard whose known concentration is about 50 μg SO_2 per ml. To prepare the calibration standards, take 1·0, 2·0, - - - - - 6·0 ml of the primary standard, add 4 ml of 0·1N sodium hydroxide and dilute to 100 ml with 10% sucrose solution. Prepare also a zero standard omitting the primary standard solution.

To 10 ml aliquots of the calibration standards add the solutions B and C and measure the optical densities as in the standard method.

Effect of sodium hydroxide treatment

A solution containing about 250 μg sulphur dioxide and 40 g sucrose per 100 ml may be prepared, without sodium hydroxide treatment, by addition of 5 ml of the primary sulphite standard to 95 ml of 40% sulphite free sucrose.

If, without any sodium hydroxide addition, 10 ml aliquots of this solution are treated with reagents B and C then it is found, on development of the colour as in the standard method, that the optical density is greatest for aliquots taken immediately after preparation of the sugar-sulphite mixture, and the optical density decreases with increasing contact time before addition of the chromogenic reagents to approach a constant value when the contact time is in excess of 10 minutes.

This fall-off in colour yield is not due to oxidation or irreversible loss of sulphur dioxide because, when the aliquots are taken at the same time intervals but are treated with 0·04 meq of sodium hydroxide immediately before addition of the chromogenic reagents, the colour yield is almost constant regardless of the time of contact before the sodium hydroxide addition.

A typical pattern for this effect of contact time, either with or without a subsequent addition of sodium hydroxide, is illustrated in Fig. 1. Either water, 0·4 ml, or 0·1N

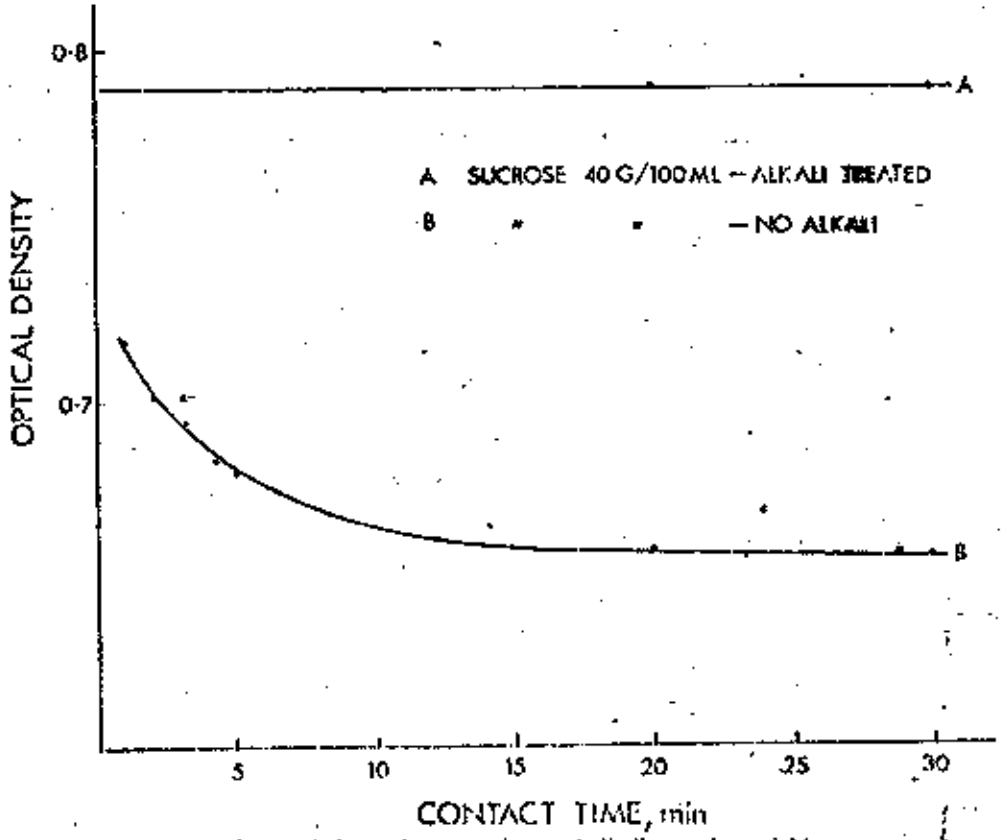

Fig. 1. Effects of contact time and alkali on colour yield

sodium hydroxide, 0·4 ml. was added immediately before the addition of reagents B and C to each aliquot so that the final volumes of all the solutions were equal.

At lower concentrations of sucrose, the pattern for the reduction in colour yield is similar to that recorded for 40% sucrose, but the difference between the colour yield with and without sodium hydroxide treatment becomes less as the sucrose concentration decreases.

From these results it is apparent that if calibration standards were prepared without sodium hydroxide treatment, and the chromogenic reagents were added within 5 minutes of preparation, then the absorption curves would be erratic unless the contact times were such that the colour yield fell off to an equivalent extent in each standard. This situation would not normally arise because the time required for preparation of the various calibration standards would generally give sufficient contact time for the colour yields to fall to the constant values. Under these circumstances, however, the colour yields for any given sulphite addition would be successively reduced as the sucrose concentration in the standards was increased.

Absorption curves prepared without sodium hydroxide treatment from calibration standards at sucrose concentrations of 40, 20 and 10 g per 100 ml are recorded in Fig. 2, showing the reduction in colour yield with increasing sucrose concentration. The recorded optical densities at each sugar concentration have been corrected for the optical density of the corresponding zero calibration standard. The absorption curve prepared with sodium hydroxide treatment at a sucrose concentration of 40 g per 100 ml is also recorded in Fig. 2. The corresponding curves for 20 and 10 g sucrose per 100 ml with sodium hydroxide treatment were not distinguishable from this curve.

If sodium hydroxide treatment is omitted, the potential error due to fall-off in colour yield becomes of increasing significance with increasing sucrose concentration in the calibration standards. This source of error is eliminated by the sodium hydroxide treatment.

In the determination of sulphur dioxide in white sugar, without added sulphite, omission of the sodium hydroxide treatment also leads to a lower colour yield than is obtained by the standard method. Comparative values for different sugars, with and without sodium hydroxide treatment, are recorded in Table I. The optical densities for both treatments were converted to sulphur dioxide concentrations using the calibration curve obtained by the standard procedure with sodium hydroxide.

Table I

Effect of alkali treatment in the determination of sulphur dioxide in white sugar

Sample	Sucrose concentration in test solution (g/100 ml)	Apparent SO_2 without alkali (p.p.m.)	Total SO_2 alkali treated (p.p.m.)	Increase from alkali treatment
A	20	3·1	3·8	22%
B	20	3·0	4·1	37%
C	20	3·2	3·7	16%
D	20	8·3	9·1	10%
E	20	8·5	10·2	20%
F	10	18·5	20·3	10%
G	10	17·7	19·8	12%

Superficially this effect appears similar to the fall-off in colour yield which was demonstrated with the calibration standards in the absence of sodium hydroxide. If the effects were identical, it might have been possible to determine the total sulphur dioxide in production sugars by omitting the sodium hydroxide and using a standard absorption curve prepared also without alkali but with the same sugar concentration. Such a procedure would not be satisfactory because, as shown in Table I, the additional sulphur dioxide liberated by the alkali treatment does not represent a constant proportion of that obtained without alkali, even when comparing sugars with closely similar sulphur dioxide contents.

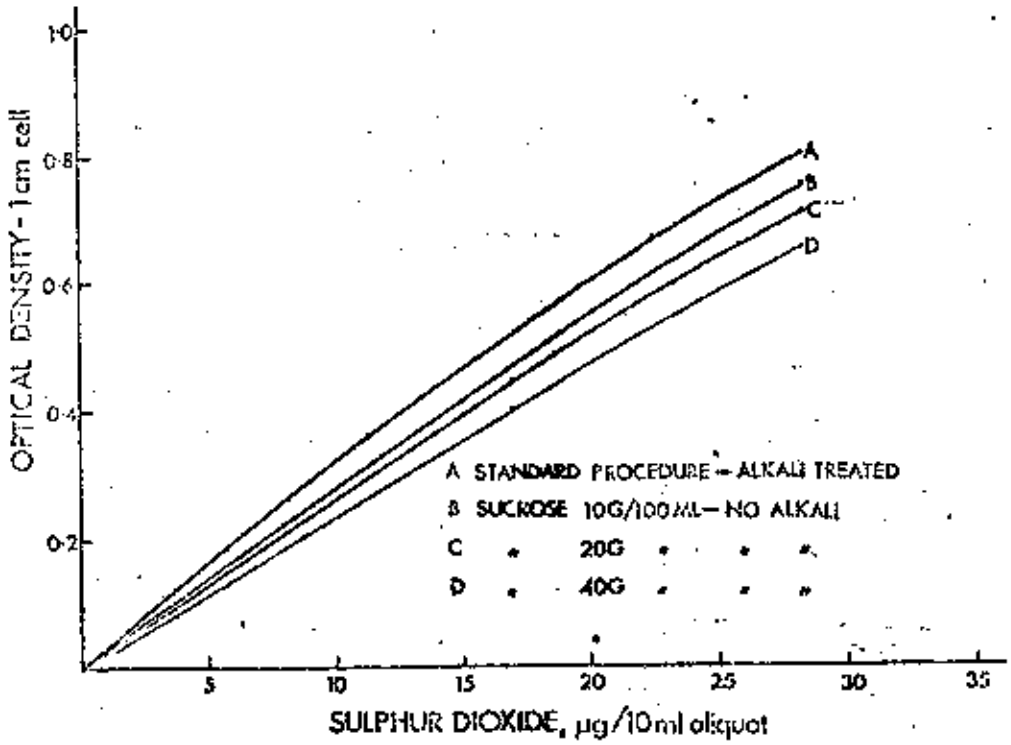

Fig. 2. Effects of sucrose concentration and alkali on colour yield

Moreover, when alkali treatment is omitted, the rate of fall-off in colour yield immediately after dissolving solid production sugars appears to be of a much smaller magnitude than that recorded in Fig. 1 for sulphite added to calibration standards. For example, finely ground sugar was dissolved at a concentration of 30 g per 100 ml and the chromogenic reagents, without sodium hydroxide, were added to the first aliquot within 2 minutes of adding water to the solid sample. The colour yield after this short interval was only 2% higher than when the time interval was increased to 20 minutes, whereas the colour yield was increased by 25% when the alkali treatment was included. Presumably the sulphur dioxide is not completely ionic even in the solid sugar.

It may be that the colour from production sugars without alkali treatment could be regarded as a measure of ionic sulphite in the solid sugar, provided the value was converted to sulphur dioxide content by reference to the standard absorption curve prepared with sodium hydroxide treatment. Such a value is probably of less commercial interest than a measure of total available sulphur dioxide and in consequence sodium hydroxide treatment of the sugar solution is used in the standard procedure.

The concentration of sodium hydroxide is not critical provided that the sugar solution is adjusted at least to pH 11 and also that the amount is not so excessive as to reduce sensibly the acidity of the bleached rosaniline. The timing of the sodium hydroxide addition is also not critical. The test solutions and standards may be handled in neutral or acidic conditions if required, provided the solutions are made alkaline before addition of the bleached rosaniline; the time required for adjustment to volume and pipetting the solutions gives adequate contact time with the alkali.

Glucose α-hydroxy sulphonate must be present in any neutral solution of sugar containing both glucose and sulphur dioxide. This compound is slowly hydrolysed in hot acid solution so that the sulphur dioxide from this source is distilled, though somewhat slowly,[8] in the Monier-Williams procedure. The sulphonate does not react with iodine in mildly acid solution, but the compound is determined by iodine titration if the solution is pre-treated with sodium hydroxide or sodium carbonate solution. The colour yield in the rosaniline procedure, without alkali, was found to be very low, presumably representing only that proportion which has hydrolysed in the cold acid medium. In alkaline solution, glucose α-hydroxy sulphonate is very rapidly hydrolysed and the compound was found to be estimated quantitatively by the rosaniline procedure with alkali treatment as suggested for white sugar. The presence of glucose α-hydroxy sulphonate however is not the predominant cause of the extra colour yield from calibration standards when treated with alkali, because addition of as much as 1% of hydrolysed sucrose to the calibration standards did not increase the difference between the two colour yields.

Experimental Results

For maximum precision a spectrophotometer would normally be used to determine the optical densities in this type of determination. Since the colorimetric method makes possible the routine determination of sulphur dioxide in the sugar factory, and factories are more frequently equipped with absorptiometers than with spectrophotometers, all the results recorded in this paper have been measured with a Spekker Absorptiometer using a Kodak No. 5 filter.

Duplicate determinations with the same solution of sucrose are reproducible with a scatter of less than 0·3 μg (i.e. better than 0·3 p.p.m. with a sucrose concentration of 10 g/100 ml and better than 0·1 p.p.m. at 40 g/100 ml). The results of recovery experiments are recorded in Table II; the amount of added sulphur dioxide was determined by the same procedure as was employed in preparation of calibration standards.

Table II
Recovery of sulphur dioxide added to white sugar

Sucrose concentration in test solution (g/100 ml)	*Sulphur dioxide (p.p.m.)*			
	Initial Value	*Added*	*Found*	*Recovery*
40	0·49	1·40	1·82	98%
40	0·49	4·98	5·32	97%
10	10·2	19·9	30·5	102%

The results of the colorimetric determination were compared with those obtained by the normal routine procedure as employed at the Central Laboratory of the British Sugar Corporation. This latter method consists of a direct iodine titration of a solution of the sugar after addition of sodium carbonate and phosphoric acid. The Central Laboratory selected 10

Table III
Sulphur dioxide in white sugar by direct iodine titration and by the rosaniline procedure

Sample	*Sulphur dioxide (p.p.m. in white sugar)*	
	Iodine titration	*Rosaniline procedure*
H	1·4	0·2
I	2·9	0·7
J	0·7	1·1
K	4·1	3·7
L	6·4	5·5
M	8·0	7·3
N	9·0	8·3
O	10·7	10·2
P	13·6	12·3
Q	14·8	12·3
R	1·0	0·3
S	4·6	3·5
T	4·6	3·8
U	5·4	3·8
V	6·0	4·1
W	10·2	9·1
X	19·2	19·8
Y	20·4	20·7
Z	22·4	22·6
Z'	30·6	28·4
Mean	9·8	8·9

[8] Isbell: *Australian J. Chem.*, 1961, 14, 302.

samples representing current production sugars (H to Q) and 10 samples of sugars which had been stored for up to 12 months (R to Z), the samples being chosen so as to give as wide a range of sulphur dioxide as possible. The sulphur dioxide contents were determined by the rosaniline procedure and the results are recorded in Table III. The iodine titration values are those measured by the Central Laboratory; for the stored samples these represent the concentration as redetermined on selection and not the concentration at the time of production.

The correlation between the two sets of data is very good though of course the percentage differences are greater for those sugars which contained less than 5 p.p.m. because the end-point error for direct titration is high at low sulphur dioxide contents. On average the rosaniline determination gave values which were 9% lower than the direct iodine titration which is reputed to give slightly excessive results for the estimation.

Summary

Neither distillation procedures nor direct titration methods have sufficient intrinsic precision for the determination of sulphur dioxide in those samples of white sugar in which the concentration approaches the lower limits of commercial practice. A simple and rapid colorimetric method, using acid bleached rosaniline and formaldehyde, is described for the determination of sulphur dioxide. The method is applicable to sugars containing from less than 1 p.p.m. to more than 30 p.p.m. of sulphur dioxide. The solution of the sugar is treated with dilute alkali before addition of the chromogenic reagents to liberate lightly bound sulphur dioxide which would not otherwise be determined by the procedure.

Acknowledgment

The authors wish to thank Mr. D. HIBBERT and the staff of Central Laboratory for selecting and titrating the sugar samples for the comparison recorded in Table III.

THE RESISTANCE HEATER FOR THE PREPARATION OF LOW GRADE MASSECUITES FOR CONTINUOUS CENTRIFUGALS

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Paper presented to the 12th Congress, I.S.S.C.T., 1965

Introduction

MODERN methods of processing low-grade massecuite require that the material from the pans be crystallized by controlled cooling in stirred water-cooled vessels for periods of 24 to 72 hours, and then conditioned to a suitable viscosity for satisfactory functioning of the centrifugals. Conditioning is usually done by reheating the massecuite, since this has been shown[1] to be preferable to the addition of water or diluted molasses to the cooled massecuite.

However, as pointed out by GUNDU RAO and SASTRY[2], FOSTER, SOCKHILL and RELF[1] and others, the reheating methods using water-heated coils in minglers, or the coils of the crystallizer itself, tend to promote considerable re-solution of sugar in the massecuite. These methods are dependent on heat transfer to a viscous massecuite film and usually require a long residence time of massecuite in the reheating unit. The control of this type of heating is difficult as the temperature of the massecuite is non-uniform, and water leakage into the massecuite with consequent drastic re-solution losses has been known to take place.

Interest was aroused in alternative means of reheating massecuite following a paper presented to the 9th I.S.S.C.T. Congress by DOSS and VISHNU[3] in 1956. These workers had examined the possibility of electrical methods of heating and were impressed by the possibility of the simplest type, that of electrical resistance heating, where the massecuite itself becomes the resistance between two metal electrodes, and heat is generated in the material without any heat transfer problems. WALLACE[4], working at the Sugar Research Institute, reported favourably on experience with a modified version of the heater of DOSS and VISHNU and pointed out the application of this reheating method to the continuous low-grade centrifugalling process. Renewed interest in the method was shown when several continuous centrifugals were scheduled for trial in Queensland mills and the design of factory scale heaters was attempted. The original prototypes were tested in conjunction

[1] FOSTER *et al: Proc. 25th Conf. Queensland Soc. Sugar Cane Tech.*, 1958, 179-188; *I.S.J.*, 1959, 61, 84.

[2] *Proc. 8th Conv. Deccan Sugar Tech. Assoc.* (India), 1951, 183-194.

[3] *Proc. 9th Congr. I.S.S.C.T.*, 1956, 2, 323; *I.S.J.*, 1956, 58, 303-305.

[4] *Proc. 27th Conf. Queensland Soc. Sugar Cane Tech.*, 1960, 133-138; *I.S.J.*, 1960, 62, 254.

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The Reaction of the Disulphide Groups of Insulin with Sodium Sulphite

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(*Received* 7 *December* 1959)

The reaction of simple disulphides with sulphite has been studied in some detail in recent years. Stricks & Koltkoff (1951) showed that, with cystine and oxidized glutathione, the equilibrium

$$RS{\cdot}SR + SO_3^{2-} \rightleftharpoons RS{\cdot}SO_3^- + RS^-$$

is dependent on pH, as this affects the relative proportions of $RS^- + RSH$ present. Cecil & McPhee (1955) and McPhee (1956) studied the kinetics of the reaction of sulphite with a number of simple disulphides. They observed that under the conditions used reaction took place with SO_3^{2-} but not with HSO_3^- ions. The net charge on the disulphide molecule was found to have a considerable effect on the rate of reaction with SO_3^{2-} ion, the positively charged compounds reacting rapidly and the negatively charged ones slowly. All disulphides investigated reacted to completion under suitable conditions.

In the present work, the behaviour of disulphide bonds in insulin has been studied in the light of the earlier investigations. Insulin was chosen as a suitable protein for various reasons. At the time the work was started it was the only protein in which both the arrangement of amino acids and the position of the disulphide bonds were known (Ryle, Sanger, Smith & Kitai, 1955). Fig. 1, showing the structure of the molecule, is reproduced from this paper. It will be seen that there are three disulphide bonds, two joining the A and B chains and

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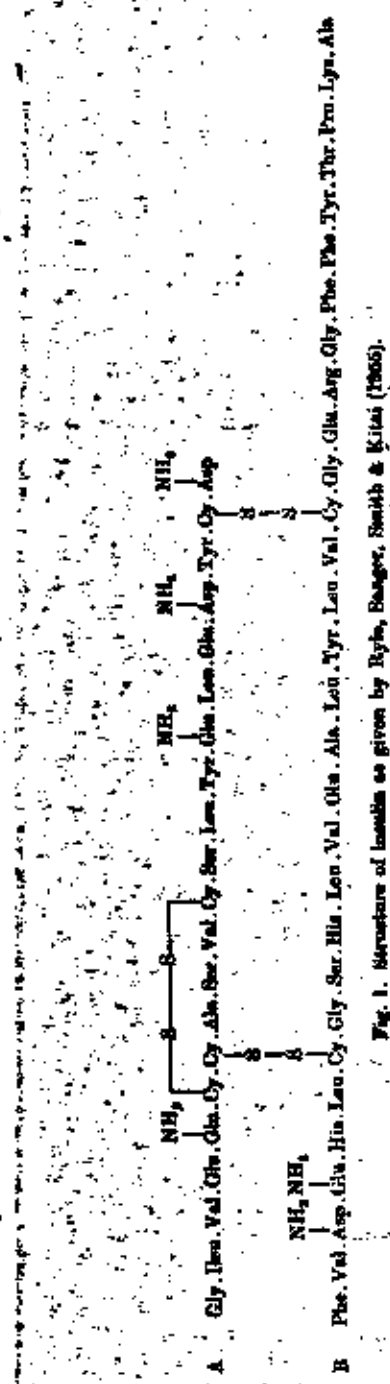

Fig. 1. Structure of insulin as given by Ryle, Sanger, Smith & Kitai (1955).

the third forming an internal ring between residues 6 and 11 in the A chain. There are no free thiol groups.

No attempt has been made to study the kinetics of these reactions in this preliminary survey. There are difficulties due to the presence of more than one reacting group on the same molecule and also to the fact that the reaction products are often insoluble. Because of this, only the extent of the reaction was studied under different conditions.

The phrase 'extent of reaction' requires some explanation. Three reactions have to be taken into account:

$$RS{\cdot}SR + SO_3^{2-} \rightleftharpoons RS{\cdot}SO_3^- + RS^-, \quad (1)$$

$$RS^- + H^+ \rightleftharpoons RSH, \quad (2)$$

$$SO_3^{2-} + H^+ \rightleftharpoons HSO_3^-. \quad (3)$$

At neutral pH most of the thiol formed is in the un-ionized form (pK_{RH} is normally in the region 8–10) and reaction (1) goes to completion. As the pH is increased above neutrality ionization of the thiol takes place and the equilibrium position of reaction (1) is displaced to the left. This is shown by allowing the reaction to reach equilibrium and acidifying to approx. pH 2 (which freezes the equilibrium) before estimating the amount of thiol formed (Cecil & McPhee, 1953). If the titration is carried out at the reaction pH, then removal of thiol from the system (as mercaptide) causes the reaction to 'follow' the titration and go to completion.

If the pH is reduced below neutrality the ratio of HSO_3^- to SO_3^{2-} ions increases (3); the rate of reaction falls rapidly and it may be difficult to reach equilibrium.

Very large differences have been observed in the second-order rate constants of positively and negatively charged disulphides with SO_3^{2-} ions and it is possible for a positively charged disulphide to react to completion under conditions under which a negatively charged compound would remain virtually intact. This could apply to a protein molecule containing several disulphide bonds of different properties.

A short account of some of this work has already appeared (Cecil & Loening, 1957).

EXPERIMENTAL

Reagents

Insulin. Crystalline ox insulin was obtained from Boots Pure Drug Co. Ltd., Nottingham. Two batches were used, nos. 4245 LL and 8011 G, with identical results. They were stored at 2–3° in a vessel containing a saturated solution of $CaCl_2.6H_2O$, which gives a relative humidity of 40% (*Handbook of Chemistry and Physics*, 1951–52). The moisture content of insulin stored in this way was found to

to 9·0%. Solutions (mM-0·1 mM) were made up in 5 mM-HCl as required.

Phenylmercuric hydroxide. Solutions were prepared and analysed as described by Allison & Cecil (1958).

Nitric acid. Dilute solutions were prepared according to Cecil (1950).

Other reagents. These were of normal reagent grade.

Reaction conditions

Insulin (50–500 μM) was allowed to react with Na_2SO_3 at 37° over the range pH 2·5–9·5. The concentration of Na_2SO_3 was such that the molar ratio Na_2SO_3:insulin was 100 or greater. Since Na_2SO_3 was always present in large excess it was also used as the buffer over the range pH 6·5–7·5 (pK_2=7, Tartar & Garretson, 1941), dil. HCl or HNO_3 being added to obtain the required pH. Phthalate was used between pH 2·5 and 6·5 and borate between pH 6·5 and 9·5. The concentration of both these buffers in the reaction mixtures was approximately 10 mM.

One source of error was the loss, by aerobic oxidation, of the insulin-SH groups formed; O_2 was therefore excluded either by the use of Thunberg tubes flushed with N_2 or by passing a slow stream of N_2 through the reaction mixture. In certain cases the reaction was carried out in the presence of an excess of phenylmercuric hydroxide, which itself prevented oxidation of SH groups.

Analytical methods

The extent of reaction of insulin with Na_2SO_3 was determined by measuring the amount of SH produced. This was done by amperometric titration with phenylmercuric hydroxide in the absence of O_2 with the dropping-mercury indicator electrode. The reasons for using this reagent to estimate SH groups in proteins are discussed by Allison & Cecil (1958) and Cecil & McPhee (1959*a*). The apparatus used was described by Allison & Cecil (1958).

Titrations were carried out over the range pH 2–9. They were followed by plotting the first reduction wave of phenylmercuric hydroxide (Benesch & Benesch, 1951). The potentials used, which are always referred to the saturated-calomel electrode, varied from −0·15 v at pH 2 to −0·5 v at pH 9. The correct potential under each set of conditions was determined by plotting the relevant current–voltage curves (see Figs. 2 and 3).

Below pH 5 KNO_3 (0·2 M) was used as the supporting electrolyte because of the insolubility of phenylmercuric chloride in acid solution. For the same reason chloride was excluded from the reaction mixtures. Above pH 5 KCl (0·2 M) was used. If KNO_3 was used between pH 5·5 and 8 trouble was experienced, owing to a flattening of the first reduction wave of phenylmercuric hydroxide. This was thought to be due to a separation of the adsorption and diffusion components of this reduction wave (see Benesch & Benesch, 1951).

Errors due to non-specific binding of phenylmercuric hydroxide by insulin (i.e. by groups other than SH) were prevented by the high concentration of Na_2SO_3 present. This forms a complex with phenylmercuric hydroxide which is easily reducible at the dropping-mercury electrode (see discussion in Cecil & McPhee, 1959*a*).

Insulin, phenylmercuric hydroxide and the mercaptides of insulin that has reacted with sulphite are surface-active and tend to compete with one another in forming a layer on the surface of the mercury drops. The residual current (which depends on the capacitance of the mercury drops) may therefore change during the course of a titration as the relative proportions of these reagents change. A value taken from the buffer solution or buffer plus insulin may not be correct for the end of a titration. The intersection of the residual-current line and excess-reagent line (see Fig. 2) was taken, at which point all the insulin-SH groups had been

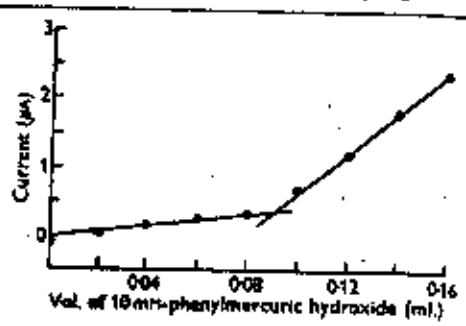

Fig. 2. Amperometric titration of an insulin–sulphite reaction mixture with phenylmercuric hydroxide. The reaction mixture was as follows: insulin, 0·285 mM; Na_2SO_3, 28 mM; potassium hydrogen phthalate, 14 mM; H_2SO_4, 0·013 N; pH, 5·15. After reaction for 6 hr. at 37° a 3 ml. sample was acidified with 2 ml. of 0·1 N-H_2SO_4 (pH 1·85) and then titrated with 0·8 mM-phenylmercuric hydroxide at −0·1 v.

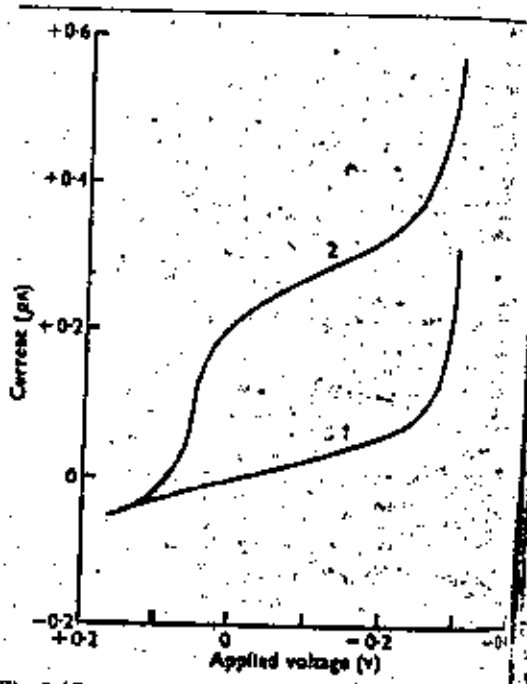

Fig. 3. Current–voltage curves of phenylmercuric hydroxide in the presence of an insulin–sulphite-reaction mixture. Curve 2 is that of the reaction mixture described in Fig. 2, acidified to pH 1·85, before titration. Curve 1 was taken at the end of the titration.

converted into mercaptide and there was no excess of phenylmercuric hydroxide. When the reaction was carried out in the presence of an excess of phenylmercuric hydroxide, the residual current had to be determined in a separate experiment.

Octan-2-ol, which is also surface-active, was originally used to prevent frothing when N_2 was bubbled through the solutions. It was found to cause errors, particularly at low pH, and its use was discontinued. Instead a ring of silicone grease was smeared round the top half-inch of the titration vessel.

RESULTS

Reaction of insulin with sulphite

The time course of the reaction was followed at a range of pH values at 37°. This was done in order to find the time required for complete reaction under various conditions. Samples from the reaction mixtures were removed at intervals, acidified with dilute nitric acid to approx. pH 2, and titrated with phenylmercuric hydroxide at −0·15v. Typical results are given in Fig. 4.

Above pH 6·5 the reactions are rapid and true equilibrium is reached within about 20 min. Below pH 6·5 the reaction is slower and the plateau reached after 3–6 hr. is taken as the extent of reaction. It is probable that there is a further very slow reaction. Oxidation, which is difficult to prevent over long periods, tends to mask this. The plateau does not therefore represent a true equilibrium.

Insulin is sparingly soluble in the range of pH 3–6 and was sometimes present as a suspension. The products of reaction are also sparingly soluble and tended to precipitate during the reactions, particularly at low pH and high sulphite concentrations.

The extent of reaction, expressed as the number of S·S bonds/molecule of insulin, is shown in Fig. 5 (curve 1) as a function of pH. The reaction goes furthest at pH 6·5–7·0. Even so, only two-thirds of the S·S bonds react. Further experiments with a wide range of reaction times (4 min.–several hours) and of sulphite concentrations (100–270 moles of sulphite/mole of insulin) confirmed this result.

The fall in the extent of reaction above pH 7 is similar to that observed with simple disulphides, and the equilibrium position varies with the sulphite concentration. If the titrations were carried out at the same pH as the reaction then, as explained earlier, the reaction 'follows' the titration and an end point corresponding to two-thirds of the S·S bonds was obtained. Between pH 3 and 5·5, one-third of the S·S bonds were split. This result was independent of the sulphite concentration (above 100 moles of sulphite/mole of insulin) and was not influenced by the pH of titration between pH 1·8 and 6.

The most significant result of these experiments is that only two-thirds of the S·S bonds present reacted with sulphite under conditions in which the simple disulphides reacted completely. Conditions were therefore sought under which the remaining one-third of the S·S bonds would react.

Reaction of insulin with sulphite in the presence of urea

A series of experiments similar to those described above were carried out with the difference that the solvent was 6M-urea instead of water. It was found that the reaction time required to reach a plateau at any given pH was approximately one-quarter of that required in the absence of urea, and that the products of reaction were soluble. The results are given in Fig. 5 (curve 2). It will be seen that a maximum of 2·4–2·5 S·S bonds react. If the titrations were carried out at the reaction pH, an uptake of phenylmercuric hydroxide corresponding to

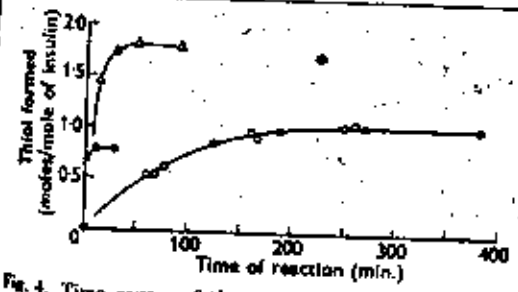

Fig. 4. Time course of the reaction between insulin and sulphite at 37° and at different values of pH. Three typical examples are given at different points in the pH range. The concentrations of insulin and Na_2SO_3 were 0·26 and 20 mM respectively with other substances as follows: ○, H_2SO_4; △, pH 6·0, 0·01M-H_2SO_4; ●, pH 9·0, 14 mM-$Na_2H_2O_4$.

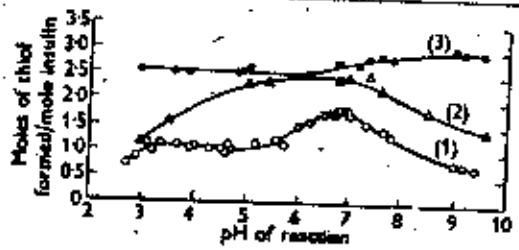

Fig. 5. Extent of reaction between insulin and sulphite under various conditions. The time course of the reactions at different values of pH was determined as shown in Fig. 4 and the maximum extent of reaction plotted against pH. Curve 1 (○), reaction with sulphite alone; curve 2 (△), reaction with sulphite in the presence of 6M-urea; curve 3 (●), reaction with sulphite in the presence of phenylmercuric hydroxide. A small excess of phenylmercuric hydroxide (3·5 moles of phenylmercuric hydroxide/mole of insulin) was used.

2·8 S·S bonds was obtained. It is clear therefore that all three S·S bonds are involved in the reaction in the presence of urea. Similar results were obtained in the presence of 2M-guanidine.

Reaction in the presence of excess of phenylmercuric hydroxide

These experiments were originally started in an attempt to prevent SH–S·S-exchange reactions. It was then found that the reaction went further in the presence of phenylmercuric hydroxide than in the presence of sulphite alone.

The compositions of the reaction mixtures were the same as those for insulin and sulphite alone except that a small excess of phenylmercuric hydroxide (i.e. > 3 moles/mole of insulin) was added. It was important to add the phenylmercuric hydroxide to the insulin before the sulphite for reasons given below.

The titrations were carried out at the reaction pH since there was no question of a shift in equilibrium. Since there was always some excess of phenylmercuric hydroxide present at the end of the reaction the value of the residual current was found from a separate experiment. For this an insulin–sulphite reaction mixture containing slightly less than one equivalent of phenylmercuric hydroxide was used (see under Analytical methods). If the excess of phenylmercuric hydroxide at the end of the reaction was small, further increments were added and the excess reagent line was extrapolated back to the residual current line. If a large excess of phenylmercuric hydroxide was present it was back-titrated with cysteine or homocysteine.

The results at different values of pH are given in Fig. 5 (curve 3). At pH 9 the uptake of phenylmercuric hydroxide corresponded exactly to the reaction of all three S·S bonds with sulphite but between pH 3 and 6 the reaction takes 24 hr. and the number is reduced to 2·5. If insulin is allowed to react with sulphite at pH 7–9 for a few minutes before adding the phenylmercuric hydroxide only 2·4–2·6 S·S bonds react. Presumably SH–S·S interchange reactions are rapid at this pH and a different reaction product results.

It was necessary to establish that the increased uptake of phenylmercuric hydroxide at pH 9 was due to reaction of the S·S bonds with sulphite and not phenylmercuric hydroxide. Heavy-metal ions, such as Ag^+ and Hg^{2+} ions, react with simple disulphides according to the equation (representing the metal ion as M^+):

$$2\ RS{\cdot}SR + 3\ M^+ + 2H_2O \rightarrow 3\ RSM + RSO_2H + 3H^+.$$

Thus 1 RS·SR ≡ 1·5 RSM (Cecil & McPhee, 1957, 1959*b*), whereas if the disulphide is first split by sulphite and the thiol formed reacts with heavy-metal ion then 1 RS·SR ≡ 1·0 RSM. If the reactions are comparable in rate then the possibility of a mixed end point exists. R. Cecil (unpublished work) has found that phenylmercuric hydroxide reacts with some simple disulphides.

Insulin (0·1 mM) and phenylmercuric hydroxide (max), buffered with phosphate at pH 7 or borate pH 9, were incubated at 37° for periods up to 3 hr. Samples were removed at intervals and acidified with dilute nitric acid. The free phenylmercuric hydroxide present was estimated by potentiometric titration with potassium bromide (Allison & Cecil, 1959). These experiments showed that there was no detectable reaction of phenylmercuric hydroxide with the S·S bonds of insulin.

There remained the possibility that the reactivity of some of the insulin S·S bonds towards phenylmercuric hydroxide might be increased after others had reacted with sulphite. If this were so the reaction of phenylmercuric hydroxide with the insulin–sulphite mixture would be dependent on the initial concentration. Experiments in which the amount of phenylmercuric hydroxide used was varied between 3 and 5 moles/mole of insulin showed that the uptake was constant. It may be assumed therefore that the S·S bonds react with sulphite and that the only function of the phenylmercuric hydroxide is to react with the SH groups so formed.

It was reported earlier that when insulin had been allowed to react with sulphite at pH 9 and titrated with phenylmercuric hydroxide at the same pH only two S·S bonds reacted. The reason for the difference between this result and those reported in this section lies in the fact that the concentration of free phenylmercuric hydroxide during a titration is small and the time it is in contact with insulin is short, when compared with the experiments just described.

Resolution of the reaction products of insulin and sulphite

The evidence obtained so far is that two-thirds of the S·S bonds of insulin react with sulphite under the same conditions as do simple disulphides, whereas the remaining third reacts only in the presence of phenylmercuric hydroxide, urea or guanidine. The simplest assumption is that two of the three S·S bonds present in insulin react normally with sulphite and that, for some reason, the third bond does not. Referring again to Fig. 1, two of the bonds act as covalent links between the A and B chains and there is no obvious reason why they should not react with sulphite. The other bond forms part of a closed-ring structure and to that extent differs from the simple disulphides studied by Cecil & McPhee (1959*a*) and McPhee (1959), which were all open-chain compounds.

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If indeed it is the two inter-chain bonds that react with sulphite at pH 7 then the insulin molecule would be split into the two chains. It should then be possible to resolve the reaction products into two fractions corresponding to the two chains. One fraction should contain a disulphide bond, which will react with sulphite plus phenylmercuric hydroxide.

It is important, in any experiment designed to test this hypothesis, that the amount of SH-S·S interchange should be kept to a minimum. This was done by using a high concentration of sulphite and a short reaction time. The composition of the reaction mixture used was as follows: insulin, 0·18 mM; HCl, 0·04 M; Na_2SO_3, 0·1 M; pH 0·9. The sulphite and insulin+HCl solutions were each heated to 37° before mixing. The reaction was complete (i.e. two S·S bonds were split) in 4 min., after which time the pH was lowered to 5 by the addition of acetic acid. A precipitate formed which was separated by centrifuging.

The A and B chains each contain two tyrosine and no tryptophan residues (see Fig. 1) and so should have the same molar absorption at 280 mμ. This provides a simple method of determining the distribution of protein between the precipitate and supernatant.

The filtrate contained a high concentration of sulphite and the absorption due to this was determined in a separate experiment. The figures in a typical experiment were as follows: supernatant, E 1·105; control (i.e. reaction mixture less insulin), E 0·683; therefore for soluble protein fraction, E is 0·422. The precipitate was dissolved in 0·01 M-sodium borate, the volume being the same as the original reaction mixture. The absorption at 280 mμ was 0·675. Thus approximately 40 % of the insulin appeared in the filtrate and 60 % was precipitated. The precipitate was not washed as this caused a loss of protein. Comparison of the absorption value of the fractions with that of the original insulin gave a recovery figure of 110 %, showing that the error caused by sulphite adhering to the precipitate was small.

In some experiments 2 moles of phenylmercuric hydroxide/mole of insulin were added after acidification in order to prevent oxidation of the SH groups. This did not affect the results.

These experiments showed that there were two reactions and so these were examined further.

Analysis of the thiol groups in the reaction products

The supernatant and precipitate were separated by centrifuging and analysed immediately. Separate experiments were done with each fraction to avoid delay and minimize loss of SH due to oxidation. Washing of the precipitate was omitted for the same reason.

The supernatant was titrated (at pH 5) with phenylmercuric hydroxide at −0·35 v after the addition of KCl to 0·1 M. The precipitate was suspended in 4 ml. of a solution of the same composition and titrated with phenylmercuric hydroxide in the same way.

One mole of insulin gave 0·6–0·7 mole of SH group in the supernatant and 1·0–1·1 mole in the precipitate. These proportions are similar to those obtained by ultraviolet absorption. This suggests that each fraction contains one SH group and that some of the soluble fraction is carried down with the precipitate.

Analysis of the disulphide bonds in the reaction products

The remaining S·S bonds will only react with sulphite plus phenylmercuric hydroxide at pH 9.

Two equivalents of phenylmercuric hydroxide were added to the reaction mixture after acidification to prevent oxidation of SH groups (this is rapid at pH 9).

Supernatant (3 ml.) was added to the following in the polarographic cell, which was kept at 37°: 0·5 ml. of 0·6 N-NaOH, 0·1 ml. of saturated KCl, 150 mg. of $Na_2B_4O_7$. N_2 was bubbled through for 10 min., after which time the current at −0·43 v was noted. 0·01 M-Phenylmercuric hydroxide (0·1 ml.) was then added. The current at −0·45 v (corresponding to the first reduction wave of phenylmercuric hydroxide) remained constant with time, indicating that there was no reaction. It was therefore concluded that there was no S·S present in the soluble fraction.

The precipitate was dissolved in the following solution, previously deoxygenated, in the polarographic cell at 37°; 3 ml. of 0·1 M-$Na_2B_4O_7$, 1 ml. of 0·2 M-Na_2SO_3, 0·1 ml. of saturated KCl. The current at −0·45 v was noted and 0·2 ml. of 0·01 M-phenylmercuric hydroxide added. The reaction was followed by observing the fall in the current, at −0·45 v with time. It was complete in 40 min. at 37°. The excess of phenylmercuric hydroxide at the end of the reaction was calculated from the current at −0·45 v. The uptake corresponded to 0·85–0·95 bond/mole of protein in different experiments.

It appears therefore that the precipitate contains the whole of the residual S·S.

End-group analyses of the reaction products

The two protein fractions were allowed to react with 1-fluoro-2:4-dinitrobenzene in an ethanol-water solution of bicarbonate (Sanger, 1949). After hydrolysis the dinitrophenyl (DNP)-amino acids were separated by paper chromatography (Blackburn & Lowther, 1951). DNP-Gly and DNP-Phe [for definitions of these abbreviations see *Biochem. J.* (1957), 66, 6] were run on the papers as

markers. After drying the paper at 105°, the spots were cut out and eluted by soaking in 3 ml. of methanol + 1 drop of conc. HCl. The solutions were clarified by centrifuging and the absorption at 360 mμ was measured. No attempt was made to measure absolute quantities since a knowledge of the relative amounts of DNP-Gly and DNP-Phe was all that was needed.

Insulin. Control experiments were carried out with insulin to see whether the correct ratios of DNP-Gly and DNP-Phe were obtained with the procedure adopted. DNP-insulin was prepared according to Sanger (1945). It was hydrolysed with 6N-HCl in a sealed tube at 105° for 8 hr. The hydrolysate was diluted with water and extracted with ether. After evaporation of the ether the extract was taken up in a small amount of methanol and loaded on to the paper. Two spots corresponding to DNP-Gly and DNP-Phe (together with a trace of 2:4-dinitrophenol) were obtained. The absorptions at 360 mμ were: DNP-Gly, 0·120 and DNP-Phe, 0·118. This experiment was repeated a number of times and the ratio of the two terminal amino acids was always 1:1 within ±5%, indicating that the destruction of DNP-Gly during hydrolysis is negligible under these conditions.

Soluble fraction. The supernatant contained a large amount of sulphite which had to be destroyed before reaction with 1-fluoro-2:4-dinitrobenzene. This was done by the addition of a slight excess of 30% (w/v) hydrogen peroxide. Bicarbonate and ethanol were then added and the reaction with 1-fluoro-2:4-dinitrobenzene carried out in the usual way. The DNP derivative is soluble over the whole pH range and so cannot be recovered, as with insulin, by precipitation at pH 5. Instead the solution was dried *in vacuo* and the solid extracted with ethanol in which the DNP derivative is, rather surprisingly, quite soluble. The ethanolic extract was dried *in vacuo* and extracted with ether to remove dinitrophenol. It was then dissolved in 0·5 ml. of 6N-HCl and hydrolysed for 8 hr. at 105°.

Chromatography showed DNP-Phe but no DNP-Gly. To confirm this another run was done with the paper more heavily loaded. The DNP-Phe spot had an absorption at 360 mμ of 1·25 and there was still no detectable DNP-Gly.

It appears therefore that the soluble fraction must consist almost entirely of B chain. This accords with the absence of any reaction for S·S bonds.

Precipitate. The precipitate was washed twice with water and the DNP derivative prepared in the same way as DNP-insulin. Chromatographic analysis of the hydrolysate gave the following absorptions at 360 mμ: DNP-Gly, 0·220; DNP-Phe, 0·550.

The other results suggest that 60–70% of B chain is in the filtrate and the precipitate sho therefore contain all the A chain and the remain of the B chain. It was to be expected therefore t the ratio DNP-Gly:DNP-Phe would be at least 2 Instead the reverse was found.

It was thought that this discrepancy might due to the presence of free SH groups in the reac products. Accordingly, similar experiments w carried out with DNP-insulin to which cystine a cysteine (approx. 20%, w/w) were added bef hydrolysis. The extinctions at 360 mμ with add cystine were 0·156 for DNP-Gly and 0·048 DNP-Phe; those with added cysteine were 0· and 0·045 respectively. In both cases there appe to have been a loss of DNP-Phe, whereas, with A chain, there appears to have been a loss of DN Gly. The quantitative aspects of end-group analy must therefore be interpreted with caution.

Relative rates of reaction of the inter- and intra-chain disulphide bonds

It has been shown that, when insulin reacts wi sulphite, the two inter-chain S·S bonds are spl There is the possibility that, in the presence phenylmercuric hydroxide, when the rever reactions are inhibited, the 6–11 intra-chain bo might react as fast as, or faster than, the inter-cha bonds. If this were so, then the insulin would n split into two components after only two bon were split.

The composition of the reaction mixture wa insulin, 0·185 mM; HCl, 0·03M; phenylmercu hydroxide, 0·37 mM (2 moles/mole of insulin Na_2SO_3, 0·05M; pH, 6·0. The reaction was co tinued until all the phenylmercuric hydroxide ha been taken up, 7 min. at 37°, when it was stoppe by acidification with acetic acid to pH 5. Th precipitate formed was separated by centrifugi and dissolved in a volume of 10 mM-$Na_2B_4O_7$ equ to that of the original reaction mixture. The ab sorption at 280 mμ was: supernatant, 0·25 control (i.e. reaction mixture less insulin), 0·21 soluble fraction, 0·070; precipitate in 10 mM $Na_2B_4O_7$, 0·980. Since only 7% of the protei remained in the supernatant (as compared wit 30–40% after reaction with sulphite above) it likely that one of the two inter-chain bonds intact. Reaction must therefore have taken plac with one inter-chain bond and with the intra-chai bond.

DISCUSSION

One of the objects of this work was to find th extent to which the S·S bonds of insulin resembl those of the simple disulphides. The behaviour o two S·S bonds of insulin above pH 7, where th extent of reaction decreases as the pH is increased

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is similar to that found by Stricks & Kolthoff (1951), Cecil & McPhee (1955) and McPhee (1956) with all the simple disulphides investigated.

The behaviour at pH 3–5, where reaction is effectively confined to one S·S bond, can also be accounted for by reference to work with simple disulphides. McPhee (1956) showed that the differences in second-order rate constants between positively and negatively charged disulphides was sufficiently large to allow for complete reaction of a positively charged compound under conditions where a negatively charged compound would be almost unaffected. These studies did not extend below pH 4·5 but Cecil (1960) has since studied the behaviour of the positively charged compound, 2:2′-diaminodiethyl disulphide (cystamine), at pH 3–5. This compound reacted slowly with HSO_3^- at low pH in phthalate buffer, but not in formate. Whether or not insulin reacts with HSO_3^- ions can only be determined by kinetic studies. It may be tentatively concluded, however, that one of the insulin S·S bonds has a positively charged environment.

The difference between insulin and the simple disulphides lies in the complete failure of one of the S·S bonds to react with sulphite. Since it has been shown that insulin splits into the A and B chains after reaction with sulphite it follows that the inter-chain S·S bonds have reacted and the 6–11 intra-chain bond in the A chain has not.

This bond has been shown to react with sulphite in the presence of urea, guanidine and phenylmercuric hydroxide. It is possible to explain these facts on the assumption that the configuration of the 6–11 ring is stable and that this stability depends on hydrogen bonding and not on the S·S bond. This would have the effect of shifting the equilibrium of reaction (1) to the left; i.e. of increasing the rate of the reverse reaction relative to the forward reaction. The effect of urea and guanidine is explained by a reduction in the stability of the ring due to interference with the hydrogen bonding. This would reduce the rate of the reverse reaction. Phenylmercuric hydroxide, on the other hand, combines with the SH groups to form undissociated mercaptide and so must inhibit the reverse reaction.

The reason why only 2·5 S·S bonds react with sulphite plus phenylmercuric hydroxide between pH 3 and 6 is obscure. The products of SH–S·S-exchange reactions, obtained by allowing insulin to react with sulphite alone for a time before adding phenylmercuric hydroxide, behave similarly. Kolthoff, Anastasi & Tan (1959) have observed a similar phenomenon with bovine-serum albumin.

The rate of the forward reaction of the 6–11 bond with sulphite was compared with that of the two inter-chain bonds by allowing only two S·S bonds to react with sulphite in the presence of phenylmercuric hydroxide. In the absence of phenylmercuric hydroxide under these conditions the two inter-chain bonds react and the insulin is split into the A and B chains. In the presence of phenylmercuric hydroxide, however, the insulin does not split into two components after two bonds have reacted. It follows that the 6–11 bond and one inter-chain bond, probably that carrying a positive charge, must have reacted.

It appears therefore that, given suitable conditions, the 6–11 S·S bond is highly reactive and, moreover, is probably not required to maintain the configuration of the ring. Lindley's (1955) observation that the 6–11 bond alone is reduced by lithium thioglycollate at pH 5 is consistent with this view. These facts, together with the similarity of the 6–11 ring to those of oxytocin and vasopressin (Tuppy, 1953; du Vigneaud, Rossler & Trippett, 1953; Popenoe & du Vigneaud, 1954), suggest that this reactive S·S bond might be intimately concerned in the biological action of insulin.

If the 6–11 S·S bond is essential to the biological activity of insulin, it can only be one of several different groups so involved; thus it has long been known that insulin is inactivated if the tyrosine residues are modified (Harington & Neuberger, 1936; Stern & White, 1937).

Fisher and Zachariah (see Addendum) found that the fraction that was precipitated at pH 5 after reaction with sulphite showed greatly decreased insulin-like activity. Since this contained all the A chain and some of the B chain, it appears that the separated chains are probably without biological activity.

The properties of the separated A and B chains are interesting. Fractionation based on the difference in solubilities was all that was attempted in order to avoid loss of material and errors due to oxidation of SH groups. It was established on the basis of SH, S·S and end-group analysis that the soluble fraction consisted of almost pure B chain and the precipitate of all the A chain, together with 20–30% of B chain. The inter-chain S·S bonds react in such a way that each chain carries a total of one SH group and presumably one $-S{\cdot}SO_3^-$ group. It has not yet been established whether this represents one SH and one $-S{\cdot}SO_3^-$/chain molecule or whether there is a mixture of products which average one group/chain. The fact that some B chain is associated with the A chain suggests that there may be a mixture in which one of the B-chain components is less soluble than the remainder. The S·S bond in the 6–11 ring of the separated A chain maintains its unique properties and will only react with sulphite in the presence of phenylmercuric hydroxide.

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The relative solubilities of the two chains require comment. Sanger (1949) found that, when prepared from insulin by oxidation with performic acid, the A chain was soluble over the whole pH range, whereas the B chain was precipitated at pH 6·5. In this case, all the sulphur was in the form of $SO_3^{=}$ ion. When insulin is split by reaction with sulphite at pH 7, the A chain can be precipitated at pH 7 whereas the B chain is soluble over the whole pH range. The DNP-substituted B chain, when prepared in this way, is soluble in ethanol.

Kolthoff, Anastasi & Tan (1958, 1959) found that the S·S bonds in bovine-serum albumin show many similarities to the 6–11 bond of insulin. There are 17 S·S bonds in the molecule. In the native protein, none of these reacts with sulphite. When the protein is denatured with urea or guanidine, the extent of reaction is dependent on pH, but at pH 4–6 reaction is virtually complete. If the native protein was allowed to react with sulphite in the presence of a small excess of mercuric chloride all the S·S bonds reacted.

Hunter & McDuffie (1959) reduced bovine albumin with thioglycollate in the presence of both urea and sodium dodecylsulphate, under which conditions all the S·S bonds were reduced. They found that the molecular weight of the reduced protein was the same as that of the native protein. It appears likely therefore that the molecule consists of one long peptide chain with a number of intra-chain S·S bonds. Thus the behaviour of the S·S bonds of bovine albumin with sulphite could be explained on the same basis as that of the 6–11 S·S bond of insulin.

The same type of structure has been demonstrated for lactogenic hormone (Li, Cole & Coval, 1957; Cole, Geschwind & Li, 1957; Li, 1957) and for ribonuclease (Moore & Stein, 1958; Anfinsen, 1958; Hirs, Stein & Moore, 1958). Bailey (1957) and Bailey & Cole (1959) have studied the reaction of both insulin and ribonuclease with sulphite, but only in the presence of 8M-urea and an oxidizing agent. Under these conditions all the disulphide bonds react to give S-sulphonate. No studies have been reported on the reaction of lactogenic hormone with sulphite.

The S·S bonds of keratin show a similar type of behaviour to those of serum albumin. Approximately half of the S·S bonds present react with sulphite at pH 5 (see review by Lindley, 1959). If the reaction is carried out in the presence of Hg^{2+} ion and urea (Leach, 1959) then all the bonds react.

The explanation proposed for the behaviour of the 6–11 S·S bond of insulin accounts for most of the observed facts and could apply to S·S bonds in other proteins. Until this is established, however, the possible effects of other factors, such as the influence of side chains, must not be ignored.

SUMMARY

1. The reaction of the disulphide bonds of insulin with sulphite has been studied under a range of conditions.

2. Two of the three disulphide bonds react with sulphite in a manner comparable with simple disulphides. Under suitable conditions they react to completion.

3. One of these two disulphide bonds reacts with sulphite at low pH and is likely to have a positively charged environment.

4. If insulin is allowed to react with sulphite under conditions in which two disulphide bonds are split, but thiol–disulphide-exchange reactions are kept to a minimum, the reaction product can be resolved into the A and B chains. This establishes that it is the two inter-chain disulphide bonds that have reacted.

5. The intra-chain bond in the A chain will only react with sulphite in the presence of urea, guanidine or phenylmercuric hydroxide. An explanation for this behaviour is suggested which could also apply to apparently unreactive disulphide bonds in other proteins.

One of us (U.E.L.) is indebted to the Agricultural Research Council for a training grant and to the John Innes Horticultural Institute for support in the later stages of the work. We are grateful to Dr R. B. Fisher for many helpful discussions and to Miss Sybil Read for skilled technical assistance. This work was supported by a grant from the Rockefeller Foundation to the Department of Biochemistry, University of Oxford.

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ADDENDUM

Determination of the Insulin-like Activity of the A-chain of Insulin

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(*Received 7 December 1959*)

The procedure used to detect insulin-like activity depends on the observation that insulin, in very low concentration, increases the permeation of the rat heart by non-metabolized sugars.

The A chain of insulin was a sample supplied by Cecil & Loening (1960) prepared by reaction of insulin with sulphite. It probably contains approximately 20% of B chain as impurity.

The isolated rat heart was perfused, by techniques similar to those described by Fisher & Lindsay (1956), with Krebs-bicarbonate medium containing 30 mM-L-arabinose and 10 mM-raffinose, together with the material suspected of insulin-like activity. By the use of the raffinose as an indicator of extracellular water, it was then possible to determine the intracellular arabinose concentration.

After removal from the animal, the hearts were rendered insulin-free by perfusion for 45 min. with the sugar-free perfusate. They were then perfused for 15 min. with a medium containing the sugars and the sample to be tested. The arabinose penetration is expressed as the percentage of intracellular water in equilibrium with the perfusate after perfusion for 15 min. (Table 1).

Table 1.

Sample	Concn. (μmM)	No. of expts.	Mean penetration of L-arabinose (%)
Nil	—	2	12·96
A chain	4	2	13·61
A chain	40	3	19·20
Insulin	4	2	20·21
Insulin	8	8	35·60

(8 μmM-Insulin is equivalent to 1 milliunit/ml.).

The insulin-like activity of the sample of A chain is certainly less than one-tenth of that of unchanged insulin.

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Analyse hémocytochimique de l'acide ascorbique des éléments cellulaires du sang périphérique dans certaines maladies professionnelles (saturnisme, silicose, intoxication par cyanures et par anhydride sulfureux).

Par MM.

A. NUNZIANTE CESÀRO, G. SAITTA et D'URSO

(Messine).

Une méthode pour mettre en évidence l'acide ascorbique dans les éléments cellulaires du sang périphérique a été élaborée, d'après la réaction de Szent Gyorgi, par Nunziante Cesàro, Saitta et D'Urso. Cette méthode a été appliquée dans de précédentes recherches aux sujets adultes normaux.

On a pu établir d'après cette recherche que les globules rouges donnent toujours une réaction négative, tandis que, dans les leucocytes, où elle est positive, l'intensité de la réaction décroît dans l'ordre suivant : granulocytes éosinophiles, monocytes, lymphocytes, granulocytes neutrophiles.

Enfin, pour une évaluation quantitative, on a étudié la formule suivante qui donne l'*index leuco-ascorbique du sang ILAS* :

$$ILAS = \frac{0(A) + 1(B) + 2(C) + 3(D)}{100}$$

Les lettres *A*, *B*, *C*, *D*, indiquent le nombre des cellules respectivement négatives, positives avec une réaction faible, moyenne ou intense. Les coefficients 0, 1, 2, 3, sont appliqués dans le même ordre.

On dira pourtant que l'index leuco-ascorbique du sang est une évaluation numérique de l'intensité moyenne de réaction, dans un élément leucocytaire du sang périphérique.

Cet index chez les sujets adultes normaux est de 1,26 environ (de 0,99 à 1,52).

Il faut souligner que d'autres index ont été élaborés dans de précédentes recherches de notre Institut et que en général leur emploi devient nécessaire quand l'évaluation histospectrophotométrique n'est pas possible pour des causes techniques.

Pour ce qui concerne l'acide ascorbique dans la pathologie professionnelle faut-il remarquer que Rozera, avec la méthode de Giroud et Leblond, a mis en évidence une diminution de cette vitamine dans les cellules de la moelle osseuse des lapins dans l'intoxication benzolique chronique.

MÉTHODE

Les recherches ont été effectuées dans les maladies professionnelles suivantes : saturnisme, silicose, intoxications par acides cyanhydrique et anhydride sulfureux.

Pour chaque maladie on a examiné le sang périphérique de 10 sujets.

La méthode élaborée pour mettre en évidence l'acide ascorbique dans les éléments du sang périphérique a été la suivante :

1° Les frottis de sang sont laissés à température ambiante jusqu'à dessèchement ;

2° On place les frottis pendant 30 minutes dans la solution :

$AgNO_3$	5 g
H_2O	100 ml
Ac. acétique	5 ml

en la tenant toujours dans l'obscurité.

3° Passage pendant 5 minutes dans une solution d'ammoniaque 5 p. 100 toujours en obscurité complète ;

4° Rinçage en *aqua fontis* ;

5° Passage : 2 minutes en May-Grünwald ;

6° Dilution du May-Grünwald avec *aqua fontis* pendant 2 minutes ;

7° Rinçage en eau courante ;

8° Coloration avec Giemsa (1 goutte par ml d'eau, pendant 10 minutes ;

9° Rinçage en *aqua fontis* ;

10° Coloration avec hématéin de Carazzi et 3) pendant 1 minute ;

11° Rinçage en *aqua fontis* pendant 5 minutes ;

12° Observation au microscope après dessèchement.

Superposés à la coloration de l'appareil on voit de petits points noirs, qui résultent de la réduction de l'$AgNO_3$ par l'acide ascorbique.

Il s'agit d'une coloration de durée illimitée.

Pour d'autres détails voir la publication déjà citée de Nunziante Cesàro, Saitta et D'Urso.

RÉSULTATS

Saturnisme professionnel. — La réaction s'est montrée toujours positive dans les granulocytes éosinophiles ; positive dans 9 cas, dans les monocytes ; souvent négative dans les lymphocytes et dans les granulocytes neutrophiles.

Fig. 1. — Acide ascorbique dans les leucocytes du sang périphérique. (Méthode de Nunziante Cesàro, Saitta et D'Urso).

On observe en haut 1 lymphocyte et au centre 2 granulocytes neutrophiles avec réaction positive.

Ces derniers leucocytes, au contraire des autres, n'ont jamais d'éléments avec une réaction intense.

En général l'acide ascorbique apparaît diminué dans les leucocytes du sang périphérique.

L'« Has » a eu une valeur moyenne de 1,16 avec oscillation de 0,91 à 1,28 (valeur inférieure à la normale) (tableau p. 616).

Silicose. — Les résultats sont voisins de ceux du saturnisme.

Dans un seul cas on a mis en évidence des granulocytes neutrophiles à réaction intense.

L'« Has » a une valeur moyenne de 1,11 avec oscillation de 1 à 1,21 et il indique une diminution de l'acide ascorbique analogue à celle du saturnisme (tableau p. 618).

Intoxication cyanhydrique. — La réaction apparaît très fréquemment positive. Les granulocytes éosinophiles et les monocytes sont toujours positifs.

Souvent les granulocytes neutrophiles et les lymphocytes sont négatifs et ceux qui sont positifs présentent une réaction faible ou moyenne, et dans des cas très rares une réaction intense.

Dans la plupart des cas les granulations sont très petites et dans les lymphocytes elle forment juste un anneau périnucléaire. On ne voit jamais de formes en chapelet.

En général les cellules présentent un comportement réactionnel très variable.

L'« Has » oscille de 0,8 à 1,64 avec une valeur moyenne de 1,34 légèrement inférieure à la normale (tableau p. 650).

Intoxication par anhydride sulfureux. — Il s'agit d'ouvriers qui travaillent dans une raffinerie et qui depuis plusieurs années inhalent des poussières et des vapeurs de soufre.

La réaction est presque toujours positive et les éléments à réaction faible ou moyenne sont très rares.

Les éosinophiles et les monocytes se présentent toujours positifs.

Des formes annulaires sont visibles dans les lymphocytes.

L'« Has » a une valeur moyenne de 1,69 avec une oscillation de 1,50 à 1,73.

L'intoxication par le soufre est la seule qui présente une augmentation de l'acide ascorbique dans les leucocytes du sang périphérique (tableau p. 652).

CONCLUSION

La recherche histochimique de l'acide ascorbique dans les éléments cellulaires du sang périphérique a été effectuée dans la silicose, le saturnisme, l'intoxication par les cyanures et par le soufre.

Elle a démontré une diminution de la vitamine C dans la silicose et dans le saturnisme. Une faible diminution est aussi évidente dans l'intoxication par les cyanures.

Au contraire dans l'intoxication par le soufre on observe une augmentation de l'acide ascorbique.

Il faut se souvenir que la disposition périnucléaire ou chapelet est formée par de petits corpuscules qui conservent leur individualité, tandis que la disposition annulaire est formée par des granulations qui ont perdu cette individualité, à cause de leur fusion.

RÉSUMÉ

On a étudié le comportement de l'acide ascorbique dans les éléments cellulaires du sang périphérique de sujets atteints des maladies professionnelles suivantes : silicose, saturnisme, intoxication par les cyanures et intoxication par le soufre.

SATU

	Réaction négative	Réaction positive			Total
		faible	moyenne	intense	
CAS I					
Granulocytes neutrophiles	17	30	3		70
Granulocytes éosinophiles			3		3
Granulocytes basophiles	3	7	10	3	23
Lymphocytes			4		4
Monocytes	20	57	20	3	100
Pourcentage					
ILAS					= 1,06
CAS II					
Granulocytes neutrophiles	4	45	14	1	64
Granulocytes éosinophiles		1	3		4
Granulocytes basophiles					
Lymphocytes		20	9	1	30
Monocytes		2			2
Pourcentage	4	68	26	2	100
ILAS					= 1,26
CAS III					
Granulocytes neutrophiles	9	38	18		65
Granulocytes éosinophiles			3		3
Granulocytes basophiles					
Lymphocytes	3	14	7	4	28
Monocytes			4		4
Pourcentage	12	52	32	4	100
ILAS					= 1,23
CAS IV					
Granulocytes neutrophiles	18	32	4	1	55
Granulocytes éosinophiles			2		2
Granulocytes basophiles					
Lymphocytes		12	18	1	31
Monocytes		2			2
Pourcentage	18	46	24	2	100
ILAS					= 1
CAS V					
Granulocytes neutrophiles	8	48	18		74
Granulocytes éosinophiles			2		2
Granulocytes basophiles					
Lymphocytes	1	5	9	4	22
Monocytes		2			2
Pourcentage	12	55	29	4	100
ILAS					= 1,25

NISME

	RÉACTION négative	RÉACTION POSITIVE faible	moyenne	intense	TOTAL
CAS VI					
Granulocytes neutrophiles	16	48	4		68
Granulocytes éosinophiles			2		2
Granulocytes basophiles	6	14	10		30
Lymphocytes					
Monocytes	22	62	16		100
Pourcentage					
ILAS					= 0,94
CAS VII					
Granulocytes neutrophiles	6	48	15		69
Granulocytes éosinophiles			2		2
Granulocytes basophiles		1			1
Lymphocytes	6	12	8		26
Monocytes			2		2
Pourcentage	12	61	27		100
ILAS					= 1,15
CAS VIII					
Granulocytes neutrophiles	10	45	17		72
Granulocytes éosinophiles		2			2
Granulocytes basophiles					
Lymphocytes	8	9	6	1	24
Monocytes			2		2
Pourcentage	18	56	25	1	100
ILAS					= 1,09
CAS IX					
Granulocytes neutrophiles	10	50	4		70
Granulocytes éosinophiles				1	1
Granulocytes basophiles					
Lymphocytes	8	7	10	1	26
Monocytes		3			3
Pourcentage	24	60	14	2	100
ILAS					= 0,94
CAS X					
Granulocytes neutrophiles	7	37	21		65
Granulocytes éosinophiles				1	1
Granulocytes basophiles					
Lymphocytes	2	18	12		32
Monocytes			2		2
Pourcentage	9	55	35	1	100
ILAS					= 1,10

31

	Réaction négative	Réaction positive faible	Réaction positive moyenne	Réaction positive intense	Total
CAS I					
Granulocytes neutrophiles	10	50	8		68
Granulocytes éosinophiles			2	1	3
Granulocytes basophiles					
Lymphocytes	5	10	12	1	28
Monocytes			1		1
Pourcentage	15	60	23	2	100
ILAS					= 1,12
CAS II					
Granulocytes neutrophiles	8	49	8		65
Granulocytes éosinophiles		1	1		2
Granulocytes basophiles					
Lymphocytes	2	9	21		32
Monocytes	1				1
Pourcentage	11	59	30		100
ILAS					= 1,19
CAS III					
Granulocytes neutrophiles	13	41	7		61
Granulocytes éosinophiles		1	2		3
Granulocytes basophiles	1				1
Lymphocytes	8	10	12	1	31
Monocytes		3	1		4
Pourcentage	22	55	22	1	100
ILAS					= 1,02
CAS IV					
Granulocytes neutrophiles	13	51	8		72
Granulocytes éosinophiles		3			3
Granulocytes basophiles					
Lymphocytes	2	12	6		20
Monocytes		4	1		5
Pourcentage	15	70	15		100
ILAS					= 1
CAS V					
Granulocytes neutrophiles	8	45	6		59
Granulocytes éosinophiles			1	1	2
Granulocytes basophiles					
Lymphocytes	8	15	12		35
Monocytes	2	2			4
Pourcentage	18	62	19	1	100
ILAS					= 1,03

COSTE

	Réaction négative	Réaction positive			Total
		Intense	Moyenne	Faible	
CAS VI					
Granulocytes neutrophiles	5	41	13	2	61
Granulocytes éosinophiles			4		4
Granulocytes basophiles					
Lymphocytes	7	19	6	1	33
Monocytes			2		2
Pourcentage	12	60	25	3	100
HAS					= 1,19
CAS VII					
Granulocytes neutrophiles	12	45	7		64
Granulocytes éosinophiles		1	2		3
Granulocytes basophiles					
Lymphocytes	7	10	11		28
Monocytes		2	3		5
Pourcentage	19	58	23		100
HAS					= 1,60
CAS VIII					
Granulocytes neutrophiles	6	42	20		68
Granulocytes éosinophiles			1		1
Granulocytes basophiles					
Lymphocytes	2	18	11		31
Monocytes					
Pourcentage	8	60	32		100
HAS					= 1,24
CAS IX					
Granulocytes neutrophiles	11	31	26		68
Granulocytes éosinophiles				1	1
Granulocytes basophiles					
Lymphocytes	3	18	8		29
Monocytes			2		2
Pourcentage	14	49	36	1	100
HAS					= 1,24
CAS X					
Granulocytes neutrophiles	15	32	18		65
Granulocytes éosinophiles			2		2
Granulocytes basophiles					
Lymphocytes	10	11	8		32
Monocytes			1		1
Pourcentage	25	46	29		100
HAS					= 1,81

INTOXICATION

	Réaction négative	Réaction positive			Total
		faible	moyenne	intense	
CAS I					
Granulocytes neutrophiles	9	35	14	1	59
Granulocytes éosinophiles			1		1
Granulocytes basophiles					
Lymphocytes	2	29	7	2	40
Monocytes					
Pourcentage	11	64	22	3	100
ILAS					= 1,17
CAS II					
Granulocytes neutrophiles	2	18	16	6	42
Granulocytes éosinophiles		1	4	4	9
Granulocytes basophiles					
Lymphocytes	3	20	22	3	48
Monocytes			1		1
Pourcentage	5	39	43	13	100
ILAS					= 1,61
CAS III					
Granulocytes neutrophiles	10	45	10		65
Granulocytes éosinophiles			2		2
Granulocytes basophiles					
Lymphocytes	1	15	15	1	32
Monocytes			1		1
Pourcentage	11	60	28	1	100
ILAS					= 1,10
CAS IV					
Granulocytes neutrophiles	8	27	33	1	69
Granulocytes éosinophiles			1	1	2
Granulocytes basophiles					
Lymphocytes	4	12	10	3	29
Monocytes					
Pourcentage	12	39	44	5	100
ILAS					= 1,12
CAS V					
Granulocytes neutrophiles	3	37	9		69
Granulocytes éosinophiles		1			1
Granulocytes basophiles					
Lymphocytes		18	9	2	29
Monocytes			1		1
Pourcentage	3	76	19	2	100
ILAS					= 1,20

CYANHYDRIQUE

	Réaction négative	Réaction positive			Total
		faible	moyenne	intense	
CAS VI					
Granulocytes neutrophiles	26	31	4		61
Granulocytes éosinophiles			1	1	2
Granulocytes basophiles					
Lymphocytes	3	25	7	1	36
Monocytes		1			1
Pourcentage	29	58	12	2	100
ILAS					= 0,88
CAS VII					
Granulocytes neutrophiles	3	27	23	3	56
Granulocytes éosinophiles					
Granulocytes basophiles					
Lymphocytes		19	16	7	42
Monocytes			2		2
Pourcentage	3	46	41	10	100
ILAS					= 1,56
CAS VIII					
Granulocytes neutrophiles	1	36	29	7	73
Granulocytes éosinophiles	1			1	2
Granulocytes basophiles					
Lymphocytes	1	11	7	5	24
Monocytes				1	1
Pourcentage	3	47	36	14	100
ILAS					= 1,61
CAS IX					
Granulocytes neutrophiles	2	48	19	1	70
Granulocytes éosinophiles			2		2
Granulocytes basophiles					
Lymphocytes	2	11	11	3	27
Monocytes			1		1
Pourcentage	4	59	33	4	100
ILAS					= 1,37
CAS X					
Granulocytes neutrophiles	2	31	14	2	49
Granulocytes éosinophiles				1	1
Granulocytes basophiles					
Lymphocytes	4	27	14	4	49
Monocytes			1		1
Pourcentage	6	58	29	7	100
ILAS					= 1,37

INTOXICATION

	Réaction négative	Réaction positive faible	Réaction positive [illegible]	Réaction positive intense	Total
CAS I					
Granulocytes neutrophiles	2	21	32	1	56
Granulocytes éosinophiles			1	1	2
Granulocytes basophiles					
Lymphocytes	2	12	20	4	38
Monocytes		2	2		4
Pourcentage	4	35	55	6	100
ILAS					= 1,63
CAS II					
Granulocytes neutrophiles	4	20	28	4	65
Granulocytes éosinophiles		1	1	1	3
Granulocytes basophiles					
Lymphocytes	2	19	15	3	39
Monocytes		2			2
Pourcentage	6	42	44	8	100
ILAS					= 1,51
CAS III					
Granulocytes neutrophiles	4	23	34	2	63
Granulocytes éosinophiles			2		2
Granulocytes basophiles					
Lymphocytes	6	8	9	4	27
Monocytes			3		3
Pourcentage	10	30	48	6	100
ILAS					= 1,50
CAS IV					
Granulocytes neutrophiles	5	28	26	5	64
Granulocytes éosinophiles				1	1
Granulocytes basophiles					
Lymphocytes		18	10	4	32
Monocytes			3		3
Pourcentage	5	46	39	10	100
ILAS					= 1,51
CAS V					
Granulocytes neutrophiles	5	27	30	4	66
Granulocytes éosinophiles		1		1	2
Granulocytes basophiles					
Lymphocytes	4	10	9	6	29
Monocytes			3	1	3
Pourcentage	9	38	41	12	100
ILAS					= 1,50

PAR LE SOUTIER

	RÉACTION négative	RÉACTION POSITIVE			TOTAL
		faible	moyenne	intense	
CAS VI					
Granulocytes neutrophiles	10	20	27	4	61
Granulocytes éosinophiles				2	2
Granulocytes basophiles	6	7	12	8	33
Lymphocytes		1	3		4
Monocytes	10	28	42	14	100
Pourcentage					
ILAS					= 1,54
CAS VII					
Granulocytes neutrophiles	3	20	30	6	68
Granulocytes éosinophiles			2	2	4
Granulocytes basophiles	2	6	7	10	25
Lymphocytes			3		3
Monocytes	5	35	42	18	100
Pourcentage					
ILAS					= 1,73
CAS VIII					
Granulocytes neutrophiles	4	30	41	1	76
Granulocytes éosinophiles				1	1
Granulocytes basophiles		2	11	7	20
Lymphocytes		2	1		3
Monocytes	4	34	53	9	100
Pourcentage					
ILAS					= 1,67
CAS IX					
Granulocytes neutrophiles	7	19	32	6	64
Granulocytes éosinophiles			1	1	2
Granulocytes basophiles	1				1
Lymphocytes	4	6	14	6	30
Monocytes		1	2		3
Pourcentage	12	26	49	13	100
ILAS					= 1,63
CAS X					
Granulocytes neutrophiles	7	25	30	6	68
Granulocytes éosinophiles				1	1
Granulocytes basophiles		10	12	8	30
Lymphocytes			1		1
Monocytes	7	35	43	15	100
Pourcentage					
ILAS					= 1,60

La méthode de coloration a été celle mise au point par Nunziante Cesàro, Saitta, D'Urso basée sur la réaction de Szent-Gyorgy.

On a observé une évidente diminution de l'acide ascorbique dans la silicose et le saturnisme.

Dans l'intoxication par les cyanures, cette diminution est plus faible, tandis que dans celle par le soufre on a trouvé une nette augmentation de la vitamine C.

Institut de Médecine du Travail de l'Université de Messine
(*Directeur : Professeur A. Nunziante Cesàro*).

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A Field Test for the Approximate Estimation of Sulphur Dioxide in Fruits Preserved for Jam Manufacture

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(*British Food Manufacturing Industries' Research Association*)

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IN 1954, a simple and rapid test was developed on behalf of the Prepared Fruit Section of the Food Manufacturers' Federation, Inc., by Dr. D. Dickinson, of the Fruit and Vegetable Canning and Quick Freezing Research Association, for the estimation of the strength of sulphur dioxide solutions used in preserving fruit for jam manufacture. Equipment and chemicals for the test were put on the market under the name "The Sulphite Preservative Solutions Test". The test has since been further developed in the laboratories of the B.F.M.I.R.A.* to make it suitable for application to fruits preserved in sulphur dioxide. This further development of the test is described below. It can be regarded as an extension of the Sulphite Preservative Solutions Test, sets of equipment for which are obtainable through the Food Manufacturers' Federation, Inc.*

It will be noted that additional chemical reagents and apparatus are required for the present test. These are readily available from the usual laboratory supply firms.

Description of Test

Reagents:

+ Potassium Iodate Iodide Tablets.
+ Concentrated Hydrochloric Acid Solution.

Sodium Hydroxide Solution, 5 per cent. Prepared as follows:

Weigh 125 g. of flake caustic soda into a large beaker, stir on distilled or tap water carefully until the soda is dissolved, allow to cool for a short time, then pour into a Winchester quart bottle and fill up with water (tap water or distilled). Insert a cork and mix. (This solution is corrosive and should not be allowed to come into contact with the skin or clothes.)

Solution of Soluble Starch, 1 per cent. Prepared as follows:

Weigh out 1 g. of soluble starch into a beaker, add 100 ml. water, heat to boiling with stirring, and cool.

Distilled water.

Apparatus:

Measuring cylinder, 10 ml.
" " 50 ml.
" " 500 ml. (preferably stoppered)
Conical flask, 300 ml.

Procedure:

Place 50 ml. of the 5 per cent sodium hydroxide solution in the 500 ml. measuring cylinder, add 150 ml. of the SO_2-preserved fruit (or, with strawberries, the liquor strained off through a coarse sieve), and well mix. Dilute to the 300 ml. mark and again mix thoroughly. (If the cylinder is not a stoppered one, this can be done by placing a thin rubber pad over the top, pressing on firmly with the hand, and shaking.)

In the conical flask dissolve one of the potassium iodide iodate tablets in about 50 ml. of distilled water, and add 10 ml. of the hydrochloric acid to liberate iodine. To this solution add the alkaline fruit mixture gradually, while shaking, until the colour of the iodine has nearly gone. Then add a few ml. of the starch solution, and add further fruit mixture, a little at a time, with shaking, until the blue colour of the starch disappears, leaving only the colour of the fruit. Note the number of ml. of fruit mixture used up (i.e., the 300 ml. initial volume minus the volume of the unused mixture). Read off the approximate amount of SO_2 in the SO_2-preserved fruit from the table in col. iii.

ml. of Fruit Mixture Used	SO_2 Content of SO_2-preserved Fruit in p.p.m.
60	2,900
65	2,680
70	2,480
75	2,320
80	2,180
85	2,050
90	1,930
95	1,830
100	1,740
110	1,580
120	1,450
130	1,340
140	1,240
150	1,160
160	1,090
170	1,020
180	970
190	920
200	870
220	790
240	730
260	670
280	620
300	580

Acknowledgment

The experimental work involved in developing the test was carried out by Miss E. A. Flack.

The thanks of the authors are due to the Director and Council of the British Food Manufacturing Industries' Research Association for permission to publish this test

* *The price of these sets of equipment is £1 10s. per set—inclusive of delivery in the British Isles. Orders should be made payable to "Food Manufacturers' Federation, Inc." and sent together with the order, to the Federation at 6, Lower Place, London, N.W.1.*

* *These are contained in the sets of equipment for the Sulphite Preservative Solutions Test referred to above.*

British Institute of Management National Conference

The British Institute of Management has announced that its National Conference, the tenth to be held since its inauguration, will this year be held at Harrogate from November 2nd to 4th, when the theme will be "The Impact of Science on Management in the Future". Speakers who have already accepted invitations to address the conference include John Diebold, American pioneer of the application of "automation", Sir Geoffrey Vickers, V.C., and Sir Charles Goodeve, O.B.E., F.R.S., Director of the British Iron and Steel Research Association.

Provision is being made for 1,000 top managers to be received at Harrogate as delegates to the conference and a programme is being prepared which will include two plenary sessions and twenty-four sectional meetings devoted to specialized aspects of management.

Zdravookhranenie 12 (1969)

Гигиена, эпидемиология
и организация здравоохранения

УДК 612.36—099

33

ВЛИЯНИЕ ВИНОГРАДНОГО СОКА, СОДЕРЖАЩЕГО СЕРНИСТЫЙ АНГИДРИД, НА ОРГАНИЗМ ЖИВОТНОГО

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Из Молдавского научно-исследовательского института гигиены и эпидемиологии (директор — к. м. н. А. П. Дисколенко)

Из производных сернистой кислоты, используемых для консервирования пищевых продуктов, все шире применяется сернистый ангидрид (SO_2). По данным А. Д. Игнатьева (1964) сернистый ангидрид обладает неспецифическими фунгицидными и бактерицидными свойствами, однако микроорганизмы довольно быстро привыкают к нему. В консервированных продуктах сернистый ангидрид разрушает витамин B_1, но благодаря кислотным свойствам способен сохранить витамин С.

Влияние сернистого газа на организм при поступлении через дыхательные пути хорошо изучено (И. В. Сидоренков, 1950; Н. В. Волкова, 1956; Т. А. Быстрова, 1957; И. К. Атякина, 1959; А. С. Ан, 1960). Исследования, посвященные изучению влияния на организм препаратов сернистой кислоты при пероральном введении, единичны. Не изучен механизм действия сернистого ангидрида при поступлении в организм, нет сведений относительно влияния данного консерванта на молодой растущий организм. Последнее весьма важно, если учесть, что сернистый ангидрид предлагается для консервирования виноградного сока, который, как известно, предназначается в основном для детского питания.

Целью настоящей работы было изучение влияния виноградного сока, сульфитированного сернистым ангидридом, на организм животных в эксперименте. В опытах были использованы 3 партии виноградного сока: сульфитированный 100 мг/л сернистого ангидрида (остаточное количество SO_2 70,4 мг/л), сульфитированный 50 мг/л сернистого ангидрида (остаточное количество 43,5 мг/л) и несульфитированный. Опыты проводились на 4 группах животных (80 крыс и 80 мышей), три из которых получали виноградный сок в течение 6 месяцев из расчета среднего количества жидкости, потребляемой в сутки. 4-я группа служила контролем. У крыс периодически (1 раз в месяц) исследовали морфологический состав крови (количество гемоглобина, эритроцитов, лейкоцитов, эозинофилов), определяли газообмен по количеству выделенной организмом углекислоты. Вели наблюдение за динамикой веса, общим состоянием, поведением, взятием корма. По истечении 6 месяцев животных забивали и определяли содержание аскорбиновой кислоты в надпочечниках, гликоген печени, щелочную фосфатазу крови, оксидазную активность, общий белок и белковые фракции крови, фагоцитарную активность лейкоцитов, весовые коэффициенты внутренних органов. У мышей вели наблюдение за общим состоянием, взятием корма, динамикой веса, выживаемостью, а также определяли газообмен (по количеству выделенной CO_2). Полученные данные подвергали вариационно-статистической обработке с использованием формул «малой выборки» (И. А. Ойвин, 1960; Е. Л. Ноткин, 1962). Органы забитых и по-

PURPOSES OF STUDY OR RESEARCH IN LIEU OF LENDING THE ORIGINAL.

гибших животных были подвергнуты патоморфологическому исследованию.

Первая группа животных получала виноградный сок, сульфитированный сернистым ангидридом из расчета 100 мг/л.

На протяжении первых 4 месяцев с момента получения сока в состоянии крыс произошли незначительные изменения. С пятого месяца у них появилась вялость, у большинства обнаруживались нагноительные процессы (абсцессы в области шеи, грудной клетки). Подопытные животные отставали в весе по сравнению с контрольными. Имелась тенденция к лейкопении, на четвертом месяце выявлена эозинопения. Существенные сдвиги наблюдались в тканевом субстрате органов: повышение содержания аскорбиновой кислоты в надпочечниках, в печени, уменьшение количества гликогена (см. таблицу).

Группа животных	Аскорбиновая кислота в надпочечниках, мг %	Гликоген печени, мг %	Активность щелочной фосфатазы	Оксидазная активность крови
	M±m	M±m	M±m	M±m
Контроль	333,6±12,8	2887,8±305,0	24,9±4,2	18,8±2,67
Первая	537,0±22,5	1477,7±221,4	13,4±2,7	15,3±1,4
Вторая	433,7±41,6	1823,7±262,0	16,5±1,25	16,6±0,75
Третья	348,8±14,0	2445,5±481,3	16,9±1,9	18,4±1,2

У мышей изменения были более выраженные. Начиная со второго месяца и до конца опыта отмечалось уменьшение веса тела ($P<0,05$—0,001). Уже с третьего месяца они становились вялыми, неопрятными, у некоторых из них наблюдались поносы. С пятого месяца мыши заболели электромелией и к концу опыта все погибли, что указывает на понижение иммунобиологической реактивности организма. Последнее подтверждается тем, что, несмотря на одинаковые условия содержания в виварium, заболела лишь данная группа подопытных животных. Макроскопически у всех животных этой группы (забитых и погибших) выявлено полнокровие внутренних органов. В легких отмечались подплевральные кровоизлияния. Желудок был вздут, его слизистая отечна, гиперемирована. Наиболее выраженные гистологические изменения обнаружены в основном в желудке и печени. Отмечено слущивание железистого эпителия желудка, местами в виде целых пластов, разрыхление и лимфогистиоцитарная инфильтрация стромы (рис. 1). У мышей круглоклеточная инфильтрация не ограничивалась стромой слизистой и мышечного слоя желудка, а распространялась и на строму пищевода. В последнем была обнаружена усиленная десквамация пластов выстилающих его ороговевших эпителиальных клеток.

Рис. 1

Печень у крыс и особенно у мышей подвергалась белковой зернистой дистрофии. На рис. 2 видно мутное набухание печеночных клеток, отдельные эпителиальные клетки печени лишены ядер. Ряд

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клеток, расположенных главным образом вблизи центральных вен, просвет которых, как правило, расширен, претерпевали довольно глубокие дистрофические изменения — вплоть до растворения ядра и плазморексиса. Указанные изменения сопровождались диффузной инфильтрацией стромы печени круглыми лимфоидными и гистиоцитарными клетками, расширением сосудов и атрофией печеночных клеток (рис. 3). О последнем свидетельствовало значительное истончение ряда печеночных балок, особенно у мышей. Со стороны надпочечников было обнаружено утолщение гломерулярной и пучковой зон коркового слоя, вакуолизация протоплазмы клеток мозгового вещества.

Вторую группу составили животные, получавшие виноградный сок, сульфитированный сернистым ангидридом из расчета 50 мг/л.

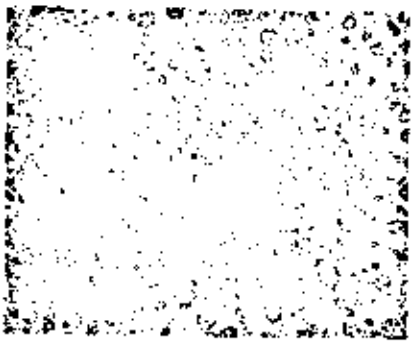

Рис. 2

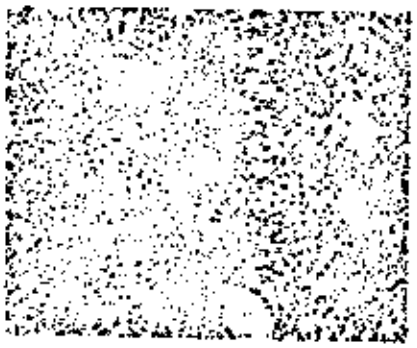

Рис. 3

Наблюдения за животными показали, что опытные крысы прибавляли в весе медленнее, чем контрольные. Потребление кислорода с первого по третий месяц опыта повышалось ($P<0,05—0,001$). Биохимическое исследование указывало на уменьшение содержания гликогена в печени (см. таблицу). У мышей на третьем месяце проведения опыта отмечалось понижение веса тела ($P<0,05—0,01$), на пятом месяце наблюдалось повышение газообмена ($P<0,05$).

При макроскопическом исследовании у крыс и мышей отмечено полнокровие внутренних органов. Слизистая оболочка желудка отечна, гиперемирована. Микроскопические изменения органов аналогичны изменениям, наблюдавшимся у животных первой группы, но по сравнению с ними менее выражены.

Третья группа животных получала несульфитированный виноградный сок. У мышей из этой группы на третьем, а у крыс на пятом месяце отмечалось понижение веса тела. По сравнению с опытными животными других изменений не обнаружено.

Макроскопические исследования показали слабовыраженное полнокровие внутренних органов, гистологические — небольшое слущивание эпителия слизистой желудка.

Таким образом, результаты исследований указывают на то, что виноградный сок, сульфитированный сернистым ангидридом в количестве 50 и 100 мг/л, вызывает значительные изменения в организме животных. Это дает основание считать указанные концентрации SO_2 неприемлемыми для консервации виноградного сока.

Поступила 28/XII 1967 г.

FOR PURPOSES OF STUDY OR RESEARCH IN LIEU OF LENDING THE ORIGINAL.

Ann. Biol. anim. Biech. Biophys., 1965, 5 (2), 267-281.

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LE MÉTABISULFITE DE POTASSIUM
ÉTUDE DE TOXICITÉ A LONG TERME SUR LE RAT

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SOMMAIRE

Pendant 20 mois, deux lots de 80 rats (40 mâles et 40 femelles) ont été soumis à un même régime alimentaire équilibré et ont reçu respectivement, à titre de boisson, soit de l'eau distillée, soit une solution de métabisulfite de potassium à 1,2 g par litre.

Aucune différence n'a été observée entre les deux lots, en ce qui concerne la mortalité, l'évolution pondérale et la consommation de nourriture, les manifestations cliniques, les formules sanguines (sauf en ce qui concerne le nombre des leucocytes), le poids de différents organes (sauf en ce qui concerne celui de la rate), et l'étude anatomique — macroscopique et microscopique — de tous les viscères prélevés à l'issue de l'expérience (œsophage, estomac, grêle, côlon, foie, reins, cœur, poumons, rate, pancréas, surrénales, gonades, fragments de muscles, encéphale, trachée).

Les seuls effets particuliers notés dans le lot d'animaux qui a reçu du métabisulfite de potassium sont : une augmentation du nombre des leucocytes (significative chez les mâles seulement) et une augmentation du poids de la rate (chez les femelles seulement).

D'autre part, au cours de deux reproductions successives, on a observé, dans le lot qui recevait du métabisulfite de potassium, une diminution du nombre moyen des jeunes par portée et de la proportion des mâles dans les portées, par rapport aux résultats obtenus dans le lot témoin. Cependant, la croissance des jeunes — soumis eux-mêmes au régime de leurs parents — a été identique dans les deux lots durant trois mois de détermination.

Le caractère limité des différences observées entre les deux lots et les difficultés de leur interprétation ne permettent pas de conclure actuellement à la nocivité du métabisulfite de potassium dans les conditions où il a été administré. Des recherches complémentaires seront indispensables, en particulier dans le domaine de la reproduction.

INTRODUCTION

L'anhydride sulfureux et quelques-uns des sels qui en dérivent (notamment le métabisulfite de potassium) figurent parmi les antiseptiques les plus anciennement et les plus largement employés en technologie alimentaire (TRCHAUT, 1955 et 1962). En France, on connaît en particulier leurs utilisations en œnologie, en cidrerie,

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en brasserie, dans l'industrie des jus de fruits, pour la conservation des poissons séchés, des crevettes roses, des fruits secs, des pommes de terre grattées, etc. (Truhaut et Souverain, 1963).

Cependant, les effets physiopathologiques de l'ingestion de sulfites font encore l'objet de diverses incertitudes, dues au petit nombre des observations expérimentales approfondies et aux résultats discordants des travaux publiés.

La plupart de ces travaux datent de plus d'un demi-siècle. Le plus ancien est dû à Laveri (1895) qui a administré à une trentaine de personnes diverses des doses de SO_2 dans une petite quantité de vin (30 à 50 ml) ; il observa des troubles divers (irritation de la gorge, brûlures d'estomac, migraines) pour les doses de SO_2 libre égales ou supérieures à 45 mg. Walbaum (1906) a également signalé une irritation du tractus digestif chez des sujets qui avaient reçu quatre fois par jour, pendant quatre jours, des doses de sulfite de sodium comprises entre 310 et 620 mg. D'autre part, Wiley (1907) a étudié le problème chez douze sujets qui avaient reçu pendant vingt jours des doses croissantes de SO_2 en solution aqueuse (80 à 100 mg) ou sous forme de sulfite de sodium en capsules (115 à 760 mg) ; il signala — outre des symptômes subjectifs tels que céphalées, étourdissements, sensations de faiblesse — des troubles rénaux et une anémie hypochrome. Il en conclut à la nécessité de proscrire SO_2, sous toutes ses formes, de toutes les denrées alimentaires.

A la même époque, quelques travaux ont été effectués sur le Chien. Kionka (1896) et Schulz (1902) ont observé des effets nocifs — en particulier des hémorragies — après administration prolongée de faibles doses de sulfites. En revanche, Wildenrath (1906), Lehmann et Treuherz (1909), Rost et Franz (1913) ont conclu à l'innocuité, pour le Chien, de doses de SO_2 allant jusqu'à 100 et même 200 mg par kg ; les derniers auteurs cités ont fait subir ce traitement à leurs animaux pendant un an sans voir apparaître aucune lésion macroscopique ou microscopique.

Les conclusions de certains de ces travaux — et singulièrement celles des observations sur l'Homme — ayant ému les milieux viticoles, une Commission scientifique fut constituée en 1910, à Bordeaux, pour reprendre l'étude du problème dans ses aspects œnologiques. Sous l'impulsion de cette commission, une première série de recherches fut effectuée sur des chiens qui reçurent quotidiennement, durant un mois, 10 ml par kg de poids, d'un vin contenant 100 mg de SO_2 libre ou 400 mg de SO_2 total (dont 300 mg à l'état combiné) par litre. En aucun cas, on n'observa de troubles gastriques, rénaux ou sanguins ; à l'autopsie, tous les organes paraissaient normaux (Gautrelet, 1910 *a* et 1910 *b*).

Une seconde série d'expériences fut tentée sur six hommes qui, pendant plusieurs semaines, ingérèrent chaque jour 15 à 20 ml par kg de poids, d'un vin renfermant 400 mg de SO_2 par litre (dont 300 mg à l'état combiné) ; aucun sujet ne fut atteint de troubles rénaux ou sanguins (Gautrelet, 1910 *a* et 1910 *b*).

Bien que la durée de ces expériences ait été trop courte pour conduire à des résultats probants, il a fallu attendre près de 40 ans pour voir apparaître de nouvelles publications sur le même problème. En 1946, Fitzhugh, Knudsen et Nelson ont publié le compte rendu d'une étude de toxicité à long terme sur des rats dont le régime contenait de 0,05 à 2 p. 100 de métabisulfite de sodium (soit 0,03 à 1,2 p. 100 de SO_2). Pour les doses de métabisulfite égales ou supérieures à 0,10 p. 100, ils signalent un ralentissement de la croissance et le développement de troubles macroscopiques et microscopiques : « yeux à lunettes », crises de polynévrite, atrophie de

différents organes, calcifications rénales, fibrose et hypertrophie épithéliale gastrique « squameuse », altérations de l'utérus, dépigmentation des incisives, etc. Une partie de ces phénomènes pouvait s'expliquer par une carence en thiamine ; on sait en effet que cette vitamine est détruite en présence de sulfites. Toutefois, l'administration d'un supplément de thiamine laissait subsister la plupart des troubles et des lésions, les crises de polynévrite étant seules à disparaître.

En 1955, nous avons effectué, en collaboration avec FLANZY, une étude de caractère préliminaire sur les effets respectifs, chez le Rat, d'un vin de Muscadet naturel, du même vin sulfité à l'avance par addition de 400 mg de métabisulfite de potassium par litre (25 p. 100 de SO_2 ainsi ajouté passant à l'état combiné), et du même vin sulfité à la même dose au moment même de l'administration. Les sujets qui, pendant 6 mois, recevaient les vins sulfités, ont pris du poids moins rapidement que ceux qui recevaient le vin naturel ; c'est le vin sulfité au moment même de l'emploi qui a entraîné le ralentissement de la croissance le plus marqué. En outre, ce vin a provoqué une nette hypertrophie du foie.

En revanche, HÖRZIG (1961-1962) n'a observé aucune modification du gain de poids, de l'indice de consommation et du poids de différents organes chez le Rat soumis pendant 100 jours à un régime alimentaire équilibré, additionné de doses de sulfites correspondant à 400 mg de SO_2 par kg de poids corporel.

Enfin, alors que l'étude qui fait l'objet de ce mémoire prenait fin, une communication sur le même sujet a été présentée par LANTEAUME et *al.* (1965) devant la Société des Experts-Chimistes de France. Ces auteurs ont administré quotidiennement par intubation gastrique à divers lots de rats, pendant 2 ans, 3 ml pour 100 grammes de poids de l'un des liquides suivants :

— eau pure ;

— solution aqueuse de métabisulfite de potassium contenant 450 mg de SO_2 par litre ;

— vin rouge de coupage contenant 110 mg de SO_2 par litre ;

— même vin contenant 450 mg de SO_2 par litre.

L'anhydride sulfureux n'a provoqué ni modifications de la croissance, ni troubles de la reproduction, ni lésions organiques décelables macroscopiquement ou microscopiquement.

PROTOCOLE EXPÉRIMENTAL

Les méthodes d'étude mises en œuvre s'inspirent largement du protocole expérimental recommandé par la F.A.O. (1958) pour l'étude toxicologique des additifs chimiques et sont proches aussi de celui qui a été décrit dans une récente circulaire du Ministère de la Santé Publique et de la Population (1963).

Animaux

L'étude a porté sur 160 rats *Wistar* (80 mâles et 80 femelles), provenant de l'élevage du laboratoire. Au début de l'étude, ils étaient âgés de 28 à 32 jours et pesaient de 50 à 60 grammes. Leur état sanitaire pouvait être considéré comme satisfaisant (courbes de croissance conformes à celles obtenues habituellement au laboratoire, pas d'infection apparente).

Tous ces animaux ont été soumis à un régime alimentaire complet et équilibré. Mais la moitié d'entre eux a reçu comme boisson de l'eau distillée (lot 1) et l'autre moitié une solution de métabisulfite de potassium à 1,2 gramme par litre, soit environ 700 mg de SO_2 par litre (lot 2).

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Régime alimentaire

Les animaux ont été soumis pendant la durée de l'expérience à un régime alimentaire équilibré :

Peptone pancréatique	18
Huile d'arachide (1)	9
Amidon de froment	40
Saccharose	24
Mélange salin de HUBBEL et coll., sans carbonate de calcium (2)	2
Levure de bière sèche	5
Agar-agar	7

Ce régime a été distribué sous forme de bouillie obtenue par addition de 350 ml d'eau distillée à 1 kg du mélange précédent.

Administration du métabisulfite de potassium

Le mode d'administration du métabisulfite de potassium (en solution dans l'eau de boisson) a été choisi en vue d'éviter la destruction partielle de la thiamine du régime par SO_2 avant l'ingestion. Malgré l'odeur et la saveur désagréables de cette solution, — nettement perceptibles pour le consommateur humain —, les rats l'ont acceptée sans difficulté (tabl. 1).

TABLEAU I

Consommation de boisson des animaux d'expérience

	Consommation moyenne de boisson (ml par jour)		
	1er-15e jours	2e mois	A partir du 9e mois
Lot 1 (eau)			
Mâles	16,2	16,0	18,1
Femelles	15,3	16,0	17,8
Lot 2 (solution sulfitée)			
Mâles	14,3	16,0	19,2
Femelles	15,3	16,3	18,1

Pour permettre de comparer les doses de SO_2 ingérées par les rats du lot 1 et celles administrées par d'autres expérimentateurs, nous avons rapporté ces doses aux quantités de nourriture consommées et au poids corporel (tabl. 2).

Les différences liées au sexe résultent du fait que les femelles ingèrent autant de boisson que les mâles, mais consomment moins de nourriture et prennent moins de poids.

Durée de l'étude

L'étude a commencé le 14 juin 1962, et le sacrifice des animaux a été échelonné sur la période allant du 1er février au 1er mars 1964. Elle a donc duré environ 20 mois.

(1) Additionnée de 1 p. 100 d'huile de foie de morue et de 0,015 p. 100 d'α-tocophérol.
(2) Le calcium est fourni par les autres constituants du régime, en particulier par la peptone.

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Observations

Les observations essentielles ont porté sur les points suivants :
— mortalité,
— évolution pondérale,
— consommation de nourriture,
— manifestations d'ordre clinique ;
— nombre de portées, leur constitution, développement pondéral des jeunes (deux générations successives) ;

TABLEAU 2

Quantités de métabisulfite de potassium et de SO_2 ingérées en moyenne par les animaux du lot 2
(rapportées aux quantités de régime consommé ou au poids corporel)

	Pour 100 g de régime consommé		Pour 100 g de poids corporel	
	Métabisulfite de K (mg)	SO_2 (mg)	Métabisulfite de K (mg)	SO_2 (mg)
Mâles				
1er-15e jours	163	95	38	19,0
2e mois	149	86	10	5,8
9e mois	157	91	6,1	3,5
20e mois	—	—	5,0	2,9
Femelles				
1er-15e jours	173	100	33	19,0
2e mois	189	110	13,5	7,8
9e mois	187	106	9,3	5,4
20e mois	—	—	7,0	4,0

— études hématologiques sur 100 sujets de la première génération (25 mâles et 25 femelles de chaque lot) : des numérations d'hématies et de leucocytes ont été effectuées au moment du sacrifice des animaux, et une étude complémentaire a porté sur des frottis de moelle osseuse et sur des frottis de sang, les résultats étant comparés à ceux des numérations ;

— étude anatomique des animaux de la première génération morts pendant l'expérience et des 100 sujets sacrifiés à son issue (par le gaz d'éclairage), cette étude comportant la recherche de lésions macroscopiques, la pesée de certains organes et l'étude microscopique des viscères suivants, après fixation dans le formol à 10 p. 100, inclusion à la paraffine et coloration par l'hématéine-éosine avec, à la demande, coloration élective : œsophage, estomac, grêle, côlon, foie, reins, cœur, poumons, rate, pancréas, surrénales, gonades, fragments de muscles, encéphale, trachée.

RÉSULTATS

Mortalité

La mortalité en cours d'expérience a atteint :

— 9 sujets du *lot 1* (témoins), dont 6 mâles et 3 femelles : soit 12 p. 100 de l'effectif total du lot ;

— 11 sujets du *lot 2* (recevant SO_2), dont 8 mâles et 3 femelles : soit 14 p. 100 de l'effectif total du lot.

Dans les deux lots, cette mortalité est due presque entièrement à une affection des voies respiratoires qui, en juin 1963 a frappé brutalement l'animalerie du laboratoire, en provoquant d'ailleurs une diminution marquée de la consommation de nourriture et une chute de poids chez de nombreux sujets. Des injections intramusculaires d'oxytétracycline (2,5 mg p. 100 g de poids pendant trois jours consécutifs) ont été pratiquées sur tous les animaux sans distinction et ont enrayé les progrès de l'épidémie. La perte de poids a été compensée et l'expérience s'est poursuivie sans nouvel incident pendant les 7 derniers mois.

Si l'on défalque des valeurs citées plus haut les décès observés en juin 1963, la mortalité a été :

dans le lot 1, de 3 sujets (1 mâle et 2 femelles) ;
dans le lot 2, de 3 sujets (1 mâle et 2 femelles).

Il ne semble donc pas qu'il ait y lieu de lui accorder une signification particulière dans le cadre de l'expérience.

Evolution pondérale

Ne pouvant reproduire ici les courbes de poids complètes, nous nous bornerons à indiquer les gains de poids à divers stades de l'expérience (tabl. 3).

Les faibles différences observées entre les deux lots ne sont pas significatives.

TABLEAU 3

Gains de poids des animaux d'expérience

	Gains de poids moyens (g)			
	Après 1 mois	Après 2 mois	Après 6 mois	Après 20 mois
Mâles				
Lot 1 (eau)	86	154	275	398
Lot 2 (solution sulfitée)	88	150	274	392
Femelles				
Lot 1 (eau)	62	102	151	237
Lot 2 (solution sulfitée)	68	106	151	233

Consommation de nourriture

La consommation de nourriture a été déterminée à plusieurs reprises au cours de l'expérience, chaque fois pendant une période de 2 semaines. Aucune différence significative n'a été observée entre les deux lots.

Manifestations d'ordre clinique

En dehors de l'affection des voies respiratoires déjà signalée, un certain nombre de sujets ont présenté, au cours de la deuxième année, des tumeurs sous-cutanées volumineuses siégeant en particulier au niveau de l'abdomen (deux dans le lot

et deux dans le lot 2). En revanche, les crises de polynévrite et le syndrome des « yeux à lunettes » signalées par FITZHUGH, KNUDSEN et NELSON (1946) n'ont été observés chez aucun de nos animaux.

Reproduction

A l'âge de neuf mois, un certain nombre de femelles, choisies au hasard dans chaque lot, ont été mises au mâle (dans une même cage : 3 femelles avec 2 mâles du même lot).

Les animaux de la deuxième génération ont été soumis dès le sevrage (3[illegible] jours) au régime de leurs parents. Lorsqu'ils ont atteint l'âge de trois mois, ils ont été mis en couple, et les animaux de la troisième génération ont été soumis au régime de leurs parents depuis le sevrage jusqu'à l'âge de trois mois.

Les résultats obtenus sont mentionnés dans le tableau 4.

TABLEAU 4

Reproduction des animaux

	Reproduction des sujets de la 1re génération		Reproduction des sujets de la 2e génération	
	Lot 1 (eau)	Lot 2 (SO_2)	Lot 1 (eau)	Lot 2 (SO_2)
Nombre de femelles accouplées	22	21	23	20
Nombre de femelles n'ayant pas donné de portée	0	4	2	3
Durée écoulée entre la mise en couple et la mise bas (jours) (*)	30,7 ± 2,2	33,1 ± 2,5	35,2 ± 0,9	30,3 ± 2,5
Nombre de jeunes par portée (*)	7,5 ± 0,5	6,1 ± 0,4	8,8 ± 0,5	7,8 ± 0,6
Proportion de mâles sur l'ensemble des jeunes (p. 100)	52	45	53	39
Proportion des portées où les mâles sont les plus nombreux (p. 100)	59	42	53	21
Gain de poids des jeunes mâles en 3 mois (g)	240	239	214	219
Gain de poids des jeunes femelles en 3 mois (g)	133	143	126	136

(*) La seconde valeur citée dans chaque colonne de chiffres représente l'erreur standard de la moyenne :

$$E = \sqrt{\frac{\Sigma (x - \bar{x})^2}{N (N - 1)}}$$

Il existe une différence significative entre les deux lots en ce qui concerne le nombre de jeunes par portée au cours de la première reproduction : en moyenne, il est diminué de près de 20 p. 100 dans le lot 2 (différence significative au seuil de $P = 0,05$). Une différence du même genre est observée au cours de la seconde reproduction, mais elle est moins marquée et non significative.

D'autre part, la proportion des mâles dans le lot 2 est moins élevée que dans

le lot 1. La comparaison des résultats obtenus dans les deux lots — par la méthode dite du χ^2 — prouve que la différence observée au cours de la première reproduction peut être due aux hasards de l'échantillonnage, mais que celle observée au cours de la deuxième reproduction est hautement significative (au seuil de $P = 0.01$).

Toutes les autres déterminations donnent des résultats identiques pour les deux lots.

Étude hématologique

a) Le nombre des hématies et des leucocytes contenus dans le sang des animaux à la fin de l'expérience est indiqué dans le tableau 5.

Tableau 5

Numérations globulaires (*)

	Hématies (millions par mm³)	Leucocytes (milliers par mm³)
Mâles		
Lot 1 (eau)	8,94 ± 0,12	8,79 ± 0,74
Lot 2 (SO_2)	8,90 ± 0,28	11,12 ± 0,91
Femelles		
Lot 1 (eau)	8,35 ± 0,37	6,44 ± 0,54
Lot 2 (SO_2)	8,25 ± 0,31	7,17 ± 0,52

(*) La seconde valeur citée dans chaque colonne de chiffres représente l'erreur standard de la moyenne :

$$E = \sqrt{\frac{\Sigma (x - \bar{x})^2}{N (N - 1)}}$$

Dans le cas des hématies, la faible différence observée entre les deux lots n'est pas significative. Le taux des leucocytes est plus élevé dans le lot 2 (SO_2) mais la différence n'est significative que chez les mâles (au seuil de $P = 0.05$).

b) Certains animaux présentaient des anomalies de leur formule sanguine : ainsi a-t-on observé une hyperleucocytose avec polynucléose neutrophile (hyperleucocytose atteignant parfois 16 000 à 19 000), en rapport avec des lésions pulmonaires retrouvées à l'examen anatomique. Ces animaux étaient un peu plus nombreux dans le lot 2 (SO_2). D'autre part, quelques animaux présentaient une leucopénie, mais ceci avec une fréquence égale dans les deux groupes.

c) L'étude des frottis médullaires n'a pas montré de différence entre les deux groupes (tabl. 6). La lecture des lames médullaires a cependant été techniquement difficile, du fait de la richesse en graisse de la moelle du Rat, ce qui a rendu délicat le problème de la coloration des lames.

TABLEAU 6

Myélogrammes (valeurs moyennes)

	Mâles		Femelles	
	Lot 1 (eau) (%)	Lot 2 (SO_2) (%)	Lot 1 (eau) (%)	Lot 2 (SO_2) (%)
Myéloblastes	3	2	2,5	3
Promyélocytes	4,5	5,5	4	4
Myélocytes neutrophiles	25	26	25	24,5
Myélocytes éosinophiles	5	4,5	5	6
Polynucléaires jeunes	6	5,5	5,5	7,5
Polynucléaires neutrophiles mûrs	40,5	41	37	39
Polynucléaires éosinophiles mûrs	3	2,5	4	1
Polynucléaires basophiles mûrs	0	0	0	0
Lymphocytes	3	3	3	1
Monocytes	2	2	4	2
Hématies nucléées	8	8	10	9
	100	100	100	100

Poids de divers organes

Le poids moyen de divers organes, rapporté à celui du corps, figure dans le tableau 7.

La comparaison des valeurs obtenues dans les deux lots ne permet de relever

TABLEAU 7

Poids de quelques organes
(pour 100 g de poids corporel) (*)

	Mâles		Femelles	
	Lot 1 (eau)	Lot 2 (SO_2)	Lot 1 (eau)	Lot 2 (SO_2)
Cœur (g)	0,78 ± 0,027	0,80 ± 0,028	0,41 ± 0,015	0,37 ± 0,011
Foie (g)	4,37 ± 0,14	4,33 ± 0,12	3,81 ± 0,10	3,61 ± 0,11
Rate (g)	0,17 ± 0,012	0,18 ± 0,007	0,17 ± 0,021	0,21 ± 0,025
Reins (g)	1,03 ± 0,05	1,05 ± 0,05	1,02 ± 0,04	0,97 ± 0,03
Surrénales (mg)	11,7 ± 0,80	13,0 ± 0,86	21,4 ± 0,91	20,7 ± 1,09
Testicules (g)	0,74 ± 0,03	0,76 ± 0,03	—	—
Ovaires (mg)	—	—	27,4 ± 3,7	23,7 ± 1,5

(*) La seconde valeur citée dans chaque colonne de chiffres représente l'erreur standard de la moyenne

$$E = \sqrt{\frac{\Sigma (x - \ )^2}{N (N - 1)}}$$

qu'une seule différence significative : elle concerne le poids de la rate, qui est plus élevé chez les femelles du lot 2 que chez celles du lot 1 (différence significative au seuil de P = 0,05).

Étude anatomique : observations macroscopiques

Les principales observations macroscopiques sont résumées dans le tableau 8. Bien que certaines lésions n'aient été observées que dans le lot 2, aucune d'entre elles n'a été suffisamment fréquente pour qu'on puisse conclure à l'existence de différences entre les deux lots.

TABLEAU 8

Étude anatomique : observations macroscopiques

	Nombre de cas (pour 50 rats par lot)	
	Lot 1 (eau)	Lot 2 (SO_2)
Tumeurs sous-cutanées (siégeant en particulier au niveau de l'abdomen)	2	2
Abcès aux poumons	4	4
Kystes dans les muscles de la cuisse	0	4
Abcès aux reins	0	1
Kystes dans le pancréas	0	1

Étude anatomique : observations microscopiques

a) Au niveau du *foie*, ont été observées :

— une dilatation sinusale très fréquente dans les deux lots, plus ou moins marquée, à disposition centro-lobulaire, en rapport avec le mode de sacrifice sans aucun doute ;

— une stéatose se répartissant comme l'indique le tableau 9.

TABLEAU 9

Répartition des cas de stéatose hépatique

	Nombre de rats	Nombre de cas			
		Stéatose nulle	Stéatose discrète	Stéatose nette	Stéatose marquée
Mâles					
Lot 1 (eau)	25	12	7	5	1
Lot 2 (SO_2)	25	13	6	3	3
Femelles					
Lot 1 (eau)	25	10	9	5	0
Lot 2 (SO_2)	25	14	8	3	0

Cette stéatose, sans particularité histologique, se retrouve avec une fréquence comparable dans les deux groupes d'animaux.

b) Au niveau de l'*œsophage*, on a trouvé, chez un mâle qui avait reçu du métabisulfite, une ulcération du bas œsophage. Cette ulcération ne peut vraisemblablement être retenue comme lésion due au sulfite, car de multiples facteurs peuvent expliquer une telle lésion chez un rongeur ; d'autre part, elle a été rencontrée une seule fois.

Aucune différence n'est à signaler entre les deux lots dans la structure de la portion œsophago-gastrique et les régions voisines. Dans les deux lots, l'épithélium malpighien a paru légèrement hyperplasique dans cette région.

c) Au niveau des *reins*, on a trouvé parfois des cylindres hyalins, des cylindres granuleux, des dilatations tubulaires kystiques (tabl. 10). Mais la répartition de ces lésions est identique dans les deux lots.

TABLEAU 10

Répartition de diverses lésions rénales

	Nombre de rats	Nombre de cas		
		Cylindres hyalins	Cylindres granuleux	Dilatations kystiques
Mâles				
Lot 1 (eau)	25	4	1	1
Lot 2 (SO_2)	25	6	1	3
Femelles				
Lot 1 (eau)	24	4	2	1
Lot 2 (SO_2)	25	3	0	1

Quelques images de hyalinisation des basales ont été observées, mais le phénomène restait limité et se retrouvait dans les deux lots. Ces modifications sont à rattacher probablement à des phénomènes de vieillissement.

Il n'a pas été observé de calcification.

Une fois, chez un mâle du lot 2 (SO_2), il a été observé des images de régression tubulaire, paraissant plus en rapport avec des phénomènes infectieux que toxiques (abcès rénal constaté à l'autopsie).

d) Au niveau des *poumons*, de nombreuses lésions inflammatoires à prédominance péribronchique, de caractère aigu au subaigu, ont été retrouvées, en rapport avec des phénomènes cliniques signalés précédemment. Leur répartition était la suivante :

Mâles, lot 1 (eau) ..... 8 cas
Mâles, lot 2 (SO_2) ..... 12 cas
Femelles, lot 1 (eau) ..... 12 cas
Femelles, lot 2 (SO_2) ..... 11 cas

La répartition n'a donc aucun caractère particulier.

c) *Les autres viscères* ont fait l'objet d'une étude systématique, sans qu'il soit possible de mettre en évidence des différences entre les deux lots, ni même des altérations.

L'étude de l'appareil digestif a été particulièrement soignée au niveau de l'estomac, notamment en ce qui concerne la forme et la répartition des éléments (cellules mucipares, bordantes, principales). Quelques images de nécrose ont été observées dans les deux lots au niveau des couches cellulaires superficielles de la muqueuse, elles étaient peut-être en rapport avec les techniques de sacrifice et de fixation des pièces. Ces aspects sont restés très limités. Les infiltrats inflammatoires ont été recherchés, ainsi que les aspects éventuels d'hyperplasie ou d'hypoplasie de la muqueuse.

DISCUSSION

I. — Des observations rapportées ci-dessus, il résulte qu'aucune différence n'a été observée entre les deux lots en ce qui concerne la mortalité, l'évolution pondérale, la consommation de nourriture, les manifestations cliniques, les formules sanguines (sauf en ce qui concerne le nombre des leucocytes), les myélogrammes, le poids de différents organes (sauf en ce qui concerne celui de la rate), l'étude anatomique des viscères.

Les effets de l'administration prolongée de sulfites ont donc été limités :

— au cours des essais de reproduction, on observe une diminution de l'importance numérique des portées et de la proportion des mâles dans les portées ;

— à la fin de l'expérience, le nombre moyen des leucocytes contenu dans le sang est plus élevé que chez les témoins ;

— le poids moyen de la rate des femelles est également plus élevé que chez les témoins.

La signification de ces faits est d'interprétation difficile.

Les observations effectuées au cours des essais de reproduction ne se sont accompagnées apparemment d'aucune malformation chez les jeunes, dont la croissance a été d'autre part normale. L'existence bien connue, en clinique et en pathologie expérimentale, des effets de certains toxiques sur la reproduction, conduit cependant à attacher de l'importance à tout effet particulier constaté dans ce domaine. Dans le cas présent, il sera nécessaire de chercher si la diminution du nombre des jeunes par portée est due à une action sur les gamètes, à des avortements ou à une résorption embryonnaire.

Quant à l'accroissement du nombre des leucocytes dans le groupe qui a reçu du métabisulfite de potassium, il convient de souligner qu'il n'est significatif qu'chez les mâles ; le nombre des leucocytes dans le sang du Rat adulte varie d'ailleurs normalement entre 6 000 et 18 000 par ml (Chesnoy et *al.*, 1962), et il suffit d'un choc émotionnel, ou d'un effort de l'animal qui se débat pendant quelques secondes pour provoquer une leucocytose. Comme on l'a vu, il reste toutefois que le cas d'hyperleucocytose avec polynucléose neutrophile, en rapport avec des lésions

pulmonaires retrouvées à l'examen anatomique, sont un peu plus nombreux chez les animaux qui ont reçu du métabisulfite de potassium que chez les témoins.

Enfin, l'augmentation du poids de la rate dans le groupe qui a reçu du métabisulfite de potassium ne se manifeste que chez les femelles. Un très petit nombre d'animaux en est responsable ; l'omission de 2 d'entre eux (sur 25) suffirait à rendre non significative la différence entre les deux lots.

II. — Les résultats de notre travail concordent avec ceux d'autres études signalées au début de notre exposé mais diffèrent de ceux obtenus par FITZHUGH et *al.* (1946) et par FRANZY et *al.* (1955).

a) Rappelons que les premiers, au terme d'une triple expérimentation effectuée en trois époques différentes et en utilisant divers protocoles dont l'un visait à déterminer la part de l'avitaminose B_1 dans les troubles observés, concluaient au rôle certain des sulfites dans l'apparition des troubles suivants : polynévrite, yeux à lunettes, dépigmentation des incisives, modification de l'utérus, atrophie de différents viscères, calcification rénale tubulaire, atrophie osseuse, nécrose myocardique localisée et hyperplasie squameuse épithéliale gastrique.

Un certain nombre de lésions n'ont pas été recherchées dans notre étude : polynévrite [1], atrophie osseuse, altération de l'utérus. Mais nous n'avons pas retrouvé les autres altérations signalées par FITZHUGH et *al.* Plusieurs raisons peuvent être invoquées pour cela, et en particulier les différences dans les conditions d'expérience.

Nous pensons que bon nombre d'altérations signalées dans le travail de FITZHUGH et *al.*, correspondent en fait à des modifications assez souvent rencontrées chez le rat âgé et que le nombre d'animaux utilisés, tant chez les témoins que chez les animaux traités, ne permet peut-être pas de conclure avec certitude à leur fréquence plus grande chez les animaux traités. C'est le cas en particulier pour les lésions dites de stéatose hépatique qui, dans le dernier travail de FITZHUGH et *al.*, se répartissaient comme suit :

Témoins	5 cas pour 8 rats
0,012 5 p. 100 de métabisulfite de sodium	6 cas pour 9 rats
0,025 p. 100	3 cas pour 8 rats
0,05 p. 100	10 cas pour 16 rats
0,25 p. 100	6 cas pour 9 rats
1,00 p. 100	5 cas pour 10 rats

Il est vrai que pour d'autres altérations le pourcentage chez les animaux traités paraît un peu plus grand que pour les témoins, mais jamais le nombre de témoins utilisé ne permet d'affirmer avec certitude que les aspects observés (assez banals en eux-même) sont valablement plus fréquents chez les animaux traités que chez les témoins.

Enfin, dans un domaine aussi difficile que celui de l'appréciation de modifications statistiquement significatives dans les études de toxicité chronique, il serait bon de pouvoir confronter des images histologiques de ce que les auteurs considèrent comme des altérations pathologiques. Il est vrai cependant que la majorité

(1) Signalons cependant qu'aucune crise de polynévrite n'a été observée.

des altérations signalées dans le travail de FITZHUGH et *al.*, ne serait qu'une survenue plus fréquente, chez les animaux traités, de modifications assez courantes en elles-mêmes et ne nécessitant pas, peut-être, la présentation de micro-photographies.

b) L'absence d'effet du métabisulfite sur le développement pondéral des animaux et sur le poids du foie paraît en discordance avec les résultats que deux d'entre nous avaient obtenus antérieurement avec PLANCY (1955), résultats rappelés dans l'introduction de ce travail. Il est possible que la divergence soit due aux formes d'administration respectives des sulfites (dans le vin, ou en solution aqueuse). Mais il se peut aussi qu'elle résulte, pour une part, des conditions d'alimentation différentes dans les deux expériences : dans notre travail de 1955, les animaux étaient soumis à un régime ne contenant que 2 mg de thiamine par kg, alors que dans la présente étude, le régime en contenait environ le double. Or, HÖTZEL (1961-1962) a constaté que des doses de SO_2 qui n'ont aucune action sur la croissance du Rat soumis à un régime alimentaire largement pourvu en thiamine, peuvent ralentir le développement pondéral lorsque le régime est peu riche en cette vitamine (1).

III. — Il y a lieu de rappeler que les doses de SO_2 administrées pendant la plus grande partie de l'épreuve atteignaient approximativement 40 mg par kg de poids corporel. Or, un homme de poids moyen (65 kg) consommant quotidiennement 1 litre de vin sulfité à la dose licite maximum, recevrait ainsi 7 mg de SO_2 par kg de poids. Le rapport entre les valeurs 40 et 7 peut paraître élevé ; mais il est bien inférieur au rapport entre doses non toxiques pour les animaux d'expérience et doses réellement ingérées par l'Homme pour que la garantie d'innocuité puisse être jugée suffisante (2).

D'autre part, notre travail, du fait même qu'il portait sur des animaux, n'a pas permis de vérifier l'existence de certains symptômes subjectifs dont se plaint parfois le consommateur humain après ingestion d'aliments ou de boissons fortement sulfités (irritation du tractus digestif, céphalées).

Reçu pour publication en janvier 1965.

SUMMARY

POTASSIUM METABISULPHITE : LONG TERM STUDY OF TOXICITY IN THE RAT

For 20 months 2 groups of 80 rats, 40 male and 40 female, were fed on the same balanced ration, and to drink they were given either distilled water or a solution of potassium metabisulphite, 1.2 g per litre.

There was no difference between the groups in mortality, changes in weight or feed intake, nor in clinical signs, blood picture, except for number of white cells, or weight of different organs except for spleen. Macroscopic and microscopic examination of viscera removed at the end of the trial, oesophagus, stomach, small intestine, colon, liver, kidneys, heart, lungs, spleen, pancreas, adrenals, sections of muscle, encephalus and trachea showed no difference between the groups.

(1) Cette constatation suggère que les sulfites peuvent détruire la thiamine *in vivo* — comme ils la détruisent *in vitro* — ou entraver son utilisation par l'organisme.

(2) Dans le cas présent, il n'était pas possible d'expérimenter avec des solutions sulfitées beaucoup plus concentrées, qui auraient été refusées par les rats.

The only effects seen in the animals given potassium metabisulphite were an increase in number of leucocytes, significant in males only, and an increase in weight of spleen, significant in females only.

On the other hand, during two successive generations the group given potassium metabisulphite produced a smaller number of young per litter, with a smaller proportion of males, than in the control group. Growth of the young, maintained in the same way as their parents, was the same in the two groups during the three months it was recorded.

The restricted nature of the differences seen between the two groups and the difficulty of interpreting them does not allow for the present any conclusion about the harmfulness of potassium metabisulphite given in the way it was. Complementary research will be necessary, particularly as regards the effects on reproduction.

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Internat. Z. Vit. Forschung 40 (1970)
Eingegangen am 28. Januar 1970

Thiaminmangel
Thiaminausscheidung
Enzymaktivität

Sulfitstoffwechsel
Sulfittoxizität
Sicherheitsfaktor Mensch-Tier

Thiaminmangel und Unbedenklichkeit von Sulfit für den Menschen

4. Mitteilung:

Schlussfolgerungen und Zusammenfassung der Ergebnisse eines Stoffwechselversuchs am Menschen

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(Direktor: Prof. Dr. H.-D. Cremer)

Zusammenfassung: Bei 12 männlichen und weiblichen Versuchspersonen wurde durch Gabe einer thiaminarmen Kost (120 mcg Thiamin pro 1000 Kalorien während 50 Tagen) eine biochemisch deutlich nachweisbare, aber erwartungsgemäß noch nicht zu klinischen Ausfallserscheinungen führende Thiaminunterversorgung erzeugt. Durch eine orale Belastung mit Sulfit (400 mg SO_2 pro Versuchsperson pro Tag während 25 Tagen) wurde der Thiaminstoffwechsel (Thiaminblutspiegel, Thiaminausscheidung, Aktivität thiaminabhängiger Enzyme) nicht signifikant beeinflusst. Unter diesen Bedingungen wurde auch die Sulfitoxidation nicht gestört (Sulfit in Blut und Harn nicht nachweisbar, Sulfit/Sulfat-Bilanz). Umfassende klinische, neurophysiologische und klinisch-chemische Untersuchungen ergaben keinen Hinweis auf Funktionsstörungen durch die Sulfitaufnahme.

Aus den Versuchen lässt sich ableiten, dass auch unter verschärften Bedingungen (Thiaminmangel) die gesundheitlich unbedenkliche Sulfitaufnahme bei mindestens 40 mg SO_2 pro Person und Tag, wahrscheinlich aber höher liegt. Aus eigenen Tierversuchen (wachsende Ratten), die unter vergleichbaren Bedingungen durchgeführt worden waren, errechnet sich bei Einbeziehung eines Übertragungs- und Sicherheitsfaktors von 100 ein deutlich niedrigerer Wert. Das

[1] Jetzige Anschrift: Institut für Ernährungswissenschaft der Universität Bonn (Direktor: Prof. Dr. D. Hörzel).

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zeigt die Schwierigkeiten bei der Ableitung duldbarer Fremdstoffaufnahmen für den Menschen aus den Ergebnissen von Tierversuchen.

Die Festlegung der duldbaren Höchstmengen eines fremden Stoffes in Lebensmitteln erfolgt meist auf der Grundlage der Ergebnisse von Tierversuchen. Bei der Übertragung tierexperimenteller Befunde auf die Verhältnisse bei Menschen findet in der Regel ein Sicherheitsfaktor von 1 : 100 Anwendung, der die Unterschiede zwischen Mensch und Versuchstier ausgleichen und das Übertragungsrisiko abdecken soll.

Die Anwendung eines Sicherheitsfaktors ist unumgänglich erforderlich, auf der anderen Seite jedoch sehr formal und oft nicht sachgerecht. Soweit die toxikologischen Eigenschaften eines fremden Stoffes es zulassen, sollten daher tierexperimentelle Untersuchungen über die Unbedenklichkeit eines Fremdstoffes durch Versuche am Menschen verifiziert werden. Die dabei erforderlichen hohen Dosen und das damit verbundene Risiko für die Versuchspersonen stehen diesem Wunsch oft entgegen.

In Anbetracht dieser Schwierigkeiten wurde von unserem Arbeitskreis ein verschärfter Test entwickelt, der nachteilige Wirkungen eines Fremdstoffes in einer Reihe von Fällen sehr frühzeitig, bei relativ geringen Dosen und damit noch vor Eintritt eines Allgemeinschadens zu erfassen gestattet. Bei dieser Versuchsmethode wird die Intensität des Stoffwechsels durch Beschränkung der Versorgung mit «kritischen» Nährstoffen reduziert, wodurch die Entgiftungskapazität meist vermindert wird und die Tiere bzw. Versuchspersonen empfindlicher reagieren (Hötzel 1962; Hötzel 1969). Über die Anwendung dieses Verfahrens soll am Modellfall «Thiaminmangel – Sulfittoxizität» berichtet werden.

In früheren Versuchen mit wachsenden Ratten hatte sich ergeben, dass sich die Toxizität von Sulfit bei unzureichender Versorgung mit B-Vitaminen mehr als verzehnfacht (Hötzel 1961). Als entscheidender Faktor wurde die Thiaminversorgugn ermittelt (Cremer und Hötzel 1966).

In Versuchen mit Thiaminmangeltieren ergab sich, dass die toxische Grenzdosis deutlich unter 40 mg SO_2/kg Körpergewicht/Tag liegt, während bei einer bedarfsdeckenden Thiaminzufuhr auch 400 mg SO_2/kg Körpergewicht/Tag ohne erkennbare Schäden vertragen wurden. Leitet man aus diesem Ergebnis einen Wert für Menschen ab, so errechnet sich bei Einbeziehung des üblichen Sicherheitsfaktors 1 : 100, dass die Tagesaufnahme von Menschen an Sulfit unter 0,4 mg SO_2/kg Körpergewicht und Tag liegen sollte, soweit nicht eine ausreichende Thiaminversorgung sichergestellt ist. Erhebungen zeigen aber, dass bei einem Teil der Bevölkerung die Thiaminaufnahme hinter den Empfehlungen

zurückbleibt (Kraut 1963; Aebi 1963 u. a.). Die SO_2-Aufnahme von Menschen übersteigt wiederum bei einem Teil der Bevölkerung den Grenzwert von 0,4 mg/kg Körpergewicht/Tag deutlich.

Aufgrund dieser Zusammenhänge erscheint eine drastische Beschränkung des SO_2-Gehaltes von Lebensmitteln (speziell Wein) geboten. Das hätte jedoch weitreichende praktische Konsequenzen, so dass man sich nicht allein auf die Ergebnisse von Tierversuchen verlassen möchte. Es erschien somit notwendig, eine Untersuchung an Menschen selbst über die Wirkung von Sulfit im Thiaminmangel durchzuführen.

1. Versuchs-Fragestellungen

In unserem Versuch mit Menschen wurden folgende Fragen bearbeitet:

1.1 Erzeugung und Nachweis einer subklinischen Thiaminverarmung.

1.2 Erfassung von Veränderungen des Thiaminstatus durch SO_2-Gaben im Mangel.

1.3 Beeinflussung der Sulfit-Oxidation (Entgiftung) durch die Höhe der Thiaminversorgung.

1.4 Einfluss von Thiaminzufuhr und Sulfitgabe auf Enzymaktivitäten.

1.5 Klinische und klinisch-chemische Untersuchungen, die insbesondere Rückschlüsse auf eine Beeinflussung der Leberfunktion und der Funktion von peripheren motorischen Nerven durch Thiaminzufuhr und Sulfitbelastung erlauben.

2. Ergebnisse

2.1 Es gelang durch Verabreichung der Thiaminmangelkost (120 mcg Thiamin/1000 Kalorien während 50 Tagen), eine Thiaminverarmung der Versuchspersonen herbeizuführen, wie sich anhand folgender Kriterien ergab: 1. Abnahme der Thiaminausscheidung im Harn (Gesamtausscheidung pro Tag und Thiamin-Kreatininverhältnis), 2. Absinken des Thiaminblutspiegels, 3. Verminderung der Aktivität der Transketolase in hämolysiertem Vollblut, Erhöhung der Aktivierbarkeit der Transketolase durch Thiaminpyrophosphat (TPP-Effekt). – Klinisch nachweisbare Mangelerscheinungen wurden bei den gewählten Versuchsbedingungen nicht beobachtet.

2.2 Durch Gaben von Sulfit (400 mg SO_2/Versuchsperson/Tag während 25 Tagen) wurde der Thiaminstoffwechsel nicht signifikant beeinflusst (Thiaminausscheidung im Urin, Thiaminblutspiegel, Transketolaseaktivität, TPP-Effekt). Bei den gewählten Versuchsbedingungen ergaben sich somit keine Anhaltspunkte

für einen Eingriff von Sulfit in den Thiaminstatus. Insbesondere liess sich eine Zerstörung von Thiamin durch Sulfit in vivo in erkennbarem Umfang *nicht* nachweisen.

2.3 Weder im Thiaminmangel noch bei ausreichender Zufuhr liess sich nach Belastung mit Sulfit im Serum oder Harn Sulfit nachweisen. Die Sulfitoxidation kann demnach bei den vorliegenden Versuchsbedingungen durch den Thiaminmangel nicht nennenswert beeinflusst worden sein.

Die Sulfatausscheidung im Harn stieg bei Belastung mit Sulfit signifikant an. Die Höhe der Mehrausscheidung entsprach rechnerisch der applizierten Sulfitmenge + Mehraufnahme an Sulfat. Als Folge der Sulfitbelastung stieg auch die Taurinausscheidung im Harn an. Diese Differenz konnte wegen der grossen Streuung der Einzelwerte nicht statistisch gesichert werden. Die Höhe der Taurinausscheidung im Harn besagt für eine Schwefelbilanz auch nicht viel, da der grössere Teil von Taurin mit der Galle ausgeschieden wird.

2.4 Im Thiaminmangel wird nicht nur die Aktivität der Transketolase vermindert und ihre TPP-Aktivierbarkeit erhöht (siehe 2.1). Auch bei drei untersuchten Dehydrogenasen konnte während der Gabe der thiaminarmen Versuchskost ein Aktivitätsabfall in hämolysierten Erythrozyten um 10 bis 40 % und z. T. ein Wiederanstieg nach zweimaliger Thiamingabe beobachtet werden. Statistisch signifikant war der Aktivitätsabfall der NADH-Diaphorase und der LDH, ebenso der Wiederanstieg der LDH nach Thiamingabe. Der Aktivitätsabfall der G-6-PDH von 10–20 % liess sich wegen der grossen Streubreite der Einzelwerte nicht sichern. Eine Hemmung der Enzymaktivität durch die Sulfitbelastung deutete sich lediglich bei der NADH-Diaphorase an, konnte jedoch nicht gesichert werden. Die ausbleibende Hemmung von Dehydrogenasen durch Sulfit wird durch den Befund erklärt, dass nach oraler Belastung Sulfit auch nicht in Spuren im Serum nachgewiesen werden konnte.

2.5 Allgemeine klinische Untersuchungen zu Beginn und zu Ende des Versuchs, in denen insbesondere nach gastrointestinalen und hepatologischen Störungen gefahndet wurde, blieben ohne Befund. Klinisch-chemische Untersuchungen (GOT, GPT, LDH, α-HBDH und alkalische Phosphatase im Serum, Serum-Elektrophorese, Thymol-Trübungstest, Haemoglobin, Haematokrit und Zahl der Erythrozyten) ergaben keine signifikante Änderung der Enzymkonzentrationen oder der Zusammensetzung von Blut und Serumeiweiss, die als Folge des Thiaminmangels oder der Belastung mit Sulfit angesehen werden könnten.

Da unser Arbeitskreis kürzlich an Versuchstieren bei Thiaminmangel eine Verlangsamung der peripheren Nervenleitungsgeschwindigkeit beobachtet hatte (Kunze 1969; Muskat 1969), wurde bei allen Versuchspersonen eine zwei-

malige neurophysiologische Untersuchung mit Messung der motorischen Nervenleitungsgeschwindigkeit durchgeführt. Die neurologische Untersuchung liess keine Änderung im Reflexverhalten oder Störungen der Sensibilität erkennen. Die motorische Nervenleitungsgeschwindigkeit wurde nicht beeinflusst.

3. Schlussfolgerungen

Durch thiaminarme Kost (120 mcg/1000 Kalorien während 50 Tagen) konnte bei 12 Versuchspersonen ein biochemisch nachweisbarer Thiamin-Mangelzustand hervorgerufen werden. Klinisch manifeste Mangelsymptome traten dabei erwartungsgemäss nicht auf. Die Versuchsbedingungen waren damit so beschaffen, dass eine weitere Verstärkung des biochemisch nachgewiesenen Thiaminmangels durch Sulfit experimentell erkennbar werden musste, wenn ein derartiger Effekt im praktisch relevanten Bereich (maximal mögliche Sulfitaufnahme aus Lebensmitteln und Ausmass der Thiaminunterversorgung in der Ernährungspraxis) bei Menschen überhaupt auftritt. Eine signifikante Verstärkung des Thiaminmangels durch Belastung mit Sulfit (400 mg SO_2/Vpn/Tag während 25 Tagen) liess sich jedoch nicht nachweisen.

Die Oxidation des Sulfits (Entgiftung) wurde durch den Thiaminmangel bei den vorliegenden Versuchsbedingungen nicht erkennbar beeinflusst, wie die Aufstellung der Sulfit/Sulfat-Bilanz ergab. Wenn überhaupt eine derartige Beziehung besteht, dann ist sie nach dem Ergebnis des vorliegenden Versuchs für die Praxis nicht relevant (Sulfitmenge, Ausmass der Thiaminversorgung).

Unabhängig vom Grad der Thiaminversorgung hat sich bei Belastung mit 400 mg SO_2/Versuchsperson/Tag während 25 Tagen aufgrund klinischer, biochemischer und neurologischer Untersuchungen kein Hinweis dafür ergeben, dass mit Sulfitaufnahmen in dieser Grössenordnung gesundheitliche Nachteile für Menschen verbunden sind.

Bei der Ableitung gesundheitlich unbedenklicher Tagesaufnahmen aus dem Ergebnis von Versuchen an Menschen berücksichtigt man üblicherweise einen Sicherheitsfaktor von 10. Danach ergibt sich aus unserem Versuch eine unbedenkliche Tagesaufnahme von 40 mg SO_2 pro Person. Da der Versuch unter verschärften Bedingungen durchgeführt worden war (Thiaminmangel), scheint die Anwendung eines noch kleineren Sicherheitsfaktors vertretbar, wonach sich eine noch höhere duldbare Tagesaufnahme ergibt. Der Versuch zeigt damit auch, dass bei der Errechnung von duldbaren Tagesaufnahmen aus dem Ergebnis von Tierversuchen im Falle von Sulfit zu niedrige Werte erhalten werden.

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Summary: A thiamine deficient diet (120 mcg/1000 calories) produced in human volunteers (males and females) within 50 days significant biochemical signs of thiamine deficiency, but no clinical symptoms. Oral administration of sulfite (400 mg SO_2/person/day) had no significant effect on thiamine status (thiamine blood content, thiamine excretion, activity of thiamine dependant enzymes). Oxidation of the administered sulfite was not influenced under this experimental conditions, as demonstrated by the sulfite/sulfate-balance and sulfite estimations in plasma and urine. Even no traces of sulfite could be detected in body fluids. Extensive clinical, neurophysiological and biochemical investigations delivered no sign for any disturbance of functions by the intake of sulfite.

On the basis of our experiment with humans the daily tolerable intake of sulfite can be calculated as 40 mg SO_2 per person, and even more because the study was conducted under stress conditions (thiamine deficiency). The calculation for the daily tolerable intake of sulfite on the basis of the results of studies with growing rats, which we hold under comparable experimental conditions, led to significant smaller amounts. The discrepancy of both results demonstrates the difficulty in estimating safe intakes of food additives for humans from the results of experiments with animals.

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Note de laboratoire

Sur l'inefficacité des procédés existants de séparation de l'anhydride sulfureux libre et combiné dans les vins et de l'anhydride sulfureux total dans certains jus de raisin

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L'extraction sélective de l'anhydride libre des vins à l'aide des procédés utilisant l'entraînement à l'azote ou à l'air, à volume constant, ne peut être quantitative en raison de la décomposition des combinaisons formées par l'anhydride sulfureux avec les substances colorées à la suite de l'acidification du milieu. De ce fait les teneurs exactes en anhydride libre et combiné ne sont pas connues avec précision. De plus les modes opératoires de ces procédés ne permettent pas d'obtenir une désulfitation complète des jus de raisin riches en substances colorées et iodophiles.

On the inefficiency of existing methods of separating free and combined sulphur dioxide in wines and total sulphur dioxide in certain grape juices

The selective extraction of free sulphur dioxide in wines, using processes involving the drawing-in of nitrogen or air, at constant volume, cannot be quantitative because of the break-down of the combinations formed by the sulphur dioxide with the coloured substances as a result of the acidification of the medium. The exact free and combined sulphur dioxide contents are thus not accurately known.

In addition, the methods of carrying out these processes do not allow complete desulphitation of grape-juices rich in coloured and iodophilous substances.

INTRODUCTION

En principe, la désulfitation à volume constant et à des températures graduées, utilisant l'entraînement par un gaz inerte, tel que l'azote ou par l'air purifié, constitue un moyen idéal pour séparer l'anhydride sulfureux libre, combiné ou total dans les jus de raisin et les vins.

L'œnochimie analytique possède un certain nombre de procédés pour obtenir ces séparations. Malheureusement leur efficacité laisse à désirer, ce que nous allons démontrer dans ce qui suit.

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I. — SÉPARATION DE L'ANHYDRIDE SULFUREUX LIBRE

Comme on le sait depuis de longue date, la concentration d'ions-hydrogène dans les solutions à désulfiter doit être suffisamment élevée (aux environs de *p*H 1) pour assurer, d'une part, la stabilité de l'anhydride libre, et, d'autre part, son élimination rapide. Tout récemment, Aulenrach et Balnat (1) ont confirmé que la désorption de l'anhydride sulfureux libre, par le courant d'azote, est effective à condition que le *p*H de la solution soit compris entre les limites 1 et 2.

C'est en vertu de cette obligation que les techniques analytiques de Paul (2) et de Deibner (3), concernant le dosage de l'anhydride libre, préconisent l'acidification des jus de raisin et des vins de manière à obtenir la concentration des ions-hydrogène correspondant au *p*H indiqué. Par conséquent, ces techniques négligent la labilité des complexes formés par l'anhydride sulfureux avec les substances colorées des jus et des vins rouges et blancs. Comme l'ont démontré expérimentalement Timberlake et Bridle (4), ces complexes sont détruits aux *p*H 1,0-1,5. Par conséquent, l'acidification des jus de raisins et des vins provoque leur enrichissement en anhydride libre aux dépens de l'anhydride combiné avec les substances colorées. Cette circonstance était entrevue il y a plusieurs années par J. Ribéreau-Gayon et Peynaud (5). Dans ces conditions, les procédés actuels, basés sur l'utilisation de l'entraînement par un gaz, ne peuvent pas doser l'anhydride libre vraiment existant dans les vins. Par conséquent, le problème de sa séparation reste entier et doit être repensé et revu sous un angle bien différent. D'autre part, la révision du procédé de Paul [*op. cit.* (2)], admis comme méthode usuelle internationale dans le Recueil des Méthodes Internationales d'Analyses des vins (6), est actuellement bien souhaitable.

Pour notre part, nous considérons nécessaires les nouvelles études afin de mettre au point une technique de séparation de l'anhydride sulfureux libre destinée à remplacer celle proposée par l'un de nous [*op. cit.* (3)], devenue imparfaite à la suite des constatations expérimentales de Timberlake et Bridle [*op. cit.* (4)]. Il faut souligner que cette imperfection concerne à la fois les dosages de l'anhydride libre, dont la teneur se trouve augmentée, et de l'anhydride combiné, diminuée dans la même proportion.

L'ajustement du mode opératoire doit porter également sur l'augmentation du volume de la prise d'essai ce qui permettrait de rendre plus réguliers et plus sûrs les résultats du dosage iodométrique terminal. En contrepartie, on risquerait d'allonger quelque peu la durée des opérations en raison des servitudes accrues de la désulfitation de volumes plus importants.

II. — SÉPARATION DE L'ANHYDRIDE COMBINÉ OU TOTAL DANS CERTAINS JUS DE RAISIN

Lors de l'étude comparative des procédés de séparation de l'anhydride sulfureux total, combiné par distillation (7) (8) et par entraînement à l'azote [*op. cit.* (3)] ou à l'air [*op. cit.* (2)], nous avons constaté l'existence des jus de raisins rebelles à leur désulfitation exécutée selon les prescriptions de ces techniques.

Les jus de raisin rouges expérimentés étaient très colorés en ce qui concerne les échantillons originaires de l'Afrique du Nord, contenant 205-210 g de sucre par litre; par contre, les jus de raisin de macération, obtenus au Domaine Expérimental de la Station, possédaient une couleur très prononcée (rouge vif), leur richesse en sucre étant moins importante, de l'ordre de 150 g par litre.

Les dosages de l'anhydride combiné ou total étaient effectués selon le procédé de Deibner-Benard [*op. cit.* (7, 8)], accepté comme méthode de référence internationale pour la détermination de l'anhydride total [*op. cit.* (6), A 17-*e*, p. 1-4], et suivant la technique de Paul [*op. cit.* (2)] admise comme méthode usuelle par l'Office International de la Vigne et du Vin [*op. cit.* (6), A 17-*e*, p. 4-7]. Le procédé rapide d'essai de Ripper (9) était exécuté sous sa forme simple [*op. cit.* (6), A 17-*e*, p. 7-8] et amélioré d'après le mode opératoire de Jaulmes et Dieuzeide (10).

Les résultats des dosages comparatifs sont consignés dans le *tableau*.

L'essai n° 1 montre clairement l'imperfection saisissante des deux variantes du procédé de séparation par entraînement, le déficit de séparation étant de l'ordre de 78 à 65 %. Par contre, le titrage direct selon Ripper accuse un excédent d'anhydride sulfureux facile à expliquer par la présence des substances iodophiles en nombre.

L'influence de la dilution a été étudiée sur un autre jus de raisin nord-africain ; d'ailleurs cette dernière n'apporte aucun avantage sensible à la technique d'entraînement (essai n° 2). D'après les résultats de l'essai n° 3, l'augmentation de la durée de désulfitation jusqu'à 30 mn pour un volume de 10-15 ml paraît intéressante, sans donner, toutefois, entière satisfaction.

Dans le cas d'un jus de raisin de macération (essai n° 4) la dilution (1 : 1) rend la technique d'entraînement aussi

TABLEAU

Comparaison des procédés de séparation de l'anhydride total, combiné, dans les jus de raisin rouges.

N° Essai	Nature Origine	Distillation (Deibner-Benard)				Entraînement										Directe	
		Iodométrie				Iodométrie (Deibner)					Acidimétrie (Paul)					Acidimétrie (Ripper)	
		Dilution	Prise d'essai ml	SO_2 mg/l Essai	SO_2 mg/l Moyenne	Dilution	Prise d'essai ml	Durée mn	SO_2 mg/l Essai	SO_2 mg/l Moyenne	Dilution	Prise d'essai ml	Durée mn	SO_2 mg/l Essai	SO_2 mg/l Moyenne	Simple	Double
1	Rouge (Afrique du Nord)	— 1 : 1 »	100 100 100	33,4 30,4 32,6	32,1	—	10	15	6,4 4,5 9,6 8,3	7,2	—	10	15	11,2	—	44,8	80,6
2	Rouge (Afrique du Nord)	1 : 1 1 : 1	50 50	20,8 18,4	19,6	1 : 1	10	15	10,2								
3	Rouge (Afrique du Nord)	1 : 1 1 : 1	100	15,3 17,2	1,62	— — —	10	15 20 30	6,4 6,4 12,8								
4	Rouge de macération 1966, Pech-Rouge (Aude)	— 1 : 1	50 100	49,9 40,3	— —	— — 1 : 1	10 — 10	15 — 15	25,6 28,8 — 40,9	27,2 —							
5	Rouge (Afrique du Nord)											10 10 10 10 10	15 30 15 15 30	4,8 6,4 1,9 3,52 3,84	27 ml H_3PO_4 à 25 %		

efficace que celle de distillation avec une seule différence que la distillation fournit des résultats comparables dans les cas des jus utilisés tels quels ou dilués sans aucune ambiguïté. Par conséquent le dosage de l'anhydride sulfureux total ou mieux combiné dans les jus de raisin ne peut se faire que par la méthode de distillation. La technique de séparation de l'anhydride total par entraînement à l'azote ou à l'air convient seulement aux vins.

De ce fait elle perd son caractère universel. Il faut ajouter que nos constatations se rapportent aux techniques anciennes de Woidich (11), Sumuleanu, Botezatu et Nicolau (12), de Frolov-Bagreev et Agabaliantz (13) et aux procédés modernes de Paul [*op. cit.* (2)], de Deibner [*op. cit.* (3)] et *a priori* également à la technique de Tanner (14). Il faut dire que cette dernière se distingue avantageusement des précédentes par l'importance des prises d'essai garantissant mieux la régularité des résultats de dosage. Toutefois, on peut penser que la durée de désulfitation du volume de la prise d'essai (50 ml) additionné du même volume de méthanol soit insuffisant surtout dans le cas de faibles teneurs en anhydride sulfureux.

Ne possédant pas l'appareil de désulfitation de Tanner, nous avons effectué quelques dosages de l'anhydride total d'un jus rouge nord-africain, selon la technique de Paul [*op. cit.* (2)].

On constate (voir l'essai n° 5) que la durée prolongée de désulfitation serait en mesure d'améliorer les résultats et qu'une acidification plus grande du milieu paraît avoir le même effet. Il est certain que les essais plus nombreux et diversifiés soient indispensables pour confirmer ou infirmer les premiers résultats obtenus.

Nous sommes conscients de la nécessité d'amplifier les comparaisons des deux méthodes de dosage de l'anhydride combiné dans les jus de raisin de différentes origines. Mais déjà les résultats partiels obtenus par nous sont suffisamment instructifs pour être portés à la connaissance des analystes.

CONCLUSION

Il serait bien téméraire de surestimer la valeur analytique des techniques actuelles utilisant l'entraînement à l'azote ou à l'air, à volume constant, pour séparer l'anhydride sulfureux libre et combiné dans les vins ainsi que l'anhydride sulfureux combiné total dans les jus de raisin.

Si la séparation convenable de l'anhydride total des vins au moyen de ces procédés reste en général réalisable, elle s'avère impossible à réussir dans certains jus de raisin. Dans ce cas la technique de Deibner et Benard [*op. cit.* (7)] basée sur la distillation du vin à l'abri de l'air, proposée en 1937 par Dubaquié (11), reste seule valable et sûre. Cette technique est également efficace pour séparer l'anhydride total des vins. Dans ces conditions on voit mal l'utilité d'avoir deux techniques de séparation de l'anhydride sulfureux total : une faisant appel à l'entraînement pour les vins et la seconde distillatoire pour les jus de raisin. A ce propos, on est amené à modifier les applications du procédé de l'un de nous [*op. cit.* (3)] prévues pour les vins et les jus de raisin. Actuellement, ce procédé convient seulement pour les vins.

Cette restriction concerne également les procédés de Paul [*op. cit.* (2)] et de Tanner [*op. cit.* (4)]. Il est extrêmement important de mettre les utilisateurs en garde contre l'emploi de ces procédés analytiques dans les transactions internationales en ce qui concerne les jus de raisin. En effet, si l'on se contentait des indications fournies par la méthode de Paul [*op. cit.* (2)] ou par une autre technique, utilisant la désulfitation à volume constant par entraînement gazeux, les jus de raisin étudiés par nous seraient censés contenir moins d'anhydride sulfureux contrairement aux résultats plus élevés trouvés par la méthode de référence éprouvée [*op. cit.* (6, 7, 8)].

Un autre enseignement auquel a abouti notre expérimentation concerne les difficultés, souvent malaisées à prévoir ou à éviter, qui entourent la mise au point des techniques analytiques de caractère universel réputées sûres et valables dans tous les cas. Or, l'existence des cas inconnus aux auteurs des procédés analytiques et classés, d'ailleurs, sans raison évidente, comme exceptionnels, sont nécessaire une collaboration internationale plus développée.

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Dosage iodométrique de l'anhydride sulfureux libre et combiné dans les vins et les jus de raisin après séparation par entraînement à l'azote et fixation dans la solution alcaline

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III. — EXPOSÉ DU MODE OPÉRATOIRE UTILISÉ POUR LA SÉPARATION DE L'ANHYDRIDE SULFUREUX LIBRE ET COMBINÉ CONTENUS DANS LES VINS ET LES JUS DE RAISIN SUIVI DE LEUR DOSAGE IODOMÉTRIQUE

A. — PRINCIPE DE LA TECHNIQUE

L'anhydride sulfureux libre est extrait, par un courant d'azote, d'une prise d'essai de 10 ml de vin ou de jus de raisin acidifiée de manière à obtenir le *p*H 0,85-0,90 et maintenue dans un bain-marie porté à la température constante de + 10° C. Le gaz sulfureux entraîné est absorbé par une solution d'hydroxyde de sodium 0,25 *N* refroidie par un mélange d'eau et de glace. La durée de désulfitation est de 15 minutes pour un volume total de 15 ml. La libération de l'anhydride sulfureux combiné est obtenue consécutivement sur la même prise d'essai en la portant à l'ébullition pendant 15 minutes, la suite des opérations étant la même.

On effectue sur les deux solutions alcalines obtenues le titrage iodométrique de l'anhydride sulfureux libre, l'acide acétaldéhydesulfureux subissant une décomposition complète.

B. — MODE OPÉRATOIRE

a) **Séparation de l'anhydride sulfureux libre et combiné**

1° Réactifs utilisés

— Solution aqueuse d'acide chlorhydrique 0,6 *N*.
— Solution aqueuse d'hydroxyde de sodium 0,25 *N*.
— Complexon III (Versénate ou sel bisodique de l'acide éthylènediamine tétracétique).

2° Appareil de distillation

L'ensemble de l'appareil (*fig. 1*) comprend un ballon à fond rond 1, un réfrigérant ascendant à reflux (conden-

p 143 — 147.

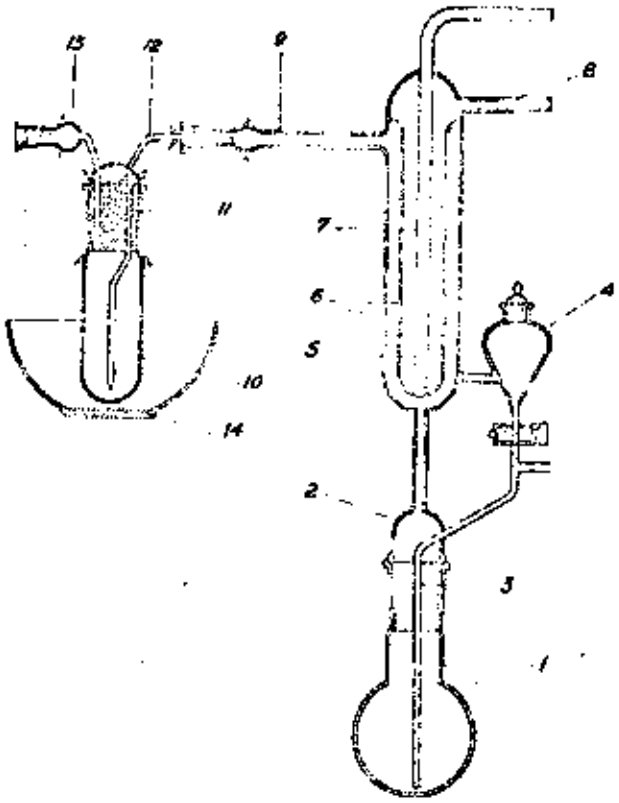

Fig. 1. — Appareil pour la séparation et la fixation de l'anhydride sulfureux.

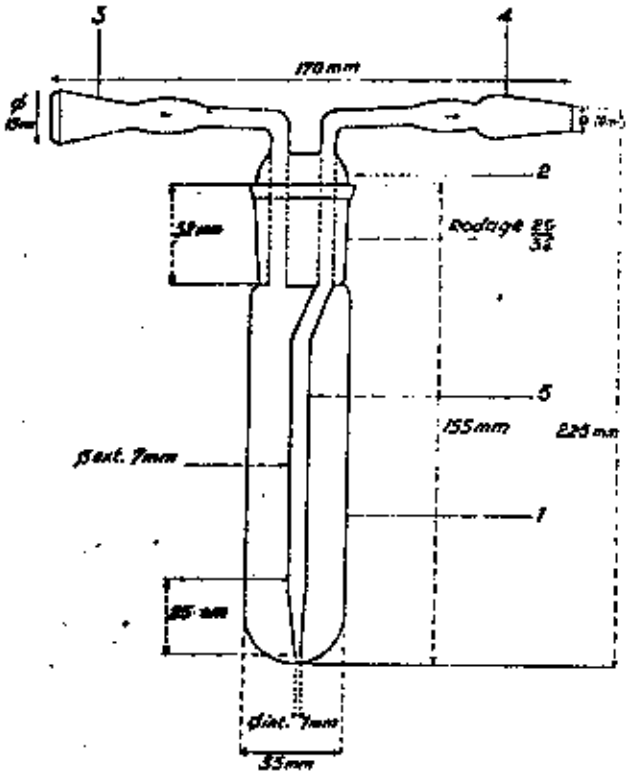

Fig. 2. — Absorbeur à tube de dispersion.

seur type Hopkins) 3, un absorbeur à tube de dispersion 10 (voir également la *fig. 2*).

— *Ballon :* D'un volume utile (sans col) de 125 ml (capacité du ballon et du col : 190 ml, rodage compris), il est plongé pendant l'extraction de l'anhydride libre, dans l'eau glacée maintenue à la température de + 10° C (la température de la prise d'essai à l'intérieur du ballon est de l'ordre de + 12° C). Par contre, pour l'extraction de l'anhydride combiné (ou total) il est nécessaire d'enlever le bain-marie et de poser le fond du ballon sur une ouverture circulaire de 5 cm de diamètre pratiquée dans une épaisse plaque d'amiante (0,5 cm) disposée à son tour sur une plaque de toile métallique.

— *Réfrigérant :* Le réfrigérant à reflux 5 (longueur totale : 25 cm; utile : 22 cm; capacité intérieure : 130 ml) est solidaire d'un rodage 2 et d'une ampoule 4 à robinet au-dessous de laquelle se trouve un tube d'amenée de l'azote 3 (diamètre intérieur 5 mm) pénétrant dans le ballon jusqu'à son fond. Le tube latéral 9, muni d'un rodage (il est préférable de disposer de rodage mâle), permet l'évacuation du courant de gaz vers l'absorbeur 10.

— *Absorbeur :* L'absorbeur est muni d'un tube de dispersion (diamètre extérieur : 7 mm, diamètre intérieur de la partie effilée : 1 mm). Il est préférable d'employer les absorbeurs (*fig. 2*) cylindriques, d'un volume utile de 90 ml, qui permettent d'obtenir une hauteur de 45 mm pour un volume de solution alcaline de 30 ml. L'absorbeur, refroidi par un bain d'eau glacée et de nombreux glaçons (température du liquide alcalin est de l'ordre de + 9° C), est relié à un flacon de garde et à la trompe à eau qui assure le courant de gaz dans l'appareillage. Un manomètre à vide à cuvette, type mural (Société Prolabo), installé dans un support spécial en bois et placé en dérivation entre le flacon de garde et l'absorbeur, permet de contrôler la constance de la dépression utilisée (50 mm de mercure) à l'aide d'une pince à vis de Mohr, disposé sur un tube de caoutchouc à l'entrée du flacon de garde.

Bien entendu, il est possible d'utiliser l'appareil distillatoire de Lieb et Zacherl à condition de veiller particulièrement au lavage soigné de la plaque en verre poreuse suivi de l'expulsion totale du liquide au moyen d'une soufflerie.

— *Dispositif de contrôle du débit de courant d'azote.* L'azote pur (l'azote R, Société l'Air Liquide) nécessaire pour effectuer l'extraction de l'anhydride sulfureux et son transfert vers l'absorbeur est fourni par une bouteille de gaz comprimée; son débit constant (40 litres/heure) est obtenu par un pointeau pour réglage des débits gazeux (Société Prolabo) et contrôlé par un débitmètre à tubes capillaires (Société Prolabo, n° 4396).

3° Conduite des opérations

On introduit dans le ballon 5 ml de solution d'acide chlorhydrique 0,6 N et 3 grains de pierre ponce; on raccorde le ballon au réfrigérant dans lequel circule un fort courant d'eau. Après avoir reçu 30 ml de solution d'hydroxyde de sodium de 0,25 N et 10 mg de complexon III, l'absorbeur est mis en circuit, toujours plongé dans un mélange d'eau glacée et de glace.

On désaère l'appareil par un courant d'azote (débit : 40 litres/heure) pendant 3 minutes. Ensuite, sans arrêter le courant gazeux, on abaisse légèrement le ballon de manière à rendre possible l'introduction d'une prise d'essai de 10 ml, mesurée exactement à l'aide d'une pipette de précision à longue tige, et de suite on raccorde le ballon au réfrigérant. Pendant cette opération l'air ne pénètre pas dans l'intérieur du ballon.

Nous avons vérifié que ce mode d'introduction est meilleur par rapport à celui utilisant l'ampoule à robinet 4 et 2 circuits d'arrivée d'azote : en effet, le lavage de l'ampoule et du tube d'amenée d'azote demande au moins 5 ml d'eau ce qui détermine l'augmentation du volume à désulfiter.

Le ballon reste tout le temps plongé dans un bain-marie, dont la température est maintenue à + 10° C.

L'introduction de la prise d'essai est suivie de la mise en marche de la trompe à eau et de l'ajustement de la dépression à 50 mm de mercure et du débit de l'azote à 40 litres/heure, ce qui constitue un début de désulfitation qui dure 15 minutes. Au bout de 15 minutes, on débranche l'absorbeur et on le remplace par un autre contenant le même volume de solution d'hydroxyde de sodium. Remarquons que le ballon et les absorbeurs peuvent être refroidis préalablement, surtout en été, chacun à la température indiquée précédemment.

On enlève le bain-marie, dans lequel est plongé le ballon, et on pose ce dernier sur l'ouverture circulaire d'une plaque d'amiante supportée par une plaque en toile métallique. Ensuite on chauffe rapidement le ballon avec la flamme d'un bec réglé de manière qu'elle lèche la surface libre de son fond. Dès que le liquide entre en ébullition, on procède à une nouvelle désulfitation au même *p*H (0,85-0,90) pendant 15 minutes dans les conditions identiques à celles de la première désulfitation. Finalement on est en présence de deux solutions alcalines contenant respectivement de l'anhydride libre et combiné.

Bien entendu, il est possible d'effectuer la séparation de l'anhydride total en utilisant une nouvelle prise d'essai.

(b) Dosage iodométrique de l'anhydride sulfureux dans les solutions sulfitiques

1° Réactifs

— Solution aqueuse d'acide sulfurique 0,75 N.
— Solution aqueuse d'iodure de potassium à 10 %.
— Solution aqueuse d'iode N/50.
— Solution aqueuse d'hyposulfite de sodium N/50. L'étalonnage des solutions d'iode et d'hyposulfite s'effectue suivant les indications publiées précédemment (*op. cit.* (15) (39)).
— Empois d'amidon de blé 0,2 % fraîchement préparé selon Chadon et Bézier (86). On délaie 0,2 g dans un peu d'eau distillée et on verse le mélange dans 100 ml d'eau en ébullition que l'on prolonge pendant 3 minutes après sa reprise; après refroidissement on filtre le liquide après décantation. Employer 5 ml par 100 ml de solution à titrer.

2° Titrage iodométrique

La solution alcaline bien froide, ainsi que les eaux de lavage de l'extérieur et de l'intérieur du tube de dispersion, sont versées très lentement dans le bécher de 250 ml de capacité, contenant un morceau de glace préparée à partir de l'eau distillée, un excès d'acide sulfurique, 18 ml ou 30 ml de solution 0,75 N (suivant le volume de solution alcaline utilisé : 30 ou 50 ml), 5 ou 10 ml de solution d'iode N/50 et 10 ml de solution d'iodure de potassium. Au cours de cette addition le contenu du bécher, plongé dans l'eau glacée contenant des glaçons, est constamment agité à l'aide d'un agitateur. Les eaux de lavage (30 ml) de l'intérieur de l'absorbeur sont également versées dans le bécher. Le volume obtenu est de l'ordre de 90 ml. Afin d'éliminer l'effet d'oxydation de l'iodure de potassium et de la volatilisation d'iode, l'ordre d'addition des réactifs est le suivant (*op. cit.* (39)) :

— refroidir la solution acide bien avant le titrage;
— juste avant le titrage ajouter les solutions d'iode et d'iodure de potassium.

Tout de suite après l'addition de la solution sulfitique on retire le bécher de son bain-marie glacé et on titre l'excès d'iode par la solution d'hyposulfite de sodium, en présence d'empois d'amidon ajouté en quantité requise vers la fin du titrage.

Le virage du bleu à l'incolore est observé en lumière réfléchie à l'aide d'un comparateur décrit précédemment (84) (*op. cit.* (15)). Compte tenu que 1 ml de solution d'iode N/50 consommé dans la réaction correspond à 0,64 mg d'anhydride sulfureux, il est facile de calculer sa teneur dans la prise d'essai de 10 ml, ensuite dans un litre de vin ou de jus de raisin.

Tableau IX

Dosage iodométrique de l'anhydride sulfureux libre et combiné dans quelques vins naturels. Résultats en mg d'anhydride sulfureux par litre.

N°	Vins analysés	Libre	Combiné	Total
1	Rouge, Corbières (Aude) 1963	5,8	103,9	109,7
2	Sauterne	11,5	292,5	304,0
3	Muscat de Frontignan	5,2	234,9	238,1

C. — RÉSULTATS OBTENUS

Nous avons effectué le dosage de l'anhydride sulfureux libre et combiné dans quelques vins en utilisant le mode opératoire mis au point. Les résultats obtenus sont consignés dans le *tableau IX*.

On remarquera que les teneurs en anhydride sulfureux libre sont faibles dans les vins analysés.

D. — VALEUR ANALYTIQUE DE LA TECHNIQUE

Comme on le sait, l'obtention de résultats reproductibles en volumétrie implique avant tout que l'on observe des conditions identiques dans l'établissement du titre des solutions et dans l'exécution des titrages (*cf. op. cit.* (15)). On se servira toujours des mêmes récipients jaugés et on effectuera le prélèvement des solutions à la burette de précision avec la même vitesse d'écoulement; la lecture du volume se fera toujours dans les mêmes conditions (2 minutes après la fin du titrage pour la lecture en centièmes de ml).

Nous employons des burettes de 10 ml de capacité, de diamètre intérieur de 7-8 mm, divisées en 1/20 de ml, munies de robinets et de pointes fines. D'après Van Nieuwenburg (85), pour ces burettes, l'adhérence du liquide aux parois est telle que deux minutes d'attente suffisent pour la lecture en centièmes de millilitre et 4 minutes pour celle en demi-centièmes. La pointe fine de nos burettes permet un débit, à chute libre, de 3 gouttes par une division de 0,05 ml ce qui signifie la possibilité d'évaluer 0,0109 mg d'anhydride dans 10 ml de prise d'essai, soit 1,09 mg par litre. Pratiquement les teneurs de 2-3 mg par litre sont dosables à condition de prendre un certain nombre de précautions (*op. cit.* (67)). En particulier, en ce qui concerne l'observation du virage d'empois d'amidon, il est indispensable d'obtenir une décoloration complète du liquide très lentement, en agitant constamment. Le virage du bleu à l'incolore est observé en lumière réfléchie, à l'aide d'un comparateur à deux compartiments aménagés pour recevoir 2 béchers. La paroi arrière de ce comparateur dont l'intérieur est peint en blanc brillant est constituée par une plaque d'opaline blanche.

Un des principaux avantages du procédé décrit consiste en ce qu'il permet d'effectuer la séparation successive de l'anhydride sulfureux libre et combiné sans pertes par l'entraînement à l'aide du courant d'azote.

L'anhydride sulfureux, capté dans la solution d'hydroxyde de sodium, 0,25 *N*, en présence de versénate, ne s'oxyde point; cette solution décompose complètement l'acide acétaldéhydesulfureux et neutralise les acides volatils et les traces d'acide chlorhydrique entraînées par le courant gazeux. L'oxydation de l'anhydride libre par l'iode en milieu acide est instantanée ce qui exclut la recombinaison de l'acétaldéhyde et l'anhydride sulfureux pendant le titrage.

La rapidité, la simplification des manipulations au cours de l'extraction de l'anhydride sulfureux doivent beaucoup à l'agencement de l'appareil distillatoire qui est moins fragile, plus simple et moins cher que l'appareil de Lieb et Zacherl. En plus l'influence de certaines substances volatiles iodophiles, autres que l'hydrogène sulfureux et les thiols, est sans effet en raison des conditions de l'extraction de l'anhydride sulfureux.

Finalement le dosage iodométrique de l'anhydride sulfureux est très précis même si la solution en contient quelques milligrammes par litre. C'est ainsi que, par exemple, pour 8-10 mg d'anhydride par litre, le dosage s'effectue avec une précision de $\pm$ 3 %; elle est de $\pm$ 0,3 % pour les teneurs de l'ordre de 225 mg par litre. L'évaluation de petites teneurs en anhydride ne présente pas de grandes difficultés d'autant plus qu'il est possible d'augmenter l'exactitude des résultats et d'éviter les calculs par l'emploi des solutions d'iode et d'hyposulfite de sodium *N*/64.

Nous avons effectué les 6 dosages iodométriques de l'anhydride libre et combiné, dans le même vin rouge, enrichi en anhydride sulfureux, au cours de la même journée, du 16 mai 1964. Les résultats obtenus sont consignés dans le *tableau X*.

Tableau X

Dosages iodométriques effectués le même jour avec le même échantillon de vin rouge Cintaudt 1964, enrichi spécialement en anhydride sulfureux.

Température du Laboratoire matin + 26,5° C, après-midi + 28,7°. Résultats exprimés en mg de SO_2 par litre.

N°	Doses		Total calculé
	Libre	Combiné	
1	10,24	224,64	234,88
2	10,24	224,64	234,88
3	10,88	224,64	235,52
4	10,24	223,36	233,60
5	10,88	223,36	234,24
6	10,88	223,36	234,24
Moyenne	10,56	224,00	

L'utilisation du calcul statistique appliqué à ces résultats nous a renseigné sur le degré de l'exactitude des résultats fournis par notre technique. Voici les chiffres obtenus. Pour l'anhydride libre la somme des carrés des écarts $\Sigma (x - \bar{x})^2$ étant de 0,64, la variance V est égale à 0,350, et l'écart type de la moyenne de 6 mesures $\sigma_m = \frac{V}{\sqrt{n}} = 0,156$. D'autre part, on sait que pour $n - 1 = 5$ et pour la probabilité de 95 % ($P = 0,05$) la table de Fischer indique pour le coefficient $t = 2,571$. Il s'ensuit que la valeur moyenne vraie a 95 % de chances de se trouver dans les limites $\bar{x} \pm \sigma_m t$, soit $10,56 \pm 0,156$

γ 2,57, c'est-à-dire entre les deux valeurs limites 10,96 et 10,16. Aucune des valeurs obtenues, indiquées dans le *tableau X*, ne se trouve en dehors de ces limites. La valeur moyenne a 95 % de chances d'être connue avec une exactitude égale à $\pm$ 3,8 %.

Quant à l'anhydride sulfureux combiné, la somme des carrés des écarts est de 2,458, la variance de 0,700, l'écart type de la moyenne de 0,312. Finalement la valeur moyenne vraie a 95 % de chances de se trouver dans les limites 224,0 $\pm$ 0,312 $\times$ 2,57, c'est-à-dire entre les deux valeurs limites 224,8 et 223,3. Les valeurs trouvées se trouvent dans ces limites. D'autre part, la valeur moyenne a 95 % de chances d'être connue avec une exactitude égale à $\pm$ 0,3 %.

Comme nous l'avons vue précédemment, les teneurs en anhydride sulfureux libre dans les vins sont, en général, très faibles. Par exemple, les vins du *tableau IX* contiennent de 3,2 à 11,5 mg d'anhydride libre par litre. Or, le recueil des méthodes internationales d'analyses des vins (87), ainsi que celui des méthodes officielles françaises d'analyses des vins et des moûts (88), admettent que les résultats peuvent être connus avec l'exactitude de 10 mg par litre, ce qui équivaut dans le cas des vins cités, à une erreur admissible de 300 à 100 %. Dans ces conditions la révision de cette tolérance pourrait être envisagée pour l'anhydride sulfureux libre.

En ce qui concerne la durée totale des manipulations relatives à l'extraction de l'anhydride sulfureux libre et combiné et au dosage iodométrique de ces deux formes, elle ne dépasse pas 45 minutes ce qui n'est nullement excessif.

Pour terminer il faut remarquer que la nouvelle technique constitue un complément, un prolongement au procédé du dosage iodométrique de l'anhydride sulfureux total, séparé par distillation, de sorte qu'actuellement on dispose d'un ensemble de techniques iodométriques permettant les dosages de différentes formes de l'anhydride sulfureux libre, combiné, total, dans les vins et les jus de raisin après leur séparation, soit par entraînement à l'azote, soit par simple distillation. D'ailleurs, le procédé d'entraînement à l'azote pourrait être parfaitement utilisé pour le dosage rapide de l'anhydride sulfureux total sans offrir, toutefois, la possibilité de doser aussi exactement, comme c'est le cas du procédé du dosage iodométrique de l'anhydride sulfureux total, les sulfates dans le résidu de distillation.

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Dosage iodométrique de l'anhydride sulfureux libre et combiné dans les vins et les jus de raisin après séparation par entraînement à l'azote et fixation dans la solution alcaline

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La possibilité d'utiliser la méthode iodométrique pour le dosage de l'anhydride sulfureux libre, combiné et total dans les vins et les jus de raisin présente un grand intérêt, tant au point de vue pratique qu'au point de vue de l'uniformisation des méthodes analytiques œnochimiques.

Les recherches effectuées ont fourni des enseignements concernant, d'une part, l'extraction sélective de l'anhydride libre et combiné par entraînement à l'azote, au moyen d'un appareil de distillation simple et peu coûteux, et, d'autre part, sa fixation par une solution alcaline, sans aucune perte par volatilisation ou par oxydation. La résolution de ces problèmes analytiques a permis de mettre au point le mode opératoire du dosage iodométrique de l'anhydride sulfureux libre et combiné dans les vins et les jus de raisin dont l'efficacité a été contrôlée par le calcul statistique.

Le dosage iodométrique exact de l'anhydride libre et combiné dans les jus de raisin et les vins restait jusqu'ici un problème analytique non résolu, ce qui a favorisé l'utilisation de différentes techniques acidimétriques, basées sur la transformation de l'anhydride sulfureux en acide sulfurique, à l'aide du perhydrol. Ces techniques acidimétriques sont sujettes à l'influence perturbatrice de différentes substances acides volatiles contenues dans les vins et entraînées par le courant d'air, d'où la nécessité de l'appareillage plus compliqué.

Les recherches effectuées ont abouti à la mise au point du mode opératoire de dosage iodométrique de l'anhydride sulfureux libre et combiné, extraits par le courant d'azote au moyen d'un appareil distillatoire simple et ensuite fixés sans pertes par une solution alcaline.

I. — INTRODUCTION

A notre connaissance, le dosage iodométrique de l'anhydride sulfureux libre et combiné dans les vins et les jus de raisin, séparés par entraînement à l'azote, avec l'emploi du réfrigérant ascendant, et reçus dans une solution d'hydroxyde de sodium, n'a pas été pratiqué en entier dans le passé. A juger d'après l'excellente revue bibliographique donnée par Monier-Williams (1), concernant la période avant 1926-1927, c'est Ripper en 1892 (2), qui a proposé la séparation de l'anhydride sulfureux total à l'aide d'un courant d'hydrogène et

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la réception du distillat dans la solution normale d'hydroxyde de potassium; après acidification le titrage de l'anhydride est effectué à l'aide de la solution d'iode. Les résultats obtenus concordent bien avec ceux fournis par le procédé de Haas (3), préconisant la séparation de l'anhydride en absence du réfrigérant, la solution d'iode étant utilisée comme liquide de fixation, sans effectuer le titrage iodométrique comme c'est le cas dans le procédé Wartha (4) bien défectueux; Gayon et Laborde (1), ensuite Ferré (6) ont cherché à améliorer l'efficacité de cette technique, entre autres, par l'emploi d'un réfrigérant ascendant. Par la suite l'emploi du perhydrol, proposé par Franz et Sonntag (7), s'est répandu de plus en plus, étant repris par Frobœse (8), par Von der Heide et Schmitthenner pour les vins (9) et après par Monier-Williams (*op. cit.* (1)), dont la technique a été modifiée et complétée par d'autres auteurs (10) (11) (13) (14). Nous avons signalé en 1959 un certain nombre de publications concernant ce sujet (15). Depuis 1927, l'utilisation du perhydrol a pris un essor considérable à juger d'après le nombre de mémoires parus concernant le dosage de l'anhydride sulfureux libre et total dans les vins et les substances alimentaires. En 1932 Photiadis (16) propose une technique comprenant la distillation en présence du gaz carbonique et l'élimination de l'excès de l'eau oxygénée, à l'aide d'une solution d'hydroxyde d'ammonium; l'excès de chlorure de baryum, après la précipitation du sulfate, est déterminé iodométriquement par l'intermédiaire du chromate d'ammonium et d'iodure de potassium. L'exemple des travaux de Sumuleanu et de ses collaborateurs montre l'absence de l'unité analytique dans les dosages de différentes formes de l'anhydride sulfureux. C'est ainsi que Sumuleanu et Ghimicescu (17 *a*) séparent l'anhydride total par entraînement à l'hydrogène et, après sa fixation dans une solution d'hydroxyde de potassium, le dosent iodométriquement après l'avoir introduit dans une solution d'iode acidifiée. Quant à l'anhydride sulfureux libre, il est séparé par les courants hydrogène ou de gaz carbonique et reçu soit dans une solution d'iode (17 *b*), soit dans une solution du perhydrol (18); cette dernière technique fait revivre le procédé de Wartha (*op. cit.* (4)), comme l'ont fait Burroughs et Sparks (19) pour le dosage de l'anhydride total dans des cidres avec l'emploi d'une solution tampon destinée à décomposer l'acide acétaldéhyde-sulfureux.

Une des modifications du procédé de Monier-Williams, due à Chandler (20), a été appliquée aux vins par Petrocini (21) sans succès à cause de l'influence perturbatrice des acides volatils passant dans le distillat malgré l'emploi du réfrigérant ascendant. Comme l'a montré Paul (22), il est possible d'éviter cet entraînement des acides par l'utilisation de l'appareil de Lieb et Zacherl (23), muni d'un réfrigérant spécial à double jaquette. D'ailleurs cet appareil distillatoire constitue l'amélioration de celui de Friedemann et de ses collaborateurs (24) destiné au dosage de l'acide lactique par l'intermédiaire de l'acétaldéhyde formé. Kielhöfer et Aumann (25) ont repris l'étude, effectuée par Paul, en notant ses défauts et en faisant une critique de la méthode iodométrique. En 1958 Paul (22 *b*) a publié la technique revue et améliorée du dosage acidimétrique de l'anhydride sulfureux libre et total. Considérant le dosage acidimétrique comme moins spécifique, Reith et Willems (26) ont préféré le titrage complexométrique de l'acide sulfurique transformé en sulfate de baryum. L'appareil est robuste avec un réfrigérant de Liebig, ce qui constitue, d'après Nehring (27), un défaut en raison de l'insuffisance du refroidissement; pour cette raison l'auteur emploie un réfrigérant à boules, comme d'ailleurs Zonneveld et Meyer (28), Tanner (29 *a*, *b*), dont la technique analytique comprend la distillation de l'anhydride en présence d'un courant d'azote, le vin étant additionné du méthanol et de l'acide phosphorique, et le titrage acidimétrique de l'acide sulfurique formé. En présence du méthanol le point d'ébullition est ramené à + 70° C ce qui diminue la formation des substances gênantes pendant le chauffage. D'après Tanner (*op. cit.* (29 *b*)) le procédé de Paul (*op. cit.* (22)) fournit des résultats plus élevés en présence de plus de 0,8 g d'acidité volatile par litre.

Rankine (30) fait appel à l'appareillage construit à l'aide de la verrerie interchangeable M. Société Quickfit; le réfrigérant employé est à double chemise, de 150 mm de longueur utile et d'une surface interne de 200 cm². Dans ces conditions l'influence de l'acidité volatile devient moins forte puisque son effet perturbateur se fait sentir à partir de 1,2 g par litre exprimé en acide acétique. Rankine effectue un essai à blanc après l'oxydation de l'anhydride sulfureux par du perhydrol. Or, dans ces conditions, les différents constituants du vin sont également oxydés d'où le risque de formation de composés volatils acides ou susceptibles de produire les acides en présence du perhydrol (*op. cit.* (15)). Tout dernièrement Burroughs et Sparks (31) ont décrit pour les cidres une technique du dosage acidimétrique de l'anhydride sulfureux libre, entraîné sous vide, par le courant de l'azote. Quant au procédé de Ares Pons (32), il sépare l'anhydride sulfureux à l'aide d'un courant de gaz inerte dans l'appareil spécial, muni d'un réfrigérant ascendant, et reçoit le distillat dans une solution de periodate de sodium alcalinisée. Le dosage iodométrique s'effectue en présence d'acide et d'iodure de potassium. En ajustant le *p*H de la prise d'essai d'abord à 1,5 à l'aide de l'acide oxalique, puis à un *p*H plus petit avec de l'acide sulfurique on obtiendrait deux fractions de l'acide sulfureux :

1° libre, combiné à l'éthanol et au glucose,
2° combiné à d'autres substances.

L'emploi des solutions d'iodate ou de periodate alcalin, comme liquide de réception de l'anhydride sulfureux, remonte aux travaux de Gudeman (33 *a*) et de Schumacher et Feder (33 *b*). Cette technique s'apparente à celles basées sur l'emploi des oxydants comme le perhydrol, et par conséquent sujettes à l'influence des substances de caractère acide ou susceptibles de produire les composés à fonction acide en présence des oxydants; dans ces conditions l'efficacité du réfrigérant ascendant joue un grand rôle.

Tout récemment le dosage iodométrique de l'anhydride sulfureux libre dans les boissons a été remis à

l'honneur par Lloyd et Cowl (35). La désorption de l'anhydride s'effectue à la température ambiante à l'aide d'un courant d'azote, le *p*H du liquide étant porté à 1-2. L'anhydride extrait est reçu dans le mélange, à volumes égaux, du glycérol et de la solution décinormale d'hydroxyde de potassium; le débit du gaz est de 90 litres/heure, la durée totale de désulfitation est de 15 minutes, pour le volume de 25 ml. Le liquide de réception est acidifié et l'anhydride est titré par une solution d'iode. Rappelons que Brown en 1910 (36) a constaté que l'addition de 5 % de glycérol diminue considérablement l'oxydation de l'anhydride sulfureux et qu'il n'est pas nécessaire de verser la solution sulfureuse dans la solution d'iode acidifiée, le titrage direct étant aussi exact. Pour les teneurs en acide sulfureux inférieures à 50 mg par litre Lloyd et Cowl utilisent la méthode colorimétrique. Jones (36) a adopté ce procédé au dosage de l'anhydride sulfureux dans les bières et les vins en y apportant quelques modifications : l'emploi du réfrigérant, le chauffage à l'aide d'une jaquette thermostatique et l'augmentation de la durée de désulfitation. Toutefois, la description de la technique modifiée est peu explicite. Cette publication clôt la liste des travaux cités dans notre mémoire. Leurs enseignements positifs ou négatifs nous ont montré l'utilité de faire appel à la méthode iodométrique pour le dosage de l'anhydride sulfureux libre et combiné dans les vins et les jus de raisin.

II. — RECHERCHES EFFECTUÉES

Le dosage iodométrique correct de l'anhydride sulfureux libre et combiné dépend de plusieurs facteurs et, en particulier, des conditions de la libération de l'anhydride sulfureux libre et de la stabilité de ses différentes formes organiques. Par conséquent, la connaissance des propriétés de l'anhydride et de ses composés d'addition présente un grand intérêt. Depuis la publication de notre mémoire en 1959 la parution de quelques publications importantes nous autorise actuellement de compléter l'exposé fait il y a 6 ans.

A. — ANHYDRIDE SULFUREUX LIBRE

Depuis les constatations faites en 1958 par Falk et Giguère (57) nous savons que les solutions aqueuses de l'anhydride sulfureux ne contiennent pas de quantités mesurables d'acide sulfureux, H_2SO_3. Dans ces solutions les molécules de l'anhydride existent en majorité comme telles, peut être sous forme d'hydrates labiles, $SO_2.nH_2O$, à côté des faibles concentrations d'ions bisulfitiques HSO_3^-, pyrosulfitiques $HS_2O_5^-$ résultant des équilibres

$$SO_2 + 2\,H_2O \rightleftharpoons HSO_3^- + H_3O^+$$
$$HSO_3^- + SO_2 \rightleftharpoons HS_2O_5^-.$$

Les ions sulfitiques SO_3^{--} peuvent exister en très petite quantité. De toute façon seulement 10 % de l'anhydride sulfureux dissous est sous forme ionique. Dans ces conditions le diagramme donné par Vas et Ingram (58), très utile au point de vue pratique, ne correspond pas exactement à la réalité.

Rappelons qu'après les définitions admises en œnochimie, on appelle anhydride sulfureux libre l'anhydride à l'état dissous et à l'état de combinaisons minérales, hydrogénosulfite et sulfite.

En ce qui concerne l'oxydation de l'anhydride sulfureux dans ses solutions aqueuses et dans les solutions aqueuses de ses sels, nous avons donné un grand nombre de renseignements dans nos publications précédentes (*op. cit.* (15) (39)), ce qui nous dispense de les répéter ici. Si la stabilité de l'anhydride sulfureux en présence de l'oxygène est nette dans les milieux acides au *p*H 1, par exemple, sa désorption quantitative au moyen de l'azote est également complétée au *p*H compris entre 1 et 2, comme l'ont montré Aulenbach et Balmat (40). Dans ces conditions la dissociation de sulfonates des glucides est négligeable (41).

B. — ANHYDRIDE SULFUREUX COMBINÉ

a) Bilan des composés organiques d'addition dans les vins

Depuis les constatations de Ripper en 1892 (42) et de Roques en 1898 (43), concernant la combinaison de l'anhydride sulfureux avec l'acétaldéhyde et les glucides dans les vins, les premiers bilans des substances susceptibles de former avec l'anhydride des composés d'addition sont dus à Kerp (44) (45), à Farnsteiner (46). Le premier a étudié les combinaisons de formaldéhyde, d'acétaldéhyde, de benzaldéhyde, d'acétone et de glucose, tandis que le deuxième auteur a constaté que l'arabinose possède une forte affinité pour l'anhydride suivi par le mannose, galactose, glucose, par les disaccharides tels que le maltose, le lactose, le trisaccharide comme le raffinose, et le fructose étant très peu réactifs. Enfin l'acide furfuralsulfureux a fait l'objet de travaux de Kerp et Wöhler (47), de Stutzer (48); les composés sulfureux des aldéhydes non saturés, comme le citronellal, ont été étudiés par Kerp et Wöhler (49). Nous avons tenu à rappeler toutes ces publications puisque l'époque actuelle a tendance de les oublier et de redécouvrir les faits établis il y a 50 ans.

Les vins contiennent d'autres corps bien définis capables de fixer l'anhydride sulfureux, en particulier, les acides pyruvique et alphacétoglutarique, dosés pour la première fois par Egorov et Borissova (50), en France par Peynaud et Blouin (51) et par Deibner et Mirelle

Cabibel-Hugues (12). Tout récemment Rodopoulo et Egorov (53) ont trouvé dans le vin, en plus de deux acides cités, trois cétoacides inconnus et dans le jerez, obtenu à partir de ce vin, quatre acides également inconnus. Parmi d'autres corps il faut citer l'acétylméthyl-carbinol, le diacétyle, les acides galacturonique, déhydroascorbique, la xylosone. D'ailleurs, ces substances se prêtent sans grande difficulté à la préparation de leurs dérivés bisulfitiques.

Le problème devient plus difficile en ce qui concerne les anthocyannes puisque les connaissances concernant leurs combinaisons avec l'anhydride sulfureux sont rares : Grafe (54) les a obtenues pour quelques anthocyanosides. D'après Hennig et Burkhardt (55), l'anhydride sulfureux se combine avec les tanins condensés, avec les dérivés de l'acide cinnamique.

Les recherches œnochimiques de Kielhöfer et Würdig (56) (57) (58), dont l'importance et l'intérêt scientifique et pratique sont bien évidents, ont comblé en grande partie une lacune dans la meilleure connaissance des substances réagissant avec l'anhydride sulfureux, en complétant en même temps leur inventaire.

Une autre importante contribution à l'étude de ces substances, présentes dans les jus de pomme et dans les cidres, a été faite par Burroughs et Whitting (59), par Burroughs et Sparks (60 *a*), par Whitting et Goggins (61) en ce qui concerne la L-xylosone. Enfin Burroughs et Sparks (60 *b*) ont effectué des recherches d'une grande valeur concernant l'identification des combinaisons organiques de l'anhydride sulfureux dans les jus de pomme et les cidres, séparées à l'aide des résines échangeuses d'ions et de la chromatographie sur papier.

Le cidre normal contient les substances suivantes réagissant avec l'anhydride sulfureux : glucides, acétaldéhyde, xylosone, acides galacturonique, pyruvique et α-cétoglutarique. Le cidre, obtenu à partir de pommes endommagées, est riche en produits métaboliques carbonylés. En effet, les beaux travaux effectués à l'Agricultural and Horticultural Research Station à Long Ashton (Bristol) ont apporté une contribution importante dont les résultats et les conclusions seront profitables aux études œnochimiques.

A la lumière de tous les enseignements actuellement existants, il est impossible d'accepter d'emblée la classification de Kielhöfer (*op. cit.* (56), (62), suivant laquelle des différentes formes organiques de l'anhydride sulfureux sont divisées en deux groupes : ses combinaisons avec l'acétaldéhyde, le glucose et l'arabinose, d'une part, et le restant des composés réunis sous le vocable Rest-SO_2. Or, dans ce dernier groupe entrent les substances bien connues comme les cétoacides, l'acétylméthylcarbinol, le diacétyle, le furfurol, l'oxyméthylfurfurol, les dérivés de l'acide cinnamique (63), dont les combinaisons bisulfitiques peuvent être préparées. Donc, le groupe Rest-SO, peut être allégé de ces substances et comprendre seulement celles, connues, dont la préparation des dérivés bisulfitiques n'est pas au point, et d'autres vraiment peu connues.

L'étude de la séparation de l'anhydride sulfureux libre et combiné sous forme organique n'est pas concevable sans la mise en œuvre de quelques composés d'addition de l'anhydride sulfureux. C'est pour cette raison que nos recherches ont débuté par la préparation de l'acétaldéhydesulfureux, du glucosesulfureux et des dérivés bisulfitiques des acides pyruvique et α-cétoglutarique.

b) Préparation des combinaisons bisulfitiques organiques

En raison des circonstances matérielles nous avons pu procéder seulement à la préparation de quatre dérivés bisulfitiques à partir de l'acétaldéhyde, du glucose, du pyruvate de sodium et de l'acide α-cétoglutarique.

1° Alpha-hydroxysulfonate de potassium

L'acétaldéhydesulfonate de potassium ou l'alpha-hydroxyéthanesulfonate de potassium a été préparé pour la première fois par Bunte (64) et ensuite par Kerp (*op. cit.* (44 *a*, *b*, *c*)). Nous avons obtenu le sel potassique de l'acide acétaldéhydesulfonique en utilisant le mode opératoire préconisé par Diemair, Koch et Hess (65) et décrit dans notre publication précédente (66), ce qui nous dispense de le reproduire dans ce mémoire.

Le dosage iodométrique de l'anhydride sulfureux combiné dans le sel obtenu s'effectuait à la température ambiante, de 18°-20° C, sur les solutions aqueuses contenant 2 g d'acide tartrique et 2 g de bitartrate de potassium par litre, sans l'addition de l'éthanol, responsable de la formation des troubles. Tout d'abord on évaluait la teneur en anhydride sulfureux libre et ensuite la teneur en anhydride combiné, en milieu rendu alcalin, suivant le mode opératoire publié en 1953 (67), et en 1955 (68). A cet effet on verse le volume de 10 ml de la solution à doser dans un liquide obtenu par mélange de 80 ml d'eau, de 5 ml de solution d'acide sulfurique 6 *N*, de 5 ml de solution d'iode *N*/50 et de 5 ml de solution d'iodure de potassium à 21 % et 20 mg de complexon III. L'excès d'iode est titré par une solution d'hyposulfite de sodium *N*/50 en présence d'empois d'amidon de blé 0,2 % ajouté en raison de 5 ml par 100 ml de solution. Le bécher de 250 ml de capacité doit être maintenu dans le mélange d'eau et de glace. Pour le titrage de l'anhydride sulfureux combiné on neutralise le liquide avec une solution d'hydroxyde de sodium 2 *N* jusqu'au virage au rose de phénolphtaléine (2 gouttes d'une solution alcoolique à 1 %); on fait disparaître la coloration rouge avec quelques gouttes de solution normale d'acide sulfurique et on ajoute 20 ml de solution saturée de borate de sodium ce qui est suffisant pour faire apparaître le virage au rose : on additionne le volume supplémentaire d'empois d'amidon (2,0-2,5 ml) et ensuite on verse lentement, goutte à goutte, en agitant régulièrement, de la solution d'iode jusqu'à l'apparition de la coloration bleue stable.

Le dosage du sel potassique de l'acide acétaldéhydesulfonique a montré qu'en solution acide de *p*H 1 ou de *p*H 3, on trouve pour 1 g de sel, dissous dans 1 litre, 4,9 mg d'anhydride sulfureux libre et 390,5 mg d'anhy-

..e combiné. Bien entendu, la teneur en anhydride est variable. Les circonstances matérielles ne nous pas permis de préparer le sel complètement débar- de son anhydride libre.

..LUCOSE-HYDROXYSULFONATE DE POTASSIUM

..ur la première fois le glucosesulfonate de sodium obtenu par Kerp (*op. cit.* (44)). La préparation améliorée par Neuberg (69), mais c'est Braverman ..a préparé à l'état cristallisé (70). On trouvera les ..ignements utiles dans les publications de Joslyn (51), ..ehman et Osman (72), de Joslyn et Braverman (73). 959, Ingles (74) a indiqué une technique améliorée préparation des glucose-hydrosulfonates de sodium potassium.

nom systématique de ces composés est le D-gly- D-ido-1,2,3,4,5,6, hexahydroxyhexyl sulfonate de ..sium ou de sodium, suivant les règles de la nomen- re des glucides (75). D'après Ingles, l'utilisation ..sulfite de potassium à la place du sel sodique faci- ..a préparation des composés cristallins, à partir ..doses; c'est ainsi que le glucose en solution aqueuse ..t, à la température ambiante, avec le bisulfite de ..tium (rendement de 50 %). Sa recristallisation, sous ..e monohydrate, n'est pas possible dans le méthanol mais dans le méthanol contenant 10 % d'eau.

..s avons essayé de préparer les sels de glucose- ..te suivant les recettes d'Ingles (*op. cit.* (77 *a*)) ou ..averman (*op. cit.* (70) (75)), en utilisant du glucose ..re, mais nous n'avons pas obtenu des résultats ..ptés. Au fait, il est indispensable d'effectuer ..plement, au moins pendant 15-24 heures, l'hydra- du glucose anhydre. Finalement nous avons pré- recette de Diemair, Koch et Hess (*op. cit.* (65)) : ..soit 158 g de monohydrate de glucose (Société Suisse) dans 60 ml d'eau, en chauffant légèrement, ..g de pyrosulfite de potassium dans 150 ml d'eau : ..nit ces solutions et on chauffe le mélange dans un muni d'un réfrigérant ascendant, à 40° C, en ..nt au moyen de l'agitateur magnétique pendant ..res : ensuite on y ajoute 150 ml de méthanol en ..t et on laisse au repos pendant trois jours; la masse ..ne obtenue contient le pentahydrate de glucose- ..ulfonate de potassium, accompagné des sulfates. ..ution séparée produit un deuxième précipité ..n, qui, après lavage avec du méthanol à 75 %, ..ue une fraction n° 2. Par refroidissement de la ..n n° 1 et du filtrat de la fraction n° 2 à la tempé- — 15° C, on obtient des cristaux de dihydrate ..cosesulfonate de potassium.

..s avons préféré utiliser le premier précipité en ..mettant au lavage avec du méthanol à 75 % la disparition du glucose constatée avec la liqueur ..., du sulfate avec la solution de chlorure de ..et du sulfite avec une solution d'iode acidulée ..ence d'empois d'amidon.

..opérations sont nécessaires puisque le méthanol à côté du sulfonate, le glucose et le bisulfite ..binés (cf. *op. cit.* (73)).

Le sel ainsi purifié est séché dans un dessiccateur rempli de chlorure de calcium anhydride, sous un vide créé par une trompe à eau.

Le dosage iodométrique du glucosesulfonate de potassium était effectué suivant le mode opératoire pour l'acétaldéhydesulfonate de potassium. Notre préparation dissoute dans la solution tartrique indiquée plus haut n'accusait pas la présence de l'anhydride sulfureux libre. Ingles (*op. cit.* (74 *b*)) a montré que le glucosesulfonate de potassium s'hydrolyse dans l'eau à 85 %; par contre, ce sel est plus stable dans la solution d'acide acétique à 10 % (1,43 N). D'après Braverman (*op. cit.* (70)), la décomposition est complète à *pH* 7, tandis qu'à *pH* 2 la décomposition est presque nulle.

Nous avons constaté que 1 g de notre préparation contenait 192 mg d'anhydride sulfureux contre 164 mg, si le sel obtenu correspondait au pentahydrate de glucose-sulfonate de potassium $C_6H_{13}SO_8K, 5\,H_2O$ (P. M. 390). Par conséquent, dans notre cas il s'agissait plutôt de monohydrate.

A côté d'autres substances d'addition préparées par nos soins, seul le glucosesulfonate ne contenait pas d'anhydride sulfureux libre.

3° SULFONATES DES ACIDES PYRUVIQUE ET ALPHA-CÉTOGLUTARIQUE

On sait depuis les travaux de Long (76) que le rendement de la réaction entre les acides pyruvique et alpha-cétoglutarique et les sulfites acides alcalins est de l'ordre de 96,5 % pour le premier et de 82 % pour le deuxième acide, ce qui rend peu satisfaisant leur dosage iodométrique.

Nous avons préparé les sulfonates de ces acides en nous inspirant partiellement du mode opératoire utilisé pour la préparation de l'acétaldéhydesulfonate.

2) Alpha-sulfonate de potassium du pyruvate sodique

On dissout 2,5 g de pyruvate de sodium pur dans 10 ml d'eau et 2,5 g de pyrosulfite de potassium pur également dans 10 ml d'eau, ensuite on mélange ces deux solutions; le liquide obtenu est laissé au repos pendant la nuit. Le lendemain on y ajoute 95 ml d'éthanol et on abandonne la solution au repos pendant 2-3 jours, le précipité cristallin obtenu est séparé par filtration sur un petit filtre Büchner muni d'un filtre de papier; le poids de poudre était de 4,5 g. Le dosage iodométrique de l'anhydride libre et combiné dans le produit obtenu, dissous dans une solution tartrique contenant 300 mg de sel par litre, nous a montré la présence de 28,8 mg d'anhydride sulfureux libre et 49,5 mg d'anhydride combiné. En principe la teneur en anhydride combiné devait être de 83,5 mg. Le lavage de 2 g de la préparation avec 2 litres d'éthanol n'a pas permis d'éliminer l'excès de l'anhydride libre, puisque 300 mg du produit lavé et séché sous vide en présence de chlorure de calcium, anhydre contenaient en vin 25,6 mg d'anhydride libre et 48,64 mg d'anhydride combiné. Par conséquent

DOSAGE DE

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comme nous le

3) Sulfonate de po

En utilisant l
haut, nous n'av
l'acide alpha-cé
cristallisé se fai
méthanol pur. I
c'est le cas pou
dosage iodomét
90 mg d'anhydr

D'une façon
a pas permis d'
cétoniques à l'
glucosesulfonate
celles d'autres
grand intérêt, c
cours des nouve
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vins.

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Les quatre c
sulfureux décrit
de la séparatio
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distillation util
gaz d'entraîne
une parfaite ab
liquides alcalins

a) Appareils d

Au début d
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Parallèlement il
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Sa robustesse, s
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reil est muni

notre préparation n'était pas pure, mais cette circonstance n'a pas empêché de faire des constatations intéressantes, comme nous le verrons dans ce qui suit.

β) Sulfonate de potassium de l'acide alpha-cétoglutarique

En utilisant le même mode opératoire, indiqué plus haut, nous n'avons pas réussi à préparer le sulfonate de l'acide alpha-cétoglutarique. La précipitation du sel cristallisé se fait bien si l'on remplace l'éthanol par le méthanol pur. Le produit obtenu n'est pas pur, comme c'est le cas pour le sulfonate de l'acide pyruvique. Le dosage iodométrique a montré que 1 g de sel contient 90 mg d'anhydride libre et 109,2 mg de combiné.

D'une façon générale, le manque de temps ne nous a pas permis d'obtenir les dérivés sulfitiques des acides cétoniques à l'état de pureté comparable à celle de glucosesulfonate. En effet, leur préparation, ainsi que celles d'autres combinaisons d'addition présentant un grand intérêt, doivent constituer un sujet de choix au cours des nouvelles recherches nécessaires à entreprendre pour une meilleure connaissance de leurs propriétés et des particularités de leur comportement dans les vins.

C. — SÉPARATION DE L'ANHYDRIDE SULFUREUX LIBRE EN PRÉSENCE DE DIFFÉRENTES FORMES ORGANIQUES DE L'ANHYDRIDE COMBINÉ

Les quatre combinaisons d'addition de l'anhydride sulfureux décrites plus haut ont été utilisées pour l'étude de la séparation de l'anhydride sulfureux libre. Cette étude tenait compte des particularités des appareils de distillation utilisés, des caractéristiques du courant de gaz d'entraînement et des conditions nécessaires pour une parfaite absorption de l'anhydride extrait dans les liquides alcalins de réception.

a) Appareils de distillation utilisés

Au début de nos recherches nous avons utilisé l'appareil de Lieb et Zacherl construit il y a une dizaine d'années par les Établissements Volck à Strasbourg. Parallèlement il nous servait pour l'étude du procédé acidimétrique de l'anhydride sulfureux capté dans une solution de perhydrol. Sa fragilité, la présence de la plaque poreuse nous ont incité à l'abandonner au profit de l'appareil de distillation que nous avons utilisé pour la séparation de l'acide cyanhydrique dans les vins (77). Sa robustesse, sa simplicité, l'agencement du réfrigérant ascendant, du type de condenseur de Hopkins, l'indiquaient tout naturellement pour la séparation de l'anhydride sulfureux suivie de son dosage iodométrique dans le liquide alcalin de réception. L'absorbeur de cet appareil est muni d'un tube simple, sans plaque poreuse.

b) Extraction de l'anhydride sulfureux libre

L'extraction de l'anhydride sulfureux libre, contenu dans une prise d'essai, à côté des combinaisons d'addition du même anhydride, dépend de plusieurs facteurs, tels que le *p*H du liquide à désulfiter, du débit de l'azote utilisé pour l'extraction de l'anhydride, de la dépression créée à l'intérieur du circuit, de la température de la prise d'essai.

En ce qui concerne le *p*H du liquide à désulfiter, il est de l'ordre de 0,85-0,90 ce qui assure la stabilité des combinaisons organiques de l'anhydride sulfureux, la désorption quantitative de l'anhydride sulfureux libre ainsi que sa stabilité en présence de l'oxygène. Le volume des liquides à désulfiter est de 15 ml, soit la prise d'essai de 10 ml et 5 ml de solution d'acide chlorhydrique 6 N.

Le débit de l'azote, la dépression à utiliser, la durée de désulfitation pour un volume donné ont fait l'objet de nombreux essais. Tout d'abord, il faut préciser que l'azote-R (Société l'Air Liquide) sort de sa bouteille sous une pression minimale au-dessous de 0,5 bar puisque l'aiguille du deuxième manomètre (l'ensemble manométrique de S. A. F.) dont une division correspond à un bar, ne décolle pas de son point zéro. Ensuite nous avons choisi une dépression égale à 50 mm de mercure puisqu'elle nous permet d'obtenir un barbotage suffisant de l'azote liquide à désulfiter. Déjà la dépression de 25 mm est insuffisante dans le cas de notre appareil : en effet, le diamètre intérieur du tube plongeant dans le liquide à désulfiter est de 5 mm. Le mode opératoire de Paul (*op. cit.* (22)) fait appel à une dépression moindre, de l'ordre de 18,5-22 mm de mercure, soit 250-300 mm d'eau, ce qui est en relation avec le diamètre intérieur nettement plus petit du tube d'amenée du gaz, de 1,5 mm. Les dépressions plus grandes jusqu'à 100 mm sont acceptables; au-delà les pertes sont plus fréquentes.

La dépression est obtenue par une trompe à eau; son réglage est généralement obtenu au moyen d'un tube de Mariotte dans lequel le niveau d'eau est réglé par un dispositif à vases communicants. Pour amortir les petites variations de pression produites par les bulles d'air barbotant dans ce régulateur, un flacon de 5 à 10 litres, rempli de gaz choisi, sert de volant. La valeur de la dépression est lue sur un manomètre à eau ou à mercure placé entre le flacon et l'absorbeur. De tels dispositifs à dépression constante sont employés lors des mesures de la viscosité (79). Remarquons que Paul (*op. cit.* (22)) utilise seulement un dispositif à dépression constante sans contrôler cette dépression par un manomètre et par un réglage de la dépression fournie par la trompe à eau. D'ailleurs nous réglons la dépression à l'aide d'une pince de Mohr à vis, placée sur un tube de caoutchouc. Il faut souligner que l'emploi du dispositif à pression constante doit s'insérer dans l'ensemble de l'appareillage destiné à obtenir une dépression constante. Déjà le diamètre du tube d'admission de l'air dans le dispositif à pression constante joue un grand rôle dans l'établissement d'une dépression fixe et suffisante.

Les essais de l'extraction de l'anhydride à partir des solutions de sulfite acide, contenant environ 500 mg

d'anhydride par litre, ont été faits dans l'appareil de distillation à reflux simple. Le débit de l'azote, mesuré à l'aide d'un débitmètre à capillaires, est de 34,5 litres/heure, l'appareil marque le débit de 41,4 litres/heure, sous l'effet de la dépression utilisée. Le ballon distillatoire est plongé dans un bain-marie porté à + 100° C, tandis que l'absorbeur, contenant 30 ml de solution d'hydroxyde de sodium 0,25 N, est refroidi avec un mélange d'eau et de glace.

Il ressort des résultats obtenus, consignés dans le *tableau I*, qu'il est préférable d'utiliser le débit d'azote de 40 litres/heure, la dépression de 50 mm de mercure et la durée de désulfitation égale à 15 min, pour un volume total du liquide à désulfiter de 15 ml.

TABLEAU I

Essais	Débit d'azote (litre/h)	Dépression (mm Hg)	Durée (mn)	Résultats obtenus	
				SO_2 libre (mg/litre)	Erreur (%)
A) Titre de la solution	—	—	—	497,9	—
— essai n° 1	40	50	15	497,9	—
— essai n° 2	20	50	15	497,9	—
— essai n° 3	20	50	10	485,1	— 2,4
B) Titre de la solution	—	—	—	487,7	
— essai	40	50	10	485,1	— 0,5

Quant à l'influence du complexon III utilisé suivant les indications de Jaulmes et Mlle Hamelle (79), le produit, ajouté à raison de 5 mg dans le ballon et dans l'absorbeur, ne paraît pas jouer un rôle utile dans le cas de deux solutions d'hydrogénosulfite de potassium contenant 487,7 et 11,5 mg d'anhydride par litre. Même avec un vin rouge Cinsault 1964, Pech-Rouge (Aude), nous avons obtenu les résultats semblables, en présence ou en absence du complexon.

c) Fixation de l'anhydride sulfureux extrait

Au début de nos essais nous avons utilisé la solution d'hydroxyde de sodium binormale préconisée par nous-mêmes pour le dosage de l'anhydride sulfureux total. Rapidement nous avons constaté que l'absorption de l'anhydride gazeux se faisait moins bien dans les solutions alcalines concentrées, d'autant plus que le lavage convenable de la plaque poreuse de l'absorbeur de l'appareil de Lieb-Zachert nécessitait l'emploi d'une soufflerie pour faciliter l'expulsion des eaux de lavage. Comme le montrent les données du *tableau II*, les solutions décinormales et 0,25 N étaient tout indiquées puisqu'en même temps elles rendaient possible l'amélioration du virage iodométrique ; c'est ainsi que dans le cas de la solution alcaline 0,25 N il faut seulement 4 gouttes de solution d'iode N/50 pour passer de la coloration bleue à l'incolore pour un volume à titrer de 100 ml, le volume d'empois d'amidon de blé (0,2 %) compris (5 ml pour 100 ml de solution).

TABLEAU II

Influence de la concentration de la solution d'hydroxyde de sodium sur l'efficacité de l'absorption de l'anhydride sulfureux. Solution d'hydrogénosulfite de potassium à 450 mg d'anhydride sulfureux par litre. Résultats du dosage iodométrique en mg de SO_2 par litre. Volume utilisé 50 ml, appareil de Lieb-Zachert.

Concentration en normalité (N)	Pertes en %	Observations
2	0,8 (1)	Virage iodométrique très long.
1	2,8 (2)	
1	0,6 (1)	
0,5	0,3 (1)	
0,25	0,15 (2)	Virage iodométrique très net et rapide.
0,25	Néant (1)	*Id.*
0,1	Néant (1)	*Id.*

(1) Lavage de la plaque poreuse avec l'emploi de soufflerie.
(2) Lavage normal.

D'autres essais effectués, dont les résultats sont consignés dans le *tableau III*, nous ont montré que la solution d'hydroxyde de sodium 0,25 N assurait une décomposition complète de solution contenant 450 mg d'anhydride combiné sous cette forme par litre, tandis que la solution alcaline décinormale n'était pas efficace, ce qui a déterminé notre choix de la première solution.

Pour faciliter l'absorption de l'anhydride sulfureux il est indispensable de refroidir la solution alcaline à + 8°-+ 9° C, à l'aide de la glace et de l'eau. En effet, on sait que la solubilité du gaz sulfureux est plus grande à + 10° C (56,6 vol. pour 1 vol. d'eau) qu'à + 20° C (39,4 vol. pour 1 vol. d'eau). Les essais effectués ont montré que le tube effilé peut remplacer une plaque poreuse. Par précaution, nous avons préféré augmenter la hauteur du liquide dans l'absorbeur en lui donnant la forme d'un cylindre étroit. L'absence des pertes en anhydride sulfureux par entraînement a été contrôlée à l'aide d'une solution de perhydrol additionnée de quelques gouttes de l'indicateur de Tashiro (0,1 g de rouge de méthyle et 0,05 g de bleu de méthylène pour 100 ml de solution alcoolique à 50°). L'absorbeur rempli avec cette solution était placé à la suite du premier absorbeur.

Tableau III

Essais de séparation avec l'appareil de Lieb-Zuckerl.
Résultats du dosage iodométrique en mg de SO_2 par litre.
Solutions utilisées : hydrogénosulfite et acétaldéhydesulfonate de potassium.

N° Essai	Solution alcaline utilisée	Titre des solutions			Après la distillation			
		Libre	Combiné	Total	Libre	Combiné	Total	
							Teneur	Erreur (%)
A	NaOH — 0,1 N — 50 ml							
1	»	1,92	464,64	466,56	459,52	1,28	460,80	— 1,2
2	»	1,92	449,28	451,20	448,00	3,20	451,20	—
3	»	1,92	448,00	449,92	432,00	9,60	441,60	— 1,8
B	NaOH — 0,25 N — 50 ml							
1	»	1,92	452,48	454,40	—	—	454,4	—
2	»	5,12	352,00	357,12	5,12	352,00	357,12	—
3	»	50,56	401,28	451,84	50,56	400,64	451,20	— 0,16
4	»	109,44	373,12	482,56	109,44	373,12	482,56	—
5	»	438,4	—	438,4	437,76	—	437,76	— 0,15
6	»	450,56	—	450,56	450,56	—	450,56	—
7	»	5,76	—	5,76	5,76	—	5,76	—
8	»	3,20	—	3,20	3,20	—	3,20	—

D'ailleurs les pertes par entraînement sont les seules possibles dans les conditions de notre mode opératoire. En effet, si une légère dépression permet de faire circuler plus facilement l'azote à l'intérieur de l'appareil, en contre-partie elle provoque des rentrées d'air en raison de l'étanchéité imparfaite des rodages, ce qui a été montré grâce à l'emploi du dispositif pour contrôler la désaération décrite en 1956 (81). D'autre part, une surpression de l'azote, évitant la pénétration de l'air, faciliterait les pertes en anhydride sulfureux plus grandes que celles qu'aurait provoquées l'oxygène de l'air. Or, l'expérience montre que la pénétration de l'air ne détermine pas l'oxydation du sulfite de sodium; sans doute, le mélange gazeux obtenu est trop riche en azote pour provoquer cette réaction. D'ailleurs l'addition du complexon III paraît inutile, si l'on en juge d'après les résultats comparatifs obtenus. Bien entendu, le courant d'air est nuisible pour l'intégrité chimique de la solution sulfitique.

Après avoir déterminé les conditions de l'extraction totale de l'anhydride sulfureux libre, de sa fixation complète par la solution alcaline choisie et de la stabilité du sulfite de sodium obtenue, nous étions amenés à étudier le comportement des combinaisons organiques de l'anhydride sulfureux, préparées précédemment par nos soins, au cours de l'extraction de l'anhydride sulfureux libre.

d) Comportement des combinaisons organiques de l'anhydride sulfureux pendant l'extraction de l'anhydride sulfureux libre

Les résultats du *tableau III* ont déjà montré que la présence de l'acétaldéhydesulfonate n'influence pas, dans les conditions de notre mode opératoire, l'extraction et la fixation de l'anhydride sulfureux libéré à partir des solutions sulfitiques.

D'autres essais ont confirmé non seulement cette première constatation, mais également ont mis en évidence la stabilité d'autres combinaisons organiques d'addition, au *p*H 2,9 et à la température de + 10° C. Les *tableaux IV* et *V* présentent les résultats de nombreux dosages de l'anhydride sulfureux libre extrait à partir des solutions synthétiques, contenant les différentes combinaisons d'addition, ou des jus de raisin.

Contrairement aux observations de Blouin et Sudraud (82) et de Sudraud (83), nous n'avons pas constaté, dans les conditions de notre mode opératoire, la décomposition du sulfonate de l'acide pyruvique. Par contre, comme le montrent les résultats des dosages consignés dans le *tableau VI*, l'acétaldéhydesulfonate subit une légère décomposition à la température de + 28° C, tandis que le pyruvate sulfonate devient moins stable à la température de + 25° C et même avant.

Tableau IV

...sultats du dosage iodométrique de l'anhydride sulfureux libre après sa séparation en présence de glucosesulfonate et de l'acétaldéhydesulfonate en mg par litre. Condition de désulfitation : débit d'azote 40 litres/heure, dépression 50 mg Hg, température — 10° C, durée 15 minutes, volume du liquide 15 ml, à pH 0,9, réception dans 50 ml de NaOH 0,1 N, appareil de Lieb-Zacherl pour les essais 1, 2, 3, appareil à reflux simple, pour l'essai 4.

...ature des solutions expérimentées	Titre des solutions	Dosage de l'anhydride libre dans le récepteur
1° Glucosesulfonate de potassium	499,40	Néant
Id.	48,00	»
Id.	10,24	»
2° *Mélange*		
Hydrogénosulfite de potassium	11,52	11,52
Glucosesulfonate de potassium	353,20	—
3° *Mélange*		
Hydrogénosulfite de potassium	60,80	60,80
Acétaldéhydesulfonate de potassium	200,00	—
Glucosesulfonate de potassium	203,20	—
4° *Mélange*		
Hydrogénosulfite de potassium	156,80	156,80
Acétaldéhydesulfonate de potassium	100	—
Glucosesulfonate de potassium	100	—
Sulfonate du pyrovate de sodium	100	—
5° Sulfonate de l'acide alpha-cétoglutarique	81,9	Néant

Tableau V

...ssais sur un jus de raisin blanc. Résultats des dosages iodométriques en mg de SO_2 par litre. Appareil de Lieb-Zacherl. Teneur en anhydride sulfureux libre, 3,2 mg/litre, déterminée selon le procédé de Ripper.

SO_2 ajouté			Après la séparation	Observations
Combiné		Total	Libre	
...étaldéhyde	Glucose			
—	100	100	Néant	
100	100	200	»	
100	100	200	»	Alcoolisé à 15° C. I.

Tableau VI

Influence de la température sur la stabilité des combinaisons de l'anhydride sulfureux à pH 0,9. Résultats du dosage iodométrique en mg de SO_2 par litre.

Composé utilisé	Température (°C)	Titre des solutions		Après la séparation	Appareil utilisé
		Libre	Combiné	Libre	
Acétaldéhydesulfonate	20	—	451,2	—	Appareil Lieb-Zacherl
»	28	—	451,2	1,92	»
Glucosesulfonate	20	—	454,4	—	»
»	28	—	454,4	—	»
Pyruvate sulfonate	10	28,8	49,28	28,8	Appareil à reflux simple
»	10	28,8	49,92	28,8	»
»	20	28,8	49,28	29,44	»
»	25	28,8	49,28	33,28	»
Sulfonate de l'acide alpha-cétoglutarique	10	67,2	81,9	67,2	»

On peut penser que le barbotage de l'azote dans le liquide facilite, à la température supérieure à + 20° C, la décomposition des combinaisons de l'anhydride sulfureux, car l'étalonnage direct de leurs solutions aqueuses effectué à la température ambiante, de 18° — 22° C, ne décèle aucune décomposition.

Finalement nous avons obtenu la certitude que les modalités de notre mode opératoire permettent d'obtenir une extraction convenable de l'anhydride sulfureux libre en présence simultanée des différentes combinaisons organiques de l'anhydride sulfureux. Il nous restait à vérifier son efficacité lors des dosages de l'anhydride libre et combiné.

e) Vérification de la technique mise au point ; comparaison avec le procédé acidimétrique

Comme nous l'avons vu, l'extraction de l'anhydride sulfureux libre s'effectue à la température de + 10°-12° C sans la décomposition des combinaisons complexes sulfureuses. L'extraction de ces dernières nécessite la température élevée, de l'ordre de 100° C, toutes les conditions restant sans aucune modification.

Les résultats du *tableau VII*, qui d'ailleurs complètent ceux du *tableau III*, nous montrent une bonne efficacité du mode opératoire mis au point, même si l'on diminue le volume de solution alcaline dans l'absorbeur destiné à capter l'anhydride sulfureux véhiculé par le courant d'azote (de 30 ml à 30 ml).

TABLEAU VII

Résultats du dosage iodométrique de l'anhydride libre et combiné, après séparation à + 10° C et à l'ébullition, en mg par litre de la solution bitartrique de pH 3,1. Appareil à reflux simple.

N°	Solution alcaline (ml)	Solutions sulfureuses utilisées				Après la séparation			
		Nature	Libre	Combiné	Total	Libre	Combiné	Total	
								Teneur	Erreur (%)
1	50	Hydrogénosulfite	449,28	—	449,28	448,64	—	448,64	0,14
2	»	*Id.*	454,4	—	454,4	454,4	—	454,4	—
3	»	Acétaldéhydesulfonate	1,92	474,88	476,8	—	—	473,2	0,7
4	»	*Id.*	3,20	470,4	473,6	—	—	473,6	—
5	50	Hydrogénosulfite	462,1	—	462,1	461,44	—	461,44	0,14
6	»	Acétaldéhydesulfonate	2,56	460,8	463,36	—	—	463,36	—
7	»	Mélange : — acétaldéhydesulfonate 50 %. — glucosasulfonate 50 %	8,96	405,76	414,7	8,96	406,4	415,4	—

TABLEAU VIII

Comparaison des procédés iodométrique et acidimétrique. Résultats exprimés en mg de SO_2 par litre.

N° Essai	Vin	Gaz utilisé	Lieb-Zacherl						A reflux simple						A chemise double		
			Acidimétrie			Iodométrie			Acidimétrie			Iodométrie			Acidimétrie		
			libre	comb.	total	libre	comb.	total	libre	comb.	total	libre	comb.	total	libre	comb.	total
1	*Vin rouge des Corbières*, 1964.	air	2,56	9,6	12,16	—	—	—	2,56	22,4	24,96	—	—	—	2,56	9,6	12,16
2		air	—	—	—	—	—	—	2,56	14,72	17,28	—	—	—	—	—	—
3		azote	—	—	—	—	—	—	2,56	8,96	11,52	—	—	—	—	—	—
4		azote	—	—	—	—	—	—	—	—	—	2,56	9,6	12,16	—	—	—
5	*Vin rouge Cinsault*, Preb-Rouge (Aude), 1964 préparé au Laboratoire et enrichi en anhydride.	air	9,6	220,8	230,4	—	—	—	9,6	219,5	229,1	—	—	—	—	—	—
6		azote	—	—	—	9,6	221,4	231,4	—	—	—	9,6	220,8	230,4	—	—	—
7	*Vin rouge Alicante-Bouschet* Azens (Aude), 1963, préparé au Laboratoire sans l'anhydride sulfureux.	azote	—	—	—	—	—	—	—	—	—	néant	néant	néant	—	—	—

comparaison des procédés iodométriques et acidi-ques nous a permis de disposer de constatations ssantes. En effet, il ressort de l'examen des résultats us, consignés dans le *tableau VIII*, que notre mode toire et la technique acidimétrique de Paul (*op. b*)) fournissent des résultats comparables. Comme voit, la première technique est tributaire d'un rant spécial soit à double chemise, soit à circu-intense d'eau, nécessaire pour faire retourner e ballon de distillation, non seulement les substan-ides normales du vin, mais également celles qui nt s'y former sous l'action d'un courant d'air, e le démontrent les résultats des essais n° 1, 2, 3, és avec le vin rouge des Corbières. Certes, la du vin joue son rôle à juger d'après les résultats ns avec le vin rouge Cinsault enrichi en anhydride.

enseignements des travaux, signalés dans la ere partie de notre mémoire, indiquent que l'in-e de l'acidité volatile se fait sentir dès qu'une ne teneur est dépassée d'où la nécessité d'un essai t. A ce propos remarquons que l'emploi du per-pour oxyder l'anhydride sulfureux n'est pas é en raison de la formation des substances acides es.

contré, notre technique iodométrique est insen-l'influence des composés acides volatils, et cet ge permet de nous contenter d'un appareil distillatoire, munis d'un condenseur à simple reflux, plus robuste, moins compliqué et moins coûteux.

En plus, nous n'avons pas constaté l'influence perturbatrice des substances volatiles iodophiles sur les résultats du dosage iodométrique, effectué suivant notre mode opératoire. En effet, l'exemple d'un vin rouge Alicante Bouschet, 1965 (essai n° 7), élaboré sans anhydride sulfureux, est intéressant à citer puisque notre procédé pour le dosage de l'anhydride sulfureux total (*op. cit.* (68) (15)) a décelé la présence des substances iodophiles correspondant à 1,3 mg d'anhydride par litre. Sans doute, ces substances sont moins volatiles dans les conditions de l'entraînement par le courant d'azote. On peut supposer que la servitude d'emploi de ce gaz peut paraître à certains comme un inconvénient majeur, mais vraiment il est difficile d'admettre ce raisonnement comme valable, compte tenu d'autres avantages que procure aux œnochimistes avertis le procédé iodométrique, même sous la forme de son mode opératoire actuel. D'ailleurs il serait très instructif de pouvoir préparer d'autres combinaisons d'addition afin de vérifier leur influence sur le dosage de l'anhydride sulfureux libre et ainsi améliorer la technique proposée. Dans ce qui suit nous allons exposer en détail le mode opératoire du dosage iodométrique de l'anhydride sulfureux libre et combiné ainsi que les résultats de l'évaluation statistique de sa valeur analytique.

(à suivre)

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heating. This suggests degradation of the molecule with the formation of a higher alkyl iodide. The presence of the methoxyl group was established by the formation of phenyl trimethylammonium iodide in good yield.

At room temperature the pigment is readily soluble in acetone and dimethyl formamide, fairly soluble in methanol, ethanol, ether, ethyl acetate and dioxane, difficultly soluble in carbon disulphide and benzene, and insoluble in carbon tetrachloride, petroleum ether and water. It is extractable from ether with sodium hydroxide solution but not with aqueous sodium carbonate. It gives a black colour with ferric chloride in alcohol.

Catalytic reduction of the pigment in methanol in the presence of platinum stops with the formation of a yellow dihydro derivative, $[\alpha]_D^{25}$ + 24·6° (c, 0·8 in dimethyl formamide). In the crude form, the dihydro derivative no longer shows the 3145 A. peak but does exhibit strong absorption at 2370 A. (E_{1cm} 35,200).

Further studies are in progress, the details of which will be reported elsewhere.

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Factors Involved in 'Chemotactism' of Leucocytes *in vitro*

THE polymorphonuclear leucocytes isolated from blood or an inflammatory exudate are able to respond to chemotactic stimuli ('tactism'). This property is readily demonstrated by the following technique[1]. A suspension of starch granules, isolated from potatoes, is spread on microscope slides and the films fixed by drying. A few drops of a suspension of leucocytes are placed on a slide so prepared, a cover-slip superimposed and the edges sealed with 'Vaseline' and wax. The preparation is then placed on the stage of a microscope in a hot-box at 37° C. Almost at once the leucocytes, attracted no doubt by soluble dextrin liberated into the medium, move towards the starch granules, and finally, within an hour, adhere to the surface of the latter in the form of regular crescents.

This phenomenon, although easy to demonstrate, only occurs under certain specific conditions. It is precisely the determination of these that has been the subject of our investigation. Our experiments, the most recent of which will be reported in detail elsewhere, have shown that at least two conditions are indispensable. First, the leucocytes must be suspended in a favourable medium; and secondly, the cells themselves must be in a suitable state as regards surface properties and enzymatic equipment.

(1) *The medium.* In our experience, the migration of leucocytes towards the starch granules never occurs in a medium which is simply mineral, such as normal saline or Ringer's solution[1]. Serum must be present; this need not necessarily be homologous (for example, guinea pig leucocytes can be suspended in rabbit serum). It is, however, obvious that a serum naturally toxic for the cells under investigation must be avoided (for example, guinea pig leucocytes must not be suspended in human serum[2]). We have further found that at least two of the normal constituents of serum must be present in sufficient concentration, namely, complement and inorganic calcium.

(a) *Complement.* Leucocytes suspended in serum, the complement of which has been eliminated by age, heating or absorption with bacteria[2], remain alive but do not move towards the starch granules. Similar results are obtained with serum the complement of which has been partially destroyed by fresh yeast or ammonia. It would thus appear that all four constituents of complement are indispensable for the production of 'tactism' *in vitro*.

(b) *Calcium ions.* In a serum medium from which the calcium has been precipitated by the addition of 10 mgm. per ml. of sodium citrate or oxalate, the leucocytes continue to form a few pseudopodia, but they do not migrate towards the starch granules[1]. Since citrate inactivates complement, the inhibition of 'tactism' caused by addition of sodium citrate might result from this effect, rather than from the precipitation of calcium. The same, however, cannot be said of oxalated serum. Furthermore, the simple addition of calcium to either of these media is sufficient to re-establish the phenomenon.

(2) *Condition of cells.* We have found it possible to inhibit completely the phenomenon of 'tactism' even in a serum medium rich in complement and calcium ions by modifying directly the state of the leucocytes.

(a) *Surface.* Leucocytes, which after being in contact with olive oil for two hours are re-suspended in serum, appear morphologically normal, but remain round in the presence of starch granules towards which they no longer migrate. Although we have been able to show that the electric surface charge of the cells has not changed, it seems likely that the observed inhibition of 'tactism' is due to a modification of the cell surface.

(b) *Cell enzymes.* One of us (J. Lebrun) has shown that the addition of certain enzyme inhibitors to the medium, without, of course, killing the leucocytes, suppresses their capacity to respond to chemotactic stimuli. The following fourteen inhibitors, in the concentrations indicated, have been found to have this effect: sodium fluoride ($M/4$), sodium pyrophosphate ($M/200$), cysteine ($M/10$), thioglycollic acid ($M/33$), sodium fluoroacetate ($M/20$), malonic acid ($M/7$), sodium cyanide ($M/90$)[3], sodium azide ($M/7$), hydroxylamine ($M/15$), sodium diethyldithiocarbamate ($M/3$), dithizone (half saturated), *ortho*phenanthroline (one-third saturated), sodium bisulphite ($M/8$), urethane ($M/5$).

Of these inhibitors, the first two also precipitate calcium and the next four inactivate complement. It is possible, therefore, that with these compounds the suppression of 'tactism' is secondary to their effect on the medium. The remaining eight inhibitors, however, have no such effects, and it seems probable that they act directly on the enzymes of the leucocytes. A diminution in the phagocytic power and respiration of the latter sometimes, but not always, accompanies the inhibition of 'tactism'.

In the light of these results, it would appear that enzyme reactions play an important part in the phenomenon of 'tactism' *in vitro*. It is conceivable, for example, that the abundance of peroxidase in polymorphonuclear cells[4] as compared with lymphocytes is related to their migratory power. The nature

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No. 4254 May 12, 1951 NATURE 775

of the enzymes involved, however, remains an open question and will form the basis for future investigations.

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Effect of Partial Acetylation on Jute

PARTIAL acetylation as a means of arresting the development of the yellow to brown colour, produced by photochemical reactions on irradiating bleached jute with a mercury vapour lamp, has been suggested by Peill[1], and Callow and Speakman[2]. The development of this undesirable shade in untreated and bleached jute has been postulated by the latter as mainly due to a sequence of reactions involving the lignin molecule, presumably through the formation of *o*-phenols and subsequent conversion to *o*-quinones. These authors are also of opinion that some discoloration may be caused by the polymerization of furfural liberated from pentosans, or result from condensation reactions between furfural and phenolic groups of lignin. On acetylation, however, the phenolic and alcoholic hydroxyl groups in jute are blocked, and consequently the possibility of the above reactions leading to discoloration is avoided.

During partial acetylation of a cellulosic fibre, the reaction is supposed to take place in a topochemical fashion, and unless the acetylated layer is removed by some suitable solvent, the reaction is expected to be confined to the surface layer of the fibre. Jute is basically composed of cellulose, hemicelluloses, and lignin, and the relative proportion of their hydroxyl groups occurring at the surface layer, which are free for acetylation, is not known. It was therefore considered of importance to determine the relative amounts of acetyl groups which reacted with individual fibre components during the acetylation of jute.

The untreated or bleached jute fabric (3 gm.), when subjected to partial acetylation by refluxing the material with a mixture of acetic anhydride (30 gm.) and xylene (30 gm.) in the presence of sulphanilic acid (0·06 gm.) as a catalyst for four hours, during which time a definite equilibrium was reached, showed that a high content of acetyl had been introduced. Under the same conditions, however, only a smaller fraction of the available acetyl reacted either with cotton or α-cellulose isolated from jute. This greater affinity of jute for acetyl groups is therefore most likely due to the presence of hemicelluloses or lignin or both. Since holocellulose isolated by two successive treatments with sodium chlorite solution (0·7 per cent) at *p*H 4, at 100° C., takes up a greater amount of acetyl (see table) than when the hemicelluloses are eliminated from jute fabric by boiling with 10 per cent caustic soda solution, which also removes only a small quantity of lignin, it seems likely that when untreated or bleached jute is acetylated, the hemicelluloses are responsible for more of the acetyl groups attached compared with lignin. Furthermore, as the isolated jute α-cellulose is capable of reacting with only a small amount of acetyl, it is probable that, during acetylation of untreated or bleached jute, the cellulosic component plays a less important part compared with hemicelluloses and lignin.

Sample	Loss in wt. due to chemical treatment (per cent)	Increase in wt. due to acetylation (per cent)*	Initial acetyl content of the material (per cent)*	Introduced acetyl content (per cent)*
Purified cotton	—	4·3	Nil	4·36
Jute α-cellulose	30·7	3·3	0·79	4·03
Degumed untreated jute	—	11·9	5·94	13·55
Jute bleached by treating successively with $NaClO_2$ and H_2O_2	4·2	13·5	3·00	14·52
Jute holocellulose	16·8	11·7	4·06	14·25
Degumed jute fabric boiled with 10% NaOH	26·6	10·8	0·34	11·05

* Expressed on bone-dry non-acetylated material.

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'Lampbrush' Fibres in the Chromosomes of *Chrotogonus incertus* Bolivar

THE meiotic chromosomes of the grasshopper, *Chrotogonus incertus* Bolivar (collected at Allahabad), assume typical 'lampbrush' appearance at pachytene, which condition persists to the end of diakinesis, but disappears completely at the first metaphase. This condition has been reported by Hsu[1] in two different species of grasshoppers, and, judging from illustrations, it seems to have been observed in grasshoppers by other investigators also, even though they do not explicitly describe it[1,2]. Hsu[1] has put forward an ingenious theory, based essentially on Goldschmidt's[3] interpretation of the 'lampbrush' chromosome, which links up spiralization, heteropycnosis and the formation of 'lampbrush' processes. According to him, heterochromatinic heteropycnosis is not due, at least not entirely, to differential nucleic acid synthesis[4], but to a difference in the capacity to undergo cyclic condensation. Negative heteropycnosis results from the fact that the intracellular conditions permit the growth of 'lampbrush' fibres on the X-chromosome, whereas the autosomes become compactly coiled. Positive heteropycnosis results when the conditions are reversed; the X-chromosome undergoes condensation, but not the autosomes, which, in consequence, show the 'lampbrush' fibres. Polymerization of the nucleoprotein, he claims, causes the chromosomes either to be coiled or show 'lampbrush' fibres. Now in the spermatocytes of *Chrotogonus*, late diakinetic chromosomes, although considerably condensed and consequently much shorter than the pachytene threads, are provided with 'lampbrush' fibres similar in size and shape to those of the earlier stages. So, condensation (spiraliza-

...nac aus E. coli RL 103; Transaminase ...illium notatum ATCC 8251; Malta... ...s Bac. subtilis ATCC 9759.

...8 (1959). School Chemistry, Rio de ... E. Müller, Würzburg

... einer mit Sorbinsäure stabilisierten ...rophotometrische Amylasebestimmung. ...chen 60 und 105 Somogyi-Einheiten ... 10,6 E. In der Rückenmarksflüssig... ...n. Die Amylaseaktivität kann nicht ... ist. Man verwendet Serum oder Hepa...plasma) zur Messung, das 1:20 mit ...ckenmarksflüssigkeit verwendet man ...Lösung (siehe unten) und 1,0 ml Tris... ...st-Blutzuckergefäße, temperiert auf ...probe (Test) 0,50 ml des verdünnten ...ssflüssigkeit. Man schüttelt um und ...n (Rückenmark). Dann gibt man zu ...ämmt mit etwa 20 ml Wasser. Zu der ...nm und dann zu beiden Proben 0,5 ml ...rden bis zur Marke (25 ml) aufgefüllt, ...nabel 620 nm gegen eine Blindprobe, die ...lt ... lassen. Die Amylase-Einheiten ...K ... de — Absorpt. Test/Absorpt... ...nungsfaktor bei 1:20 = 1, 1:40 = 2, ...). Man stellt eine kalt gesätt. Lösung ...ze stehen lassen). In einem 1 l-Meß... ...ke in 50 ml Sorbinsäurelösung, setzt ... schüttelt um und füllt nach Erkalten ...ktlösung (pH 3,4) auf. In Polyäthylen... ...dan löst 19,4 g Tris(hydroxymethyl)... ...lösung mit konz. Salzsäure auf pH 7,5

...School of Med., Pittsburgh, Pa. (USA). Ursula Baumann

...schem Material wird von E. Levin[1] ...thode nach Somogyi vorgeschlagen: ...n 3 min in ein Wasserbad von 40° C. ... Man läßt 30 min einwirken und gibt ...s und gibt 0,4 ml Zinksulfatlösung zu. ...rt. Ebenso wird mit einer Kontroll... ...Falls in der Probe nur geringe Eiweiß... ...e glatte und gute Fällung zu erreichen, ...t ml alkalisches Kupferreagens nach ...asserbad in bedeckten Gläschen. Man ...ases nach Nelson zu, schüttelt durch, ...notocolorimetriert unter Verwendung ... wird aufgestellt.

...1959). Irmgard Pfitzer

Z. Anal. Chem. 178 (1961)

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Aus dem Universitäts-Institut für Lebensmittelchemie Frankfurt/M. und dem Institut für Obst- und Gemüseverwertung Geisenheim/Rhg.

Zur Bestimmung der gesamten und freien schwefligen Säure im Wein*

Von

W. Diemair, J. Koch und D. Hess**

Mit 4 Textabbildungen

(Eingegangen am 14. Juli 1960)

Gesamte schweflige Säure

Die bisher bekannten Methoden zur Bestimmung der gesamten schwefligen Säure im Wein arbeiten nach verschiedenen Prinzipien:

1. Die gesamte schweflige Säure wird abdestilliert, in der Vorlage zu Schwefelsäure oxydiert und titrimetrisch mit Natronlauge[10,14,16,17] oder gravimetrisch als Barium-[5,6,14] oder Benzidinsulfat[21] bestimmt.

2. Die durch Destillation abgetrennte schweflige Säure wird in der Auffanglösung jodometrisch erfaßt, nachdem der Aldehydkomplex aufgespalten worden ist[1,9,21].

3. Man mißt photometrisch die durch schweflige Säure bedingten Veränderungen von Malachitgrünlösungen[19] oder Formaldehyd-Fuchsinlösungen[4,15].

4. Die durch Destillation abgetrennte schweflige Säure ergibt mit Barium-[13] oder Bleisalz[13] eine Trübung, die nephelometrisch ausgewertet werden kann.

Im Allgemeinen gelangen die beiden ersten Verfahren zur Anwendung. Die alkalimetrische Titration nach Destillation wird einerseits durch die im Wein vorhandenen flüchtigen Säuren erschwert, andererseits setzt sie voraus — besonders bei geringen SO_2-Mengen — daß der Arbeitsraum vollständig frei von Säure- und Ammoniakdämpfen ist. Die gravimetrischen Methoden sind sehr zeitraubend und mit den bekannten fällungstechnischen Schwierigkeiten behaftet.

Zur schnellen und exakten SO_2-Bestimmung eignen sich besonders die jodometrischen Verfahren. Von diesen haben wir besonders die Methoden nach Weinmann[21] und nach Deibner[1] auf ihre Genauigkeit und Reproduzierbarkeit untersucht.

Die beiden Methoden unterscheiden sich vor allem in der Wahl der Auffanglösung für das abdestillierte SO_2. Weinmann benutzt eine *alkalische* Auffanglösung, wobei mit einer Oxydation des Sulfitions gerechnet werden muß, Deibner dagegen eine *saure*. Diese muß genügend gekühlt werden, um den Dampfdruck von SO_2 so weit herabzusetzen, daß

* Für die Untersuchungen wurden Mittel des Bundesernährungsministeriums zur Verfügung gestellt, wofür auch an dieser Stelle bestens gedankt sei.

** Die Arbeit stellt einen Ausschnitt dar aus der Diplomarbeit von D. Hess: Beitrag zur Bestimmung der schwefligen Säure im Wein (Universität Frankfurt/M. 1957) und der Dissertation von D. Hess: Über den Einfluß von schwefliger Säure und l-Ascorbinsäure bei der Weinbereitung (Frankfurt/M. 1960).

keine Verluste entstehen. Das Einstellen der Vorlage in Eis, wie Deibner es vorschlägt, genügt nach unseren Erfahrungen nicht. Man muß die Auffanglösung vielmehr durch Zugabe von Eisstückchen direkt kühlen (Tab. 1). Bei hohen SO_2-Konzentrationen können auch dann noch Verluste auftreten, die man jedoch durch Vergrößerung des Volumens der Auffanglösung auf einen Fehler von unter 1% herabdrücken kann. Bei der Destillation ist allgemein zu beachten, daß ein Teil der schwefligen Säure im Destillierkolben zu Schwefelsäure oxydiert werden kann. Bei einigen Verfahren[5,6,14] wird daher die Luft mit CO_2, nach Weinmann u. Deibner mit Wasserdampf, aus der Apparatur vertrieben und erst dann wird die Untersuchungslösung aus einem Tropftrichter zugegeben. Es empfiehlt sich ein Nachspülen des Tropftrichters mit luftfreiem Wasser, andernfalls Verluste bis 14% entstehen können.

Tabelle 1. *Der Einfluß der Auffanglösung bei der SO_2-Bestimmung in Natriumpyrosulfitlösungen wechselnder Konzentration*

Auffanglösung: 10 ml n NaOH-Lösung + 30 ml H_2O							
gegeben	mg/l	23,6	23,6	86,6	86,6	474,4	474,4
gefunden	mg/l	2,3	1,7	4,3	23,4	410,0	422,0
	= %	10,0	7,2	5,0	27,0	86,6	89,0
Auffanglösung: 10 ml n Salzsäure + 30 ml H_2O Kühlung durch Einstellen in Eiswasser							
gegeben	mg/l	20,7	20,7	86,6	86,6	474,4	474,4
gefunden	mg/l	19,5	20,4	81,3	80,3	427,6	433,0
	= %	94,2	98,6	93,9	92,8	90,2	91,3
Auffanglösung: 10 ml n Salzsäure + 30 ml H_2O Kühlung durch Zugabe von Eisstückchen							
gegeben	mg/l	20,7	20,7	86,6	86,6	474,4	474,4
gefunden	mg/l	20,7	20,7	86,4	85,9	468,8	467,6
	= %	100,0	100,0	99,6	99,7	98,9	98,6

Anmerkung. Die schweflige Säure wurde nach der Vorschrift von Deibner abdestilliert, jedoch in verschiedenartigen Lösungen aufgefangen.

Jeder Wein enthält mehr oder weniger große Mengen Acetaldehyd, die bei der Destillation mit übergehen und in der sauren Auffanglösung einen Teil der schwefligen Säure zu α-Oxyäthansulfonsäure binden, die nicht mehr mit Jod reagiert. Benutzt man eine alkalische Auffanglösung, so liegen die schweflige Säure bzw. Sulfit und der Acetaldehyd zwar weitgehend ungebunden nebeneinander vor, jedoch treten dann durch Luftsauerstoff bedingte Oxydationen auf.

Deibner umgeht diese Schwierigkeit, indem — nachdem die im Destillat frei vorliegende SO_2 mit Jodlösung titriert ist — auf pH 8 gepuffert

Einstellen der Vorlage in Eis, wie Deibner ...seren Erfahrungen nicht. Man muß die ... Zugabe von Eisstückchen direkt kühlen ...ntrationen können auch dann noch Ver... durch Vergrößerung des Volumens der ... von unter 1% herabdrücken kann. Bei ...u beachten, daß ein Teil der schwefligen ...schwefelsäure oxydiert werden kann. Bei ...er die Luft mit CO_2, nach Weinmann u. ... der Apparatur vertrieben und erst dann ... aus einem Tropftrichter zugegeben. Es ...des Tropftrichters mit luftfreiem Wasser, ...tstehen können.

...zung bei der SO_2-Bestimmung in Natriumpyro... ...wechselnder Konzentration

...l n NaOH-Lösung + 30 ml H_2O				
	86,6	86,6	474,4	474,4
	4,3	23,4	410,0	422,0
	5,0	27,0	86,6	89,0
...ml n Salzsäure + 30 ml H_2O ... E... ...len in Eiswasser				
	86,6	86,6	474,4	474,4
	81,3	80,3	427,6	433,0
	93,0	92,8	90,2	91,3
...ml n Salzsäure + 30 ml H_2O ...ugabe von Eisstückchen				
	86,6	86,6	474,4	474,4
	80,4	85,0	468,8	467,6
	99,0	99,3	98,9	98,6

... wurde nach der Vorschrift von Deibner ...igen Lösungen aufgefangen.

weniger große Mengen Acetaldehyd, die ...en und in der sauren Auffanglösung zu α-Oxyäthansulfonsäure binden, die ...tzt man eine alkalische Auffanglösung, ...w. Sulfit und der Acetaldehyd zwar ...ander vor, jedoch treten dann durch ...en auf.

...gkeit, indem — nachdem die im Destil... ...sung titriert ist — auf p_H 8 gepuffert

und eine abgemessene Menge Jodlösung zugesetzt wird, um nach einiger Zeit das unverbrauchte Jod in der wieder angesäuerten Lösung mit Natriumthiosulfat zu bestimmen.

Die überschüssige Jodlösung darf man keinesfalls vor dem Abpuffern auf p_H 8 zusetzen, da sonst zu hohe SO_2-Werte vorgetäuscht werden, die durch Substanzen bedingt sein müssen, die bei der Destillation des Weines gebildet werden.

Werden diese Bedingungen eingehalten, so kann man in Modellwein nach der Deibnerschen Methode 98,4—101,1% der vorgelegten SO_2-Menge erfassen, während die Weinmannsche Methode mit einem Fehler bis zu 15% arbeitet (Tab. 2).

Tabelle 2. *Bestimmung der schwefligen Säure nach Deibner und nach Weinmann in Modellwein* wechselnder SO_2-Konzentration*

Methode nach Deibner (bei intensiver Kühlung)						
gegeben mg/l SO_2	18,5	18,5	87,4	87,4	349,0	349,0
gefunden mg/l SO_2	18,2	18,6	86,6	86,3	345,2	346,4
= %	98,4	101,1	99,2	98,8	98,9	99,3
Methode nach Weinmann						
gegeben mg/l SO_2	21,2	21,2	85,2	85,2	349,0	349,0
gefunden mg/l SO_2	18,4	17,9	79,1	74,7	330,0	327,0
= %	87,3	84,9	92,8	87,8	94,6	93,8

* Der Modellwein enthielt im Liter: 2 g Weinsäure, 5 g Äpfelsäure, 2 g Calciumlactat, 0,7 g Essigsäure, 0,6 g Gerbsäure, 90 g Alkohol, 10 g Glycerin, 20 g Zucker, 0,2 g Acetaldehyd.

Aus SO_2-freiem Apfelwein, dem 26—472 mg/l SO_2 zugesetzt worden war, verminderten sich die Abweichungen bei der Bestimmung nach Deibner (bei intensiver Kühlung der Auffanglösung) sogar auf ± 0,5%.

Diese Methode, die demnach bei intensiver Kühlung der Auffanglösung zur exakten Bestimmung der gesamten schwefligen Säure gut geeignet ist, erweist sich bei Reihenuntersuchungen, infolge der erforderlichen Manipulationen bei der Bereitung der Auffanglösung und bei der Titration, als recht umständlich.

Man umgeht die Schwierigkeit der Kühlung, wenn man eine Auffanglösung von p_H 3,5 wählt, da sich bei diesem p_H-Wert das übergehende SO_2 in der Vorlage als Hydrogensulfit ansammelt und sich nicht mehr verflüchten kann. Mit einer Oxydation durch Luft braucht man innerhalb der zu einer Bestimmung erforderlichen Zeit bei diesem p_H-Wert praktisch nicht zu rechnen, da gegen Luftsauerstoff das Hydrogensulfition wesentlich stabiler ist als das Sulfition.

Der etwas umständliche Titrationsvorgang läßt sich verkürzen, wenn man analog wie bei der Acetaldehydbestimmung[11] arbeitet und das an

Aldehyd gebundene SO_2 nach Zugabe von Natriumhydrogencarbonat in schwach alkalischem Medium direkt mit Jod titriert.

Unter Berücksichtigung dieser Möglichkeiten wurde von uns eine entsprechende Methode entwickelt.

Tabelle 3. *Vergleich der neuen Methode zur Bestimmung des gesamten SO_2 in Weinen mit der Deibner-Methode*

Methode	mg/l gesamte SO_2											
Deibner	12	18	56	58	97	113	128	134	140	197	210	237
neue	12	17	54	57	96	111	127	132	140	196	210	235
Differenz	0	—1	—2	—1	—1	—3	—1	—2	0	+2	0	—2

Vergleicht man die Analysenergebnisse, die nach dieser Methode und derjenigen von Deibner (Tab. 3) erhalten werden, so sind nur geringe Differenzen zu beobachten. Da unsere Methode leichter zu handhaben ist als die von Deibner, kann sie allgemein empfohlen werden.

Freie schweflige Säure

Die Bestimmung der freien schwefligen Säure erfolgt im allgemeinen nach der Anweisung von Ripper[23], wonach eine bestimmte Menge Wein angesäuert und mit Jodlösung direkt titriert wird. Dabei bleibt unberücksichtigt, daß außer freier schwefliger Säure auch andere Jod reduzierende Substanzen, wenn auch nur in geringem Maße, im Wein vorliegen. Falls die L-Ascorbinsäure zur Weinbehandlung zugelassen wird, womit in absehbarer Zeit zu rechnen ist, muß diese Bestimmung zu völlig falschen Ergebnissen führen. Die Entwicklung einer brauchbaren Methode zur richtigen Bestimmung der freien SO_2 erschien daher dringend notwendig.

Zur Abänderung der Ripperschen Methode bieten sich zwei Möglichkeiten an. Man kann entweder die freie schweflige Säure an Aldehyde oder Ketone binden, oder sie absaugen und den verbleibenden Rest reduzierender Substanzen ermitteln.

Tabelle 4. *Veränderungen des Gehaltes an gebundener schwefliger Säure während des 15 min langen Absaugens der freien schwefligen Säure aus saurer Lösung*

Wein Nr.	bestimmt			berechnet	
	schweflige Säure in mg/l			gebunden mg SO_2/l[4]	Differenz mg/l
	frei[1]	gesamt[2]	gebunden[3]		
1	20,4	157,0	133,0	136,6	—1,6
2	47,2	287,8	238,2	240,6	—2,4
3	51,8	309,8	256,4	258,0	—1,0

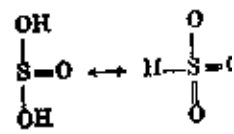
[1] nach Abzug des Blindwertes; [2] bestimmt nach Deibner; [3] im Absaugrückstand nach Deibner bestimmt; [4] berechnet aus gesamter und freier schwefliger Säure.

Unsere Untersuchung
nach Zusatz von Acet
Titration nach Ripper
läßt, was auch von Ins
Besser macht man da
bestimmt den Jodverb
saugen der SO_2 aus der
Das Ansäuern ist er
zuführen. Gleichzeitig

Aldehyd

derart verzögert, daß
Säure (15 min genügen
Säure in Freiheit gesetz
Diese Methode ist
Für besondere Untersu
Methode, die auch klei
Hier bot sich die *Polar*

Die schweflige Säure
Medium keine Stufe, e
werden nach Cosman
an der Kathode zu Dith
reduziert gemäß der Gleic
$2\,SO_2 + 2e \rightarrow S_2O_4^{2-}$.
Die Stufenhöhe soll
Cosman von der Wasser
ionenkonzentration unab
gig sein. Kolthoff fand
gegen, daß nicht nur das Re
tionspotential, sondern
der Diffusionsstrom pH-ab
gig ist und mit sinkende
wächst. Nur in stark sa
Medium (pH 0—1) wird d
samte schweflige Säure
ziert, da nur in diesem p
reich das tautomere Verh



quantitativ nach links
schoben ist.
Heyrovský und Sa
SO_2-Bestimmungen durc
zugehen.

40

... on Natriumhydrogencarbonat in
Jod titriert.
...ichkeiten wurde von uns eine

...mmung des gesamten SO_2 in Weinen
...ethode

...amte SO_2						
...	128	134	140	197	210	237
...1	127	132	140	199	210	235
...	—1	—2	0	+2	0	—2

..., die nach dieser Methode und
...en werden, so sind nur geringe
...ethode leichter zu handhaben
...ein empfohlen werden.

Säure

...n Säure erfolgt im allgemeinen
...ch eine bestimmte Menge Wein
...er... Dabei bleibt unberück-
...e... andere Jod reduzierende
... Maße, im Wein vorliegen. Falls
...g zugelassen wird, womit in
... Bestimmung zu völlig falschen
...ner brauchbaren Methode zur
...ien daher dringend notwendig.
...hode bieten sich zwei Möglich-
schweflige Säure an Aldehyde
... und den verbleibenden Rest

...dener schwefliger Säure während des
...igen Säure aus saurer Lösung

	erzeichnet	
...den²	gebunden mg SO_2/l²	Differenz mg/l
...,0	136,6	—1,6
...,2	240,0	—2,4
...,4	258,0	—1,6

...ch Deibner; ³ im Absaugrückstand
...rater und freier schwefliger Säure.

Unsere Untersuchungen zeigten, daß der Farbumschlag beim Titrieren nach Zusatz von Acetaldehyd noch „ziehender" erfolgt als bei der Titration nach Ripper, so daß sich der Endpunkt schlecht ermitteln läßt, was auch von Ingram[3] angegeben wird.

Besser macht man daher von der anderen Arbeitsweise Gebrauch und bestimmt den Jodverbrauch einmal direkt und einmal nach dem Absaugen der SO_2 aus der angesäuerten Untersuchungslösung.

Das Ansäuern ist erforderlich, um das Hydrogensulfit in SO_2 überzuführen. Gleichzeitig wird dadurch das Einstellen des Gleichgewichts

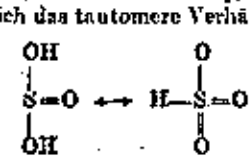
$$\text{Aldehydbisulfit} \rightleftharpoons \text{Aldehyd} + \text{Hydrogensulfit}$$

derart verzögert, daß während des Absaugens der freien schwefligen Säure (15 min genügen hierfür) praktisch keine gebundene schweflige Säure in Freiheit gesetzt wird (Tab. 4).

Diese Methode ist für die allgemeine Weinanalyse gut brauchbare. Für besondere Untersuchungen[2] fehlte uns jedoch eine noch exaktere Methode, die auch kleine SO_2-Mengen zuverlässig zu erfassen gestattet. Hier bot sich die *Polarographie* an.

Die schweflige Säure gibt nach Kolthoff[12] in alkalischem und neutralem Medium keine Stufe, eine solche erscheint jedoch in saurem Milieu. Dabei werden nach Gosman[3] nur die undissoziierten Moleküle der schwefligen Säure an der Kathode zu Dithionit reduziert gemäß der Gleichung $2\,SO_2 + 2e \rightarrow S_2O_4^{2-}$.

Die Stufenhöhe soll nach Gosman[3] von der Wasserstoffionenkonzentration unabhängig sein. Kolthoff[12] fand hingegen, daß nicht nur das Reduktionspotential, sondern auch der Diffusionsstrom p_H-abhängig ist und mit sinkendem p_H wächst. Nur in stark saurem Medium (p_H 0—1) wird die gesamte schweflige Säure reduziert, da nur in diesem p_H-Bereich das tautomere Verhältnis



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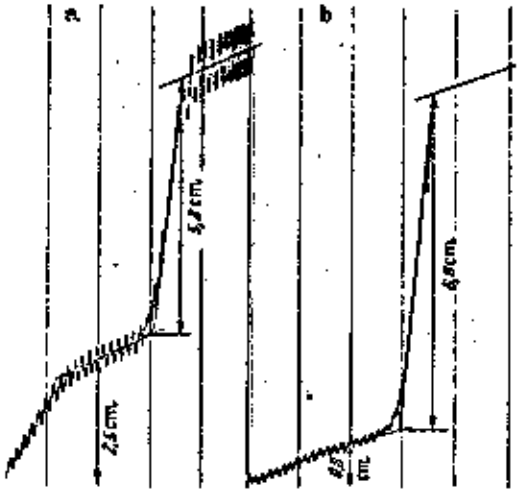


Abb. 1. Einfluß von Sauerstoff bei der polarographischen Bestimmung der schwefligen Säure in einem Wein mit 15 mg/l freier schwefliger Säure. Empfindlichkeit E 1/20. *a* vor, *b* nach Entfernung des Sauerstoffs

Heyrovský[7] und Salvarezza[22] führten im Wein polarographisch qualitative SO_2-Bestimmungen durch, ohne jedoch auf eine quantitative Bestimmung einzugehen.

Voraussetzung für die quantitative Bestimmung ist die Befreiung der Untersuchungslösung von Sauerstoff, da die Diffusionsströme des Sauerstoffs den der SO_2 beeinflussen. Mit wachsendem Sauerstoffgehalt nimmt die Sauerstoffstufe zu, die SO_2-Stufe ab (Abb. 1).

Bei der Vertreibung des Sauerstoffs durch Stickstoff empfiehlt es sich, reproduzierbar zu arbeiten, damit Einflüsse, die durch die Behandlung bedingt sind, in die Eichkurve eingehen. Zum Entfernen des Sauerstoffs genügt ein 15 min langes Durchleiten von Stickstoff (200 Blasen/min, gezählt in einer vorgeschalteten Waschflasche). Dabei werden nur sehr geringe Mengen SO_2 (maximal 1 mg/l) mitgerissen. Bei geringerer Zeitdauer und Strömungsgeschwindigkeit ist Sauerstoff polarographisch noch nachweisbar. Von einer größeren Strömungsgeschwindigkeit haben wir abgesehen, da die Zählung der Gasblasen dann nicht mehr sicher möglich war.

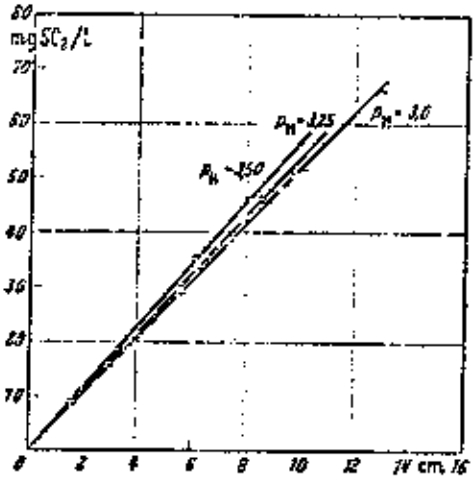


Abb. 2. Eichkurven zur polarographischen Bestimmung der freien schwefligen Säure in Wein bei pH 3,0—3,25—3,5 Empfindlichkeit $E^1/_{10}$

Unter Berücksichtigung der Entlüftungsbedingungen ergaben sich an Modellweinen* wechselnder SO_2-Konzentration (pH 3,0—3,25—3,5) Eichkurven mit geradlinigem Verlauf (Abb. 2). Die Abweichungen sind gering und betragen maximal 1 mg/l SO_2.

Wir haben dann an Weinen mit wechselndem Gehalt an freier schwefliger Säure Parallelbestimmungen polarographisch und nach der modifizierten Ripperschen Methode durchgeführt. Wie Tab. 5 zeigt, ist nur bis zu einem Gehalt von etwa 10 mg freier schwefliger Säure im Liter eine befriedigende Übereinstimmung festzustellen. Die Abweichungen nehmen darüber mit wachsender Konzentration unregelmäßig zu. Jodometrisch wird stets mehr freie schweflige Säure gefunden als polarographisch, möglicherweise dadurch bedingt, daß beim Ansäuern oder bei der Titration „labil-gebundene" schweflige Säure in Freiheit gesetzt und miterfaßt wird. Hierbei kann sich sowohl der Einfluß der Verdünnung als auch der pH-Verschiebung geltend machen.

* 0 g Weinsäure, 9 g Glycerin, 1 g Kaliumbiphosphat, 1 g Ammoniumsulfat, 0,5 g Natriumchlorid und 90 ml Äthanol werden zu 1 l gelöst; der gewünschte pH-Wert wird mit n Natronlauge elektrometrisch eingestellt.

Eine Abspaltung vo
Säure tritt nicht ein. I

Tabelle 5. *Vergleich der pola*
zur Bestimm

Wein Nr.	freie schweflige S	
	jodometrisch	polarographisch
1	1,2	1,3
2	1,9	2,0
3	3,7	3,8
4	6,8	6,5
5	8,7	8,5
6	9,9	10,2
7	11,8	12,5
8	21,1	19,0

und polarographisch pra
Säure erfaßbar. Hingeg
koordinativ angelagerte
tes Hydrogensulfit zwar
jodometrisch, nicht aber
polarographisch erfaßt
(Tab. 6).

Die polarographische
Methode ist daher als
die exaktere zur Bestim-
mung der freien schwef-
ligen Säure anzusehen.
Da man im Wein neben
der „gebundenen" und
der „freien" noch „labil
oder koordinativ gebun-
dene" schweflige Säure
in Zukunft unter der fre

1. Bestimm

In den Destillierkolben
50 ml dest. Wasser, in die V
40 ml dest. Wasser. Man er
der Vorlage keine Luftblas
Den Tropftrichter spült m
Wenn der Rückstand im D
Destillation ab und spült

...e ...timmung ist die Befreiung der ...ie Diffusionsströme des Sauer... wachsendem Sauerstoffgehalt nimmt ...e ab (Abb. 1).

...ss durch Stickstoff empfiehlt es sich, ...Einflüsse, die durch die Behandlung bedingt sind, in die Eichkurve eingehen. Zum Entfernen des Sauerstoffs genügt ein 15 min langes Durchleiten von Stickstoff (200 Blasen/min, gezählt in einer vorgeschalteten Waschflasche). Dabei werden nur sehr geringe Mengen SO_2 (maximal 1 mg/l) mitgerissen. Bei geringerer Zeitdauer und Strömungsgeschwindigkeit ist Sauerstoff polarographisch noch nachweisbar. Von einer größeren Strömungsgeschwindigkeit haben wir abgesehen, da die Zählung der Gasblasen dann nicht mehr sicher möglich war.

Unter Berücksichtigung der Entlüftungsbedingungen erwechselnder SO_2-Konzentration g... ...igem Verlauf (Abb. 2). Die ...gen ...aximal 1 mg/l SO_2.

...chseindem Gehalt an freier schwef... ...larographisch und nach der modi... ...chgeführt. Wie Tab. 5 zeigt, ist ... 10 mg freier schwefliger Säure ...stimmung festzustellen. Die Ab... ...achsender Konzentration unregel... ...mehr freie schweflige Säure ge... ...cherweise dadurch bedingt, daß ...on „labil-gebundene" schweflige ...erfaßt wird. Hierbei kann sich ...g als auch der p_H-Verschiebung

...iumbiphosphat, 1 g Ammoniumsulfat, ...rden zu 1 l gelöst; der gewünschte p_H... ...h eingestellt.

Eine Abspaltung von an Aldehydgruppen gebundener schwefliger Säure tritt nicht ein. In glucoschaltiger Modellösung sind jodometrisch

Tabelle 5. *Vergleich der polarographischen mit der modifizierten Ripperschen Methode zur Bestimmung der freien schwefligen Säure in Wein*

Wein Nr.	freie schweflige Säure in mg/l			Wein Nr.	freie schweflige Säure in mg/l		
	jodometrisch	polarographisch	Differenz		jodometrisch	polarographisch	Differenz
1	1,2	1,3	+ 0,1	9	23,0	16,8	— 6,2
2	1,9	2,0	+ 0,1	10	25,7	21,5	— 4,2
3	3,7	3,8	+ 0,1	11	32,5	26,8	— 5,7
4	6,8	6,5	— 0,3	12	39,0	32,0	— 7,0
5	8,7	8,5	— 0,2	13	47,5	36,0	—11,5
6	9,9	10,2	+ 0,3	14	54,0	43,0	—11,0
7	11,8	12,5	+ 0,7	15	61,3	51,5	— 9,8
8	21,1	19,0	— 2,1				

und polarographisch praktisch die gleichen Mengen an freier schwefliger Säure erfaßbar. Hingegen wird an Stickstoff, z.B. von Aminosäuren, koordinativ angelagertes Hydrogensulfit zwar jodometrisch, nicht aber polarographisch erfaßt (Tab. 6).

Tabelle 6. *Der Einfluß von Glucose und von Aminosäuren bei der jodometrischen und polarographischen SO_2-Bestimmung in Modellweinen*

Art und Menge des Zusatzes	freie schweflige Säure in mg/l		
	jodometrisch bestimmt	polarographisch bestimmt	Differenz
20 g/l Glucose	7,0	6,5	—0,5
20 g/l Glucose	24,3	24,2	—0,1
20 g/l Glucose	65,1	64,5	—0,6
50 mg/l Glycin	49,8	47,0	—2,8
50 mg/l Glutaminsäure	54,8	53,0	—1,8

Die polarographische Methode ist daher als die exaktere zur Bestimmung der freien schwefligen Säure anzusehen. Da man im Wein neben der „gebundenen" und der „freien" noch „labil oder koordinativ gebundene" schweflige Säure vorliegen hat, muß einmal geklärt werden, was in Zukunft unter der freien „schwefligen Säure" verstanden werden soll.

Arbeitsvorschriften

1. Bestimmung der gesamten schwefligen Säure

In den Destillierkolben (Abb. 3) gibt man 5 ml 85%ige Phosphorsäure und 50 ml dest. Wasser, in die Vorlage 10 ml Citronensäure-Phosphatpuffer p_H 3,4 und 40 ml dest. Wasser. Man erhitzt den Destillierkolben zum Sieden und läßt, wenn in der Vorlage keine Luftblasen mehr entweichen, 25 ml Wein in den Kolben tropfen. Den Tropftrichter spült man zweimal mit je 10 ml luftfreiem dest. Wasser nach. Wenn der Rückstand im Destillierkolben noch etwa 50 ml beträgt, bricht man die Destillation ab und spült den Kühler mit dest. Wasser aus. Man versetzt das

Destillat mit 1 ml 1%iger Stärkelösung und titriert mit 0,01 n Jodlösung bis zum Umschlag, setzt 1 g Natriumhydrogencarbonat zu und titriert weiter bis zur schwachen Blaufärbung, die 30 sec bestehen bleiben muß. Verbrauch: a ml.

Es ist erforderlich, täglich einen Blindversuch durchzuführen. Hierzu fügt man zu 10 ml Pufferlösung 100 ml dest. Wasser, 1 ml 1%ige Stärkelösung, 1 g Natriumhydrogencarbonat und titriert wie oben. Verbrauch: b ml.

Der Gehalt an gesamter schwefliger Säure berechnet sich dann wie folgt: mg/l $SO_2 = 12{,}8\,(a-b)$.

2. Bestimmung der freien schwefligen Säure

a) Modifizierte Ripper-Methode. In einem 100 ml-Erlenmeyer-Kolben versetzt man 25 ml Wein mit 10 ml n Salzsäure und 1 ml 1%iger Stärkelösung und titriert mit 0,01 n Jodlösung bis zur Blaufärbung, die 30 sec bestehen bleiben muß. Verbrauch: a ml.

Zur Bestimmung des Blindwertes gibt man in einen 250 ml fassenden Rundkolben 25 ml Wein und 10 ml n Salzsäure und verschließt

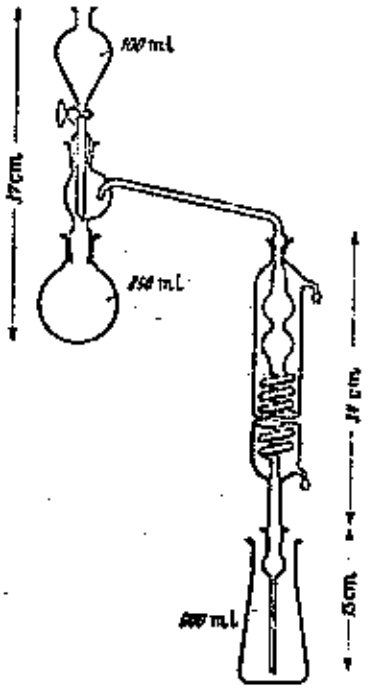


Abb. 3. Apparatur zur Bestimmung der gesamten schwefligen Säure

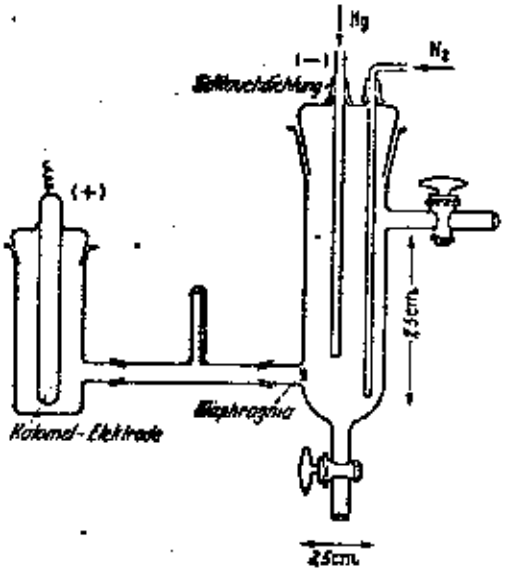


Abb. 4. Gefäß zur polarographischen Bestimmung von freiem schwefliger Säure

mit einem doppelt durchbohrten Gummistopfen. Durch die eine Bohrung führt bis zum Kolbenboden eine Capillare, die mit einer Kohlensäureflasche verbunden ist, durch die andere ein gewinkeltes Rohr, das zur Wasserstrahlpumpe führt. Man saugt 15 min lang ab, fügt einige Tropfen Stärkelösung hinzu und titriert mit 0,01 n Jodlösung, bis die Blaufärbung 30 sec bestehen bleibt (b ml). Der Gehalt an freier schwefliger Säure berechnet sich dann zu 12,8 $(a-b)$ mg/l.

b) Polarographische Methode. Das Polarographiergefäß (Abb. 4), etwa 30 ml fassend, ist mit einem Schliffstopfen mit zwei Öffnungen versehen; durch die eine Öffnung führt die Quecksilbertropfelektrode, durch die andere, ebenfalls luftdicht verschlossen, ein zur Capillare ausgezogenes Einleitungsrohr. Der seitlich angebrachte

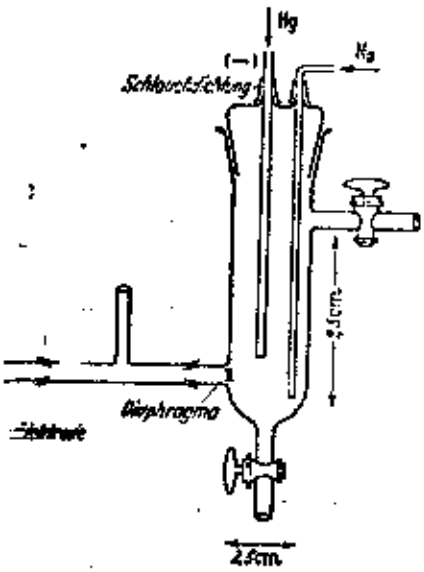
...d t...ert mit 0,01 n Jodlösung bis zum ...honat zu und titriert weiter bis zur ... bleiben muß. Verbrauch: a ml.

...rsuch durchzuführen. Hierzu fügt man ... 1 ml 1%ige Stärkelösung, 1 g Natrium... ...brauch: b ml.

...Säure berechnet sich dann wie folgt:

... schwefligen Säure

...m 100 ml-Erlenmeyer-Kolben versetzt ... 1 ml 1%iger Stärkelösung und titriert ... Jodlösung bis zur Blaufärbung, die ...stehen bleiben muß. Verbrauch: a ml. ...estimmung des Blindwertes gibt man ... 250 ml fassenden Rundkolben 25 ml ...d 10 ml n Salzsäure und verschließt



...fäß zur polarographischen Bestimmung von freier schwefliger Säure

...fen. Durch die eine Bohrung führt bis ...er Kohlensäureflasche verbunden ist, ...r Wasserstrahlpumpe führt. Man saugt ...ung hinzu und titriert mit 0,01 n Jod... ... bleibt (b ml). Der Gehalt an freier ...8 (a—b) mg/l.

...phiergefäß (Abb. 4), etwa 30 ml fassend, ...gen versehen; durch die eine Öffnung ... die andere, ebenfalls luftdicht ver... ...ltungsrohr. Der seitlich angebrachte

Schliffhahn wird nach Vertreiben des Sauerstoffs geschlossen. Seitlich unten ist ein Diaphragma eingeschmolzen, durch welches das Polarographiergefäß mit der Kalomelelektrode in gesättigter Kaliumchloridlösung in Verbindung steht. Zur Entleerung des Gefäßes dient ein unten angeschmolzenes Rohr mit Schliffhahn.

Den zur Vertreibung des Sauerstoffs erforderlichen reinen Stickstoff erhält man, indem man doppelt gereinigten Stickstoff (lieferbar durch Ges. für Lindes Eismaschinen, Hannover-Herrenhausen) durch einen auf etwa 150°C elektrisch geheizten, mit BTS-Katalysator der BASF[23] gefüllten Kupferturm schickt. Zur Überwachung der Strömungsgeschwindigkeit läßt man den Stickstoff anschließend eine Waschflasche mit alkalischer Pyrogallollösung passieren.

Alle Schlauchverbindungen bestehen aus diffusionsfestem Butylschlauch.

Man füllt das Polarographiergefäß, in einem Wasserbad auf 20°C temperiert, mit der Untersuchungslösung, leitet 15 min lang sauerstoffreien Stickstoff (200 Blasen/min) durch und polarographiert dann bei kathodisch polarisierter Quecksilbertropfelektrode von —0,3 bis —0,8 V. Der zu vermessende Diffusionsstrom ist der Konzentration an freier schwefliger Säure proportional und ist, unter Berücksichtigung des p_H-Wertes, an Hand der Eichkurve auszuwerten.

Zusammenfassung

Ein Vergleich der Methoden von Weinmann u. Deibner zur Bestimmung der gesamten schwefligen Säure zeigte, daß die letzte Methode zu exakteren Werten führt, vor allem dadurch bedingt, daß das überdestillierte Schwefeldioxyd in einer sauren gekühlten Vorlage aufgefangen wird. Ein Nachteil der Methode ist, daß bei der Titration der Vorlage mit Jodlösung der p_H-Wert mehrfach geändert werden muß. Es gelang uns, diesen Mangel ohne Einbuße an Analysengenauigkeit dadurch zu beseitigen, daß wir als Auffanglösung ein Puffergemisch (p_H 3,5) benutzen und nicht in saurem, sondern in schwach alkalischem Milieu mit Jodlösung titrieren. In dieser Form kann das Verfahren zur Bestimmung der gesamten schwefligen Säure im Wein empfohlen werden.

Zur genauen Bestimmung der freien schwefligen Säure arbeiteten wir ein polarographisches Verfahren aus, bei dem im Gegensatz zur jodometrischen Methode die koordinativ gebundene schweflige Säure nicht miterfaßt wird. Der verhältnismäßig große apparative Aufwand bei der Polarographie spricht jedoch gegen die Anwendung dieses Verfahrens in der routinemäßigen Weinanalyse. Es wird deshalb eine vereinfachte, aber recht genaue jodometrische Methode zur Bestimmung der freien schwefligen Säure vorgestellt, die geeignet ist, Aufnahme in die neue „Allgemeine Verwaltungsvorschrift für die Untersuchung von Wein und ähnlichen alkoholischen Erzeugnissen sowie von Fruchtsäften*" zu finden, die an die Stelle der alten Amtlichen Anweisung getreten ist.

* Allgemeine Verwaltungsvorschrift (Beilage zum Bundesanzeiger Nr. 86 vom 5. Mai 1960).

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Aus dem Universitäts-Institut für Lebensmittelchemie Frankfurt a. M. und dem Institut für Obst- und Gemüseverwertung Geisenheim/Rhg.

Die polarographische Bestimmung von l-Ascorbinsäure im Wein*

Von

W. Diemair, J. Koch und D. Hess**

Mit 1 Textabbildung

(Eingegangen am 14. Juli 1960)

Das klassische Verfahren zur Bestimmung von Ascorbinsäure beruht auf der Titration mit 2,6-Dichlorphenol-indophenol nach Tillmans[23]. Diese Methode mit all ihren Modifikationen[2,14,15] ist beim Wein infolge der störenden Wirkung der freien schwefligen Säure nicht anwendbar. Auch die Titration mit N-Bromsuccin-

* Für die Untersuchungen wurden Mittel des Bundesernährungs-Ministeriums zur Verfügung gestellt, wofür auch an dieser Stelle bestens gedankt sei.

** Die Arbeit stellt einen Ausschnitt dar aus der Dissertation von D. Hess: Über den Einfluß von schwefliger Säure und l-Ascorbinsäure bei der Weinbereitung (Universität Frankfurt am Main 1960).

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Received 23 February 1966

SUMMARY

Hygienic Significance of the Use of Tetracyclines in Poultry Farming

V.E. Shneiderman

The paper deals with certain problems connected with the feeding of poultry with high doses of tetracycline. The findings confirmed the ability of chlortetracycline (CTC) to accumulate in the organs and tissues of ducks due to its long-term use. CTC proved to be present in the organs and tissues on the 7th day after ceasing feeding. The boiling of ducks' organs and tissues for a period of 90 minutes did not produce complete inactivation of the traces of CTC present in the raw products. A definite relationship was established between the relatively high retention of CTC in the organs and tissues and the amount of water present in the product after its boiling for one hour and a half. It is suggested that tissue proteins may have a protecting effect on CTC during the thermal treatment.

UDC 616-006.4-02:664.8.035-092.9

EFFECT OF CERTAIN FOOD PRESERVATIVES ON THE DEVELOPMENT OF MALIGNANT PROCESSES IN MICE
(Vliyanie nekotorykh pishchevykh konservantov na razvitie zlokachestvennogo protsessa u myshei)

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Institute of Nutrition of the Academy of Medical Sciences of the USSR, Moscow

There has been a sharp increase in the use of various chemicals in the food industry. We possess only scant knowledge of the possible toxic

and carcinogenic effects of these additives to food products when they penetrate the human organism for prolonged periods of time.

Sodium bisulfite is used in the treatment of fruit, vegetables, and small berries for the prevention of mold. Its permissible residual concentration is up to 300 mg/1 kg. Benzoic acid is used mainly in the treatment of the same products and their preserves in order to prevent fermentation; its concentration does not exceed 0.2% of the product by weight. Both preservatives have been in use for about half a century.

Until recently, sodium bisulfite and benzoic acid were regarded as relatively harmless noncarcinogenic substances (Kiechebusch and Lang; Hosino; Lockett and Natoff). Recently, however, several authors have demonstrated some unfavorable effects of these substances on animals (Fitzhugh et al.; Morgan); the use of benzoic acid has therefore been prohibited in certain countries (France), and the use of sulfur compounds is under discussion at the World Health Organization.

Sorbic acid and nisin are new preservatives. Sorbic acid, originally isolated from rowan, proved to be a very efficient preservative. This is a polyunsaturated fatty acid with six carbon atoms. According to the literature (Lang), addition of 5% sorbic acid to the ration of rats did not cause any pathological changes, while addition of 8% of the same substance (Deuel et al.) increased the weight of the liver. We did not find any reports of carcinogenic properties of sorbic acid in the literature.

The antibiotic nisin was originally isolated from acid dairy products. This is a product of vital activity of lactic streptococci and has no medical value, but it lowers the resistance of spores of thermally stable bacteria to heating, so that its addition to preserves permits lower sterilization temperatures, thus improving the food value of the preserves. There are no data on carcinogenic properties of nisin.

Studies of the toxicity of benzoic acid in mice in concentrations used for human food products gave results as follows: the survival time of the mice was the lowest recorded in this series of experiments; the gain in weight was the lowest; there was impairment in the detoxifying function of the liver, and the susceptibility of the animals to the unfavorable results of cold and centrifugation (Ignat'ev) was highest.

The combined use of benzoic acid and sodium bisulfite also resulted in a markedly reduced survival time of mice, a decrease in their consumption of water and food, lag in weight gain and lowering of resistance to physical loads and starvation. There was also a hypertrophy of certain organs, especially the liver.

Sorbic acid proved to be nontoxic. Its inclusion in the animals' rations resulted in a higher survival time, more rapid increase in weight, greater consumption of food, greater phagocytic activity in comparison to the control animals, etc.

Nisin possessed the highest toxicity, alone or when mixed with sorbic acid. At the same time there was a certain antagonism in the effects of sorbic acid and nisin. On the whole, all the indices mentioned above were nearer to normal values than in the case of nisin alone, probably owing to the favorable effect of sorbic acid.

In all experiments in which mice received nisin alone or nisin in combination with sorbic acid their gain in weight was greater. In a certain series of experiments the animals were fat, the body weight exceeding 40 g in 44% of the experimental animals.

There were also cases of adiposity among animals which received sodium bisulfite in combination with benzoic acid, mainly in females (Ignat'ev).

In our experiments we used 360 mice of both sexes. They were divided into five groups, four experimental and one control. All animals received identical rations, first of natural products (according to standards established by the Academy of Medical Sciences of the USSR), later briquetted food. Experimental mice received an addition of the preservatives being investigated, 0.2% benzoic acid, 0.01% nisin, 0.2% sorbic acid or 0.4% sodium bisulfite (percentages by weight of the total rations). The doses of preservatives were the same as those used in foodstuffs for human consumption. Special groups of mice were used for studies of the combined effects of the mixtures of sodium bisulfite + benzoic acid and sorbic acid + nisin. For three months the experimental animals received these preservatives in their food in the amounts indicated above, and this was followed by intraperitoneal inoculation with Ehrlich's ascites carcinoma to the experimental and control animals.

As is known, the introduction of 50—80 million tumor cells into the abdominal cavity of mice causes the development of Ehrlich's ascites carcinoma in 100% of the experimental animals, and their survival time after the inoculation is 12 to 17 days. According to our preliminary observations, the introduction of a smaller number of tumor cells (1.2—1.25 million cells) led to the development of tumors in 70—100% of mice, and their survival after the inoculation was somewhat prolonged (21—30 days).

This was the method of inoculation used by us in order to provide a more distinct expression of the effect of the preservative on the development of ascites carcinoma. The mice were weighed before the experiment and their abdominal volume was measured. After this, measurements were performed at intervals of 4 days, beginning with the day of transplantation of the tumor and until the animals died. The observation period was 53—64 days, and then all the surviving mice were killed and autopsied. All the mice (those which died spontaneously or were killed after the termination of the experiment) were measured for the amount of ascitic fluid and its blood content. The latter was designated in arbitrary units as follows: weak pink color — 1 unit, red color — 2 units, and dark red color — 3 units. The results were statistically analyzed and the P value was determined by comparing the indices of a given group against the control.

The results are listed in Tables 1 and 2.

It will be seen from Tables 1 and 2 that the successful inoculation with tumors was more frequent in mice which received sodium bisulfite and benzoic acid on their own and less so in mice which received both substances together. The shortest survival time after transplantation of the tumor was found in mice which received sodium bisulfite and benzoic acid on their own. The volume of ascitic fluid was greatest in mice which received sodium bisulfite. The percentage of mice which developed tumors among those receiving sorbic acid on their own or together with nisin did not differ from the percentage in control mice which did not receive additions of preservatives. The survival time of these mice after the transplantation was slightly shorter than in the control mice, and the volume of ascitic fluid in mice receiving sorbic acid was the same as in the controls; it

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TABLE 1. Results of
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Sorbic acid + nisin
Control

TABLE 2. Survival
its blood content

Preservative
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Sodium bisulfite + benzoic acid
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TABLE 1. Results of inoculation of Ehrlich's ascites carcinoma in mice receiving food preservatives in ... rations

Preservative	Number of mice	Observation period, days	Number of tumors		P
			abs	%	
Sodium bisulfite	97	53	61	62.9 ± 6.5	< 0.001
Benzoic acid	90	53	62	68.8 ± 5.1	< 0.001
Sodium bisulfite + benzoic acid	22	66	9	40.9 ± 10.1	< 0.01
Sorbic acid	58	66	17	29.3 ± 6.0	
Sorbic acid + nisin	49	66	16	30.6 ± 6.1	
Control	49	66	16	32.6 ± 6.5	

TABLE 2. Survival time of mice with Ehrlich's ascites carcinoma, the mean volume of ascitic fluid and its blood content

Preservative	Time at which mice began to die, days	Mean volume of ascitic fluid, ml		Degree of hemorrhagic properties, arbitrary units
		M ± m	P	
Sodium bisulfite	12	6.5 ± 0.418	< 0.002	2.7
Benzoic acid	15	5.03 ± 0.330	< 0.001	2.6
Sodium bisulfite + benzoic acid	24	3.4 ± 0.476	0.001	2.5
Sorbic acid	21	5.08 ± 0.626	> 5	2.8
Sorbic acid + nisin	21	4.09 ± 0.446	> 5	3.0
Control	24	5.03 ± 0.86	-	2.5

Thus, the addition of sodium bisulfite and benzoic acid to the rations of the mice facilitated a more intensive development of Ehrlich's ascites carcinoma.

Studies were also made of the effect of body weight on the incidence of Ehrlich's ascites carcinoma in mice which received preservatives in the food rations. As already mentioned, the addition of sorbic acid and nisin, and in isolated cases also sodium bisulfite with benzoic acid, to rations of experimental mice led to a tendency towards a pronounced increase in body weight of the animals. The body weight was found to exceed 40 g in 40% of mice and in isolated cases it reached 50 – 60 g.

According to the literature, adiposity of mice caused by aurothioglucose is accompanied by a more intensive development of malignant tumors. For instance, according to Wexler spontaneous tumors of the mammary gland occurred earlier and more frequently in mice of strain A with adiposity due to aurothioglucose.

The same results were also attained with respect to the development of spontaneous hepatoma in male mice of the strain C_3H.

In our experiments, 90 mice were given either a mixture of sorbic acid + nisin or a mixture of sodium bisulfite + benzoic acid in doses indicated above after a period of 8 months. Adiposity incidence (percentages) differed according to the sex (occurring more frequently in females), and therefore our experiment included both males and females. The experiments included both adipose mice and mice of ordinary weight. For this purpose, the mice were divided into 9 groups, 10 animals in each group (males and females or ordinary weight, males and females of excessive weight, and the control group).

The experiment was designed to provide an answer to the question of whether the addition of the preservatives to the rations facilitated a more intensive development of the malignant tumor or whether this effect was secondary, coming as a corollary of adiposity due to the preservative used in the experiment.

All mice were given intraperitoneal doses of 1.2 – 1.25 million cells of Ehrlich's ascites carcinoma. We did not succeed in observing any difference in the development of the tumors in mice of the different groups, yet the survival time of the animals and the volume of the ascitic fluid were not identical. Thus, the survival time after transplantation of the tumor was somewhat shorter (11 – 13 days) in mice treated with the mixture of sodium bisulfite and benzoic acid, in comparison to the other groups, while the volume of their ascitic fluid was somewhat larger (9 – 11 ml), as against 15 days and 7 – 8 ml, respectively, in the control. The difference was statistically insignificant, but the trend described a higher malignancy of Ehrlich's ascites carcinoma in mice treated with sodium bisulfite in combination with benzoic acid. This effect is obviously unrelated to the capacity of these preservatives for causing adiposity.

The time at which the mice began to die, indicating the intensity of development of the tumor, the volume of ascitic fluid and its blood content are shown in Table 2.

Conclusions

1. When Ehrlich's ascites carciroma was transplanted to mice which received the older preservatives sodium bisulfite and benzoic acid with their food, the percentage of tumors which developed was the greatest. The survival time of these mice was the shortest, while the volume of ascitic fluid was the largest. These data indicate a higher malignancy of the tumors which developed in comparison to control mice.

2. The percentage of mice which developed tumors among those treated with sorbic acid and nisin, as well as the degree of malignancy of the tumors, was the same as in the control mice.

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SUMMARY

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Received 8 July 1964

SUMMARY

Effect of Certain Food Conservants on the Development of Malignant Processes in Mice

A.A. Dinerman, A.D. Ignatiev

In tests performed over 365 mongrel mice of both sex was studied the effect of certain food conservants (sodium bisulfite, benzoic acid, sorbic acid, nizine) on the development of ascetic Erlich cancer. Those mice, that received sodium bisulfite and benzoic acid, presented a higher percentage of developed tumours, a shorter life length and, a larger amount of ascetic fluid than the mice, receiving sorbic acid and nizine as well as the control animals. The fattening caused by the conservants had no effect on the development of the malignant processes.

J. Dental Res. 26 (1947)

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THE INFLUENCE OF SODIUM BISULPHITE ON ACID PRODUCTION IN SALIVA[1]

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The rôle of acids in tooth decalcification (1) is now generally accepted. As a result recent attempts at dental caries control have been in the direction of decreasing the rate of acid formation in the mouth, and/or increasing the rate of acid neutralization in regions associated with the caries susceptible portions of the teeth (2). Since the latter has been considered difficult to achieve, methods and means of decreasing the rate of acid formation in the mouth have been the aim of most of the recent investigations in this field. Controlled diet, oral hygiene, and interference with the enzyme or coenzyme systems necessary for carbohydrate degradation have each presented distinct possibilities. Chemical inhibitors of enzyme systems has been studied by Stephan (3) using urea, Zander (4) using ammoniacal silver nitrate, Dean, Jay, Arnold, and Elvove (5) using the fluoride ion, and most recently Burrill, Calandra, Tilden, and Fosdick (6) using synthetic vitamin K. These investigators have all succeeded in reducing significantly the rate of acid formation in the saliva.

The work of Burrill, Calandra, Tilden, and Fosdick (6) was particularly interesting because a 2-methyl 1,4-napthoquinone-sodium bisulphite addition compound was employed. The results were attributed to the quinone which was regarded by these investigators as the active ingredient, the sodium bisulphite being used for the purpose of rendering the 2-methyl 1,4-napthoquinone water soluble so it would be more rapidly absorbed (7).

Sodium bisulphite, however, has had some use in medicine as an antiseptic and an antifermentation agent (8). The possible effect of sodium bisulphite on the results obtained by Burrill, Calandra, Tilden, and Fosdick stimulated investigation in regard to the action of this salt on the process of acid production in saliva. Since Burrill and his associates used less than 0.75 mg. sodium bisulphite in their study with 2-methyl 1,4-napthoquinone (6), a series of preliminary tests were made using varying concentrations of sodium bisulphite. It was found that values ranging from 2.5 mg. to 10 mg. showed a definite interference with the acid formation, the most efficient concentration ranging from

[1] These studies were supported by grants from Gelatin Products Company, and the American Dried Milk Institute. Received for publication December 2, 1946.

We wish to acknowledge the assitance of Miss Thelma Skinner in making the dental caries activity determinations.

[2] Associate Professor of Biochemistry, Medical College, University of Alabama, Birmingham, Alabama.

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5.0 mg. to 7.5 mg., quantities much above those used by Burrill, Calandra, Tilden and Fosdick. No significant effect was observed with values below 1.0 mg. indicating, therefore, that the presence of sodium bisulphite in the form of an addition compound probably did not influence the results obtained by Burrill and his associates.

MATERIALS AND METHODS

Selection of Patients: Fifteen patients were selected from the personnel of the Nutrition Clinic, the Jefferson-Hillman Hospital of Birmingham, Alabama, and from patients referred to the Dental Department of the Nutrition Clinic by local practitioners. Each of these patients had been studied in the Clinic for the 2 preceding years and records of their dental caries activity during this period of time were available. The saliva of 11 of them had been consistently positive, 3 had been negative and one plus/minus. The patients, who appeared to be in good health, ranged in age from 13 years to 38 years, and included 13 females, one of whom was colored, and 2 white males.

Method of Study: Forty cc. of paraffin stimulated saliva were collected from each patient between the hours of 8:00 and 10:00 in the morning. Three different methods were used to test the saliva samples for dental caries activity. They were 1, the method of Fosdick, Hansen, and Epple (9), based on the difference in the amount of soluble calcium in saliva samples before and after incubation, 2, the method of Hadley (10), which determines the number of *Lactobacillus acidophilus* per cc. of saliva, and 3, the method of Dreizen, Mann, Cline, and Spies (11), which measures the buffer capacity of the saliva. One cc. of saliva was used for the bacteriological test, 5 cc. for the buffer capacity test, and 30 cc. for the calcium test.

The 30 cc. of saliva used for the calcium test were divided into 3 parts, each containing 10 cc. One part was immediately tested for calcium, the second part was incubated in the presence of tricalcium phosphate and dextrose for 4 hours at 37° C. in a shaker apparatus suspended in a water bath, and 7.5 mg. of crystalline sodium bisulphite were added to the third part which was also incubated under the same conditions. Following the incubation period the 2 samples of saliva so treated were tested for soluble calcium by titrating against 0.01 N $KMnO_4$ using the method of Fosdick, Hansen, and Epple (9).

To determine the effect of the salt in saliva treated in vivo, 7.5 mg. of crystalline sodium bisulphite were incorporated in a paraffin block. The patients were permitted to rest from ½ to 1 hour after the collection of the first sample, following which 30 cc. of saliva were again collected, using the bisulphite-containing paraffin block as a secretory stimulant. The subjects were cautioned not to swallow any of this saliva during the period of collection. The 3 different laboratory tests for caries activity were then repeated on the saliva thus obtained.

OBSERVATIONS

No significant differences were observed in the buffering capacity of the saliva in any of the 15 patients when the saliva samples containing sodium bisulphite

were compared with those free of bisulphite. Neither were there any appreciable differences in the lactobacillus counts in the bisulphite-treated and untreated salivas.

The results obtained with the chemical tests, however, showed a striking difference. As may be seen from Table I, the production of acid in the saliva was interfered with in every case in which sodium bisulphite was added. This phenomenon was also manifested in the salivas collected by stimulation with the sodium bisulphite-containing paraffin blocks. Regardless of the dental caries activity of the untreated saliva, all but one of the sodium bisulphite samples gave a negative reaction when tested. In the one instance in which a

TABLE I

*Results of chemical test for dental caries activity on untreated saliva and saliva treated with sodium bisulphite in fifteen patients**

PATIENT	UNTREATED SALIVA		SALIVA TREATED IN VITRO†		SALIVA TREATED IN VIVO‡	
	Activity	pH reading after incubation	Activity	pH reading after incubation	Activity	pH reading after incubation
1. T. J. McK.	++++	4.55	—	6.61	—	6.75
2. E. R.	+++	4.71	—	7.00	±	6.55
3. E. S.	+++	4.61	—	6.59	—	6.15
4. E. M.	+++	4.48	—	6.39	—	6.40
5. B. C. C.	+++	4.88	—	6.81	—	6.00
6. T. A. S.	++	5.01	—	7.02	—	7.12
7. M. H.	++	4.51	—	6.30	—	6.70
8. J. C.	++	5.06	—	7.06	—	6.80
9. L. M.	++	5.15	—	7.31	—	6.02
10. A. B. H.	++	4.88	—	6.90	—	6.63
11. S. W.	+	4.95	—	6.90	—	6.00
12. M. F. H.	±	5.82	—	6.90	—	7.08
13. A. L. B.	—	6.51	—	7.00	—	6.80
14. S. D.	—	6.77	—	7.30	—	7.09
15. C. B.	—	6.08	—	6.91	—	6.81

* The chemical test used is that of Fosdick, Hansen, and Epple.
† Saliva samples to which 7.5 mg. sodium bisulphite were added directly.
‡ Saliva stimulated by paraffin containing 7.5 mg. sodium bisulphite.

plus/minus value was obtained, the patient admitted having swallowed some of the sodium bisulphite despite advance instructions not to start mastication of the paraffin block until it had been sufficiently softened by the warmth of the mouth.

To emphasize further the influence of the sodium bisulphite upon the acid production of the saliva of these patients, pH readings were taken of the saliva samples removed from the water bath at the end of the 4-hour period of incubation. A Beckman pH Meter was used to make the determinations. These results are also summarized in Table I where it is noted that in every instance the untreated salivas had a lower pH reading than the bisulphite-treated specimens of the same saliva.

DISCUSSION

The findings obtained with the chemical test for caries activity indicate that the sodium bisulphite exhibited an inhibitory effect on acid production in the saliva at levels above 1.0 mg. In attempting to explain the mechanism of the action of sodium bisulphite, it is obvious that the reduced acid production could not be attributed to dilution as the salt was added in the crystalline form. The action was not a neutralization as the salt is normally acid in reaction, 7.5 mg. dissolved in 10 cc. of distilled water having the pH of 5.1. As the addition of the sodium bisulphite did not change the buffer capacity of the saliva, the decreased acid production could not be accounted for on this basis. The fact that the bacteria persisted in their growth in the presence of sodium bisulphite, indicates that the action is not a germicidal one. Curtis and Scott (12) point out that sulphites and hyposulphites, while maintaining their chemical composition as such, are practically devoid of either germicidal or antiseptic properties. The possibility of the sulphite and bisulphite ions drawing calcium out of solution and thereby producing "false" negatives was investigated, but did not occur with the concentrations used in this study.

The decreased acid production can, however, be interpreted as resulting from an interference with the carbohydrate degradation system in which the *Lactobacillus acidophilus* and the other acidogenic organisms present in the mouth take part. Sodium bisulphite is a reducing agent (8), and this property may conceivably account for the reduced acid formation in the saliva since reducing agents are classified as inhibitors of enzyme action (13). Sulphites,* even in minute amounts, are also known to interfere with the action of ferments (14). They modify the process of fermentation in part by uniting with aldehydes which interferes to a marked degree with the aldehyde serving as a hydrogen acceptor in the development of pyruvic acid, the hydrogen going instead to the formation of glycerol (15). The fixation of glyceraldehyde, which is produced in the primary decomposition of the glucose molecule, would of necessity interfere with the acid production in saliva since the glyceraldehyde is believed to be modified to methyl glyoxal hydrate, the mother substance for all subsequent changes leading to the formation of lactic acid (16). Should interference with co-carboxylase alone have occurred as might have been expected by sulphite splitting of the thiamin moiety, increased acid production instead of the observed decrease would have been the result. These findings can in no way be interpreted as demonstrating any clinical usefulness of this product. Possible clinical applications are now under consideration.

SUMMARY AND CONCLUSIONS

1. The dental caries activity of untreated saliva and of saliva treated with sodium bisulphite of 15 patients was determined by 3 different laboratory tests.

* The significance of the sulphite radical was demonstrated when sodium pyrosulphite was substituted for sodium bisulphite in 7 cases. All cases positive without the pyrosulphite were rendered negative (Poedick, Hansen, and Epple) with the addition of pyrosulphite.

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2. The addition of sodium bisulphite produced a marked decrease in the acid production of the saliva of each patient.

3. The sodium bisulphite apparently acts by interfering with the enzyme or coenzyme systems involved in carbohydrate degradation process. The sulphite radical combines with the aldehydes formed in the breakdown of carbohydrates and prevents the formation of lactic acid.

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Farmakol, Toksikol. (1968)

сукцинатдегидрогеназы в настоящее время трудно объяснимо. Предположение о том, что фтор-ион взаимодействует с SH-группами фермента, не получило подтверждения в наших опытах, поскольку изменения содержания их в крови при остром отравлении животных обнаружить не удалось.

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Выводы

1. При остром отравлении животных фтористым натрием наблюдаются отчетливые нарушения углеводно-фосфорного обмена, выражающиеся в гипергликемии, гиперлактацидемии, увеличении содержания фруктозо-1,6-дифосфата и снижении уровня макроэргов в крови и гликогена в печени.

2. Активность фосфопируватгидратазы в тканях головного мозга, сердечной и скелетной мышц не изменяется, а активность сукцинатдегидрогеназы в мышце сердца несколько угнетается.

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Поступила 28/XII 1966 г.

DISTURBED CARBOHYDRATE-PHOSPHATE METABOLISM IN ACUTE SODIUM FLUORIDE POISONINGS

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In animals poisoned with lethal doses of sodium fluoride one could see a substantial rise in the glucose, lactic acid and fructose-1,6-diphosphate levels with simultaneous drop of the ATP blood content and of glycogen in the liver too. This was not attended by any substantial modification of the phosphopyruvate-dihydratase (enolase) activity in the brain, myocardium and skeletal muscles. The activity of the rabbit's blood serum aldolase slightly increased, while that of succinate-dehydrogenase went down.

УДК 615.777.33-089

МАТЕРИАЛЫ К ТОКСИКОЛОГИИ МЕТАБИСУЛЬФИТА НАТРИЯ

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Сульфиты давно и широко применяются в различных областях промышленности (химическое производство, фотография и т. д.), производстве пищевых продуктов (мясные продукты, сухофрукты, виноде-

лие), а также встречаются при промышленных загрязнениях воздуха и воды. Токсикологические исследования, касающиеся главным образом введения ингаляционно и через рот, дали разноречивые результаты. Кроме того, основное внимание в них было уделено местному, контактному действию сульфитов.

Между тем резорбтивное действие сульфитов весьма интенсивно сказывается при массированной или хронической интоксикации. Целью данной работы является изучение действия сульфитов при парентеральном введении, где проявляется главным образом резорбтивный эффект.

Методика

Исследовали метабисульфит натрия «чистый», серия № 270.

Приготовляли растворы в концентрации от 0,5 до 20%. Действие препарата изучали в острых и хронических опытах при внутривенном и внутрибрюшинном введениях. В острых опытах на мышах регистрировали токсичность (LD_{50}), на кошках — артериальное давление, ЭКГ и дыхание. В хронических опытах на крысах и кроликах изучали влияние препарата на кровь, мочу, вес и поведение животных; затем животных забивали и исследовали патологоанатомически. В опытах на собаках изучали влияние однократного внутривенного введения препарата на биохимический состав и функциональное состояние крови.

Результаты

При определении LD_{50} использовали метод Беренса. Белым мышам весом 18—21 г внутрибрюшинно вводили раствор метабисульфита натрия возрастающих концентраций в объеме 1 мл. LD_{50} оказалась равной 0,91±0,07 г/кг (табл. 1). Гибель животных наступала в течение 30—40 мин.; животные, пережившие час, выжили и в дальнейшем были здоровыми.

Таблица 1

Испытание токсичности метабисульфита натрия на белых мышах

Концентрация растворов (в %)	Доза (в г/кг)	Погибло / Выжило	Накопленные частоты	% смертности
1,0	0,5	0/6	0/13	0
1,5	0,75	1/5	1/7	12,5
2,0	1,0	4/2	5/2	71,5
2,5	1,25	6/0	11/0	100

LD_{50} = 0,91±0,07 г/кг

Особое внимание уделяли изучению влияния возрастающих доз метабисульфита натрия при внутривенном введении. Шести кошкам под эфирным оглушением вводили 0,5—1—2—5 и 10% растворы препарата в дозах соответственно 7,5—15—30—75 и 150 мг/кг с промежутками 20 мин. При этом было найдено, что доза 30 мг/кг дает кратковременное (20—30 сек.) учащение дыхания без заметных изменений артериального давления и ЭКГ. При дозе 75 мг/кг, кроме учащения дыхания, отмечали падение артериального давления в среднем до 60 мм, державшееся 7—8 мин., урежение пульса до 80 в минуту, нарушение проводимости и токсические изменения в миокарде (увеличение *PQ*, понижение вольтажа *QRS*, появление отрицательного зубца *T*). Основные изменения ЭКГ и гемодинамики при этих дозах восстанавливались через 20 мин. Летальный исход обычно наступал при суммарной дозе препарата 300 мг/кг.

Внутривенное введение собакам метабисульфита натрия в дозе 30 мг/кг, оказавшейся максимально переносимой без заметного эффекта, не вызвало существенных изменений в биохимическом составе крови и функциональном состоянии ее элементов. Пробы крови брали до инъекции препарата и через час после нее (табл. 2).

Таблица 2

Влияние однократного внутривенного введения метабисульфита натрия в дозе 30 *мг/кг* на функциональное состояние крови у собак

Исследуемый показатель	Собака А		Собака Б	
	фон	опыт	фон	опыт
Резистентность эритроцитов (в %)	0,38—0,52	0,40—0,52	0,44—0,54	0,44—0,50
Гематокрит (в %)	16	48	—	—
Свертываемость	3 мин. 10 сек.	2 мин. 50 сек.	3 мин. 20 сек.	2 мин. 40 сек.
Лейкоцитарная активность	12/50	15/50	14/50	15/50
Фибриноген	8 мин. 27 сек.	4 мин. 40 сек.	7 мин.	8 мин. 10 сек.
	18 *мг*	15 *мг*	14 *мг*	12 *мг*
Сахар крови (в *мг*%)	79	89	90	95
Билирубин (в *мг*%)	0,47	0,59	0,58	0,8

Эту же дозу использовали и для хронических опытов. В течение 2 недель препарат вводили 3 крысам внутрибрюшинно и 2 кроликам внутривенно. Наибольшие изменения отмечены со стороны красной крови: снижение числа эритроцитов и содержания гемоглобина, особенно к концу 1-й недели затравки. В течение 2-й недели отмечалась тенденция к их повышению, причем одновременно увеличилось число ретикулоцитов. Аналогично изменялся и вес животных. Лейкоцитарная формула колебалась в нормальных пределах. В моче отсутствовали белок и сахар. В месте введения препарата также не найдены какие-либо специфические изменения.

Гистологическое исследование органов животных, забитых после 2-недельной затравки, обнаружило умеренно выраженное вакуольное перерождение и дистрофические изменения в печени. В почках, селезенке, сердце и нервных клетках в области продолговатого мозга изменений не найдено.

Аналогичные, но менее выраженные явления найдены у кроликов.

Обсуждение

Литературные данные о токсичности сульфитов разноречивы. Ряд авторов (Hoffmann и Sonntag; Rost) отметил, что введение через рот собакам сульфитов в дозах 0,5—1,2 г/кг не вызвало тяжелых явлений интоксикации, отмечено лишь солевое, осмотическое действие. В то же время у людей сульфиты в дозах 200—300 мг в ряде случаев вызывают понос, жжение в желудке, головную боль (Lafontaine и Goblet). Диета с содержанием 0,25% сульфитов вызвала у крыс задержку роста, атрофию костного мозга, полиневрит (Fitzhugh и соавторы), в то же время не было отмечено существенных токсических явлений при длительной затравке крыс, кроликов и собак большими дозами сульфитов (Steinhoff и Marquardt).

Данные экспериментов показывают, что сульфиты обладают умеренной токсичностью и быстро инактивируются в организме, на что указывают сроки восстановления сердечной деятельности у кошек и выживания у мышей. Действие SO_2 и сульфитов при введении через рот и ингаляционно на первый план выдвигает локальные явления — раздражение слизистой оболочки дыхательных путей и пищеварительного тракта. В данной работе не наблюдалось раздражения брюшины при внутрибрюшинной затравке крыс.

При обсуждении возможных механизмов резорбтивного действия сульфитов предполагали (Fitzhugh и соавторы), что они разрушают тиамин, однако данные И. В. Сидоренкова и К. В. Слободкиной пока-

зали, что решающее значение имеет угнетение тканевого дыхания путем связывания продуктов углеводно-жирового обмена, а также повреждение тиоловых групп ферментов.

Разноречивы данные о влиянии сульфитов на кровь. И. В. Сидоренков отмечает их анемизирующее действие, Л. С. Митина — увеличение гемоглобина и эритроцитов. Как видно из полученных результатов, разноречивость может быть объяснена моментом наблюдения, так как в первый период выражена анемизация, во второй нарастают репаративные процессы. Можно предполагать, что сульфиты оказывают анемизирующее действие, соединяясь с липопито-полисахаридной частью оболочки эритроцитов и вызывая гемолиз (Г. И. Езриелев). Не было отмечено угнетения гемопоэза, а также угнетающего действия на живые клетки крови — лейкоциты.

При исследовании гемодинамики у кошек при внутривенной затравке отмечено 3 периода: падение артериального давления, брадикардия с нарушением (замедлением) проводимости и период восстановления (или паралича). Можно предполагать, что описанные изменения связаны с нарушением энергетического обмена в активно работающем миокарде, вызванным тем, что сульфиты связывают метаболиты углеводно-жирового обмена.

Судьба сульфитов в организме изучена недостаточно, но имеются указания (Rost и Jürss), что они быстро и почти полностью превращаются в сульфаты.

Выводы

1. Метабисульфит натрия является веществом с умеренной токсичностью и быстро инактивируется в организме.

2. Токсический эффект складывается из контактного раздражения слизистой оболочки и резорбтивного действия, осуществляемого главным образом путем связывания метабисульфитом натрия альдегидо-кетонных звеньев углеводно-жирового обмена.

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Поступила 24/I 1967 г.

MATERIALS CONTRIBUTIVE TO TOXICOLOGY OF SODIUM METABISULFITE

G. I. Ezrieley

Toxicity and resorptive action of sodium metabisulfite with its intravenous and intraperitoneal administration were investigated on experimental animals. In acute tests the preparation was found to become quickly inactivated in the organism with subsequent restoration of hemodynamic disorders produced by it. In chronic tests notice was taken of anematizing action exerted by sodium metabisulfite coupled with a progressive intensification of reparative processes at the end of poisoning. A histological examination of the liver disclosed moderate dystrophic manifestations and vacuolar degeneration.

ten ebenso wie die Bern-Lötschberg-Simplon-Bahn durch den Verkehrsausfall empfindliche Verluste erleiden.

Die Bahn soll folgendermassen geführt werden: von Aosta (583 Meter) das Tal der Doire (Dora Baltea) aufwärts 32 Kilometer bis nach Pré-Saint-Didier (990 Meter), von dort über Courmayeur (1178 Meter) nach dem Südostfuss des Montblancmassives (Entrèves im Winkel der aus dem Val Veni und dem Val Ferret zusammenfliessenden Zweige der Doire) und in 14 500 Meter langem Tunnel ins Tal der Arve nach Chamonix (1039 Meter). Das geologische Prognostikon lautet günstig, da das zu durchfahrende Gebirge aus Granit besteht, der den Erbauern keine unliebsamen Überraschungen bereiten wird wie seinerzeit der Simplontunnel. Die Weiterleitung der Linie hätte nach französischer Auffassung in Richtung Annecy—Aix-les-Bains zu erfolgen unter Umgehung von Genf; die Zuführung von Mailand nach Aosta bedeutet eine schwere Bedrohung der Interessen Turins.

Eine Vollendung dieser Bahn, die eine unmittelbare Verbindung der beiden Hauptstädte Rom und Paris bringen würde, bedeutet gleichzeitig eine günstige Verbindung zwischen den Kanalhäfen und dem Orient sowie zwischen Odessa und Bordeaux, dem nächsten atlantischen Hafen, so dass ein scharfer Wettbewerb zwischen der unmittelbaren Ausfuhr Russlands nach dem Atlantik auf dem Seeweg und der mittelbaren Ausfuhr via Bordeaux entstehen dürfte.

Bei dem Interesse, das Italien gegenwärtig für seine Bahnprojekte aufbringt, ist es nicht ausgeschlossen, dass trotz der entgegenstehenden finanziellen Hindernisse — der Tunnelbau allein wird einige hundert Millionen Lire kosten — der Plan sehr gefördert werden wird. z.

SO_2 libre et combiné dans les Denrées alimentaires.

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Par A. Farine Dr ès Sciences.

La conservation des denrées périssables est un problème qui ne saurait être négligé: c'est pourquoi l'on a été amené à faire usage de conservateurs et d'antiseptiques. Parmi ceux-ci nous trouvons fréquemment l'acide sulfureux. Il entre dans la préparation de beaucoup d'aliments. C'est un des trois conservateurs classiques avec le sel de cuisine et le fumage. De tous temps on l'a utilisé dans la préparation des vins. En Amérique il est considéré comme un poison redoutable occasionnant entre autre de l'albuminurie et des troubles gastriques.

Le but que l'on se propose avec l'adjonction de conservateurs est varié: d'une part SO_2 confère aux aliments une durée plus grande grâce à son action sur les organismes inférieurs, d'autre part l'acide sulfureux donne à certains aliments, par exemple aux fruits, un aspect de fraîcheur, et à la viande une couleur rouge fraîche.

Parallèlement le chimiste analyste s'est occupé de la recherche qualitative et quantitative de l'acide sulfureux dans les denrées alimentaires l'intérêt de cette recherche est accru par le fait que les médecins attribuent des propriétés néfastes aux aliments qui en contiennent.

Un fait curieux mérite d'être mentionné ici: la conservation de la viande au moyen de sulfites fut combattue pour la bonne raison que des essais bactériologiques ont démontré que la viande ainsi traitée conserve pendant un temps assez long l'aspect extérieur de la fraîcheur, mais qu'il est possible, par addition de sulfite, de faire disparaître l'aspect désagréable et la mauvaise odeur de la viande qui commence à se gâter. Intérieurement cependant la putréfection suit lentement mais sûrement son cours.

La détermination de l'acide sulfureux était accompagnée au début de grandes difficultés. On n'a pas tardé à reconnaître qu'il existe à l'état „libre" et „combiné" dans les denrées alimentaires. Le SO_2 libre est facilement titrable avec une solution d'iode, il en est autrement de l'acide combiné à certains corps qui sont:

pour le vin *l'acétaldéhyde* (travaux de Schmitt & Ripper),
pour le moût *le sucre* (Beythien, Fresenius, Kerp, etc.).

Ripper a proposé pour la détermination du SO_2 combiné, pour ce qui concerne le vin, de mettre l'acide en liberté au moyen d'un alcali et de titrer par l'iode. Haas, au contraire, proposa d'acidifier au moyen de l'acide phosphorique puis de distiller dans un courant de CO_2 et titrer ensuite SO_2 dans le distillat à l'état de SO_4H_2 après oxydation.

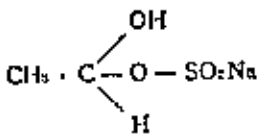
Ces méthodes cependant ne permettaient pas une détermination exacte. Les travaux de *Furnsteiner* et *W. Kerp* qui furent publiés dans le courant de l'année 1904 constituaient un progrès important tant au point de vue chimique qu'hygiénique. Ils avaient trait à la combinaison de SO_2 avec les *aldéhydes*, les *cétones* et les *sucres*. W. Kerp a continué ces travaux. Il ressort de ses recherches combien l'étude de la chimie-physique fut utile, car elle a permis d'élucider beaucoup de problèmes.

Ce n'est qu'à partir du moment où l'on a réussi à éclaircir la constitution des sulfites „combinés" en solution aqueuse que l'on put établir l'action pharmacologique de ces sels.

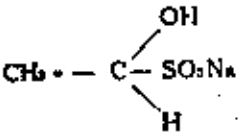
W. Kerp a démontré le premier qu'il y a de l'acétaldéhyde dans le vin et ceci d'une manière exacte. Il est évident que dans ce cas SO_2 sera combiné à l'aldéhyde. Nous allons retracer rapidement quelques travaux de Kerp, spécialement ceux qui concernent les produits d'addition d'aldéhydes et de cétones avec SO_2. Ils constituent la base de nos connaissances au sujet de la manière d'être de l'acide sulfureux dans les denrées alimentaires de même qu'au point de vue hygiénique.

Theorie de la dissociation du complexe des acides sulfureux combinés.

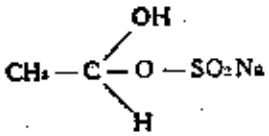
Si l'on ajoute de l'acétaldéhyde à une solution aqueuse de sulfite acide de sodium il se forme le produit d'addition suivant:



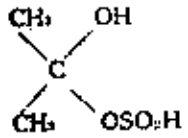
Autrefois on supposait que l'addition de SO_2 aux aldéhydes et cétones donnait lieu à la formation d'acide *α-oxysulfoniques*. Le sel sodique aurait la constitution:



D'après cela l'atome de soufre serait lié directement à l'atome de carbone. Cependant en traitant ces produits d'addition par des acides dilués ou de la soude il y a facilement régénération d'aldéhyde ou de cétone. De plus cette scission se fait déjà en solution aqueuse. Il en découle donc que la formule suivante est la plus probable:

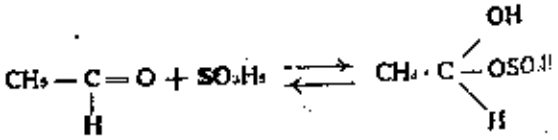


SO_2 peut de même s'additionner aux cétones. Si nous prenons le cas de l'acétone nous obtiendrons l'acide suivant:



D'ailleurs cette addition n'est pas complète. Si l'acide sulfureux ou les sulfites sont mis en présence des aldéhydes ou cétones en quantités moléculaires, un certain pour cent reste non combiné. Nous pouvons donc représenter le phénomène d'addition de la manière suivante:

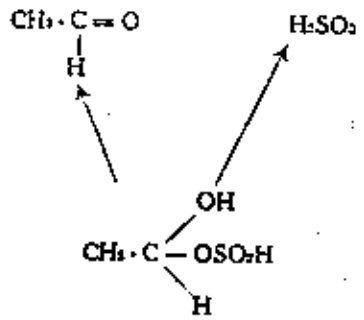
Dans le cas de l'acétaldéhyde:



Il s'agit donc d'une réaction réversible: il y a un état d'équilibre. D'autre part si on dissout un sel de ces combinaisons aldéhydiques ou cétoniques dans l'eau, il y a également une scission en aldéhyde ou cétone et sulfite acide. Le „complexe" a été scindé.

A coté de cette scission il faut considérer la dissociation électrolytique: ces acides-aldéhydes et acides cétones sont des acides assez forts. Il s'en suit que dans les solutions aqueuses de ces combinaisons nous avons un système compliqué de divers équilibres. Dans le cas de l'acétaldéhyde nous pouvons schématiquement représenter la constitution de ces acides, en solution aqueuse, comme suit:

1. Le „complexe" Aldéhyde · SO_2 se scinde en CH_3CHO et SO_3H_2:



Cette réaction est réversible, comme nous l'avons vu précédemment.

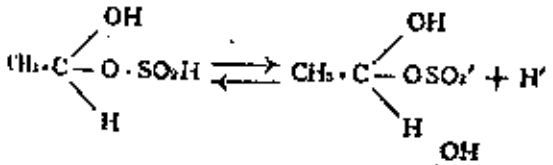
2. dissociation électrolytique de SO_3H_2 (1er stade):

$$H_2SO_3 \rightleftarrows HSO_3' + H'$$

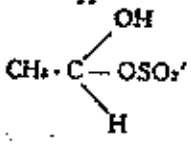
3. Scission de l'acide sulfureux en solution aqueuse:

$$H_2SO_3 \rightleftarrows H_2O + SO_2$$

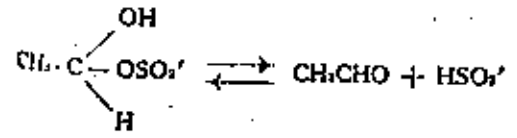
4. dissociation électrolytique du „complexe" Acétaldéhyde · SO_2.



5. Scission du complexe



selon le schéma:



6. dissociation électrolytique de l'ion négatif de SO_3H_2 (2e phase).

$$HSO_3' \rightleftarrows SO_3'' + H'$$

De ces 6 équations c'est spécialement la Ière et Ve qui nous intéressent. Pour faciliter posons pour l'aldéhyde le signe: A.
Nous obtenons pour la formule I:

$$A \cdot SO_2H_2 \rightleftarrows A + H_2SO_3$$

Si nous appliquons la loi d'action de masse de Guldberg-Waage nous aurons la relation: „le produit de la concentration moléculaire de A et de SO_3H_2, divisé par la concentration moléculaire de l'acide combiné ASO_3H_2 est constant et indépendant de la dilution. Ce rapport s'exprime par l'équation:

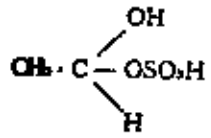
$$\frac{[A] \cdot [H_2SO_3]}{[AH_2SO_3]} = K_I$$

de même pour V nous aurons:

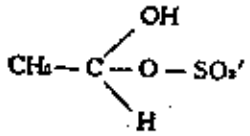
$$\frac{[A] \cdot [HSO_3']}{[AHSO_3']} = K_V$$

Les constantes KI et KV de ces deux équations indiquent les quantités de SO_2 libre et combiné présentes dans la solution.

Par SO_3 combiné il faut entendre d'une part celui qui fait partie de l'acide:



non dissocié et celui qui fait partie de l'anion de cet acide, soit le complexe:



Il est facile au moyen de ces constantes de déterminer la quantité de SO_2 libre et combiné, si on connaît la quantité totale d'acide sulfureux présente dans la marchandise soumise à l'examen.

Effet pharmacologique des sels acides combinés.

Il y a un fait intéressant à signaler, c'est que l'effet pharmacologique de ces sels est en accord parfait avec leur action chimique. Comme l'on sait l'acide sulfureux est un poison assez fort, aussi pour l'animal supérieur; il est indifférent qu'il soit absorbé par voie intraveineuse, donc directement par le sang, ou bien par l'estomac.

Le sulfite acide de Sodium est plus toxique que le sel neutre. Pour déterminer la toxicité de l'acide sulfureux combiné il s'agissait tout d'abord d'examiner si les combinaisons complexes ont une action spécifique. Ce n'est pas le cas. Elles semblent avoir la même action que SO_2 lui-même. Il y avait lieu de supposer que le SO_2 engendré par la scission du complexe provoque l'action toxique et que le sel qui présente une constante de scission très grande possède une toxicité en rapport.

Les essais entrepris par *Rost* et *Franz* confirment cette supposition comme le montre le tableau suivant:

Nature du sel	La mort survient après injection des cmc sol. à 5% de SO_2	après minutes	Quantité mortelle pour 1 kg (poids du corps) après injection dans l'estomac:
Formaldéhyde-sulfite de Na . .	44 cmc	42	1,85 gr. SO_2
Acétaldéhyde-sulfite de Na	11 cmc	25	1,22
Sulfite neutre de Na	8 cmc	17	0,65
acétone-sulfite de Na	7—8 cmc	8—11	0,63
glucose-sulfite de Na	4—5 cmc	10—11	0,58
Sulfite acide de Na	3—4 cmc	6—8	0,45

Ces essais ont une certaine importance quant à l'appréciation des denrées alimentaires, car SO_2 est lié à divers corps, tels que: l'acétaldéhyde dans le vin, le sucre dans les fruits etc.

Diderot disait: „dans la société nous avons deux sortes de personnes: les bons médecins et les mauvais cuisiniers: — les premiers travaillent à conserver notre santé, — les derniers travaillent à la détruire. Les derniers sont plus sûrs de leurs faits que les premiers."

Et Diderot n'avait pas tort.

Die Entwicklung der organischen Chemie bis 1800.

IV.

Wasserabspaltungen.

Wiewohl durch die Untersuchungen von Williamson 1850 dargetan wurde, dass die Bildung des Äthyläthers nicht auf einer wasserabspaltenden Wirkung der Schwefelsäure beruhe, so mögen die wichtigsten historischen Daten dieser Verbindung gleichwohl an dieser Stelle angeführt werden. Auf das anlässlich der Esterifizierung schon mitgeteilte möge hier nochmals kurz hingewiesen werden; es handelte sich für die alten Chemiker nicht darum, eine neue Substanz herzustellen, sondern die Schwefelsäure zu „versüssen", und die Angaben hierüber in den Büchern unter dem Namen des Raymundus Lull (XIII. Jahrhundert) und des Basilius Valentinus (XVI. Jahrhundert) beziehen sich jedenfalls nur auf die sog. „versüsste Schwefelsäure", d. h. auf ein Gemisch von Alkohol, Äthyläther, Wasser und vielleicht noch schweflige Säure (Lull in „Epistola accurtationis lapidis benedicti", an den König Robert von Schottland gerichtet († 1329) und Basilius V. in der „Wiederholung des grossen Steines der uralten Weisen").

Ein solches Gemisch wird auch der „spiritus vitrioli antepilepticus" des Paracelsus gewesen sein, das von dem grossen Mediziner gegen die Fallsucht empfohlen wurde.[1]) Derselbe soll auch die schlafmachende Wirkung dieses „spiritus" an Hühnern festgestellt haben[2]). Entgegen der bisher allgemein angenommenen Priorität des Valerius Cordus hinsichtlich der Entdeckung des reinen Äthyläthers muss nach Hermann Peters[3]) dieselbe bis auf weiteres auf Johann Ralla, einen Oheim des Cordus, zurückgeführt werden, welcher Besitzer einer Apotheke in Leipzig war und bei welchem Cordus die „Apothekerkunst" erlernte

J. Pharmacol. & Exp. Therap.
86(1) 1946

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THE CHRONIC TOXICITY OF SULFITES

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For many years the process of sulfurizing has been the principal commercial method of preserving dried fruits and for an equal number of years the question of the harmful potentialities of sulfites in food products has lacked a definite answer based on scientific experimentation and observation. The work of Wiley (1) on human volunteers, which was performed toward the beginning of the present century, indicated but produced no conclusive evidence that metabolic disturbances resulted from the addition of salts of sulfurous acid to foods. The older experiments of Kionka (2) and of Lehmann (3) showed that large, although unspecified amounts of sulfites, added to meat diets of dogs produced diarrhea and kidney damage. Rost and Franz (4) failed to confirm the results of Kionka. They gave 40 dogs, both growing and mature, oral doses of different sulfites varying from 0.05 to 1 gram per day for periods of from 1 month to over a year without visible macroscopic or microscopic tissue changes. Larger doses caused vomiting; however, no other symptoms appeared. In humans they found that doses of 1 gram of sodium sulfite per day decreased the utilization of protein and fat. Gastrointestinal symptoms seldom occurred at this dose, but with doses of from 4 to 5.3 grams per day there occurred abdominal pains and vomiting.

Later experiments of Morgan and her associates (5, 6) showed the ability of sulfites to destroy thiamine. This fact has been of such importance that in any calculation of the daily allowance of this vitamin the theoretical amount in dried fruits has had to be totally disregarded. Recently, in order to supply the special food requirements of troops fighting in very hot climates, it was necessary to sulfurize fruits to an extent where any toxic hazard inherent in the process, in addition to the destruction of thiamine, became an important problem. The experimental work reported below was conducted for the purpose of evaluating the toxicity of various levels of sulfites when added to the diet. Since sulfites may be converted to sulfides and sulfates these were included in our studies as well as diets containing sulfite but low in thiamine and diets with sulfites and added thiamine.

EXPERIMENTAL. Rats from our colony of Osborne-Mendel strain have been used in 3 experiments on the chronic toxicity of sulfites. The ration used in each experiment was composed of corn starch 60%, casein 18%, corn oil 6%, brewer's yeast 5%, whole liver powder 5%, salt mixture (U.S.P. XII No. 2) 4%, and cod liver oil 2%. All diets were refrigerated until used. The rats were kept in individual cages in a room with the temperature and humidity controlled.

The first experiment hereinafter referred to as the 1940 experiment was designed to study the effect of thiamine on the toxicity of sulfites. The supply of thiamine for the rats was either increased above or decreased below that found in the regular diet. The increase in available thiamine was by intramuscular injection of 100 micrograms twice

weekly. The available thiamine was decreased by autoclaving the yeast before adding it to the diet. The sulfite was added as sodium bisulfite and the sulfite values referred to throughout are to this salt ($NaHSO_3$) and not to sulfur dioxide. In this experiment 3 levels were used: 0.5%, 1% and 2%, in 3 different types of diets. The first diet had sulfite added, the second contained sulfite and was supplemented with thiamine, and the third contained sulfite but had its thiamine content reduced by autoclaving the yeast component. A fourth unsulfited diet was used as a control thus making a total of 10 diets. This experiment utilized a balanced incomplete block design where each of 5 diets was fed to each of 3 rats from the same litter. The design was replicated for male and female rats. Thus 3 male and 3 female rats were taken from each of 50 litters at 3 weeks of age and placed at random on the proper diet according to the design.

Almost a year later when it appeared that aging of the diets containing sulfites was having some effect, a second experiment hereinafter referred to as the 1941 experiment was begun to compare the effects of the various levels of sulfite in diets prepared weekly with the effects of the same levels in diets prepared in sufficient quantities to last five or six weeks and stored under refrigeration. These diets, designated "old" and "new", were used for the controls and the 1% and 2% sulfite levels. Two lower levels of sulfite, namely 0.1% and 0.25%, were also included on the "old" diet basis. Since sulfite is sometimes oxidized to a sulfate, two levels of sodium sulfate (0.25% and 1%) were included in this experiment. Thus again, 10 diets were used in the same type of balanced incomplete block design as utilized in the 1940 experiment.

A third experiment hereinafter referred to as the 1942 experiment was begun in which lower levels of sulfite were included (0.025%, 0.05%, 0.05% and 2%). Since diarrhea with black feces resulted from feeding sulfites at the high levels it was considered possible that a sulfide might be contributing to the toxicity of the sulfite; therefore, two levels of sodium sulfide (0.25% and 1%) were fed. Seven diets including the control were used in this experiment. The proper replicated balanced incomplete block design utilized 28 litters with 3 males and 3 females from each litter and resulted in 12 males and 12 females on each diet.

RESULTS. *The effect on growth.* Usually comparisons of effects of various diets on growth over a period of time are made by comparing the gains in weight. However, this results in a loss of much information on the growth curve of the rat as given by the weekly weighings. In the following analyses an increase in information on the growth effects has been obtained by including both the gain in weight and the average weight for the individual animal over the period of study. Since the rats from each litter on this experiment are assigned to the diets at random and since the initial weights are within rather small limits, no correction for initial weight is necessary.

Analysis of the growth curves for this study was divided into 2 parts: (1) the first 12 weeks and (2) the first year. For the first 12 weeks, mathematical curves were initially fitted to the weekly weights of each rat. These curves were of such a type (orthogonal polynomials) that the several coefficients for one curve were independent of each other (7). The first coefficient is the average weight of the rat over the period; the second coefficient is proportional to the average gain in weight; the third coefficient is proportional to the average change in gain in weight, etc. In this study, the first two coefficients were the only ones that showed consistent trends with increased doses of sulfites. This, therefore, is equivalent to analyzing the effects of the various levels of sulfites in the diet by comparing average weights, and by comparing gains in weight.

Such comparisons are made in this study. For the 52-week period, the design of the experiment was upset by the death of a large number of animals, and conclusions are based only on those rats that survived. Since no deleterious effects on growth were caused by the sulfates or sulfides, analysis will be shown on the sulfites only.

The comparisons are presented graphically. Figure 1 shows the average weights over 12 weeks according to diet, as labeled; males and females are given separately since they have decidedly different averages and standard deviations. Limits are shown bracketing the average weights. The limits are to be used in

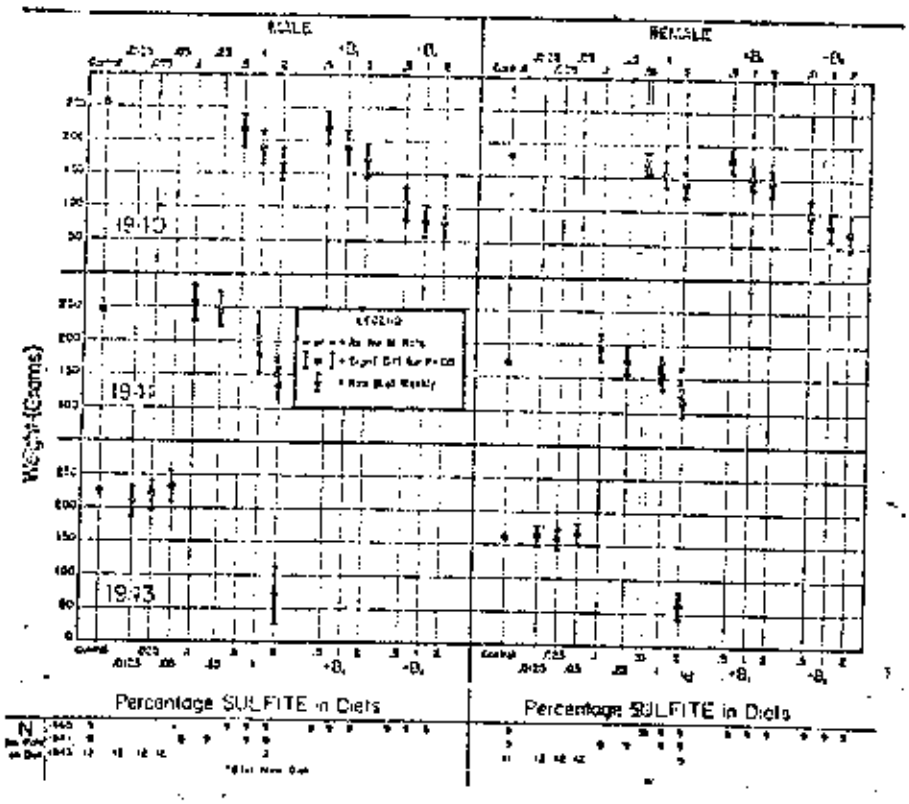


FIG. 1. AVERAGE WEIGHTS OF RATS OVER THE PERIOD OF THE FIRST 12 WEEKS ON DIETS CONTAINING VARIOUS AMOUNTS OF SULFITES—3 EXPERIMENTS

comparing the average weight for each experimental diet with the average weight for the control group, and show the size of difference from the control that would be statistically significant ($p = 0.05$) in this experiment. Where the numbers of experimental and control animals are the same, as is the case of most of the 12 week data, the brackets can also be used for judging the significance of the difference between two test diets. The limits are calculated using the

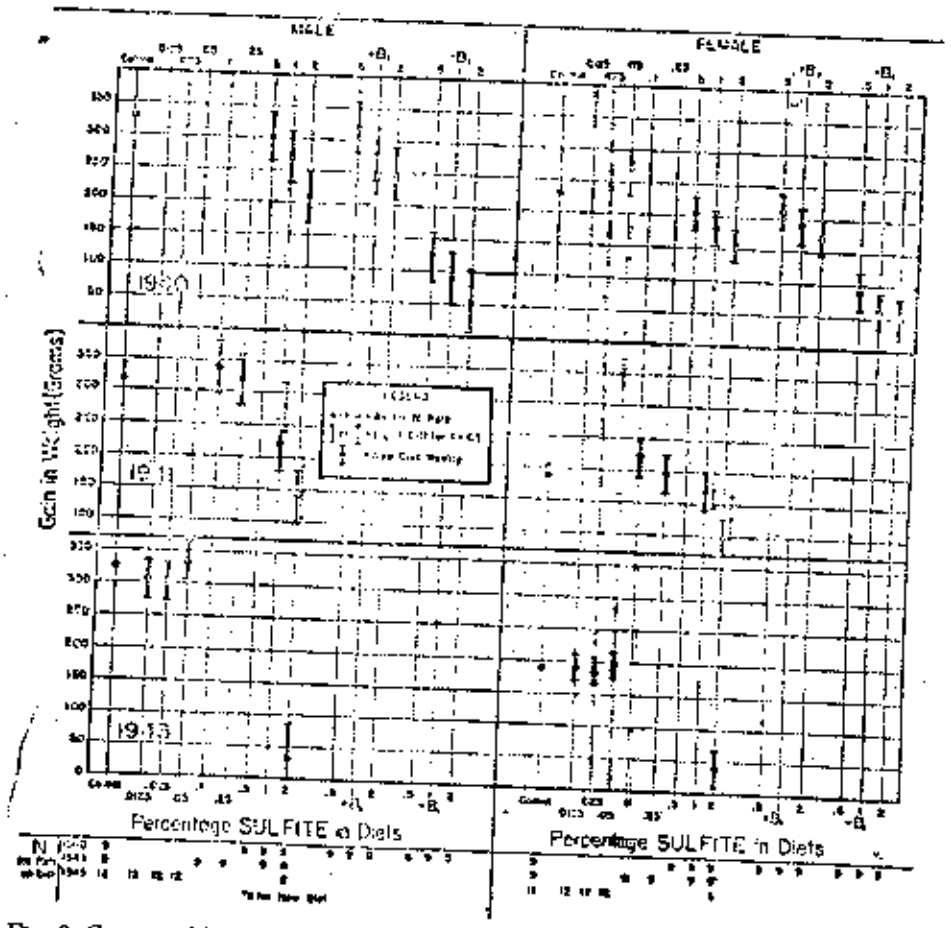


FIG. 2. GAINS IN WEIGHT OF RATS OVER THE PERIOD OF THE FIRST 12 WEEKS ON DIETS CONTAINING VARIOUS AMOUNTS OF SULFITES—3 EXPERIMENTS

formula for the "t" test.[1] If the average for the controls falls within the limits designated on either side of the average for a particular level of sulfite, (as it does

[1] In application of the formula for the "t" test,

$$t = \frac{\bar{x}_c - \bar{x}_t}{\sqrt{\frac{\sigma_c^2}{N_c} + \frac{\sigma_t^2}{N_t}}}$$

$\bar{x}_c$ and $\bar{x}_t$ are the average weights for N_c and N_t animals on the control and experimental diet "t" respectively. Also σ_c^2 and σ_t^2 are equal and are the pooled variances (squares of the standard deviations) for all groups where there is no correlation between the average and the variance. (The square of the standard deviation is calculated for the average weights on each experimental and the control diets. These variances are then each multiplied by the number of animals N in each group and sum of these products is divided by the total

in all instances for the 0.0125% of sulfite) this can be interpreted to mean that in this experiment there is no significant difference between the average weights for the control and that level of sulfite. If the average for the control falls outside the limits a statistically significant difference exists.

The charting of results in this manner points out not only significant differences from the control for all diets simultaneously but also any trends of average weight or gain in weight with increasing dosages of sulfite. Moreover, visual comparisons can be made of results for several experiments.

Figures 1 to 4 show the following about the average weights and the average gains in weight of the rats on these three experiments:

1. There is a very definite trend toward smaller average weights and smaller gains in weight with an increase of sulfite in the diet above 0.25%.

2. The rats on the diets containing 0.07% and 0.1% sulfite grew more rapidly and had greater gains in weights for the first 12 weeks than the rats on the control diet. The average weights and gains in weight shown on figures 1 and 2 and the average weights shown on figure 3 are slightly higher for the animals on the above levels than the control for both male and female rats. Considering the 12 week data first there are 8 comparisons (figures 1 and 2) between the control group and the experimental groups fed these two levels of sulfite and as can be seen from the figures all differences are in the same direction. These early gains were not maintained up to the end of the first year. Thus at 52 weeks the male rats on the 0.1% sulfite-containing diet had a gain in weight significantly smaller than the control as can be seen on figure 4. The female rats also had smaller gains in weight for the 52 week period though the difference is not significant. Rats on the 0.05% sulfite level had gains in weight which did not differ significantly from the controls at 52 weeks.

3. The results on the series having supplemental injections of thiamine were about the same as on those without the added thiamine.

4. The rats on diets from which the greater part of the thiamine had been removed had the lowest series of average weights and gains in weight. However, there is also a downward trend with increased amounts of sulfite in this diet.

5. For the 12 week period rats on the "new" diet had larger average weights and gains in weight than those on the "old" diet. Although these differences are not individually significant, they were all in the same direction.

The effect on survival time. The increased amounts of sulfite in the diet affect not only the growth of the rats on these diets but also the survival time. Since

number of animals in all the groups used to obtain the pooled variances σ^2.) The limit $\bar{x}_c - \bar{x}_i$ for $p = 0.05$ used on figures 1, 2, 3, and 4 is found by transposing the above formula to

$$\bar{x}_c - \bar{x}_i = t\sigma\sqrt{\frac{1}{N_c} + \frac{1}{N_i}}$$

and the value of t is obtained from a "t" table for $p = 0.05$ and degrees of freedom equal to $N_c + N_i - 2$. A "t" table is given in almost any statistical text or book of statistical tables (8).

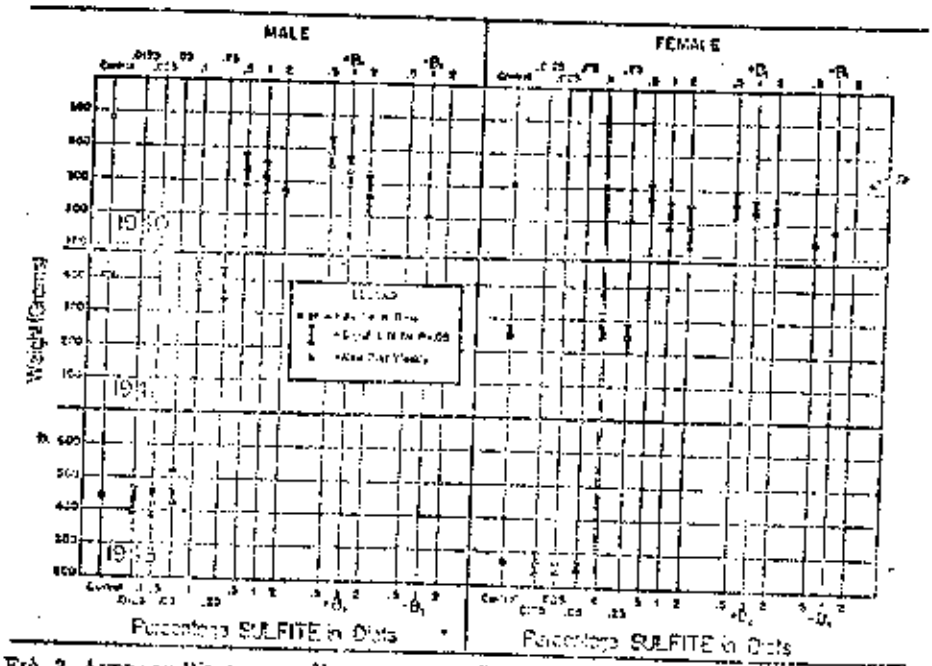


FIG. 3. AVERAGE WEIGHTS OF RATS OVER THE PERIOD OF THE FIRST 52 WEEKS ON DIETS CONTAINING VARIOUS AMOUNTS OF SULFITES—3 EXPERIMENTS

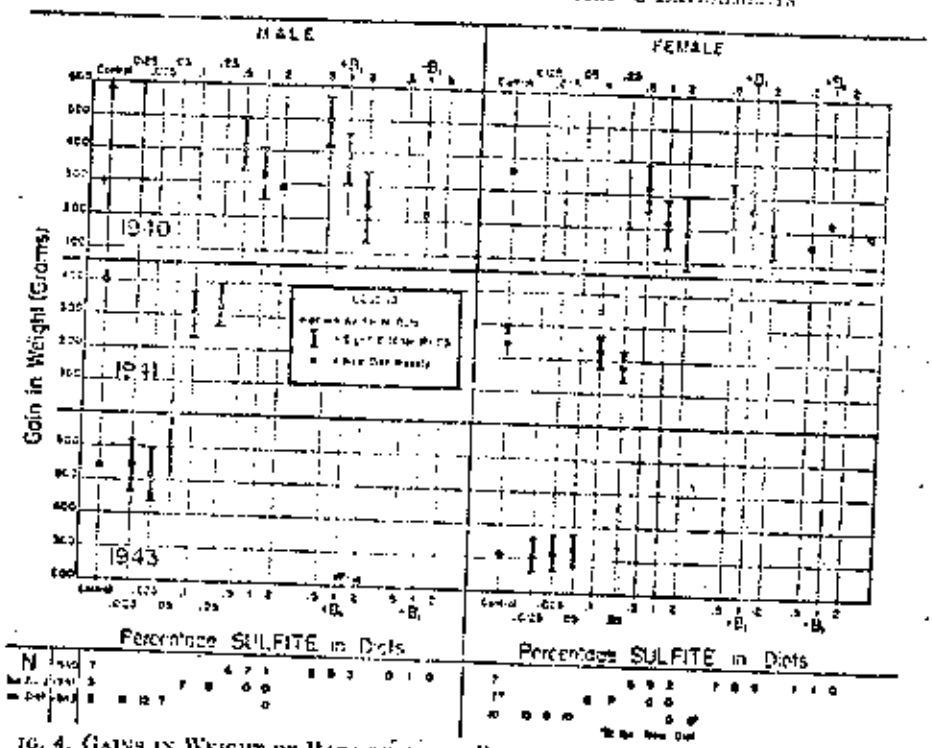
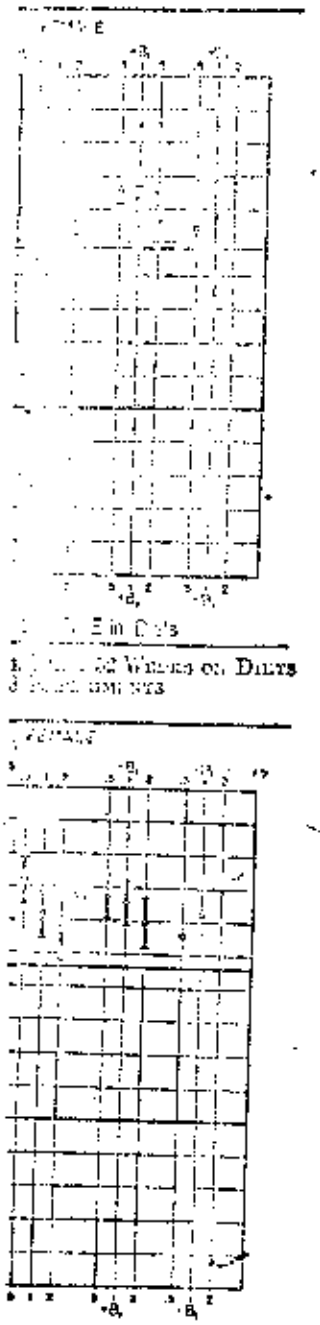


FIG. 4. GAINS IN WEIGHT OF RATS OVER THE PERIOD OF THE FIRST 52 WEEKS ON DIETS CONTAINING VARIOUS AMOUNTS OF SULFITES—3 EXPERIMENTS



First 52 Weeks on Diets
Experiments

the addition of sulfates and sulfides has no effect on the survival time of the animals, analyses are shown only on the sulfites. Figure 5 shows the diets in the same order as in figures 1 to 4, with the results for male and female rats shown separately. In figure 5 each symbol represents one rat and the averages of results on each diet are indicated by horizontal lines. No significant limits are indicated since the rats that did survive were killed at the end of that experi-

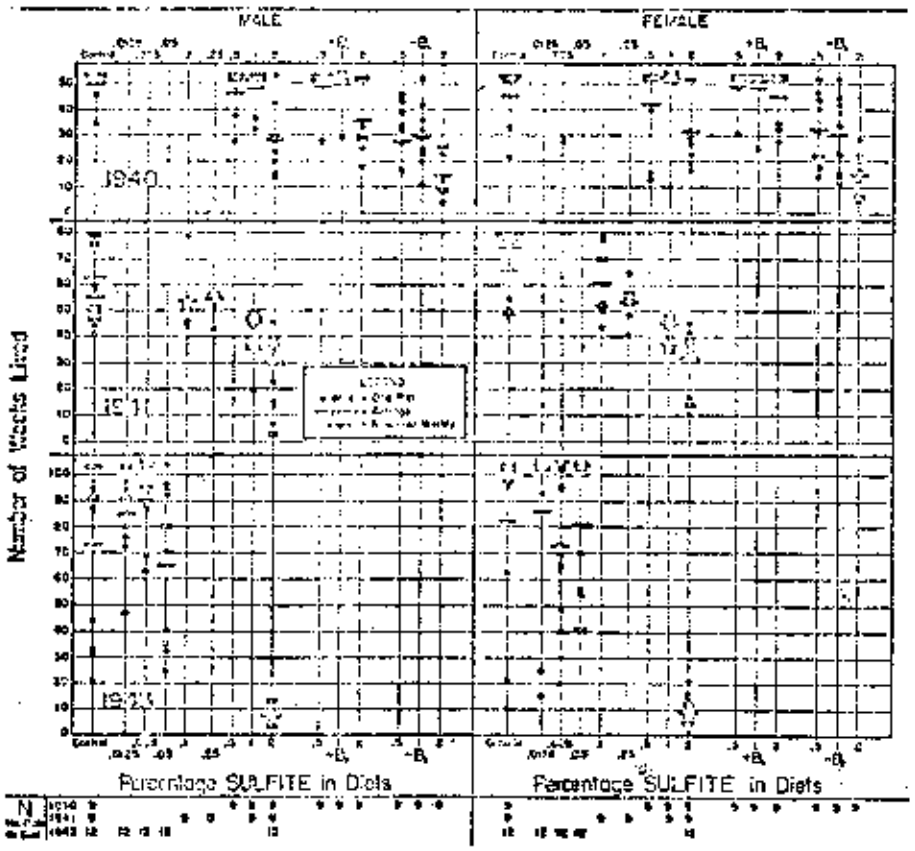


FIG. 5. SURVIVAL TIME FOR RATS ON DIETS CONTAINING VARIOUS AMOUNTS OF SULFITES —3 EXPERIMENTS

ment as shown by the bunching of symbols at the top of the 1940 and the 1943 parts of the figure and since the distribution of survival times followed none of the commonly recognized types. Each of the 3 experiments was continued for a different length of time, the 1940 experiment for one year, the 1941 experiment for one and one-half years, and the 1943 experiment for 2 years. However, the trends shown are very similar to those shown in figures 1 to 4. In all cases the rats on a diet containing 2% of sulfite had a short average survival time. In

1941 even the 0.25% sulfite in the diet seems to have shortened the lives of the female rats and the 0.1% sulfite in the diet also produced an average survival time less than the control. No trends are evident for survival times of rats on diets containing less than 0.1% sulfite. The results on diets supplemented by thiamine seem to be about the same as those not supplemented, whereas the reduction of the thiamine in the diet resulted in a sharply decreased average survival time.

Pathology. Of the 504 rats started on these experiments, 70 were not sent to the pathology laboratory for one reason or another. Of the remaining 434, 233 were sectioned microscopically. Sections were made routinely of lung, heart, liver, spleen, pancreas, stomach, small intestine, kidney, adrenal and testis, with other structures such as ovary, uterus, colon, brain, thyroid, bone, bone marrow and leg muscles being sectioned less frequently. In addition, certain other animals had confirmatory sections made of spontaneous tumors and other noticeable conditions, but these are not considered as sectioned animals.

Rats fed high doses of sulfite in the diet frequently showed stunting of growth, clinical polyneuritis, "spectacle" eyes, bleached incisor teeth, brown uteri, atrophy of various viscera, calcified renal tubular casts, and atrophy of bone marrow and bones; less frequently they showed focal myocardial necrosis and fibrosis, and gastric squamous epithelial hyperplasia (table 1). With a decrease in the percentage of sulfite from 2% to 0.5% or even to 0.25% there was not a great deal of change in the lesions observed; the animals simply lived longer and finally attained about the same condition that those on higher dosage levels did in a shorter period. This is, however, a broad general statement and will not be exact for each individual lesion. The level of sulfite in the diet which would begin to cause histological changes in rats would appear to lie at approximately 0.1% on the basis of these studies.

Aging of the diet had a definite effect on incidence of lesions of the teeth and uteri, while it had no effect on the polyneuritis; this indicates a sharp separation in etiology.

The following remarks are intended to help clarify the nature and degree of involvement of some of the lesions listed in table 1. Hyperplasia of the gastric squamous epithelium (usually near its junction with the glandular portion of the stomach) somewhat paralleled the browning of the uteri, although not closely. The lesion was slight or moderate in degree in all instances. "Myocardial fibrosis" included focal lesions in all stages from recent necrosis to replacement by fibrous tissue. The left ventricle was chiefly affected, the right ventricle less so, and the atria scarcely at all. The lesion was slight or moderate in degree in all instances. Fatty degeneration of the liver was very slight or slight in all instances except a few of moderate degree. It will be noted that the condition was more frequent in the control, sulfate, sulfide, and low dose sulfite rats than in rats receiving high doses of sulfite. There was no apparent relation between thiamine and this condition in the present material. Calcified renal tubular casts occurred chiefly around the corticomedullary junction. Very little tubular atrophy occurred as a result of the process. Brown pigment in the renal convoluted

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TABLE 1

Summary of gross and microscopic pathology in rats fed sulfite, sulfate or sulfide

Dosage*	Animals in group	Polyneuritis	Renal calculi	Hydronephrosis	Testicular atrophy	Fatty degeneration of liver	Hyperplasia of glandular stomach	Myocardial necrosis	Fatty degeneration of liver	Myocardial necrosis of right ventricle cells	Renal tubular atrophy	Tubular casts in urine
1940 series												
2% − B_1	18	16	0	4	0/18	2/18†	4/13	3/15	0/15	11/15	0/15	Mod. to marked
2%	18	2	3	9	0/18		1/13	4/12	2/13	10/12	2/12	Mod. to marked
2% + B_1	18	0	8	5	1/18		0/15	2/12	0/12	9/12	6/12	Mod. to marked
1% − B_1	18	17	0	0	0/13		0/18	4/12	0/12	10/10	2/10	Mod. to marked
1%	18	0	1	1	3/17		0/17	1/10	0/10	7/10	9/10	Mod. to marked
1% + B_1	18	0	0	0	0/18		0/18	0/10	0/10	7/10	8/10	Mod. to marked
0.5% − B_1	18	16	0	0	0/17		0/17	3/11	1/11	10/11	0/11	Mod. to marked
0.5%	18	0	0	1	1/17		0/17	0/10	1/10	5/10	3/10	Mod. to marked
0.5% + B_1	18	0	0	0	1/18		0/18	2/11	2/11	5/11	0/11	Mod. to marked
Control	18	0	0	0	0/13		0/16	1/13	4/13	1/13	1/13	Slight
1941 series												
2%, "old" diet	18	7	7	2	4/17	†	0/17	2/11	1/11	9/11	2/11	Mod. to marked
2%, "new" diet	18	9	0	1	0/17		2/17	2/10	0/10	9/10	0/10	Mod. to marked
1%, "old" diet	18	5	0	1	7/18		1/18	1/9	0/9	1/9	1/9	Mod. to marked
1%, "new" diet	18	8	0	2	1/17		1/17	0/9	1/9	8/9	0/9	Mod. to marked
0.25%, "old" diet	18	0	0	0	1/17		0/17	0/9	3/9	5/9	1/9	Mod. to marked
0.1%, "old" diet	18	5	0	1	0/18		7/18	0/9	2/9	8/9	0/9	Mod. to marked
Sulfate 1%	18	0	0	0	0/16		0/13	5/7	3/7	4/7	0/7	Moderate
Sulfate 0.25%	18	0	0	0	0/17		0/17	0/6	4/6	1/6	0/6	Slight
Control, "old" diet	18	0	0	0	0/9		1/9	1/7	5/7	2/6	1/6	Slight to moderate
Control, "new" diet	18	0	0	0	0/9		1/9	0/6	3/6	2/6	1/6	Slight to moderate
1943 Series												
0.05%	24	0	0	0	0/22	‡	0/22	2/10	10/10	3/10	0/10	Slight
0.025%	24	0	0	0	0/17		0/17	3/8	8/8	0/8	0/8	Slight
0.0125%	24	0	0	0	0/19		0/20	0/9	0/9	1/9	1/9	Slight
Sulfide 1%	24	0	0	0	0/16		1/15	0/10	5/10	2/10	0/10	Slight
Sulfide 0.25%	24	0	0	0	0/17		1/17	1/9	6/9	1/9	0/9	Slight
Control	24	0	0	0	0/13		0/13	2/8	5/8	0/8	0/8	Slight

* Sulfite unless otherwise specified.

† In 1943 and 1941 series present in from ⅓ to all of autopsied rats in all groups except 2% − B_1. More frequent in second series than in first.

‡ Present in from ⅓ to ½ of autopsied rats in all groups of 1943 series.

tubules, most of which did not react for iron with acid ferrocyanide, was most frequently seen in the groups receiving added thiamine. The condition was always of slight or moderate degree. Testicular tubular atrophy of itself showed no special features. Interlobular edema was seen almost entirely in the first series and principally in the sulfite and sulfite plus thiamine groups. On a dosage level of 2% the majority of the testes in these two groups showed from slight to marked edema.

Lung, adrenal, pancreas, small intestine, colon, ovary, and spleen showed no distinct differences between test and control animals attributable to the sulfite, the only changes being those of inanition. A few small ulcers in the proventriculus were scattered among the various groups; in all except one of these stomachs hair balls were present. A few each of brain, sciatic nerve and thyroid were sectioned. These were negative except for one brain showing hemorrhages and one with foci of softening, in animals with polyneuritis. Tibia and femur, including the bone marrow and the surrounding leg muscles, were sectioned more frequently; the only changes observed here were those of inanition.

The animals receiving sulfates and sulfides in their diets showed no distinct differences from the controls (table 1) as a result of the treatment.

Discussion. The greater share of the deleterious effect of sulfites in the diet is probably due to the destruction of vitamins. In studies on the chemistry of thiamine, Williams, Waterman, Keresztesy and Buchman (9) reported that sulfite split the molecule and destroyed the antineuritic properties of this vitamin. This observation would explain growth changes and much of the pathology observed in the present experiment. The destruction of the thiamine in the diet caused the polyneuritis and most, if not all, of the focal myocardial necrosis and fibrosis. Since a lack of thiamine decreases the desire for food (10, 11), the inanition which produced the stunting of growth and atrophy of the individual organs and tissues can be considered to be a result of destruction of the vitamin by the sulfite. Depigmentation of the teeth, brown uteri and "spectacle" eyes have not been reported to be caused by a thiamine deficiency. Moore (12) stated that either a vitamin E or vitamin A deficiency will cause dental depigmentation in the rat. The fact that aging of the diets had no greater effect on growth indicates that the diets were not low in vitamin A. In this connection Morgan (5) reported that the process of sulfurizing dried fruits did not destroy vitamin A. Further studies are being made on the effects of sulfite on the teeth and the findings will be reported later. According to Mason (13) female rats with a vitamin E deficiency of long duration have brown uteri and the intensity of the color increases as the deficiency continues. Although the brown uteri in the present experiment were grossly and microscopically of the type generally considered typical of vitamin E deficiency, they did not have the intense color which we have observed in rats with definite vitamin E deficiency (14). No other uncomplicated histopathological evidence of vitamin E deficiency was seen. "Spectacle" eyes in rats have been reported to occur in biotin deficiency (15). Since the rats on the levels of sulfite which caused this symptom also had extreme diarrhea, either the diarrhea or the condition in the intestines that produced the

diarrhea may have prevented the synthesis or absorption of the biotin in the intestinal tract.

Chemical analyses of the diets showed that up to 75% of the sulfite disappeared when the diets were left open to the air at room temperature for a week. A part of this was accounted for by an increase in sulfate sulfur in the diet and the other part probably escaped as sulfur dioxide. Less than 20% was lost from diets refrigerated for as much as 6 weeks. Since the diets were exposed in the food cups to the air for varying lengths of time up to a week, these facts indicate that the animals obtained only part of the sulfite originally added to the diet.

It is observed from the experiment with the added thiamine that a depletion of the antineuritic vitamin is not the only factor affecting growth rate. These rats had no polyneuritis; however, they showed a distinct growth retardation. A deficiency either of vitamin E or of biotin, or a combination of the two, may have produced this effect. With the possible exception of a reduction in the amount of biotin made available to the animal as a result of feeding sulfite, our results support the finding of Kline (16) who stated that only thiamine of the vitamin B complex was destroyed during the process of treating dried yeast with a solution of sodium sulfite.

Testicular atrophy, renal tubular calcified casts and gastric squamous epithelial hyperplasia had a complex etiology in this study, with inanition, vitamin deficiency and perhaps direct sulfite toxicity all being partially responsible for the conditions observed. Hair balls in the stomach were unusually frequent in these animals, even in the controls. They are rarely seen in control animals of other experiments and indicate some induced deficiency in all diets, as do the mild fatty degeneration of the liver and the testicular atrophy. The diarrhea observed in all rats on concentrations of sulfite of 0.1% or more was specific for the sulfite, and resulted possibly from an irritation of the gastrointestinal tract. It is difficult to draw a line of demarcation between the effects due to ingestion of the sulfite residues and those due to the consumption of a diet rendered deficient by the addition of sulfite. Contrary to our expectations neither sulfate nor the sulfide showed any laxative action.

SUMMARY

1. Sulfite in concentrations of 0.1% (615 p.p.m. as SO_2), or more, in the diet was toxic to rats. Amounts of sulfite less than 0.1% (615 p.p.m. as SO_2) in the diet had no significant effect on growth. The rats on the 0.05% (308 p.p.m. as SO_2) sulfite containing diet grew more rapidly and had greater gains in weight for the first 12 weeks; however, these gains were not maintained up to the end of the year.

2. There was a definite trend toward smaller average weights and smaller gains in weight of the animals as the sulfite in the diet increased from 0.1% to 2%.

3. Rats fed diets containing sulfite with the addition of thiamine given hypodermically showed growth curves very similar to those of rats on these same diets without the added thiamine.

4. The addition of sulfite to the diets produced deleterious effects over and above those produced by the removal of the main source of thiamine.

5. Sulfite in concentrations of 0.25% (1538 p.p.m. as SO_2), or more, decreased the average survival time.

6. The lowest level of sulfite in the diet which produced histopathological changes was 0.1% (615 p.p.m. as SO_2).

7. The gross and microscopic pathological changes in the rats on 0.25% (1538 p.p.m. as SO_2) or more, of sulfite in the diet included stunting of growth, clinical polyneuritis, "spectacle" eyes, bleached incisor teeth, brown uteri, atrophy of various viscera, calcified renal tubular casts, atrophy of bone marrow and bone, and less frequently focal myocardial necrosis and fibrosis and gastric squamous epithelial hyperplasia.

8. Aging of the sulfite-containing diets caused a decrease in growth rate during the first 12 weeks and an increase in the incidence of lesions of the teeth and uteri, while it had no significant effect on the incidence of polyneuritis.

9. Sulfate and sulfide in the concentrations used in this experiment produced no growth or histopathological changes.

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Analyst 69 (1944)

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Notes

THE DETERMINATION OF SULPHUR DIOXIDE IN GELATIN

The published methods stipulate soaking the gelatin until swollen in water containing acid and then removing the sulphur dioxide from the boiling liquid in a current of carbon dioxide, oxidising it in the distillate, and determining it either volumetrically (iodimetric or acidimetric) or gravimetrically ($BaSO_4$). The soaking of the gelatin and the slow distillation add considerably to the time needed for the determination. In industrial laboratories where many samples may have to be tested in a day there is need for a simple, rapid and accurate method. The following method has been found to meet these requirements. It depends for its success on (1) rapid heating of the liquid to b.p. by a current of steam; (2) rapid expulsion of air from the flask by the steam; (3) the fact that solution of the gelatin is delayed until the liquid is at, or nearly at, b.p., when the air will have been swept from the flask by the steam. Thus the sulphur dioxide is held by the gelatin until the latter begins to dissolve at the boiling-point.

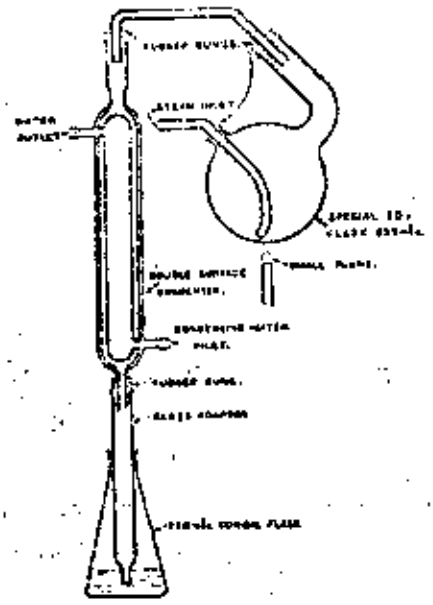

Fig. 1

METHOD—*Reagents*—Sulphuric acid: 50 ml of H_2SO_4 (sp.gr. 1·84) diluted to 250 ml with water; hydrogen peroxide: 10 vol. strength; sodium hydroxide, *N*/20; indicator: 1 g of methyl orange and 1 g of Xylene Cyanol FF, dissolved in 500 ml of 50% alcohol.

Apparatus—A special flask (500 ml), round-bottomed, with long neck bent at a right angle and hole in bulb, with fittings (see Fig. 1); double surface condenser (10 in.); a 10-ml burette reading to 0·05 ml; a conical flask (250 ml) as receiver.

Procedure—Place 75 ml of water in the flask without wetting the neck, add 20 g of the gelatin (broken sheet or powder) followed by 25 ml of the dil. sulphuric acid, and swirl. Fit the flask to the condenser, put 20 ml of hydrogen peroxide (neutralised, if necessary, to the indicator) in the receiver, and adjust the receiver so that the end of the adapter is covered by the liquid. Immediately pass a rapid current of steam into the flask and collect 100 ml of distillate within 10 min., assisting the distillation by heating the flask with a small flame. Titrate the distillate with *N*/20 sodium hydroxide, using the indicator recommended, to a neutral-grey end-point (1 ml = 0·0016 g of SO_2 or 80 p.p.m.)

NOTES—(1) Sulphuric acid is not carried over by entrainment with the steam.

(2) Addition of sulphuric acid in excess of the quantity stated gives slightly high results, possibly owing to slight charring of the organic matter, with consequent reduction of the H_2SO_4 to SO_2.

(3) The use of phosphoric acid in place of sulphuric acid yields low results.

(4) Hydrochloric acid is unsuitable, as it is carried over with the steam. It can, however, be used if the sulphur dioxide is received in *N*/20 iodine and determined either volumetrically or gravimetrically; if volumetrically, the receiver must be stoppered and fitted with a trap containing glass-wool moistened with potassium iodide soln.

(5) The end-point can be judged with an accuracy of at least 0·1 ml. This corresponds with 8 p.p.m. of sulphur dioxide.

(6) Results agree well with those obtained by the gravimetric method in which hydrochloric acid and carbon dioxide are used.

(7) The method is satisfactory for gelatin in sheet or powder form.

(8) It is important to bend the end of the steam delivery tube so that the flow of steam into the liquid is directed obliquely against the walls of the flask. This gives a rotatory motion to the liquid, reduces frothing and prevents splashing.

The special "SO_2 flask" may be obtained from Messrs. Stanley Belcher & Mason, Ltd.

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OXIDATION OF SULPHUR COMPOUNDS WITH IODINE IN ALKALINE SOLUTION

ALTHOUGH the oxidising power of hypiodites has long been known, their use for the volumetric determination of sulphur compounds does not seem to have been recently investigated. Methods requiring back-titration have been described for sugars (Hinton and Macara, ANALYST, 1924, 49, 2), formaldehyde (Romijn, Abst. ANALYST, 1897, 22, 221) and thiourea (Cuthill and Atkins, *J. Soc. Chem. Ind.*, 1937, 56, 57), but inorganic sulphides are usually determined with iodine in acid solution, when $S'' \equiv 2I$. The advantages of using iodine in alkaline solution are that $S'' \equiv 8I$, that the titration of alkali soluble sulphides is possible, and that the reagent, iodine in potassium iodide, is stable compared with hypochlorites and hypobromites.

ARSENIC—Oxidation of As_2S_3 to arsenate and sulphate occurs in strongly alkaline soln. (above about 4 *N*), so that $As \equiv 14I$. Methods involving back-titration necessitate acidification of the reaction mixture and the heat developed tends to cause loss of iodine. This is accentuated by the effervescence upon subsequent addition of sodium bicarbonate, whilst sodium acetate gives variable results. After a large number of oxidation-reduction indicators and dyestuffs of various types had been unsuccessfully tested, I found that a 0·02% soln. of aligarin or quinalizarin in dilute sodium hydroxide soln. could be used as an internal indicator permitting direct titration. As an alkaline quinalizarin soln. is soon decolorised in air, alizarin is to be preferred.

Z. Lebensm.-Unters.-Forsch. 140 (1969)

Zur Bestimmung geringer Mengen schwefliger Säure in Lebensmitteln

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Eingegangen am 20. August 1968

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Determination of Sulfurous Acid in Small Quantities in Foods

Summary

An indirect and a direct method for determination of sulfur dioxide in low concentrations (1—4 μg/25 ml) are described. The indirect method can be used for at least 1 mg sulfur dioxide to be destillated and showed a standard deviation of 2—4% (potatoes 10%). For the direct method the standard deviation was 1—6%; the latter therefore is considered to be especially suitable.

Zusammenfassung

Es wird eine Arbeitsvorschrift zur Bestimmung geringer Konzentrationen (etwa 1—40 μg/25 ml Untersuchungslösung) Schwefeldioxid mit Pararosanilin angegeben.

2. An reinen Sulfitlösungen wird das Pararosanilinverfahren sowohl bei direkter als auch indirekter Anwendung (Bestimmung des Schwefeldioxids nach destillativer Abtrennung) überprüft.

3. Das indirekte Verfahren läßt sich einsetzen, wenn die zu destillierenden Schwefeldioxidmengen wenigstens 1 mg betragen.

4. Die Freisetzung und destillative Abtrennung des Schwefeldioxids aus Lebensmitteln wird in einer modifizierten Reith-Willems-Apparatur vorgenommen, wobei ein Zusatz von Ascorbinsäure empfohlen wird.

5. Das direkte Verfahren eignet sich für die Bestimmung von Schwefeldioxid in Fruchtsäften, Weinen, Pektin, Sultaninen, Trockenaprikosen und Stärkesirup; seine Anwendung bei Kartoffeln, Meerrettich, Marmelade und Gelatine führte zu unbefriedigenden Ergebnissen.

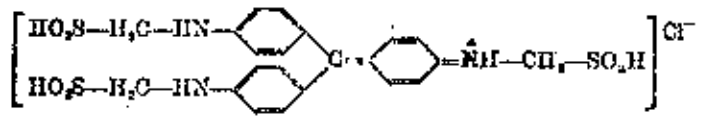
6. Die Standardabweichungen der Schwefeldioxidbestimmung mit Pararosanilin in Lebensmitteln betragen beim direkten Verfahren 1—6%, beim indirekten Verfahren 2—4% (Kartoffeln 10%).

7. Die direkte Bestimmung ist einfach sowie schnell durchführbar und eignet sich besonders für die Erfassung geringer Mengen.

Schwefeldioxid wird in großem Umfang zur Oberflächenkonservierung von Lebensmitteln, Unterbrechung der Gärung und zur Hemmung enzymatischer und nichtenzymatischer Bräunungsprozesse, insbesondere bei Obst- und Gemüseerzeugnissen, eingesetzt. Die Anwendung von Schwefeldioxid birgt aber sowohl die Gefahr einer nicht unwesentlichen Zerstörung bzw. Inaktivierung von wertvollen Lebensmittelinhaltsstoffen (z. B. Vitamin B_1) (1, 2) in sich als auch bei mengenmäßig beachtlichem Verzehr derselben eine in Erwägung zu ziehende toxische Wirkung auf den menschlichen Organismus (3).

Der quantitativen Erfassung von schwefliger Säure kommt somit in der Lebensmittelanalytik große praktische Bedeutung zu. Über die Möglichkeiten zur Bestimmung von Schwefeldioxid in Lebensmitteln haben u. a. JOSLYN u. BRAVERMAN (4), KRÖLLER (5) sowie SCHROETER (6) zusammenfassend berichtet. Auf Grund unserer Literaturrecherchen (7, 8) erschien es uns interessant und lohnend, das auf den Arbeiten von STEIGMANN (9) und GRANT (10) aufbauende, von WEST u. GAEKE (11) zur Schwefeldioxidbestimmung in Luft angewendete Pararosanilinverfahren eingehend auf seine Brauchbarkeit zur Bestimmung geringer Mengen Schwefeldioxid in Lebensmitteln zu untersuchen. Im Prinzip handelt es sich bei diesem Verfahren, das sich gegenüber anderen Methoden durch seine relativ hohe Spezifität auszeichnet, um

die Anlagerung von Formaldehyd und Schwefeldioxid an mit Salzsäure entfärbtes Pararosanilin zu einem rotvioletten Farbstoff [Absorptionsmaximum bei 570 nm] (12).



I. Untersuchungen an reinen Sulfitlösungen

1. Direkte Schwefeldioxidbestimmung

Auf Grund systematischer Untersuchungen (7) mit dem Ziel, die Pararosanilinmethode von West u. Gaeke (11) im Hinblick auf Empfindlichkeit, größtmögliche Stabilität der Reagentien und Einfachheit in der Ausführung zu verbessern, ist die nachfolgende Arbeitsvorschrift entwickelt worden.

Arbeitsvorschrift

1—5 ml der zu untersuchenden Probe (1—40 µg Schwefeldioxid werden in ein 25 ml-Meßkölbchen, das 10 ml Quecksilberchlorid-Stabilisatorlösung enthält, pipettiert. Dann gibt man 4 ml Formaldehyd-Reagens und 5 ml Pararosanilinlösung zu und füllt mit dest. Wasser auf. Eine Blindprobe wird parallel hergestellt. Die Proben bleiben bei Zimmertemperatur verschlossen stehen. Nach 25 min ist die maximale Farbtiefe erreicht, die nach 40 min langsam wieder abnimmt. Die Messung der Lichtabsorption erfolgt innerhalb dieser 15 min mit dem VSU 1 der Firma Carl Zeiss, Jena, bei 570 nm.

Reagentien

Pararosaniliureagens. 100 mg Pararosanilinhydrochlorid DAB 6 werden mit 200 ml bidest. Wasser in einen 1 l-Meßkolben überführt. Man setzt sofort 100 ml verd. Salzsäure (80 ml konz. Salzsäure + 80 ml dest. Wasser) zur Entfärbung zu und füllt mit bidest. Wasser bis zur Marke auf. Die Salzsäurekonzentration beträgt jetzt 6%. Vor seiner Benutzung bleibt das Reagens über Nacht stehen und wird weiterhin in dunkler Flasche aufbewahrt.

Formaldehydreagens. 10 ml 36%igen Formalins werden mit bidest. Wasser auf 1 l aufgefüllt. Der Reagenstiter darf um nahezu 50% abnehmen (entsprechend einem zu fordernden Gehalt von 0,021—0,037% Formaldehyd in der Meßlösung), ohne daß das Analysenergebnis wesentlich beeinflußt wird. Die umständliche tägliche Titerkontrolle kann man dadurch umgehen, daß man alle 3 Wochen eine frische Lösung herstellt und diese im Dunkeln aufbewahrt.

Quecksilberchlorid-Stabilisatorlösung. 27,2 g Quecksilber(II)-chlorid pro anal. und 11,7 g Natriumchlorid pro anal. werden in bidest. Wasser gelöst und auf 1 l aufgefüllt.

0,1 n-Sulfitlösung. Die Lösung wird aus Natriumdisulfit ($Na_2S_2O_5$) mit bidest. Wasser hergestellt und im Dunkeln aufbewahrt. Der Titer nicht stabilisierter Lösungen muß täglich jodometrisch bestimmt werden.

0,1 n-Jodlösung.

0,1 n-Natriumthiosulfatlösung.

Beim Verdünnen der Sulfitstammlösung mit bidest. Wasser zum Aufstellen der Eichkurve wird zur Stabilisierung Natriumtetrachloromercurat (12 ml/100 ml Sulfitlösung) zugegeben.

Die Eichkurve bildet im Bereich bis 40 µg Schwefeldioxid in 25 ml Lösung eine Gerade (Standardabweichung ± 2%). Für höhere Konzentrationen ist das Lambert-Beersche Gesetz nicht mehr erfüllt. Alle Proben müssen deshalb vor der Farbreaktion in diesen Bereich verdünnt werden. Ein Verdünnen nach bereits erfolgter Farbreaktion führt zu falschen Resultaten.

Die Empfindlichkeit des Verfahrens ist außerordentlich groß. Da die zu messenden Proben grundsätzlich nur einen Schwefeldioxidgehalt bis 40 µg in 25 ml aufweisen dürfen, kann bei sulfitreichen Substraten durch entsprechendes Verdünnen u. U. ein relativ großer Fehler entstehen, so daß es in derartigen Fällen vorteilhafter sein dürfte, weniger empfindliche Methoden zur Bestimmung heranzuziehen. Das beschriebene Pararosanilinverfahren ist also bevorzugt für die Erfassung geringer Mengen Schwefeldioxid geeignet.

2. *Schwefeldioxidbestimmung nach destillativer Abtrennung*

Da in manchen Lebensmitteln auf Grund ihrer Eigenschaften und Inhaltsstoffe (z. B. ungenügende Löslichkeit, schwer entfernbare Trübstoffe) eine direkte Schwefeldioxidbestimmung nicht möglich ist, muß in derartigen Fällen die schweflige Säure durch Destillation vor der eigentlichen Bestimmung abgetrennt werden. Für diese destillative Abtrennung eignet sich die Apparatur nach REITH u. WILLEMS (13), wenn sie statt mit einem Rezipienten mit mindestens 2 verbunden ist, da andernfalls die Absorption des Schwefeldioxids nicht vollständig ist (7).

Die nachfolgende Arbeitsvorschrift hat sich auf Grund der eigenen Erfahrungen zur destillativen Abtrennung des Schwefeldioxids aus reinen Sulfitlösungen bestens bewährt.

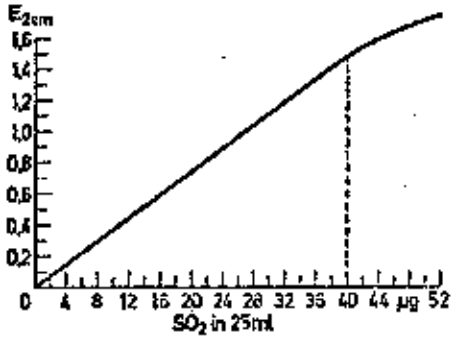


Abb. 1

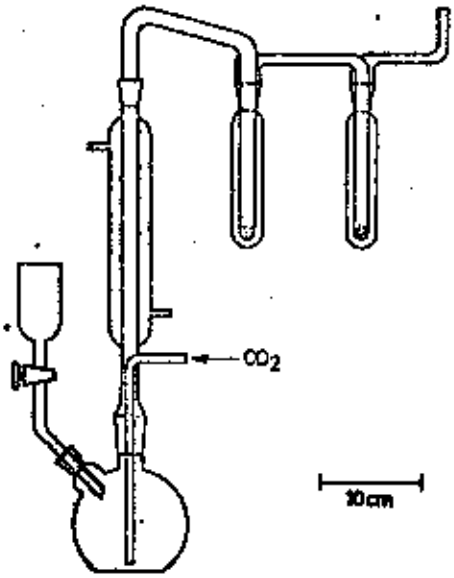


Abb. 2

Abb. 1. *Eichkurve zur Schwefeldioxidbestimmung mittels Pararosanilin*

Abb. 2. *Modifizierte Apparatur nach* REITH-WILLEMS *zur destillativen Abtrennung von Schwefeldioxid*

Arbeitsvorschrift

In die beiden Absorptionsvorlagen der Apparatur werden je 15 ml Quecksilberchlorid-Stabilisatorlösung und in den Stehkolben 50 ml bidest. Wasser gefüllt. Danach wird 15 min Kohlendioxid über eine Waschflasche mit Kaliumpermanganatlösung durch die Apparatur geleitet, der Gasstrom unterbrochen und die sulfithaltige Lösung durch den Trichter in die Apparatur gegeben. Nun wird so viel 15%ige Salzsäure zugesetzt, daß die Gesamtlösung 1,8% Salzsäure enthält (die Salzsäure läßt sich prinzipiell durch Phosphorsäure ersetzen). Nach Einstellen des Kohlendioxidstromes auf eine Blasenzahl von 50—70/min wird unter gleichmäßigem Erhitzen des Destillationskolbens 20 min destilliert. Der Inhalt der Absorptionsvorlagen wird in einen 100 ml-Meßkolben gespült. Nach dem Verdünnen in den Meßbereich der Eichkurve wird die Farbreaktion ausgeführt.

Tab. 1 zeigt, daß die Schwefeldioxidbestimmung durch eine dazwischengeschaltete Destillation praktisch nicht beeinträchtigt wird. Die Stabilität des Schwefeldioxids während der Destillation ist durch eine Kohlendioxidatmosphäre gesichert. Für eine quantitative Absorption sind zwei Rezipienten mit Brausevorrichtung ausreichend (Fritteneinsätze setzen dem schwachen Kohlensäurestrom einen zu großen Widerstand entgegen).

Die Reproduzierbarkeit der durch Destillation erhaltenen Werte ist gut und weicht nicht signifikant von der der direkten Bestimmung ab. Bei Einwaagen von 1—30 mg

Schwefeldioxid ist mit Sollwertabweichungen von maximal ± 9 % zu rechnen. Bei sehr kleinen zu destillierenden Sulfitmengen wird das Ergebnis unsicher. Die untere Grenze ist mit etwa 1 mg Schwefeldioxid festzulegen. Bei Destillation von Schwefeldioxidmengen unter 1 mg werden auch die Sollwertabweichungen größer, während die der direkten Bestimmung selbst bei 0,03 mg Schwefeldioxid nur etwa ± 4 % betragen.

Tabelle 1. *Wiedergefundenes Schwefeldioxid nach destillativer Abtrennung mit Salzsäure aus reinen Sulfitlösungen*

Schwefeldioxid eingesetzt mg	Schwefeldioxid wiedergefunden %
25,6	99,2
22,8	100
16,6	99,4
13,6	101,4
9,2	105,4
2,9	108,2
1,5	96,6
0,3	89

II. Untersuchungen an Lebensmitteln

Die Auswahl der Lebensmittel erfolgte anhand der Konservierungsmittelanordnung [DDR-GBl. II 13 (1967)] und entsprechender TGL-Normen, wobei nur solche Lebensmittel herangezogen werden, die auch tatsächlich in der Praxis mit Schwefeldioxid behandelt werden. Folgende Lebensmittel werden untersucht: Fruchtsäfte Rot- und Weißweine, flüssiges Pektin, Konfitüren und Marmeladen, Trockenobst, geriebener Meerrettich, Stärkesirup und Gelatine, außerdem werden noch laugengeschälte Kartoffeln eingesetzt.

Tabelle 2. *Wiedergefundenes Schwefeldioxid vom zugesetzten in ungeschwefelten Lebensmitteln (Bestimmung nach destillativer Abtrennung)*

Lebensmittel	Schwefeldioxid zugesetzt mg	Schwefeldioxid wiedergefunden %
Pflaumenmost	25,1	99,2
	19,5	94,5
	13,4	96,3
	5,3	102,7
	1,5	92,0
Apfelmost	26,9	96,8
	20,6	99,0
	11,78	98,3
	8,8	91,5
	2,94	90,5
Apfelwein	14,3	99,3
	11,4	101,8
	10,3	95,6
	8,3	100,0
	5,67	96,9
Obstpektin, flüssig	27,6	92,7
	6,1	103,3
	5,1	101,5
	3,0	96,4
Meerrettich, frisch	14,3	94,4
	10,8	99,1
	7,2	102,8
	5,5	93,6
Kartoffeln, laugengeschält	13,9	94,0
	13,4	95,5
	10,4	89,5
	10,4	88,5

1. *Bestimmung des Schwefeldioxids nach destillativer Abtrennung*

Zunächst werden Versuche zur Schwefeldioxidbestimmung nach destillativer Abtrennung mit Salzsäure durchgeführt; der Anteil aus Anthocyan-Schwefeldioxid-Additionsverbindungen wird hierbei miterfaßt (14). Die für Modell-Lösungen mitgeteilte Arbeitsvorschrift (vgl. Abschnitt I, 2) gilt grundsätzlich für alle Lebensmittel mit dem Hinweis, daß 1–40 mg Schwefeldioxid destilliert werden und die Destillationszeit 45 min beträgt. Abweichungen davon werden an den entsprechenden Stellen vermerkt.

Die Untersuchungen werden an ungeschwefelten und industriell geschwefelten Lebensmitteln – teilweise unter weiterem Zusatz von Schwefeldioxid – durchgeführt. Die hier angegebenen Werte sind als repräsentativ aus über 300 Einzelbestimmungen ausgewählt worden. Auf Grund von Literaturangaben (5) und orientierender Vorversuche hat es sich als zweckmäßig erwiesen, zur Stabilisierung des Schwefeldioxids bei Marmeladen und Kartoffeln vor der Destillation eine Spatelspitze Ascorbinsäure zuzusetzen. Die untersuchten Marmeladen, Konfitüren, Rosinen sowie die Gelatine werden vor der Destillation nicht zerkleinert, während die Kartoffeln gestückelt und der Meerrettich gerieben werden.

Tabelle 3. *Wiedergefundenes Schwefeldioxid vom zugesetzten in geschwefelten Lebensmitteln (Bestimmung nach destillativer Abtrennung)*

Lebensmittel	ursprünglicher Schwefeldioxidgehalt mg/kg	Schwefeldioxid zugesetzt mg/kg	Schwefeldioxid wiedergefunden %
Sultaninen			
A	320	289	99,7
	320	250	95,3
B	460	470	95,2
	460	500	96,9
Heidelbeermarmelade	142	100	93,4
	142	160	94,0
Heidelbeerkonfitüre			
A	90	114	100,0
	90	114	98,7
B	98	74	99,2
	98	72	95,8
C	110	115	95,3
	110	115	98,2
Aprikosenmarmelade	8	100	91,7
	8	100	100,0

Zusammenfassend zeigen die durchgeführten Untersuchungen, daß die Bestimmung geringer Mengen schwefliger Säure nach destillativer Abtrennung mittels Salzsäure in der modifizierten Apparatur nach Reith u. Willems (13) in Lebensmitteln mit der Pararosanilinmethode zu empfehlen ist. Zugesetztes Schwefeldioxid wird mit 88,3–103,3 % wiedergefunden. Die größte Standardabweichung ist mit ± 4 % und der Streubereich bei 95 % Sicherheit mit maximal ± 8,5 % anzusetzen. Lediglich bei laugengeschälten Kartoffeln weichen die Werte stärker ab (wiedergefunden 88,5 % bis 95,5 %; Standardabweichung ± 10 %; Streubereich ± 22,5 %). Bei allen Lebensmitteln kann zur Stabilisierung des Schwefeldioxids ein Zusatz von Ascorbinsäure empfohlen werden, da andernfalls u. U. zu niedrige Werte (z. B. bei Kartoffeln und Marmeladen) erhalten werden.

Tabelle 4. *Schwefeldioxidgehalt ungeschwefelter und geschwefelter Lebensmittel (Bestimmung nach destillativer Abtrennung)*

Lebensmittel		Schwefeldioxidgehalt
ungeschwefelte Lebensmittel		
Pflaumenmost		0
Apfelmost		0
Apfelwein		0
Obstpektin, flüssig		0
Meerrettich		0
Kartoffeln		0
geschwefelte Lebensmittel		
Bulgarischer Rotwein	A	42 mg/l
	B	53 mg/l
	C	114 mg/l
Obstpektin, flüssig	A	11 mg/kg
	B	19 mg/kg
	C	217 mg/kg
	D	13 mg/kg
Meerrettich	A	172 mg/kg
	B	277 mg/kg
Kartoffeln (laugengeschält)	A	38 mg/kg
	B	42 mg/kg
Sultaninen	A	320 mg/kg
	B	490 mg/kg
	C	903 mg/kg
	D	210 mg/kg
Trockenaprikosen		2100 mg/kg
Heidelbeermarmelade		143 mg/kg
Aprikosenmarmelade		8 mg/kg
Heidelbeerkonfitüre	A	90 mg/kg
	B	95 mg/kg
	C	110 mg/kg
Speisegelatine		401 mg/kg

2. *Direkte Schwefeldioxidbestimmung*

Die Schwefeldioxidbestimmung nach destillativer Abtrennung ist zwar universell anwendbar, doch sind diesem Verfahren hinsichtlich seiner Empfindlichkeit Grenzen gesetzt. Geringe Sulfitkonzentrationen können z. T. vorteilhafter direkt mit der Pararosanilinmethode bestimmt werden.

Ziel der nachfolgenden Untersuchungen ist festzustellen, bei welchen Lebensmitteln eine direkte Schwefeldioxidbestimmung möglich ist. Anorganisch gebundenes und freies Schwefeldioxid werden in den Proben direkt ermittelt, während organisch gebundenes Schwefeldioxid vorher mit Lauge zum entsprechenden Sulfit umgesetzt werden muß.

Arbeitsvorschrift zur direkten Bestimmung des Gesamt-Schwefeldioxidgehaltes

10 ml des Lebensmittels (verdünnte Probe bzw. wäßriger Auszug), in denen sich 30—300 µg Schwefeldioxid befinden sollen, werden in einem 100 ml-Meßkolben nacheinander mit 5 ml Quecksilberchlorid-Stabilisatorlösung und 5 ml 0,1 n-Natronlauge versetzt. Nach 30 sec wird mit 5 ml 0,1 n-Schwefelsäure neutralisiert und mit dest. Wasser zur Marke aufgefüllt. 10 ml dieser Lösung werden mit 4 ml Formaldehydlösung und 5 ml Pararosanilinreagens versetzt und auf 25 ml aufgefüllt. Nach 30 min wird die gefärbte Lösung gegen einen Blindwert (A) (10 ml Stabilisatorlösung, 4 ml Formaldehydlösung, 5 ml Pararosanilinreagens und 6 ml dest. Wasser) gemessen.

Bei gefärbten Lebensmitteln empfiehlt es sich, auf Grund eigener Untersuchungen zur Herstellung eines Blindwertes (B) wie folgt zu verfahren:

10 ml der Probe werden zur Entfernung des Schwefeldioxids mit 10 ml 0,1 n-Schwefelsäure versetzt und 15 min zum Sieden erhitzt. Man läßt abkühlen, neutralisiert mit 10 ml 0,1 n-Natron-

lauge, füllt mit bidest. Wasser auf 100 ml auf und verfährt mit 10 ml dieser Lösung wie im Hauptversuch (vgl. o.). Der zwischen den Blindwerten A und B gemessene Extinktionsunterschied wird bei gefärbten Lebensmitteln in Abzug gebracht.

Tabelle 5. Wiedergefundenes Schwefeldioxid vom zugesetzten in ungeschwefelten Lebensmitteln (Direkte Bestimmung)

Lebensmittel	Schwefeldioxid zugesetzt mg/100 ml	Schwefeldioxid wiedergefunden %
Pflaumenmost	0,28	92,9
	0,85	101,3
	1,41	100
	1,97	97,5
	2,63	98,4
Apfelwein	0,59	98,3
	0,89	96,2
	1,19	100,8
	1,48	101,4
Obstpektin, flüssig	5,38	100,5
	6,80	99,5
	8,23	90,4
	10,00	99,0
Capillärsirup	7,1	108,5
	7,1	106,3
	10,34	93,6
	10,34	100,1
	14,10	102,8
	14,10	102,1

Tabelle 6. Wiedergefundenes Schwefeldioxid vom zugesetzten in geschwefelten Lebensmitteln (Direkte Bestimmung)

Lebensmittel	ursprünglicher Schwefeldioxidgehalt	Schwefeldioxid zugesetzt	wiedergefunden %
Bulgarischer Rotwein B	52,4 mg/l	11,2 mg/l	94,0
	52,4 mg/l	16,5 mg/l	98,8
Sultaninen D	200 mg/kg	250 mg/kg	103,4
	200 mg/kg	250 mg/kg	98,9
Trockenaprikosen	2070 mg/kg	3060 mg/kg	105,9
	2070 mg/kg	3060 mg/kg	93,7

Tabelle 7. Direkte Bestimmung von Schwefeldioxid in Wein nach der Pararosanilin- und Jodmethode

	Gesamtes Schwefeldioxid		Freies Schwefeldioxid	
	Pararosanilinmethode mg/l	Jodmethode mg/l	Pararosanilinmethode mg/l	Jodmethode mg/l
Bulgarischer Weißwein	97,2	85,7	6,8	7,1
Sowjetischer Bowlenwein	212	180	8,0	8,3

Eine direkte Schwefeldioxidbestimmung in *Trockenobst* mittels Pararosanilin wurde bereits von NURY u. BOLIN (13) beschrieben. Bei den eigenen Versuchen an Sultaninen und Trockenaprikosen wird auf eine Feinzerkleinerung des Untersuchungsmaterials verzichtet.

Tabelle 8. *Vergleich des indirekten und direkten Verfahrens zur Schwefeldioxidbestimmung mittels Pararosanilin in Lebensmitteln*

Lebensmittel		Schwefeldioxidgehalt nach destillativer Abtrennung	Schwefeldioxidgehalt direkt
ungeschwefelte Lebensmittel			
Pflaumenmost		0	0
Apfelmost		0	0
Apfelwein		0	0
Obstpektin, flüssig		0	0
geschwefelte Lebensmittel			
Bulgarischer Rotwein	B	53 mg/l	52 mg/l
	C	114 mg/l	122 mg/l
Obstpektin, flüssig	C	217 mg/kg	195 mg/kg
	D	18 mg/kg	16 mg/kg
Sultaninen	D	210 mg/kg	209 mg/kg
Trockenaprikosen		2100 mg/kg	2070 mg/kg

Tabelle 9. *Reproduzierbarkeit der Ergebnisse bei der Bestimmung von schwefliger Säure mittels Pararosanilin in Lebensmitteln*

Lebensmittel	Schwefeldioxid-konzentration mg/100 ml	Standard-abweichung %	Streubereich bei 95% statistischer Sicherheit %
Direktbestimmung			
Apfelsaft	1—12	± 2,8	± 4
Heller Apfel- bzw. Traubenwein	10(0,6*)	± 4,0(± 2,3*)	± 2,4(± 4,6*)
Rotwein	1—5	± 4,3	± 9
Obstpektin, flüssig	8—20	± 2,5	± 5,4
Stärkesirup	7—14	± 1,2	± 2,6
Sultaninen	20	± 5,7	± 14
Trockenaprikosen	200	± 2,7	± 0,6
Bestimmung nach Destillation			
Moste	3—50	± 3,6	± 8,2
Weine	4—5	± 2,9	± 4,5
Obstpektin, flüssig	1—2	± 3,0	± 7
Sultaninen	30—90	± 3,0	± 0,7
Marmeladen und Konfitüren	10—30	± 2,9	± 6,1
Meerrettich	15—30	± 2,8	± 6,4
Gelatine	40—80	± 2,4	± 5,4
Kartoffeln	3—6	± 10	± 22,5

* = Werte gelten für freie schweflige Säure.

Anmerkung: Die Standardabweichung wurde im allgemeinen berechnet nach $s = \sqrt{\frac{\Sigma \Sigma (x_{ij} - \bar{x}_j)^2}{N - M}}$, der Streuwert wurde nach $x = t(F, \alpha) \cdot s$ ermittelt (17).

Die Proben (20 g) werden über Nacht unter einer Kohlendioxidatmosphäre mit einer definierten Menge Wasser (100—200 ml) gequollen. Ein aliquoter Teil der filtrierten klaren wäßrigen Lösung wird entsprechend seinem Schwefeldioxidgehalt verdünnt und dann für die Bestimmung verwendet.

Zur Bestimmung des freien Schwefeldioxids in *Weißwein* mittels Pararosanilin werden 1—2 ml Wein eingesetzt.

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In Abänderung der gebräuchlichen Arbeitsvorschrift zur jodometrischen Bestimmung des freien Schwefeldioxidgehaltes in Weißwein (16) wird mit 0,01 n-Jodlösung titriert.

Versuche, Schwefeldioxid direkt in *Marmelade, Konfitüre, Meerrettich, Gelatine* und *Kartoffeln* zu bestimmen, führen zu unbefriedigenden Ergebnissen. Die Ursachen hierfür sind primär in rein technischen Schwierigkeiten, die sich u. a. aus der ungünstigen Konsistenz sowie Löslichkeit des Probenmaterials ergeben, zu suchen.

Zusammenfassend zeigen die Untersuchungen, daß die direkte Schwefeldioxidbestimmung mit Pararosanilin bei einigen der hier eingesetzten Lebensmittel mit befriedigender Genauigkeit und Reproduzierbarkeit möglich ist. Ihr Vorteil gegenüber der Schwefeldioxidbestimmung nach destillativer Abtrennung ist in der einfachen Arbeitsweise, der Kürze der Analysendauer sowie der Tatsache zu sehen, daß wesentlich geringere Sulfitgehalte erfaßt werden können bzw. nur sehr geringe Mengen an Untersuchungsmaterial benötigt werden.

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Zur Kenntnis der antimikrobiellen Wirkung von Biphenyl und Derivaten des Biphenyls

VII. Mitteilung

Wirkung auf den Stickstoffwechsel*

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Mitteilung aus der Deutschen Forschungsanstalt für Lebensmittelchemie, München

Eingegangen am 21. Mai 1968

About the Antimicrobial Effect of Biphenyl and Its Derivates

VII. Effect on the Nitrogen Metabolism

Summary

Experiments with sodium-o-phenyl-phenolate (SOPP) and biphenyl inhibited cells of *E. coli* showed strong inhibition of glutamic dehydrogenase and weak inhibition of glutamic-oxalic-

* Die Untersuchungen wurden im Rahmen eines Grant des US. Department of Agriculture Nr.: FG-Ge-102 durchgeführt. Fräulein Chr. Waibel danken wir für die sorgfältige technische Hilfe.

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The amino-acids first react with part of the reducing sugars in the dough, notably glucose and fructose, which are the first sugars to be fermented in panary fermentation.[7] This reaction accounts for the decrease in gas production during the early stages of fermentation where the concentration of these sugars is limiting. As maltose is produced in the doughs, the amino-acids react with it to form compounds of glycosylamine type. The formation of these compounds seems to be favoured when maltose is in the 'nascent' state, i.e., when maltose is produced by β-amylase activity in the presence of amino-acids, because inclusion of a solution of glucose-maltose in synthetic doughs does not result in a depression of fermentation in the presence of amino-acids even though the water content of the synthetic doughs is low enough. The depression of fermentation is shown only when maltose is formed in the synthetic doughs by β-amylase activity. Apparently the amino-acids do not react to a noticeable degree with the maltose pre-existing in the freshly mixed dough.

It is assumed, therefore, that depression of fermentation in the presence of glycine and lysine occurs because part of the maltose formed by β-amylase activity becomes unavailable for fermentation. Although the possibility cannot be excluded that glycosylamine compounds in small amounts have a toxic effect on yeast and that osmotic effects may be contributing factors, in the opinion of the author the main reason for the depression of fermentation on addition of amino-acids to doughs is the formation of non-fermentable glycosylamine compounds.

Acknowledgments

The author wishes to thank Dr. J. W. Lee and Mr. M. V. Tracey, of the Wheat Research Unit, C.S.I.R.O., North Ryde, for the stimulating discussions during the later stages of this work and during the preparation of the manuscript, and Dr. E. F. L. J. Anet, of the Division of Food Preservation, C.S.I.R.O., Homebush, for helpful discussion. The author is grateful to Miss Carolyn Marshall for skilled technical assistance.

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Received 8 May, 1961

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ASSESSMENT OF THE 'MELLOENE' TREATMENT OF BISCUIT FLOURS

By D. M. FREELAND

A rapid chemical test for the degree of treatment of biscuit flours with 'Melloene' was sought. The reduction of the optical density of a solution of Astrazone Pink FG (Bayer) under specified conditions has been effective.

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Introduction

When a stream of wheat flour flows into an atmosphere containing sulphur dioxide and water vapour, changes in the quality of the gluten occur which cause a loss of tenacity (strength) of a dough made with it. This is the basis of the 'Melloene' treatment of flour. A similar weakening may be brought about by treatment with bisulphites. The effect of this treatment is related to the original strength of the gluten and to the concentration of sulphur dioxide applied, in terms of g. of sulphur dioxide per sack (280 lb.) of flour. Applications of up to 8 g./sack may be usual but there are cases when higher rates of treatment are desired—a quantity of up to 20 g./sack even may be indicated. Flours so weakened can be applied to the making of biscuits.

Although recently Matsumoto *et al.*[1] have discussed the effect of bisulphite on the disulphide linkage in wheat proteins and quote many previous references to work on the sulphydryl-disulphide linkage in proteins, there is no absolute explanation of the reaction the proteins undergo when so treated. The linkages of the affected groups are not permanently ruptured, for a treated flour will revert to a stronger condition within 3 weeks after treatment and by the end of this time it may regain three-quarters or more of the lost strength as indicated by the water absorption. Thus an untreated flour absorbing water to 14·6 gal./sack, and requiring 11 gal./sack after treatment, reverted in 4 weeks to show absorption of 14·2 gal./sack. The tests to be described also respond to the reversion in strength of a treated flour.

The effect of a high rate of 'Melloene' treatment results in an enhanced and peculiar curve on the 'Research' Extensometer. This instrument is suitable for finding the strength of treated flours if the usual procedure is modified. The graphs penned show the rheological properties of a dough in terms of resistance to applied stress and elongation because of it. Such tests take too long when speedy answers are desired, e.g., when variations in strength occur between bags of a delivery of flour. A rapid method of testing was desired and a chemical rather than a physical approach was preferred.

Although the lability in the combination of sulphur dioxide with flour has been mentioned, it is bound to such a degree that only a small fraction of the SO_2 applied is detected when a suspension of treated flour is steam distilled. Acid hydrolysis effects the release of SO_2 in amount comparing favourably with the amount added. An attempt to displace SO_2 from a heavily treated flour by passing dry nitrogen through a column of flour connected to a scrubber containing hydrogen peroxide and barium chloride was unsuccessful.

It was shown by Liddell[2] that the dye Astrazone Pink FG (Bayer) is capable of being reduced by very small amounts of SO_2 in the atmosphere. When an alkaline solution of the dye was applied to a treated flour, the colour changed from a full pink, through a faint pink and on to yellow as time advanced. This transformation provided a means of assessment of the degree of treatment shown by biscuit flours treated with SO_2, either as a rapid sorting test or quantitative procedure. With biscuit flours it appears there is a threshold level of treatment to about 2–4 g. of 'Melloene'/sack below which the tint of the colour is unaltered when the test is applied. With flours for bread- or cake-making the test does not respond, even with treatment with 20 g./sack. Results upon such flours are given later.

Experimental

Materials and methods

Astrazone Pink reagent.—Dissolve 0·1 g. of Astrazone Pink FG (Bayer)* in 20 ml. of ethanol (96% vol. I.M.S.) as a stock solution.

(1) *'Spot' or pellicle test*

To 5 ml. of an aqueous mixture, containing 5% by wt. of sodium bicarbonate and 24% by vol. of glycerol, add 0·1 ml. of Astrazone Pink reagent and dilute to 20 ml. with distilled water. This dilution contains 0·005% of the dye and it should show 23–26% light transmission by EEL absorptiometer, in an 0·5 cm. cell, 604 filter, against water as a blank. This solution should be freshly prepared for a series of tests, for at this dilution the colour fades in the presence of alkali.

* Obtainable from Hopkin & Williams Ltd., Chadwell Heath, Essex.

Compact the flour to be tested to present a smooth surface. (The porcelain powder trays and presser used with a Lovibond Tintometer are ideal for this purpose.) Add one drop of the test solution by means of a piece of glass tubing drawn to a capillary tip and note the time taken for the colour to fade or alter. This transformation may not be uniform; yellow at edges with pink centre may arise from a flour of medium degree of treatment. A reference standard may be obtained by spotting untreated flour. The approximate rate of treatment by 'Melloene' is indicated by the time measured for change of tint. Complete alteration to yellow in 3-5 min. is given by flour treated to the order of 16–18 g./sack; 5-10 min. by medium treatment of 5–10 g./sack.

The suitability of the flour for specific usage and variations between bags from the same milling are quickly evident if the treatment has not been evenly accepted by the flour. The test cannot be made rigidly quantitative, for the tint presented depends on variables, e.g., the nature of the protein in the wheats entering the grist; the reception of the SO_2 by the flour, which may not agree regularly with the rate of gas applied as metered on the plant; the degree of reversion ensuing after treatment, for sometimes bags of flour from an earlier milling are included in a delivery of newly milled wheat. From this it is evident that it is unlikely that correlation of the results in a comparative test will exist between a given treatment under laboratory conditions and that applied to the same degree by the miller.

(2) *Light transmission test*

Astrazone Pink Solution.—Dilute 0·5 ml. of the Astrazone Pink stock solution to 2·5 ml. with distilled water and adjust the dilution, if necessary, so that 20–23% of light is transmitted via a 606 filter (0·5 cm. cell against water) by the EEL absorptiometer.

Procedure

Place 1 g. of flour in a 15-ml. tapered polythene centrifuge tube. Add 8 ml. of distilled water and stir the flour into it with a thin glass rod having a blob at an end. Shake the mixture for 30 sec. and centrifuge at about 2000 r.p.m. for 5 min. Filter the supernatant fluid through a 5½-cm. Whatman No. 1 paper, collect 6 ml. of filtrate, and divide this between two 0·5 cm. absorptiometer cells. With 'Emil' micro-pipettes, add 0·1 ml. of 5% (w/v) sodium bicarbonate solution and 0·2 ml. of Astrazone Pink solution to one of the cells. To the other cell add only the sodium bicarbonate. With a 604 filter, take readings of the percentage light transmission in the EEL absorptiometer through both cells containing the filtrate against a 0·5 cm. cell containing water (100% light transmission). It will be found that the transmission through the coloured flour extract increases with time to a maximum and thereafter decreases gradually. The peak transmission is recorded. Near to the attainment of this reading, take a further reading of the tinted extract against the untinted filtrate set to read 100% transmission or the maximum degree thereto.

If x is the transmission of tinted cell against water blank 100%,
y the transmission of flour extract and alkali against water blank 100%,
z the transmission of tinted extract against $y = 100\%$,
then $100x/y$ should equal z if y has yielded a reading of 100%.

If y is too dense to produce a setting of 100%, expand the reading of the tinted extract by dividing it by $y/100$ to equate z.

Because of the variables associated with flour described in the 'spot' test, and the difficulty in obtaining samples of flour treated at a scale of levels from a commercial mill, it is only possible to present the results for an untreated biscuit flour as treated in the laboratory. Values of the specific recovery test of Clewlow & Dixon[3] are also tabled (Table I).

In commenting upon the results of the specific recovery test, Clewlow observes that the lower rates of treatment have a marked effect upon the recovery properties of the dough which are progressively reduced as the treatment increases to 12 g./sack. A specific recovery figure of 12 represents the shrinkage of the dough piece due entirely to the removal of moisture and not to residual elasticity of the gluten. The lower figure of 9 shown for treatment at 20 g./sack is not particularly significant and occurs because the centre of the dough piece dries out and

Table I

Effect of level of 'Melloene' treatment on Astrazone Pink transmission and specific recovery of flour

'Melloene' treatment, g./sack	Light transmission, %	Specific recovery
0	26·5	18·5
4	27	15·5
8	30	14
12	37	12·5
16	41	12
20	61	9

cracks away from the periphery. The Astrazone tests show an increase in the light transmission at the 12 g./sack level and the two tests taken together show where the change in the chemical effect of the treatment on the gluten occurs.

Judged by the results of the Astrazone tests upon flours treated in bulk, the values of the laboratory treatments are lower. Thus, biscuit flours treated commercially at the rate of 16–20 g./sack yield readings around 60–85%, those at 7 g./sack, 30–50%, and those at 4 g./sack the same as, or a little more than, the light transmitted by a blank solution of water, sodium bicarbonate and dye.

In relation to the uses of flour treated at the mill, those giving a high light transmission should result in flour suitable for hard-dough biscuits and those yielding a lower order of transmission could be used for making the softer types of biscuits.

When protein precipitants, e.g., sodium tungstate or zinc ferrocyanide, are added to the flour extract, the filtrate from it shows no significant reduction in colour intensity of the Astrazone Pink when the test procedure is applied. The colour reaction of the normal test is therefore intimately connected with further water-soluble protein brought into solution by the action of sulphur dioxide. This extra water-soluble protein is, therefore, precipitated in common with the normal water-soluble proteins of the flour prior to the testing of the filtrate.

In Table II, is recorded the transformation of insoluble to soluble protein consequent upon treatment, based on flours of 15% moisture content.

Table II

Gain in proteins of water-soluble extract as percentage of total proteins of untreated flour

Treatment, g./sack	Water-soluble proteins, % gain
16	4·62
8	2·03
7	1·30

(3) *Relation of Astrazone Pink test to extensometry of biscuit flours*

If the procedure suggested for extensometry of bread flours is followed when testing 'Melloene'-treated flours, the dough is apt to prove difficult to manipulate and the curves obtained are small in stature. By working in a room maintained at 75° F, and with alterations to the doughing and resting times, a larger curve results. With treatments of 16–18 g./sack the contour of the curve shows an exaggerated resistance for a short period which departs rapidly as stress continues. Such curves correspond to 70–80% light transmission by the Astrazone Pink test. Flours treated with 6–7 g. of 'Melloene' per sack produce graphs with a reduced initial resistance and with much the same extensibility until resistance is zero, after which extension continues. At rates below 4 g./sack, a lower degree of resistance is maintained with more extension, and E values of 23 and under are given. Some typical light transmission values of flours treated at the mill against the average resistance (R) and the extensibility (E), as the average reading at which the pen reaches the abscissa, are shown in Table III.

Thus there is no definite relation between the respective values of the chemical and physical test. The results shown were derived from samples of flour from different grists and from different millers and it is evident, therefore, that the quality of the gluten controls the ultimate

Table III

Light transmission values (Astrazone Pink test) and extensometer values for various flours treated with 'Melloene'

Light transmission, %	R	E	Light transmission, %	R	E
		Blanks (water, dye, alkali) 21–33			
82·5	260	7·8	43·5	98	6·5
76	221·8	6·1	36·5	130	9·8
75·5	215	7·1	34·5	210	23
67·5	250	8·0	33	140	20·8
64·5	258	7·5	31	175	15·6
49	85	6·0	23	100	20·3
47·5	110	7·2	21	212	24·2

results. But with knowledge of the degree of treatment expected at a mill it is possible to reconcile the values obtained in one or other of the tests with the quality of deliveries.

(4) *Reaction of bread and cake flour to the Astrazone Pink test*

In a private communication Clewlow describes a modified test with Astrazone Pink on a baker's flour treated in his laboratory with increments of 'Melloene' up to 20 g./sack. Against this are shown his results from a biscuit flour tested by the same procedure (Table IV).

Table IV

Comparative results for bread and biscuit flours after 'Melloene' treatment

Sample	Total light transmission, %		Light transmission, % after deducting blank	
	Baker's	Biscuit	Baker's	Biscuit
Blank	31·75	28	—	—
Untreated	31·75	29·5	0	1·5
Treated 4 g./sack	32·25	29·5	0·5	1·5
" 8 "	32·25	33·25	0·5	7·25
" 12 "	33	40·5	1·25	12·5
" 16 "	32·25	44·5	0·5	16·5
" 20 "	31·5	54	0·25	26

A cake flour, milled from a grist of Manitoba No. 2 Koga, Capelle and Australian wheats, was treated with 'Melloene' by Clewlow. Astrazone Pink tests by the method described and extreme extensometer results are indicated in Table V.

Table V

Effect of treatment of a cake flour treated with 'Melloene' on light transmission (Astrazone Pink test) and extensometer readings

Sample	Light transmission against water = 100%	Extensometer R	Extensometer E
Untreated	25·5	465	19·3
Treated 2 g./sack	25·25		
" 4 "	22·5		
" 8 "	23·5		
" 12 "	25·5		
" 16 "	25·5		
" 20 "	24	190	25·1

While the extensometer offers evidence of reduction in strength, no indication is afforded by the change in colour of Astrazone Pink. Hence strong flours can take treatment with more than 20 g./sack without response to the test, whereas weaker flours used for biscuits respond when a threshold value corresponding to 'Melloene' treatment of about 4 g./sack is exceeded.

Summary and conclusions

1. For biscuit flours subjected to 'Melloene' treatment a simple test based on the reduction of Astrazone Pink FG is described.

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2. The test may be applied qualitatively as a sorting test, or quantitatively, provided that it is carried out without delay to cause reversion of the extract from the sample.

3. A threshold value of about 2–4 g./sack of treatment has to be passed before appreciable values arise in the test results.

4. When the test is applied to stronger flours used by bakers there is little or no response to the test, even when as much as 20 g./sack treatment has been given to them.

5. The test therefore depends on the nature or quality of the proteins of the flour as combined with sulphur dioxide. Physical tests on strong flours show that there is a reduction in strength despite the ineffectiveness of the chemical test.

6. Regain in the strength of treated biscuit flour with the passing of time is indicated by the results of the test as also by extensometry.

7. The relation of the results of the test to those given by the extensometer for samples of flours taken from different mills applying varying rates of 'Melloene' treatment to biscuit flour is shown.

Acknowledgments

The author is indebted to Mr. G. Clewlow, of Wallace & Tiernan Ltd., for his practical assistance and comments, and to the Directors of Macfarlane, Lang & Co. Ltd., for permission to publish this account of work carried out in their Laboratory.

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CHEMICAL STUDIES ON THE HERRING *(CLUPEA HARENGUS)*. VI.—Carbonyl Compounds Formed during the Heat Processing of Herring

By R. B. HUGHES

Herring were cooked at 100° under a stream of nitrogen and the resulting vapour passed through a solution of 2,4-dinitrophenylhydrazine. The main components of the resulting mixture of 2,4-dinitrophenylhydrazones were shown to be the derivatives of acetaldehyde, propionaldehyde, isobutyraldehyde, 2-methylbutyraldehyde and acetone.

Introduction

The importance of certain carbonyl compounds as flavour components in a variety of foodstuffs is becoming increasingly apparent, but little is yet known of their nature and behaviour in fish during storage and under various processing conditions. Formaldehyde has received some attention by reason of its production from trimethylamine oxide under certain conditions. The presence of traces of formaldehyde in canned fish products has been reported by Biegler,[1] and by Lunde & Mathieson,[2] who also found traces in fresh fish. Tanikawa *et al.*[3] detected

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Kurze Originalmitteilungen

Stabilität der Bindung von SO_2 an Inhaltsstoffe des Weines

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Mitteilung aus dem Institut für Chemie und Technologie der Bundesforschungsanstalt für Lebensmittelfrischhaltung, Karlsruhe

Eingegangen am 1. August 1968

Bei den Diskussionen über die ernährungsphysiologische Bedeutung des zur Behandlung von Lebensmitteln in großem Umfange verwendeten Schwefeldioxids wird immer wieder die Frage aufgeworfen, ob das im Lebensmittel enthaltene SO_2 nicht zum Teil so stark an bestimmte Lebensmittelinhaltsstoffe gebunden ist, daß es im Magen gar nicht freigesetzt werden würde und damit seine schädigenden Wirkungen auf den Organismus nicht ausüben könne. Der Magensaft enthält bekanntlich in erheblichen Mengen freie Salzsäure, und zwar beim normalen erwachsenen Mann etwa 50 mval/l, bei der erwachsenen Frau etwa 38 mval/l. Der pH-Wert liegt im Durchschnitt bei 1,2 ± 0,3 für reinen, durch eine Magenfistel entnommenen Saft. Er beträgt 1 Std nach einer sogenannten Ewaldschen Probemahlzeit im Durchschnitt 1,7 ± 0,1. Bei Kindern liegen diese Werte im allgemeinen etwas höher (1).

Zur Austreibung des SO_2 bei seiner analytischen Bestimmung in den einzelnen Lebensmitteln werden je nach der angewendeten Methode verschieden starke Säuren verwendet. In zahlreichen Versuchen konnten wir feststellen, daß abhängig vom pH-Wert der Destillationslösung in manchen Fällen unterschiedliche Ergebnisse erhalten werden. So ergaben sich bei Anwendung einer salzsauren Destillationsflüssigkeit mit einem pH-Wert von 0,35 je nach untersuchtem Produkt häufig höhere Werte für SO_2, als wenn Phosphorsäure mit einem pH-Wert von 1,5 bis 1,6 zur Destillation verwendet wurde (2) (3).

Mit Phosphorsäure erhält man also Destillationsbedingungen, die hinsichtlich des pH-Wertes in etwa den Verhältnissen im Magen entsprechen. Mit gewissen Einschränkungen ist daher der Schluß gestattet, daß im Magen gleiche Mengen an SO_2 aus Verbindungen mit irgendwelchen Lebensmittelbestandteilen freigesetzt werden dürften wie bei der Destillation mit Phosphorsäure.

Bei der Schwefelung von Wein hat man beobachtet, daß insbesondere Weine aus edelfaulen Trauben, also sogenannte Spitzenweine, in vielen Fällen relativ hohe Mengen an Gesamt-SO_2 benötigen, damit die für die Stabilität des Weines erforderlichen Mengen an freiem SO_2 vorhanden sind. In diesem Zusammenhang wird diskutiert, ob in diesen Weinen das sogenannte Rest-SO_2 so stark an Weininhaltsstoffe gebunden ist, daß es im Magen nicht freigesetzt wird.

Wir haben deshalb insgesamt 16 Weinproben auf den SO_2-Gehalt untersucht, und zwar nach der Methode Reith-Willems (vgl. 2) unter Verwendung von Salzsäure (pH 0,35 in der Destillationslösung) und Phosphorsäure (pH 1,5—1,6 in der Destillationslösung). Es wurden nach Möglichkeit „Normal"- und Spitzenweine desselben Anbaugebietes geprüft. Die Ergebnisse dieser Untersuchungen sind in der Tabelle enthalten.

Tabelle. *SO_2-Bestimmung im Wein.* Methode nach Beyer-Willems bei verschiedenem pH-Wert in der Destillationslösung; Doppelbestimmungen

Weinbaugebiet und Weinsorten	Gehalt an SO_2/kg nach Destillation mit HCl (pH 0,35) in mg	H_3PO_4 (pH 1,5—1,6) in mg	freie schwefl. Säure [jodometrisch nach (4)]
Weinbaugebiet Rheinhessen:			
1953er Trockenbeerenauslese	118,3—117,7	109,4—108,9	
Spätlese (Jahrgang unbekannt)	227 —229	224 —225	
Weinbaugebiet Spanien:			
Art Trockenbeerenauslese (Jahrgang unbekannt)	2,5— 2,2	0,6— 0,6	nicht bestimmt
Art Mistella (Jahrgang unbekannt)	1,9— 1,9	0,0— 0,6	
Weinbaugebiet Pfalz:			
1960er aus Neuzucht, 50% Edelfäule	142,3—142	137 —136,4	
1960er aus Neuzucht, 10% Edelfäule	131,9—135,2	133,3—134	
Weinbaugebiet Nahe:			
1961er Riesling	154,8—153,1	152,0—152	17,2—17,4
1961er Botrytis-Auslese	275 —276	272 —271	23,0—23,0
Weinbaugebiet Franken:			
1962er Rieslaner Normalese	181 —180,2	175,3—175	nicht bestimmt
1962er Rieslaner Cabinet	362,5—361	335,5—336,1	38,1—37,7
1964er Rieslaner Normalese	193,2—197,7	194,5—193,2	28 —28,1
1964er Rieslaner Auslese	477 —470	464 —462	63,7—64
1965er aus Neuzucht, Normalese	324 —323	319 —318	36,8—36,5
1965er aus Neuzucht, Auslese	200,3—200,3	195,8—194,3	28,2—23,1
1966er Normalese	184 —183,3	180,5—180,1	30,9—30,7
1966er Beerenauslese	577 —573	533 —551	67,9—67

Aus den hierin eingetragenen Werten geht eindeutig hervor, daß bei der Destillation mit Phosphorsäure in allen Fällen praktisch gleiche Werte für den Gesamt-SO_2-Gehalt gefunden wurden, als wenn die Destillation mit Salzsäure pH 0,35 erfolgte.

Zusammenfassung

Diese Untersuchungen zeigen, daß bei Weinen, selbst bei solchen aus edelfaulen Trauben, die Bindung des SO_2 an Weininhaltsstoffe nicht so stark ist, als daß sie nicht bei einem pH-Wert, der dem im Magensaft vorhandenen entspricht, aufgespalten werden könnte. Demnach kann, unter der Voraussetzung, daß die Verhältnisse bei der Destillation bei pH 1,5—1,6 mit denen im Magen in etwa gleichsetzbar sind, das gesamte in Wein enthaltene SO_2 physiologisch wirksam werden. Weitere Untersuchungen sind vorgesehen.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 236, No. 6, June 1961
Printed in U.S.A.

Detection of Free Radicals Generated during Enzymic Oxidations by the Initiation of Sulfite Oxidation*

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(Received for publication, October 10, 1960)

The initiation of chain reactions by free radicals can serve as the basis for sensitive detectors of the occurrence of these radicals. Thus, the polymerization of vinyl compounds has been used to detect free radicals in Fenton's reagent (1, 2), in illuminated solutions of chlorophyll (3) and in solutions of xanthine oxidase acting on formaldehyde (4). The utility of the aerobic oxidation of sulfite as a free radical-detecting chain reaction has been demonstrated in several systems (5–7). In view of the demonstration of unpaired electrons in many enzyme-substrate systems, by the technique of electron spin resonance spectrometry (8–10), it appeared of interest to survey several oxidative enzyme systems for the ability to initiate the aerobic oxidation of sulfite when acting on their respective substrates. The nature of the sulfite-O_2 chain reaction has been described previously (6).

EXPERIMENTAL PROCEDURE

Materials and Methods—The ability of various enzymes to initiate the oxidation of sulfite when acting on their substrates was examined in Warburg respirometers in the manner previously used with milk xanthine oxidase (5). All experiments were conducted in 0.05 M potassium phosphate, pH 7.8, containing 0.005% Versene Fe-3, and the flask contents totaled 2.2 ml. Because extensive chain breaking (11) by the enzyme preparation or its substrate could mask chain initiation, systems to be tested for the initiation of sulfite oxidation were first tested for their ability to inhibit the oxidation of sulfite as initiated by the milk xanthine oxidase system. Only those enzyme and substrate combinations in which the components exhibited negligible inhibition in this system were tested for their own ability to initiate sulfite oxidation.

Moccasin venom L-amino acid oxidase was prepared according to Singer and Kearney (12). Aldehyde oxidase was prepared from rabbit liver by the procedure of Hurwitz (13) and was further purified by Dr. K. V. Rajagopalan by treatment with protamine sulfate and by adsorption-elution on alumina C_γ gel. Pig heart cytochrome oxidase was prepared according to Smith and Stotz (14). Microsomes were prepared from rabbit liver according to Mitoma, Posner, Peitz, and Udenfriend (15). Calf liver xanthine oxidase was prepared according to Kielley (16) by Bernard Jacobson, who also examined its ability to initiate sulfite oxidation. We are indebted to Dr. A. Meister and Dr. D. E. Green for their generous gifts of rattlesnake venom L-amino acid oxidase (17) and purified, soluble cytochrome oxidase, respectively. Some of the properties of this preparation were described by Criddle and Bock (18). Tyrosinase, horse-radish peroxidase, catalase, milk xanthine oxidase, and lipoxidase were products of the Worthington Biochemical Corporation. Cytochrome c was obtained from the Sigma Chemical Company and was reduced with dithionite and dialyzed against cold buffer before use. Linoleic acid was a product of the Hormel Foundation, Minneapolis, Minnesota. Suspensions in buffer were prepared immediately before use.

RESULTS

Limited Initiation of Sulfite Oxidation by H_2O_2—Before examining peroxide-producing or peroxide-consuming enzymes as initiators of sulfite oxidation, we investigated the ability of hydrogen peroxide, *per se*, to initiate sulfite autoxidation. A continuous supply of hydrogen peroxide, such as would be provided by a peroxide-generating enzyme, was simulated by placing hydrogen peroxide in the center well of Warburg vessels and permitting gaseous diffusion to carry the peroxide into the main chamber. In control experiments, the center well contained 0.05 ml of 30% hydrogen peroxide, whereas the main compartment contained 2.15 ml of buffer which was sampled and assayed for hydrogen peroxide by the iodometric method of Patrick and Wagner (19). This arrangement was found to provide a linear rate of transport of hydrogen peroxide from the center well into the main compartment over the course of 1 hour.

Hydrogen peroxide proved to be a poor initiator of the autoxidation of sulfite. The introduction, by diffusion, of 0.25 μmole of peroxide per 10 minutes resulted in the aerobic oxidation of only 0.7 μmole of sulfite in the same interval, *i.e.* about 3 sulfite ions oxidized per hydrogen peroxide molecule. In contrast, xanthine oxidase causes the oxidation of approximately 10,000 sulfite ions per molecule of xanthine oxidized under the same conditions.

Dehydrogenases and Oxidases—After correction for the effects of hydrogen peroxide generation, the L-amino acid oxidases from rattlesnake and moccasin venoms were found not to initiate the oxidation of sulfite in the presence or absence of L-leucine. Mushroom tyrosinase, with or without L-tyrosine, was also inactive. Additional enzymes which have been tested for their ability to initiate the oxidation of sulfite in the presence of their respective substrates and which were previously reported to be inactive (5) include succinic dehydrogenase, glucose oxidase,

* This study was supported in part by Contract AT-(40-1)-289 between Duke University and the United States Atomic Energy Commission and by Grant C-91 from the National Institutes of Health, United States Public Health Service.

† Senior Research Fellow, National Institutes of Health, United States Public Health Service.

amino acid oxidase, alcohol dehydrogenase, lactic dehydrogenase, and uricase.

Calf liver xanthine oxidase was found to initiate sulfite oxidation in a manner analogous to that previously observed with milk enzyme (5). Rabbit liver aldehyde oxidase, when on *N*-methyl nicotinamide, initiated sulfite oxidation as effectively as xanthine oxidase.

Metalloporphyrin Enzymes—Catalase did not initiate the

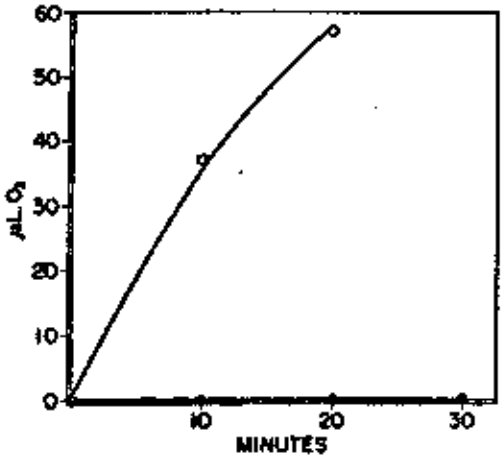


FIG. 1. Initiation of the aerobic oxidation of sulfite by azide-catalase and hydrogen peroxide. In addition to 100 μg of dialyzed catalase and 20 μmoles of sodium sulfite, each flask contained: ●——●, 0.030 ml of 30% hydrogen peroxide in the center well or 1 μmole of sodium azide; ○——○, 0.030 ml of 30% hydrogen peroxide in the center well and 1 μmole of sodium azide.

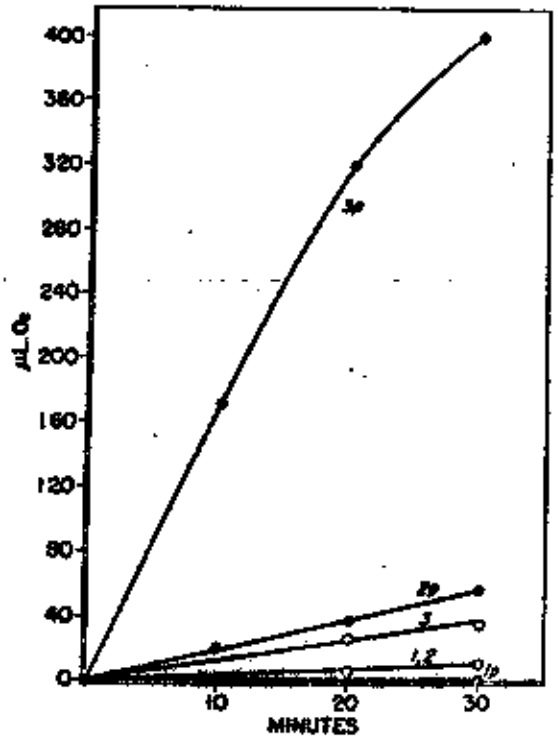


FIG. 2. Initiation of sulfite oxidation by the peroxidase system. All flasks contained 40 μmoles of sodium sulfite and the following as indicated: *1*, 0.05 ml of 10% hydrogen peroxide (center well); *2*, 5 μg of horse-radish peroxidase; *3*, 0.05 ml of 10% hydrogen peroxide (center well) and 5 μg of horse-radish peroxidase. The [illegible]tions, *1p*, *2p*, and *3p*, correspond to *1*, *2*, and *3*, but each contained in addition 5 μmoles of phenol.

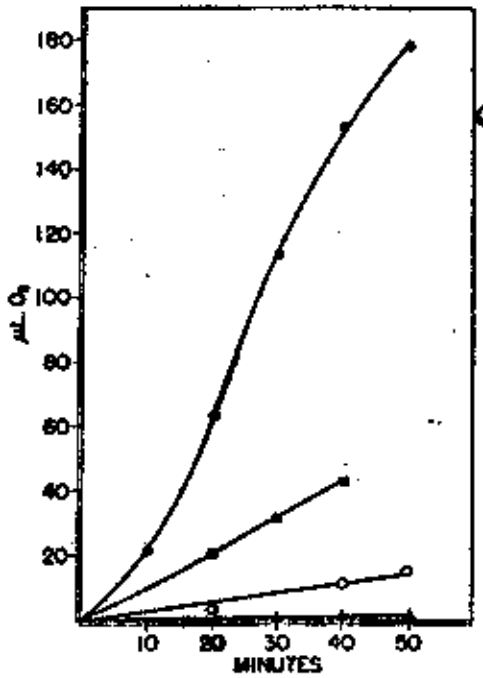


FIG. 3. Initiation of the oxidation of sulfite by the cytochrome oxidase system. Contents of the flasks were: ▲——▲, 0.035 μmole of ferrocytochrome *c* plus cytochrome oxidase; ○——○, 0.035 μmole of ferrocytochrome *c* or cytochrome oxidase, plus 20 μmoles of sodium sulfite; ●——●, 0.035 μmole of ferrocytochrome *c* plus cytochrome oxidase and 20 μmoles of sodium sulfite; ■——■, same as ●——● plus 0.08 μmole of sodium cyanide. The cytochrome oxidase catalyzed the oxidation of ferrocytochrome *c* at a rate of 0.00065 μmole per minute per flask.

oxidation of sulfite, either in the presence or in the absence of hydrogen peroxide. Azide-catalase initiated the oxidation of sulfite but only in the presence of hydrogen peroxide. These results are shown in Fig. 1. As shown in Fig. 2, horse-radish peroxidase initiated the oxidation of sulfite only in the simultaneous presence of hydrogen peroxide and a peroxidizable substrate such as phenol.

Fig. 3 indicates that neither cytochrome oxidase nor ferrocytochrome *c*, tested separately, significantly initiated sulfite oxidation, whereas vigorous sulfite oxidation was initiated during the aerobic oxidation of ferrocytochrome *c* by cytochrome oxidase. This could be demonstrated equally well with the particulate enzyme of Smith and Stotz (14) or with a highly purified soluble preparation (18). Cyanide strongly inhibited the initiation of sulfite oxidation by this system.

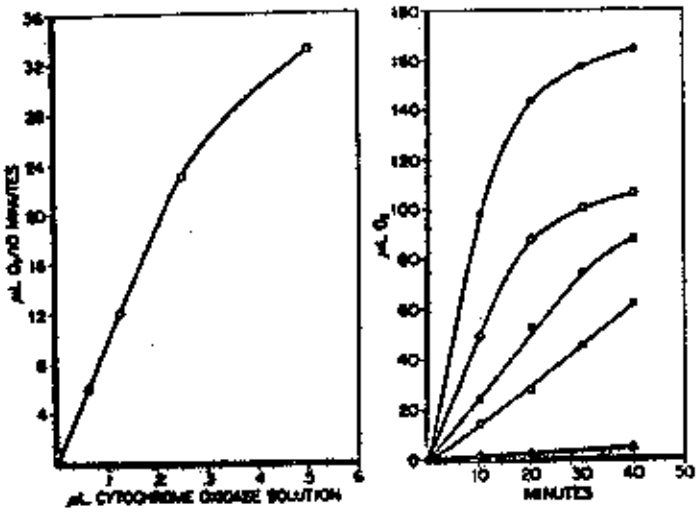
As shown in Fig. 4, the rate of sulfite oxidation was proportional to the concentration of cytochrome oxidase only at very low concentrations of the enzyme. Within the linear portion of this curve, the ratio of the atoms of oxygen consumed to the molecules of cytochrome *c* oxidized is approximately 5000. The variation of the rate of initiated sulfite oxidation with the concentration of ferrocytochrome *c* was also investigated. The half-maximal rate of oxygen uptake was observed at 2.4×10^{-6} *M* ferrocytochrome *c*. Although not readily interpretable as a binding constant (20), this result was in reasonable agreement with results obtained by conventional manometric assays of cytochrome oxidase (21).

Lipoxidase—As shown in Fig. 5, dilute suspensions of linoleic acid effectively initiated sulfite oxidation. Addition of lipoxidase which, of itself, did not initiate sulfite oxidation, greatly

augmented the rate of sulfite oxidation observed in the presence of linoleate.

Microsomes—Rabbit liver microsomes and TPNH were found to act as chain breakers in the oxidation of sulfite initiated by the xanthine oxidase test system. Of necessity, therefore, attempts to demonstrate initiation of sulfite oxidation by a microsomal hydroxylation system were performed at low concentrations of the essential components. Microsomes equivalent to 15 mg (wet weight) of liver were found to initiate the aerobic oxidation of sulfite in the presence of 4.5×10^{-4} M TPNH, in the absence of other substrates. DPNH did not effectively replace TPNH.

Mechanism of Initiation of Sulfite Autoxidation—It has previously been suggested that the product of the univalent reduction of oxygen, *viz.* the superoxide anion, $O_2^{\cdot-}$, may be the agent responsible for initiation of sulfite oxidation in enzymatic systems. That this free radical can serve in this regard was demonstrated directly by electrolytic initiation of aerobic sulfite oxidation in a Warburg vessel with sealed-in platinum electrodes. The production of $O_2^{\cdot-}$ at an inert cathode has been indicated by Kolthoff and Jordan (22). When a potential difference of 0.8 volt was impressed across the electrodes, sulfite oxidation was initiated. The rate of sulfite oxidation observed was proportional to the size of the cathode and independent of the size of the anode. As shown in Fig. 6, when the rate of sulfite oxidation was plotted as a function of the impressed voltage, a polarographic reduction wave for oxygen was obtained. Oxidative enzymes which effect the univalent reduction of oxygen, therefore, might also be expected to initiate the oxidation of sulfite under appropriate conditions.



FIGS. 4 AND 5

FIG. 4 (*left*). Relationship between the concentration of cytochrome oxidase and the initiated rate of sulfite oxidation. Each flask contained 40 μmoles of sodium sulfite, 0.017 μmole of ferrocytochrome *c* and the indicated volume of a stock cytochrome oxidase preparation which catalyzed the oxidation of 1.8 μmoles of ferrocytochrome *c* per ml per minute under these conditions.

FIG. 5 (*right*). Initiation of sulfite oxidation by the lipoxidase system. Each flask contained 20 μmoles of sodium sulfite and, except for flask △, 0.05 ml of a 0.25% suspension of linoleic acid in 0.5% crystalline bovine serum albumin. Soybean lipoxidase was present in the following amounts: ●——●, 6 μg; ○——○, 3 μg; ▣——▣, 1.5 μg; △——△ and □——□, 0.0. Control flasks, containing linoleate and 6 μg of lipoxidase, but no sulfite, exhibited no detectable oxygen uptake.

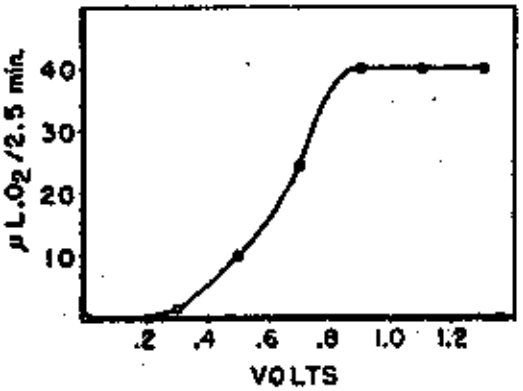


FIG. 6. Electrolytic initiation of sulfite oxidation. The indicated rates of initiated sulfite oxidation correspond to the rate of oxygen uptake observed at each impressed voltage corrected for the rate of oxygen uptake observed in the absence of an impressed voltage.

DISCUSSION

Of the flavoproteins investigated, only the xanthine oxidases of milk and calf liver and the aldehyde oxidase of rabbit liver were found to initiate the oxidation of sulfite. Beinert (23) has presented evidence for the oxidation and reduction of the prosthetic groups of several flavoenzymes by successive univalent steps with a semiquinoid form of the flavin as an intermediate.

Nevertheless, it appears that, other than xanthine oxidase and the aldehyde oxidase, the flavoproteins examined by the present technique do not generate radicals *which are capable of initiating the sulfite-oxygen chain reaction.* The uniqueness of the xanthine and aldehyde oxidases among the flavoproteins in the present series appears to relate to the possibility that their metal components may, to some extent, be involved in transporting electrons directly to oxygen. The observation that xanthine oxidase, when acting on hypoxanthine in the presence of luminol or lucigenin, causes the emission of light (24) has been confirmed in this laboratory. It appears likely that the mechanism of this induced luminescence is related to that of the initiation of sulfite oxidation and depends upon radicals which are generated by the enzyme, in agreement with the suggestions of Totter *et al.* (25).

The generation of reactive radicals by the xanthine oxidase system results in phenomena which may be readily misinterpreted. Thus, Westerfeld *et al.* (26) reported that addition of glutathione to xanthine oxidase caused enhancement of the rate of oxygen uptake, and concluded that glutathione "enhances" the activity of this enzyme. But glutathione has no effect on the activity of xanthine oxidase when assayed by spectrophotometric determination of urate production, as described by Kalckar (27). The apparent enhancement of xanthine oxidase activity by glutathione or cysteine observed in the manometric assay appears to be due to oxidation of these sulfhydryl compounds induced by the radicals generated by the enzyme system.

The iron of catalase has been shown to be in the ferric state and is thought not to be reduced by interaction with hydrogen peroxide. Strong reductants such as dithionite do not effect the reduction of this iron (28). The complete inability of catalase to initiate the aerobic oxidation of sulfite in the presence or absence of hydrogen peroxide is in accord with these observations. Keilin and Hartree (29) reported that the iron of azide-treated catalase is reduced to the ferrous state by hydrogen peroxide and that ferrous azide-catalase is readily reoxidized by oxygen. The latter reaction would be expected to generate the superoxide

ion which should initiate the oxidation of sulfite, in agreement with the observations reported above.

Peroxidase initiates the oxidation of sulfite only in the presence of both hydrogen peroxide and a peroxidizable substrate, but not in the presence of either alone. Hence, it appears likely that the interaction of peroxidase with hydrogen peroxide involves transfer of an electron pair and does not generate radicals, whereas oxidation of peroxidizable substrates by the oxidized enzyme or enzyme-peroxide complex proceeds by successive, univalent electron transfer and, therefore, generates radical intermediates. This concept is in accord with the mechanism postulated by George (30). Free radicals produced by the action of peroxidase plus hydrogen peroxide on hydroquinone or ascorbate have been detected by electron spin resonance spectrometry (10). Initiation of sulfite oxidation by linoleate and its marked enhancement by lipoxidase is in accord with suggestions that free radical intermediates occur in the autoxidation of unsaturated fatty acids (31) and in the enzyme-catalyzed oxidation (32, 33). The ability of lipoxidase to catalyze the co-oxidation of a variety of compounds when acting on linoleic acid (34–37) provides further support for the generation of radicals by this enzyme system.

Studies of hydroxylation reactions caused by model systems composed of hydrogen peroxide, ferrous salts, and ascorbate have led to the proposal that the hydroxylating species in these systems is the hydroxyl-free radical (38, 39). The ability of rabbit liver microsomes to initiate sulfite oxidation in the presence of TPNH, and *in the absence of a hydroxyl acceptor*, indicates the generation of reactive radicals, *e.g.* $HO_2\cdot$ or $HO\cdot$, by this system and strongly suggests that hydroxylations effected by microsomes in the presence of TPNH may be mediated by hydroxyl or perhydroxyl radicals.

The pronounced initiation of sulfite oxidation observed during aerobic oxidation of ferrocytochrome *c* by cytochrome oxidase indicates the participation of free radical intermediates in this process, and suggests that the reduction of oxygen by this enzyme proceeds by successive univalent steps yielding oxygen radicals, probably enzyme bound, which are responsible for the observed initiation of sulfite oxidation. The co-oxidation of 3,4-benzpyrene to hydroxylated and quinoid derivatives by the cytochrome oxidase system as reported by Pihar (40) probably also relates to the reactivity of such oxygen radical intermediates. The appearance of molecular species bearing unpaired electrons in the cytochrome oxidase system has been demonstrated by electron spin resonance spectrometry (8). The initiation of sulfite oxidation by cytochrome oxidase plus ferrocytochrome *c* is not inhibited by 3×10^{-4} M bathocuproine sulfonate, a copper chelating agent indicating that either the copper moiety is not the locus of oxygen binding and reduction or, as recently suggested (41), that the copper is unavailable for reaction with this chelating agent. A mechanism involving the successive univalent reduction of oxygen by cytochrome oxidase has been proposed by King and Lee (42).

Neither uricase nor tyrosinase initiate sulfite oxidation when acting upon their substrates. Uricase activity has been found to result in peroxide formation (43). Studies with H_2O^{18} have indicated that uricase catalyzes a 2-electron transfer from substrate to molecular oxygen (44). Since uricase, which contains 1 atom of copper per mole, catalyzes a 2-electron transfer, it has seemed likely that alternate reduction and reoxidation of the enzymic copper does not occur in the catalytic mechanism (45). The failure of uricase to initiate the oxidation of sulfite, when acting upon uric acid, suggests that reactive radicals are not produced as intermediates during the catalytic action of this enzyme, in accord with the conclusions of Mason (46).

Kubowitz (47), who found that enzyme-bound copper is essential to the activity of tyrosinase, suggested that the copper undergoes alternate reduction and reoxidation. However, from the behavior of tyrosinase in the presence of a biquinoline-acetic acid reagent specific for the cuprous form, Kertesz (48) concluded that the copper of tyrosinase is in the reduced state and does not change valence in the course of its catalytic activity. This concept is supported by the failure of tyrosinase to initiate the oxidation of sulfite, indicating that this enzyme probably does not produce reactive oxygen radicals when acting upon tyrosine. It would be of interest to examine laccase in similar fashion since, in contrast to tyrosinase which has 1 copper atom per mole and is pale yellow (49), laccase is stated to contain 4 atoms of copper per mole and its blue color is bleached by anaerobic addition of substrate (50).

SUMMARY

Initiation of the aerobic oxidation of sulfite has been used to demonstrate free radicals generated by the catalytic action of several oxidative enzymes. Xanthine oxidase, liver aldehyde oxidase, cytochrome oxidase, lipoxidase, peroxidase, and liver microsomes in the presence of reduced triphosphopyridine nucleotide were found to generate radicals in the course of their respective catalytic actions, whereas diverse other oxidative enzymes failed to do so. The significance of these findings with respect to the mechanism of action of each of these enzymes is discussed.

Acknowledgment—Our thanks are due to Mr. Lorrance Greenlee who performed the experiments on the electrolytic initiation of sulfite oxidation.

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Gazeta Cukrownicza 77(1) 1969

Oznaczania śladowych ilości dwutlenku siarki w cukrach białych

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Zawartość śladów dwutlenku siarki w cukrze możemy tłumaczyć stosowaniem środków do odbarwiania soków cukrowniczych: dwutlenku siarki, podsiarczynu sodowego czyli hydrosulfitu, węgla aktywowanego i innych środków bielących. Ślady dwutlenku siarki w cukrze mogą pochodzić również z gazu saturacyjnego.

Poglądy co do szkodliwości kwasu siarkawego dla organizmu ludzkiego są podzielone. Według niektórych autorów [1, 2, 3] kwas siarkawy w małych ilościach jest zupełnie nietoksyczny, gdyż ulega w przewodzie pokarmowym szybkiemu utlenieniu, po czym zostaje wydalony z ustroju. Natomiast innego zdania jest Cremer [4], który w doświadczeniach na królikach stwierdził, że wprowadzenie bardzo małych ilości kwasu siarkawego powoduje znaczne obniżenie odporności krwi na gronkowce.

Niezależnie od ewentualnej szkodliwości kwasu siarkawego dla organizmu ludzkiego zawartość jego w artykułach żywnościowych musi być ograniczona, chociażby tylko z tego powodu, że nadmiar SO_2 wpływa niekorzystnie na właściwości smakowe i zapachowe tych produktów.

W cukrach zawartość dwutlenku siarki powoduje ponadto inwersję cukru, szczególnie po utlenieniu dwutlenku siarki w wilgotnym powietrzu na kwas siarkowy.

Znane powszechnie metody oznaczania dwutlenku siarki w produktach żywnościowych, jak jodometryczna i destylacyjna, podawane przez wielu autorów [5, 6, 7, 8], nie nadają się do oznaczania nieznacznych ilości SO_2.

Natomiast wizualne metody kolorymetryczne, czy też instrumentalne, przydatniejsze do oznaczania śladowych zawartości różnych substancji były stosowane już w 1920 r. [9] do oznaczania dwutlenku siarki w lekach i w niektórych artykułach owocowo-spożywczych.

Najczęściej stosowano metodę opartą na odbarwianiu zieleni malachitowej dwutlenkiem siarki. Ekstynkcję roztworów mierzono fotometrem Pulfricha.

W późniejszych latach (1957 r.) zaproponowano metodę kolorymetryczną opartą na zasadzie reakcji dwutlenku siarki z fuksyną w obecności formaldehydu i kwasu mineralnego [9]. Ekstynkcję tego związku o barwie intensywnie fioletowej mierzono również fotometrem Pulfricha. Według danych z literatury metoda ta dawała dobre rezultaty.

W angielskiej literaturze opisane są inne metody kolorymetryczne oznaczania dwutlenku siarki, mianowicie oparte na zastosowaniu nie tylko fuksyny, lecz i innych barwników, np. para-rozaniliny i chlorowodorku rozaniliny [10].

Jako metodę oznaczania dwutlenku siarki w cukrach białych w warunkach polskich można polecać metodę kolorymetryczną z użyciem fuksyny zasadowej i zastosowaniem fotokolorymetru Spekol.

Metodę tę opracowano, wzorując się na metodzie oznaczania dwutlenku siarki w cukrach białych podanej przez A. Carruthersa, R. K. Heancya i I. F. T. Oldfielda [10].

Odczynniki

1. Sacharoza cz.d.a. 40-procentowy wodny roztwór.
2. Podstawowy roztwór dwutlenku siarki: ok. 1,0% roztwór siarczynu sodowego ($Na_2SO_3 \cdot 7 H_2O$ cz.d.a. w 40% wodnym roztworze sacharozy wolnej od SO_2).
3. Ściśle 0,01 n roztwór jodu w KJ (1 ml 0,01 n odpowiada 0,32 mg SO_2).
4. Wzorcowy roztwór roboczy dwutlenku siarki: 5 ml roztworu podstawowego rozcieńcza się 40-procentowym roztworem sacharozy w kolbie miarowej 100 ml.
5. Odbarwiona fuksyna.
   Zawiesinę fuksyny o stężeniu około 1 g w 100 ml wody destylowanej, ogrzewa się do około 50° i pozostawia na 48 godzin, niekiedy wstrząsając, po czym sączy się przez zwykłą bibułę filtracyjną. Następnie do 4 ml tak otrzymanego przesącza dodaje się 6 ml stężonego kwasu solnego, miesza i rozcieńcza wodą destylowaną do 100 ml. Odbarwienie roztworu fuksyny nie zachodzi natychmiast, lecz dopiero po upływie około 1 godziny przy tym nie jest całkowite; utrzymuje się kolekko zielonkawy.
6. Wodny 0,2-procentowy roztwór formaldehydu.
7. Wodorotlenek sodu 0,1 n.

Przygotowanie skali wzorców

W celu wykreślenia krzywych wzorcowych ustala się dokładne stężenie podstawowego roztworu dwutlenku siarki przez miareczkowanie ściśle 0,01 n roztworem jodu w KJ, a następnie przygotowuje wzorzec roboczy (Odczynniki p. 4).

Jeżeli stężenie SO_2 w podstawowym roztworze nie jest ściśle 23,264 mg/100 ml, należy skorygować stężenie, dodając wody lub Na_2SO_3 i ponownie sprawdzić.

...adnie ilości 1 ml, 2 ml, 3 ml, 4 ml, 5 ml, 6 ml, 8 ml, 10 ml, 20 ml, 25 ml, co odpowiada w ...ym przypadku stężeniom: 0,01163 mg; 0,02325 mg, ...189 mg, 0,04652 mg, 0,05815 mg, 0,06978 mg, ...904 mg, 0,1163 mg, 0,2326 mg i 0,29075 mg SO_2.

...dstawowy roztwór siarczynu zawierał bowiem ...aszym przypadku 23,264 mg SO_2 w 100 ml. Do ...lej kolbki dodaje się po 4 ml 0,1 n NaOH i uzu...ia do 100 ml 40-procentowym roztworem sa...rozy.

...rzygotowuje się również wzorzec „O" — 4 ml ...n NaOH uzupełnione do 100 ml 40-procentowym ...worem sacharozy.

...następnie z kolbek 100 ml pobiera się do suchych ...bówek po 10 ml roztworów wzorcowych, dodaje ...ejno 2 ml odbarwionej fuksyny i 2 ml 0,2-pro...centowego roztworu formaldehydu. Miesza się i po...zwia w temperaturze pokojowej przez 30 ± 5 min.

...ierzy się przepuszczalność optyczną (*T*) próbek ...rcowych barwy fioletowej fotokolorymetrem ...kol przy długości fali światła 545 nm, używając ...vet na 1 ml. Pomiary wykonuje się w odnie...iu do wody destylowanej.

...omiary powtarza się i w przypadku zgodności ...ików wykreśla krzywą zależności przepuszczal...ci optycznej (*T*) w % od stężenia SO_2 w 100 ml.

...W celu sprawdzenia dokładności wyników wykre...się również zależność ekstynkcji ($E = \lg \frac{1}{T}$) ...stężenia SO_2 w mg/100 ml. Zależność ta jest pro...niowa. Wartości ekstynkcji można obliczyć lub ...zytać z tablic fizykochemicznych podanych w Po...niku Fizykochemicznym [11].

...ęść doświadczalna — oznaczanie SO_2 w polskich cukrach białych.

...W opisany wyżej sposób wykonane zostały pomiary ...zbędne do sporządzenia krzywych wzorcowych. ...niki tych pomiarów podane są w tablicy.

...Krzywe wzorcowe podano na wykresie: a. Zależ...ć T % = *f* (c), b. Zależność $E = f \cdot (c)$.

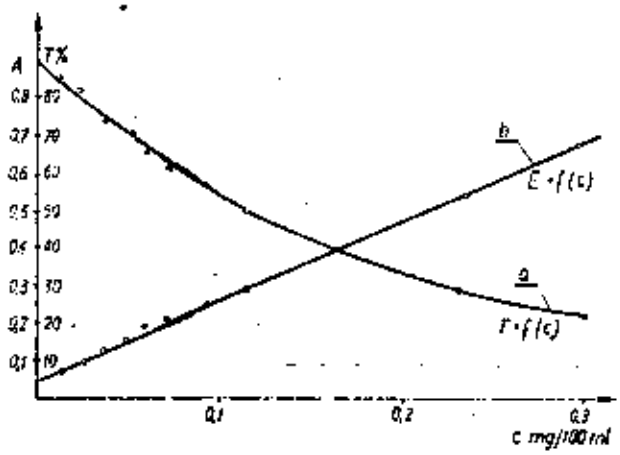
...0 g cukru rozpuszczano w wodzie destylowanej ...kolbie miarowej 100 ml, dodawano 4 ml 0,1 n ...OH i uzupełniano do kreski. Następnie odmierza...do probówki 10 ml roztworu, dodawano 2 ml od...wionej fuksyny i 2 ml roztworu formaldehydu. ...szano i pozostawiano w temperaturze pokojowej ...cz 30 ± 5 min. Mierzono przepuszczalność optycz...(*T*) badanej próbki analogicznie do sposobu, w ...i sporządzono skalę wzorców. Z krzywej wzorco...i podanej na wykresie, odczytywano zawartość ...SO_2 w 40 g badanego cukru, a następnie prze...no na 1000 g badanego cukru. W ten sposób ...ono zawartość dwutlenku siarki w 220 prób...ch z różnych cukrowni polskich w okresach kam...alnych 1965/66, 1966/67 i 1967/68.

...Uzyskane wyniki wahały się od 0 do 0,9 mg/kg, ...tylko w nielicznych przypadkach zawartości SO_2 ...y wyższe: 1,9; 2,2; 4,7; 8,8 i 10,4 mg/kg.

...Jednocześnie nie zostały jeszcze opracowane polskie

Wyniki pomiarów

Ilość ml pobranych z wzorca roboczego	Stężenie SO_2, w mg/100 ml	T %	E
0	0,0	99	0,0087
1	0,012	93	0,0315
2	0,023	80	0,0969
3	0,035	71	0,1306
4	0,047	70	0,1550
5	0,058	65	0,1869
6	0,070	61	0,2116
8	0,093	56	0,2519
10	0,116	50	0,3010
20	0,233	28	0,5528
25	0,291	21	0,6778



Rys. Przykład krzywej wzorcowej: c — stężenie SO_2, mg/100 ml, *T* — przepuszczalność optyczna, %, E — ekstynkcja w 545 nm

normy, ani międzynarodowe na zawartość dwutlenku siarki w cukrze. Projekty norm, wpływające z różnych państw do Komitetu Redakcyjnego Kodeksu Żywnościowego FAO, wysuwają propozycje dopuszczające zawartość dwutlenku siarki w cukrze maksymalnie 20 mg/kg. Jak widzimy, wszystkie polskie cukry pod tym względem mieszczą się zupełnie swobodnie w ramach proponowanych norm.

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Rev. Port. Farm. 15 (1965)

O ANIDRIDO SULFUROSO NAS AGUARDENTES E UM MÉTODO DE DETERMINAÇÃO

por
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O anidrido sulfuroso pode ser reduzido pelo cloreto estanoso a ácido sulfídrico, e este, pela acção da p-aminodimetilanilina e do cloreto férrico, transformado em azul de metileno, avaliável espectrofotomètricamente.

O ácido sulfídrico já existente pode ser deduzido por um ensaio prévio, embora, no nosso caso, a sua influência seja pràticamente nula.

Este método, pela sua sensibilidade e especificidade, supera os da prática corrente, incluindo os de oxidação pelo iodo que actuam indiscriminadamente, oxidando não só o anidrido sulfuroso como muitos outros compostos, não prèviamente eliminados.

A figuração teórica e a experimentação já verificada das possibilidades de combinação do enxofre com numerosíssimos compostos orgânicos, abre um vasto campo de pesquisa na bromatologia.

Desdobram-se perante nós sèries infindáveis de complexos cuja importância no metabolismo vegetal e animal está em estudo ou por averiguar: sulfónios, sulfóxidos, sulfuretos, mercaptanos, ácidos aminosulfídicos, são representativos dos grupos mais importantes.

Já tivemos ocasião de tratar dos mercaptanos nas aguardentes (*). Nessa altura, como era lógico, pretendeu-se também averiguar da existência de compostos mais simples que tivessem uma acção básica no processamento dos complexos enxofrados. Fomos naturalmente levados à pesquisa e determinação do ácido sulfídrico e do anidrido sulfuroso.

Dos ensaios analíticos deste último apresentamos hoje a estas Jornadas um resumo.

A razão de existir anidrido sulfuroso nas aguardentes encontra-se, implícita, da sua presença nos vinhos sujeitos a destilação.

A importância a dar aos compostos enxofrados — pelo menos de muitos deles — na organolèptica das aguardentes, foi causa primária do nosso estudo.

A influência das aguardentes na qualidade do vinho do Porto é facto a que se tem que dar justo relevo.

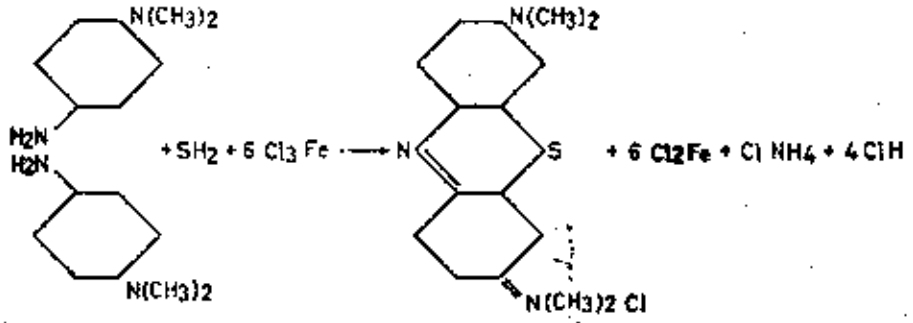
(*) «Sobre mercaptanos nas aguardentes vínicas» — Trabalho apresentado nas III Jornadas Farmacêuticas Portuguesas.

I. MÉTODO

O método baseia-se na redução do anidrido sulfuroso pelo cloreto estanoso

$$8\ Cl_2\ Sn + SO_2 + 6\ ClH \longrightarrow SH_2 + 2\ OH_2 + Cl_4\ Sn$$

e transformação do ácido sulfídrico em azul de metileno pela acção do p-amino-dimetilanilina em presença do cloreto férrico.



Os valores de absorção do azul de metileno verificam-se a 745 mμ em padrões que inicialmente correspondem a 0,2 — 0,4 — 0,5 — 0,8 — 1,0 — 1,2 mcg de SH_2/ml (Fig. 1).

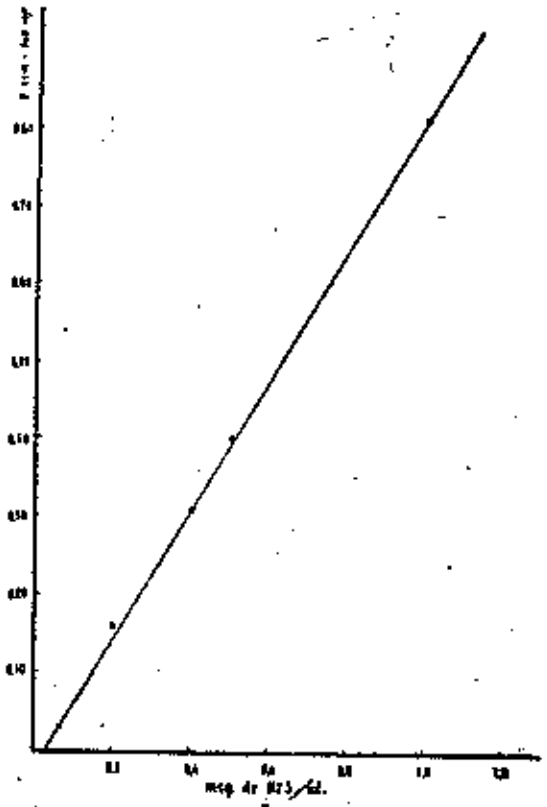


FIG. 1 - CURVA DE CALIBRAÇÃO DO AZUL DE METILENO DERIVADO DO H_2S

2. REAGENTES

1) Solução de Cl_2 Sn, 2 OH_2 (100 g de Cl_2 Sn, 2 OH_2 em 700 ml de Cl H conc. e OH_2 q. b. p. 1000 ml).
2) Solução tampão de pH=7 de fosfato biácido de sódio (PO_4 H_2 Na, OH_2) 0.5 M.
(69 g de fosfato em 750 ml de OH_2; ajustar, potenciomètricamente a pH=7 com uma solução a 20 % de OH Na e completar a um litro).
3) Solução de sulfato de p-aminodimetilanilina (150 mg em 150 ml de SO_4 H_2 (2+1); conservar sob refrigeração.
4) Solução de Cl_3 Fe (2,7 g de Cl_3 Fe, 6 OH_2 em 50 ml de ClH e diluir a 100 ml).
5) Solução a 2 % de acetato de zinco.

3. APARELHAGEM

Consta de:

Uma série de três frascos lavadores de 250 ml cada um, e uma proveta de rolha esmerilada.

Os dois primeiros frascos lavadores contêm 100 ml da solução clorídrica de cloreto estanoso; o terceiro contém 5 ml da solução-tampão em 95 ml de água destilada.

A proveta de recepção contém 40 ml de solução de acetato de zinco.

4. MODO OPERATÓRIO

Os frascos lavadores estão ligados por tubo de plástico, adaptando-se ao último frasco (com a solução-tampão) também por meio de um tubo de plástico um tubo de vidro de ponta afilada que possa ser destacável, mergulhado no líquido da proveta. Quando destacado, o tubo de vidro deve caber dentro da proveta arrolhada.

Introduz-se no primeiro frasco lavador a amostra a analisar, em quantidade tal que o SO_2 presente esteja compreendido entre 5 e 100 mg e faz-se passar uma corrente de N ou CO_2.

No nosso trabalho e em relação à aguardente, servimo-nos do CO_2 em débito moderado e durante 3 a 4 horas, pois só desta maneira conseguimos recuperações aceitáveis. Como complexante usamos o Titriplex III, quer nas soluções clorídricas do Cl_2 Sn, quer na solução-tampão do frasco lavador.

A solução de Cl_2 Sn do primeiro frasco lavador conserva a sua eficiência durante alguns ensaios e a do segundo frasco por uma semana.

A solução-tampão terá que ser renovada ou apenas reforçada, após cada ensaio, pela adição de 5 ml da solução tampão fresca, para reter os vapores de ClH que são arrastados pela corrente gasosa e que libertariam o SH_2 na proveta de recepção.

Deve-se empregar água bidestilada em aparelhagem de vidro, pois a presença de cobre na solução tampão e nos frascos lavadores, num teor de 0,1 p. p. m., é suficiente para se cometer um erro de 1 p. p. m. em relação ao SO_2.

No final do ensaio, a proveta, rolhada e contendo o tubo destacável, mergulha-se em água com gelo, sendo-lhe adicionados 5 ml da solução de sulfato de p-aminodimetilanilina; agita-se e adiciona-se 1 ml da solução de cloreto férrico, agitando novamente.

Depois da proveta permanecer 1 hora à temperatura ambiente, perfaz-se o volume de 50 ml e faz-se a leitura no espectrofotómetro a 745 mμ.

Executa-se um ensaio em branco.

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Com o valor de SH_2 obtido no gráfico, procede-se ao cálculo do SO_2.

$$\text{meq de } SH_2 \times \frac{64}{34} \times \frac{1000}{V} \times \frac{1}{1000} = \text{mg de } SO_2 ‰$$

Nos ensaios efectuados de recuperação, com adição de complexantes, obtiveram-se resultados que podemos considerar satisfatórios: recuperou-se 90,5 a 95,6 do anidrido sulfuroso existente.

RESULTADOS

Valores de anidrido sulfuroso encontrados em aguardentes

N.º de Ordem	SO_2, mg ‰	N.º de Ordem	SO_2, mg ‰
1	1.50	11	0.00
2	5.64	12	0.00
3	0.52	13	1.50
4	0.50	14	1.50
5	0.27	15	71.00
6	0.40	16	97.00
7	0.15	17	65.00
8	0.20	18	84.50
9	0.15	19	15.80
10	0.00	20	32.94

CONCLUSÕES

Os quantitativos de anidrido sulfuroso nas aguardentes vínicas podem atingir números relativamente altos (97 mg/l), embora também haja aguardentes que não o acusem.

Este facto, digno de se ponderar, pela influência prejudicial que pode ter nos vinhos onde ela é incorporada, cria a necessidade da eliminação prévia do anidrido sulfuroso nos vinhos a destilar.

SUMMARY

The sulphurous anhydride in brandies and a determination method

It is acceptable the influence of the sulphurous anhydride who exists in the brandy added to Port wine; this action should attain the organoleptic properties not only in a direct way, as by means of derivative compounds. This fact is responsible for the necessity of determine precisely its rate. The best method of determination of sulphurous anhydride is that which is based on its transformation into hydrogen sulphide by adding stannous chloride, and adding then p-dimethylanilina and ferric chloride to produced methylene blue, whose concentrations are assayed spectrophotometrically.

Many analytical results are included.

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(Trabalho realizado no Instituto do Vinho do Porto)

Anal. Chem. 19 (1947)

54

Colorimetric Determination of Sulfur Dioxide

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The test based on a chromogenic reaction of sulfur dioxide with fuchsin and formaldehyde in acid has been adapted to quantitative colorimetric determination of 0 to 10 micrograms of sulfur dioxide in 1 ml. of solution with an error of less than 0.1 microgram for pure solutions. Methods have been devised for direct application to alkaline extracts of animal tissues, including blood, employing mercuric chloride to remove sulfhydryl interference. For determination in fruit, preliminary distillation is used to remove interfering substances.

THE sensitive color reaction for sulfurous acid described by Steigmann (*8*) can be utilized for the quantitative determination of this substance in concentrations of 0 to 10 micrograms per milliliter of solution or of an alkaline extract or distillate of certain biological materials. In comparison with the methods which are commonly employed, involving distillation followed by iodometric titration, the present method of analysis offers advantages in simplicity and sensitivity. The reaction described by Steigmann consists of the production of a red color by the action of sulfur dioxide on fuchsin in the presence of formaldehyde and a strong mineral acid. The reaction is relatively insensitive to aldehydes other than formaldehyde. It was pointed out by Steigmann that, although a similar color is produced by sulfhydryl compounds and thiosulfate, the test could be made specific for sulfur dioxide by preliminary removal of interfering substances by treatment with mercuric chloride.

It has been ascertained that treatment with mercuric chloride reduces to a reasonably low value the blank for several biological materials of animal origin. The natural coloring of fruits and vegetables is not removed by this treatment. Conditions suitable for quantitative measurement of sulfur dioxide by means of Steigmann's color reaction have been determined and appropriate methods of application have been devised for determination of sulfur dioxide in different types of animal tissue, exemplified by cornea, conjunctiva, aqueous humor, and blood. For the ocular tissues mentioned, which were studied in an investigation of injuries from sulfur dioxide, it was found possible to apply the color reaction directly to a mercuric chloride-treated extract. For blood, which is also of toxicological interest, a modification of the procedure used on ocular tissues permitted removal of the large amount of soluble protein present and direct application of the color reaction. Determination of sulfur dioxide in fruit, which may be of use in the study of the sulfite treatment of foodstuffs, was not practical by a direct method, but could be accomplished by employing a distillation procedure.

REAGENTS AND STANDARDS

Color reagent is prepared by diluting 11 ml. of concentrated sulfuric acid with 231 ml. of water and adding 4 ml. of a 3% solution of fuchsin (basic indicator, National Aniline and Chemical Co.) in alcohol. One milliliter of 40% formaldehyde solution is then added to give a faint pink color to the brownish solution. A fine dark sediment appears in the course of several hours, but this has little effect on the sensitivity of the supernatant reagent. This reagent was prepared daily with little variation in color production.

Standard sulfur dioxide solution is made from iodometrically standardized water solutions of sulfur dioxide gas or of solid sodium bisulfite (Coleman and Bell Co.). With titrimetrically equivalent solutions no difference in color production was observed and the bisulfite standard is preferred on the basis of convenience. No variation in the composition of the solid bisulfite was observed during several weeks.

Colorimeter calibration is performed by mixing 4 ml. of color reagent with 1 ml. of each of a series of dilutions of standard sulfur dioxide solution as well as with 1 ml. of water for a blank. When mercuric chloride is to be used to remove interfering substances, as in the biological applications described below, an equal concentration of mercuric chloride is included in the standard solutions. After 5 minutes at room temperature the colors are measured with a Ceneo Photelometer using its green filter and setting the galvanometer at 100 with the blank mixture. The color-concentration relationship follows Beer's law over most of the range of 0 to 10 micrograms.

PROCEDURES

For analysis of simple aqueous solutions containing no protein or colored substances, such as samples obtained by absorption of sulfur dioxide in alkaline solution in air analysis, the same procedure is applied as for the standards in colorimeter calibration, using samples suitably diluted to contain 0 to 10 micrograms of sulfur dioxide per ml. When sulfhydryl compounds or thiosulfates are present, the sample is mixed with an equal volume of saturated mercuric chloride and centrifuged before mixing with the color reagent. Comparison is made with standards containing an equal concentration of mercuric chloride. For biological samples the following procedures may be used.

Animal Tissues with Little Soluble Protein. Materials such as cornea, conjunctiva, or aqueous humor are treated with a solution of 1% potassium hydroxide in 10% alcohol which has been recommended for extraction of sulfur dioxide from sulfited food products (*2*). Approximately 1 ml. of this extractant is used per 20 mg. of cornea or conjuctiva or per 0.15 ml. of aqueous humor. It was found that a single extraction consisting of soaking solid tissue in the extractant for 10 minutes with occasional stirring removed over 95% of the total sulfur dioxide extractable by two successive treatments. After the extraction period, the mixture is made neutral with respect to phenolphthalein by titration with 1 *N* hydrochloric or acetic acid, and 1 ml. of saturated mercuric chloride is added per milliliter of extractant. Precipitation of proteins is hastened by centrifuging for 2 to 3 minutes and 1 ml. of the supernatant or of an appropriate dilution of supernatant is mixed with 4 ml. of color reagent and after 5 minutes is compared colorimetrically with standards containing an equivalent concentration of mercuric chloride.

Blood. To 0.15 ml. of blood are added 0.5 ml. of 1% alcoholic potassium hydroxide and 2.35 ml. of water. Then, without neutralization, the solution is mixed with 1 ml. of saturated mercuric chloride solution and centrifuged. One milliliter of the supernatant, or of an appropriate dilution thereof, is mixed with 4 ml. of color reagent and the color is measured in the usual way.

Fruit. Approximately 250 mg. of fruit are macerated with 3 ml. of 1% potassium hydroxide in 10% alcohol. A 2-ml. aliquot is placed in the side bulb of a vacuum distillation apparatus previously described (*1*) and a sodium hydroxide pellet of roughly 50 mg. is placed in the condenser portion. The material in the bulb is frozen by immersion in a dry ice–acetone mixture and approximately 0.05 ml. of concentrated sulfuric acid is added to it. The apparatus is evacuated, sealed, and then allowed to warm to room temperature to permit the fruit extract to melt and react with the sulfuric acid. This mixture in the side bulb is then refrozen to prevent bumping before immersing the condenser portion of the apparatus in dry ice mixture for distillation. The volatile constituents are allowed to distill without heating. When distillation is complete, the distillate is allowed to melt and dissolve the sodium hydroxide pellet, but air is not admitted for 30 minutes in order to absorb remnants of sulfur dioxide still in the gas phase. The distillate is analyzed by the procedure described for sulfur dioxide solutions without interfering substances.

EXPERIMENTAL

The composition of the color reagent and the concentration of mercuric chloride used in the procedures outlined were determined

Table I. Determination of Sulfur Dioxide

Sample	Sulfur Dioxide, Micrograms: Added	Found	Average recovery
Cornea, cattle, 40 mg.	0	0.4, 2.4, 3.0	
	20	23.8	23.4
	40	42.2, 43.8, 45.0	42.6
Conjunctiva, cattle, 40 mg.	0	3.5, 1.7	
	15	18	15.4
	40	40	37.4
Aqueous humor, cattle, 0.3 ml.	0	0, 0.1	
	20	19.8	19.8
Blood, rabbit, 0.15 ml.	0	1, 1	
	5	5.6, 6.2	4.4
	10	9.8, 11.8	9.8
	20	17.4	16.4
	40	38.4	37.4
Peach, fresh, 250 mg.	0	0	
	200	180	180
	400	400	400
	500	440, 530	485

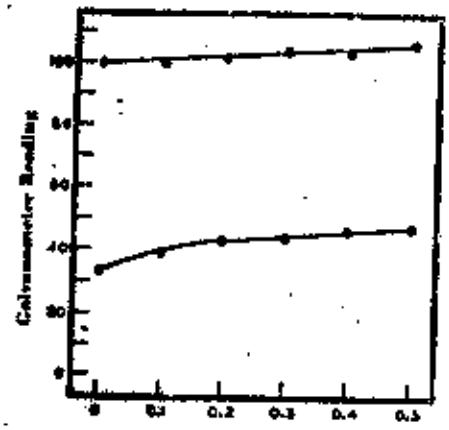

Figure 1. Colorimeter Values of 5-Ml. Color Reaction Mixtures

0 (upper curve) or 5 (lower curve) micrograms of sulfur dioxide and varying quantities of saturated mercuric chloride solution

experimentally to be approximately optimal for the applications described. While a considerable increase in sensitivity of the color reaction is obtainable in the absence of mercuric chloride by using as little as 0.5 ml. of color reagent with 3.5 ml. of sulfur dioxide solution, the color formation in this dilute mixture is seriously reduced by addition of mercuric chloride. At the concentration of color reagent recommended above, the color production is less seriously affected by this addition and shows little variation with change in the amount of mercuric chloride present in the range employed in the analysis of biological materials. Such independence of small changes in mercuric chloride concentration is desirable because of the indefinite proportion lost by precipitation with proteins. The effect of variation of mercuric chloride concentration on color production was determined by comparing the transmission of color reaction mixtures of the usual 4 ml. of color reagent with 1-ml. aqueous samples which contained either 0 or 5 micrograms of sulfur dioxide and from 0 to 0.5 ml. of saturated mercuric chloride solution. The results are shown in Figure 1, where the transmission of these mixtures is compared with that of the blank without sulfur dioxide or mercuric chloride.

The efficiency of removal of sulfhydryl interference by mercuric chloride treatment was determined by analysis of samples containing 0, 2, 4, 6, and 8 micrograms of sulfur dioxide per ml. of 0.01 *M* cysteine hydrochloride solution. These samples were neutralized, immediately mixed with an equal volume of saturated mercuric chloride solution, and centrifuged. Analysis of the supernatant fluid by reaction with the color reagent gave values which were all in agreement within 0.1 microgram of the standard values determined in the usual way.

The manner of separation of sulfur dioxide and interfering substances from animal tissues appears to be determined by the relationship of the pH to fixation of sulfur dioxide by proteins and binding of sulfhydryls by mercury. It was found that when a considerable amount of soluble protein is present, as in blood, it is necessary to effect precipitation of the protein in an alkaline medium in order to prevent excessive binding of sulfur dioxide by the protein. When various methods of acid precipitation were tried, recoveries averaged only 50 to 60% of the sulfur dioxide added. Alkaline or neutral solution also favors reaction of sulfhydryls with mercury, but excessive alkalinity must be avoided in order to prevent precipitation of mercuric oxide. The supernatant must be perfectly clear after mercuric chloride treatment before mixing with the color reagent.

The influence of addition to the chromogenic reaction mixture of small amounts of alkali, such as might be present in experimental sulfite samples, was determined by measuring color production by samples containing 3 micrograms of sulfur dioxide and 0 to 0.25 *M* potassium hydroxide. No significant differences were found.

It has been found advisable to minimize the time between the addition of mercuric chloride and color reagent, since there is progressive loss of sulfur dioxide at the rate of approximately 1% per minute when 5 micrograms are originally present. Similarly, a constant time of reaction of the color reagent with the sulfur dioxide was employed, for mixtures which stood 15 minutes instead of 5 showed a decrease of 6% in apparent concentration.

RESULTS

Determination of sulfur dioxide in pure solution was found to be accurate to within 0.1 microgram in the range of 0 to 10 micrograms per ml. and similar accuracy has already been noted for solutions of sulfur dioxide in 0.01 *M* cysteine when assessing the effectiveness of mercuric chloride in removing interference by this sulfhydryl compound. Determination of sulfur dioxide added in known amounts to cornea, conjunctiva, aqueous humor, blood, and peach were also carried out using the procedures described. The sulfur dioxide was added to an alkaline suspension in the case of the peach. The results of these measurements on biological materials are given in Table I.

DISCUSSION

Comparison of the results obtained in determining sulfur dioxide in pure solution or in cysteine solution with the results for determinations on protein-containing samples shows a difference in the efficiency of removal of interfering substances and in the constancy of recovery of sulfur dioxide. It appears that mercuric chloride treatment is not so completely effective in removing interference from animal tissues as in removing a simple sulfhydryl compound. The source of the small amount of color in the blanks of the tissues tested is uncertain, but it seems unlikely that it represents any naturally occurring sulfur dioxide.

It appears that adequate separation of sulfur dioxide from interfering substances of peach is obtained by the distillation procedure which was employed. However, determinations on peach attempted at lower concentrations of sulfur dioxide than shown in the table gave progressively poorer recoveries as the concentration was decreased, although control distillations of sulfur dioxide in water did not give comparably diminishing returns. Thus the procedure described for fruit, while simple and specific, is not comparable in sensitivity with the methods given for animal tissues.

ACKNOWLEDGMENT

The author wishes to acknowledge the valuable technical assistance of Helen H. Pentz.

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(2) Iokhel'son, D. B., and Novstruyeva, A. I., *Veterinary Pilnaiya*, 9, 25 (1940).
(3) Steigmann, A., *J. Soc. Chem. Ind.*, 61, 18 (1942).

Arch. Ophthalmol. 38(6) (1947)

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OCULAR INJURY DUE TO SULFUR DIOXIDE

II. Experimental Study and Comparison with Ocular Effects of Freezing

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THE ACCIDENTAL injuries of the eye produced by liquefied sulfur dioxide which have been reported by myself[1] and others have usually been severer than those produced by other substances with similar moderately acidic properties. To account for the greater toxicity of sulfur dioxide, previous observers have proposed some rather unusual mechanisms based on the physical and chemical peculiarities of the noxious agent. Experiments carried out in this laboratory indicate that a relatively simple mechanism, different from those previously proposed, is responsible in large measure for the peculiar toxicity of sulfur dioxide.

The following two hypotheses have previously been advanced to explain the severity of ocular injury produced by liquid sulfur dioxide. It has been assumed by some investigators that injury of the eye is due to freezing produced by the rapid evaporation of the liquefied gas (Kennon,[2] Clark[3]), while others have assumed that injury is caused by the oxidative formation of sulfuric acid from sulfur dioxide on the moist mucous membrane surfaces (Flurry and Zernik,[4] Rost[5]). That sulfurous acid, which is formed directly on contact of sulfur dioxide with water, could itself be sufficiently injurious to produce the damage observed has not been seriously considered, perhaps because sulfurous acid is a relatively weak acid, similar to phosphoric acid, and might therefore not be expected to cause the serious disturbances observed clinically.

From the Howe Laboratory of Ophthalmology, Harvard Medical School, and the Massachusetts Eye and Ear Infirmary.

1. Grant, W. M.: Ocular Injury Due to Sulfur Dioxide: I. Report of Four Cases, Arch. Ophth. this issue, p. 755.

2. Kennon, B. R.: Report of a Case of Injury to Skin and Eyes by Liquid Sulfur Dioxide, J. Indust. Hyg. 9:486-487, 1927.

3. Clark, C. P.: Accidental Freezing of the Eye by Sulfur Dioxide, Am. J. Ophth. 19:881-884, 1936.

4. Flurry, F., and Zernik, F.: Schädliche Gase, Dampfe, Nebel, Rauch und Staubarten, Berlin, Julius Springer, 1931, p. 143.

5. Rost, E., in von Bock, J., and others: Handbuch der experimentellen Pharmakologie, Berlin, Julius Springer, 1927, vol. 3, pt. 1, pp. 389-417.

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The evidence cited to support the idea that frostbite is an important factor is the indefinite report by Carroll[6] of a case in which partial epithelial denudation of the cornea and temporary conjunctival congestion developed after exposure to a cold wind on two successive days. Similarly, keratitis epithelialis, resolving completely in three days, was reported by Colombo[7] as occurring in an aviator exposed to wind and low temperature for several hours. However, there seems to be little similarity to injury with sulfur dioxide in either case. Furthermore, there appears to be no experimental information on the effects of freezing of the eye which could be used as a basis for comparison.

The evidence that the injury results from oxidation of sulfur dioxide to sulfuric acid is also unsatisfactory. It is based on the fact that the major portion of sulfurous acid or its salts (bisulfite or neutral sulfites) which are ingested or injected can be accounted for in the form of sulfate in the urine (Rost and Franz[8]) and that little sulfite can be found in the circulating blood after the ingestion of large amounts of the sulfite. These observations led to the assumption that the oxidation to sulfate occurred in the lining of the gastrointestinal tract, but it is noteworthy that sulfite introduced directly into the blood stream also disappears rapidly.[8] From such evidence it has been inferred that oxidation of sulfur dioxide similarly occurs on the mucous surfaces of the respiratory system and elsewhere, with production of enough sulfuric acid to account for the lesions produced by exposure to sulfur dioxide. However, the formation of sulfuric acid in significant amounts on mucous membrane surfaces has not been demonstrated directly.

Previous experiments on ocular lesions due to sulfur dioxide have been confined to cursory observations and limited to the effect of exposure of animals to mixtures of the gas and air. Ogata[9] reported clouding of the corneas of rabbits, guinea pigs and mice after exposure for four hours to a concentration of 400 parts per million of air by volume and clouding in 1 guinea pig after exposure for one hour to a concentration of 1,000 parts per million. Weedon and associates[10] mentioned the occurrence of corneal opacification in mice and guinea

6. Carroll, F.: Frost Bite of the Cornea, Am. J. Ophth. 16:994, 1933.

7. Colombo, G. L.: Bilateral Changes in the Corneae Following Exposure to Cold in an Airman, Brit. J. Ophth. 5:553-558, 1921.

8. Rost, F., and Franz, F.: Vergleichende Untersuchung der pharmakologischen Wirkungen der organische gebundenen schwefligen Säuren und des neutralen Schwefligsäuren Natriums: II., Arb. a. d. k. Gesdhtsamte. 43:187-303, 1912.

9. Ogata, M.: Ueber die Giftigkeit der schwefligen Säure, Arch. f. Hyg. 2:223-245, 1884.

10. Weedon, F.; Hartzell, A., and Setterstrom, C.: Effects on Animals of Prolonged Exposures to Sulfur Dioxide, Contrib. Boyce Thompson Inst. 10:281-324, 1939.

pigs exposed to concentrations of 110 to 300 parts per million of air for approximately three days, and McNally[11] noted severe conjunctivitis and corneal opacity in guinea pigs killed by exposure to 10,000 parts of sulfur dioxide per million of air for fifteen minutes. No experimental investigation of the effect of exposure to liquid sulfur dioxide has been made.

Because of this lack of information on the possible physical and chemical effects of liquid sulfur dioxide on the eye, and the consequent uncertainty regarding the mechanism of production of the lesions observed clinically, the following experimental investigation and comparison of these effects was undertaken.

EXPERIMENTAL INVESTIGATION

Effect of Freezing.—Owing to the paucity of information on the consequence of brief freezing of the living eye, observations were made on the effect of exposing the rabbit eye to a refrigerant liquid. In order to permit clear differentiation of physical and chemical effects in these experiments, use was made of a chemically inert, relatively nontoxic refrigerant, "freon-12" (dichlorodifluoromethane). This refrigerant has physical properties similar to those of sulfur dioxide but a relatively innocuous chemical nature, as confirmed by the observation that exposure of a rabbit's eye to a rapid stream of the pure gas at room temperature for ninety seconds caused no irritation or injury of the eye apparent grossly, on application of fluorescein or on biomicroscopic examination during the ensuing three days. Liquefied "freon," on the other hand, may be assumed to produce a temperature as low as, or lower than, that produced by sulfur dioxide when permitted to evaporate in contact with the eye, since at atmospheric pressure its boiling point is —28 C., as compared with that of —10 C. for sulfur dioxide.

Experiments with freezing were first carried out with a mixture of liquefied "freon" and oil, analogous to the mixtures of sulfur dioxide and oil to which the patients were exposed in the accidents previously reported.[1] Each of 2 rabbit eyes was held open by means of a speculum and was subjected at a distance of about 6 inches (15 cm.) to the full blast of a spray obtained by opening wide the valve on an inverted tank of the "freon"-oil mixture. The duration of exposure to the spray was approximately one second, which is presumably somewhat longer than a human being would keep his eye open in similar circumstances. The corneas and margins of the lids became white and hard during the spraying, but regained their normal appearance during the next five or ten seconds. Biomicroscopic examination revealed no

11. McNally, W.: The Use of Sulfur Dioxide as a Refrigerant, Indust. Med. 8:234-238, 1939.

abnormality immediately. During the next few hours there appeared slight vascular congestion and edema of the nictitating membrane and conjunctiva. Moderate edema of the corneal epithelium gradually developed, progressing to denudation of a small central area in twenty-four hours. Within seventy-two hours of the exposure and thereafter the eyes appeared normal, both grossly and microscopically.

Freezing of the eye for a few seconds, such as might conceivably occur in an accidental exposure, thus produced disturbances which were mild and transient. For more prolonged freezing, liquid "freon" was sprayed onto 1 rabbit eye for five seconds and onto another eye for thirty seconds. The corneas of both eyes became immediately opaque, dense white and hard. The nictitating membrane and the lids were solid. In one to two minutes the tissues thawed and the normal transparency of the cornea and appearance of the eyes were completely regained. However, in about five minutes the corneal endothelium of the eye which had been exposed for thirty seconds was seen on biomicroscopic inspection to have become gray with dark round and oval defects. There rapidly followed in this eye shedding of most of the endothelium in sheets, which could be seen settling slowly through the aqueous humor. In the eye exposed for five seconds, gray opacity and numerous defects of the endothelium were apparent in fifteen minutes, but shedding was less extensive. At this time, chemosis of the conjunctiva and injection of the iris were apparent in both eyes, but the corneas did not stain. At thirty to forty-five minutes thickening of the corneas and faint diffuse staining with fluorescein were observed. During the next several hours the corneas became swollen to several times their normal thickness and acquired a translucent, bluish appearance. They continued to take a diffuse stain with fluorescein. The conjunctiva, nictitating membrane and margin of the lids of the eye exposed for thirty seconds underwent a severe reaction, with partial necrosis and considerable purulent discharge. Within ten days the cornea of this eye became opaque, yellow and sloughed. The reactions were considerably milder in the eye exposed for five seconds. This eye showed improvement in ten days and appeared practically normal in six weeks. The cornea was clear except for a small gray patch in the center, where some edema of the stroma and epithelium persisted. There was little increased vascularity of the cornea at the limbus.

Effect of Sulfur Dioxide Without Freezing.—The effects of sulfur dioxide were next examined under conditions in which there could be no question of freezing. Exposures of rabbits and guinea pigs to mixtures of sulfur dioxide and air at room temperature in a constant flow exposure chamber confirmed the production of keratitis and corneal clouding under conditions of time and concentration similar to those reported by other investigators. The results are given in table 1.

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by Friedenwald, Hughes and Herrmann[12] as characteristic of severe acid burns. The only possibly distinguishing feature of sulfur dioxide burns was a striking predominance of eosinophils in the corneal infiltrate at all stages. Moreover, there was relatively little iritis in these eyes. As described for the acid burns, the corneal endothelium was not obviously damaged.

Effect of Hydration Products of Sulfur Dioxide.—Information on the chemical mechanism by which sulfur dioxide may cause the changes observed in the preceding experiment was sought by investigation of some of the chemical transformations which might be involved. It would be expected that sulfur dioxide in moist buffered tissue would primarily be converted into sulfurous acid, acidic bisulfites and neutral sulfites in proportions determined by the effectiveness of buffering, and that there might be differences in the toxicity of these various forms. Furthermore, the possibility of oxidation to a stronger acid was to be considered.

Since it was not feasible to determine the amount of each of these derivatives formed in the cornea on exposure to sulfur dioxide, the total sulfur dioxide in all forms was measured. It was then possible to introduce similar quantities of bisulfite and neutral sulfite alone into other corneas and to compare their toxicities. The rate of disappearance of sulfur dioxide and its derivatives was also measured, and tests were made for the formation of sulfuric acid.

The total quantity of sulfur dioxide present in all forms was measured in rabbit corneas excised immediately after exposure for five seconds to sulfur dioxide gas. Extracts of individual corneas in alcoholic potassium hydroxide solution were made and analyzed by a colorimetric method (Grant[13]). The concentrations of sulfur dioxide found immediately and at intervals after exposure are shown in the chart. The concentrations found in the conjunctiva and the aqueous are given in table 2 for comparison.

It may be observed from these data that the concentration of sulfur dioxide in all forms in the living cornea decreases exponentially with time, with a half-life of approximately fifteen minutes. Furthermore, the rise in concentration of sulfur dioxide in the aqueous humor immediately after exposure suggests that the cornea and conjunctiva are readily permeable to sulfur dioxide.

Qualitative testing of the neutralized corneal extracts by means of barium chloride failed to reveal an increase of sulfate such as might be found if a significant proportion of the sulfur dioxide were oxidized to sulfuric acid.

12. Friedenwald, J. S.; Hughes, W. F., and Herrmann, H.: Acid Burns of the Eye, Arch. Ophth. 35:98-108 (Feb.) 1946.

13. Grant, W. M.: Colorimetric Determination of Sulfur Dioxide, Indust. & Engin. Chem. (Anal. ed.) 19:345-346, 1947.

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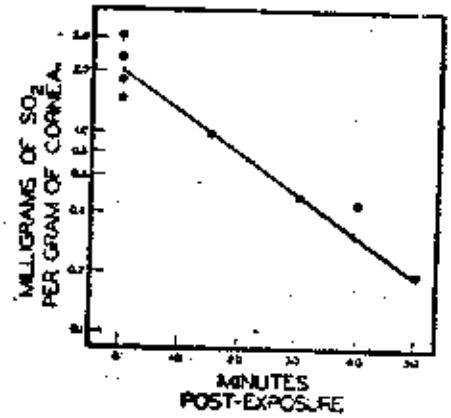
Comparative experiments to assess the relative rates of diffusion of sulfur dioxide from cornea into the air or into aqueous bathing fluids were performed by exposing buttons cut from beef corneas to sulfur dioxide gas for five seconds and then measuring the amounts residual after various periods of standing in air, water or a 5 per cent solution of sodium bicarbonate at room temperature. The half-life of the sulfur dioxide in air-bathed corneal buttons was approximately 240 minutes, while in those bathed by water or sodium bicarbonate it was 5 to 10 minutes. Thus, it appears that during the rapid disappearance of sulfur dioxide from the cornea in vivo diffusion into the air must be an insignificant factor.

To determine the toxicity of sulfur dioxide in the form of sulfite, a neutral 1.4 molar solution of sodium sulfite was dropped onto 2 rabbit eyes continuously for thirty minutes. Analysis of 1 cornea showed



Time relation of decrease in concentration of sulfur dioxide in all forms in the corneas of rabbits following acute exposure to the gas.

that a concentration was achieved equivalent to 2.15 mg. of sulfur dioxide per gram of cornea, equaling the average concentration found immediately after the exposures to the gas. Although this treatment caused intense conjunctival injection and chemosis, loss of epithelium from the center of the cornea and the presence of aqueous flare, no opacity was produced and the eye was apparently normal in two days.

The toxicity of bisulfite was investigated by irrigation of rabbit eyes for thirty minutes with 3.5 molar sodium bisulfite at its natural p_H of 3.8, producing a concentration in the cornea equivalent to 4.63 mg. of sulfur dioxide per gram of cornea. This concentration was somewhat greater than the highest concentration achieved with exposures to the gas. The damage caused by this treatment resembled that resulting from sulfur dioxide itself but was considerably milder than that produced by an exposure for five seconds. There were severe con-

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junctival injection and chemosis, with grayness and irregularity of the corneal epithelium and swelling of the stroma. However, the ground glass appearance of the epithelium, which was not altered by glycerin and the translucent opacity of the stroma cleared completely without vascularization in ten days.

By comparison, it appears that sulfur dioxide must exert its injurious effect principally in the form of sulfurous acid, since neither the neutral sulfite nor the bisulfite produced injury of the severity observed in exposures to sulfur dioxide and the formation of sulfuric acid was negligible. The mechanism by which sulfurous acid might produce these injuries was, therefore, next to be investigated.

Effect of Sulfur Dioxide (or Sulfurous Acid) on Corneal Catalase.—An indication of whether exposures of the cornea to sulfur dioxide may be attended by inactivation of corneal enzymes was sought through

TABLE 2.—*Concentration of Sulfur Dioxide in Ocular Tissues*

Time Following Exposure	Cornea, Micrograms per Gm.	Conjunctiva, Micrograms per Gm.	Aqueous, Micrograms per Gm.
Immediate (30-40 sec.)	1,800	2,250	340
	1,470	2,920	434
	3,170	627	157
	2,410	1,790	39
Average	2,227	1,894	255
15 minutes	978	200	533
30 minutes	465	378	236
40 minutes	456	...	430
60 minutes	198	...	52
90 minutes	108	50	35

measurement of the activity of a single enzyme, catalase. The behavior of this enzyme was investigated only to obtain information on the nature of the changes in the cornea, and not to attempt identification of what specific enzymes might be involved in production of injury.

The catalase activity of single minced rabbit corneas was estimated by volumetric determination of the rate of liberation of oxygen from dilute hydrogen peroxide, using a micromodification of the method of Thompson.[14] One eye of each rabbit was exposed to pure sulfur dioxide gas for five seconds in the manner already described, and the other eye was used as an unexposed control. The corneas were excised, and catalase assay was performed immediately after exposure. The rate of liberation of oxygen ranged from 393 to 840 (average 650) cu. mm. per minute per gram of cornea for 5 normal corneas, whereas with 2 exposed corneas there was no measurable liberation of oxygen in fifteen minutes. These results are considered to show that an exposure

14. Thompson, R.: Simplified Apparatus for Catalase Determination, Indust. & Engin. Chem. (Anal. ed.) 14:585, 1942.

to sulfur dioxide which will result in serious injury is immediately attended with changes within the cornea sufficiently drastic to cause inhibition of enzymes. However, it cannot be concluded that the injury to the cornea is due to inactivation of catalase alone, for in separate experiments in which corneal catalase was inhibited by means of hydroxylamine no opacification of the cornea resulted.

Comparative experiments to determine the effect of freezing on corneal catalase were carried out on excised cattle eyes. No significant difference was found between the activity of control corneas and that of corneas of eyes frozen with "freon" for either five or thirty seconds.

Effect of Sulfur Dioxide on Swelling Properties of the Cornea.—For the purpose of determining whether sulfur dioxide may produce, in addition to inhibition of enzymes, a gross alteration in the physical characteristics of the corneal stroma which is not revealed with the usual histologic technic, measurements were made of the turgescence properties of normal and of exposed corneas, employing the technic of Kinsey and Cogan.[15] Excised cattle eyes were used in these experiments, and the swelling of exposed corneal buttons was allowed to take place in running water so that residual acid would be removed. Five pieces of cornea were used for each determination, and the weights were compared after two thousand six hundred minutes was allowed for swelling. Normal corneal buttons increased in weight 530 per cent, while corneal buttons exposed for ten to fifteen seconds to sulfur dioxide gas increased only 134 per cent. In another set of measurements, the control corneal buttons increased 470 per cent in weight, while buttons from corneas exposed for five seconds to the gas increased 159 per cent. Thus, a length of exposure such as causes a severe corneal injury produces a significant alteration in the turgescence properties of the stroma.

This inhibition of turgescence was found to be persistent even though, on washing in water, the analytic value for sulfur dioxide was reduced to a quantity not significantly different from the blank value for cornea, which corresponded to approximately 0.5 per cent of the immediate post-exposure concentration. Since it has previously been ascertained that essentially complete recoveries of sulfur dioxide are obtained with the method of analysis employed, it appears that the amount of residual or fixed sulfur dioxide which could be associated with the inhibition of turgescence was not greater than approximately 30 micrograms per gram of cornea.

To determine whether sulfur dioxide solution differs notably from other acids in its ability to inhibit corneal swelling, the effect on swelling of exposures to several other acids was compared. Sets of 5 corneal buttons each were exposed by dipping for two minutes in 0.1 molar

15. Kinsey, V. E., and Cogan, D. G.: The Cornea: III. Hydration Properties of Excised Corneal Pieces, Arch. Ophth. 28:272-284 (Aug.) 1942.

solutions of seventeen different acids, including sulfurous acid, all adjusted to a p_H of 2.0 with either hydrochloric acid or sodium hydroxide. The extent of swelling in running water for two thousand six hundred minutes was then measured. The results are given in table 3. The differences observed are no doubt dependent to some extent on differences in rate of penetration, and this factor will be further considered later. However, it is apparent from these measurements that sulfur dioxide solution has no outstanding effectiveness over solutions of other acids in impairing corneal turgescence when the acids have direct contact with the corneal stroma.

Determination of Permeability of the Cornea to Sulfur Dioxide.—A comparison was made of the permeability of the cornea to sulfur dioxide and to other acidic substances in order to determine whether the toxicity of sulfur dioxide to the eye may be enhanced by any superior properties of penetration. Such a possibility is suggested by the rapid

TABLE 3.—*Effect of Exposure to Various Acids on Subsequent Swelling of Cornea in Running Water*

Acid	Corneal Swelling *	Acid	Corneal Swelling
Control	420	Formic	160
Phenol	286	Phosphoric	158
Trichloroacetic	273	Lactic	150
Boric	208	Oxalic	150
Hydrochloric	195	Monochloroacetic	144
Nitric	187	Acetic	143
Sulfuric	185	Maleic	143
Sulfurous	183	Sulfosalicylic	139
Nicotinic	168	Propionic	119

* The corneal swelling is expressed as the final weight in terms of the percentage of the original weight.

penetration of sulfur dioxide into the aqueous humor of exposed rabbit eyes apparent from the measurements of concentration given in table 2. Excised cattle corneas were fastened to the ends of glass tubes in the manner described by Cogan and Kinsey[16] to form a membrane separating fluid in a tube from fluid bathing the outside. Comparisons of the rate of penetration of acids into the cornea were made by covering the epithelial surfaces with 2 cc. of sulfurous acid or other acid while periodically determining the p_H of 2 cc. of water bathing the other side of the cornea. The times required for various acids to lower the hydrogen ion concentration on the other side of the cornea to p_H 5 were determined, and after each experiment the integrity of the epithelial barrier of each cornea was checked by estimation of its permeability to fluorescein. In order to facilitate comparison of the acids, they were all used in 0.1 molar concentration and were adjusted with either hydrochloric acid

16. Cogan, D. G., and Kinsey, V. E.: The Cornea: I. Transfer of Water and Sodium Chloride by Osmosis and Diffusion Through the Excised Cornea, Arch. Ophth. 27:466-476 (March) 1942.

or sodium hydroxide to p_H 1.0 in one set of measurements and to p_H 2.0 in another. The results of these two sets of timing measurements are given in table 4, together with the dissociation constants of the acids employed.

It may be concluded from examination of these data that the cornea has a relatively high permeability to sulfurous acid which cannot be related simply to its dissociation characteristics. This high permeability can be explained, however, by the fact that sulfur dioxide, which in aqueous solution is in equilibrium with sulfurous acid, has considerable lipid solubility to facilitate its transfer through cellular membranes. Measurement of the distribution of sulfur dioxide between water and a typical lipid, tributyrin, showed that sulfur dioxide is, in fact, preferentially soluble in the lipid. Further evidence indicating that penetra-

TABLE 4.—*Comparison of Rates of Penetration of the Cornea by Various Acids in Vitro*

Acid	Time of Penetration (Min.)		Dissociation Constant
	p_H 1.0	p_H 2.0	
Sulfurous	[illegible]	125	1.7×10^{-2}
Trichloroacetic	162	>400	2.0×10^{-1}
Monochloroacetic	129	165	1.4×10^{-3}
Formic	156	115	1.76×10^{-4}
Phosphoric	172	360	1.1×10^{-2}
Hydrochloric	157	>400	
Propionic	162	180	1.4×10^{-5}
Acetic	198	>400	1.75×10^{-5}
Maleic	250	>400	1.5×10^{-2}
Sulfuric	300	>400	
Sulfosalicylic	---	>400	
Nitric	---	>400	
Lactic	---	>400	1.38×10^{-4}

tion of sulfurous acid is effected through a lipid-soluble phase was obtained in examination of the penetration of "latex" membranes, which unreported experiments by Dr. D. G. Cogan have shown to resemble the cornea in being most readily permeable to substances having a definite lipid-soluble phase. Sulfurous acid was found to pass through a "latex" membrane many times faster than phosphorus acid at a comparable concentration and hydrogen ion concentration.

COMMENT AND CONCLUSIONS

Observations made in this study on the effect of freezing and of high concentrations of sulfur dioxide at room temperature leave little doubt that it is the chemical properties of liquid sulfur dioxide which are primarily responsible for corneal injury.

The injurious action of solutions of sulfur dioxide on tissue other than the eye has been known since Pfeiffer[17] (1890) described the

17. Pfeiffer, L.: Zur Kenntniss der giftigen Wirkung der Schwefligen Säure und ihrer Salze, Arch. f. exper. Path. u. Pharmakol. 27:261-296, 1890.

production of protein coagulation, interference with blood supply and necrosis by the substance. The irritant effect and bactericidal action of sulfur dioxide have been noted in numerous subsequent reports. That sulfur dioxide exerts its toxicity principally in the form of sulfurous acid, as concluded in the present experiments on the eye, was previously indicated by Jacobj and Walbaum [18] in a consideration of gastric irritation and was clearly demonstrated by Rahn and Conn [19] in a study on yeast. The conclusion that sulfur dioxide produces an "acid burn" in the eye is supported by the similarity of the histologic changes to those of other acid burns.

When an explanation is sought for the greater toxicity of sulfurous acid than of similar acidic substances, it is observed that sulfurous acid does not have outstanding protein-denaturing properties as compared with other acids, and that, although it does inhibit enzymes, it may do so merely by lowering the p_H, as claimed by Pfeiffer.[17] In fact, the toxicity of sulfurous acid is particularly notable only where cellular structures are concerned, as in the cornea. According to the foregoing experiments, the peculiar susceptibility of cells to sulfurous acid may be explained by the ready penetration of sulfur dioxide through cell membranes, due to its considerable lipid solubility. Thus it appears that the severity of injury with liquid sulfur dioxide is in large part due to the easy penetration of a high concentration of sulfur dioxide through the semipermeable cellular barriers of the cornea, giving access to vital structural and enzyme proteins, which it denatures.

What specific reactions of sulfur dioxide are involved in its devitalizing action are undetermined, but it does seem possible to conclude, by comparison of the relative rates and p_H optima, that its reactions with disulfide linkages (Elsworth and Phillips [20]), thiamine (Williams and associates,[21] 1935) or with aldehydes are probably not the basis of its injurious effect.

SUMMARY

Freezing of the anterior portion of the living rabbit eye for several seconds produces mild and transient disturbance, while freezing for thirty to ninety seconds causes loss of the endothelium with consequent

18. Jacobj, C., and Walbaum, H.: Zur Bestimmung der Grenze der Gesundheitsschädlichkeit der schwefligen Säure in Nahrungsmitteln, Arch. f. exper. Path. u. Pharmakol. 54:421-438, 1906.

19. Rahn, O., and Conn, J. E.: Effect of Increase in Acidity on Antiseptic Efficiency, Indust. & Engin. Chem. 36:185-187, 1944.

20. Elsworth, F. F., and Phillips, H.: The Action of Sulfites on the Cystine Disulfide Linkages in Wool: II. The Influence of Temperature, Time and Concentration on the Reaction, Biochem. J. 35:135-143, 1941.

21. Williams, R. R.; Waterman, R. E.; Keresztesy, J. C., and Buchman, E. R.: Studies of Crystalline Vitamin B: III. Cleavage of Vitamin with Sulfite, J. Am. Chem. Soc. 57:536-537, 1935.

edema of the stroma and epithelium, changes unlike those produced in accidental ocular injury with liquid sulfur dioxide.

Lesions similar to those caused by liquid sulfur dioxide can be produced by several seconds' exposure to the pure gas at room temperature, a condition in which there is no question of freezing.

The concentration of sulfur dioxide in all forms in the rabbit cornea was measured at intervals after exposure and was found to decrease exponentially, with a half-life of approximately 15 minutes.

Introduction of neutral sulfite or bisulfite into the rabbit cornea in concentrations comparable to the total sulfur dioxide concentration of gas-exposed corneas does not produce comparably severe injury, and it is concluded that sulfur dioxide exerts its toxic effect principally in the form of sulfurous acid. No significant production of sulfuric acid was detected.

Sulfur dioxide impairs the turgescence properties of the corneal stroma, as well as inactivates corneal catalase.

The epithelium and stroma of the cornea have a relatively high permeability to sulfur dioxide, a property which is related to the lipid solubility of sulfur dioxide.

It is concluded that the production of severe ocular injury by liquid sulfur dioxide is not due to freezing or to the formation of sulfuric acid, as has formerly been postulated, but is the result of its ready penetration of the corneal epithelium in high concentrations and its action, principally as sulfurous acid, in denaturing corneal proteins, including enzymes.

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Arch. Opthalmol.
38 (6): 755-761
(1947)

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OCULAR INJURY DUE TO SULFUR DIOXIDE

I. Report of Four Cases

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SULFUR dioxide in high concentration in the form of the liquefied gas commonly used in domestic refrigerators is an occasional cause of serious ocular injury. Accidental spraying of liquefied sulfur dioxide into the eyes of persons working on refrigeration machines has in some instances permanently reduced vision to light perception through production of corneal opacification and vascularization. The manner in which sulfur dioxide causes such severe damage is conjectural. In the present report, 4 cases of ocular injury due to sulfur dioxide are described, and in a subsequent report an experimental study will be presented aimed toward an elucidation of the means by which sulfur dioxide causes the damage.

Several previous descriptions of ocular injury due to liquid sulfur dioxide have been presented. A report of an accident involving squirting of liquid sulfur dioxide into the face was given by Kennon[1] (1927), who noted recovery of 20/15 vision following superficial corneal and conjunctival injury. He drew the conclusion that sulfur dioxide is relatively innocuous, but this impression was not confirmed in the experience of Clark,[2] who described 2 cases in which exposure to liquid sulfur dioxide resulted in serious corneal opacification and loss of vision.

Other instances of injury by sulfur dioxide refrigerant, with the outcome varying from complete recovery to dense corneal opacification, were noted, but not described in detail, by Shafer (cited by Clark[2]) and Goldburgh and Gouley.[3] A single instance of injury of the cornea and conjunctiva from liquid sulfur dioxide which was successfully treated with an amniotic membrane graft is included in a series of cases of caustic

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1. Kennon, B. R.: Report of a Case of Injury to Skin and Eyes by Liquid Sulfur Dioxide, J. Indust. Hyg. 9:486-487, 1927.

2. Clark, C. P.: Accidental Freezing of the Eye by Sulfur Dioxide, Am. J. Ophth. 19:881-884, 1936.

3. Goldburgh, H., and Gouley, B.: Sulfur Dioxide Chemical Pneumonia: Report of a Case with Recovery Following Accidental Explosion of a Refrigerator, Unit. Ann. Int. Med. 18:237, 1943.

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burns studied by Sorsby and Symons.[4] Inquiry among a number of other ophthalmologists revealed that several had seen patients with ocular injuries from sulfur dioxide. Presumably, owing to the occurrence of a considerable number of unreported accidents, the presence of an ocular hazard appears to be generally recognized among persons using liquid sulfur dioxide in the refrigeration, as well as in the petroleum industry.

When only the gaseous form of sulfur dioxide is employed, as in magnesium foundries, ocular reactions are, by contrast, relatively mild. This difference is no doubt due to the warning characteristics of the gas, which permit avoidance of excessive exposure. Whereas accidental contamination with the liquid occurs with such suddenness as to preclude effective reflex self protection, exposure to the gas produces symptoms which make its presence obvious, and even unbearable, at lower concentrations than those which cause ocular damage. The warning symptoms and the concentrations at which they occur have been determined by a number of observers (Ogata,[5] Lehmann,[6] Ronzani[7] and Holmes and others[8]) and are considered to consist of a characteristic pungent smell, appreciable at a concentration of 3 to 5 parts per million of air by volume; irritation of the throat, at a concentration of 8 to 12 parts per million, and coughing, painful breathing and a sensation of burning of the eyes, at a concentration of 20 parts per million. On account of these unpleasant symptoms, it is considered that 10 parts of sulfur dioxide per million of air is the maximum concentration tolerable for long exposures and that 50 to 100 parts per million is the maximum for exposures of one-half to one hour. However, an appreciable increase in tolerance is noted among persons chronically exposed.

Conjunctival inflammation has often been mentioned as a frequent consequence of prolonged exposure to high concentrations (Flurry and Zernik,[9] Kehoe and associates[10] and Hamperdinck[11]) and has at times

4. Sorsby, A., and Symons, H. M.: Amniotic Membrane Grafts in Caustic Burns of the Eye, Brit. J. Ophth. 30:337-345, 1946.

5. Ogata, M.: Ueber die Giftigkeit der schwefligen Säure, Arch. f. Hyg. 2: 223, 1884.

6. Lehmann, K. B.: Experimentelle Studien über den Einfluss technisch und hygienisch wichtiger Gase und Dämpfe auf den Organismus, Arch. f. Hyg. 18: 180-191, 1893.

7. Ronzani, E.: Ueber den Einfluss Einatmungen reizender Gase der Industrien auf den Verteidigungskräft des Organismus gegenüber den infektiven Krankheiten, Arch. f. Hyg. 67:255, 1908.

8. Holmes, J. A.; Franklin, E. C., and Gold, R. A.: Report of the Selby Smelter Commission, Bulletin 98, United States Department of Interior, Bureau of Mines, 1915, pp. 172-175.

9. Flurry, F., and Zernik, F.: Schädliche Gase, Dämpfe, Nebel, Rauch- und Staubarten, Berlin, Julius Springer, 1931, p. 143.

been sufficient to necessitate change of occupation (Lewin and Guillery[12]). However, permanent ocular damage from occupational or accidental exposure to gaseous sulfur dioxide appears to be rare and is apparently unlikely to occur even in acute, nearly lethal exposures to the gas. Such conclusions may be drawn from the report of Rostoski and Crecelius[13] on the consequences of exposure of 33 men to sulfur dioxide for eight to forty-five minutes in high enough concentrations to cause the death of 10 and to leave 18 with chronic bronchitis, emphysema and bronchiectasis. Although all the men were said to have had severe inflammation of the eyes and oral mucous membrane immediately after the accident, a persistent ocular disturbance ("conjunctival catarrh") was noted in only 1 survivor. In other instances of accidental exposure to gaseous sulfur dioxide little attention has been paid to the ocular inflammation, whether acute or chronic (Humperdinck,[11] Wiese,[14] Goldburgh and Gouley[7]).

In contrast to the reports already mentioned is that of Strebel,[15] in which emphasis was placed on the presence of a superficial central punctate keratitis characteristically occurring in the manufacture of viscose and attributed by him to the action of sulfur dioxide. As pointed out by Knapp,[16] Strebel was apparently in error as to the cause of the disturbance. Sulfur dioxide is not ordinarily employed in the manufacture of viscose, and none of the commoner symptoms of exposure to sulfur dioxide were noted. Furthermore, it appears that the condition described by Strebel was identical with that subsequently considered by other investigators to be due to the hydrogen sulfide which is formed in the viscose process (Hortsch,[17] Barthelmy,[18] Rodenacker[19]).

10. Kehoe, R. A.; Machle, W. F.; Kitzmiller, K., and LeBlanc, T. J.: On the Effects of Prolonged Exposure to Sulfur Dioxide, J. Indust. Hyg. 14:159-173, 1932.

11. Humperdinck, K.: Zur Frage der chronischen Einwirkung von Schwefeldioxydgasen, Arch. f. Gewerbepath. u. Gewerbehyg. 10:4-18, 1940.

12. Lewin, L., and Guillery, H.: Die Wirkungen von Arzneimitteln und Giften auf das Auge; Handbuch für die gesamte ärztliche Praxis, Berlin, A. Hirschwald, 1905, vol. 1 and 2.

13. Rosteski, O., and Crecelius: Zur Kenntnis der Sulfitgasvergiftungen, Deutsches Arch. f. klin. Med. 168:107, 1930.

14. Wiese, E.: Untersuchungen in einer Leichtmetallgiesserei, abstracted, Zentralbl. f. Gewerbehyg. 28:109, 1941.

15. Strebel, J.: Durch SO_2 verursachte Augenschädigungen (spez. zentrale punktförmige Viskoseverätzung der Hornhaute): Schutz durch Maskenbrille mit Zinkkohlefilter, Schweiz. med. Wchnschr. 53:560-561, 1923.

16. Knapp, P.: Zur Frage der Keratitis traumatica infolge Einwirkung von Gasen, Schweiz. med. Wchnschr. 53:702, 1923.

17. Hortsch, W.: Schwefelwasserstoff die Ursache der Augenerkrankungen in der Viskose-Kunstseidenindustrie, Veröffentl. a. d. Geb. d. Volksgesundhdienst. 47: 669-734, 1937.

(Footnotes continued on next page)

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REPORT OF CASES

Case 1.—In August 1941, E. L., a man aged 27, with previously normal eyes and normal health, received a spray of sulfur dioxide and oil in both eyes from the explosion of a refrigerator on which he was working. There was immediate blurring of vision, but little discomfort. His eyes and face were washed with water, but the attention of an ophthalmologist was sought only after four days, when ocular pain and photophobia became considerable. Vision at that time permitted counting of fingers at 8 feet (240 cm.) with the right eye and at 5 feet (150 cm.) with the left eye. There was gray opacity of both corneas with some conjunctival edema and erosion, especially in the right eye. Despite treatment with a bland ointment and atropine, the photophobia, epiphora and intense conjunctival injection persisted, but vision in the left eye improved to 20/40 in the next four months. Vision in the right eye never improved beyond 20/200 and in four years was reduced to distinguishing light and dark. Loss of vision in the right eye was due to corneal opacity, which in the early stages had appeared diffuse and infiltrative, but after four years was characterized by a dense central plaque, surrounded with a halo of gray infiltration and extensive superficial and deep vascularization. A small central area stained with fluorescein at times. Because of incapacitating discomfort from severe photophobia and a sensation of burning, the cornea of the right eye was covered with a conjunctival flap, giving the patient nearly complete relief for the first time in four and one-half years.

Case 2.—H. B., a man aged 52, in normal health except for his eyes, was seen for the first time ten years after the explosion of a sulfur dioxide refrigerator in his face. His face and eyes had been heavily contaminated with oil and sulfur dioxide, which he could make little attempt to wash off because the accident occurred at some distance from running water. He had immediate serious diminution in vision, which did not improve. Ten years after the accident he could perceive only hand movements at 5 feet with the right eye and at 3 feet (90 cm.) with the left eye but had good light projection. Leukomas of both corneas, with superficial vascularization, were present. There was pronounced symblepharon of both eyes, with thick bands of tissue extending from the fornices to the cornea. The appearance at this stage is shown in the figure. An optical iridectomy was done in the left eye several years after the accident. Later, a resection of a portion of the corneal scar in the right eye and transposition of the symblepharon tissue, followed by roentgen irradiation of the vascularized cornea, was of no apparent benefit.

Case 3.—F. K., a man aged 21, and his brother, A. K. (case 4), received a spray of sulfur dioxide and oil in both eyes from a refrigerator on which they were working. Since discomfort was slight, although vision was blurred, efforts at decontamination consisted principally in wiping their faces and eyes. Two hours later there were edema of the lids, blepharospasm and conjunctival edema, with grayness of the corneas, which stained faintly with fluorescein in the area of the palpebral fissures. In addition to injuries of the eyes, both patients received superficial burns of the mucous membranes of the lips, tongue and nose, with temporary loss of sense of taste and smell, as well as some tracheal irritation.

18. Barthelmy, H. L.: Ten Years Experience with Industrial Hygiene in Connection with Manufacture of Viscose Rayon, J. Indust. Hyg. 21:141-151, 1939.

19. Rodenacker, G.: Die gewerblichen Gefahren des Schwefelwasserstoffes, Med. Klin. 37:215-217, 1941.

In case 3 the immediate signs were all more prominent in the left eye than in the right. Three days after exposure vision was 20/30 in the right eye and 20/40 in the left eye. There was some superficial opacity of the left cornea, and both corneas stained in part. The conjunctivas were still intensely injected and edematous, with a number of small hemorrhages and pale areas of vascular thrombosis, particularly in the left eye.

By the end of the first week the relatively mild degree of involvement of the right eye was obvious, for by then the cornea was practically normal, and in two or three weeks after injury this eye had entirely regained its normal appearance. On the other hand, the more serious nature of the injury to the left eye was suggested during the first week by the appearance of irregularity of the endothelium, slight stromal edema and some opacification. Increased prominence of the corneal nerves was also noted in this eye and the cornea was definitely anesthetic.

For two to three weeks after injury small areas of the cornea of the left eye stained irregularly, and opacification gradually increased in both the superficial and the deep stroma of the temporal portion. This was soon accompanied with super-

Appearance of the eyes ten years after injury by sulfur dioxide (case 2).

ficial and interstitial vascularization. During the first two months the central and temporal portions of the corneal stroma became heavily vascularized. However, during the third to the fifth month there was appreciable clearing.

At no time did the patient have any real pain, although in the first several weeks there were considerable photophobia and some scratching sensation. Mydriasis was maintained with atropine, and subsequently with scopolamine for four months. During this time there was no elevation of intraocular pressure or evidence of iritis.

Case 4.—A. K., a man aged 28, received a spray of sulfur dioxide and oil in his eyes in the same accident as that sustained by his brother (case 3). He experienced the same relatively slight discomfort but had more pronounced immediate blurring of vision. Two hours later there were severe conjunctival edema, blepharospasm and grayness of the corneas, which stained faintly with fluorescein. The lids were edematous but did not appear to be injured. Three days later both corneas were gray with a grossly irregular, lusterless surface, more noticeable in the right eye. Both corneas stained moderately with fluorescein and were completely anesthetic. The conjunctivas were edematous and pale, with thrombosis

of all visible vessels. Vision was reduced to the perception of hand movements in the right eye and to counting fingers at 3 feet in the left eye.

By the end of the first week the dense grayness and irregularity of the anterior surface of the corneas were lost centrally, and vision was considerably improved. However, the stroma of both corneas was still moderately thickened, and the endothelium was gray and irregular. The corneal nerves were abnormally prominent. The conjunctival vessels were still thrombosed; but many small hemorrhages had now appeared, so that the original pallor of the conjunctivas was replaced with a beefy appearance.

During the remainder of the first month the corneas remained fairly clear, although there was persistent staining, more conspicuous in the right eye, and faint laminate opacities could be seen in the stroma, both at the anterior surface and in the central portion. Swelling of the stroma did not progress, and the grayness and irregularity of the endothelium were gradually replaced with a granular appearance. In this period the patient complained of severe photophobia and frontal headaches.

During the second month there were increasing infiltration and opacification of both corneas, with occasional remissions and progressive, dense interstitial vascularization. The anterior surfaces continued to be grossly uneven and to stain irregularly with fluorescein. The photophobia and headache subsided.

In the third month after injury the conjunctivas, which were still severely injected, began to overgrow the corneas, but there appeared to be no tendency to formation of symblepharon.

By the fourth month vision was reduced to perception of hand movements as shadows, owing to corneal opacification and extensive superficial and interstitial vascularization.

During the period described the patient was treated with atropine and then with scopolamine, as was his brother (case 3). At no time was the ocular tension found to be elevated, and no deposits were observed on the posterior surface of the cornea.

COMMENT

From the cases described here, it appears that sulfur dioxide causes almost immediate optical alterations in the cornea, most obviously due to changes in the corneal epithelium, which, though opacified, remains adherent to the stroma, as in acid burns. At first there is only minor discomfort, even with serious injury, probably because of damage to the corneal nerves and resultant anesthesia. The patient may become aware of the seriousness of his injury only during the next several hours or days, when there may be further decrease in vision. There are then considerable swelling of the lids and thrombosis of the conjunctival vessels. Less of the opaque corneal epithelium in several days permits somewhat better vision and in cases of severe injury reveals more clearly the evidences of deeper injury, in the form of stromal edema, opacity of the corneal nerves and a grayness and irregularity of the endothelium. Such deeper changes resemble those produced by the penetrating action of alkalis, and, similarly, the subsequent course may be characterized by the occurrence of stromal infiltrates and interstitial vascularization. In the eyes observed, serious stromal changes of this nature occurred

with only moderate thickening of the stroma, suggesting that minor impairment of semipermeability of the endothelium may be involved. In these cases there was no problem of either iritis or glaucoma. Except for corneal scarring, conjunctival overgrowth of the cornea or formation of symblepharon appears to be the most frequent complication.

In their principal features, the cases reported here are similar to those of other authors. Thus, the slight degree of injury observed by Kennon [1] in his single case has counterparts in the left eye in case 1 and in the right eye in 3, while the effects described by Clark [2] resemble those observed in some of the other eyes in the present cases. The interpretations of these authors, however, find no support in the present observations. The belief that sulfur dioxide is relatively innocuous is certainly unfounded, while the claim that sulfur dioxide produces its ocular injuries through freezing could not be substantiated without considerable additional information, which is unavailable clinically.

To supply some of the information necessary for evaluation of the mode of action of sulfur dioxide in producing ocular injuries, an experimental investigation has been undertaken and will be reported separately.

SUMMARY

Ocular injuries resulting from spraying of liquid sulfur dioxide and oil into both eyes in 4 cases were characterized in most instances by immediate damage to the corneal epithelium, as in acid burns, with underlying stromal and endothelial injury, as in alkali burns. In the cases of mildest injury the outcome was complete recovery, while in the cases of severest damage dense opacification followed corneal infiltration and interstitial vascularization.

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Sulfur Dioxide: Sulfite

Interaction With Mammalian Serum and Plasma

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The chemistry of sulfite—bisulfite (the hydrate of sulfur dioxide in mammalian plasma and serum was investigated in vitro and in vivo. The longevity of sulfite in contact with mammalian plasma and known components of blood was determined by adaptation of a colorimetric method for sulfite analysis. Evidence from all experiments indicated that, under physiological conditions, sulfite reacts reversibly with disulfide bonds present in the plasma resulting in formation of S-sulfonates (sulfitolysis). Free sulfite was not detected in plasma of rabbits immediately following exposure to approximately 25 ppm SO_2, but there was good evidence for substantial elevation of plasma and serum S-sulfonate content. Reactivity of sulfite with plasma constituents may protect many body tissues from the insult of relatively high concentrations of sulfite and may facilitate prolonged exposure to very low levels of sulfite.

SULFUR DIOXIDE is one of the earliest recognized and most widely studied air pollutants. Among the biological effects of SO_2 on man and other mammals, the most thoroughly investigated have been those related to pulmonary mechanics. In addition, SO_2 has been shown to affect antibody production and enzymatic activity of mammals exposed to the gas.[1] Several workers[2-6] using SO_2 labelled with radioactive sulfur (^{35}S) ($^{35}SO_2$) to study the absorption, distribution, and retention of SO_2 in the mammalian body have found, in general, that ^{35}S is absorbed into the bloodstream from all portions of the respiratory tree and is distributed essentially to all body tissues including the brain. The sulfur from inhaled SO_2 remains in the body tissues for an appreciable length of time, but its chemical nature has not been thoroughly investigated. Bystrova[2] observed that some of the inhaled SO_2 appeared to combine with the tissue proteins, however, the specific nature of the binding was not investigated.

Previous studies utilizing ^{35}S have given little information concerning the chemical behavior of SO_2, or its hydrate (bisulfite), once it has come in contact with the body tissues and fluids. Work by Frank et al[6] showed that some $^{35}SO_2$ was liberated from the bloodstream of dogs following exposure of their surgically isolated upper airways. Elfimova and Pushkina[7] detected free sulfite in the blood of children living in a "polluted" area.

The purpose of this study is to investigate the reactivity of sulfite or bisulfite (hereafter referred to simply as sulfite) with mammalian plasma and serum under physiological conditions.

Materials and Methods

The reactivity of sulfite added to serum or plasma in vitro was followed by monitoring the concentration of the sulfite in the reaction mixture. This was accomplished by adaptation of the West and Gaeke[8] method for sulfite analysis.

Reagents.—Para-rosaniline hydrochloride (PRA) was purchased from a commercial manufacturer. Insulin was obtained from a commercially available preparation of beef insulin, and a sample of crystalline bovine zinc insulin was shipped directly to us. All other chemicals were reagent grade. All solutions were prepared with deionized water. Reagent

Submitted for publication May 29, 1970; accepted June 1.

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solutions used for sulfite analysis and for standardization of sulfite were prepared according to guidelines in a Public Health Service Publication.[9]

Procedure 1, Collection of Blood.—All plasma and serum samples were collected from the marginal ear vein of adult New Zealand white rabbits with the aid of a suction device or from an arm vein of adult humans with evacuated test tubes. Edetate disodium (disodium ethylenediaminetetraacetate) at 2 mg/ml was used as an anticoagulant in the plasma samples.

Procedure 2, Analysis of Sulfite.—Ten 200µl of aqueous standard sulfite or unknown solution containing approximately 0.3 to 5.2 nanomols of sulfite, 20µl of PRA and then 20µl of formaldehyde reagent were added. After 20 minutes, the optical density of this mixture, termed a "standard reaction solution" at 560 mµ, was measured.

Procedure 3, Analysis of Sulfite in Presence of Plasma or Serum.—To 200µl of solution containing 40µl of serum or plasma, approximately 6 microequivalents of hydrochloric acid, and 0.3 to 5.2 nanomols of sulfite, 20µl of PRA and 20µl of formaldehyde reagent are added. A serum or plasma reagent blank was prepared at the same time by omitting the sulfite, or by including 20µl of 0.0030% hydrogen peroxide in a 200µl acidified serum (plasma)-sulfite solution. The optical density was measured against this blank after 20 minutes at 560 mµ.

Procedure 4, In Vitro Reaction of Sulfite With Plasma or Serum.—Serum or plasma was incubated with a known amount of sulfite in a nitrogen atmosphere at 98.6 ± 2 F (37 ± 1 C), pH 7.4 (0.11M tromethamine [Tris] hydrochloride), using a ratio of 1 volume of serum (plasma) to 1 volume of buffer and sulfite. These conditions are referred to as standard pH 7.4 incubation conditions. After incubation for the desired period of time, the amount of sulfite remaining in the mixture was analyzed according to procedure 3, adding sufficient excess HCl to destroy the buffering effect of the tromethamine present in the incubation mixture. This determination indirectly gives the amount of sulfite that "disappeared" from the incubation mixture and which was assumed to have reacted with the serum (plasma).

Procedure 5, Recovery of Reacted Sulfite by Treatment With Alkaline Cyanide.—Plasma or serum was incubated with a known amount of sulfite (standard pH 7.4 incubation) until at least 95% of the sulfite had reacted. To this mixture, sufficient alkaline sodium cyanide solution was added in order that the resulting mixture was 0.0208M in cyanide and at approximately pH 10.2 This mixture was incubated under nitrogen for 60 minutes at 98.6 ± 2 F (standard alkaline cyanide incubation conditions). Then, the contents were transferred quantitatively to a seamless cellulose dialysis bag (one half-inch inflated diameter), and dialyzed at approximately 39 F (4 C) against 4 ml of 0.0664M glycine-sodium hydroxide buffer at pH 10.2 for 2½ hours. (Autoxidation of sulfite under these conditions is insignificant.) The dialysate and the dialyzed solution both were analyzed for sulfite, and the total amount detected was compared with the amount added initially to the pH 7.4 incubation mixture.

Comments on Procedures.—Volumetric polyethylene micropipettes were used to make up all solutions for incubation and for sulfite analysis. The standard sulfite curve prepared by procedure 2 was in accordance with Beer's law over the range 0 to 5.2 nanomols sulfite per standard reaction solution (240µl).

There is measurable reagent blank interference in the spectrophotometric analysis for sulfite in the presence of serum or plasma. This interference, which is unstable and increases with time even after 30 minutes, stems from the light-scattering effect of the plasma protein, the absorption by plasma pigments, and, most importantly, absorption by a purple coloration arising from the reaction between PRA, formaldehyde, and thiol groups in the plasma.[10] After 20 minutes, the interference of a given plasma reagent blank solution prepared as outlined in procedure 3 was equivalent to about 1 nanomol of sulfite. The exact magnitude of the interference of various serum or plasma reagent blank solutions, however, varies not only as a function of time but also slightly from one serum or plasma sample to another. Therefore, to cancel out this interference, plasma (serum) reagent blanks must be prepared at the same time and from the same plasma (serum) sample that is used to make up the plasma-sulfite solutions.

The HCl included in the reaction solution given in procedure 3 is necessary to destroy the buffering capacity of the serum or plasma, thus producing a reaction solution pH (1.4) which is the same as that of the standard reaction solution (from procedure 2). Constancy of pH of the reaction solution is essential because both molar absorptivity at 560 mµ and the absorption spectrum of the sulfite-PRA-formaldehyde reaction product are pH-dependent.[11]

Three quantities of sulfite, 1.7, 3.6, and 5.2 nanomols, were analyzed in the presence of serum collected from an unexposed rabbit as described by procedure 3. Each analysis was replicated six times, and the percentages of added sulfite detected were 97 ± 1 (m ± SD), 97 ± 2, and 102 ± 3, respectively; the standard

deviation of values obtained in the corresponding analyses of the same quantities of aqueous sulfite (six replications) were 2%, 2%, and 3%, respectively.

The reason for adding hydrogen peroxide to the plasma (serum) reagent blank of procedure 3 is to oxidize any sulfite present. Previous tests with nine samples of rabbit plasma taken from three unexposed rabbits had shown that 20μl of 0.0316% hydrogen peroxide were sufficient to oxidize 2.2 nanomols of sulfite added to a 40μl aliquot of plasma, although the exact amount of hydrogen peroxide required was somewhat variable. An excess of hydrogen peroxide, produced by doubling the concentration, had no effect on the analysis.

Apparatus for Exposure of Rabbits to SO_2.—A plastic glove box with approximately 8 cu ft of space was used as an SO_2 exposure chamber for rabbits. Sulfur dioxide was metered into a 12-inch polyethylene tube (⅜-inch inside diameter[ID]) where it mixed with ambient air. The mixture was "pulled" through the chamber at a rate of 2.4 cu ft/min (1 air change every 3.4 minutes) by a rotary, vane type pump. The concentration of SO_2 in the chamber was determined by the West and Gaeke method.[8] The distribution of pollutant within the chamber proved satisfactory, however, there was considerable fluctuation in SO_2 concentration over an extended period of time due to imperfect regulation of SO_2 input. The average SO_2 concentration was 23.5 ± 5 ppm.

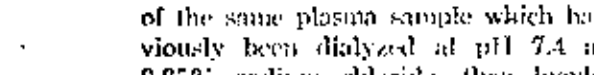
The rabbits weighed between 3 and 4 kg and were 4 to 22 months old. One rabbit was exposed at a time. Food and water were continuously available before, during, and after exposure.

Results and Comment

In Vitro Reaction of Sulfite and Plasma (Serum).—Figure 1, *triangle line*, illustrates the reaction between a given sample of rabbit plasma and 50μM sulfite according to standard pH 7.4 incubation conditions. The *circle line* of Fig 1 represents the reaction, under identical circumstances, with portions of the same plasma sample which had previously been dialyzed at pH 7.4 against 0.85% sodium chloride, then lyophilized, and finally reconstituted to original volume with deionized water. Direct comparison of both lines of Fig 1 makes clear that a portion of the reactivity of rabbit plasma for sulfite is dialyzable, however, most of the reactivity is in the nondialyzable fraction. Reaction of rabbit plasma, both dialyzed and unaltered, with an excess of sulfite showed that approximately two thirds of the total reactivity of this sample was associated with the nondialyzable fraction. Comparison of a dialyzed and nondialyzed human serum sample gave similar results.

In a closely related experiment, the reaction between portions of a human serum sample and 390μM sulfite was followed for three hours. The reaction reached either an end point or a steady state equilibrium after approximately one hour and with about 81% of the sulfite still in the free form. The quantity of free sulfite remained constant between one and three hours of incubation—evidence that there was no loss of sulfite by autoxidation in the system. This situation might be likened to in vivo conditions in which autoxidation to sulfite does not occur at a significant rate for two reasons: (1) the presence in mammalian tissue fluid of substances which act as chain breakers of the free radical autoxidation chain; and (2) the very low level of free metal ions which catalyze the autoxidation of sulfite.[12]

Nature of Reaction Between Sulfite and Plasma (Serum).—The technique used for following the sulfite concentration in incubation mixtures obviously does not identify the reactions occurring. The data given have shown, however, that sulfite is not undergoing a rapid oxidation and that most of the reactivity of the plasma is located in the nondialyzable fraction. Such observations lead to the speculation that sulfite could be reacting with disulfide bonds present in plasma, largely in the plasma proteins, with the resulting formation of S-sulfonate residues (reaction 1).



$$(1)\quad R\text{-}S\text{-}S\text{-}R + SO_3^{=} \rightleftharpoons R\text{-}S\text{-}SO_3^{-} + R\text{-}S^{-}$$

This is a well-known and thoroughly documented reaction[12] which is often utilized as a gentle method for breaking the protein into fragments (sulfitolysis). The reaction has rarely been used or studied under physiological conditions, however, and usually entails the use of extremely high concentrations of sulfite, a wide range of pH values, and often denaturing agents.

The cyanide ion is more thiophilic than is

sulfite and it will attack the S-sulfonate disulfide and under alkaline conditions (reaction 2).

$$(2)\quad R\text{-}S\text{-}SO_3^- + CN^- \rightarrow R\text{-}S\text{-}CN + SO_3^{=}$$

Sulfite generated from S-sulfonate can then be analyzed by the modified West and Gaeke method previously described. This reaction provides a logical technique for determination of the extent of S-sulfonate formation in an incubation mixture. To test this procedure, 54 nanomols of sulfite was allowed to react with 480μl of rabbit plasma for 15 minutes using standard pH 7.4 incubation conditions (56μM sulfite). At least 95% of the sulfite had reacted after this incubation. Three separate analyses showed that 94%, 100%, and 105% of the sulfite added to the three incubation mixtures was recovered in the free state by treatment with alkaline cyanide. This sulfite is termed "cyanolytic sulfite," although, in this case, a small amount of sulfite which had not reacted at pH 7.4 is included in the cyanolytic sulfite recovered. The complete, or near complete, recovery of reacted sulfite by this procedure is evidence that S-sulfonates are the dominant and probably the only reaction product formed in standard pH 7.4 incubation mixtures.

Exposure of Rabbits to SO_2 and Analysis for Cyanolytic Sulfite.—Four rabbits were exposed individually to SO_2 at 23 ± 5 ppm. Figures 2 and 3 show the content of plasma and serum cyanolytic sulfite before and for an extended period after exposure. For clarity of illustration no data points are presented for samples collected between approximately 30 and 200 hours postexposure. Rabbits 1 and 2 (Fig 2) were exposed continuously for 14 hours and rabbits 3 and 4 for 62 hours (Fig 3). Two or three 1-ml aliquots from each plasma and serum sample were incubated according to standard alkaline cyanide conditions. Liberated sulfite was recovered by repeated dialysis for 2¾-hour periods under conditions described in procedure 5. Note the sharp increase in cyanolytic sulfite detected immediately after SO_2 exposure in all four rabbits. In this analytical procedure, however, any free sulfite present in the plasma would be determined as cyanolytic sulfite. Thus, three aliquots of the plasma sample taken from rabbit 3 immediately after exposure were treated with an alkaline solution containing no cyanide (pH 10.2) and analyzed in the usual fashion for liberated sulfite; none was detected. These data, then, support the supposition that S-sulfonates are formed in the plasma and serum as a direct result of SO_2 exposure.

Cyanolytic sulfite in the plasma immediately following 62 hours of exposure was greater than that observed after 14 hours, indicating that the tissues had not reached equilibrium with the approximately 25-ppm atmospheric SO_2 even after 14 hours of continuous exposure. The decay of plasma and serum cyanolytic sulfite following exposure can be explained by reversal of the initial reaction (represented by reaction 1) brought about by diffusion of sulfite from the bloodstream and subsequent oxidation to sulfate. Oxidation of sulfite is most likely accomplished by the enzyme sulfite oxidase (sulfite: oxygen oxidoreductase E.C. 1.8.3.1). This enzyme has been shown to occur in various tissues of several mammals.[14,15] The approximate times required for the exogenous plasma cyanolytic sulfite levels of rabbits 1 to 4 to decay to one half their immediate postexposure values are 30, 10, 30, and 5 hours, respectively. The level and half-life of cyanolytic sulfite in the blood and other tissues of animals following exposure to SO_2 is an area which warrants more extensive and carefully controlled investigation.

Serum samples were collected and analyzed simultaneously with plasma samples from rabbits 1 and 4. The concentration of cyanolytic sulfite was always greater in the plasma than in the corresponding serum sample. This might be explained by the presence in the plasma fibrinogen of a large number of disulfide bonds. For example, human fibrinogen has been shown to contain 56 half-cystine residues per molecule, many of which are part of the N-terminal "disulfide knot."[16] The molecular structure of fibrinogen for other mammalian species investigated is similar.

Endogenous Cyanolytic Sulfite.—Cyanolytic sulfite was present in the plasma and serum of all four rabbits prior to exposure. Seven preexposure plasma samples were

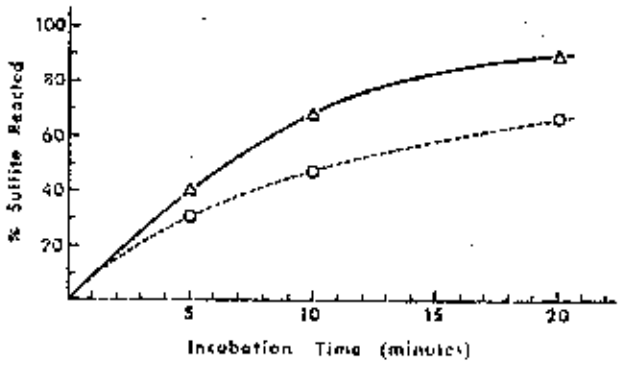
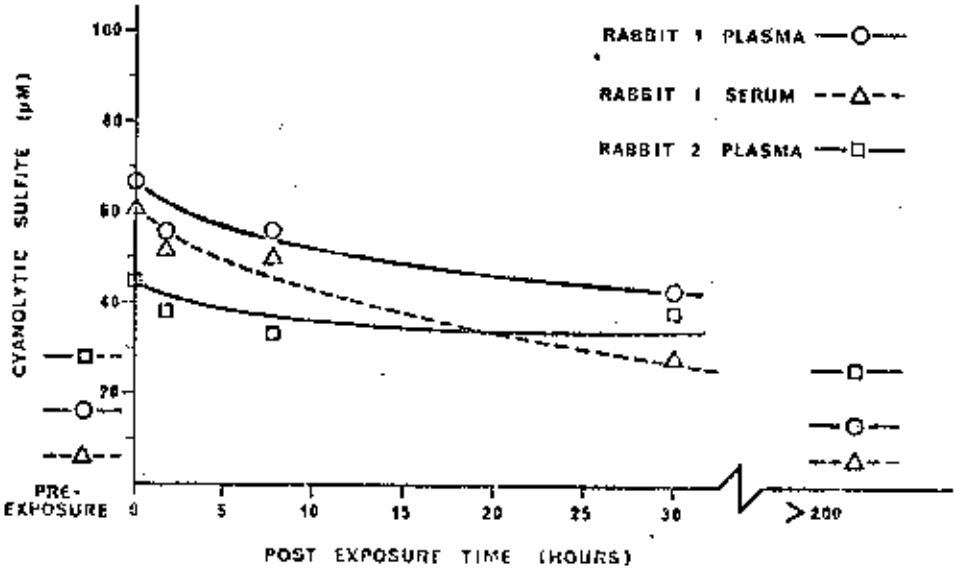


Fig 1.—In vitro reaction of rabbit plasma with 50μM sulfite according to standard pH 7.4 incubation conditions. Each point is mean of three separate incubations. Triangle line, unaltered plasma; circle line, dialyzed, lyophilized, and reconstituted plasma.

Fig 2.—Serum and plasma cyanolytic sulfite concentration of rabbits 1 and 2 before and after exposure to 23.5 ± 5 ppm SO_2 for 14 hours.



taken from rabbit 2 over a period of ten days; each was analyzed in triplicate. Two collection times were used: 9:30 am and 3:30 pm. The cyanolytic sulfite concentration averaged 27.4μM ± 3.4μM and showed no significant diurnal nor day-to-day variation over the ten-day sampling period.

No endogenous cyanolytic sulfite was detected in samples of human plasma from three men (25 to 32 years of age). The diversity observed between rabbit and human plasma regarding apparent endogenous S-sulfonates may reflect a possible difference in sulfite oxidase activity between the two species; this in turn would determine the availability of endogenous sulfite for reaction with disulfide groups.

Free Sulfite in Plasma of Exposed Rabbits.—Analysis for plasma sulfite of rabbits 3 and 4 immediately after their removal from the exposure chamber disclosed a sulfite level of about 0.016 mg/100 ml (2μM). This value is no different than those obtained using plasma from unexposed rabbits

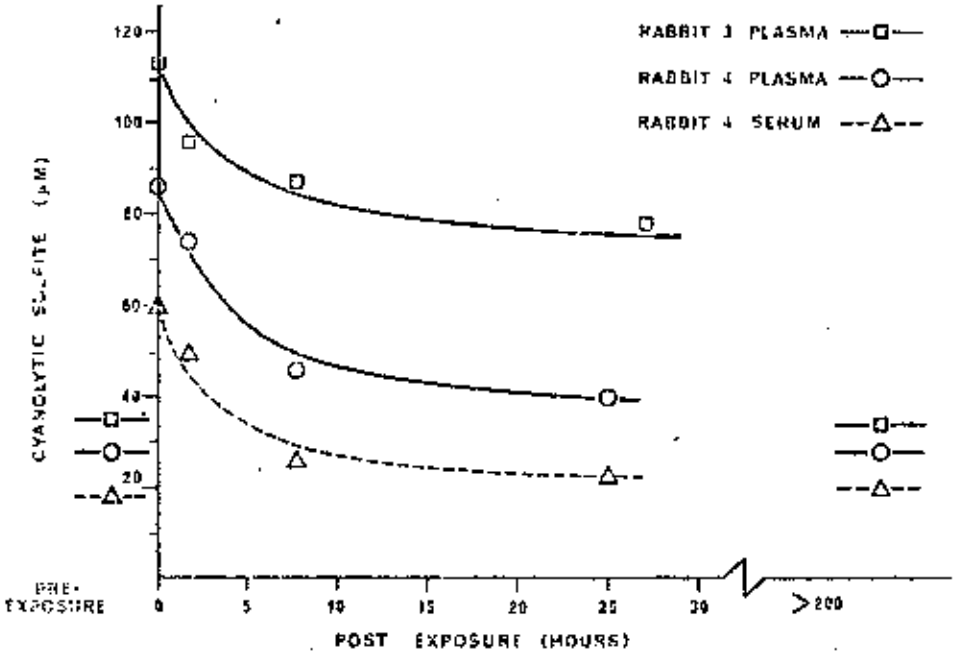


Fig 3.—Serum and plasma cyanolytic sulfite concentration of rabbits 3 and 4 before and after exposure to 23.5 ± 5 ppm SO_2 for 62 hours.

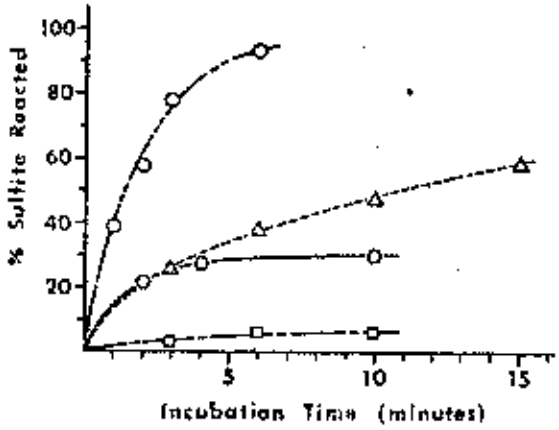


Fig 4.—In vitro reaction of 50μM sulfite with selected compounds known to be present in mammalian blood. Each point is mean of three to six separate incubations. Circle line, 340μM cystine; triangle line, 340μM oxidized glutathione; hexagon line, 340μM cysteine and 75μg/100 ml of cupric ion; square line, 170μM insulin.

and is below the minimum concentration of plasma sulfite which can be accurately determined by the methods used. These data, in conjunction with the data presented for plasma cyanolytic sulfite content, show that the equilibrium position of sulfite in plasma, in vivo, lies far to the right or combined state (reaction 1) for relatively small amounts of sulfite.

The above conclusion is not entirely consistent with data presented by Elfimova and Pushkina.[7] These workers reported free sulfite in the blood of children from a "polluted" area ranging from "traces" to 0.12

mg/100 ml, as contrasted with a lack of sulfite in the blood of children from a control area. The discrepancy noted could possibly stem from the difference in species tested. It seems more likely, however, that detection of sulfite by Elfimova and Pushkina[7] was an artifact of the analytical method used. This method (Grant[17]) utilizes an alkaline protein precipitation step with alcoholic potassium hydroxide and mercuric chloride. These conditions would tend to reverse the equilibrium established at pH 7.4 between sulfite and plasma constituents probably resulting in the formation of dichlorosulfitomercurate (II), a complex that would be determined as sulfite in the ensuing analysis.[18] Thus, it is possible that sulfite detected by Elfimova and Pushkina[7] existed in vivo as S-sulfonate. A similar upset of the equilibrium established between sulfite and plasma at pH 7.4 probably does not occur when sulfite analysis is performed by the method presented in this paper. The first step in this analytical procedure is acidification. There is good evidence that acidification freezes the equilibrium position of reaction 1.[19,20]

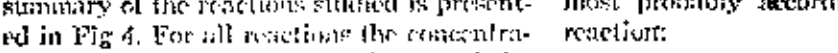
In Vitro Reaction of Sulfite With Compounds of Physiological Importance.—The reactions of sulfite with selected physiologically important compounds present in blood, most containing one or more disulfide bonds, were pursued in vitro in order to characterize their reactivity. Standard pH 7.4 incubation conditions were used except that the plasma or serum was eliminated. A summary of the reactions studied is presented in Fig 4. For all reactions the concentration of sulfite was 50μM and that of the reactant being studied was 340μM, unless otherwise specified.

The reaction of cystine with sulfite is relatively rapid (Fig 4, *circle line*); half of the sulfite reacted within about 1½ minutes. Presumably the reaction involved is sulfitolysis of the disulfide bond. Treatment of the incubated mixture with alkaline cyanide under standard conditions yielded 100% recovery of the added sulfite. Although the concentration of cystine in this incubation mixture was about 16 times that normally found in rabbit plasma,[21] the relatively rapid rate of reaction suggests that cystine is a very important component in the overall reactivity of plasma with sulfite. The difference in reactivity between dialyzed and normal plasma (Fig 1) can be accounted for by the expected cystine content of rabbit plasma. The *triangle line* of Fig 4 represents the reaction between oxidized glutathione, a cystine-containing peptide, and sulfite; half of the sulfite reacted within 10 to 11 minutes.

Sulfitolysis of the interchain disulfide bonds of insulin, which inactivates the molecule, has been demonstrated at pH 7.4.[19] The rate of sulfitolysis and the equilibrium position attained (Fig 4, *square line*) indicate that this reaction is of no significance at physiological conditions. Equilibrium was reached after incubation for six minutes at which time only about 6% of the sulfite had reacted. The concentration of insulin used was 170μM, or 340μM in interchain disulfide bonds; this is thousands of times greater than physiological concentrations in the blood of mammals.

There are many reasons to conclude the potential for physiologically important sulfitolysis of hormones containing disulfide bonds (for example, insulin, oxytocin, vasopressin) under in vivo conditions is nil. Most important is the very low concentration of the active species of any hormone in the bloodstream or body tissues at a given time. This, in combination with the low concentration of sulfite, makes any significant reaction improbable.

Cysteine reacted with sulfite in the presence of cupric ion, 75μg/100 ml (11.8μM) most probably according to the following reaction:



$$(3)\quad R\text{-}SH + SO_3^{=} + 2Cu^{++} \rightarrow R\text{-}S\text{-}SO_3^{-} + 2Cu^{+} + H^{+}$$

The reaction is depicted in Fig 4, *hexagon line*. Elimination of cupric ion from the incubation mixture prevented the reaction while increasing cupric ion to 750μg/100 ml resulted in the complete loss of sulfite within four minutes. Since cupric ion also catalyzes the autoxidation of sulfite, care was taken to use adequate control incubations.

The normal concentration of copper in human adult plasma is 115μg/100 ml, but much less than 4% of this is dialyzable, the bulk being attached to plasma proteins.[22] In rabbit plasma, there is approximately [illegible]

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of copper per 100 ml. The concentration of free cupric ion in the plasma of most species is probably too low to function in the reaction of sulfite with SH groups. Other oxidants may also function in reaction 3, for example, ferric iron. However, as with plasma copper, nearly all the ferric ion is associated with transport proteins. Edetate disodium (4 mg/ml) was added to a portion of a rabbit serum sample and its reactivity was compared with the untreated portion of the same sample. The addition of edetate, which would create a complex with any free cupric or ferric ions present, did not affect reactivity of the serum with sulfite.

Conclusions

The evidence presented supports the theory that sulfite which enters the bloodstream during exposure of a mammal to atmospheric SO_2 forms S-sulfonate groups with constituents of the plasma, probably exclusively by sulfitolysis of disulfide groups. The composite reaction involved apparently reaches a position of equilibrium with very close to 100% of the sulfite in the oxidized or sulfonate form, provided that the capacity of the reactive groups of the plasma has not been exceeded by an excess of sulfite. This interaction quite probably affords protection to many tissues of the body from the insult of high concentrations of sulfite. The thought also occurs that this same phenomenon may in addition facilitate prolonged exposure to a very low level of sulfite, as sulfite oxidase, maintaining a low concentration of sulfite in the body, indirectly causes the release of sulfite from S-sulfonate linkage.

This project was sponsored under PHS grant AP 00022 through the Center for Air Environment Studies of the Pennsylvania State University.

This article is paper 3703 in the journal series of the Pennsylvania Agricultural Experiment Station.

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Kvasný průmysl

ODBORNÝ ČASOPIS PRO PRACOVNÍKY V KVASNÝCH PRŮMYSLECH

58

Rychlá a přesná metoda stanovení obsahu SO_2 ve chmelových hlávkách

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Síření chmele je jedním z nejrozšířenějších konzervačních způsobů chmele. Kysličník siřičitý, adsorbovaný v nepatrných koncentracích v hlávkách chmele, má příznivý vliv na stabilitu hořkých látek [1] a svými redukčními vlastnostmi také příznivě ovlivňuje barevný vzhled chmele, kde napomáhá k vyrovnání různých nepříznivých poškození barvy hlávek.

Adsorbovaný kysličník siřičitý se zejména při vyšších koncentracích z části oxyduje za vzniku kysličníku sírového, který s vodními parami atmosféry dává kyselinu sírovou, jež působí nepříznivě na kvalitu hlávek, především na jejich barvu. Proto je třeba v některých případech kontrolovat síření a stanovit jeho stupeň v zájmu zpracovatelnosti jednotlivých partií chmele. Vzhledem k velmi malému obsahu kysličníku siřičitého, který zůstává po síření ve chmelu, je velmi obtížné běžnými analytickými vážkovými metodami stanovit jeho množství. Tyto metody selhávají, zejména u starších chmelů, kde je podstatná část kysličníku siřičitého již zoxydována [2].

Nejběžněji používanou metodou stanovení obsahu kysličníku siřičitého v sířeném chmelu je metoda s chloridem barnatým. Při izolaci tohoto plynu se využívá jeho těkavosti s vodní párou a jímání do roztoku jódu, kde současně probíhá jeho oxydace na kysličník sírový, dávající s vodou kyselinu sírovou. K jejímu vážkovému stanovení se využívá reakce síranů s barnatými ionty. Citovaná metoda je zdlouhavá a vzhledem k malým množstvím kysličníku siřičitého ve chmelu, značně nepřesná. To platí i o ostatních vážkových metodách (benzidinové atd.).

Rychlou metodou pro stanovení kysličníku siřičitého, je nepřímá titrační jodometrická metoda, založená na tom, že se přidává vodný roztok siřičitanů do nadbytečného roztoku jódu o známé normalitě a zpětnou titrací thiosulfátem sodným se zjišťuje množství jódu, který se zúčastnil reakce s kysličníkem siřičitým. Nevýhodou je, že kysličník siřičitý se vytěsňuje vodní párou a spolu s ním těkají i chmelové silice. Tento způsob stanovení kysličníku siřičitého je nevhodný, protože při extrakci získaného destilátu organickými rozpouštědly se nepodaří kvantitativně oddělit chmelové silice od alkalického roztoku kysličníku siřičitého (prakticky siřičitanu). Většina látek obsažených ve chmelové silici je nenasyceného charakteru reagující s jódem a podstatně tak zvyšuje jeho spotřebu, a tím znemožňuje použít tohoto způsobu pro stanovení obsahu kysličníku siřičitého ve chmelu.

Z dalších metod, využívajících fyzikálních přístrojů k stanovení tohoto plynu, možno uvažovat ještě metodu polarografickou, avšak pro běžné použití v praxi je není možno brát v úvahu, poněvadž podstatná část provozních laboratoří není vybavena tímto přístrojem.

Z uvedených příčin byla vypracována přesnější a expeditivnější metoda, která vykazuje větší přesnost a není náročná na laboratorní vybavení. Požadované podmínky má metoda kolorimetrická [3], kterou autoři upravili pro potřeby stanovení kysličníku siřičitého ve chmelu.

Experimentální část

Princip metody

Rozemletá chmelová drť se vpraví do destilační baňky, přídavkem kyseliny fosforečné se vytěsní kysličník siřičitý, který se jímá v absorpčním roztoku. Absorpční roztok, ve kterém je obsažen SO_2, dává s fuchsin-formaldehydovým činidlem červenofialové zabarvení, jehož intenzita je přímo úměrná koncentraci kysličníku siřičitého. Z kalibrační křivky se vyčte koncentrace SO_2. Tímto způsobem lze podat i kvalitativní důkaz SO_2 ve chmelu. Metoda

má vysokou citlivost a je jí možno zjistit 0,5 gama SO_2.

Potřebná činidla:

1. Kyselina fosforečná 85 % p. a.

2. Absorpční roztok + 0,2 N NaOH s přídavkem 5 % glycerinu (antioxydační činidlo, aby se zabránilo oxydaci SO_2). Do odměrné baňky na 1000 ml se odváží 8 g NaOH p. a. a přidá se 50 g glycerinu p. a., destilovanou vodou se doplní po značku a promíchá.

Příprava reagenčního roztoku:

Roztok fuchsinu: odváží se 0,10 g bazického fuchsinu, rozpustí se v 10 ml etanolu, přidá se 44 ml konc. H_2SO_4 p. a. a 735 ml destilované vody a po 3 až 4 dnech se přefiltruje.

Roztok formaldehydu: 5 ml 40% formaldehydu se zředí na 100 ml destilovanou vodou a promíchá.

Před použitím se smíchá 10 dílů roztoku fuchsinu s 1 dílem formaldehydu.

Příprava kalibrační křivky:

Základní roztok se připraví ze siřičitanu. Odváží se 0,1094 g siřičitanu sodného p. a. (*Merck*) o hodnotě 98 % a rozpustí se v 500 ml 0,2 N NaOH s obsahem 5 % glycerinu. Roztok obsahuje v 1 ml 100 gama SO_2. Standardy se připraví tím způsobem, že základní roztok se zředí 0,2 N NaOH s přídavkem glycerinu tak, aby výsledné roztoky obsahovaly 1, 3, 6, 9 gama SO_2/ml. Z těchto roztoků se odměří po 1 ml do zkumavek, každá zkumavka se doplní na 15 ml absorpčním roztokem s přídavkem glycerinu, promíchá a přidá se 10 ml reagenčního roztoku. Nechá se asi 20 min stát až se ustálí zabarvení a pak se měří na fotokolorimetru filtrem 500 mμ. Naměřené hodnoty se vynesou do souřadnicového systému, a tím se získá kalibrační křivka pro SO_2.

Vlastní stanovení

Do zábrusové baňky obsahu 750 ml se vpraví 10 g chmelové drtě, přilije se 250 ml destilované vody, přidá se 10 ml 85% kyseliny fosforečné, baňka se napojí na chladič s alonží, jejíž konec musí zasahovat do nádobky s absorpčním roztokem (asi 50 ml). Konec alonže se ponoří asi 1,5 až 2 cm. Od začátku varu se destiluje ½ h. Po této době se nejprve odstaví baňka s destilátem, ponechá se asi 5 min odkapat (aby se propláchl chladič), nesmí se přerušit var. Potom se převede nadestilovaný roztok do odměrné baňky na 200 ml a doplní absorpčním roztokem po rysku a promíchá. Takto připravený roztok se použije k vlastnímu stanovení obsahu kysličníku siřičitého.

Pro velkou citlivost reakce se objeví zabarvení s fuchsin-formaldehydovým činidlem i u chmelů nesířených avšak hodnota extinkce je vždy pod hranicí nejnižšího standardu. U chmelů starších, sířených, je třeba brát pouze 1 ml základního roztoku, u chmelů čerstvě sířených postačí 0,1 ml základního roztoku. U chmelů nesířených se odpipetuje ze základního roztoku celých 15 ml. Postupuje se tak, že přesně odměřené množství, např. 0,5 ml základního roztoku se doplní na 15 ml absorpčním roztokem, čili přidá se 14,5 ml abs. roztoku, promíchá se a přidá 10 ml reagenčního roztoku a opět promíchá. Musí se vždy dodržovat poměr 2 : 3 (tj. 3 díly základního a absorpčního roztoku : 2 díly reagenčního roztoku). Tato reakce může sloužit i ke kvalitativnímu zjišťování přítomnosti SO_2 a ověřovat, zda chmel byl či nebyl sířen.

Informační stanovení obsahu SO_2 u starších chmelů

K tomuto účelu se použilo chmele sířeného (*tabulka 1*), jehož vzorek byl vyseknut z balotu a chmele nesířeného, který byl směsí typových vzorků (*tabulka 2*). Zároveň se stanovil i obsah veškeré síry, tedy i té, která je vázána v rostlinné hmotě. Rostlinná hmota se spálila mokrou cestou. Výsledky stanovení jsou uvedeny v *tabulkách 1 a 2*.

Tabulka 1

Obsah SO_2 a veškeré S u staršího chmele (z balotu)

Vzorek	Obsah SO_2 v %	Obsah veškeré S v %
1	0,005	0,398
2	0,009	0,451

Tabulka 2

Obsah SO_2 a veškeré S u nesířeného chmele

Vzorek	Obsah SO_2 v %	Obsah veškeré S v %
1	0,0002	0,238
2	0,0002	0,394
3	0,0002	0,219
4	0,0002	0,249

Hodnoty vyjadřující procento SO_2 jsou zaokrouhleny. Z uvedených čísel je vidět, že obsah SO_2 u chmelů starších se pohybuje řádově okolo 0,005 %, u chmelů nesířených o celý řád níže a sice 0,0002 %. Každé uvedené číslo je průměrem ze čtyř stanovení. Shodu vidíme i u obsahu celkové síry, kde hlávky nesířené mají opět nižší obsah v poměru k sířeným. Kolísání ve výsledku se pohybuje v průměru ±5 %.

Vliv doby sírení na obsah SO_2 ve hlávkách chmele

Za tímto účelem se sířily vzorky chmele po 30, 45, 60 a 90 min. Po této době se vzorky zabalily do modrých papírů a druhý den zpracovaly podle výše uvedené metodiky. Výsledky jsou uvedeny v *tabulce 3*.

Tabulka 3

Vliv doby sírení na obsah SO_2 ve hlávkách chmele

Doba sírení v min	Obsah SO_2 v %
30	0,005
45	0,006
60	0,010
90	0,002
kontrola	0,0002

Závěr

Byla vypracována nová, přesná a rychlá metoda stanovení obsahu SO_2 ve hlávkách, která plně nahrazuje dosud používanou obtížnější vážkovou metodu.

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Došlo do redakce 19. 8. 1963.

СКОРОСТНОЙ МЕТОД ТОЧНОГО ОПРЕДЕЛЕНИЯ СОДЕРЖАНИЯ SO_2 В ХМЕЛЕВЫХ ШИШКАХ

Была разработана новая колориметрическая метод определения содержания SO_2 в хмелевых шишках, требующий меньше времени и дающий более точные результаты чем применяемый в настоящее время весовой анализ. Принцип описываемого метода заключается в реакции абсорбированного раствора содержащего SO_2 и фуксинформальдегидного реактива. Метод отличается выдающейся чувствительностью и дает возможность определить наличие незначительных количества SO_2

SCHNELLE UND EXAKTE METHODE ZUR FESTSTELLUNG DES SO_2-GEHALTS IN HOPFENDOLDEN

Es wurde eine kolorimetrische Methode zur SO_2-Bestimmung in Hopfen ausgearbeitet, die schneller und genauer als die bisher benützte gravimetrische Methode ist. Das Prinzip der kolorimetrischen Methode besteht auf der Farbreaktion der SO_2-haltigen Absorptionslösung mit der Fuchsin-Formaldehyd-Reagenz. Die neue Methode ist sehr empfindlich und ermöglicht die Feststellung von bis 0,5 Gramm SO_2.

TIME SAVING, ACCURATE METHOD FOR DETERMINING SO_2 CONTENT IN HOP CONES

A new colorimetric method has been elaborated for determining SO_2 in hop cones. The method is more time saving and accurate than weighing analysis which is now chiefly used. The principle of the suggested new method is in a colour reaction between an absorbing solution containing SO_2 and fuchsine formaldehyde reagent. The method is extremely sensitive and permits to determine very small quantities of SO_2 present in the investigated material.

Ekonomické hvozdění sladu

VÁCLAV VLČEK, Potravinoprojekt Brno

663.43

V několika článcích, publikovaných již dříve v tomto časopise, byly autorem diskutovány výsledky technologického a ekonomického průzkumu hvozdění sladu, podložené teoretickými rozbory a provozními zkouškami. Týkaly se v podstatě přednosti a nedostatků způsobů hvozdění na dvouliskových hvozdech, a to tzv. klasického způsobu s odsušením 2 × 12 hodin proti odsušení 2 × 24 hodin.

Přesto, že výsledky druhého způsobu byly po stránce kvality sladu i po stránce ekonomie výroby jednoznačně příznivé a byly potvrzeny jak provozní praxí, tak i výsledky zkoušek brněnského pracoviště VÚPS, je a jejich správnosti mnohde pochybováno. V tomto příspěvku jsou proto uvedeny konkrétní údaje, dosažené v provozu sladovny jihomoravských pivovarů n. p. v Brně, kde již velké poslední tři sladovací kampaně, tedy dobu již dostatečně dlouhou pro posouzení dosažených výsledků, používají způsobu odsušení 2 × 24 hodin. Současně je poukázáno na chyby, kterých se někteří uživatelé tohoto způsobu dopouštějí.

Jako podkladů byla použita data z měsíčních hlášení jak co do množství vyrobeného sladu, tak i co do množství spotřebované páry k otopu hvozdu, odebírané z veřejné teplárny. Tato pára je měřena samostatným, cejchovaným paroměrem, který slouží jen pro registraci spotřeby páry pro hvozd a jeho údaje jsou občasně interně prověřovány měřením kondenzátů v rozměrné odměrné nádobě na 100 hl obsahu.

Poněvadž teplárna dodává páru o tlaku asi 6 atp, jsou odpočty paroměru násobeny koeficientem 1,05. Tím se dostává přepočet na tzv. normální páru, tj. páru, jejíž jedna tuna má tepelný obsah 640 000 kcal a toto množství je účtováno za cenu 34,— Kčs/t. Pro velikou vzdálenost mezi pivovarem a teplárnou pivovar kondenzáty nevrací. Jejich průměrná teplota činí asi 40 °C.

Jestliže teplárna dodává v některém období páru o nižším tlaku, než je garantováno, je tím pochopitelně jak odpočet na paroměru, tak i množství páru dodávaného tepla zkresleno. V tom případě poskytuje teplárna bonifikaci, vyjádřenou jak v množství, tak i v ceně v měsíčních fakturách.

Vzhledem k tomu, že kampaň 1962/63 proběhla, hlavně pokud jde o dodávku páry, za mimořádně těžkých podmínek (mrazová kalamita), ale také proto, že byl již používán pozměněný způsob klíčení ječmene proti dvěma předcházejícím kampaním (podle zlepšovacího návrhu *Kral - Vlček*), kdy klíčící ječmen byl v poslední dny klíčení prováděn v bubnech nevlněným vzduchem (tj. byl válečkován), byl za těchto podmínek nadměrný zelený slad na hvozd podstatně sušší, a tím byla snížena i spotřeba topné energie při hvozdění. Z uvedených důvodů byla tato kampaň vynechána z celkového porovnání, přestože dosahované výsledky byly velmi příznivé.

Pro celkové porovnání jsou tedy brány výsledky dvou sladovacích kampaní, a to 1960/61 a 1961/62, kdy bylo pracováno za normálních a výrobně stejných podmínek.

Váženým průměrem z obou těchto kampaní dostáváme tyto výsledky:

Vyrobeno sladu 89 820,75 q,

spotřebováno 9 697,90 tun normální páry, tj. 108,08 kg normální páry na 100 kg sladu, což přepočteno po 640 kcal na 1 kg normální páry, udává spotřebu tepla na 100 kg sladu 69 171 kcal měřeno v páře.

Předpokládáme-li, že je tato pára vyráběna v parním kotli 75% účinnosti (kterou výrobci nových kotlů zaručují), činila by kalorická spotřeba 92 228 kcal na 100 kg sladu, měřeno v palivu spotřebovaném pod kotlem.*)

Je nutno ještě konstatovat, že byl ve všech kampaních vyráběn slad velmi dobré jakosti, s vlhkostí nepřesahující v průměru 4 %, s nízkou barvou a se zcukřením do 10 minut, i při poměrně dlouho vydržované dotahované teplotě. Proti dřívějším letům je příznivější i dinastická malátnost asi o 20 až 30 jednotek i sladovací výtěžek.

Z uvedených výsledků je patrno, že se dá na našich dvouliskových hvozdech, vytápěných párou

*) Teoreticky by 5,25 q spotřeb, odpovídajícím od topného obsahu normální páry (640 kcal/kg) tedy tepla od kondenzátu (40 °C) kcal/kg, tj. 40 kcal na každý kg normální páry, nebyl vyrobena vlastně jen 4,1 q tepla z normální páry a spotřeba tepla by potom byla 61 512 kcal měřeno v páře, resp. 66 461 kcal měřeno ve spotřebu v palivu pod kotlem na 100 kg sladu.

Vol. 39, No. 1, 1970 (BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS)

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THE MUTAGENIC ACTION OF SODIUM BISULFITE

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Received February 26, 1970

Summary. This paper describes the mutagenic activity of sodium bisulfite toward phage λ. The frequency of mutation of the c gene of λ was increased about 10 times as much as that of spontaneous mutation by treatment of the phage with 3 M $NaHSO_3$ solution of pH 5.6 at 37° for 1.5 hrs. The mutagenesis could be related to the cytosine derivative specific reaction of sodium bisulfite.

In our current studies on the interactions of chemicals with nucleic acids and their components, we have discovered that sulfite ions react with a variety of pyrimidine bases[1-3]. Sodium bisulfite forms an addition compound with uracil, 5,6-dihydrouracil-6-sulfonate[2]. Cytosine and sodium bisulfite form an unstable adduct, dihydrocytosine-6-sulfonate, which is hydrolyzed to give 5,6-dihydrouracil-6-sulfonate. Uridine and cytidine have been found to undergo similar addition reactions with sodium bisulfite[3]. 5-Methylcytosine is converted into thymine by treatment with bisulfite[3]. These reactions occur at 37° and at pH around 6. The dihydrocytidine-6-sulfonate readily regenerates cytidine in its neutral solution. The 5,6-dihydrouridine-6-sulfonate is convertible into uridine on treatment with weak alkali. No reaction has so far been detected with other major nucleosides and nucleotides when they were treated with sodium bisulfite under similar conditions.

From these findings, it may be expected that cytosine (and 5-methylcytosine) residues in DNA will react with sodium bisulfite. Such reactions,

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if they take place in vivo, may exhibit mutagenic or inactivating action on the organisms whose genetic informations are encoded in their DNA.

A mutant of phage λ which possesses a mutation at the c genes forms a "clear" plaque instead of a normal "turbid" one. By measuring the induction of clear mutations of phage λ mutagenic action of sodium bisulfite was found.

Materials and Methods

E. coli K12 used as the indicator for scoring c mutants of λh was N14-4[4] which is a derivative strain of W3623 try⁻ gal⁻ sm^r (Lederberg). General methods of handling of phage and media used were described in a previous paper[5]. 0.5 ml of solution of sodium bisulfite or control buffer and 0.01 ml of suspension of phage with the titer of 2.3×10^{10} were mixed and incubated at 37°. After various times, an aliquot was taken, diluted and plated to measure the mutation frequency. The sodium bisulfite solution was prepared by mixing 3 M $NaHSO_3$ and 3 M Na_2SO_3 solutions in 3:1 (v/v) ratio. The pH was 5.6. The composition of the control buffer is 2.3 M in NaCl and 1 M in sodium phosphate buffer, the final pH being 5.6. Mutagenesis by bisulfite was compared with that by hydroxylamine. The c mutation was induced by treatment of λ with 1 M solution of hydroxylamine for 6 hrs at pH 7.5 and 37°. Treated λ phages were plated with indicator bacteria to give about 1,000 plaques in one plate. The term, frequency of mutation, is used to indicate the frequency of mutants among surviving phages.

Results and Discussion

The numbers of plaques screened and that of mutants found are shown in Table I, together with the frequency of mutation with 95 % probability level. The frequency of c mutation increased as a function of time of treatment with bisulfite, and the maximum value was obtained by the treatment for 90 min. Longer treatment did not seem to further increase the frequency. The absence of the increase of mutation frequency by the

Table I Frequency of c mutation induced by various agents.

Agent	Treated time min	Survival of λ	No. of plaques screened	No. of mutants found	Frequency observed units 10^{-5}	Frequency with 95 % probability units 10^{-5}
Sodium bisulfite	0	1	206,000	52	25	19- 34
	60	1.4×10^{-1}	42,600	69	160	130-200
	90	4.8×10^{-2}	40,800	107	260	220-320
	180	2.0×10^{-3}	36,100	101	280	230-340
Control	90	2.2×10^{-2}	83,700	27	32	22- 47
Hydroxylamine	360	1.1×10^{-1}	23,100	98	420	340-510

incubation in the control buffer eliminates the possibility that the mutation might have been caused by factors other then the bisulfite ions such as pH or high salt concentration. The potency of sodium bisulfite as a mutagen is comparable to that of hydroxylamine under the test conditions employed.

From these observations, we conclude that sodium bisulfite possesses a mutagenic activity.

Little can be stated at this stage of work about the mechanism of the mutagenesis caused by the bisulfite treatment. A transition mutation, C-G ⟶ T-A, would be brought about by bisulfite-modification of cytosine (and/or 5-methylcytosine[6]). A preliminary experiment has shown that DNA, when treated, in vitro, with $H^{35}SO_3^-$, does incorporate the ^{35}S into its molecule, and, furthermore, this label can be completely removed by treatment with alkali (pH 12, 37°, 1 hr). This observation is just as expected from the experiments carried out with cytosine. In addition, when the DNA, which had been treated with bisulfite and then with alkali, was hydrolyzed with perchloric acid and the hydrolysate analysed by paper chromatography, a spot of uracil was detected. Uracil was not detectable in the hydrolysate of DNA that had not been treated with sulfite. These observations are in favor of the view that the mutation is caused by the modification of cytosine (and/or 5-methylcytosine) by bisulfite.

Chemical mutagens often exhibit cancer inducing activities. It would be interesting to examine if bisulfite ions could cause cancer in some organism. Such studies are of importance because of the air pollution by sulfur dioxide and of the sulfite salts being used as the food additives.

Acknowledgements

We thank Professor T. Ukita of the University of Tokyo and Dr. S. Nojima, Head, Department of Chemistry, National Institute of Health of Japan, for their giving us the opportunity of carrying out the present work and for their kind encouragement throughout it. Thanks are also

due to Professor J. Tomizawa of the University of Osaka for his critical reading of the manuscript.

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Gastrointestinal Gas Production in Rats as Influenced by Some Animal and Vegetable Diets, Sulfiting, and Antibiotic Supplementation[1]

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The increased use of dehydrated foods as a convenience factor for military rations has elicited a number of informal observations concerning digestive disturbances and increased production of flatulent gases. This is of particular interest where individuals are obliged to operate in confined spaces. Determination of the validity of these observations is also important to commercial interests in view of the increasing use of dehydration as a food processing method.

The relationship between diet and intestinal gas formation has been investigated for a numer of foods fed to man and dogs. Most investigators have observed that intestinal gas production increased with feeding vegetable diets (Kirk, '49; Askevold, '56; Blair et al., '47). Correlation has also been established for foods high in crude fiber (Kodama and Miura, '49). On the other hand, Kirk ('49) reported no significant change in the composition or the amount of gas when milk or lactose were fed. Blair et al. ('47) reported that the proportion of protein, fat, and carbohydrate in diets has a negligible effect on the volume of intestinal gas.

In a previous report from this laboratory (Hedin and Adachi, '62), the development of a routine procedure with laboratory animals for assessing the magnitude and composition of gastrointestinal gases was described. Analysis of samples from the stomach and intrestine was accomplished at intervals for several days to determine production patterns for two diets expected to differ markedly in repsonse. The daily peak production of stomach gases occurred at approximately two hours after feeding, and the peak production of intestinal gases occurred at 4 hours. A casein diet produced relatively small quantities of gases. When the casein diet was fed for several days and then replaced by a red bean diet, increased hydrogen and carbon dioxide production was observed and the total amount of intestinal gas also increased. From the second through the eleventh day, there was a gradual decrease in the hydrogen production, while the percentage of carbon dioxide rose to 90 and the total gas production remained constant. This report describes further studies with this procedure for the assessment of gas production arising when several products of animal and plant origin were fed to rats. The effect of sulfiting the vegetable products, the supplementation of several diets with wide spectrum antibiotics, and the production of gases from a highly purified diet is also reported.

EXPERIMENTAL

Food items. The pork and beef were cooked until tender, frozen at −10°F, and dehydrated without thawing to a residual moisture content of 2%. The eggs were precooked, scrambled, frozen at 0°F and dehydrated without thawing to a residual moisture content of 2%. The skim milk powder was a commercial, instant, spray-dried product. The carrots, cabbage, and lima beans were air-dried at 140 to 160°F

Received for publication March 31, 1962.

[1] This paper reports research undertaken at the Quartermaster Food and Container Institute for the Armed Forces, Q&E Research and Engineering Command, U. S. Army, and has been assigned no. 2204 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or endorsement of the Department of Defense.

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for 4 hours and at 120°F for 15 hours to a residual moisture content of 2%. The three vegetable products were obtained in both sulfited and nonsulfited forms. To accomplish the sulfiting, each vegetable was dipped in dilute sodium sulfite solutions for predetermined time periods.

Diets. The casein and red bean diets were described previously (Hedin and Adachi, '62). The food items were compounded into diets so that they comprised 40% of the total weight. Also included were 8% of refined cottonseed oil,[1] 5% of USP salts 14, 4% of non-nutritive cellulose,[2] 1% of a B-vitamin mixture and 42% of corn starch. The amino acid diet and the wheat gluten diet were previously described by Hepburn et al. ('60). Where indicated, a mixture of 3.2 gm of neomycin sulfate and 800,000 units of nystatin[4] was added to each kg of diet.

Methodology. Male albino rats, 200 to 250 gm, were trained to consume 10 gm of diet in a one-hour period. The animals were maintained with the experimental diet for 7 days and killed 4 hours after the final daily feeding. Sample collection, microbiological assays, and analysis procedures were described previously (Hedin and Adachi, '62).

RESULTS AND DISCUSSION

Animal product diets. Gas volumes and compositions for the stomachs and intestines of rats fed 5 animal product diets are summarized in tables 1 and 2. Except for the greater gas volume found in the stomach of rats fed the dehydrated beef diet no marked dietary response was evident. A similar observation was made previously in this laboratory with the casein and a red bean diet for a number of time periods. With the intestinal gases, dietary responses appear both for volume and for composition, however. Over 7 times as much gas was found in rats fed the dried skim milk diet as compared with rats fed the casein diet. This observation is in contrast to that of Kirk ('49) who found no enhancement in gas production where milk or lactose were fed. Approximately twofold increases were observed with the egg or beef diets, but no change of consequence occurred with rats fed the pork diet. The chief dietary response for composition also occurred with rats fed the dried skim milk diet. The

[1] Wesson Oil. The Wesson Oil Company, New Orleans, Louisiana.
[2] Alphacel. Nutritional Biochemicals Corporation, Cleveland.
[4] Polyene antifungal antibiotic produced by *Streptomyces noursei* (Mycostatin, E. R. Squibb and Sons, New York.

TABLE 1

Total volume and composition of stomach gases from rats fed 5 dehydrated animal diets[1]

Diet	Total volume	Hydrogen	Oxygen	Nitrogen	Carbon dioxide
	ml	%	%	%	%
Casein	1.34 ± 0.05[2]	0.2 ± 0.1	8.2 ± 2.1	69.6 ± 3.6	22.0 ± 4.7
Dried skim milk	1.18 ± 0.24	2.6 ± 1.2	4.3 ± 0.9	73.4 ± 2.8	19.8 ± 3.0
Dried scrambled egg	1.50 ± 0.05	1.6 ± 0.8	6.0 ± 0.7	78.3 ± 1.7	14.1 ± 1.4
Dehydrated beef	2.49 ± 0.18	1.4 ± 0.5	8.9 ± 1.2	78.1 ± 1.9	11.6 ± 0.8
Dehydrated pork	1.10 ± 0.16	3.9 ± 2.6	6.6 ± 2.6	74.4 ± 3.8	15.1 ± 2.9

[1] Animals killed 4 hours after seventh feeding.
[2] Mean and standard error of the mean; represents at least 6 observations.

TABLE 2

Total volume and composition of intestinal gases from rats fed 5 dehydrated animal product diets[1]

Diet	Total volume	Hydrogen	Oxygen	Nitrogen	Carbon dioxide
	ml	%	%	%	%
Casein	0.86 ± 0.14[2]	7.8 ± 3.1	9.8 ± 3.4	57.4 ± 8.1	25.0 ± 7.8
Dried skim milk	6.41 ± 0.68	37.3 ± 1.9	0.3 ± 0.1	8.5 ± 1.6	53.9 ± 8.1
Dried scrambled egg	1.81 ± 0.32	8.1 ± 2.3	3.9 ± 2.8	33.9 ± 8.0	54.1 ± 8.1
Dehydrated beef	1.91 ± 0.25	2.6 ± 0.4	2.4 ± 0.3	48.5 ± 3.2	46.5 ± 2.5
Dehydrated pork	1.05 ± 0.12	1.2 ± 1.0	4.1 ± 0.7	53.4 ± 3.4	41.3 ± 2.4

[1] Animal killed 4 hours after seventh feeding.
[2] Mean and standard error of the mean; represents at least 6 observations.

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hydrogen was increased to approximately 37%, whereas no other diet obtains a hydrogen content of greater than 8%. Since there was no absolute decrease in the nitrogen content, an absolute increase in the hydrogen, and to a lesser extent, the carbon dioxide is indicated. The decrease in the oxygen content is attributed to uptake by the microfloral population.

Vegetable product diets. Gas volumes and compositions for the stomachs and intestines of rats fed three sulfited vegetable product diets and their nonsulfited controls are summarized in tables 3 and 4. Rats fed the sulfited cabbage diet refused to consume more than 5.6 gm during the allotted period. To further observe an apparent toxicity manifestation, the experiment was repeated and the animals killed 4 hours after the third daily feeding with the voluntary consumption reduced to 8.2 gm. Normal consumption was observed with all other diets.

The total stomach gas content was lower for the carrot and cabbage diets than for the animal product diets. The content for the lima bean diet was similar to that of the casein diet and also to that of the red bean diet previously reported. Sulfiting further decreased the gas content for each product. The composition of gases did not appear to be affected by diet except for the decrease in the carbon dioxide content in rats fed the sulfited diets. The total intestinal gas content was higher for the vegetable products than for the animal products except with the dried skim milk diet. This substantiates the observations of other investigators (Kirk, '49; Askevold, '56; Blair, '47). The values for carrots and

TABLE 3

Total volume and composition of stomach gases from rats fed sulfited and nonsulfited vegetable product diets

Diet	Total volume	Hydrogen	Oxygen	Nitrogen	Carbon dioxide	Methane
	ml	%	%	%	%	%
Carrots[1]	0.31±0.10[2]	0.0	9.2±1.2	81.4±0.8	9.4±1.8	0.0
Carrots, sulfited[1]	0.16±0.07	0.0	10.9±2.5	75.8±2.4	13.3±3.7	0.0
Lima beans[1]	0.97±0.25	0.4±0.1	7.8±1.1	80.4±0.5	11.4±1.0	0.0
Lima beans sulfited[1]	0.78±0.12	1.2±1.0	8.7±0.4	78.7±1.8	13.4±0.8	0.0
Cabbage[1]	0.85±0.09	4.0±1.8	7.8±2.8	60.0±9.1	28.2±7.7	1.1±1.0
Cabbage, sulfited[3]	0.32±0.06	0.0	11.5±1.6	75.7±0.7	12.8±2.4	0.0
Cabbage, sulfited[4]	0.37±0.05	3.8±1.1	14.9±2.1	69.4±5.8	11.9±1.5	0.0

[1] Animal killed 4 hours after seventh feeding.
[2] Mean and standard error of the mean; represents at least 6 observations.
[3] Animals killed 4 hours after the third daily feeding. Animals refused to consume more than an average of 8.2 gm on the third day.
[4] Animals killed 4 hours after the seventh daily feeding. Average feed consumption on the seventh day was 5.6 gm.

TABLE 4

Total volume and composition of intestinal gases from rats fed sulfited and nonsulfited vegetable product diets

Diet	Total volume	Hydrogen	Oxygen	Nitrogen	Carbon dioxide	Methane
	%	%	%	%	%	%
Carrots[1]	2.70±0.31[2]	11.6±3.4	0.5±0.1	10.8±2.2	77.1±5.7	0.0
Carrots, sulfited[1]	2.59±0.33	10.6±3.6	0.5±0.1	13.5±4.0	75.4±3.7	0.0
Lima beans[1]	2.73±0.23	10.3±2.6	0.2±0.1	4.3±0.9	85.2±3.3	1.4±0.7
Lima beans, sulfited[1]	2.61±0.25	4.3±1.0	0.7±0.3	9.3±3.4	85.7±2.9	4.3±1.2
Cabbage[1]	2.05±0.20	4.2±1.6	1.6±0.8	14.5±1.4	79.7±5.5	3.5±2.7
Cabbage, sulfited[3]	0.72±0.10	2.4±0.3	2.6±0.7	30.7±2.8	64.3±4.5	0.0
Cabbage, sulfited[4]	0.83±0.07	2.5±0.6	1.4±0.3	43.9±1.9	52.2±3.1	0.0

[1] Animal killed 4 hours after seventh feeding.
[2] Mean and standard error of the mean; represents at least 6 observations.
[3] Animals killed 4 hours after the third daily feeding. Animals refused to consume more than an average of 8.2 gm on the third day.
[4] Animals killed 4 hours after the seventh daily feeding. Average feed consumption on the seventh day was 5.6 gm.

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lima beans were similar to that of the red bean diet (2.76 ml) reported previously (Hedin and Adachi, '62). It was somewhat unexpected that the volume for cabbage was lower than for the other vegetables in view of its odorous character. The carbon dioxide also was increased both on a percentage and an absolute basis for the vegetable diets as compared with the animal product diets (except with dried skim milk). The increase is sufficient to account for the increased volume and probably indicates increased microfloral activity.

After sulfiting, a marked depression of gas formation was noted with the cabbage diet. The sulfiting levels were fixed by commercial practice. Residue analysis gave the following results: cabbage 2,110 ppm; lima beans, 119 ppm; and carrots 2,070 ppm. If the depression in gas formation observed for cabbage were to be attributed wholly to the sulfite, a similar depression would be expected for carrots. Because no such depression occurred, and in view of the decrease in food consumption of rats fed the sulfited cabbage diet, an interaction of sulfite with cabbage but not with carrots to form a toxic product may be indicated.

Purified diets. The results obtained from a study of the gastrointestinal gas production of rats fed two purified diets is summarized in table 5. The amino acid diet had been shown satisfactory for growth by Hepburn et al. ('60) as had the wheat gluten diet when supplemented with lysine, histidine, methionine, threonine and tryptophan. These diets were fed in the expectation that a lowered intestinal gas production from that of the casein diet might occur and result in selection of a more favorable reference diet. As shown in table 5, the intestinal gas production with both diets was higher than for any of the other diets tested except dried skim milk. The unexpected results were confirmed in a second experiment. The intestinal gas composition for both diets also compares closely with the skim milk diet (table 2) except for the absence of methane in the latter. Hydrogen largely accounts for the added volume produced by these three diets over that of the vegetable product diets, whereas carbon dioxide accounts for the difference in volume of vegetable products over the other animal product diets. Because the casein diet, a poor gas producer, contains a mineral mixture starch, refined cottonseed oil and cellulose, the inclusion of these ingredients in the three high gas-producing diets cannot be implicated. The only apparent common factor remaining is the free amino acid content. However, the other food items that produced less intestinal gas also would be

TABLE 5

Gastrointestinal gas production of rats fed two purified diets[1]

Diet and gas origin	Total volume	Hydrogen	Oxygen	Nitrogen	Carbon dioxide	Methane
	%	%	%	%	%	%
Amino acid, stomach	1.59±0.23[2]	0.6±0.4	12.5±2.4	66.2±5.6	15.6±3.6	5.1±5.0
Amino acid, intestinal	4.86±0.60	26.5±4.8	0.6±0.3	7.0±1.1	60.7±3.2	5.0±3.0
Amino acid, intestinal[3]	5.53±0.98	34.9±0.9	0.7±0.2	10.8±1.5	52.5±1.6	1.1±0.7
Wheat gluten, stomach	1.03±0.20	0.2±0.1	12.8±1.9	73.2±1.1	13.7±2.2	0.1±0.1
Wheat gluten, intestinal	4.14±0.41	25.7±6.0	0.4±0.2	6.2±1.0	55.8±3.7	11.9±4.6
Wheat gluten, intestinal[3]	4.62±0.53	29.0±3.2	0.5±0.1	14.2±4.5	51.0±3.5	5.3±1.4

[1] Animal killed 4 hours after seventh feeding.
[2] Mean and standard error of the mean; represents at least 6 observations.
[3] Repeat experiment.

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expected to contain some free amino acids. It appears, nevertheless, that the high volume production with the amino acid diet and, to a lesser extent, with the wheat gluten diet should be attributed to the amino acid content. This observation does not appear to have been previously reported in the literature. Whether the high production with the skim milk diet should be attributed to the lactose content despite Kirk's ('49) evidence to the contrary or to another factor, possibly the amino acid content, or both, cannot be determined from the available data. The data obtained for stomach gases do not suggest any marked dietary effect.

Dietary antibiotic supplementation. In table 6 are summarized the results obtained from the incorporation of a mixture of 3.2 gm of neomycin sulfate and 800,000 units of nystatin[1]/kg of diet. Neither agent is appreciably absorbed from the intestinal tract. Gastrointestinal gas volumes and compositions were obtained for the casein, red bean, and skim milk diets, with and without antibiotic supplementation. No marked dietary differences were observed with the stomach gases. However, a number of changes occurred with the intestinal gases as a consequence of antibiotic supplementation.

Contrary to the expectation that the gas content would be reduced to a low level, an increase was observed for the rats fed the casein diet, whereas there was no appreciable change with the red bean diet and the content for rats fed the skim milk diet fell only to the approximate level of the other two. A factor contributing to the intermediate value was the several-fold enlargement of the intestinal tract of rats fed the antibiotic supplements. The cecum was found to be as large or larger than the stomach even directly after feeding. This enlargement has been reported only for chickens administered penicillin (Anderson et al., '52) with concomitant disappearance of coliforms, lactobacilli and enterococci.

The most marked change observed for the composition of the intestinal gases after antibiotic supplementation is the decrease in the relative proportion of carbon dioxide with the accompanying increase in nitrogen. Hydrogen is decreased with the casein and the skim milk diet but increased for the red bean diet. Also there is an increase in the relative proportion of oxygen after antibiotic supplementation. Normally, the oxygen content decreases to near zero in the intestinal tract and this decrease has been attributed to uptake of oxygen by the microflora (Blair, '47; '49). To provide more direct evidence, microbiological assays were performed on the cecal contents for total aerobes, total aciduric organism, and for coliforms by procedures reported previously (Hedin and Adachi, '62). A marked decrease in organisms was observed for all classes except molds after antibiotic supplementation. It thus appears that microorganisms were largely responsible for the production of carbon dioxide. The available results are not sufficiently conclusive to assign hydrogen production to the activity of the microflora, however.

SUMMARY

The magnitude and composition of gastrointestinal gas production in rats fed

[1] See footnote 4.

TABLE 6

Effect of a neomycin sulfate-nystatin mixture on the production of intestinal gases in rats[1]

Diet	Total volume	Hydrogen	Oxygen	Nitrogen	Carbon dioxide
	ml	%	%	%	%
Casein	0.80 ± 0.21[2]	12.4 ± 2.1	2.5 ± 0.6	38.1 ± 3.5	47.0 ± 5.6
Casein + antibiotics[3]	2.52 ± 0.48	2.2 ± 1.3	3.8 ± 0.3	84.0 ± 1.3	10.0 ± 0.7
Dried skim milk	6.41 ± 0.66	37.3 ± 1.9	0.3 ± 0.1	8.5 ± 1.6	53.9 ± 8.1
Dried skim milk + antibiotics[3]	2.68 ± 0.37	20.4 ± 4.1	2.3 ± 1.3	56.1 ± 5.4	21.2 ± 1.6
Red bean	2.76 ± 0.23	12.0 ± 3.5	0.6 ± 0.2	4.5 ± 0.9	82.9 ± 2.7
Red bean + antibiotics[3]	3.08 ± 0.27	34.7 ± 7.8	3.6 ± 2.5	29.3 ± 5.2	32.4 ± 5.8

[1] Animal killed 4 hours after seventh feeding.
[2] Mean and standard error of the mean; represents at least 6 observations.
[3] 3.2 gm neomycin sulfate and 800,000 units nystatin/kg of diet.

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several products of animal and plant origin is reported. The effect of sulfiting the vegetable products, the supplementation of several diets with wide spectrum antibiotics, and the production of gases from a diet of known chemical composition is also discussed. Dietary effects were evaluated at the 40% level of the diet 4 hours after the seventh daily feeding.

The highest production of intestinal gases was observed for rats fed the dried skim milk. The production from beef, pork, egg and casein was less than one-third that of the dried skim milk. The production of intestinal gases by carrots, cabbage and lima beans was higher than for the 4 animal products with the increased production attributed to an increase in carbon dioxide. Sulfiting reduced the production particularly from the cabbage diet where an apparent manifestation of toxicity occurred.

An amino acid diet and an amino acid supplemented, wheat gluten diet gave a higher gas production than did the vegetable products. The increase with these diets and also with the dried skim milk diet was largely attributed to increased hydrogen production.

Supplementation with a neomycin-nystatin mixture decreased carbon dioxide production. A volume decrease was observed with the dried skim milk diet, but was not seen with the casein or red bean diet because of an increased intestinal (cecal) size.

Dietary responses for the stomach gases were minimal.

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J. Biol. Chem. 204 (1953)

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THE ENZYMATIC OXIDATION OF SULFITE*

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(Received for publication, March 6, 1953)

It has been suggested that the formation of inorganic sulfate from methionine and cysteine by liver preparations proceeds by stepwise oxidations through cysteinesulfenic and cysteinesulfinic acids (2–4). Chatagner *et al.* have demonstrated the presence in rabbit liver of a transaminase which catalyzes amino transfer from cysteinesulfinic acid to α-ketoglutarate (5). The resulting sulfinopyruvic acid yields SO_2 and pyruvate, but it is not known whether this reaction is spontaneous or enzymatically catalyzed.

Although sulfite is oxidized to sulfate *in vivo* (6), it had not been demonstrated that this process is enzymatic. This report describes the enzymatic formation of inorganic sulfate from sulfite, various carbonyl-bisulfite addition compounds (α-hydroxysulfonic acids), and derivatives of aminomethanesulfonic acid by preparations obtained from the livers and kidneys of various mammals and from several microorganisms.

Results

Oxidation of Substituted Aminomethanesulfonic Acids—During an investigation of the sarcosine oxidase of rat liver homogenates, the sulfonic acid analogue of sarcosine, *N*-methylaminomethanesulfonic acid (*N*-methyl-AMSA), was tested as a possible inhibitor. Instead, however, oxygen consumption was increased in proportion to the amount of added sulfonic acid, and, in flasks containing no sarcosine, approximately 3 atoms of oxygen were consumed per molecule of sulfonic acid.

The products of the oxidation of aminomethanesulfonic acid (AMSA) and a series of related compounds by the soluble and insoluble phases of rat liver homogenates were then investigated. These results are sum-

* This work was supported by a grant from the National Institutes of Health, United States Public Health Service.

A preliminary report was presented before the American Society of Biological Chemists, New York, April, 1952 (1).

† Predoctoral Fellow of the National Institutes of Health. The data in this report are taken in part from a dissertation presented to the Graduate Council of Duke University in partial fulfilment of the requirements for the degree of Doctor of Philosophy, June, 1952. Present address, Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Missouri.

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marized in Table I and Fig. 1. Both washed residue and supernatant fluid oxidized AMSA and its *N*-methyl, ethyl, isopropyl, *n*-propyl, and *n*-butyl derivatives as well as hydroxymethanesulfonic acid (HMS). Oxidation of each substrate yielded inorganic sulfate and formaldehyde which was further oxidized to formate by liver supernatant fluid. As reported by Bernheim (7) addition of aminoguanidine sulfate permitted oxidation of formaldehyde to formate by washed liver residue. AMSA and its *N*-substituted derivatives released ammonia or the corresponding amine in the presence of rat liver supernatant fluid. With washed residue an additional atom of oxygen was consumed by the further oxidation of *n*-butylamine and *n*-propylamine but not of their lower homologues in

TABLE I

Enzymatic Oxidation of Substituted Methanesulfonic Acids by Preparations from Rat Liver

Liver preparation	Substrate	O_2 uptake	O_2 per mole	SO_4^{--}	NH_3 or amines	HCHO
		μl.		μM	μM	
Residue	*N*-Methyl-AMSA	51	0.91	4.3	2.5	+
	N-Ethyl-AMSA	55	0.99		4.4	+
	N-*n*-Butyl-AMSA	118	2.10	4.2		+
	HMS	51	0.91	4.6	0	+
Supernatant fluid	*N*-Methyl-AMSA	112	2.00	5.5	4.7	−
	N-*n*-Butyl-AMSA	120	2.20	5.0	4.4	−
	HMS	109	1.97	4.9	0	−

Each vessel contained 5.0 μM of substrate and 1.0 ml. of liver preparation in a final volume of 2.2 ml. of phosphate buffer, pH 7.8.

accordance with the known specificity of liver amine oxidase (8). HMS yielded sulfate and formaldehyde with washed liver residue and sulfate and formate with supernatant fluid.

Oxidation of α-Hydroxysulfonic Acids and of Sulfite—The α-hydroxysulfonic acids formed by addition of bisulfite to formaldehyde, acetaldehyde, pyruvic, oxalacetic, and α-ketoglutaric acids were all oxidized in the presence of washed rat liver residue, although the corresponding carbonyl compounds were not themselves oxidized by this preparation. 1 mole of CO_2 was produced per mole of oxalacetate or its bisulfite addition compound; no decarboxylation of pyruvate, α-ketoglutarate, or of their bisulfite addition compounds was observed. The bisulfite addition compounds formed by *n*-propionaldehyde, isopropionaldehyde, *n*-butyraldehyde, and acetone were resistant to oxidation as were cysteic acid, taurine, *N*-methyltaurine, methanesulfonic and bromomethanesulfonic acids, and glycine.

In control vessels containing no liver it was observed that the bisulfite addition compounds of α-ketoglutarate and pyruvate spontaneously autoxidized more rapidly than did that of oxalacetate, whereas the addition compounds prepared from formaldehyde and acetaldehyde were entirely stable. This behavior suggested that the autoxidizable compounds dissociated to the equivalent carbonyl compounds and bisulfite which, as shown in Fig. 2, is rapidly autoxidizable. When the various hydroxysulfonic acids or bisulfite was incubated with heat-inactivated liver residue, serum albumin, or ethylenediaminetetraacetate (EDTA), autoxidation was almost entirely suppressed, presumably owing to binding of the metal

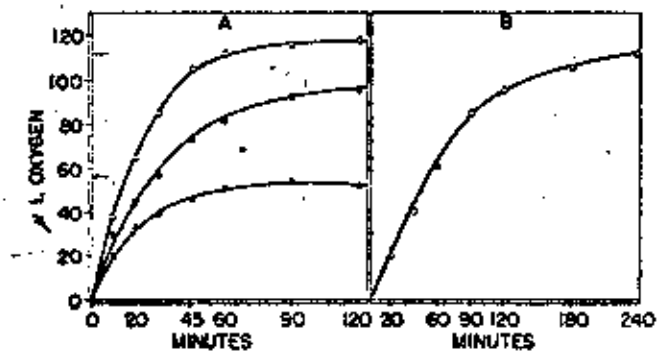

FIG. 1. Oxidation of substituted methanesulfonic acids. *A*, by supernatant fluid from rat liver homogenates. ○, *N*-*n*-butyl-AMSA; ●, *N*-*n*-propyl-AMSA; ▲, average curve for AMSA, *N*-methyl-AMSA, *N*-ethyl-AMSA, *N*-isopropyl-AMSA, and HMS. *B*, by washed insoluble residue from rat liver homogenates. This is a typical curve given by each of the substrates tested with the supernatant fluid. The arrows indicate theoretical oxygen uptake.

ions which catalyze bisulfite autoxidation. When incubated with unheated rat liver residue, however, all of these compounds, *including inorganic bisulfite*, were oxidized. The rates of oxidation of bisulfite and the autoxidizable addition compounds in the presence of EDTA and liver residue were essentially identical and significantly exceeded that of the non-autoxidizable addition compounds corresponding to formaldehyde and acetaldehyde or the various aminomethanesulfonic acids. These relations are summarized in Fig. 3.

Inhibitors—Oxidation of HMS and bisulfite by rat liver residue was not inhibited by 0.01 M fluoride, azide, cyanide, iodoacetate, hydroxylamine, semicarbazide, phenylhydrazine, phenylthiourea, phenyldithiocarbamate, or dinitrophenol. These findings have been interpreted as indications that heme, copper, sulfhydryl, or carbonyl groups are not essential to the activity of this system.

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Stability of Sulfite Oxidase—The enzyme system (or systems) responsible for the oxidation of bisulfite and the various substituted methanesulfonic acids is unusually stable. Little or no loss of activity has been observed under the following conditions: storage at −18° of fresh frozen rat liver for 4 months, storage of washed rat liver residue at 0–3° for 1 week, dialysis of rat liver residue against water or 0.02 M phosphate, pH 7.4, for 1 week, storage at 0–3° for several days in the range pH 5.5 to 10.5, heating to 58° for 3 minutes. The enzyme is destroyed by exposure to temperatures above 60° for several minutes.

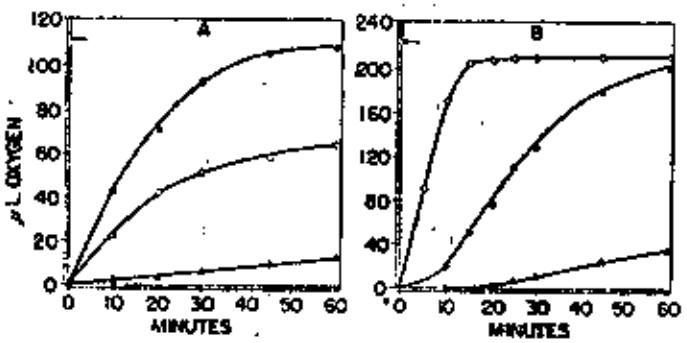
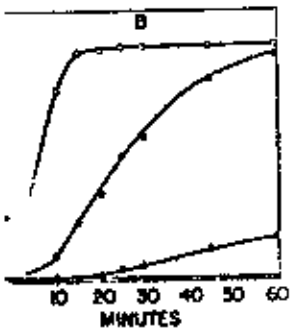


FIG. 2. Oxidation of pyruvate-bisulfite (*A*) and inorganic bisulfite (*B*) by rat liver residue (●) and autoxidation in the presence of buffer (○) or boiled liver residue (▲). Each vessel contained 10 μM of pyruvate-bisulfite or 20 μM of bisulfite in a final volume of 2.2 ml. in 0.05 M phosphate buffer, pH 7.5. The arrows indicate theoretical oxygen uptake. The autoxidation of ketoglutarate-bisulfite was somewhat faster than that of pyruvate-bisulfite, while that of oxalacetate-bisulfite was appreciably slower.

Preliminary Purification of Sulfite Oxidase—An extract of rat liver acetone powder was used as starting material and was fractionated by heating and ammonium sulfate precipitations. The most active fraction thus far obtained exhibits 4 times the specific activity of the crude extract (Table II). Specific activity has been defined as microliters of oxygen uptake per minute per mg. of protein nitrogen, determined manometrically in air at 37.5°. The initial slope of the curve obtained by plotting oxygen uptake against time was used in calculating specific activities.

Properties of Sulfite Oxidase—The most purified preparation (Fraction IV) was a clear, pale yellow solution which did not oxidize xanthine or DL-alanine. The optimal pH of Fraction IV for sulfite oxidation was 9.3, whereas oxidation of HMS was most rapid at pH 8.3 (Fig. 4). With bisulfite as substrate the theoretical oxygen uptake was observed. With HMS, oxygen uptake commenced rapidly but soon subsided and stopped at approximately 25 per cent of the theoretical uptake. That this is due

system (or systems) respon- various substituted methane- or no loss of activity has been orage at −18° of fresh frozen rat liver residue at 0–3° for 1 water or 0.02 M phosphate, pH days in the range pH 5.5 to zyme is destroyed by exposure tes.



inorganic bisulfite (*B*) by rat liver buffer (O) or boiled liver residue bisulfite or 20 μM of bisulfite in a fer, pH 7.8. The arrows indicate ketoglutarate-bisulfite was some- ie that of oxalacetate-bisulfite was

se—An extract of rat liver ace- and was fractionated by heat- The most active fraction thus activity of the crude extract efined as microliters of oxygen rogen, determined manometri- the curve obtained by plotting calculating specific activities. purified preparation (Fraction ch did not oxidize xanthine or IV for sulfite oxidation was 9.3, id at pH 8.3 (Fig. 4). With n uptake was observed. With but soon subsided and stopped etical uptake. That this is due

to poisoning of the enzyme by the formaldehyde released during the oxidation of HMS was shown as follows: Further addition of 20 μM of substrate after oxygen uptake had ceased (about 30 minutes) failed to elicit additional oxygen uptake. Addition of 10 μM of formaldehyde to the flask prior to addition of the enzyme prevented any oxygen uptake upon substrate addition. Formaldehyde-binding agents such as hydroxylamine

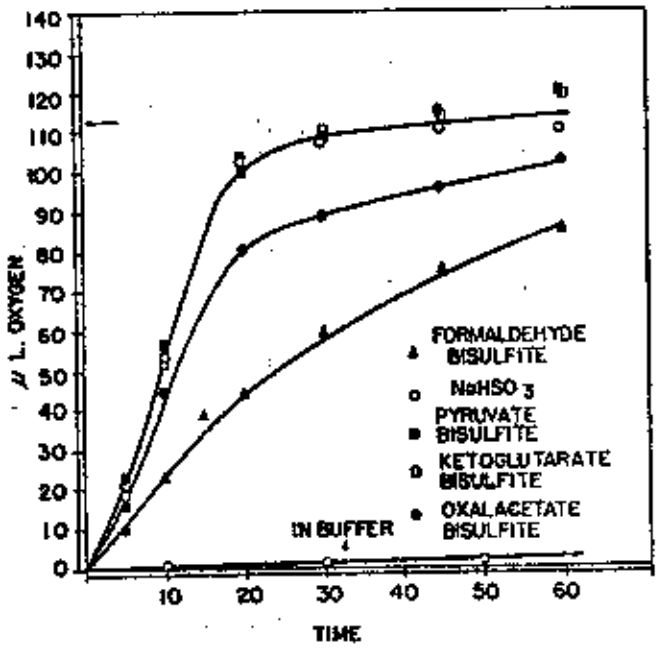


FIG. 3. Oxidation of bisulfite and some carbonyl-bisulfite addition compounds by rat liver residue in the presence of EDTA. O represents the autoxidation of bisulfite under these conditions. Each flask contained 10 μM of substrate and 1 ml. of enzyme preparation in a total volume of 2.2 ml. of 0.05 M phosphate buffer containing 0.2 mg. of EDTA. The arrow indicates theoretical oxygen uptake.

or inert protein (human serum albumin) prevented poisoning of the enzyme and permitted almost theoretical oxygen consumption. Hydroxylamine inhibited the enzymatic activity of Fraction IV towards bisulfite, although it had no inhibitory action with rat liver residue. This is probably due to binding of the hydroxylamine by the inert proteins and perhaps carbonyl compounds present in the rat liver residue, since inert protein (human serum albumin) greatly decreased the inhibitory action of hydroxylamine towards Fraction IV.

Substrate Specificity of Fraction IV—This fraction catalyzed the oxidation of each of the compounds earlier noted to be oxidized by washed liver

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residue. In addition, metabisulfite ($S_2O_5^{=}$) was oxidized with an oxygen uptake of 2 atoms of oxygen per molecule of metabisulfite. Cysteinesulfinic acid was also oxidized but it is not clear whether the same enzyme

TABLE II

Purification of Liver Sulfite Oxidase

Fraction No.	Specific activity*	Total units	Recovery
			per cent
I	1.15	462	
II	1.84	338	73
III	2.57	360	78
IV	4.00	141	31

* Microliters of O_2 uptake per minute per mg. of protein N.

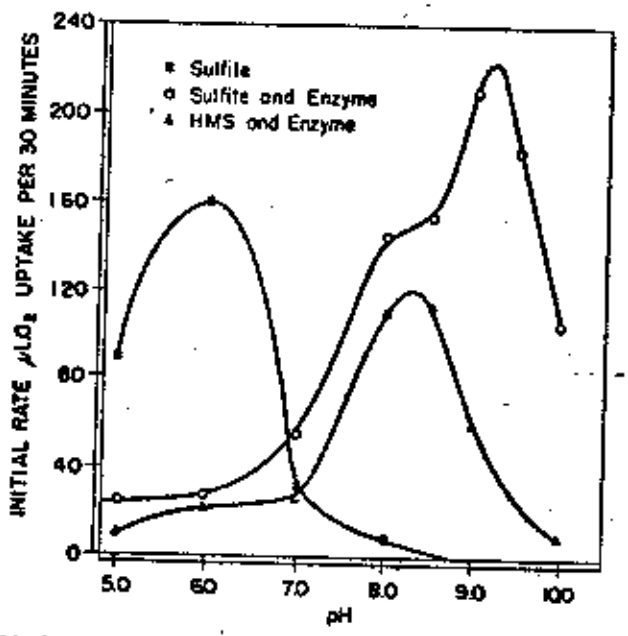


FIG. 4. Oxidation of bisulfite (○) and of hydroxymethanesulfonic acid (formaldehyde-bisulfite) (△) by Fraction IV and autoxidation of sulfite (■) as a function of pH. Each flask contained 5 μM of substrate and 1.0 ml. of Fraction IV in 2.2 ml. of buffer containing 2 mg. of EDTA. The buffers used were 0.04 M acetate, phosphate, and borate.

systems were involved. Thiosulfate ($S_2O_3^{=}$) and dithionate ($S_2O_6^{=}$) were not oxidized.

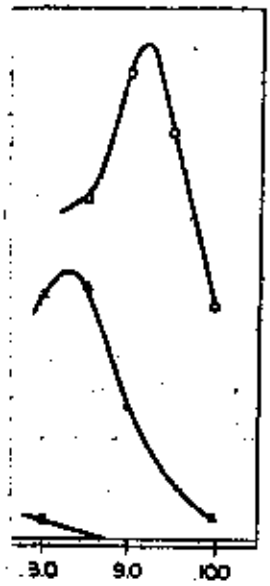
Catalytic Reduction of Methylene Blue—In view of the evidence that the sulfite oxidase is not a copper- or hemoprotein and has no readily dialyzable component, it appeared of interest to determine whether this system

$_4^-$) was oxidized with an oxygen
ecule of metabisulfite. Cysteine-
s clear whether the same enzyme

ulfite Oxidase

Total units	Recovery
	per cent
462	
338	73
360	76
141	31

g. of protein N.



oxymethanesulfonic acid (formalde-
ation of sulfite (□) as a function of
1.0 ml. of Fraction IV in 2.2 ml. of
used were 0.05 M acetate, phosphate,

$^-$) and dithionate ($S_2O_6^-$) were

n view of the evidence that the
tein and has no readily dialys-
determine whether this system

would catalyze the reduction of methylene blue. As shown in Fig. 5, in the absence of enzyme, bisulfite but not HMS slowly reduced methylene blue anaerobically. Fraction IV markedly accelerated decolorization of methylene blue by bisulfite but, with HMS as substrate, decolorization did not occur for 10 to 20 minutes when it commenced abruptly and the rate attained was about 50 per cent of that with bisulfite.

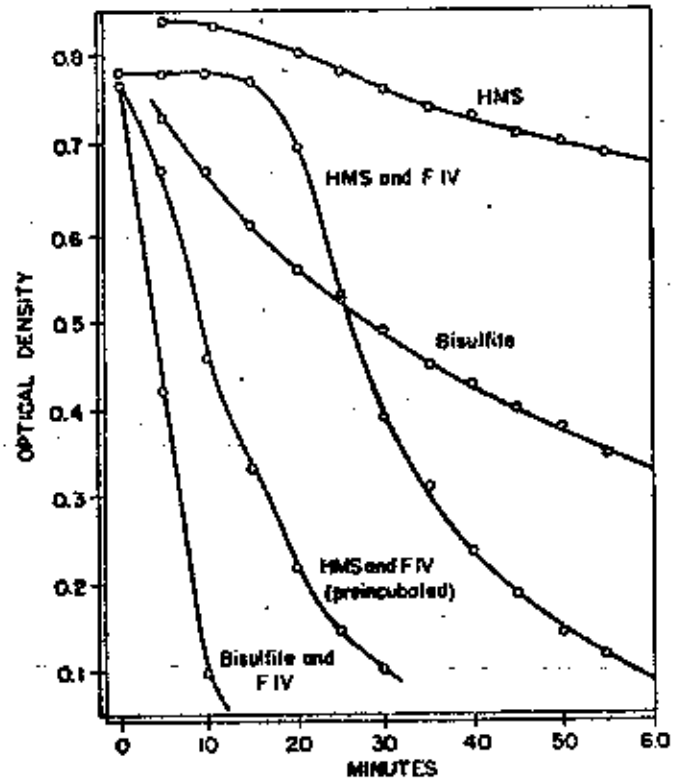


FIG. 5. Enzymatic anaerobic reduction of methylene blue by bisulfite and HMS. Each Thunberg tube contained 0.1 μM of methylene blue, 40 μM of substrate, and, when present, 1.0 ml. of Fraction IV in a final volume of 5 ml. of 0.05 M phosphate buffer, pH 7.8.

The lag time in the enzymatic reduction of methylene blue by HMS suggested that two enzymatic steps were involved: preliminary cleavage of HMS to formaldehyde and bisulfite and enzymatic reduction of methylene blue by the latter. This possibility was tested by anaerobically preincubating HMS with Fraction IV for 30 minutes and then adding methylene blue. As shown in Fig. 5, this procedure abolished the lag time. Essentially similar data were obtained with AMSA, *N*-methyl-, and *N*-butyl-AMSA.

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That HMS oxidation requires some enzyme in addition to the sulfite oxidase was also shown as follows: Fraction IV was brought to pH 5.3 for 10 minutes, the precipitate was removed, and the supernatant fluid restored to pH 7.8. The supernatant fluid exhibited no loss of activity towards sulfite during this procedure, while the rate of HMS oxidation was reduced about 50 per cent.

Catalysis of Bisulfite Oxidation by Methylene Blue—The slow reduction of methylene blue by bisulfite, observed in the absence of enzyme, suggested that this compound might catalyze the aerobic oxidation of bisulfite in

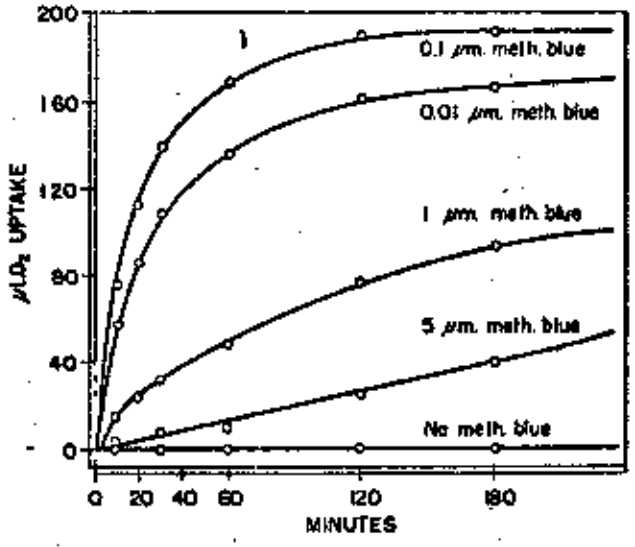

FIG. 6. Catalysis of bisulfite oxidation by methylene blue. Each flask contained 10 μM of bisulfite and 0.2 mg. of EDTA in final volume of 2.2 ml. of 0.05 M phosphate buffer, pH 7.8. The methylene blue content per flask is shown in the diagram.

the presence of EDTA. This proved to be the case; the rate of O_2 consumption increased as the methylene blue concentration increased up to 0.1 μM per flask. Without explanation is the finding that methylene blue concentrations greater than this were inhibitory as shown in Fig. 6.

Effect of Enzyme Concentration on Oxygen Consumption—Another surprising aspect of this enzyme system is the nature of the curves obtained when bisulfite or HMS is oxidized in the presence of lesser amounts of Fraction IV. These are illustrated in Fig. 7 and are not presently understood. Adequate explanation awaits purification of the responsible enzyme and understanding of its mode of action.

Distribution of Enzymes—Systematic studies of the biological distribu-

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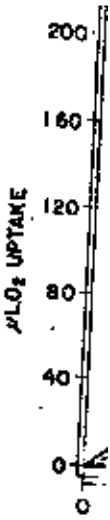

FIG. 7. Effect of enz
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ethylene Blue—The slow reduction of
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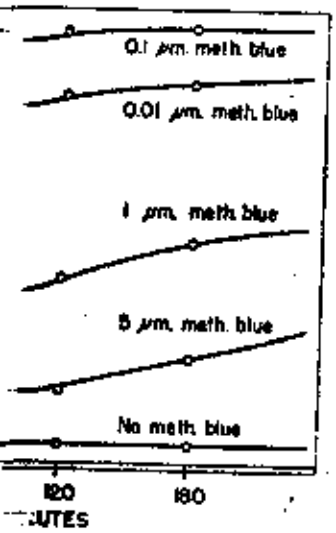

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Oxygen Consumption—Another sur-
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studies of the biological distribu-

tion of these enzymes have not yet been performed. Oxidation of bisulfite is catalyzed by both supernatant fluid and washed residues of homogenates of rat liver and kidneys and liver homogenates from pigs, mice, hamsters, and guinea pigs. HMS and *N*-methyl-AMSA were not oxidized by preparations of rat heart, spleen, brain, or skeletal muscle. Of all preparations tested only the supernatant fluids from liver homogenates have oxidized formaldehyde. HMS is oxidized by *Escherichia coli* and *Aerobacter aerogenes* but not *Staphylococcus albus* or *Saccharomyces*

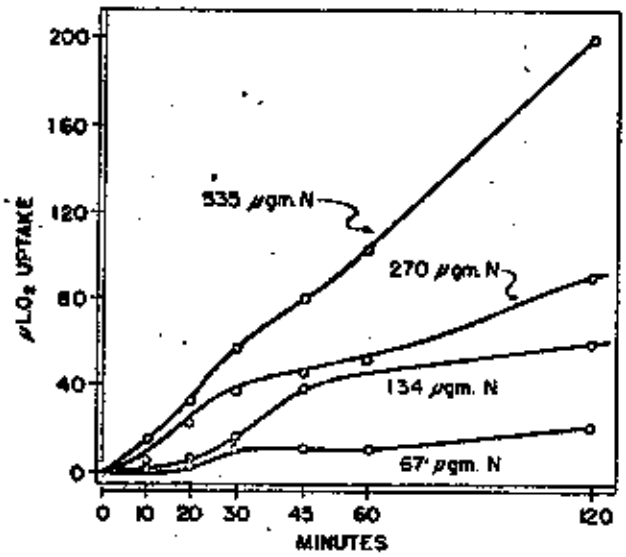

FIG. 7. Effect of enzyme concentration on oxidation of bisulfite. Each vessel contained 5 μM of bisulfite in 2.2 ml. of 0.05 M phosphate buffer, pH 7.8, and 0.2 mg. of EDTA. The flask content of Fraction IV is shown on the corresponding curve.

cerevisiae. This represents oxidation of the bisulfite moiety rather than of the formaldehyde, since *A. aerogenes* and *S. albus* catalyzed formaldehyde oxidation while *E. coli* and *S. cerevisiae* did not. These studies were performed before the possibility that HMS oxidation may require two or more enzymes was recognized and thus need not necessarily reflect the distribution of the bisulfite oxidase itself.

As a result of the observations of the oxidation of the carbonyl bisulfites, it was thought of interest to determine whether bisulfite could replace the requirement for arsenate or phosphate in the oxidation of 3-phosphoglyceraldehyde by crystalline triosephosphate dehydrogenase. However, oxidation of 3-phosphoglyceraldehyde did not occur when bisulfite was employed in place of phosphate or arsenate.

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EXPERIMENTAL

Methods and Materials

Fresh tissues were washed with ice-cold 0.05 M potassium phosphate buffer, pH 7.8, weighed, and homogenized with a plastic Potter-Elvehjem blender. 2 ml. of buffer per gm. of tissue, wet weight, were employed. The homogenate was then strained through several layers of muslin. Heart and skeletal muscle was ground with buffer and sand in an ice-cold mortar, and then strained through muslin. The homogenates were centrifuged at 2000 × *g* and 0–5° for 15 minutes. The residues were washed twice with phosphate buffer, resuspended in the original volume of buffer, and rehomogenized. Oxidation was observed manometrically in the Warburg apparatus at 37.5°. Autoxidation of bisulfite was followed by placing solid $NaHSO_3$ in the side arm to prevent reaction during the equilibration period. In all later experiments, when autoxidation was undesirable, the buffer solution was made with demineralized water containing 0.01 per cent of tetrasodium EDTA.

Formaldehyde was quantitatively determined by the method of MacFadyen (9). Recrystallized formaldehyde-bisulfite was employed as the analytical standard. Qualitative tests for formate were performed by this method after reduction with magnesium and HCl. Inorganic sulfate was determined by a modification of the benzidine method (10), after precipitation of protein and inorganic phosphate with uranium acetate. Sulfite was determined by iodometric titration, or manometrically, under conditions suitable for autoxidation to sulfate. Ammonia and volatile amines were measured by the micro diffusion method of Conway (11).

Synthesis of Substituted Methanesulfonic Acids

Formaldehyde-bisulfite was prepared and recrystallized several times from dilute ethanol. The bisulfite addition products of pyruvate, oxalacetate, and α-ketoglutarate were not isolated as the crystalline material. Rather, equimolar amounts of the neutralized carbonyl compound and $NaHSO_3$ were allowed to stand in solution. Aliquots were removed as required. The bisulfite addition products of acetaldehyde, *n*-propionaldehyde, *n*-butyraldehyde, and acetone were prepared from $NaHSO_3$ and excess carbonyl compound and recrystallized from dilute ethanol.

Aminomethanesulfonic acid was prepared by the method of Raschig and Prahl (12) in which HMS is shaken with aqueous ammonia at room temperature for ½ hour; the solution is then chilled and brought to pH 4.0 with 10 N H_2SO_4. The precipitate is collected, washed with ethanol, and recrystallized from water. The various *N*-alkylated derivatives were prepared by suitable modifications of this procedure. The only note-

worthy exceptions were *N*-ethyl- and *N*-methyl-AMSA which could only be obtained after removal of the excess amine with Dowex 50 in the hydrogen form. Some properties of these derivatives are summarized in Table III.

DL-Glyceraldehyde-3-phosphate, as the 1-bromide-3-phosphoric acid (dimer) dioxane addition compound, was generously donated by Dr. Erich Baer. This compound was dissolved in ice-cold water and dilute alkali was added to bring the pH to 7.0 immediately before use. Crystalline triosephosphate dehydrogenase was prepared by Dr. R. G. Parrish by the procedure of Cori, Slein, and Cori (13). Activity of the enzyme was assayed spectrophotometrically, and the increased absorption at 340 mμ due to the reduction of DPN was measured. A Beckman model DU photoelectric quartz spectrophotometer was employed. Cysteinesulfinic

TABLE III

N-Alkyl Derivatives of Aminomethanesulfonic Acid

Alkyl substituent	Structure	N calculated	N found	M.p., uncorrected	Recrystallizing solvent
		per cent	*per cent*	°C.	
Methyl	$C_2H_7NO_3S$	11.19	11.05	167–168	H_2O-ethanol
Ethyl	$C_3H_9NO_3S$	10.05	9.85	151–152	95% ethanol
Isopropyl	$C_4H_{11}NO_3S$	9.15	8.90	170–173	H_2O
n-Propyl	"	9.15	9.00	145–147	Absolute ethanol
n-Butyl	$C_5H_{13}NO_3S$	8.39	8.30	136–130	" "

acid was prepared by Mrs. Florapearl Cobey as described by Toennies and Lavine (14, 15). Cysteic acid was kindly provided by Dr. James Andrews. The inorganic sulfur compounds tested were commercial preparations, as were methanesulfonic acid, taurine, and *N*-methyltaurine.

Cultures of *E. coli*, *A. aerogenes*, and *S. albus* were obtained from the Department of Bacteriology, Duke University School of Medicine. *S. cerevisiae* Y-567 was obtained from the culture collection, Northern Regional Research Laboratory, United States Department of Agriculture, Peoria, Illinois.

Purification Procedure—All manipulations were performed in the cold (0–5°). Rat liver acetone powder was extracted with 10 volumes of 0.05 M phosphate buffer, pH 7.8, for 30 minutes with constant stirring. The suspension was centrifuged at 2000 × *g* for 20 minutes, the residue reextracted with 5 volumes of fresh buffer, and the extracts pooled and designated Fraction I. Fraction I was rapidly heated, with continuous stirring, to 58° and then cooled in an ice bath. The precipitate formed during heating was removed by centrifugation and the supernatant solu-

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tion designated Fraction II. The pH was then adjusted to 5.5 by dropwise addition of 0.1 N HCl with continuous stirring, followed by centrifugation and readjustment of the pH of the supernatant solution to 7.8 with dilute KOH. This was designated Fraction III and was brought to 0.5 saturation with respect to ammonium sulfate by the slow addition of an equal volume of a saturated solution. The precipitate which formed was collected by centrifugation, dissolved in a minimal volume of phosphate buffer, and exhaustively dialyzed against changes of buffer. The resultant solution was termed Fraction IV and was frozen and stored.

Enzyme activity was determined manometrically with the Warburg micro respirometer. Each flask contained 1 ml. of enzyme solution, usually 20 μM of substrate, and sufficient buffer to bring the final volume to 2 ml. 0.2 ml. of 20 per cent KOH was placed in the center well. Protein nitrogen was determined by a micro-Kjeldahl procedure (11).

Decolorization of methylene blue was observed in Thunberg tubes. Each tube contained 1 ml. of Fraction IV, 0.1 μM of methylene blue, and 40 μM of substrate. The final volume was made up to 5.0 ml. with phosphate buffer. The tubes were alternately evacuated and flushed with moistened tank nitrogen three times and incubated at room temperature after mixing. Reduction of methylene blue was followed in a Coleman junior spectrophotometer at 650 mμ.

DISCUSSION

These data appear to establish the existence in mammalian liver and kidney of an enzyme capable of catalyzing the oxidation of sulfite to sulfate. From the studies of Chatagner *et al.* (5), there is little doubt that the metabolism of cysteinesulfinic acid leads to production of SO_2 which, on hydration at the pH of body fluids, results in a mixture of sulfite and bisulfite ions. While readily autoxidizable in the presence of traces of polyvalent metal ions, autoxidation of these anions is not to be expected in biological systems, since, as shown by the behavior of heat-inactivated liver and of serum albumin, proteins completely inhibit this autoxidation, perhaps by virtue of their ion-binding capacity. Thus, the formation of sulfate from the sulfur-containing amino acids requires enzymatic catalysis of this last oxidative step.

The presently known properties of this enzyme are unusual, and understanding of its mode of action awaits further purification. Studies with the usual inhibitors indicate that it is not a copper- or hemoprotein nor has it a component readily removable by dialysis. Like the dehydrogenases, it catalyzes reduction of methylene blue by its substrate. But at the optimal pH of the enzyme, 9.3, its substrate may be presumed to be the sulfite ion, since at this pH the second dissociation of sulfurous acid is

complete (pK_2 = 7.2) and dehydrogenation of the few bisulfite ions which may exist at this pH seems unlikely. In any case, the enzymatic oxidation is in contrast to autoxidation which is most rapid at pH 6 and negligible at the optimal pH for the enzymatic process (Fig. 4). Fraction IV, although unable to oxidize xanthine or D-alanine, is highly colored and after acid hydrolysis yields riboflavin which was identified by paper chromatography. Because of the relatively crude state of this preparation, no significance may yet be attached to this observation.

The collected evidence strongly suggests that oxidation of the various α-hydroxy- and α-aminosulfonic acids by liver preparations is not directly catalyzed by the sulfite oxidase itself, and that preliminary enzymatic cleavage to the corresponding carbonyl compounds and bisulfite is required. This evidence may be summarized as follows: The optimal pH for sulfite oxidation differs from that for HMS oxidation. The enzymatic oxidations of sulfite and the autoxidizable α-hydroxysulfonic acids proceed at equal rates which exceed those of the stable α-hydroxy- and α-aminosulfonic acids all of which, in turn, are also oxidized at essentially the same rate. Enzymatic reduction of methylene blue commences immediately in the presence of sulfite but only after a significant lag time in the presence of α-hydroxy- or α-aminosulfonic acids. This lag time is abolished by preincubation of substrate and enzyme. The abrupt manner in which reduction occurs after the lag period is, however, surprising, since a sigmoidal curve would have been expected. Removal from Fraction IV of protein insoluble at pH 5.3 is without effect on sulfite oxidation yet decreases that of HMS by almost 50 per cent.

If such a desmolytic enzyme exists, its normal metabolic substrate is unknown. Conceivably, bisulfite arising from the metabolism of cysteine might condense with the many carbonyl compounds now recognized in intermediary metabolism and this enzyme may be required for the further metabolism of the addition compounds. The metabolic origin and nature of other α-hydroxysulfonic acids are presently unknown. Further study is also required to establish whether the same enzyme is also responsible for scission of the α-aminosulfonic acids.

SUMMARY

Evidence is presented indicating the existence in the liver and kidney of various mammals and in several bacteria of an enzyme which catalyzes the oxidation of sulfite to sulfate. The properties and preliminary purification of this enzyme are described and its metabolic significance discussed. Oxidation by rat liver of a series of α-hydroxy-, α-amino-, and *N*-substituted α-aminosulfonic acids is reported, together with evidence suggesting the presence of an enzyme which catalyzes the scission of these compounds to bisulfite and the corresponding carbonyl compounds.

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Internat. Z. Vit. Forschung 39 (1969)
Eingegangen am 7. November 1969

Sulfittoxizität
Thiaminmangel
Thiamin-Mangelkost
Klinische Untersuchungen

Neurophysiologische Untersuchungen
Leberfunktion

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Thiaminmangel und Unbedenklichkeit von Sulfit für den Menschen[1]

1. Mitteilung:

Problemstellung, Versuchsplanung, Klinische Untersuchungen

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unter Mitwirkung von
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(Direktor: Prof. Dr. H.-D. Cremer)

Zusammenfassung: Tierversuche der Verfasser (Cremer und Hötzel) hatten gezeigt, dass die Toxizität von Sulfit von der Thiaminversorgung beeinflusst wird. Während bei einer Thiaminzufuhr von nur 50 % des Bedarfs (5,5 mcg/100 g Ratte/Tag) bei Gabe von 40 mg Sulfit/kg Körpergewicht/Tag im subchronischen Versuch bereits 8 von 13 Versuchstieren starben, überlebten bei höherer Thiaminzufuhr (über den Bedarf hinausgehende Gabe) die Versuchstiere auch noch Belastungen mit 400 mg Sulfit/Körpergewicht/Tag bei normaler Entwicklung. Da Sulfit als Lebensmittelzusatzstoff Verwendung findet und da andererseits von einem Teil der Bevölkerung geringere Thiaminmengen aufgenommen werden als den Empfehlungen entspricht, erschien es wünschenswert, in einem Versuch mit Menschen zu überprüfen, ob bei Thiaminmangel Sulfitbelastungen,

[1] Für die Unterstützung dieser Untersuchungen danken wir dem Bundesministerium für Gesundheitswesen, der Deutschen Forschungsgemeinschaft, dem Weinforschungs-Institut Trier.

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Für Beratung und Mitwirkung bei elektromyographischen und neurologischen (3), bei klinischen (4) und klinisch-chemischen (5) Untersuchungen sei hiermit gedankt.

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wie sie in praxi vorkommen können, ohne gesundheitliche Störungen vertragen werden.

Der Versuch, an dem 12 klinisch gesunde Versuchspersonen (6 weibliche und 6 männliche Vpn) teilnahmen, hatte eine Dauer von 65 Tagen. Nach einer Vorbeobachtungsperiode mit Normalkost erhielten die Vpn während 50 Tagen eine Thiaminmangelkost (120 mcg Thiamin/1000 Kalorien), deren Zusammensetzung beschrieben wird. 15 Tage nach Beginn des Thiaminmangels wurde die Testgruppe während 25 Tagen mit 400 mg Schwefeldioxid/Person/Tag belastet. Das SO_2 wurde in Form von Natriumhydrogensulfit und als «Natriumglucosesulfonat» täglich mit Getränken verabreicht, die auch die Kontrollgruppe ohne Sulfitzusatz erhielt. Dann schloss sich eine zehntägige Nachbeobachtungsperiode an, in der weiterhin die Thiaminmangelkost, jedoch kein Sulfit verabreicht wurde. Zu Ende des Versuchs erhielten alle Vpn einen Thiaminstoss (2 x 100 mg/Person an aufeinanderfolgenden Tagen), um die Normalisierung thiaminabhängiger Versuchskriterien erfassen zu können und um die Vpn wieder in einen ausgeglichenen Thiaminversorgungszustand zu bringen.

Zur Prüfung der Frage, ob Sulfit bei Thiaminmangel zu klinisch manifesten Veränderungen führen kann, unterzogen sich sämtliche Vpn zu Beginn, während und am Ende des Versuchs einer Untersuchung, bei der insbesondere auf gastrointestinale und hepatologische Erkrankungen geachtet wurde. Ein klinischer Befund konnte nicht erhoben werden. Auch GOT, GPT, LDH, α-HBDH, alkalische Phosphatase im Serum, Serum-Elektrophorese, Thymol Trübungstest, Haematrokrit, Zahl der Erythrozyten und neurophysiologische Untersuchungen (Messung der motorischen Nervenleitungsgeschwindigkeit, Reflexverhalten) lieferten keinen Hinweis auf eine Störung durch die Belastung mit Sulfit.

Über den Einfluss der Thiaminversorgung und der Sulfitzufuhr auf den Thiaminstatus und auf die Aktivität von Dehydrogenasen in Erythrozyten wird ebenso wie über den Metabolismus von Sulfit bei Thiaminunterversorgung in gesonderten Mitteilungen in dieser Zeitschrift berichtet.

1. Problemstellung

Zum Schutz der Bevölkerung gegen gesundheitliche Nachteile durch Lebensmittelfremdstoffe wurden von einschlägigen Gremien ausführliche Richtlinien zur toxikologischen Überprüfung derartiger Stoffe vorgelegt. Bei den tierexperimentellen Untersuchungen im Rahmen dieser Überprüfung erhalten die Versuchstiere eine optimal zusammengesetzte Kost, der vorzüglich noch Sicherheitszuschläge von Vitaminen zugelegt werden. Bei derartigem experimentellem

Vorgehen werden Wechselwirkungen zwischen dem Stoffwechsel von im Überschuss vorliegenden Nährstoffen und der Belastung mit Fremdstoffen u. U. nicht erkannt. Da solchen Wechselbeziehungen jedoch grössere praktische Bedeutung zukommen könnte, haben wir schon vor längerer Zeit entsprechende Versuche bei beschränkter Zufuhr einzelner Nährstoffe aufgenommen. In diesen Untersuchungen wurden Wechselbeziehungen zwischen der Wirkung von einigen Lebensmittelfremdstoffen und der Nährstoffversorgung gefunden (Hötzel und Cremer, 1960; Hötzel, 1962; Hötzel, 1963; Hötzel und Weber, 1964; Hötzel, 1969).

Theoretisch interessant und praktisch bedeutsam war das Ergebnis unserer Versuche mit wachsenden Ratten, in denen sich gezeigt hatte, dass die Toxizität von Sulfit im Mangel an Thiamin wesentlich ansteigt (Hötzel, 1961). Während bei einer Thiaminzufuhr von 50 % des Bedarfs (0,1 mg Thiamin pro 100 g Kost, entsprechend 5,5 mcg pro 100 g Ratte pro Tag) bei Tagesgaben von 40 mg Sulfit pro kg Körpergewicht (berechnet als SO_2) im subchronischen Versuch bereits 8 von 15 Versuchstieren starben, überlebten bei höherer Thiaminzufuhr die Versuchstiere auch noch Sulfitgaben von 400 mg pro kg Körpergewicht pro Tag und mehr.

Die Testdosen von Sulfit wurden oral als Natriumdisulfit getrennt von der Kost verabreicht, um eine Zerstörung des Thiamins im Futter durch Sulfit zu vermeiden. In weiteren Versuchen mit parenteraler Gabe von Sulfit konnte auch im akuten Versuch eine signifikante Abhängigkeit der Sulfittoxizität von der Thiaminversorgung nachgewiesen werden. Es ergab sich weiterhin, dass die Toxizität nicht wesentlich davon abhängt, ob Schwefeldioxid in anorganischer Bindung (Natriumdisulfit) oder in organischer Bindung (Glucosesulfonat oder Azetaldehydsulfonat) verabreicht wurde (Hötzel, Cremer und Muskat, 1965).

Mit der Wechselbeziehung zwischen Sulfit und Thiamin haben sich neben unserem Arbeitskreis auch noch Jaulmes, Boucard und Laval, 1959; Bhagat und Lockett, 1964; Wilmes, 1966; Lhuissier, 1966; Causeret, 1965; Sidorenxov, 1965 und Hermus, 1969 beschäftigt. Über den Wirkungkmechanismus wurde noch keine völlige Klarheit gewonnen. Zu einem Teil kommt die Wechselbeziehung durch eine Zerstörung von Thiamin durch Sulfit im Verdauungskanal zustande, soweit die Versuchsbedingungen das ermöglichen. Darüber hinaus treten Interaktionen zwischen Thiamin und Sulfit im intermediären Stoffwechsel auf (Diskussion siehe bei Cremer und Hötzel, 1966; Hötzel, 1969). Die Untersuchungen zur Aufklärung des Wirkungsmechanismus, insbesondere über die Beeinflussung von Enzymen bzw. Enzymaktivitäten durch Sulfit in Abhängigkeit von der Thiaminversorgung, werden fortgesetzt.

In den Versuchen mit Thiaminmangeltieren ergab sich, dass die toxische Grenzdosis deutlich unter 40 mg SO_2/kg Körpergewicht/Tag liegt. Leitet man aus diesem Ergebnis einen Wert für Menschen ab, so errechnet sich unter Einbeziehung des üblichen Übertragungs- und Sicherheitsfaktors (1 : 100), dass die Tagesaufnahme von Menschen an Sulfit unter 0,4 mg SO_2/kg Körpergewicht liegen sollte, soweit nicht eine ausreichende Thiaminversorgung sichergestellt ist. Für den erwachsenen Menschen mit 70 kg Körpergewicht entspricht das einer Menge von weniger als 28 mg SO_2 täglich.

Wenn die SO_2-Höchstmengen für Lebensmittel nach dem neuen Regierungsvorschlag berücksichtigt werden, dürfte die Sulfitaufnahme der Bevölkerung bei durchschnittlich 12 mg/Tag liegen. Die aus Gründen der gesundheitlichen Unbedenklichkeit zu erhebende Forderung für die Höhe der SO_2-Aufnahme wäre damit erfüllt. Allerdings ist bei dem Wert von 12 mg nur ein durchschnittlicher Tagesverbrauch von weniger als 50 g Wein zugrundegelegt.

Dieser Mittelwert wird von einem Teil der Bevölkerung deutlich überschritten, denn schon bei Genuss von 200 ml Wein mit einem Gehalt von 200 ppm SO_2 werden 40 mg SO_2 aufgenommen. Dieser Wert übersteigt die tierexperimentell ermittelte duldbare Tagesaufnahme dann erheblich, wenn die Versorgung mit Thiamin hinter den Empfehlungen zurückbleibt. Das ist bei einem nicht unwesentlichen Teil der Bevölkerung der Fall (Kraut, 1963; Aebi, 1963; u. a.).

Aufgrund dieser Zusammenhänge erscheint eine drastische Beschränkung des SO_2-Gehaltes von Lebensmitteln (speziell Wein) unumgänglich notwendig. Eine solche Massnahme hätte jedoch weitreichende Konsequenzen, so dass man sich dabei nicht allein auf das Ergebnis von Tierversuchen verlassen möchte. Da im Einzelfall die Ergebnisse von Tierversuchen sicherlich nicht in quantitativen Massstäben auf Menschen übertragen werden können, erschien es notwendig, eine Untersuchung an Menschen selbst über die allgemeine Wirkung von Sulfit und speziell über die Sulfit-Thiamin-Wechselbeziehung durchzuführen.

2. *Versuchsbedingungen*

2.1 Zeitlicher Ablauf

Der Versuch hatte eine Gesamtdauer von 65 Tagen mit einer Unterteilung in 5 Versuchsperioden unterschiedlicher Dauer. Einen genauen Einblick in den zeitlichen Ablauf gibt Tabelle 1.

In Periode I wurden Untersuchungen zur Ermittlung der klinischen, physiologischen und biochemischen Ausgangswerte der Versuchspersonen (Vpn) durchgeführt. Periode II diente der Einleitung eines leichten Thiaminmangels und der

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Tab. 1: Aufteilung in Versuchsperioden

	Dauer (Tage)	Testgruppe	Kontrollgruppe	Versuchsziel
Periode I (15. 10. – 27. 10. 68)	13	Normalkost		Vorbeobachtung
Periode II (28. 10. – 11. 11. 68)	15	Thiamin-Mangelkost		Thiaminverarmung
Periode III (12. 11. – 6. 12. 68)	25	Thiamin-Mangelkost *mit Sulfit*	*ohne Sulfit*	Sulfitbelastung
Periode IV (7. 12. – 16. 12. 68)	10	Thiamin-Mangelkost		Nachbeobachtung
Periode V (17. 12. – 18. 12. 68)	2	Normalkost + «Thiaminetosa»		Regeneration

Vereinheitlichung der Thiamin-Körperbestände der Vpn. Die Notwendigkeit dazu ergibt sich retrospektiv aus den grossen Unterschieden der Harn-Thiaminausscheidung bei einzelnen Vpn zu Versuchsbeginn (siehe II. Mitt., Tab. 5). In Periode III – der Hauptperiode – erfolgt die Belastung mit Sulfit. Der Sulfiteinfluss kann dabei durch Vergleich zwischen beiden Gruppen in der gleichen Periode und als Periodenvergleich der Testgruppe erfolgen (Abweichungen von Periode III mit Sulfit gegenüber Periode II und IV ohne Sulfitbelastung). Periode IV diente einmal diesem Periodenvergleich an denselben Versuchspersonen, zum anderen sollten in diesem Zeitraum mögliche Spätwirkungen des Sulfit erfasst werden. In Periode V wurden bei Normalkost hohe Mengen von Thiamin verabreicht, um die Normalisierung der thiaminabhängigen Versuchsparameter zu erfassen und um den Thiaminmangel der Vpn wieder zu beseitigen.

2.2 Versuchspersonen

Die Vpn wurden nach eingehender klinischer Untersuchung am Ende der Vorperiode aus einem Kollektiv von je 10 männlichen und 10 weiblichen Probanden ausgewählt. Die Auswahl und die Verteilung auf die beiden Gruppen erfolgte innerhalb der Geschlechter streng zufällig (Los). Zu Beginn des Versuchs bestand jede Gruppe aus 3 weiblichen Vpn (17–19 Jahre alt) und 3 männlichen Vpn (22–56 Jahre alt). Der Versuch wurde von allen Vpn bis zum Ende durchgehalten.

2.3 Verabreichung von Sulfit

Die Vpn der Testgruppe erhielten während Periode III (25 Tage) pro Person und Tag 400 mg Schwefeldioxid. Davon wurden 50 mg in Form von Natrium-

hydrogensulfit («freie schweflige Säure») und 350 mg in Form von «Natriumglucosesulfonat» («gebundene schweflige Säure») verabreicht. Das Verhältnis von «freier» zu «gebundener» schwefliger Säure entspricht mit 1 : 7 etwa den Verhältnissen im Wein. Jede Vpn erhielt zu den Mahlzeiten täglich insgesamt 500 ml Traubensaft und 300 ml Wein, in denen bei der Versuchsgruppe die Testdosis von Sulfit anteilig gelöst war. Der Sulfitgehalt der Getränke wurde jeden 2. Tag nach einer Modifikation der Bestimmungsmethode von Kielhöfer und Aumann (1958) kontrolliert. Der Kontrollgruppe wurde der gleiche Saft und Wein in gleichen Mengen wie der Versuchsgruppe verabreicht, aber ohne Zusatz von Sulfit.

Bei dem Traubensaft handelte es sich um einen Kurtraubensaft, dessen nativer Sulfitgehalt bei etwa 1–2 mg im Liter lag. Der von uns verwendete Wein war ein 1967er Mosel-Wein, der nach einem Spezialgärverfahren hergestellt worden war und uns vom Weinforschungsinstitut in Trier freundlicherweise zur Verfügung gestellt wurde. Nach der angegebenen Analyse enthielt er bei einem Extraktgehalt von 25 g/Liter 7,66 % Alkohol und durch die besondere Art der Herstellung nur 1,2 mg % gesamtschweflige Säure. Wegen der Gefahr einer bakteriellen Infektion wurde er sofort nach der Gärung tiefgefroren und in diesem Zustand bis zum Verbrauch aufbewahrt.

Bei der Festlegung der Teststoffdosis wurde vom Ergebnis der erwähnten Tierversuche ausgegangen. Es hatte sich gezeigt, dass im Thiaminmangel die maximal unwirksame Dosis unterhalb 40 mg Sulfit pro kg Körpergewicht/Ratte pro Tag liegt. Die formale Umrechnung auf Menschen (Einbeziehung des Übertragungsfaktors Tier-Mensch, Ausklammerung des Sicherheitsfaktors) ergibt den Wert 4 mg/kg Mensch/Tag. Für den 70 kg schweren Menschen errechnet sich dann eine Tages-Gesamtdosis von 280 mg Sulfit. Bei dieser Gabe hätte sich im Versuch mit Menschen eine «Sulfitwirkung» einstellen müssen, wenn die formale Übertragung gerechtfertigt ist.

Im Interesse einer schärferen Versuchsaussage wurde die Testdosis noch auf 400 mg/Tag erhöht. Die experimentelle Sulfitaufnahme war damit der unfreiwilligen Sulfitaufnahme bei Genuss von etwa 1 l Wein ungefähr äquivalent (je nach Herkunft des Weines). Eine höhere Sulfitaufnahme wäre an sich im Interesse einer grösseren Sicherheit der Versuchsaussage wünschenswert gewesen (siehe IV. Mitt.: «Auswertung und Beurteilung eines Stoffwechselversuchs am Menschen), liess sich jedoch in Anbetracht der Dreifachbelastung der Versuchspersonen (Sulfit, Alkohol, Thiaminmangel) nicht verantworten.

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2.4 Versuchskost

Im Hinblick auf die Fragestellung müssen folgende Forderungen an die Versuchskost gestellt werden:

1. Übereinstimmende Energie- und Nährstoffaufnahme in beiden Versuchsgruppen (insbesondere bei Thiamin).
2. Deckung des individuellen Kalorien- und Nährstoffbedarfs der Vpn ausser bei Thiamin.
3. Suboptimale Thiaminzufuhr
   bei weibl. Vpn höchstens 350 mcg/Tag } = 140 mcg/1000 Kal.
   bei männl. Vpn höchstens 450 mcg/Tag }
4. Anlehnung an übliche Kostgewohnheiten und wenigstens ein Minimum an abwechslungsreicher Kostplangestaltung als Voraussetzungen für quantitative Aufnahme der zugeteilten Mahlzeiten und Durchhalten über 65 Versuchstage.

Diese Forderungen wurden folgendermassen realisiert:

Die wünschenswerte Nährstoffzufuhr für die Vpn wurde nach den Angaben der Deutschen Gesellschaft für Ernährung (DGE, 1962) und den Richtlinien des *Food and Nutrition Board* des *National Research Council* (NRC, 1964) ermittelt. In Tabelle 2 sind die Werte für die Hauptnährstoffe während der verschiedenen Versuchsperioden – für weibl. und männl. Vpn getrennt – aufgeführt. Da beide Versuchsgruppen gleichartig zusammengesetzt sind, stören die Unterschiede in der Nährstoffaufnahme zwischen männl. und weibl. Vpn die Vergleichbarkeit zwischen Versuchs- und Kontrollgruppen nicht.

Tab. 2: Wünschenswerte Aufnahme an Hauptnährstoffen (in g und Kalorien)

Versuchszeitraum	Geschlecht der Vpn	Eiweiss (g)	Fett (g)	Kohlenhydrate (g)	Kalorien
Periode II + IV	weibl.	80–85	115–120	215–230	2300–2400
	männl.	80–85	140–160	260–300	2700–3050
Periode III	weibl.	80–85	100–110*	335–340*	2650–2800*
	männl.	80–85	130–140*	380–400*	3100–3250

Aus der Gruppe derjenigen Lebensmittel, die aufgrund ihres Nährstoffgehaltes grundsätzlich geeignet schienen, wurden für den Versuch diejenigen mit geringem Thiamingehalt herausgesucht. Darauf aufbauend wurde in Anlehnung an Mitteilungen von Reuter und Mitarbeitern (1967) und Kraut und Mitarbeitern (1965) ein Rahmenplan (s. Tab. 3) zusammengestellt, der, soweit möglich, auch

* Die abweichenden Werte bei Fett, Kohlenhydraten und Kalorien von Periode III gegenüber Perioden II und IV ergeben sich durch die zusätzlichen Gaben von Traubensaft und Wein (teilweiser Ausgleich der Kalorienzufuhr durch verminderte Fettmenge).

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Abwechslung in der Speisenfolge erlaubte und übliche Kostgewohnheiten berücksichtigte.

Tab. 3: Rahmenplan für die Versuchskost (Tagesmengen)

Eiweissreiche Lebensmittel:

100 g Sahnequark
50 g Käse (Chester, Gouda, Schmelzkäse)
120 g Fleisch (Rind, Kalb oder Geflügel)
50 g Aufschnitt (Rauchfleisch, Hackbraten, Wurst u. a. v. Rind)

Fettreiche Lebensmittel:

25 g Sahne
20 g Kokosfett
40 g Butter (kalorische Zulage: + 40 g Butter)

Kohlenhydratreiche Lebensmittel:	*kalorische Zulage*
50 g Pumpernickel	
5 Brötchen	+ 1 Brötchen
30 g Honig	
30 g Marmelade	
30 g Zucker	
100 g Kartoffeln	+ 100 g Kartoffeln
oder entsprechende Menge Kart.-Trockenerzeugnisse	
oder 60 g Reis	+ 40 g Reis
oder 75 g Teigwaren	+ 35 g Teigwaren

Ausserdem wurden täglich verabreicht:

200 g Gemüse (bevorzugt Gurken, rote Rüben, Sellerie, Pfifferlinge, Spargel, Kopfsalat)
150 g Obst (bevorzugt Sauerkirschen, Preiselbeeren, Pfirsiche, Äpfel, Birnen)
Getränke: 2 Tassen Bohnenkaffee
2 Tassen schwarzen Tee (in der II. und IV. Versuchsperiode)
Wasser, Mineralwasser, Coca Cola und 1 weitere Tasse Bohnenkaffee waren erlaubt, mussten jedoch im Protokoll angegeben werden.

Täglich Vitaminzulage aller Vitamine ausser Thiamin in bedarfsdeckenden Mengen.

Bei der Zubereitung der Kost[7] wurden Verfahren bevorzugt, die den grösstmöglichen Verlust an Thiamin erwarten liessen: Kochen in viel Wasser ohne Mitverwendung des Kochwassers; langes Schmoren, Warmhalten und Wiederaufwärmen. Auf diese Weise konnten bei einigen Lebensmitteln analytisch kontrollierte Thiaminverluste in Höhe von 80–90 % des Ausgangswertes herbeigeführt werden. Das erlaubte eine erweiterte Verwendung auch von Gemüsearten mit relativ hohem Thiamingehalt.

[7] Der Sonderküche der Staatl. Diätschule der Med. Universitätskliniken Giessen wird für die Zubereitung gedankt.

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Die Thiaminaufnahme wurde nach Tabellen (Souci und Bosch, 1967) errechnet und analytisch nach einer modifizierten (s. II. Mitt.) Thiochrommethode (Strohecker und Henning, 1963) überprüft. Die Ergebnisse sind in Tabelle 4 zusammengefasst.

Tab. 4: Kalorien- und Thiaminaufnahme

Versuchsperiode	Geschlecht der Vpn	Kcal	Berechnet		Analysiert	
			mcg Thiamin /Tag	mcg Thiamin /1000 Kcal	mcg Thiamin /Tag	mcg Thiamin /1000 Kcal
II	weibl.	2420	466	189	295	121
	männl.	2940	533	182	355	120
III	weibl.	2840	452	151	322	112
	männl.	3360	518	155	380	112
IV	weibl.	2390	415	174	308	128
	männl.	2915	477	164	361	120
Mittelwert	weibl.	2613	450	170	311	118
	männl.	3134	516	165	371	119

Die Ergebnisse von Tabelle 4 zeigen, dass die analytisch bestimmten Thiaminmengen im Durchschnitt um etwa 40 % niedriger als die errechneten Werte liegen. Pro Person und Tag wurden von den weiblichen Vpn im Durchschnitt 311, von den männlichen Vpn 371 mcg Thiamin aufgenommen. Diese Thiaminmenge entspricht bei den männlichen und weiblichen Vpn übereinstimmend rund 120 mcg/1000 Kalorien. Zur Beurteilung der Thiaminmangelsituation der Vpn empfiehlt sich der Vergleich mit Richtlinien über die wünschenswerte Thiaminaufnahme: Die im Versuch aufgenommene Thiaminmenge beträgt (bezogen auf 1000 Kalorien) 17 % der Empfehlungen der DGE (1962), 30 % der Richtlinien des NRC (1964) und macht 60 % des «Minimalbedarfes» (NRC, 1964) aus. Die Thiaminversorgung lag damit im experimentell interessanten Bereich.

2.5 Versuchskriterien, Untersuchungsmethoden

Der Versuch wurde mit einer eingehenden klinischen Untersuchung begonnen und abgeschlossen. In der Versuchszeit wurde regelmässig der Blutstatus (Hämoglobin, Hämatokrit, Erythrozytenzahl) erfasst und Bestimmungen von Sulfit, Sulfat, Taurin, Thiamin im Harn, Sulfit und Thiamin im Blut, Transketolase, G-6-PDH, LDH, NADH-Diaphorase in Erythrozyten, GOT, GPT, LDH, alkal. Phosphatase, α-HBDH im Serum, Serumelektrophorese, Thymoltrübungstest in regelmässigen Abständen durchgeführt. Eine neurologische Untersuchung schloss

sich an. Zu Ende des Versuchs wurde ein Thiaminstoss verabreicht (2 x 100 mg/Person), um die Normalisierung bestimmter Versuchsparameter nach Beseitigung des Thiaminmangels erfassen zu können.

Über Einzelheiten der Bestimmungsmethoden und über die Ergebnisse der vorliegenden grossen Gemeinschaftsuntersuchung wird in den Mitt. II und III im Zusammenhang berichtet.

3. *Klinische Betreuung, neurophysiologische Untersuchung*

Zur Prüfung der Frage, ob Sulfit bei Thiaminmangel im Organismus zu klinisch manifesten Veränderungen führen kann, wurden klinische, klinisch-chemische und neurophysiologische Untersuchungen durchgeführt. Die Voraussetzung war, dass gesunde Versuchspersonen ausgewählt wurden, die insbesondere keine Lebererkrankungen durchgemacht hatten.

Zu Beginn des Versuchs unterzogen sich sämtliche Versuchspersonen einer klinischen Untersuchung, bei der insbesondere nach gastrointestinalen und hepathologischen Erkrankungen gefragt und der Tastbefund der Leber erhoben wurde. Zwei Auswärter mit tastbar vergrösserter Leber schieden aus.

Die folgenden klinisch-chemischen Untersuchungen wurden am Ende der Vorperiode, in der Mitte und am Ende der Hauptperiode sowie am Ende der Nachperiode durchgeführt: GOT, GPT, LDH, α-HBDH, alkalische Phosphatase im Serum, Serum-Elektrophorese, Thymol Trübungstest, Haematokrit und Zahl der Erythrozyten. Dabei ergaben sich keine signifikanten Änderungen der Enzymaktivitäten, des Haemoglobingehaltes, der Erythrozytenzahl oder des Haematokrits. Einer der Probanden, der während der Hauptperiode Sulfit erhielt, hatte von vornherein etwas erhöhte LDH-Werte, diese änderten sich jedoch im Verlauf des Versuchs nicht. Am Ende des Versuchs wurden dazu erneut Thymol-Trübungstest und Serumelektrophorese kontrolliert, wobei letztere weder eine Änderung in den Globulinfraktionen noch im Gesamteiweiss erkennen liess. Auch ergab die klinische Abschlussuntersuchung wiederum mit Bestimmung der Konsistenz und Grösse der Leber keine Abweichungen von der Voruntersuchung.

Neben diesen Untersuchungen, die auf die allgemeine Leberverträglichkeit und den Ausschluss einer toxischen Wirkung auf die Leber zielten, wurden bei allen Probanden eine zweimalige neurophysiologische Untersuchung mit Messung der motorischen Nervenleitungsgeschwindigkeit durchgeführt, da an Versuchstieren bei Thiaminmangel das Auftreten von Polyneuropathien (Kunze, 1969; Muskat, 1969) beschrieben wurde. Diese Untersuchungen fanden zu Beginn der Hauptperiode und am Ende der Nachperiode innerhalb von drei Tagen für

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die ganze Probandengruppe statt. Dabei ergab sich keine Verlängerung der motorischen Nervenleitungsgeschwindigkeit, wie auch die zugleich durchgeführte neurologische Untersuchung keine Änderung im Reflexverhalten oder Störungen der Sensibilität erkennen liess.

Auch die Beobachtung der Probanden während des Versuchs liess keine Abweichung vom Normverhalten erkennen. Einige Vpn klagten lediglich am Ende des Versuchs über eine gewisse Gereiztheit, die wir jedoch auf die lästigen und etwas unangenehmen Begleiterscheinungen des Versuchs (ständige gemeinsame Nahrungsaufnahme ohne die Möglichkeit einer zusätzlichen individuellen Gestaltung, Urinsammeln, wiederholte Blutabnahme) beziehen und nicht als eine Folge des relativ geringen Thiaminmangels oder der Sulfitapplikation deuten möchten.

Summary:

1. Object of the study, experimental design, and clinical examination

The authors' (Cremer *and* Hötzel) *previous animal experiments had shown that the toxicity of sulfite is influenced by the thiamine intake. In subchronic experiments 8 out of 13 animals, having received only 50 % of their daily thiamine requirement, died when they were administered 40 mg of sulfite per kg body weight per day, whereas animals having received higher thiamine doses than the daily requirement survived even when they were administered 400 mg of sulfite per kg body weight per day. They did not show any developmental disturbances.*

Since sulfite is used as a food additive and since large parts of the population have a lower daily intake of thiamine than recommended, it seemed advisable to investigate whether normal doses of sulfite can be tolerated by man without any health damage, also under thiamine deficiency conditiones. 12 healthy volunteers (6 males and 6 females) participated in the 65 days' experiment. In the first period they received a normal diet; during the following 50 days the diet was thiamine-deficient (120 mcg thiamine/1000 Kcal.). The composition of this diet is described in detail. 15 days after the start of the thiamine-restriction the test group received 400 mg of sulfite per person per day for 25 days. 50 mg of SO_2 were administered as sodiumhydrogensulfite and 350 mg of SO_2 as sodium-glucose-sulfonate, always dissolved in drinks. The control group received the same drinks but without sulfite added. In the third period of 10 days the volunteers were not given any more sulfite, but still received the thiamine deficient diet. At the end of the experiment, 100 mg of thiamine was administered orally on each of two days, to each person in order to normalize the thiamine status of

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Toxicität von schwefliger Säure in Abhängigkeit von Bindungsform und Thiaminversorgung*

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Mit 1 Textabbildung

(Eingegangen am 30. Juni 1965)

Bei der Beurteilung der Unbedenklichkeit von Lebensmittelfremdstoffen geht man in erster Linie von zwei Größen aus:

1. Die Menge des Zusatzstoffes, die auf der Grundlage experimenteller Erfahrungen noch keine gesundheitlichen Nachteile herbeizuführen vermag.
2. Die Höchstmenge des Fremdstoffes, die von Menschen unter ungünstigsten Umständen über längere Zeit mit Lebensmitteln aufgenommen werden kann.

Unter Zugrundelegung neuerer Untersuchungen und Erhebungen erscheint der Abstand zwischen der Menge von SO_2, mit deren Aufnahme mit der Nahrung man unter praktischen Verhältnissen heute durchaus rechnen könnte, zu der mit an Sicherheit grenzenden Wahrscheinlichkeit ungefährlichen Dosis, bedenklich gering. Im chronischen Versuch mit Ratten fanden Fitzhugh u. Mitarb. (1), daß 14 mg SO_2/kg Ratte/Tag symptomlos vertragen wurden, daß aber bei etwa 30 mg SO_2 bereits leichtere Schäden auftraten. In früheren Untersuchungen (2, 3) sind wir zu in der Größenordnung entsprechenden Ergebnissen gekommen, allerdings mit andersartiger Versuchstechnik. Bei einer Unterversorgung an Thiamin beobachteten wir bei 40 mg SO_2/kg Ratte/Tag bei wachsenden Ratten eine deutliche Verminderung von Wachstum, Futteraufnahme und Futterverwertung und das gehäufte Auftreten von Todesfällen. Als unwirksame Dosis fanden wir 1 mg SO_2/kg Ratte/Tag.

Bei Anwendung des üblichen Sicherheits- bzw. Übertragungsfaktors von 100 errechnet sich aus den Ergebnissen von Fitzhugh u. Mitarb. (1), daß Mengen von etwa 10 mg SO_2 pro Tag für den 75 kg schweren Mann noch unbedenklich sind. Bei Zugrundelegung der Ergebnisse unserer Versuche errechnen sich noch kleinere „annehmbare Tagesaufnahmen" für SO_2.

Die mittlere Tagesaufnahme der westdeutschen Bevölkerung übersteigt diese Werte. Im Jahre 1962 errechnet sich allein aus der Aufnahme von Rosinen, Trockenobst, Traubenmost und Wein (4) eine tägliche Zufuhr von 20 mg SO_2 pro Kopf der Bevölkerung. Dieser Wert spiegelt jedoch nur einen Teil der tatsächlichen SO_2-Aufnahmen wider, da Trockengemüse und Kartoffeltrockenerzeugnisse, die nach

* Dem Bundesministerium für Ernährung, Landwirtschaft und Forsten und dem Forschungskreis der Ernährungsindustrie danken wir für die finanzielle Unterstützung unserer Arbeiten.

Heintze (5) teilweise sehr hohe Restmengen von SO_2 enthalten, ebensowenig einbezogen sind wie Erzeugnisse aus Obstpülpen, Obstmark und Citrusmuttersäften. Bei gewohnheitsmäßigem Genuß von Wein liegt die SO_2-Aufnahme wesentlich über dem angegebenen Durchschnittswert.

Die zitierten toxikologischen Untersuchungen wurden jedoch mit anorganischen Verbindungen des SO_2 durchgeführt, während die schweflige Säure in behandelten Nahrungsmitteln und Wein nur zu einem Teil in freier Form vorliegt. Der größere Anteil wird an Aldehyd- und Ketogruppen von Nahrungsmittelinhaltsstoffen gebunden [Jurd (6); Tanner (7); Kieluöfer (8)]. Somit ergibt sich die Frage, ob SO_2 in den Bindungsformen, die in Lebensmitteln vorliegen, die gleichen toxikologischen Eigenschaften hat wie SO_2 aus anorganischen Verbindungen, aus denen es leicht freigesetzt wird.

Dieser Frage sind wir im vorliegenden Versuch nachgegangen. Dabei wurde die Wirkung von Glucose-Natriumhydrogensulfit und Acetaldehyd-Natriumhydrogensulfit, die als Bindungspartner des SO_2 in Nahrungsmitteln bzw. im Wein mengenmäßig größere Bedeutung haben, im Vergleich zur Toxicität von Natriumdisulfit bei vollernährten wachsenden Ratten und bei Tieren im Mangel an B-Vitaminen vornehmlich anhand des Wachstums und der Überlebensrate überprüft.

Versuchsanstellung

Tierhaltung

Als Versuchstiere fanden männliche wachsende Ratten aus einer gewerblichen Zucht* Verwendung. Nach einer kurzen Eingewöhnungsperiode wurden die Tiere in drei große Gruppen unterteilt: Mit einer halbsynthetischen Kost, die an anderer Stelle ausführlich beschrieben ist (3), erhielten die Versuchstiere entweder über den Bedarf hinausgehende Mengen von B-Vitaminen (Versuchsreihe A), oder nur 50% des Bedarfs der verschiedenen B-Vitamine (Versuchsreihe B), oder 80% des Bedarfs der verschiedenen B-Vitamine mit Ausnahme von Thiamin, das in gleichen Mengen wie bei Versuchsreihe A verabreicht wird (Versuchsreihe C). Eine zusätzliche Tiergruppe erhielt „Altromin R", ein handelsübliches Rattenfutter (Versuchsreihe D).

Die Tiere wurden zur Gewöhnung 7 Tage mit den entsprechenden Kostformen gefüttert, wobei in den Versuchsreihen B und C eine Verarmung an B-Vitaminen schon vor Beginn des Hauptversuches eintrat. Zu Beginn des Hauptversuches wurde jede Versuchsreihe in 7 Einzelgruppen aufgeteilt. Die Tiere dieser Einzelgruppen erhielten Glucose-Natriumhydrogensulfit (GNHS), Acetaldehyd-Natriumhydrogensulfit (ANHS) oder Natriumdisulfit ($Na_2S_2O_5$).

Die beiden SO_2-Additionsverbindungen sowie das Natriumdisulfit wurden in zwei verschiedenen Dosierungen, nämlich in Mengen von 50 und 300 mg/kg pro Tag (berechnet auf SO_2), verabreicht. Die verbleibenden Gruppen erhielten als Kontrollen keinen Teststoff. Diese Unterteilung in Einzelgruppen wurde bei den Versuchsreihen A, B und C gleichartig durchgeführt. Reihe D mit Altromin bestand dagegen nur aus zwei Einzelgruppen, von denen die eine als Kontrolle keinen Teststoff, die andere 300 mg pro kg/Tag SO_2 in Form von Natriumdisulfit erhielt. Der Versuch bestand also insgesamt aus 23 verschiedenen Gruppen mit durchschnittlich 10 Tieren pro Gruppe.

Zu Beginn des Hauptversuchs betrug das durchschnittliche Gewicht aller Tiere etwa 80 g (Mangelgruppen 76 g, vollernährte Tiere 90 g). Die Tiere wurden im klimatisierten und gleichmäßig ausgeleuchteten Stall in Einzelkäfigen gehalten. Futter und Trinkwasser wurden *ad libitum* verabreicht, wobei jedoch die Futteraufnahme erfaßt wurde. Die Teststoffmenge wurde nach dem Körpergewicht (auf SO_2 berechnet) alle zwei Tage für jedes Tier einzeln eingewogen. Um einer Zerstörung von Thiamin im Futter durch SO_2 vorzubeugen, wurden die Teststoffe getrennt vom Futter als Mischung von Teststoff, Glucose und Casein verabreicht. Zweimal pro Woche wurden die Tiere gewogen. Als Versuchskriterien wurden Gewichtsentwicklung, Futteraufnahme, Futterverwertung und Todesfälle erfaßt. Tote Tiere wurden seziert, die Lebern für eine Thiaminbestimmung entnommen. Abweichungen in der Verhaltensweise der Tiere wurden registriert. — Die Hauptversuchsdauer betrug 133 Tage.

* Meyer-Arend, Bad Salzuflen.

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Chemische Arbeiten

Die beiden Additionsverbindungen der schwefligen Säure wurden von uns nach den Vorschriften von KERP (9) präparativ aus analysenreinem Kaliumhydrogensulfit und SO_2 unter Verwendung von absolutem Alkohol als Entwässerungsmittel dargestellt. In beiden Verbindungen wurden sofort nach der Herstellung und dann während des Versuchs im Abstand von jeweils 2 Tagen der Gehalt an abspaltbarem SO_2 ermittelt. Zur quantitativen Bestimmung der schwefligen Säure benutzten wir eine von uns modifizierte Methode nach KIELHÖFER (10). Die dabei verwendete Apparatur nach LIEBIGER, wurde während der Destillation mit Stickstoffgas gefüllt, um eine vorzeitige Oxydation von SO_2 zu vermeiden. Zur Freisetzung des Schwefeldioxyds wurde Orthophosphorsäure verwendet. Zur Oxydation diente eine 1%ige H_2O_2-Lösung in der Vorlage. Der Gehalt an abspaltbarem SO_2 lag bei den beiden Verbindungen kurz nach der Herstellung bei 80—85% der theoretisch berechneten Menge, sank aber dann im Laufe der Zeit um 5—10% ab. Durch laufende Kontrolle des SO_2-Gehaltes konnte die auf SO_2 bezogene Einwaage der Testsubstanzen für die Versuchstiere ständig dem sich ändernden Gehalt an schwefliger Säure angepaßt werden.

Ergebnisse

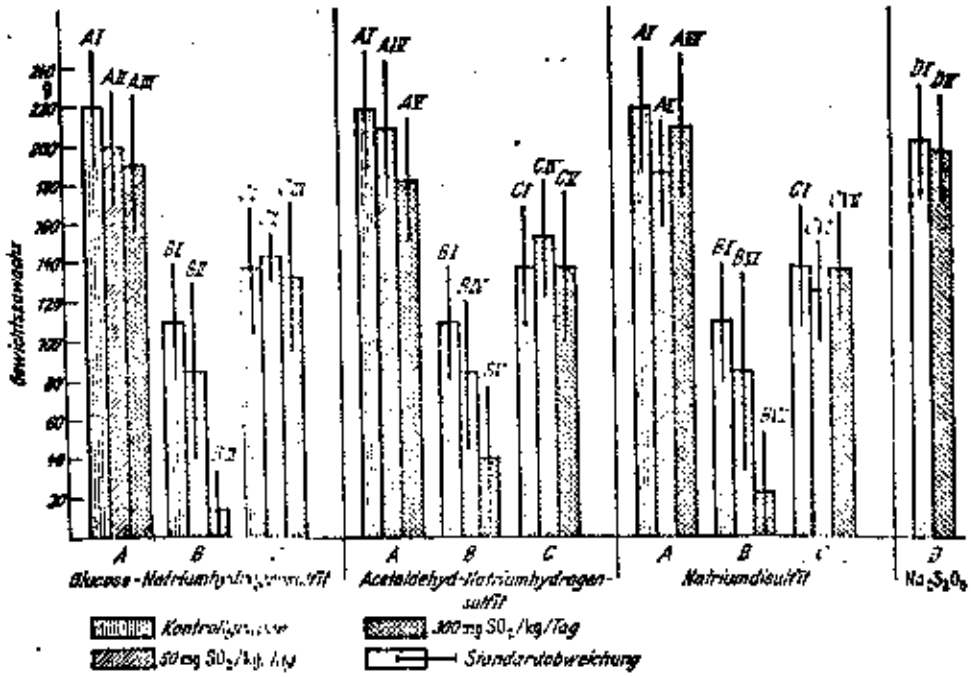

Abb. 1. Toxicität von schwefliger Säure in Abhängigkeit von Bindungsform und Thiaminversorgung

Abb. 1 ist durch senkrechte Striche in vier Spalten unterteilt. In den drei ersten Spalten sind die Werte für die verschiedenen Teststoffe angeführt, in der vierten Spalte die Werte für die beiden Vergleichsgruppen mit Altromin. Innerhalb jeder dieser Spalten sind die Wachstumswerte der vollernährten Tiere, der Tiere mit nur halber Bedarfsdeckung an B-Vitaminen und derer, die dazu noch bedarfsüberstehende Gaben von Thiamin erhalten, gegenübergestellt. Von den nebeneinanderstehenden 3 Gruppen mit gleichem Ernährungszustand erhält jeweils eine kein SO_2, die anderen 50 bzw. 300 mg SO_2 pro kg Körpergewicht pro Tag in Form des jeweiligen Teststoffes. Der Gewichtszuwachs ist als Säule entsprechender Länge dargestellt, wobei die Größe der Standardabweichung gekennzeichnet ist. Die einzelnen Gruppen sind, dem Versuchsaufbau entsprechend, mit Buchstaben Ziffern-Kombinationen gekennzeichnet.

Tabelle. *Wirkung verschiedener Sulfite in Abhängigkeit von der Thaminversorgung*

Vers.-Reihe	Zufuhr von B-Vit.	Teststoff	SO_2-Dosis mg/kg/Tag	Gruppen Nr.	Gewichtszuwachs[3] nach 6 Wochen g	Gewichtszuwachs[3] nach Vers.-Ende g	Todesfälle[1]	Überlebensrate[2]
A	Volle Bedarfsdeckung (halbsynth. Kost)	ohne	—	I	219 ± 31.5	330 ± 71.3	0/7	100.0
		GNHS	50	II	200 ± 28.0	314 ± 50.5	2/9	95.0
			300	III	191 ± 36.7	314 ± 58.5	0/10	100.0
		ANHS	50	IV	211 ± 35.6	327 ± 46.3	0/9	100.0
			300	V	182 ± 35.5	284 ± 69.9	0/11	100.0
		$Na_2S_2O_5$	50	VI	186 ± 27.4	287 ± 39.6	0/8	100.0
			300	VII	209 ± 30.0	324 ± 61.7	0/7	100.0
B	Unterversorgung an allen B-Vit.	ohne	—	I	111 ± 29.2	181 ± 71.4	0/10	100.0
		GNHS	50	II	84 ± 48.9	110 ± 25.42	6/9	73.1
			300	III	13 ± 21.8	—[4]	9/9	35.0
		ANHS	50	IV	83 ± 46.7	159 ± 56.2	5/11	87.7
			300	V	40 ± 37.1	132[5]	9/10	47.8
		$Na_2S_2O_5$	50	VI	84 ± 53.5	170[5]	10/11	72.8
			300	VII	22 ± 31.8	—[4]	11/11	37.3
C	Volle Thaminmenge jedoch Mangel an allen anderen B-Vit.	ohne	—	I	138 ± 33.0	271 ± 43.5	1/12	94.7
		GNHS	50	II	145 ± 11.1	262 ± 20.1	0/8	100.0
			300	III	135 ± 38.1	250 ± 63.7	1/10	97.8
		ANHS	50	IV	154 ± 30.8	275 ± 64.6	0/10	100.0
			300	V	138 ± 40.6	255 ± 69.1	0/9	100.0
		$Na_2S_2O_5$	50	VI	125 ± 26.0	244 ± 44.6	2/11	97.7
			300	VII	136 ± 29.2	244 ± 33.4	1/9	95.1
D	Volle Bedarfsdeckung („Altromin")	ohne	—	I	204 ± 29.2	282 ± 29.7	0/11	100.0
		$Na_2S_2O_5$	300	II	198 ± 29.0	280 ± 46.2	0/8	100.0

GNHS = Glucose-Natriumhydrogensulfit ANHS = Azetaldehyd-Natriumhydrogensulfit.
[1] Todesfälle/Tierzahl pro Gruppe.
[2] Überlebensrate: Anzahl erlebter Versuchstage in Prozent aller Versuchstage.
[3] Mittelwert und Standardabweichung.
[4] Keine Angabe möglich, da alle Tiere gestorben.
[5] Keine Angabe für Standardabweichung möglich, da nur 1 Tier überlebte.

Die statistische Sicherung der Ergebnisse erfolgte nach dem t-Test. Differenzen zwischen Gruppen mit einem p-Wert, der kleiner als 0,05 ist, werden als signifikant angesehen. Signifikante Unterschiede bestehen in der Gewichtsentwicklung nach 6 Wochen zwischen folgenden Gruppen: B I und B III, B II und B III, B I und B V, B IV und B V, B I und B VII, B VI und B VII.

Eine Auswertung von Gewichtsentwicklung und Futterverwertung ist bei den Tieren der Versuchsreihe B nur für die ersten 6 Wochen des Hauptversuchs möglich, weil später zahlreiche Todesfälle bei denjenigen Gruppen auftreten, die eine hohe Teststoffgabe (300 mg SO_2/kg/Tag) erhalten. In Abb. 1 ist daher die Gewichtsentwicklung aller Gruppen nur für die ersten 6 Hauptversuchswochen angegeben und in der Tab. wurde neben dem Ergebnis des Gesamtversuches auch der Gewichtszuwachs nach 6 Wochen angeführt, um einen Vergleich zwischen allen Gruppen zu ermöglichen.

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Es ergibt sich, daß nach 6 Wochen Versuchsdauer die Gabe von SO_2 bei vollernährten Tieren keinen systematischen Einfluß auf die Gewichtsentwicklung hat, unabhängig davon, ob die Tiere eine halbsynthetische Kost oder ein handelsübliches Fertigfutter aus „natürlichen" Kostbestandteilen (Altromin R) erhalten. Das gleiche Ergebnis wird auch mit einer Kost erzielt, die den Thiaminbedarf voll deckt, von allen anderen B-Vitaminen jedoch nur etwa 50% des Bedarfes zuführt. Wird in dieser Kost jedoch auch der Thiamingehalt auf 50% der bedarfsdeckenden Gabe reduziert, so wird durch die höhere Gabe von SO_2 die Gewichtsentwicklung signifikant vermindert, wobei nicht nur zwischen dieser Gruppe und der Kontrolle, sondern auch zwischen den beiden Dosierungen von SO_2 deutliche Unterschiede bestehen. Jedoch tritt kein gesicherter Unterschied in Abhängigkeit davon auf, ob SO_2 durch $Na_2S_2O_5$, GNHS oder ANHS zugeführt wird. Alle drei Verbindungen zeigen also übereinstimmende toxikologische Eigenschaften im Mangel an B-Vitaminen bzw. in Abhängigkeit von der Thiaminversorgung.

Das Ergebnis des Gesamtversuches ist in der Tabelle dargestellt. Es zeigt sich, daß das Zwischenergebnis nach 6 Wochen Versuchsdauer bestätigt wird: Bei den Tieren im Mangel an allen B-Vitaminen (Versuchsreihe B) sind diejenigen mit der höheren SO_2-Gabe, die bereits nach 6 Wochen ein signifikant verringertes Wachstum zeigten, bei Versuchsende mit Ausnahme eines Tieres alle gestorben. Bei den Tieren mit der niedrigeren SO_2-Dosis aus der gleichen Versuchsreihe, die nach 6 Wochen ein nicht signifikant, aber einheitlich verringertes Wachstum aufwiesen, sind die Unterschiede gegenüber der Kontrollgruppe ohne SO_2 nunmehr nach 13 Wochen Versuchsdauer signifikant. Zu Versuchsende nach 19 Wochen ist ein größerer Teil dieser Tiere ebenfalls gestorben, während die Kontrollen überleben.

Im Hinblick auf Todesfälle und Überlebensrate deutet sich ein Unterschied bei den Mangeltieren mit 50 mg SO_2 in Abhängigkeit vom verabreichten Teststoff an: Die Anzahl der Todesfälle steigt in der Reihenfolge ANHS, GNHS, $Na_2S_2O_5$ von etwa 50% gestorbener Tiere auf 90% an. In der Überlebensrate bestehen dagegen keine Unterschiede zwischen GNHS und $Na_2S_2O_5$, nur ANHS ist geringfügig besser. Diese Unterschiede lassen sich jedoch unter den Bedingungen unseres Versuches nicht sichern. Die mittlere Überlebenszeit läuft darüber hinaus mit der Anzahl von Todesfällen in den drei verschiedenen Gruppen nicht parallel.

Bei den Tieren mit voller Bedarfsdeckung an B-Vitaminen (Versuchsreihen A und D) bzw. Mangel an allen B-Vitaminen außer Thiamin (Versuchsreihe C), treten Todesfälle als Folge der Verabreichung von SO_2 in den verschiedenen Bindungsformen nicht auf. Auch in der Gewichtsentwicklung lassen sich zu Versuchsende signifikante Unterschiede bei diesen Tieren ebensowenig nachweisen wie nach einer Versuchsdauer von 6 Wochen.

Diskussion der Ergebnisse

Die Ergebnisse bestätigen unseren früheren Befund, daß die Toxicität des SO_2 im Mangel an B-Vitaminen deutlich höher ist als bei ausreichender Zufuhr [Hötzel (2)]. Dabei hat die Thiaminversorgung den entscheidenden Einfluß. Der Wirkungsmechanismus, der dem Antagonismus zwischen Thiamin und SO_2 zugrunde liegt, läßt sich zwar auf der Grundlage weiterer Befunde diskutieren [Hötzel (3); Cremer u. Hötzel (11)], ist jedoch noch nicht aufgeklärt.

Was die Toxicität der verschiedenen Testsubstanzen anbelangt, war im Falle der Glucose-Additionsverbindung zu erwarten, daß sich ihre Wirkung nicht wesentlich von der des Natriumdisulfit unterscheidet: Die Dissoziationskonstante der Glucosehydrogensulfit-Verbindung liegt nach Pollard, Burroughs und Whiting [zit.

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...wachs[2] g Vers.-Ende	Todesfälle[1]	Überlebensrate[3]
330 ± 71.3	0/7	100.0
314 ± 50.5	2/9	95.0
314 ± 58.5	0/10	100.0
327 ± 40.5	0/9	100.0
284 ± 69.9	0/11	100.0
287 ± 39.6	0/8	100.0
324 ± 61.7	0/7	100.0
181 ± 71.4	0/10	100.0
110 ± 25.42	6/9	73.1
—[4]	9/9	35.0
150 ± 56.2	5/11	87.7
132[5]	0/10	47.8
179[5]	10/11	72.8
—[4]	11/11	37.3
271 ± 43.5	1/12	94.7
262 ± 20.1	0/8	100.0
250 ± 93.7	1/10	97.8
275 ± 64.6	0/10	100.0
255 ± 69.1	0/9	100.0
244 ± 6	2/11	97.7
244 ± 4	1/9	95.1
282 ± 30.7	0/11	100.0
290 ± 46.2	0/8	100.0

Natriumhydrogensulfit.

Versuchstage.

überlebte.

Test. Differenzen zwischen ...nt angesehen. Signifikante ...schen folgenden Gruppen: ... B VII, B VI und B VII.

...erwertung ist bei den ...Hauptversuchs möglich, ...ten, die eine hohe Test- ...e Gewichtsentwicklung ...gegeben und in der Tab. ...Gewichtszuwachs nach ...uppen zu ermöglichen.

nach Tanner (7)] bei pH 3—4 bei $K = 6.4 \times 10^{-1}$. Die Additionsverbindung mit Glucose ist demnach nur wenig stabil. Wie Schanderl (12) in vivo beim Menschen und auch an Modellmagensaft zeigen konnte, wird in Abhängigkeit vom pH-Wert durch Magensaft relativ schnell SO_2 aus den Verbindungen freigesetzt, die im Wein vorliegen. Schanderl führt diesen Befund vor allem auf die hohe Zerfallskonstante der Glucose-SO_2-Additionsverbindung zurück.

Acetaldehyd-Hydrogensulfit ist mit einer Dissoziationskonstante von $2{,}54 \times 10^{-6}$ (bei pH 3,2) wesentlich stabiler als die Glucose-Hydrogensulfitverbindung. Tanner (7) errechnete, „daß die Additionsverbindung der Glucose 150000mal leichter HSO_3-Ionen abgeben kann, als unter gleichen Bedingungen HSO_3-Ionen von der Acetaldehyddisulfitanlagerung frei werden". Es war somit zu erwarten, daß die Acetaldehyd-Hydrogensulfit-Verbindung durch diese Eigenschaft deutlich weniger toxisch wirkt als die Additionsverbindung mit Glucose. Diese Annahme konnte durch das Ergebnis unseres Versuchs jedoch *nicht* bestätigt werden. Wenn überhaupt, dann ist Acetaldehyd-Natriumhydrogensulfit nur geringfügig weniger toxisch als Glucose-Natriumhydrogensulfit oder Natriumdisulfit.

Offenbar kommt es bei der chronischen Toxicität des SO_2 im Thiaminmangel nicht so sehr auf die Schnelligkeit an, mit der SO_2 aus den Additionsverbindungen freigesetzt wird, als vielmehr auf den Anteil, der bei der Magen-Darm-Passage überhaupt frei wird. Nach dem Ergebnis unserer Versuche muß das an Acetaklehyd gebundene Natriumhydrogensulfit bei der Passage von Magen und Dünndarm weitestgehend freigesetzt und dann resorbiert worden sein. Weniger wahrscheinlich ist, daß der Gesamtkomplex — wenigstens teilweise — ungespalten resorbiert und erst im intermediären Stoffwechsel zerlegt wird. Jedenfalls ist in unseren Versuchen mit Ratten bei Verabreichung gleicher Sulfitmengen die Belastung mit SO_2 bei den verschiedenen Testsubstanzen etwa gleich hoch gewesen.

Es ergibt sich nunmehr die Frage, inwieweit die Ergebnisse unserer Versuche mit Modellsubstanzen einen Rückschluß auf die Toxicität des „gebundenen" SO_2 im Wein und in anderen Lebensmitteln erlauben. Kielhöfer (8) und Heintze (13, 14) haben gezeigt, daß im allgemeinen der größere Anteil des gebundenen SO_2 als Acetaldehyd- und Glucose-Additionsverbindung vorliegt. Als weitere Bindungspartner gelten Brenztraubensäure, α-Ketoglutarsäure, L-Xyloson, Arabinose, Galakturonsäure und Xylose u. a. Die Summe der nicht identifizierten Verbindungen wird von Kielhöfer (8) als „Rest-SO_2" bezeichnet.

Die SO_2-Additionsverbindungen mit den angeführten Substanzen haben durchweg eine deutlich höhere Dissoziationskonstante als die Verbindungen mit Acetaldehyd. Der Acetaldehyd-Verbindung mit $K = 1{,}5 \times 10^{-6}$ kommt die Brenztraubensäure-Verbindung mit $K = 4.0 \times 10^{-4}$ (bei pH 3—4) am nächsten. Die anderen genannten Verbindungen haben höhere Zerfallskonstanzen [Pollard u. a., zit. nach Tanner (7)]. Die Substanzen, die den „Rest-SO_2" im wesentlichen ausmachen, haben somit insgesamt höhere Dissoziationskonstanten als die Verbindung mit Acetaldehyd. Bei den bisher nicht identifizierten Verbindungen kann man den gleichen Sachverhalt daraus schließen, daß sie als Bestandteile des „Rest-SO_2" eine ständig fließende Quelle für freies SO_2 darstellen, während die Acetaldehyd-Hydrogensulfit-Verbindung unter gleichen Bedingungen nicht gespalten wird (8).

Man darf damit feststellen, daß die SO_2-Acetaldehyd-Verbindung die stabilste Additionsverbindung unter den Substanzen ist, die als SO_2-Bindungspartner in Lebensmitteln mengenmäßig Bedeutung haben, und somit schlußfolgern, daß die Gesamtheit der SO_2-Additionsverbindungen im Wein keine geringere Toxicität als die Acetaldehyd-Verbindung aufweisen wird.

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Zu unseren Versuchsergebnissen steht nicht im Gegensatz, daß die Acetaldehyd-Verbindung bei Genuß von Wein bei anfälligen Versuchspersonen weniger leicht zu unangenehmen Erscheinungen vom Magen aus (Sodbrennen u. a.) führt, als andere, insbesondere die Glucose-Verbindung. Es ist durchaus möglich, daß der größere Anteil der Acetaldehyd-Hydrogensulfitverbindung, im Gegensatz zu Glucose-Additionsverbindungen, erst im Intestinum, und nicht schon im Magen SO_2 abspaltet. Im Zusammenhang mit der SO_2-Intoxikation ist diese Eigenschaft jedoch wenig bedeutsam, wie sich aus unseren Versuchen ergab.

Zusammenfassung

23 Gruppen wachsender Ratten erhielten mit halbsynthetischen Kostformen ausreichende Mengen von B-Vitaminen, wurden in einen Mangel an B-Vitaminen gebracht oder einer Unterversorgung an allen B-Vitaminen außer Thiamin unterworfen. Bei jedem der genannten Versorgungszustände an B-Vitaminen wurden die Tiere mit zwei verschiedenen Dosierungen von SO_2 belastet, wobei als Testsubstanzen Glucose-Natriumhydrogensulfit, Acetaldehyd-Natriumhydrogensulfit oder Natriumdisulfit Verwendung fanden. Der Versuch dauerte 133 Tage.

Es ergab sich, daß bis zu einer Dosis von 300 mg SO_2/kg Ratte/Tag bei den Tiergruppen mit ausreichender Versorgung an allen B-Vitaminen, und auch bei denen im Mangel an allen B-Vitaminen außer Thiamin, die Gewichtsentwicklung und Futterverwertung nicht signifikant beeinflußt wurden. Todesfälle durch den Einfluß des SO_2 traten nicht auf. Gleiches gilt für solche Tiere, die ein übliches Rattenfutter (Altromin R) mit voller Bedarfsdeckung an allen Nährstoffen erhielten.

Bei den Tieren im Mangel an B-Vitaminen beeinflußte im Gegensatz zu den vorher genannten Tieren bereits eine Gabe von 50 mg SO_2 pro kg Körpergewicht pro Tag Wachstum, Futterverwertung, Zahl der überlebenden Tiere und die Überlebensrate signifikant. Zwischen den beiden Dosierungen bestanden deutliche Unterschiede im Schweregrad der Schädigung. Dabei wirkten Natriumdisulfit und Glucose-Natriumhydrogensulfit in etwa gleicher Weise, die Toxicität von Acetaldehyd-Natriumhydrogensulfit war vielleicht geringfügig kleiner, was jedoch statistisch nicht gesichert werden konnte.

Aus dem Ergebnis des Versuchs wird die Schlußfolgerung gezogen, daß es vom Standpunkt der Toxikologie aus nicht angebracht ist, bei Lebensmitteln, insbesondere bei Wein, zwischen gebundener und freier schwefliger Säure zu unterscheiden. Die verschiedenen Bindungspartner des SO_2, die mengenmäßig in Lebensmitteln eine Rolle spielen, haben etwa die gleichen toxikologischen Eigenschaften wie freie schweflige Säure bzw. ihre Salze.

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werden, daß aus dem Vitamin-B_{12}-Coenzym vor der intravitalen Verteilung sehr schnell das Aquocobamid entsteht oder — weniger wahrscheinlich — das letztere vor seiner Organinkorporation noch in das Vitamin-B_{12}-Coenzym überführt wird.

Es besteht somit keinerlei Veranlassung, das stabile Cyano- oder Aquocobamid in der Prophylaxe und Therapie von Vitamin-B_{12}-Mangelzuständen durch das Vitamin-B_{12}-Coenzym zu ersetzen, da dem Nachteil der geringeren Resorbierbarkeit und Instabilität gegenüber Licht und Cyanid keinerlei Vorteile gegenüberstehen. Zudem ist bisher nicht nachwiesen und auch unwahrscheinlich, daß die B_{12}-Coenzyme „in vivo" nach parenteraler Zufuhr wirksamer als z. B. Aquocobamid oder das unnatürliche Cyanocobamid — bei der Katalyse B_{12}-bedürftiger Stoffwechselreaktionen — sind. Vielmehr dürften die Zellen der B_{12}-bedürftigen ein- und vielzelligen Organismen ohne Schwierigkeiten in der Lage sein, das oral oder parenteral aufgenommene Cyano- oder Aquocobamid nach Inkorporation in die Zelle rasch in die B_{12}-Coenzyme umzuwandeln und als solche zu speichern. Die B_{12}-Coenzyme sind daher vorläufig nur von theoretischem Interesse.

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Einfluß der Versorgung mit B-Vitaminen auf die Toxicität von Schwefeldioxyd

Von

D. Hötzel (Gießen)

Mit 2 Textabbildungen

Bei der Prüfung von Lebensmittelzusatzstoffen auf ihre gesundheitliche Unbedenklichkeit wird den Versuchstieren im allgemeinen eine optimal zusammengesetzte Kost verabreicht, die insbesondere über den Bedarf hinausgehende Vitaminmengen zuführt. Wechselwirkungen zwischen Lebensmittelfremdstoffen und Nährstoffen — gleichgültig ob sie positiv oder negativ zu bewerten sind — können bei dieser Art der Versuchsanstellung nicht sichtbar werden. Auch eine generell größere Empfindlichkeit mangelernährter Tiere kann so nicht erkannt werden.

Diese Feststellung gewinnt an praktischer Bedeutung durch die Tatsache, daß nach Erhebungen von Kraut, Bramsel, Gsell und Stiebeling Teile der Bevölkerung auch in den Industriestaaten von einigen Nährstoffen geringere Mengen aufnehmen, als allgemein empfohlen wird. Und daraus ergibt sich die Frage, ob Lebensmittelfremdstoffe bei diesen nicht optimal versorgten Menschen tatsächlich anwirken, wie es auf Grund der Ergebnisse von Versuchen mit vollernährten Tieren erwartet wird. Dieser Frage sind wir in Tierversuchen nachgegangen.

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Als Mangelfaktor wählten wir die Gruppe der B-Vitamine, weil diese als Bestandteile von Fermenten Bedeutung bei Entgiftung vorgängen haben können und weil Zusatzstoffe auf die Versorgung mit B-Vitaminen einwirken können durch Antivitaminwirkung, durch Zerstörung bzw. Schutz vor Zerstörung und durch Beeinflussung der enteralen Vitaminsynthese. Schließlich kommt Untersuchungen mit B-Vitaminen als Mangelfaktor auch praktische Bedeutung zu, weil, wie schon gesagt, die Versorgung der Bevölkerung mit Thiamin, Riboflavin und gelegentlich auch anderen B-Vitaminen nicht immer optimal ist.

In unseren bisherigen Versuchen über die Wirkung von hohen mittelfremdstoffen in Abhängigkeit von der Versorgung mit B-Vitaminen haben wir sehr unterschiedliche Wirkungsbilder gesehen. Auf Benzoesäure reagierten mangelernährte Tiere in gleicher Weise wie vollernährte, o.-Hydroxybenzoesäureäthylester beeinflußte das Wachstum bei Mangeltieren positiv, bei vollernährten Tieren dagegen negativ. Bei Schwefeldioxyd wiederum reagieren Mangeltiere sehr viel empfindlicher als vollernährte, wie Abb. 1 zeigt.

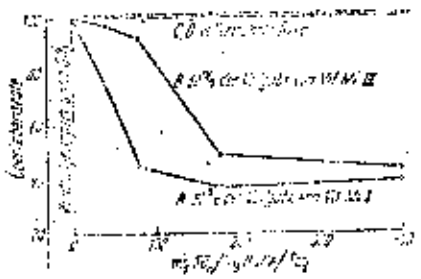

Abb. 1. Die unterschiedliche Wirkung von steigenden Substanzen bei vollernährten und Mangeltieren

Daß die Toxicität von Schwefeldioxyd in so starkem Maße von der Versorgung mit B-Vitaminen abhängig ist, hat unseres Erachtens große praktische Bedeutung, weil unter sehr ungünstigen Bedingungen die Sicherheitsspanne für die Anwendung von Schwefeldioxyd bei Konsumenten mit unzureichender Versorgung mit B-Vitaminen damit recht klein werden kann. Wir sind aus diesem Grunde der Wirkung von Schwefeldioxyd in Abhängigkeit von der Versorgung mit B-Vitaminen weiterhin experimentell nachgegangen.

Zunächst schien es uns interessant zu überprüfen, ob auch bei parenteraler Applikation die Wirkung von Schwefeldioxyd von der Versorgung mit B-Vitaminen abhängig ist. Diesbezügliche Versuche ergaben, daß bei unzureichender Versorgung mit B-Vitaminen die Hälfte der Tiere durch 408 mg SO_2 getötet wurde, während bei vollernährten Tieren dazu 452 mg SO_2 erforderlich waren. In einem zweiten Versuch wurde die DL_{50} für Mangeltiere mit 370 mg, für die vollernährten Kontrollen mit 421 mg SO_2/kg Ratte ermittelt. Ebenso wie bei oraler Gabe ist also auch bei parenteraler Applikation die Toxicität von Schwefeldioxyd bei einem Mangel an B-Vitaminen signifikant größer als bei vollernährten Ratten. Die erhöhte Giftwirkung von SO_2 im B-Mangel kann also nicht — oder doch nur zu einem geringen Teil — durch eine Beeinflussung der enteralen Vitaminsynthese zustande kommen.

Es erschien nun die Frage interessant, ob die Toxicität von SO_2 durch alle Vitamine des B-Komplexes beeinflußt wird oder ob dabei nur

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einzelne B-Vitamine Bedeutung haben. Zur Bearbeitung dieser Frage stellten wir einen weiteren Tierversuch an, in dem zunächst einmal alle Versuchsgruppen eine halbsynthetische Kost mit so geringen Mengen von B-Vitaminen bekamen, daß sie gerade nicht starben. Neben dieser Kost wurde den verschiedenen Versuchsgruppen jeweils ein B-Vitamin in größeren Mengen zugelegt. Gleichzeitig erhielten die Tiere entweder 80 mg oder 180 mg SO_2 pro kg Ratte und Tag. Der Versuch umfaßte 34 Versuchsgruppen und hatte eine Dauer von 154 Tagen. Das Ergebnis ist in der Abb. 2 für die Dosis von 80 mg SO_2/kg Ratte/Tag dargestellt.

Es ergibt sich aus den Darstellungen, daß den stärksten Einfluß auf die Toxicität von SO_2 die Versorgung mit Thiamin hat. Auch Pantothensäure entfaltet einen deutlichen Effekt. Die Wirkung der anderen B-Vitamine ist demgegenüber schon bei 80 mg SO_2 gering und verschwindet mit Erhöhung der SO_2-Dosis vollständig.

Über die Ursache der Wechselwirkungen zwischen Thiamin und SO_2 kann man sich bisher kein rechtes Bild machen. SO_2 wird im Organismus durch Oxydation zu SO_3 entgiftet. Diese Reaktion, die von Sulfitoxydasen bzw. Sulfitoxydasen vermittelt wird, läuft im Organismus ständig als beim Stoffwechsel schwefelhaltiger Aminosäuren. Es ist nun durchaus denkbar, daß die Aktivität des Sulfite oxydierenden Fermentsystems durch einen Mangel an B-Vitaminen reduziert wird. Es kann jedoch nicht erklärt werden, wieso dabei der Thiaminversorgung übergeordnete Bedeutung zukommt. Bisher ist nur bekannt, daß bei der Oxydation der Sulfite Lipoinsäure und Hypoxanthin als Cofaktoren notwendig sind.

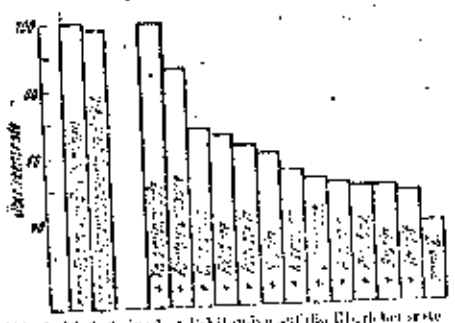

Abb. 2. Einfluß einzelner B-Vitamine auf die Überlebensrate bei Unterversorgung an B-Vitaminen und Belastung mit 80 mg SO_2 pro Tag pro kg Ratte

Es ist daher naheliegender zur Erklärung der Sulfitwirkung anzunehmen, daß durch SO_2-Gaben Thiamin zerstört wird. Es ist bekannt, daß bei pH 5 und Zimmertemperatur Thiamin durch Sulfit im Verlauf mehrerer Stunden nahezu quantitativ zerstört wird. Daß diese Reaktion auch im Organismus ablaufen könnte — wenn auch mit stark verringerter Geschwindigkeit — ließ das Ergebnis der vorhergehenden Versuche vermuten. Um darüber weitere Informationen zu erhalten, prüften wir, ob und in welchem Umfang der Thiaminbestand des Organismus durch SO_2-Gaben verändert wird.

Wie sich zeigte, wird durch Schwefeldioxyd in allen Fällen die Leberthiaminmenge deutlich verringert, was sich physiologisch bei den Mangeltieren naturgemäß sehr viel stärker bemerkbar macht als bei den vollernährten Tieren unseres Versuches, die über den Bedarf weit hinausgehende Thiaminmengen erhielten.

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Bei Anerkennung der Beobachtung, daß Thiamin auch in vitro durch SO_2 zerstört wird, läßt sich die beobachtete partielle Schutzwirkung gegen SO_2 durch Pantothensäure zwanglos erklären. Bei den durch Thiaminmangel gestörten Kohlehydratabbau kann ein Teil der energieliefernden Prozesse kompensatorisch durch Mobilisierung des Fettsäurestoffwechsels ablaufen. Dazu ist vermehrt die Gegenwart von Coenzym A erforderlich, die Kompensation kann also nur dann erfolgen, wenn ausreichende Mengen von Pantothensäure zur Verfügung stehen. — Es wäre zu erwarten gewesen, daß auch die Nicotinsäure einen Einfluß auf die Toxizität von SO_2 hat; SO_2 blockiert spezifisch bestimmte Dehydrogenasen nach Pantoxantin u. Mitarb. Da Niacin Coferment der Dehydrogenasen ist, war anzunehmen, daß beim Zusammentreffen von Niacinmangel und SO_2-Applikation es zu einer starken Veränderung der Aktivität von Dehydrogenasen kommt, die durch Zufuhr höherer Niacinmengen wieder aufgehoben werden kann. Ein derartiger Einfluß des Niacin konnte in unseren Versuchen jedoch nicht in größerem Umfang beobachtet werden, entweder, weil der Einfluß des SO_2 auf Thiamin überwiegt, oder weil bei nicht keimfrei gehaltenen Tieren ausreichende Niacinmengen durch enterale Synthese bereitgestellt werden.

Zusammenfassend darf auf Grund unserer Tierversuche festgestellt werden, daß die Toxizität von SO_2 in sehr starkem Umfang von der Versorgung mit Thiamin, weniger stark von der Pantothensäurezufuhr abhängig ist. Auch für Menschen mit suboptimaler Thiaminversorgung ist SO_2 möglicherweise nicht ganz so unbedenklich, wie sich aus Versuchen mit vollernährten Tieren ergibt. Eine Gefährdung durch SO_2 in Nahrungsmitteln ist mit Sicherheit auszuschließen wegen der nur sehr geringen Mengen, die höchst aufgenommen werden können. Die mitgeteilten Befunde können jedoch Bedeutung haben für die Festlegung der zulässigen Höchstgrenze von SO_2 in Wein bestimmter Herkunftsgebiete, denn es kann nicht ausgeschlossen werden, daß bei Genuß bestimmter Weine in größeren Mengen die zu fordernde Sicherheitsspanne überschritten wird.

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Aussprache

Herr H. Knop (Bonstrup):

Auf Grund meines hier 1957 gezeigten Stoffwechselschemas vermutete die Anreicherung von Anemin bzw. von Anemintrypophosphat in der Zelle eine Aktivierung der Phosphorylierungsvorgänge und Oxydationen durch Erhöhung des alkalischen Potentials, diese Säure muß deshalb diesem Prozeß entgegenwirken und eine sekundäre B_1-Avitaminose hervorrufen. Stare wies 1957 in seinem Referat darauf hin, daß die Carboxylase häufig bedeutend stärker wirke als das meist von den Vitaminherstellern herangezogene Amprolidyldrochlorid, wie es hier verwendet wurde.

Herr H. Kaiser (Dortmund):

Vitamin-B_1-Mangel hat sich in den Versuchen des Herrn Hörzen als ausgezeichnetes Mittel zur Erkennung des Einflusses von SO_2 erwiesen. Könnte man nicht umgekehrt durch Zufuhr von SO_2 Anhaltspunkte für einen geringen Versorgung mit B_1 erhalten?

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THE INTRAVENOUS TOXICITY OF SODIUM BISULFITE

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Received for publication September 15, 1950

The use of sodium bisulfite in solutions which may be given intravenously in relatively large quantities, e.g. protein hydrolysates, requires a knowledge of its systemic toxicity. To date, very little has been published on the intravenous toxicity of sodium bisulfite. Reiss and Gerstl (1946) reported a minimum lethal dose of 1.02 millimols (106 mgm.) of sodium bisulfite per kgm. by intravenous administration to rabbits. These investigators described a reference to the toxicity of sodium bisulfite as follows: "3–4 ml. of a *M*-aqueous solution of sodium bisulphite (3–100 mgm.) given intravenously were lethal for rabbits." As nearly as could be determined, this referred to a review of the pharmacology and toxicology of sulfurous acid (Rost, 1927) which reported that 3 to 4 ml./kgm. of a one per cent solution of sodium bisulfite injected intravenously in rabbits produced death within six to eight minutes. The original source, however, quoted the lethal dose of sodium bisulfite by intravenous injection in rabbits weighing approximately 1.5 kgm. as being 3 to 4 ml. of a solution containing 2.96 per cent SO_2 when given at a rate of 1 ml. per minute (Rost and Franz, 1904). A lethal dose range of 96 to 128 mgm./kgm. might be calculated from the latter data which values do not agree with the "3–400 mgm." cited above.

Fitzhugh, Knudsen and Nelson (1946) made an extensive study of the chronic oral toxicity of sulfites and found that concentrations of 0.1 per cent (615 p.p.m. as SO_2) or more in the diet were toxic to rats. However, these authors considered that much of the stunting of growth, clinical polyneuritis, "spectacle" eyes and various pathological changes observed in the rats were manifestations of vitamin deficiency, particularly of thiamine which is readily destroyed by sulfite. Although several reports have appeared describing the effect of sodium bisulfite on the toxicity of various pressor amine and local anesthetic solutions, these investigations were primarily concerned with the local rather than the systemic toxic effects of sodium bisulfite, *per se* (Tainter, *et al.*, 1937; Tainter, *et al.*, 1941; Richards, 1943a,b; Richards and Kueter, 1943; Richards and Kueter, 1948).

The present communication reports the results obtained in a toxicologic investigation of sodium bisulfite by intravenous injection in experimental animals in single and in repeated doses. Particular reference is made to the systemic effects of sodium bisulfite in distilled water and in an acid hydrolysate of casein (Parenamine[1]) when given by this route of administration.

METHODS. *Acute Toxicity.* The acute toxicity of sodium bisulfite in solution in distilled water was determined by intravenous injection in male, albino Webster strain mice (22 ± 2 gm.); male, Sherman strain rats (100 ± 10 gm.); golden Syrian hamsters (90 ± 10 gm.);

[1] Parenamine is the registered trade-mark of Winthrop-Stearns Inc., for protein hydrolysates for intravenous administration.

and rabbits of mixed chinchilla stock (2.5 ± 0.5 kgm.). A comparison of the acute intravenous toxicity of sodium bisulfite in distilled water and in 6 per cent Parenamine was made in mice. The acute toxicity of sodium sulfite, sodium acetone-bisulfite and sodium sulfate in aqueous solution also was determined by intravenous injection in mice for comparative purposes. The solutions prepared for injection were used within an hour of the time of preparation. The volume injected was restricted to 0.35 ± 0.15 ml. for all species except the rabbit for which a volume of 1.0 ml./kgm. was employed. The rate of injection in each instance was 1.0 ml./minute. The animals were housed in a well-lighted and ventilated room maintained at 24.5° C. and a nutritionally adequate diet and water were available at all times. The test animals were observed for acute lethal effects for several hours following injection and the LD_{50} ± its standard error (Miller and Tainter, 1944) was calculated at the end of 24 hours. Observation was continued for a period of seven days after injection for any delayed toxic manifestations.

Subacute Toxicity Test in Rabbits. Since the acute intravenous LD_{50} for sodium bisulfite was found to be approximately 60 mgm./kgm. in rabbits, three dose levels of 0.5, 1.0 and 2.0 times this value were selected, representing a total daily dosage of 30, 60 and 120 mgm./kgm. These quantities were divided into three equal parts and given intravenously three times daily, five days a week for three weeks. Each injection, equivalent to 10, 20 and 40 mgm./kgm., respectively, was given in a volume of 2 ml./kgm. of body weight at a rate of 4 ml./min. The concentrations of each of the solutions, therefore, were 0.5, 1.0 and 2.0 per cent of sodium bisulfite, respectively.

Sodium bisulfite was administered in distilled water at the above dose levels to one set of three groups of three rabbits each while a second set of three groups of three rabbits each received sodium bisulfite at the same dose levels in 6 per cent Parenamine. Two rabbits received 2.0 ml./kgm. of normal saline as a control for the first set and an additional two rabbits received a like volume of 6 per cent Parenamine without sodium bisulfite as a control for the second set. After the third day of the test the aqueous solutions were prepared daily. Sufficient amounts of the sodium bisulfite in Parenamine for the entire experiment were prepared and stored in sterile, rubber-capped vials. These solutions were kept under refrigeration and each day's supply of vials was brought to room temperature over night before injection. The rabbits were weighed three times a week and the total volume of injection adjusted after each weighing. The rabbits at the highest dose levels and the controls were sacrificed for gross and histopathologic examination at the end of the experiments.

Results. *Acute Toxicity.* These results are summarized in table 1. The intravenous LD_{50} for sodium bisulfite, both in distilled water and in Parenamine, was found to be 130 mgm./kgm. in mice. Sodium bisulfite appeared to be twice as toxic in rabbits while the susceptibility of rats and hamsters seemed to be intermediate. In mice, sodium sulfite and sodium acetone-bisulfite both were significantly less toxic than sodium bisulfite, and sodium sulfate was only one-tenth as toxic as sodium bisulfite.

Symptoms preceding death were the same for each of these substances. Respiratory depression became apparent almost immediately after injection of lethal doses. Terminal clonic convulsions and death following respiratory arrest within ten to twenty minutes after injection were observed with each of the species studied. There were no delayed deaths.

Subacute Toxicity. There were no deaths during the experiment and all of the rabbits gained weight as shown in table 2. There were no symptoms of either acute or cumulative toxicity observed in any of the rabbits. The only obvious difference noted between the two test groups was the marked irritation resulting in thrombosis of the ear veins of the rabbits receiving sodium bisulfite in distilled

water. Within a few hours after injection, the vein became occluded at the site of injection with formation of a thrombus which often extended for 2 to 3 cm. along the course of the vein. The thrombus became very brittle in 12 to 24 hours and usually began to soften by the following day. Frequently, these thrombi were observed to dislodge and enter the circulatory system without causing any

TABLE 1

Acute intravenous toxicity

SUBSTANCE	SPECIES	NUMBER OF ANIMALS	LD_{50} ± S.E.
			mgm./kgm.
Sodium Bisulfite	Mouse	30	130 ± 8
		30	130 ± 10*
	Rat	30	115 ± 10
	Hamster	30	95 ± 6
	Rabbit	15	65
Sodium Sulfite	Mouse	30	175 ± 8
Sodium Acetone-Bisulfite	Mouse	30	160 ± 10
Sodium Sulfate	Mouse	30	1220 ± 90

* Comparative assay of acute intravenous toxicity of $NaHSO_3$ in 6 per cent Parenamine vs. $NaHSO_3$ in distilled water.

TABLE 2

Subacute intravenous toxicity of sodium bisulfite administerd in divided doses 3 times/day, 5 days/week for 3 weeks in rabbits for a total of 45 injections

TOTAL DAILY DOSE	AVERAGE TOTAL OF SOLUTION INJECTED	MORTALITY AFTER 21 DAYS	INITIAL MEAN BODY WEIGHT	GAIN IN WEIGHT	
mgm./kgm.	*ml.*		*kgm.*	*kgm.*	*per cent*
Sodium Bisulfite in Distilled Water					
30	205	0/3	2.20	0.22	10
60	196	0/3	2.06	0.47	23
120	177	0/3	1.81	0.37	20
Control (Normal Saline)	203	0/2	2.28	0.09	4
Sodium Bisulfite in 6% Parenamine					
30	178	0/3	1.90	0.16	8
60	191	0/3	1.93	0.42	22
120	198	0/3	2.06	0.30	14
Control (6% Parenamine)	175	0/2	1.78	0.50	28

apparent effect on the rabbit. Solutions of sodium bisulfite in protein hydrolysate, however, did not produce any evidence of irritation. The marginal ear veins of the rabbits receiving sodium bisulfite in Parenamine were used alternately throughout the experiment without evidence of thrombus formation or any apparent injury other than slight cicatrix formation at the sites of repeated injection. No evidence of irritation was observed in the control groups.

The pH of the aqueous bisulfite solutions was observed to decrease from a pH

of 6 to approximately 3 after 72 hours while no significant change in the pH of the Parenamine bisulfite solutions (pH 5.7) was noted during the three-week test period. The odor of SO_2 became increasingly apparent as the aqueous bisulfite solutions increased in acidity. Only by preparing the aqueous solutions fresh each day and alternating the injections among the available superficial ear veins was it possible to finish the complete schedule of 45 injections. Even though these solutions were prepared fresh daily, they were still found to be irritating to the intima of the ear veins of the rabbits.

Since sodium bisulfite in aqueous solution decomposes upon exposure in air to form the acidic salt, sodium bisulfate, which in turn attacks unreacted sodium bisulfite to cause the formation of free SO_2, it would seem that the irritation might be ascribed either to acidity or to a combination of acidity plus free SO_2. Additional experiments disclosed that a volume of 2.0 ml./kgm. of a two per cent aqueous solution of sodium bisulfate (no SO_2 present) adjusted to a pH of 3.0 when injected three times daily into the right marginal ear veins of rabbits for a total of six injections failed to produce any evidence of stasis or thrombosis. In contrast to this, thrombi developed within a few hours after the second injection of a two per cent aqueous solution of sodium bisulfite which on standing had reached a pH of 3.0 and smelled strongly of SO_2.

At the end of the 21-day test the following rabbits were sacrificed: three which received 120 mgm./kgm./day of sodium bisulfite in distilled water, three which received 120 mgm./kgm./day of sodium bisulfite in Parenamine and three controls, one of which received 2 ml./kgm. of normal saline three times daily, the other two 2 ml./kgm. of 6 per cent Parenamine solution three times daily without sodium bisulfite. No gross abnormalities were found in the viscera at autopsy and histopathological examination of the heart, lungs, liver, spleen and kidneys revealed no microscopic changes attributable to the intravenous administration of sodium bisulfite. Insignificant concurrent changes, such as mild granular cylindruria and focal tubular nephritis, were encountered both in the controls and in the animals which received the sodium bisulfite.

Discussion. The acute intravenous toxicity of sodium bisulfite appeared to be rather sharply defined as judged by the magnitude of the standard errors obtained in the acute studies. The deaths occurred either immediately following injection of relatively large doses or not at all and appeared to be a result of respiratory failure. There were no apparent residual toxic effects at sublethal doses. It was interesting to note that while the acute LD_{50} for rabbits was 65 mgm./kgm., nearly double this amount could be given daily in divided doses five days a week for three weeks without any apparent evidence of either acute or cumulative toxic effects. While the acute intravenous toxicity of sodium bisulfite in rabbits appears to be greater than that reported by Rost and Franz (1904) and Reiss and Gerstl (1946), the difference may be due to the fact that the latter investigators have reported their results in terms of the lethal dose or LD_{100} as contrasted with the use of the LD_{50} value in the present communication.

The irritation phenomena observed in the rabbits receiving the aqueous bisulfite solutions appeared to be associated with the presence of free SO_2 in the

solutions. Although the aqueous bisulfite solutions became quite acid upon standing and consequently more irritating, the pH factor alone did not seem to account for the irritation seen. Thrombi quickly developed upon the intravenous injection of an aqueous solution of sodium bisulfite which had a pH of 3.0 and smelled strongly of SO_2. The absence of irritation in the rabbits receiving the Parenamine bisulfite solutions may have been due to the fact that these solutions were stored in sealed vials and hence the exposure to air and opportunity for decomposition to occur was less than with the aqueous solutions. It is possible that some decomposition did occur but the amino acids may have served to buffer the solutions so that any acid break-down products were not detectable by either pH measurement or irritation phenomena. The sodium bisulfite dissolved in Parenamine, however, was apparently capable of operating independently as a toxic agent since identical acute toxicity values were obtained in mice with solutions both in distilled water and in the hydrolyzed protein solution. Tainter *et al.* (1941) likewise observed an increase in the acidity of aqueous sodium bisulfite solutions. These investigators reported a marked increase in the incidence of trismus and of postoperative swelling at the site of injection after the use of local anesthetic solutions containing sodium bisulfite during dental surgery.

SUMMARY

1. The comparative acute intravenous toxicity in mice of sodium bisulfite in distilled water and in 6 per cent protein hydrolysate was found to be 130 ± 8 and 130 ± 10 mgm./kgm., respectively.

2. Sodium bisulfite appeared to be twice as toxic for rabbits as for mice while the susceptibility of rats and hamsters was intermediate between these two species.

3. Two-thirds of the approximate LD_{50} for rabbits was injected intravenously three times daily, five days a week for three weeks without apparent toxic effect.

4. Aqueous solutions of sodium bisulfite rapidly increased in acidity and acquired a strong odor of SO_2 on standing whereas no significant change in pH was observed with similar concentrations in protein hydrolysate over a period of three weeks.

5. Severe vascular irritation resulting in thrombus formation was observed with aqueous solutions of sodium bisulfite in rabbits while the protein hydrolysate bisulfite solutions were found to be non-irritating.

6. No evidence of cumulative toxicity by intravenous injection of sodium bisulfite, either in distilled water or in protein hydrolysate, was observed in a subacute toxicity study in rabbits.

7. Gross and microscopic pathological examination of the heart, lungs, liver, spleen and kidneys revealed no tissue changes attributable to the intravenous administration of sodium bisulfite to rabbits at total daily doses up to 120 mgm./kgm., five days a week, for three weeks.

ACKNOWLEDGEMENT: The authors gratefully acknowledge the technical assistance of Miss Christine Eddison, Mr. C. George, Mr. John Romano and Mr. D.

K. Seppolin. The authors also wish to express their grateful appreciation to Dr. Alex Lesuk for technical advice on the chemistry of sodium bisulfite in aqueous solution.

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Acta. Biol. anim., Biochi., Biophys., 1965, 5 (1), 53-59

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EFFETS DE L'INGESTION DE SULFITES SUR L'EXCRÉTION DU CALCIUM CHEZ LE RAT

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SOMMAIRE

Chez les jeunes rats soumis à un régime alimentaire moyennement riche en calcium (0,5 p. 100 sous forme de carbonate), l'introduction de 0,5 ou 1 p. 100 de métabisulfite de potassium dans le régime ne modifie pas sensiblement l'excrétion calcique fécale, mais entraîne un accroissement significatif de l'excrétion urinaire.

Inversement, chez des animaux dont le régime est riche en calcium (1 p. 100 sous forme de carbonate), l'administration de métabisulfite de potassium dans les conditions indiquées précédemment provoque une augmentation significative de l'excrétion calcique fécale, mais cette sans effet sur l'excrétion urinaire.

Ces résultats, dont la signification est discutée, attirent l'attention sur la nécessité de s'informer des répercussions éventuelles de l'ingestion des sulfites utilisés en technologie alimentaire comme agents de conservation, sur les modalités de l'excrétion du calcium chez l'Homme.

Parmi les problèmes d'ordre nutritionnel que pose l'emploi des sulfites en technologie alimentaire [1], les effets éventuels de ces agents sur le métabolisme du calcium ne paraissent pas avoir retenu l'attention jusqu'ici.

La question mérite pourtant d'être examinée. En effet, les sulfites ingérés peuvent être transformés rapidement en sulfates (SOLLMANN, 1957 ; LEHMAN, 1957), substances qui, dans certaines conditions, accroissent les pertes calciques de l'organisme :

1° Comme l'a montré KOWARSKI (1958), l'administration orale de sulfate de sodium peut entraîner, chez l'Homme, une augmentation de l'élimination fécale du calcium, augmentation qui serait due à la formation, dans le tube digestif, de sulfate de calcium peu ionisé et difficilement absorbable. Cette propriété a été mise à profit

(1) Au sujet de cet emploi, voir CHZAN, CAUSERET et HUGOT (1963).

par l'auteur et par divers autres (COUTEL et *al.*, 1962 ; LESTRADET, 1962) pour lutter contre un passage digestif excessif du calcium chez certains malades.

Dans les affections qui entraînent une élévation du taux des sulfates sériques (néphropathie chronique, anurie), les sulfates endogènes entraînés par les sécrétions digestives paraissent susceptibles de jouer le rôle du sulfate de sodium administré *per os*, leur effet se traduisant quelquefois par un bilan calcique négatif (LESTRADET, et *al.*, 1962).

2° Dans certains cas, l'ion SO_4 accroît l'élimination rénale du calcium. Chez le Rat, l'administration de sulfate de magnésium provoque ce phénomène, alors qu'il ne se produit pas avec d'autres sels magnésiens comme le carbonate, le chlorure ou le citrate (RANDOIN et *al.*, 1952) ; d'autre part, l'ingestion de sulfate de calcium provoque une perte calcique urinaire beaucoup plus élevée que celle qu'entraînent d'autres sels de calcium (carbonate, lactate, phosphate tricalcique) (CAUSERET et HUGOT, 1958).

De même, chez l'Homme, l'administration intraveineuse de sulfate de sodium accroît fortement l'élimination du calcium par le rein, cette élimination étant multipliée par 20 et même par 40 dans certaines conditions de perfusion (LESTRADET, 1962).

Il nous a donc paru utile de chercher si l'ingestion de sulfites ne pourrait pas entraîner des effets similaires.

MATÉRIEL ET MÉTHODES

1. *Animaux et régimes alimentaires*

L'expérience a porté sur 42 rats *Wistar* du sexe mâle, provenant de l'élevage du Laboratoire. Ils étaient âgés initialement de 30 jours et pesaient de 60 à 75 grammes.

Ces animaux ont été répartis en 6 lots qui ont été soumis à des régimes alimentaires dérivant tous du même mélange de base :

Caséine lavée à l'alcool	18
Huile d'arachide	9
Amidon de froment	45
Saccharose	24
Mélange salin de Hubbard sans CO_3Ca	2
Agar-agar	2

Pour chaque lot, des quantités déterminées de calcium (sous forme de carbonate) et, dans certains cas, de métabisulfite de potassium (1), ont été substituées, dans le mélange précédent, à des quantités d'amidon égales (tableau 1).

La dose de calcium la plus faible (0,5 g. 100 du régime) peut être considérée comme normale pour le Rat ; l'autre (1 g. 100) est forte. Le choix de ces deux doses est dû au fait que les conclusions d'études comparatives du genre de celle qui fait l'objet de ce travail dépendent quelquefois du niveau d'ingestion du calcium (CAUSERET et HUGOT, 1959 et 1960).

Le choix des doses de métabisulfite utilisées a été basé sur les données suivantes :

— ces doses peuvent être considérées comme non-toxiques à court terme pour le Rat ;

— les trois valeurs expérimentales du rapport $\frac{SO_4 \text{ ingéré}}{Ca \text{ ingéré}}$ (0,29 pour le lot V ; 0,58 pour les lots

(1) Avec le sulfite de sodium, le métabisulfite de potassium est le sulfite le plus employé en Industrie comme agent de conservation. Il fournit une quantité de SO_2 égale à 0,58 g. 100 de son poids.

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II et VI ; 1,15 pour le lot III) peuvent être obtenus dans l'alimentation de l'Homme ; la quantité de calcium ingéré étant supposée normale (environ 800 mg par jour), ces valeurs correspondraient à une consommation de vin — sulfité à la dose licite maximum de 450 mg par litre — égale respectivement à un demi-litre, un litre ou deux litres.

TABLEAU I

Teneur des régimes en calcium et en métabisulfite de potassium

Lots	Calcium (g. 100 du régime)	Métabisulfite de K (g. 100 du régime)	Rapport SO_2 Ca
I	0,5	0	0
II	0,5	0,5	0,58
III	0,5	1,0	1,15
IV	1,0	0	0
V	1,0	0,5	0,29
VI	1,0	1,0	0,58

Les vitamines ont été introduites dans les six régimes aux doses suivantes, pour 100 g : thiamine 0,20 mg ; riboflavine 0,30 mg ; pyridoxine 0,25 mg ; acide nicotinique 5 mg ; pantothénate de calcium 1 mg ; vitamine A 100 U I ; vitamine D_2 500 U I ; α-tocophérol 1,5 mg ; vitamine K hydrosoluble 0,1 mg.

Les régimes ont été distribués sous forme de bouillie préparée à partir de 2 parties de mélange sec pour 1 partie d'eau distillée.

2. *Déroulement de l'expérience*

Après une période d'adaptation de 3 jours, les fèces et les urines des animaux ont été recueillies durant 10 jours.

Le dispositif expérimental utilisé a été décrit dans une publication antérieure (CAUSERET, 1954).

3. *Méthode de dosage et calcul des résultats*

Le calcium a été dosé dans les régimes et dans les excreta par complexométrie, au moyen du complexon IV et en utilisant la calcéine comme indicateur (MORT, 1950). Le dosage était effectué directement sur les urines. Les régimes déshydratés, ainsi que les fèces déshydratées et pulvérisées, étaient préalablement incinérés à 550°C pendant 12 heures.

Les modalités de l'excrétion du calcium ont été déterminées par calcul des deux rapports :

$$\frac{\text{Ca fécal}}{\text{Ca ingéré}} \times 100 \qquad \frac{\text{Ca urinaire}}{\text{Ca ingéré}} \times 100$$

RÉSULTATS

Les résultats obtenus sont réunis dans le tableau 2.

On voit que, chez les animaux dont le régime renferme 0,5 p. 100 de calcium, l'administration de métabisulfite de potassium ne modifie pas sensiblement l'excrétion fécale du calcium, alors qu'elle entraîne une augmentation significative de l'excrétion urinaire (au seuil de $P = 0.01$ pour le lot II et de $P = 0.05$ pour le lot III).

TABLEAU 2. — *Résultats expérimentaux*

Lots	Numéro des rats	Ca ingéré (mg/jour)	Ca fécal (mg/jour)	Ca urinaire (mg/jour)	$\frac{\text{Ca fécal}}{\text{Ca ingéré}} \times 100$	$\frac{\text{Ca urinaire}}{\text{Ca ingéré}} \times 100$
I	1	55,1	24,3	2,0	44,1	3,6
	2	50,0	20,6	1,5	41,0	2,5
	3	62,4	29,5	1,75	47,2	2,8
	4	53,2	28,9	1,3	52,3	2,3
	5	54,6	20,0	1,0	36,6	1,8
	6	60,6	22,7	2,0	37,5	3,3
	7	55,0	23,2	1,5	42,2	2,7
	8	52,8	18,3	1,05	34,6	2,0
	Moyennes				*41,2 ± 2,3*	*2,6 ± 0,2*
II	9	58,1	21,9	1,05	45,5	2,8
	10	51,2	16,1	1,5	30,2	3,0
	11	57,1	27,9	2,5	48,8	4,4
	12	57,1	24,3	2,5	51,3	5,3
	13	50,7	20,1	4,7	44,6	7,9
	14	45,5	25,5	1,7	52,6	4,0
	15	55,4	22,0	3,7	39,7	6,7
	Moyennes				*45,9 ± 2,0*	*4,9 ± 0,7*
III	16	63,7	27,9	2,1	42,8	3,3
	17	55,9	23,1	3,55	41,7	6,2
	18	42,9	16,0	1,0	37,3	2,3
	19	63,5	19,2	2,1	30,1	3,3
	20	58,4	25,3	2,6	43,3	4,4
	21	54,3	19,6	2,8	36,2	4,8
	22	52,0	20,1	1,5	38,6	2,9
	23	52,7	25,8	1,75	49,0	3,3
	24	57,4	25,7	1,6	44,8	2,8
	Moyennes				*40,1 ± 1,9*	*3,7 ± 0,4*
IV	25	117,4	76,7	9,2	65,3	7,8
	26	123,6	78,6	8,0	63,6	6,5
	27	113,9	67,7	6,15	59,4	5,4
	28	108,4	62,0	12,25	57,2	10,4
	29	116,8	70,1	11,0	60,3	9,2
	30	85,1	53,8	9,0	63,1	10,5
	31	101,9	60,2	10,2	59,1	10,0
	32	84,4	60,8	4,3	72,0	5,1
	Moyennes				*62,8 ± 1,6*	*8,2 ± 0,8*
V	33	98,3	68,7	7,4	69,9	7,5
	34	102,5	73,7	9,8	71,8	9,6
	35	99,2	77,4	9,3	78,0	9,4
	36	95,0	75,7	6,1	80,5	6,5
	37	102,4	71,4	9,1	69,7	8,9
	38	93,4	73,2	6,1	78,4	6,6
	39	77,5	49,1	6,7	63,3	8,7
	40	82,2	60,6	5,8	73,7	7,0
	Moyennes				*73,2 ± 2,0*	*8,0 ± 0,1*
VI	41	101,8	70,6	7,7	69,4	7,6
	42	105,8	84,5	6,2	79,9	5,9
	43	84,2	62,3	9,3	73,9	11,0
	44	89,7	54,9	7,7	68,0	9,6
	45	64,1	41,9	6,0	65,4	9,4
	46	97,5	71,4	5,9	73,2	6,0
	47	81,2	54,4	4,5	66,6	5,5
	48	100,6	73,6	3,9	67,2	2,7
	49	102,8	64,9	3,6	63,1	3,5
	Moyennes				*69,6 ± 1,7*	*6,8 ± 1,1*

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Inversement chez les animaux dont le régime renferme 1 p. 100 de calcium, le sulfite accroît significativement l'excrétion fécale du calcium (au seuil de $P = 0.01$ pour le lot V et de $P = 0.02$ pour le lot VI), mais il reste sans effet sur l'excrétion urinaire.

Enfin, quel que soit l'apport alimentaire de calcium, l'excrétion calcique — fécale et urinaire — n'est pas modifiée significativement lorsque la teneur du régime en métasulfite de potassium passe de 0,5 p. 100 à 1 p. 100.

DISCUSSION

Avant de tenter d'expliquer la nature du lien observé entre la valeur de l'apport calcique et les effets de l'ingestion de sulfite sur les modalités de l'excrétion du calcium, il n'est pas inutile de rappeler que nous avons déjà signalé des liens du même genre. Ainsi :

— lorsque le taux de calcium du régime est peu élevé, la substitution de carbonate de calcium au lait dans ce régime ne modifie pas l'excrétion calcique du Rat ; mais lorsque l'apport calcique est élevé, cette même substitution accroît l'excrétion fécale du calcium et diminue l'excrétion urinaire (CAUSERET et HUGOT, 1960) ;

— lorsque le taux de calcium du régime est peu élevé, la substitution d'oxalate de calcium au carbonate dans ce régime accroît l'excrétion fécale du calcium sans modifier l'excrétion urinaire ; mais lorsque l'apport calcique est élevé, cette même substitution augmente à la fois l'excrétion fécale et l'excrétion urinaire (CAUSERET et HUGOT, 1961).

Dans le cas présent, tout se passe comme si le sulfite n'entravait l'absorption digestive du calcium que dans la mesure où l'apport calcique est excédentaire. Cette constatation s'accorde avec le fait que, chez le Rat en croissance, soumis à un régime de taux calcique « normal », l'ion SO_4 n'accroît pas l'élimination fécale du calcium (RANDOIN et *al.*, 1952 ; CAUSERET et HUGOT, 1958). Rappelons aussi que le tube digestif du Rat absorbe assez activement le calcium d'un sel peu ionisé et réputé difficilement absorbable comme l'oxalate, pourvu que le régime soit peu riche en calcium ; si l'apport calcique est élevé, la fraction du calcium alimentaire non absorbée augmente (CAUSERET et HUGOT, 1961).

Il est plus difficile d'expliquer que l'accroissement de l'excrétion urinaire du calcium sous l'influence de sulfite ne se manifeste qu'avec le régime à 0,5 p. 100 de calcium. Puisque, chez le Rat, le rein élimine difficilement le calcium, il est possible qu'un « plafond » de l'excrétion soit déjà atteint chez les sujets du lot IV, l'introduction de sulfite dans les régimes des lots V et VI ne pouvant donc plus l'augmenter encore.

L'existence d'un tel plafond conduit d'ailleurs à se demander si l'accroissement de l'excrétion de calcium fécale chez les animaux des lots V et VI est bien dû à une diminution de l'absorption digestive. Il se pourrait en effet que l'influence des sulfites sur le métabolisme du calcium s'exerce au seul niveau de la rétention, les sujets des lots V et VI rejetant dans l'intestin l'excès de calcium absorbé, parce que le rein

ne peut plus l'éliminer. Il serait intéressant d'étudier la valeur de cette hypothèse en effectuant des bilans calciques avec dilution isotopique (^{45}Ca).

Enfin, on peut s'étonner que les effets du métabisulfite de potassium sur l'excrétion calcique ne s'accroissent pas lorsque le taux de ce corps dans le régime passe de 0,5 p. 100 (lots II et V) à 1 p. 100 (lots III et VII. Toutefois, s'il est possible que les sulfites agissent sur l'excrétion du calcium par l'intermédiaire des sulfates auxquels ils donnent naissance *in vivo*, on ne doit pas perdre de vue que la possibilité qu'a l'organisme d'oxyder les sulfites est limitée : il n'est donc pas à exclure que la capacité d'oxydation maximum soit atteinte chez les animaux des lots II et V dont le régime est déjà relativement riche en sulfites.

Quoi qu'il en soit, il serait injustifié de tirer de notre travail des conclusions applicables avec certitude à l'alimentation humaine. On sait en effet que les modalités du transit du calcium dans l'organisme ne sont pas les mêmes chez le Rat et chez l'Homme. En particulier, chez ce dernier, l'absorption digestive du calcium paraît moins complète et l'excrétion du calcium par le rein représente une fraction de l'excrétion totale beaucoup plus élevée. Nous nous bornerons donc à conclure que les effets des sulfites observés chez le Rat rendent nécessaire une expérimentation sur l'Homme lui-même.

Reçu pour publication en novembre 1964.

SUMMARY

EFFECT OF INGESTION OF SULPHITES ON EXCRETION OF CALCIUM BY RATS

Ingested sulphites can be rapidly changed to sulphates within the organism. Many workers have shown, in man and rat, that when sulphates are given then can increase the amount of calcium excreted in faeces and urine. The authors therefore studied whether sulphites, substances widely used as preservatives in food technology, had a similar effect on the elimination of calcium.

Forty-nine young rats received balanced diets with 0,5 or 1,0 p. 100 calcium, as carbonate, and 0,5 or 1,0 p. 100 potassium metabisulphite was added to some diets. Excreta were collected during 10 days.

In the rats given the diet with 0,5 p. 100 calcium, 0,5 p. 100 potassium metabisulphite did not affect excretion of calcium in faeces but it caused a significant increase of the excretion in urine.

Inversely, in the animals given 1,0 p. 100 calcium in the diet, metabisulphite given in the conditions already described caused a significant increase in excretion of calcium in faeces but had no effect on the excretion in urine.

The significance of the results is discussed. They draw attention to the need for study of the eventual effect that ingestion of sulphites may have on excretion of calcium by man.

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J. Dent. Res. 31 (1952)

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SYMPTOMS OF VITAMIN A AND E DEFICIENCIES IN THE INCISOR OF THE RAT FED SODIUM SULFITE

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FOR many years sulfites have been used for food preservation. During World War II when troops were serving in tropical climates it became necessary to sulfurize dried fruit to a much higher extent than heretofore, and the question of possible toxic effects became important.

Fitzhugh, Knudsen and Nelson[3] studied the effect on rats of diets containing sulfites. They observed polyneuritis, focal myocardial necrosis and fibrosis, which they interpreted as being caused by a thiamine deficiency. The animals furthermore developed "spectacle eyes," brown uteri, atrophy of various viscera, and changes in the kidneys and bones. The changes were complex and were discussed in some detail.

During the course of these experiments, it was noticed that the incisor teeth, normally orange in color, gradually became white. This decoloration is itself of considerable interest, because it has been shown that it can be produced to a varying degree by several different experimental procedures.

A further stimulus to the present investigation was the question whether the lack of pigmentation is accompanied by histologic changes of the incisor.

MATERIAL AND METHODS

This investigation is based on the gross anatomic, roentgenologic and histologic examination of the teeth and adjacent structures of 43 of the rats used by Fitzhugh, Knudsen and Nelson[3] (Table I). At 21 days of age, weaning time, these Osborne-Mendel rats had varying amounts of sodium bisulfite ($NaHSO_3$) added to a basic diet consisting of:

Cornstarch	60 per cent	Casein	18 per cent
Corn oil	6 per cent	Brewer's yeast	5 per cent
Powdered whole liver	5 per cent	Salt mixture (U.S.P. XII No. 2)	4 per cent
		Cod liver oil	2 per cent

Eight groups of 18 to 24 animals each received an addition of 0.0125, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0 and 1.5 per cent sodium bisulfite to the basic diet. After 3 months the concentration of sulfite for the last group was increased from 1.5 to 2 per cent. A similar group of animals fed only the basic diet was kept as controls. The dry sulfite powder was mixed with the basic ration by means of a dough mixer. The diets were refrigerated until used. All animals were kept in individual cages in a room with controlled temperature and humidity and

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Received for publication, April 23, 1952.

were given free access to their respective diets and water. At weekly intervals body weights and food consumption were determined, and a supply of food sufficient to last a week was placed in each food cup. Surviving animals were sacrificed after 1 to 2 years. The heads of animals that were found dead or that were sacrificed at the end of the experiment were bisected and preserved in a 10 per cent formalin solution.

The experiment was carried out in the laboratories of the Food and Drug Administration in Washington. A selected group of 43 rat heads were sent to the Department of Histology, College of Dentistry, University of Illinois. The teeth were examined macroscopically for the degree of pigmentation. The skulls were x-rayed and were then decalcified and embedded in paraffin. Central sections of the incisor and molar teeth were stained with hematoxylin and eosin or impregnated with silver (Gomori).

FINDINGS

A. Pigmentation of the Incisors.—The normal enamel of the upper incisors of the Osborne-Mendel strain of rats is deep orange in color. After feeding sulfites there was a lack of pigmentation, the degree of which was generally speaking proportionate to the concentration of sulfite and to the duration of the experiment (Table I). However, upper and lower incisors were not always affected to the same degree. Thirteen rats showed no changes in the pigmentation and of the remaining 28 rats 20 had whiter upper incisors and normal lower incisors. Only 8 had both the upper and the lower incisors depigmented.

TABLE I

THE EFFECT OF SODIUM SULFITE ON THE UPPER INCISOR OF THE RAT

GROUP	CONCENTRATION OF SULFITE (%)	NUMBER OF ANIMALS	DURATION OF EXPERIMENT (YEARS)	NUMBER OF ANIMALS WITH: CHANGES IN INCISOR PIGMENTATION	PATHOLOGIC CHANGES IN ENAMEL ORGAN	SHORT ENAMEL MATRIX	CESSATION OF LINGUAL DENTIN FORMATION	INVAGINATION OF LINGUAL ODONTOGENIC EPITHELIUM	DISTORTED (FIELDED) ENAMEL MATRIX	KERATINIZATION IN NASOLACRYMAL DUCT	THICKENING OF FUNDIC ALVEOLAR BONE
O	–	2	1	0	0	0	0	0	0	0	0
C	0.0125	2	1½	2	2	0	0	0	0	0	0
A	0.025	6	1	1	0	0	0	0	0	(1)	0
B	0.05	5	1	2	1	0	0	0	0	0	0
D	0.1	5	1	1	2	1	0	1	0	0	1
E	0.25	3	1	0	1	1	0	0	0	(1)	0
F	0.25	2	1½	2	0	0	0	0	0	0	0
G	0.5	3	1	2	3	1	0	0	0	0	0
H	0.5	4	1½	4	4	1	2	2	1	0	1
K	1.0	3	1	3	3	1	3	3	2	3	3
L	1.0	3	More than a year	3	3	3	3	3	3	3	3
M	2.0	3	Less than a year	3	3	2	3	2	0	2	3
N	2.0	2	1	2	2	2	2	2	2	2	2

With increasing dosage the abrasion pattern especially of the lower incisors was irregular, and in several cases the upper incisors were fractured.

B. Histology of the Normal Rat Incisor.—The rat incisor is a continuously growing tooth that is covered only on the convex side with enamel, while its concave surface is covered by a thin layer of cementum. The incisors develop primarily from an epithelial base, the odontogenic epithelium. This epithelium behaves differently at the labial and the lingual side.

Lingually the epithelium consists of one to two rows of spindle-shaped cells, that are comparable to the cells of Hertwig's epithelial root sheath of teeth with limited growth. Like the latter the lingual epithelial cells have retained their qualities to induce differentiation of odontoblasts although they do not form enamel.

Labially the epithelium differentiates into the ameloblasts and the papillary layer. The latter is composed of the stratum intermedium, stellate reticulum, outer enamel epithelium of the enamel organ, and includes numerous capillary invaginations from the surrounding connective tissue (Fig. 1).

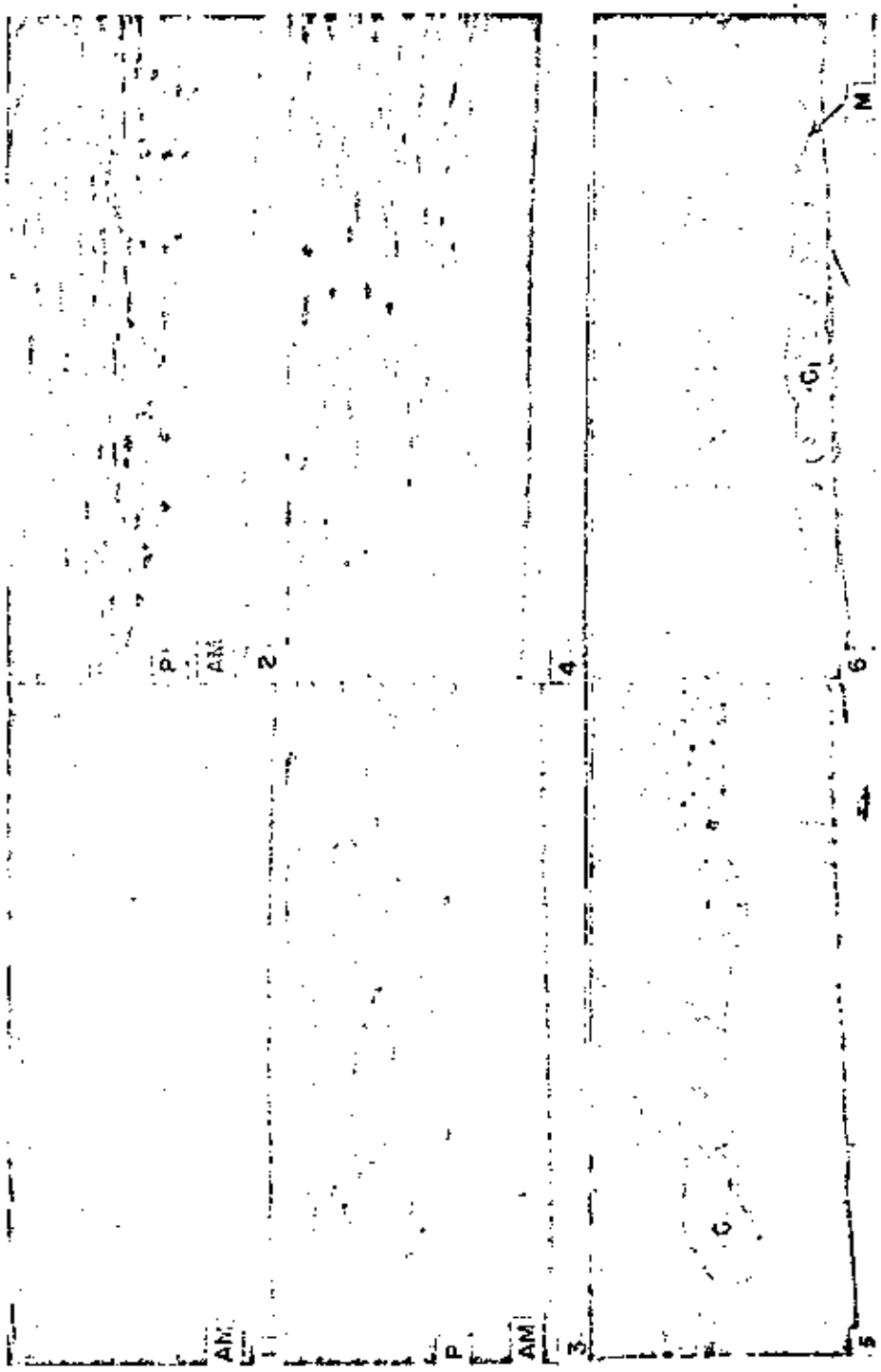
After forming the enamel matrix the ameloblast as well as the other layers of the enamel organ undergo a physiologic atrophy. In the normal adult rats of the Osborne-Mendel strain the atrophic changes can be observed about midway between the basal end of the tooth and the gingival crevice. In an incisal direction, the first sign of the atrophy is the progressive reduction of the height of the ameloblasts, that gradually assume a cuboidal shape (Fig. 2). The integrity of the papillary layer of the enamel organ is undisturbed in this area but farther incisally the folds of this layer gradually straighten out and from here on the enamel is covered by 5 to 6 layers of cuboidal to spindle-shaped epithelial cells.

The odontogenic epithelium establishes the dentino-enamel and dentino-cemental junctions and activates the adjacent cells of the pulp to differentiate into odontoblasts. Normally the widths of the labial and lingual dentin at the midlevel of the tooth are essentially equal. As a result of the continuous formation of dentin the pulp narrows toward the incisal edge.

The alveolar bone of the upper incisor consists of anterior and posterior portions which are separated by the premaxillo-maxillary suture. The posterior maxillary portion forms the fundus and the posterior third of the labial alveolar bone. The fundic bone is extremely thin and owing to its shift toward the tooth by sutural growth at the premaxillo-maxillary suture, osteoclastic resorption can be seen on the periodontal side.

C. Histologic Changes in the Incisors of Sulfite-fed Rats.

Enamel organ and enamel: In normal adult rats the enamel organ undergoes a physiologic atrophy at about the middle of the incisor. In the experimental rats the area of atrophic changes had shifted frequently to the basal third. The atrophy of the enamel organ is not only premature but also atypical. Normally the atrophy starts with a reduction of the ameloblasts in height and is followed by a reduction of the papillary layer. In the experimental rats the regression set in with a sudden reduction of the papillary layer (Fig. 3) and was then followed by atrophy of the ameloblasts. Furthermore, whereas physiologic atrophy progresses continuously and regularly until the reduced enamel



Figs. 1 to 6.—Photomicrographs of the enamel organ of the upper rat incisor. Hematoxylin-eosin stain. Orig. mag. ×400.
AM = ameloblasts, P = papillary layer.

Fig. 1.—Middle third of normal incisor.

Fig. 2.—Incisal third of normal incisor. Note the shortened ameloblasts and the reduction of the papillary layer.

Fig. 3.—Middle third of incisor of rat fed 0.1 per cent sulfite for one year. Note the shortened ameloblasts and the atrophy of papillary layer in contrast to the full ameloblasts and papillary layer in Fig. 1.

Fig. 4.—Incisal third of incisor of rat fed 0.1 per cent sulfite for one year. Note the absence of the papillary layer and ameloblasts, which have been replaced by densely and irregularly arranged polyhedral cells.

[illegible]

organ fuses with the gingival epithelium, atrophy of the sulfite-fed rats is irregular and areas of far-advanced atrophy alternate with areas of a fairly normal enamel organ.

In advanced stages of atrophy the enamel organ consists of stratified epithelium, the single cells of which are of irregular shape and densely packed (Fig. 4). In the incisal third the atrophic enamel organ consists only of 1 to 2 layers of epithelial cells.

In some of the animals the picture of pathologic atrophy was complicated by different degrees of edema in the adjacent connective tissue leading to the formation of small rounded spaces between the cells of the papillary layer (Fig. 5). Often small cystlike cavities were formed (Fig. 6) in the fluid of which macrophages could be observed.

The experimental changes in the enamel organ had occurred after completion of enamel formation. It is therefore not surprising that as a rule the enamel was normal. Enamel hypoplasia was observed in only one instance. However, the length of the nonacidsoluble enamel matrix appeared reduced in some of the animals fed lower doses of sulfite and in most of the animals fed the high doses. Concomitant with the reduction of the nonacidsoluble enamel matrix in length, a folding of the dentino-enamel junction had occurred. The severity of the folding increased with the increase in dosage of sulfite.

It must be pointed out that there was no correlation between the lack of pigmentation and the histopathologic findings in the enamel organ. In a number of instances (for example, in Group F) the incisors appeared white, whereas the enamel organ appeared normal.

Dentin: In the control animals and those fed up to 0.5 per cent sulfite, formation of dentin was normal. The odontoblasts were of normal height and appearance, and the predentin width was normal on both labial and lingual sides of the tooth.

In two of the rats fed 0.5 per cent sulfite and in all rats that were fed 1.0 per cent sulfite or more, the formation of dentin on the lingual side at the basal one-third of the tooth was severely disturbed leading to a reduction in width (Table 1). In most cases the odontoblasts had assumed a spindle shape. No predentin was present and the junction of dentin and pulp was scalloped (Fig. 7). The innermost layer of dentin was stained deeply with hematoxylin.

In all but one of the animals fed 1.0 per cent sulfite or more, some degree of dysfunction of the odontoblasts was seen also on the labial side of the tooth. The pulpal border of the dentin was irregular and covered by irregularly arranged cells.

Odontogenic epithelium: In the control animals and those fed less than 0.5 per cent sulfite, the odontogenic epithelium was entirely normal. In all but 4 of the animals fed higher doses the epithelium invaginated into the pulp cavity toward the lingual formative side (Figs. 7 and 8). In some instances this epithelium reached as far as the middle of the tooth. These epithelial islands and cords induced in some places differentiation of odontoblasts and formation of dentin in the center of the pulp.

Fig. 7.—Photomicrograph of a central section of an upper incisor of a rat fed 2.0 per cent sulfite for one year. Hematoxylin-eosin stain. Orig. mag. ×16. Note the narrow basal dentin and the invasion (*IN*) of odontogenic epithelium into the pulp.

Fig. 8.—Higher magnification of the basal dentin of the basal third of the incisor in Fig. 7. Note the narrow dentin (*D*), the scalloped border between pulp and dentin and the absence of odontoblasts. The keratinous scales of the metaplastic stratified squamous epithelium (*KE*) of the nasolacrimal duct fills partially the lumen.

The supporting structures of the upper incisor:

1. The Lingual Gingiva. In the controls and in the animals fed less than 0.5 per cent sulfite, the lingual epithelial attachment ended incisally to the alveolar crest. In most of the animals fed higher doses of sulfite, a deep pocket had developed, separating the alveolar crest from the cementum-covered surface of the tooth. In this area the periodontal surface of alveolar bone showed active resorption characterized by Howship's lacunae and large numbers of multinucleated osteoclasts.

2. The Periodontal Membrane at the Lingual Side. In a few instances the periodontal membrane showed reduction in its width and the normal division in 3 layers had disappeared. Principal fibers ran without interruption from alveolar bone to cementum.

3. The Alveolar Bone. The most striking alteration could be seen at the fundus of the alveolar socket in one of the animals of Groups D and H and in all of the animals of Groups K to N. The change consisted of a thickening of the normally extremely thin fundic bone. The apposition of bone began at the periphery and progressed toward the center of the fundic plate. The newly formed bone was separated from the old bone by a distinct resting line and was characterized by a great number of osteocytes and multiple tonguelike spicules which projected toward the tooth. Apposition of bone could also be observed on the labial periodontal surface of the premaxilla, where normally resorption occurs.

4. The Nasolacrimal Duct.* In the control animals and in all but one rat fed less than 0.5 per cent sulfite, the respiratory epithelium of this duct was entirely normal. In all the animals fed higher doses of sulfite, this epithelium was replaced by stratified squamous epithelium, which in some cases underwent keratinization. The changes became more extreme with increased dosage of bisulfite culminating in the plugging of the entire duct by a keratinized mass (Figs. 7 and 8).

DISCUSSION

Animal experiments and chemical investigations reported in the literature pointed to an indirect action of sulfites that had been added to stored foods (Fitzhugh, Kruelsen and Nelson[2]). The sulfites did not seem to be toxic in themselves but caused damage by destroying vitamins contained in the diet.

Deficiencies in 3 different vitamins were assumed. Lack of thiamin could be proved by a disappearance of polyneuritis under thiamin management. Vitamin E deficiency was consistent with the findings of "brown uteri." Vitamin A deficiency seemed probable because of lack of pigmentation of the incisors. However, no loss of vitamin A could be detected in the food and no

*In central longitudinal sections through the upper rat incisor an oval cavity is seen within the premaxilla on the convex side of the tooth. This cavity, lined by epithelium, has been variously interpreted as part of the nasal cavity or part of the maxillary sinus. Study of serial sections of the head cut on the sagittal plane has shown that this cavity is a cross section of the nasolacrimal duct. This duct starts in the anterior-most recess of the orbit, runs forward and slightly downward between the infraorbital nerve and the posterior part of the socket of the upper incisor. It then quite abruptly turns medially, crosses the incisor on its concave surface and opens into the inferior meatus under cover of the maxilloturbinal bone. The epithelium is pseudostratified ciliated columnar and many small mucous glands are found in the wall of the duct.

sign of vitamin A deficiency could be detected in the many viscera investigated. The typical keratinizing epithelial metaplasias (Wolbach[15]) were looked for in the various organs and were not present except in the lungs, where they sometimes accompanied chronic pneumonia in both treated and control rats.

The present report has shown specific histologic signs of both vitamin E and A deficiency in the rat incisor. The lack of pigment in this tooth and the folding of the dentino-enamel junction, however, are not indicative of any specific deficiency.

Lack of the yellow-brown pigment of the rat incisor has been found under many and various experimental conditions. Deficiency of iron, magnesium and tryptophane, vitamin A, vitamin D and vitamin E; intoxication with cadmium, fluorine, and strontium, and finally after parathyroidectomy.

Folding of the dentino-enamel junction has been observed in all cases where tooth eruption was retarded (hypophysectomy, vitamin A deficiency, excessive incisal stress, etc.). Retardation of tooth eruption is caused primarily by retarded growth of the basal connective tissue of the pulp. The folding of the dentino-enamel junction, therefore, is the result of a disturbance of the normal balance between the rate of growth of the connective tissue and of the enamel epithelium (Sicher[13]).

Signs of Vitamin E Deficiency.—Lack of pigmentation in vitamin E deficiency has been described by Moore[8] and Granados and Dam.[4] According to Irving[9] and Pindborg[10] the premature, sudden and atypical atrophy, the edema, and formation of cysts of the enamel organ are characteristic for vitamin E deficiency. This conclusion was confirmed by the fact that the administration of alpha-tocopherol to rats placed on a diet deficient in vitamin E led to a repair of the described characteristic lesions.

Identical findings in the enamel organs of rats fed higher doses of sodium sulfite permit, therefore, the conclusion that the vitamin E had been eliminated by the addition of the sulfite.

Signs of Vitamin A Deficiency.—It has been shown that vitamin A deficiency causes retardation in eruption (Fredericia and Gudjonsson[3]), metaplasia of different types of epithelium, characteristic changes in the rat incisor (Wolbach and Howe,[14] Schour, Hoffman, and Smith[12]) and in the apposition and resorption pattern of growing bones (Wolbach[15]).

The ciliated epithelium of the nasolacrimal duct of the normal rats had been replaced in the sulfite-fed animals by a highly keratinized stratified squamous epithelium. Desquamated horny scales might almost obliterate the lumen.

Atrophy of the odontoblasts, especially on the lingual side of the incisor, leading to retardation and irregularities of dentin formation, and invasion of the odontogenic epithelium into the pulp, have been shown to be specific for vitamin A deficiency by Wohlbach and Howe, and Schour, Hoffman, and Smith. Replacement therapy with vitamin A corrects these symptoms.

Only a few reports have dealt with the changes in the periodontal membrane and the alveolar bone of the upper incisor in vitamin A deficiency. Schour, Hoffman, and Smith[12] stated that the fundus of the alveolus appeared thicker than normally with prominent spicules of bone, and Irving[7] found a considerable

overproduction of new bone. Apposition occurred according to his findings in some areas where normally resorption takes place as has also been described by Wolbach for other regions of the skeleton. The increased apposition could be seen particularly in the fundic bone, where the new bone showed tongue-shaped outgrowths into the periodontal tissues. A direct comparison of our findings with the illustrations of Irving shows that the changes observed are identical.

Combined Effects of Vitamin A and Vitamin E Deficiency.—The combined occurrence of vitamin A and vitamin E deficiency can be understood from the result of the experiments of Davies and Moore.[4] These investigators showed that vitamin E deficiency led secondarily to vitamin A deficiency. Later it has been demonstrated that vitamin E protects vitamin A and carotene by preventing their oxidation (Quackenbush, Cox, and Steenbock[11]; György and Torravelli[5]; and Hickman, Harris, and Woodside[6]). It seems possible that during storage of the food, vitamin E was destroyed by the sulfite and that consequently vitamin A was oxidized and thus eliminated.

The results of the present experiment are surprising in that they not only confirm the assumption of vitamin E deficiency, but also demonstrate definite signs of vitamin A deficiency. These positive findings are in contrast to those reported earlier (Fitzhugh, Knudsen, and Nelson[2]) in which the evidence of vitamin A deficiency was inconclusive. The explanation for this seeming contradiction is in all probability the high sensitivity of the rat incisor to otherwise subclinical vitamin deficiencies. This susceptibility in turn seems to be founded on the fact that the rat incisor is a continuously forming, growing, and erupting tooth. This entails not only the presence of almost embryologic tissue but also its rapid growth and especially rapid processes of histodifferentiation. This high susceptibility of the rat incisor is consistent with many other observations that fast-growing tissues show a much greater sensitivity to a variety of injuries.

SUMMARY AND CONCLUSIONS

This study is based upon the anatomic and histologic changes of the upper incisors of 43 rats fed a diet containing sulfite ranging from 0.0125 to 2 per cent over a period of from 1 to 2 years.

1. Low doses (up to 0.025 per cent sulfite) produced slight deficiency of pigmentation of the incisor and slight atrophy of the enamel organ.

2. High doses (0.5 to 2.0 per cent sulfite) caused pronounced lack of pigmentation of the enamel; sudden and atypical atrophy of the enamel organ, often accompanied by edema; foldings of the dentino-enamel junction; atrophy and disturbed histodifferentiation of the odontoblasts; retardation and disturbance of dentin formation; invasion of the odontogenic epithelium into the pulp; thickening of the fundic alveolar bone; and keratinization of the epithelium of the nasolacrimal duct.

3. The findings of atypical atrophy and edema of the enamel organ are indicative of vitamin E deficiency.

4. The findings of atrophy of the odontoblasts, invasion of the odontogenic epithelium into the pulp and metaplasia of the epithelium of the nasolacrimal duct are considered to be specific for vitamin A deficiency.

5. The rat incisor is highly sensitive to otherwise subclinical vitamin deficiencies and is therefore useful in assaying different vitamin deficiencies.

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と同時に Hm が増加する．蒸留無菌は 40°C では Hd が減少しても Hm は生成されない．

終りに本研究に御指導を賜った名古屋市衛生研究所長，大山保氏に謝意を表します．

本報の要旨は日本食品衛生学会第7回学術講演会（昭和39年5月15日，名古屋市）において発表．

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拡散分析の衛生化学的応用研究（第4報）[*1]

食品中の亜硫酸塩類の定量

（昭和39年7月2日受理）

石坂音治[**]　石原英子[**]

On Application of Diffusion Analysis to Hygienic Chemistry (IV). Determination of Sulfur Dioxide in Foods

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Diffusion analysis combined with pararosaniline colorimetry has been studied for the determination of sulfur dioxide in food. After addition of 1 ml of absorbing reagent into the inner chamber and then into the outer, 1 ml of liquid sample (under SO_2 12 μg/ml) and 0.2 ml of 25% H_3PO_4 solution, standard unit are allowed to stand for 90 minutes at 20~40°C. After the lapse of time, the contents of inner chamber are treated with pararosaniline reagent and measured by colorimetry.

This method appears to be profitable comparing with the usual distillation method in that the loss deviation of sulfur dioxide can be at least kept in 5% and many samples may be treated simultaneously.

(Received July 2, 1964)

食品中の亜硫酸塩類の定量は古くから多くの方法が行なわれているが，公定法[*1]に採用されている定量法は

[*1] 第3報，名古屋市衛研，7，18（1959）．
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たとえば蒸留法[*1]には蒸留操作があるために酸化イオウの損失，多量の試料を同時に処理することができない欠点がある．他の方法としてパラローザニリン比色法が文献[*2]にみられた．

著者らはパラローザニリンが少ないこと，試料を並行

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して多数処理できることを目的として，亜硫酸塩類の定量を拡散法をクロマニックに1ヶ法律を組み合わせて行なった．基礎実験において SO_2 12 μg/ml 以下の定量が可能であることを知ったのでこの基礎資料とこの方法で数種の食品中の亜硫酸塩類の定量を行なった結果を報告する．

実験操作

1. 装置 1) 標準型拡散器，No. 1 2) 日立分光光度計 EPU-2 形 3) 10 mm セル

2. 試薬 1) 5%炭酸水素ナトリウム液 2) 吸収液：塩化第二水銀（特級）27.2 g および塩化ナトリウム（特級）11.7 g を5%グリセリンで1 L とする． 3) 拡散器の気密剤：85%リン酸（特級）．4) 25%リン酸：特級リン酸を用い毎週調製する． 5) *p*-ロザニリン・ホルムアルデヒド試液：*p*-ロザニリン塩酸塩1 g を水100 ml に溶かし，その2 ml をとり，これに塩酸6 ml を加えてから水で薄めて100 ml とし，これに新たに標定したホルマリンを用いて0.1%水溶液としたものを混和する．着色したものは使用できない．冷暗所に保存する． 6) 二酸化イオウ標準溶液：亜硫酸水素ナトリウム約0.5 g を吸収液100 ml に溶かし，その10 ml をとり，ただちに0.1 N ヨウ素液15 ml および塩酸1 ml を加え，0.1 N チオ硫酸ナトリウム溶液で滴定する．このときの滴定量を a ml とし，別に空試験を行ない，その滴定量を b ml とする．つぎにただちに亜硫酸水素ナトリウム溶液93.75/(b−a)×F ml（F は0.1 N チオ硫酸ナトリウム溶液の力価）をとり，これに吸収液を加えて100 ml とし，さらに吸収液で100倍に希釈して二酸化イオウ標準溶液とする．

二酸化イオウ標準溶液 1 ml＝0.003 mg SO_2

3. 定量操作 基本操作は衛生試験法注解一般試験法拡散分析法[1]の項に従って行なう．拡散器の内室に吸収液1 ml を，外室に液状試料（後記調製法参照）1 ml（SO_2 12 μg/ml 以下）および25%リン酸0.2 ml を入れ，ふたをし20〜40°で90分間拡散後，内室の吸収液を目盛付共セン試験管にとり，さらに内室を吸収液で洗い，洗液を合せ全量を5 ml とし，*p*-ロザニリン・ホルムアルデヒド試液1 ml を加え，すばやく混和し，20〜25°で35分間放置後，575 mμ 付近で吸光度を測定し標準曲線から含量を求める．空試験は液状試料の代りに吸収液を用い同様に操作する．

基礎資料

1. 測定波長 SO_2 6 μg/6 ml の割で呈色している溶液について吸光度を測定し Fig. 1 に示す結果を得た．最大吸収波長が575 mμ であったので本実験においてはこの波長で測定した．最大吸収波長は条件によって移動するので定量に際して最大吸収波長をさがす必要がある．

2. 拡散時間 拡散温度として室温の23°と，フ卵器中の37°について拡散時間別に吸収率を測定し Fig. 2 に示す結果を得た．23°では60分で100%，37°では30分で100%の吸収率を示し，この状態は120分経過しても変らないので，十分吸収させる意味で90分を拡散時間とし

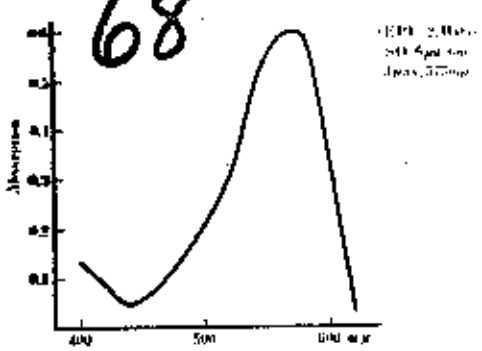

Fig. 1. Absorption spectrum of SO_2-pararosaniline colored solution

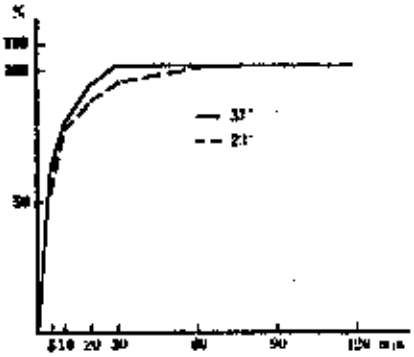

Fig. 2. Diffusion time and SO_2 absorption curve in standard unit
Inner chamber: 1 ml of the absorbent
Outer chamber: 1 ml of standard solution (SO_2 6 μg/ml) and 0.2 ml of 25% H_3PO_4

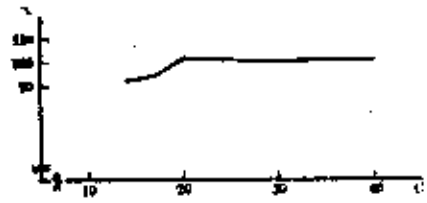

Fig. 3. Diffusion temperature (°C) and SO_2 absorption curve in standard unit
Diffusion time: 90 min
Inner chamber: 1 ml of the absorbent
Outer chamber: 1 ml of standard solution (SO_2 6 μg/ml) and 0.2 ml of 25% H_3PO_4

た。

3. 拡散温度　拡散時間を90分と定めて拡散温度別に吸収率を測定し，Fig. 3に示す結果を得た。14°で92%，17°で94%，20°以上で100%となり，40°においても変らないので拡散温度範囲を20～40°にとった。

4. 検量範囲　各濃度の二酸化イオウ標準溶液を拡散器の内室に入れ，定量操作に従って発色させ Fig. 4 の標準曲線を得たので定量範囲を SO_2 12 μg/ml 以下とした。この場合，二酸化イオウ標準溶液を直ちに発色させたものに比べてわずかに濃く呈色する傾向がある。

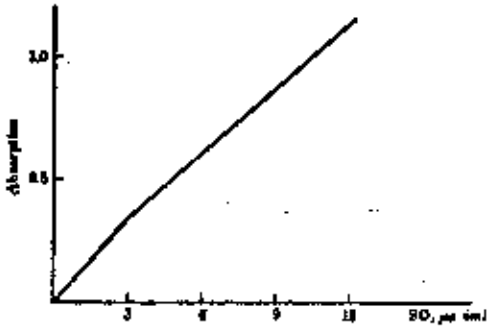

Fig. 4. Calibration curve of SO_2 at 575 mμ

食品中の亜硫酸塩類の定量

1. 検体使用量　食品中には使用規準で Table 1 のように二酸化イオウの量が限定されているので，これから SO_2 10 μg/ml の濃度になるような量を算出して使用量の目安にした。

Table 1. Available Quantity (A. Q) of Sample for 100 ml Test Solution, Calculated from the Officially Permitted Content (O.P.C) of SO_2 in Foods

Subjects	O.P.C SO_2 g/kg (1964, Jan.)	A.Q g/100 ml
Dried apricot	1.00	1.
Dried pine-apple gelatine Dried gourd shavings	0.50	2.
Syrup Millet-jelly	0.30	3.
Wine	0.20	5.
Natural fruit juice	0.13	8.
Other foods	0.03	30.

2. 液状試料の調製　液体をそのまま或は2.5%硫酸水溶液で稀釈するか，揮発性の吸収液で液状にする。ドライフルーツ，ドライヴェジタブル，ゼラチン，かんぴょうなどは細かく切って揮発性の吸収液に対し一定倍率液状にする。

3. 食品中の亜硫酸塩類の定量　食品中の亜硫酸塩類を拡散法と蒸留法で定量し Table 2 に示す結果を得た。液状試料 200 ml を作り，100 ml を蒸留法に，残りを拡散法に用いた。蒸留法の溶液については拡散法と同様にパラローザニリン法で定量した。拡散法では同一検体のものを数個並べて定量しその平均値を取っているが，ばらつきは約5%以下であった。

Table 2. Comparison of Diffusion and Distillation Methods for the Determination of SO_2 in Foods

Food Samples	A. Distillation M	B. Diffusion M	100 × B/A
Dried gourd shavings	1.560	1.773	114
Wheat-gluten A	.458	.511	112
Wheat-gluten B	.094	.137	146
Dried pine-apple	.086	.107	124
Candied apricot	.049	.060	122
White wine	.036	.043	119
Claret	0	0	—
Gelatine	0	0	—
Millet-jelly	0	0	—
Dried grape	0	0	—

結　　論

以上の結果より本法に関してつぎのことを知った。

1. SO_2 12 μg/ml 以下の定量が可能である。
2. データのばらつきは約5%以下である。
3. 検体を多数並行して処理できる。
4. 検体使用量が食品によって異なる欠点がある。

本報の要旨は日本食品衛生学会第7回学術講演会（昭和39年5月15日，名古屋市）において発表。

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Bull. OIV (1970)

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État actuel des techniques pour le remplacement de l'anhydride sulfureux

Plan de travail
établi à l'intention des rapporteurs nationaux

par le Prof. P. Jaulmes

Importance et nécessité des travaux destinés à diminuer l'emploi de l'anhydride sulfureux en œnologie.

Degré de toxicité de SO_2 libre et combiné à court et à long terme.

Vitesse de l'action de SO_2 libre et combiné sur la thiamine.

Conditions d'emploi de SO_2 seul pour en restreindre les doses :
avant la fermentation,
pendant la fermentation,
après la fermentation.

Moyens proposés pour éviter l'emploi de SO_2 ou en restreindre les quantités.

A. *Vinification des vins secs.*
1° Chauffage de la vendange.
2° Macération carbonique.
3° Vinification à l'abri de l'air.
4° Clarification des moûts.
5° Emploi de la bentonite.
6° Autres procédés : centrifugation, pasteurisation, réfrigération, enzymes réductrices, actinisation.
7° Addition de thiamine.

B. *Vinification en vue de l'obtention de vins moelleux ou doux.*

[illegible] ..., etc.

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C. *Conservation des vins secs.*
Acide ascorbique.
SO_2 et son oxydation par l'air.
Vins en cuve en vidange, gaz inertes.

CONCLUSION.

* * *

EXPOSÉ GÉNÉRAL (*)

du Prof. P. JAULMES,
Chaire de Chimie appliquée
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Faculté de Pharmacie de Montpellier,
France.

Le titre de cet exposé retenu par le Conseil de l'O.I.V. est certainement trop présomptueux. Du moins il démontre une fois de plus le grand intérêt qu'attache l'O.I.V. à la diminution de l'emploi de cet additif œnologique ou même à sa disparition si cela était possible.

J'ai accepté la charge de rédiger cet exposé, parce que depuis longtemps je m'intéresse à ce problème du point de vue du toxicologue et du bromatologue, mais il faut reconnaître qu'au point de vue de la technologie du vin il n'y a malheureusement rien de bien nouveau à vous apprendre, malgré les communications de 9 pays (*) qui m'ont bien facilité la tâche. Je remercie les collègues qui ont bien voulu rédiger ce qu'ils savaient ou ce qui est fait dans leurs pays respectifs pour réduire dans la mesure du possible les doses d'emploi de l'anhydride sulfureux en œnologie.

Je dois rappeler aussi les rapports nationaux, ainsi que le rapport général de notre excellent collègue et ami, M. Feduchy, qui ont été présentés sur ce même sujet au Congrès international de la Vigne et du Vin à Thilisi en 1962. C'est en somme le relevé des propositions et des progrès notables effectués depuis 8 ans dont je me propose de vous entretenir sans pouvoir prétendre être complet.

(*) Exposé Général présenté à la 9e Session de la Commission II (Chypre : 7-12 octobre 1970). Voir également Ordre du Jour (*Bull. de l'O.I.V.*, n° 469, 1970).

(*) [illegible] Espagne, Luxembourg, Portugal, Roumanie et U.R.S.S. [illegible] seront publiées dans les prochains numéros du *Bulletin de l'O.I.V.*

Tout d'abord pourquoi faut-il réduire les doses limites d'emploi de l'anhydride sulfureux?

Nous savons que les Comités de la Commission mixte F.A.O./O.M.S. du Codex alimentarius s'occupent depuis plusieurs années de réduire l'emploi de l'anhydride sulfureux dans toutes les matières alimentaires : jus de fruits et vins en particulier, la Commission de Toxicologie ayant indiqué que cet additif présentait une certaine toxicité et qu'il fallait en limiter l'absorption quotidienne à 20 ou 30 milligrammes par jour. A la dernière réunion de la Sous-Commission des Méthodes d'analyse M. Mattey a rappelé aux experts de l'O.I.V. combien les doses limites relativement élevées d'anhydride sulfureux prévues pour les vins dans les législations nationales inquiétaient les experts de F.A.O., O.M.S. et il insistait sur l'intérêt que présenterait la réduction de ces doses limites, étant donné qu'elles sont très rarement atteintes comme il l'avait constaté lui-même dans les vins consommés en Suisse.

Degré de toxicité de l'anhydride sulfureux

Avant d'aborder mon sujet, permettez-moi de vous donner un avis personnel sur cette toxicité. J'ai fait déterminer les toxicités aiguë et chronique par le Service d'Essais physiologiques des Matières alimentaires du Laboratoire Central de l'Intendance militaire de Paris. Les résultats de ces études ont été publiés dans 3 mémoires.

Toxicité aiguë

Les doses DL 50 ont été les suivantes :

SO_2 dilué ou salifié { 2 g/kg rat pour une concentration de 35 g/l.
1,04 g/kg rat pour une concentration de 65 g/l.

donc la *toxicité* augmente nettement avec la concentration

SO_2 combiné à l'éthanal 1,56 g/kg rat.
Éthanal libre 1,9 g/kg rat.

Les symptômes de l'intoxication par la combinaison de SO_2 avec l'éthanal sont semblables à ceux provoqués par l'éthanal et différents de ceux donnés par SO_2 libre ou salifié (sulfite).

Les sulfites ne sont donc pas plus toxiques que le carbonate monosodique (Na HCO_3) et autres sels réputés inoffensifs ou « substances légèrement toxiques » selon Hodge et Steiner.

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Toxicité chronique

Entre 1963 et 1965, on a traité 4 générations successives de rats Wistar par un liquide eau ou vin renfermant 450 milligrammes de SO_2 par litre à raison de 3 millilitres par jour et par 100 grammes de poids corporel. Aucun effet ne fut constaté sauf une légère diminution des oxydations cellulaires dans le tissu hépatique.

Ces doses de SO_2 ayant été jugées trop faibles par les hygiénistes de l'O.M.S., ces expériences viennent d'être refaites sur des rats Wistar avec des doses beaucoup plus élevées : 10 millilitres de solution à 1,2 g SO_2 par litre par jour pour chaque animal de 400 grammes depuis le sevrage jusqu'à l'âge de 5 mois. Ensuite ces mêmes rats ont absorbé journellement 10 millilitres d'une solution à 6 grammes de SO_2 par litre pendant 7 mois (cette dose quotidienne correspond à 10 g de SO_2 pour un homme de 70 kg, elle atteint 15 % de la dose DL 50).

Aucun effet nocif ne fut observé autre que la diminution des oxydations au niveau des cellules hépatiques et cela seulement dans le cas de SO_2 libre. Ce dernier effet ne fut pas observé avec SO_2 combiné à l'éthanal.

Action sur la thiamine

Étant donné que l'un des principaux griefs que l'on fait à SO_2 est son action destructrice sur la thiamine alimentaire, thiamine qui est précisément un très actif protecteur vis-à-vis de l'intoxication éthylique, on a mesuré les variations de l'excrétion urinaire de la thiamine de ces mêmes rats soumis à l'absorption de fortes doses de SO_2. Une diminution n'a été observée que dans le cas de SO_2 libre à la dose considérable de 6 grammes par litre dans l'eau de boisson. L'anhydride sulfureux combiné à l'éthanal n'a pas provoqué cet effet. Ces expériences *in vivo* ne sont pas absolument démonstratives, Lhuissier n'avait-il pas conclu d'une façon différente en 1956 dans la même recherche ? Aussi ai-je confié à l'une de mes assistantes, Mlle Bres, l'étude de la cinétique de réaction de SO_2 sur la thiamine en fonction du pH et de la concentration. Cette étude est difficile, car il faut opérer rigoureusement à l'abri de l'air. Elle a été faite avec SO_2 libre et SO_2 combiné à l'éthanal et à 37 °C. On a fait agir un excès variable d'anhydride sulfureux par rapport à la thiamine qui était dosée à 0,5 g par litre. La vitesse de destruction est d'autant plus grande que la concentration en anhydride sulfureux est plus forte. Elle varie beaucoup avec

le pH : à pH inférieur à 2 ou supérieur à 7,5 elle est faible et [illegible] par un maximum à pH 5,8.

Or, le pH du suc gastrique est voisin de 2, tandis que la bi[illegible] pH supérieur à 7, le pH du sang est de 7,35 et le pH du contenu i[illegible] est généralement compris entre 7 et 7,5. A ces pH la vitesse de [illegible] est faible : destruction inférieure à 12 p. 100 au bout de 5 heu[illegible] que la concentration de SO_2 était de 860 milligrammes par li[illegible] supérieure aux concentrations possibles dans le tube digestif.

Quant à SO_2 combiné à l'éthanal, il ne paraît pas agir sur la thiamine à pH inférieur à 7. Au pH supérieur à 7, pour lesquels la combinai[illegible] est dissociée, l'alcalinité suffit pour modifier la thiamine, d'une manière réversible tant que le pH est inférieur à 9.

Quant à l'action de la thiamine combinée au corps cétoniques, acides pyruviques, α-cétoglutarique, etc., aux sucres, etc., leur étude est entreprise. Comme ces combinaisons sont moins stables que celles contractées avec l'éthanal, il paraît probable que leur action physiologique est semblable à celle de l'éthanal libre.

Dans l'état actuel de cette étude, j'estime que la toxicité de SO_2 a été quelque peu exagérée. Toutefois soyons prudents, ce corps pourra avoir d'autres actions sur d'autres éléments du vin ou des matières alimentaires.

Il n'en reste pas moins que tout excès de SO_2 donne un goût désagréable au vin et que nombre de personnes ont une muqueuse stomacale sensible à cet additif volatil dans l'état de liberté où il se trouve dans le bol alimentaire à pH 2 ou 3 dans l'estomac, cet effet n'est pas contesté.

Il faut donc éviter tout excès de SO_2 libre ou combiné aux corps cétoniques.

Bien que SO_2 combiné à l'éthanal soit exempt de goût et d'odeur, qu'il ne soit pas libéré dans l'estomac, il faut éviter dans la mesure du possible la présence de quantités notables de combinaisons de SO_2 avec l'éthanal parce que ce dernier corps est 5 à 6 fois plus toxique que l'alcool.

Nous sommes maintenant fixés sur l'objectif à atteindre.

CONDITIONS D'EMPLOI DE L'ANHYDRIDE SULFUREUX *seul* POUR EN RESTREINDRE LES DOSES NÉCESSAIRES.

Je rappe[illegible] tout d'abord ce qui est connu sur l'emploi de SO_2 seul pour évite[illegible] excès, et qui est très justement rappelé dans les bel[illegible] rapports r[illegible] que l'O.I.V. a reçus à ce sujet.

L'anhydride sulfureux ne doit être ajouté qu'avant ou après la fermentation alcoolique :

Avant la fermentation : il sert comme antioxygène évitant la madérisation pendant les opérations précédant la fermentation. Il est indispensable dans le cas des vendanges plus ou moins pourries en tant que réducteur et inhibiteur d'enzymes oxydantes.

Il est utile comme antiseptique, il élimine les bactéries et sélectionne les levures pour ne conserver que les plus vigoureuses. Il sert de produit acidifiant favorisant la dissolution des anthocyanes et il agit aussi pour faciliter la précipitation des éléments en suspension.

Il est modérateur de la fermentation alcoolique, ce qui est indispensable en pays chauds pour éviter une élévation excessive du milieu en fermentation.

Les doses d'emploi varient avec les pays : 70 milligrammes par litre en Autriche et d'une manière facultative, tandis qu'en Afrique du Sud on estime 200 milligrammes par litre comme nécessaire.

Après la fermentation : SO_2 sert de stabilisateur du vin fait et il assure sa conservation : 10 à 20 milligrammes de SO_2 libre par litre suffisent pour protéger le vin contre la tourne, mais il en faut davantage pour le protéger contre la piqûre. Il a toutefois l'inconvénient d'empêcher la fermentation malolactique généralement souhaitable, aussi faut-il attendre que cette évolution se soit produite pour effectuer cette addition.

Pendant la fermentation : vouloir arrêter une fermentation en pleine évolution par une addition de SO_2 nécessite l'emploi d'une forte dose ajoutée en une seule fois : 200 à 300 milligrammes par litre, et il faut séparer immédiatement les levures qui ne sont qu'endormies sinon elles complexent le SO_2 par l'éthanal et c'est ce qu'il faut éviter. La seule manière rationnelle d'arrêter une fermentation est de filtrer ou de centrifuger le moût *avant* l'addition de SO_2 ou d'acide sorbique qui ne doit se faire que sur un milieu limpide.

Moyens proposés pour éviter l'emploi de l'anhydride sulfureux ou en restreindre les quantités.

A. — Vinification des vins secs.

Étudions maintenant les moyens *auxiliaires* permettant de diminuer les quantités de SO_2 nécessaires à l'obtention de bons vins quel que soit l'état ou la qualité de la vendange.

1° *Le chauffage de la vendange* remplace avec avantage l'action de SO_2 dans plusieurs de ses effets : chauffage à 70-80 °C durant quelques minutes. On obtient ainsi :

a) la dissolution des anthocyanes,

b) la destruction des germes de maladies et des ferments sauvages,

c) la destruction des enzymes oxydantes. Mais l'oxydation se produisant entre le moment du foulage, ou même de la récolte si les raisins sont blessés durant le transport, et le moment où la température atteint celle de la destruction des enzymes, il faudrait que pendant cette période la vendange soit maintenue à l'abri de l'air. Cette condition est d'autant plus intéressante que le chauffage produit le minimum d'altération du goût s'il est pratiqué sur une vendange exempte d'oxygène et à l'abri de l'air. L'emploi de l'azote ou du gaz carbonique dès l'entrée en cave s'imposerait donc, mais cette technique nécessiterait la révision complète de tous les aménagements des caves de vinification et entraînerait une augmentation importante des prix de revient.

Un autre procédé apparemment très satisfaisant est le chauffage brusque des raisins entiers par un courant de vapeur d'eau. Ainsi les enzymes oxydantes sont tuées avant d'avoir pu agir sur les composants du moût. Il est actuellement essayé à Bordeaux.

Je pense que c'est pourtant dans l'une de ces voies que l'on pourrait obtenir la suppression complète de SO_2 et une conduite rationnelle de la vinification, mais nous n'en sommes pas encore là...

2. *La macération carbonique* assure tout aussi bien la fermentation anaérobie sans anhydride sulfureux. Les raisins intacts et non foulés, sont entassés dans une cuve que l'on a préalablement remplie de gaz carbonique et où ils séjournent à l'abri de l'air pendant plusieurs jours. On obtient ainsi des vins très perfumés, colorés et souvent différents des vins obtenus par des méthodes traditionnelles. Ce procédé est déjà répandu et de nombreuses publications lui ont été consacrées, en particulier par Flanzy, Poux et leurs collaborateurs qui ont été les pionniers dans ce domaine.

3° *La vinification à l'abri de l'air sans chauffage de la vendange* a donné d'excellents résultats à divers expérimentateurs : Hernandez en Espagne, Schaeffer en Alsace, Vieira en Portugal, en Afrique du Sud. Hernandez commence la fermentation sous vide, Schaeffer sous gaz carbonique et ce dernier indique que par ce moyen il a pu diminuer de moitié l'emploi de SO_2. Le pressurage en pressoir Vialla sous CO_2 a

donné un moût dont le potentiel d'oxydoréduction était de 170 mV avant toute addition de SO_2, alors qu'à l'air il atteint 300 mV, ceci montre bien l'importance de l'oxydation lorsqu'on presse une vendange à l'air libre. Schaeffer a constaté que la fermentation malolactique était ensuite plus facile et que l'acidité volatile était plus faible. Mais le vieillissement et le développement des qualités des vins de cru sont beaucoup ralentis.

Plusieurs auteurs ont remarqué que les vins élaborés à l'abri de l'air étaient plus sensibles à la madérisation que les vins obtenus par les procédés habituels. Il semble donc que le chauffage de la vendange doive être le corollaire de la vinification à l'abri de l'air; mais à ce point de vue l'anhydride sulfureux en quantité modérée remplace le chauffage, soit par son action réductrice, soit par son action directe sur les enzymes oxydantes. Sur ce dernier phénomène, qui devrait être étudié méthodiquement, j'ai très peu de données. La vinification à l'abri de l'air résoud donc en partie le problème, puisqu'elle se traduit par une diminution de l'ordre de 50 p. 100 de la quantité d'anhydride nécessaire.

4. *La clarification des moûts* par divers moyens a donné à M. Asvany les résultats intéressants permettant de réduire la quantité d'anhydride utile. Dans son rapport (Hongrie), il insiste beaucoup sur le fait que les vins de presse sont de plus en plus riches en colloïdes pendant le cours de la pressée et que cette richesse nécessite des quantités de plus en plus élevées d'anhydride.

5. *L'emploi de la bentonite* pour le traitement des moûts a donné de très bons résultats à de nombreux œnologues, car ce traitement éliminant les substances colloïdales et une partie des enzymes permet de réduire l'emploi de l'anhydride de 25 à 50 p. 100. C'est ce que recommandent MM. Milisavljevic, Azvany, Lepedatu, Schaeffer, X... d'Espagne et bien d'autres. L'effet conjoint de la bentonite et de l'anhydride sulfureux assure une bonne vinification même dans le cas des vendanges pourries sans qu'il soit nécessaire d'employer d'aussi fortes doses d'anhydride qu'en l'absence de bentonite.

6. D'*autres procédés* ont été employés ou essayés pour éviter l'emploi de trop grandes quantités de SO_2 au moment de la vinification.

La *centrifugation*, la *filtration* pour clarifier les moûts destinés à l'élaboration des vins blancs sont recommandées par MM. F. Paul, Azvany et X... d'Afrique du Sud et d'Espagne.

La *pasteurisation* pour tuer toutes les bactéries et les levures non désirables pourrait être employée.

Le refroidissement ou *réfrigération* évite l'emploi de l'anhydride sulfureux pour ralentir la fermentation dans les pays chauds et empêcher l'élévation excessive de la température. L'emploi d'anhydride sulfureux dans ce but est à proscrire, puisqu'il provoque la formation d'éthanal et l'augmentation considérable de l'anhydride sulfureux combiné. Cet usage très répandu n'est qu'un pis-aller, une solution de facilité.

L'emploi de l'*enzyme glucoxydase + catalase* a été essayé avec un certain succès en U.R.S.S. Il doit être utilisé en l'absence d'anhydride sulfureux qui détruit l'enzyme.

L'*actinisation* (de De Stoutz) permet de chauffer le moût à 90-95 °C pendant 7 secondes sous l'action d'un rayonnement infrarouge avec échangeur de température. Ce procédé donne des résultats semblables à ceux du chauffage de la vendange. Il enlèverait même le goût de pourri au moût de raisin atteint de pourriture grise.

M. Orechkina mentionne qu'on a essayé en U.R.S.S. l'emploi des ultrasons, de l'irradiation gamma, mais ces moyens altèrent moût et vin.

7. L'addition de *thiamine* à la dose de 0,50 g par hectolitre de vendange proposée par Peynaud et Mlle Lafon-Lafourcade permet d'obtenir une fermentation alcoolique avec une formation réduite d'acide pyruvique et d'acide α-cétoglutarique qui complexent SO_2. Ce procédé est à retenir comme étant capable de réduire l'anhydride sulfureux dans les vins. Cette addition est efficace même dans le cas des moûts de raisins pourris. Les auteurs recommandent également, et dans le même but, d'éviter les fermentations à température trop élevée, à pH élevé, de supprimer les aérations et de levurer avec *Saccharomyceus ellipsoïdes* peu cetogène.

En résumé, pour la vinification des vins secs, on peut supprimer l'anhydride sulfureux en chauffant la vendange, de préférence en atmosphère inerte et en ensemençant le milieu par un pied de cuve d'une levure non cétogène et incapable de réduire les sulfates en SO_2 comme cela a été observé pour certaines espèces de levure très réductrices.

On peut vinifier avec peu d'anhydride (50 à 70 mg/l) en refroidissant le milieu en fermentation, en ajoutant de la bentonite à la vendange pour absorber protéines, dextranes, enzymes oxydantes ou en clarifiant le moût par centrifugation, filtration, lorsqu'il s'agit d'une vinification en blanc.

Ces moyens permettent de vinifier correctement même des vendanges pourries sans utiliser de doses excessives d'anhydride.

B. — VINIFICATION EN VUE DE L'OBTENTION DE VINS MOELLEUX OU DOUX

Cette vinification nécessite quelques moyens en plus de ceux que je viens de rappeler, car il est difficile de conserver une certaine quantité de sucre fermentescible.

Le principe fondamental, admis par tous, est d'arrêter la fermentation au moment voulu par filtration, clarification (voir pasteurisation) et non par la seule addition d'anhydride. Ensuite, on ajoutera de l'anhydride avec ou sans une des substances antifongiques actuellement en usage : acide sorbique ou pyrocarbonate d'éthyle.

Ces deux substances ont des avantages et des inconvénients différents. La première doit être protégée par SO_2, sinon la fermentation malolactique, qui n'est pas arrêtée par cet acide, le transforme en acide caproïque ou en aldéhyde caproïque qui donne au vin un goût de poisson ou de géranium. Son action antifongique ou plutôt fongostatique est d'ailleurs assez faible et il en faut 200 milligrammes par litre au minimum dans les vins peu alcoolisés. Cette addition ne permet pourtant que de réduire la quantité d'anhydride de 450 à 300 milligrammes par litre. M. Lepadatu recommande l'emploi simultané de bentonite, d'anhydride sulfureux et d'acide sorbique comme étant très avantageux.

Je dois signaler qu'un jeune chercheur de la Faculté de Pharmacie de Nantes, M. Sparfel, vient de faire une étude sur l'activité antifongique de divers dérivés de l'acide sorbique. Il a constaté que ses esters éthylique et propylique étaient plus actifs que l'acide libre. Peut-être y aurait-il intérêt à les essayer en œnologie.

La deuxième substance, l'ester éthylique de l'acide pyrocarbonique, a l'avantage de s'hydrolyser en quelques heures dans le vin en donnant éthanol et gaz carbonique. Il reste seulement du carbonate diéthylique à raison de 5 p. 100 de la quantité de pyrocarbonate employée. Cet « avantage » a l'inconvénient de rendre l'action de cet antiseptique très fugace. C'est une pasteurisation chimique. On ne peut l'employer qu'au moment de la mise en bouteilles. De plus nous connaissons mal l'action de cet ester sur les divers constituants du vin pendant sa courte vie.

Dans le but d'arrêter la fermentation et de stabiliser les vins doux, M. Orechkina indique qu'on a employé en U.R.S.S. le marc de moutarde contenant 1 p. 100 d'essence à la dose de 350 à 400 milligrammes par litre (soit 3 à 4 mg/l d'essence) en association avec la bentonite et l'anhydride sulfureux. Ce produit ne peut être employé que pour les vins de qualité inférieure à cause du léger goût qu'il donne au vin. Il signale égale-

ment qu'on a essayé les sels d'argent à des doses de 1 à 5 milligrammes par litre d'argent, mais sans succès, heureusement pour la santé des consommateurs

C. — Conservation des vins secs

La conservation des vins secs est le dernier problème à étudier. Il faut les protéger contre la tourne, la piqûre, l'oxydation, la madérisation, etc.

Pour éviter oxydation et madérisation, l'autorisation d'emploi de l'*acide ascorbique* à la dose généralement limitée à 100 milligrammes par litre est presque générale. Cet acide est un puissant réducteur, mais il ne faut l'employer qu'au moment de la mise en bouteille car au contact de l'air il donne des peroxydes et l'oxydation est plus profonde qu'en l'absence d'acide ascorbique. Aussi faut-il toujours le protéger par de l'anhydride sulfureux.

Pour protéger le vin contre la tourne, nous n'avons que SO_2, mais 20 milligrammes de SO_2 libre par litre suffisent pour protéger le vin si le pH n'est pas supérieur à 3,5. Il suffit donc d'entretenir une telle dose de SO_2 par addition calculée de sulfite ou d'anhydride. A cette faible dose il n'est pas sensible au goût. Pour que ce traitement soit fait sans excès il est indispensable qu'il soit précédé d'un dosage. D'une façon générale tout additif ne devrait être ajouté au vin que sous la surveillance d'un chimiste compétent et responsable. Pour le SO_2, tout autant que pour le ferrocyanure, cette nécessité est évidente.

Réalisée avec modération, cette addition à un vin fait n'a aucun inconvénient, car elle n'entraîne pas la formation de composés complexants, si bien que cet anhydride reste libre et actif. Sous cette forme, il est d'ailleurs oxydable par l'oxygène de l'air, si bien qu'on peut le faire disparaître par sa transformation en sulfates par 1 ou 2 soutirages successifs avec aération si on le désire.

Voici d'ailleurs, à titre d'exemple, quelques constatations que j'ai récemment faites pour mettre cette oxydation en évidence :

1° Un vin en vidange agité à l'air de temps en temps voit son anhydride disparaître assez rapidement. De 155 milligrammes de SO_2 total il est progressivement descendu à 29 milligrammes par litre en 15 jours, le SO_2 combiné s'étant peu à peu libéré et oxydé. A ce moment, il ne restait que du SO_2 combiné et le vin s'est piqué.

2° Si le vin n'est qu'une seule fois mis en contact avec l'air avec agitation, il dissout 8 millilitres d'oxygène à 8 °C, 6,5 ml à 20 °C. Cet

oxygène dissous peut théoriquement oxyder respectivement 46 milligrammes et 37 milligrammes de SO_2 libre. C'est la limite maximale théorique de disparition de SO_2 après une [illegible]. L'expérience montre qu'en réalité la disparition de SO_2 n'est pas aussi importante dans ce cas; dans un essai elle n'a atteint que 12 milligrammes et seul SO_2 libre a été oxydé. Il y a en quelque sorte une compétition entre SO_2 libre et les substances réductrices du vin. Si initialement le vin est très pauvre en SO_2 libre, par exemple 2 à 3 milligrammes par litre pour 235 de SO_2 total, le SO_2 combiné n'a pas été oxydé et il est resté fixe. Dans de telles conditions il n'est pas protégé contre la tourne. Cette étude mériterait d'être beaucoup approfondie, car je ne donne que quelques résultats d'expériences.

Pour éviter la piqûre des vins en cuve en vidange, le procédé traditionnel est d'introduire du SO_2 gazeux dans l'atmosphère. Ce procédé est bon, mais cet SO_2 se dissout peu à peu dans le vin et il s'oxyde. Le vin subit une certaine oxydation, une partie du CO_2 normal du vin se répand dans l'atmosphère et le vin prend le goût d'évent.

La conservation du vin en cuve en vidange reçoit une solution excellente par l'emploi des gaz inertes, azote ou gaz carbonique ou le mélange de ces deux gaz sous la pression ordinaire majorée de quelques millibars (2 ou 3). L'azote seul amène peu à peu un appauvrissement du vin en CO_2 d'autant plus marqué que le rapport volume vide/volume de vin est plus élevé. Le vin perd une partie de sa fraîcheur et de son bouquet. Le CO_2 pur donne un léger pétillant au vin qui n'est pas souhaitable pour la plupart des vins rouges, encore que le goût des consommateurs ne soit pas un facteur constant. Les vins présentant un léger pétillant sont appréciés en Italie et en Allemagne, mais non en France. Ce goût évoluera sans doute.

En utilisant un mélange des deux gaz dans des proportions calculées, on peut conserver la teneur naturelle du vin en CO_2 ou l'ajuster à sa valeur optimale selon sa nature.

Nous pensons qu'il est très important de maintenir cette atmosphère rigoureusement exempte d'oxygène car les agents de la piqûre et de la fleur sont très résistants à l'asphyxie. Le vin introduit dans une cuve est généralement chargé d'oxygène, ne l'oublions pas. Un procédé qui a donné ses preuves est l'emploi d'une cuve auxiliaire d'une vingtaine de litres contenant une solution de sulfites à pH 7 activés par des traces de cuivre, cobalt et manganèse. Cette solution fixe peu à peu l'oxygène, le gaz inerte étant propulsé d'une manière continue de la cuve principale à la cuve auxiliaire par un ventilateur de très faible puissance. Le vin se conserve ainsi aussi bien en vidange que s'il était en cuve pleine. L'acidité

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volatile n'augmente pas, le SO_2 libre et combiné du vin reste constant. Il est donc inutile de renouveler cette addition pour éviter la tourne. Le gaz carbonique reste indéfiniment constant dans le vin quelles que soient les proportions respectives de vide et de plein et la durée du séjour.

Conclusions

Tous les rapports nationaux reçus par l'O.I.V. mentionnent dès le début que l'anhydride sulfureux reste irremplaçable.

M. Vieira indique, non sans esprit, qu' « avec SO_2 n'importe quel fou incompétent peut faire du vin ». Ce sera du vin non piqué ni tourné, mais il y a peu de chances pour que ce soit du bon vin. L'abus de l'anhydride sulfureux a notablement contribué à l'abandon du vin par nombre de consommateurs.

Bien que j'aie tout lieu de penser pour l'instant que l'anhydride sulfureux ne soit pas aussi nocif qu'on le croit de nos jours, il faut en réduire le plus possible l'emploi et j'ai déclaré devant la commission des additifs alimentaires de l'O.M.S.-F.A.O. à Arnhem, en octobre 1969, que l'O.I.V. ferait tous ses efforts pour réduire l'emploi de cet antiseptique, mais qu'il fallait des délais à la viticulture mondiale pour modifier les techniques et les installations et matériel de cave afin d'arriver à ce résultat.

Une meilleure connaissance du mécanisme d'action de l'anhydride sulfureux, action d'ailleurs complexe, permet d'envisager cette réduction et peut-être même sa suppression.

Pour la vinification des vins totalement fermentés, on peut se passer des services de ce corps par le chauffage ou la pasteurisation à l'abri de l'air, par la macération carbonique. Mais ce sont là des procédés qui ne peuvent encore être généralisés. Il faut d'ailleurs mieux en connaître tous les effets et la technique.

La clarification des moûts, l'addition de bentonite à la vendange, le refroidissement du milieu en fermentation, un levurage massif, l'addition de thiamine, la vinification et le travail des vins à l'abri de l'air sont susceptibles d'entraîner une diminution importante des doses d'anhydride nécessaire pour assurer une vinification satisfaisante même dans les cas, hélas trop fréquents, de vendanges pourries.

Pour l'obtention de vins moelleux, on peut également beaucoup réduire les doses d'anhydride par l'emploi de techniques œnologiques rationnelles : filtration stérilisante, pasteurisation, clarification et stabilisation par bentonite associée à l'acide sorbique et à l'anhydride.

Pour la conservation des vins, si l'anhydride sulfureux reste indispensable à petite dose, on peut en restreindre l'emploi par l'adjonction d'acide ascorbique, de pyrocarbonate d'éthyle au moment de l'embouteillage, par la conservation des vins en vidange sous gaz inertes exempts d'oxygène.

Toutes ces techniques sont encore trop peu utilisées et de nouveaux progrès doivent être acquis. Sans doute sont-elles plus onéreuses que la solution de facilité que constituent l'emploi de doses importantes d'anhydride sulfureux. Mais la qualité des vins offerts par la viticulture et l'œnologie n'est-elle pas la seule espérance qu'elles peuvent entretenir de voir la consommation se développer et conquérir de nouveaux marchés?

Je signale pour terminer qu'en Italie, Bulgarie, Portugal, U.R.S.S., Roumanie, la dose limite d'SO_2 totale pour les vins secs est seulement de 200 milligrammes par litre. Le rapport espagnol signale que dans ce pays le prix du vin est quelque peu fonction inverse de sa teneur en anhydride sulfureux. Voilà deux bons exemples que je soumets à votre appréciation.

Au cours de l'Assemblée générale de Paris, en octobre 1969, nous avions fait le relevé de quantités limites d'anhydride sulfureux admises pour les vins dans les 15 pays représentés et nous avions été quelque peu étonnés des doses élevées admises dans la plupart d'entre eux. Ne pensez-vous pas qu'il y aurait lieu de réduire ces doses comme nous le conseillait en avril dernier, avec beaucoup de bon sens, notre excellent collègue suisse, M. Mattey?

Zur toxikologischen Bedeutung von Sulfit beim Wiederkäuer

The toxicological significance of sulphite fed to ruminants

Au sujet de l'importance toxicologique du sulfite pour les ruminants

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I. Allgemeines

Die Vorlage des Vorschlags einer Verordnung des EWG-Rates über die Festlegung von Höchstgehalten an unerwünschten Stoffen und Erzeugnissen in Futtermitteln am 18. 2. 1971 im Bundestag (1) ließ es als geboten erscheinen, die Stichhaltigkeit des im genannten Entwurf vermerkten Höchstgehaltes an schwefliger Säure in Höhe von 400 mg pro kg in Trockenschnitzeln zu überprüfen. Die schweflige Säure ist seit vielen Jahrzehnten Gegenstand einschlägiger Untersuchungen gewesen, zumal sie in technologischer Hinsicht sowohl vom Standpunkt der Anwendungstechnik aus als auch als Faktor der Abgase bei Verbrennungsvorgängen erhebliche Bedeutung besitzt. Quantitative Angaben zeichnen die in Tabelle 1 aufgeführten charakteristischen Werte ab (2).

Diese erheblichen in die Atmosphäre entweichenden SO_2-Mengen sind gleichbedeutend mit der Tatsache, daß SO_2 unter den gasförmigen phytotoxischen Luftverunreinigungen die am weitesten verbreitete Verbindung ist (2). Im Rahmen der Pflanzenpathologie ist SO_2 in den letzten Jahren erstaunlich gut und weitgehend untersucht worden. Entsprechende Untersuchungen bei Haustieren treten demgegenüber weit zurück.

Im speziellen Falle gelangt SO_2 in nennenswerten Quantitäten erst während des Trocknungsprozesses auf die Rübenschnitzel. Die Menge ist von der Art des verwendeten Holzmaterials und der Verfahrenstechnik abhängig.

II. Zur Chemie

SO_2 (Schwefligsäureanhydrid) ist ein farbloses Gas von scharfem Geruch. Das Molgewicht beträgt 64,07. Im SO_2 sind 50,05 % Schwefel und 49,95 % Sauerstoff enthalten. Bei 3 at läßt es sich zu einer farblosen Flüssigkeit komprimieren. Mit Wasser bildet es schweflige Säure (H_2SO_3), die eine schwache Säure ist. Jedoch entstehen bei der Verbrennung von Schwefel aus den SO_2-Molekülen mit Wasser direkt HSO_3^- und H_3O^+-Ionen:

$$SO_2 + 2H_2O \rightleftharpoons HSO_3^- + H_3O^+ \qquad pK_S = 1{,}96$$

Im Handel ist eine Lösung von ca. 6 % SO_2 erhältlich (3). Eine SO_2-Lösung in Wasser geht allmählich in Schwefelsäure über.

Die schweflige Säure bildet primäre (Hydrogensulfite, veraltet Bisulfite) und sekundäre (Sulfite) Salze. Die Alkalisalze der schwefligen Säure sind in Wasser leicht löslich. Es handelt sich im wesentlichen um folgende Verbindungen:

Natriumsulfit (Na_2SO_3)	alkalisch
Calciumsulfit ($CaSO_3$)	sauer
Natriumhydrogensulfit bzw. -bisulfit ($NaHSO_3$)	sauer

Natriumhydrogensulfit hat ein Molgewicht von 104,07 und enthält 61,56 % SO_2, 30,81 % S, 46,13 % O und 22,10 % Na. Es riecht nach SO_2 und wird gradiert zu Sulfat oxidiert.

SO_2 speziell in bzw. auf Rübenschnitzeln soll in Form von Sulfit und Hydrogensulfit vorliegen. Über das Vorkommen organisch gebundener Anteile liegen keine Angaben vor. Die Konzentration auf den Handelsprodukten soll zwischen 400 und 1800 mg/kg liegen.

Hierbei ist also zu beachten, daß sich an Schnitzeln ähnlich wie bei Obst usw. (4) komplexe Verbindungen bilden können, die einem mehr oder weniger schnellen Zerfall unterliegen.

Die Wirkung der komplexen Salze hängt von deren Spaltung ab. Das wirksame Agens ist der Hydrogensulfitrest. SO_3, SO_3^{--}, SO_3H^- und H_2SO_3 wirken schon in geringer Konzentration desinfizierend, und zwar sowohl gegenüber Bakterien als auch gegenüber Hefen und Schimmelpilzen.

III. Anwendung

In der Technik und im Haushalt dient SO_2 (schweflige Säure und ihre Salze) zur Textilbleichung, zur Konservierung von Nahrungsmitteln und zur Parasitenbekämpfung. Im Brauereigewerbe und in Nahrungsmittelbetrieben verwendet man es zur Desinfektion. Bekannt ist das „Ausschwefeln" der Weinfässer. In der Landwirtschaft kommt SO_2 bei der Silagebereitung zur Anwendung. Zur Konservierung von Wurst, Früchten, Sirup, Bier, Apfelwein und anderen Nahrungsmitteln findet es in Konzentrationen von 40 bis 3000 mg/kg Verwendung. Noch 1952 berichtet Gaddum (5), daß es „neben Benzoesäure das einzige in England gesetzliche Konservierungsmittel" ist. Entsprechend gibt die Verordnung über die Verwendung von Schwefeldioxid Höchstmengen als Restgehalt schwefliger Säure als SO_2 in mg/kg in Lebensmitteln an (6). Die dort verzeichneten Konzentrationen reichen bei den verschiedensten Produkten von 20 bis 2000 mg/kg. Diese Konzentrationsangaben besitzen für die nachfolgenden Aussagen nur geringen Wert, da es sich um Lebensmittel für den Verbrauch durch Menschen handelt und die menschlichen Eßgewohnheiten – speziell bei den genannten Produkten – auch quantitativ von denen der Haustiere, insbesondere der Wiederkäuer, abweichen.

Früher wurde SO_2 usw. auch zur Konservierung von Fleisch benutzt. Mellor kommt zu der bemerkenswerten Feststellung: „...selbst wenn Sulfite an und für sich nicht gesundheitsschädlich sind", ist die Anwendung unverantwortlich, weil das Aussehen verändert wird und das Fleisch ein frisches Aussehen erhält (Verschleierung von Verderb). Es sind also nicht primär toxikologische Erwägungen, son-

Tabelle 1
Schwefel- und Sulfitgehalt in Brennstoffen

Brennstoff	Gesamtschwefel %	Abgabe des Gesamtschwefels %	Abgabe als SO_2 %	Abgabe als SO_2 bzw. H_2SO_4
Braunkohle	0,3 – 6,5	50 – 80		
Steinkohle	0,5 – 2,3	60 – 90	90–99	1 – 10
Heizöl	– 5,1	60 – 80		

Tabelle 2
Beispiele für Toxizitätswerte von SO_2-Gas

Nr.	Dosis	Dauer	Tierart	Befund	Autor
1	1,0 mg/Liter	5 h	Maus	letal	Bentz (12)
2	2,0 mg/Liter	10 h	Maus	letal	Bentz (12)
3	0,001 – 0,006 Vol.-%	2 – 6 h	Maus	letal	Hauschild (7)
4	2,6 mg/Liter		Ratte	letal	Bentz (12)
5	1000 mg/kg		Ratte	letal	Merck (3)
6	0,15 Vol.-%	sehr schnell	Katze	letal	Hauschild (7)
7	3 – 5 mg/Liter		Katze	rasch tödlich	Starkenstein (zit. in (4))
	0,17 %		Katze	letal	Kisskalt (13)
8	5 – 40 mg/kg	8 h	Schwein	Reizg. am Auge und Respirationstrakt	Garner (14)
9	500 mg/kg	1 h	Pferd	gefährlich	Garner (14)
10	0,02 Gew.-%		Mensch	tödlich erträglich	Lehmann (zit. in (4))
11	10 mg/kg (0,001 Vol.-%)		(Mensch)	MAK-Wert	Hauschild (7)

dern lebensmittel-(fleisch-)hygienische Bedenken, die in Deutschland eine Verwendung als Präservesalz gem. § 21 des Fleischbeschaugesetzes, § 3 des Lebensmittelgesetzes und § 9 der Hackfleisch-Verordnung untersagen.

Nicht uninteressant ist der Hinweis von Hauschild (7) wonach ein Sulfitzusatz zu Ampullenlösungen unbedenklich ist, da schon kleinste Mengen (< 0,1 %) zur Stabilisierung oxidabler Substanzen ausreichen.

Im medizinalen Bereich findet H_2SO_3 als Spray bei Tonsillitis, bei septischer Pharyngitis und zu Zwecken der Antisepsis im Gastrointestinaltrakt Verwendung. Als gastrointestinalwirkendes Antiseptikum dient auch $NaHSO_3$. SO_2, H_2SO_3 und $NaHSO_3$ dienen zur Bekämpfung parasitärer Hauterkrankungen (3). Speziell im veterinärmedizinischen Sektor wurden Pferdeläuse mit einer 1 %igen und Räudemilben mit 4 %iger Konzentration von SO_2 begast (8). Örtlich wird es 40 %ig (1) zur Begasung am Huf eingesetzt, wo es bei Hufkrebs austrocknend und ätzend wirkt (9).

IV. Klinische Toxikologie

Klinisch-toxikologisch steht die Beschreibung von Belastungen nach Einwirken von gasförmigem SO_2 im Vordergrund der Berichte. Ganz offensichtlich scheint die Bewertung nach solchen Meßwerten zu erfolgen, die sich an den Gaskonzentrationen orientieren. Die gewerbetoxikologischen Gesichtspunkte, die zu MAK-Werten[1]) von 10 mg/kg (0,001 Vol.-%) führten, sind in diesem Zusammenhang irrelevant. Aus den gleichen Gründen ist die Beschreibung der toxikologischen Symptome zu verstehen, die sich fast ausschließlich mit jenen der Atemwege auseinandersetzt. Bei Inhalation beobachtet man bei Mensch und Tier Erscheinungen wie Rhinitis, Laryngitis, Glottiskrampf, Pharyngitis, Tracheitis, Bronchitis, Bronchopneumonie, Lungenödem und Lungenemphysem (10) (11). 400 mg SO_2/kg in der Luft wirken in wenigen Minuten letal, nicht zuletzt durch Ersticken und Laryngospasmen (11). Die „Giftwirkung" bei Begasung ist rein örtlicher Natur. Resorptivvergiftungen nach kutaner Aufnahme sind nicht bekanntgeworden (10). Als lokale Reizerscheinung ist nach Mother auch die Einwirkung auf die Schleimhäute des Respirationstraktes zu werten, die auf der Umwandlung von SO_2 in H_2SO_4 beruht. Sinngemäß wird über Verätzung der Haut und über Hornhauttrübungen am Auge und über Konjunktivitis berichtet (10). Die Schleimhautreizung beginnt bei 10 mg SO_2/kg in der Luft (11). In flüssiger Form kann es zu Blasenbildung und Epithelverlust kommen (11).

Natriumsulfit und Natriumhydrogensulfit führen nach Angaben von Hauschild (7) zu Reizerscheinungen am Magen-Darm-Kanal; jedoch kommt es nur selten zu einer Resorptivvergiftung, weil sie im Blut schnell zu Sulfaten oxidiert werden (vgl. auch (11)).

Die Daten über akut-toxische Grenzwerte sind von den verschiedensten Tierarten bekannt. Die Experimentaldaten beziehen sich jedoch zumeist auf parenterale Applikationswege, insbesondere die Inhalation. Die Werte besitzen daher im vorliegenden Zusammenhang nur für orientierende Vergleiche Bedeutung.

Die beim Verzehr von Rübenschnitzeln aufgenommenen SO_2-Mengen sind für die Auslösung einer akuten Vergif-

[1]) MAK = Maximale Arbeitsplatzkonzentration

Tabelle 3
Toxizität von H_2SO_3 und Sulfiten

	mg/kg	Applikation	Tierart	Bemerkungen	Autor
H_2SO_3	2,50	oral	Kaninchen	letal	Hauschild (7)
	12,50	oral	Kaninchen	letal, berechnet als mg SO_2	Rost (4)
	ca. 1	oral	Mensch	80 mg/Person (Röchnerin); Symptome	Braun (zit. in (15))
	ca. 3 – 10	oral	Katze	10 – 30 mg/Tier (0,2 % H_2SO_3 + 0,2 % HCl); Symptome	Jacoby (15)
	0,35 – 0,7	oral	Mensch	25 bis 50 mg/Person (0,05 % H_2SO_3)	Jacoby (15)
	3 ml	intravenös	Kaninchen	letal in 4½ h (2,6 %ige SO_2-Lösung)	Rost (4)
Na_2SO_3	175	intravenös	Maus	letal	Bentz (12)
	200	intravenös	Meerschweinchen	letal	
	200	intravenös	Kaninchen	letal	
	400	intravenös	Katze	letal	
	600	subkutan	Meerschweinchen	letal	Bentz (12)
	600	subkutan	Kaninchen	letal	
	1300 – 1600	subkutan	Katze	letal	
	1300 – 1600	subkutan	Hund	letal	
$NaHSO_3$	115	intravenös	Ratte	LD_{50}	Merck (3)
	30	intravenös	Kaninchen	letal	Hauschild (7)
	900 – 1000	subkutan	Kaninchen	letal	Hauschild (7)

lung als zu gering zu bezeichnen. Demgegenüber steht die Beobachtung, daß bei längerer Exposition und ausreichender Konzentration eine Schädigung im Sinne chronischer Vergiftungen nicht ausgeschlossen ist. Hauschild (7) erwähnt z. B., daß Affen nach Inhalation parkinsonähnliche Symptome erkennen ließen. Rost (4) verweist aber unter Zitierung zahlreicher Literaturstellen darauf, daß sich mit schwefliger Säure und ihren Salzen keine chemische Vergiftung erzielen läßt.

Rost (4) konstatierte resümierend, daß die Verwendung von schwefliger Säure oder von Sulfit in einer Höhe, wie sie zur Lebensmittelkonservierung verwendet werden, vom pharmakologischen Standpunkt aus als unbedenklich bezeichnet werden kann.

V. Experimentelle Toxikologie

Sofern toxische Werte erreicht werden, reagieren die einzelnen Tiere unterschiedlich auf SO_2 bzw. Sulfite. Diese individuellen Empfindlichkeitsunterschiede sind auf die verschiedensten Begleitumstände zurückzuführen. So konnte z. B. nachgewiesen werden, daß die SO_2-Toxizität durch Thiaminmangel beträchtlich erhöht wird (16) (17). Jedoch SO_2-Konzentrationen, wie sie beispielsweise bei konservierten Lebensmitteln vorkommen (Sulfit in Mengen von 400 mg/Tag), lösen beim Menschen, der (experimentell) an der unteren Grenze der Thiaminversorgung steht, keine Schäden aus, so daß Cremer (18) die verabfolgten Mengen für unbedenklich hält, was sich mit der oben angegebenen Ansicht von Rost (4) deckt.

Die experimentell ermittelten Einzelaussagen ergänzen das zuvor geschilderte klinisch-toxikologische Bild. Hierzu sollen die wesentlichsten Angriffsmomente zusammengefaßt abgehandelt werden:

1) Lokale Wirkungen

SO_2 wirkt weniger auf die äußere Haut als auf die Schleimhäute reizend. Am stärksten betroffen sind die Schleimhäute des Respirationstraktes. Konzentrierte SO_2-Lösungen wirken auf Gewebe nekrotisierend, verdünnte Lösungen lösen keine bemerkenswerten Effekte aus. Rost (4) vermutet, daß die fehlende chemische Wechselwirkung mit dem Protoplasma der Organzellen auf der schnellen Oxidation zum indifferenten Endprodukt beruht.

2) Orale Anwendung

Die Angaben über die Wirkungen, die nach oraler Gabe ausgelöst werden, beziehen sich im wesentlichen auf Tiere mit einhöhligem Magen. Hunde erbrechen sich nach Überschreiten bestimmter Konzentrationen von schwefligsauren Präparaten. Dosierungen, die unterhalb dieser Grenzdosis liegen, bleiben, über ein Jahr verabfolgt, verträglich (4).

H_2SO_3 führt 5 %ig in Mengen von 50 ml beim Kaninchen zu Magen-Darm-Reizung und Tod; 1 %ig erzeugt sie eine haemorrhagisch-nekrotische Gastroenteritis, 0,5 %ig ist sie über mehrere Tage verträglich. Bei Katzen bleibt eine Reizung unterhalb von 0,1 % aus (15). 80 mg H_2SO_3 (4 % SO_2) führen beim Menschen zu gastrischen Unverträglichkeiten. Sulfite führen bei höheren Dosen ebenfalls zu Reizerscheinungen. Rost (4) berichtet, daß bei Kaninchen Sulfit, Hydrogensulfit und Komplexverbindungen die Menge von 0,2 g/kg (in 50 ml) in 5 Tagen und von 0,25 g/kg bereits in 12 bis 18 Minuten tötet.

Oral an Kaninchen verabfolgtes Sulfit in Höhe von 3 g löst bei 10- bis 110maliger Gabe keine Resorptionsvergiftung und keine Diarrhoe aus (4).

Hunde, die 42- bis 418mal Natriumsulfit, Natriumhydrogensulfit oder glucosaschwefligsaures Natrium oral erhielten, zeigten weder Erbrechen noch Diarrhoe. Magenblutungen und sonstige pathologische Organveränderungen fehlten (4).

3) Resorption

Die Resorption aus dem Darm ist für Sulfite nur schwer nachweisbar und dann nur, wenn die Dosis sehr hoch liegt und die chemische Untersuchung relativ schnell nach der Eingabe erfolgt. Zumeist lassen sich nur Spuren von Sulfit (SO_3) im Blut nachweisen. Daher gelingt es als Folge der bereits im Darmkanal einsetzenden Oxidation auch nicht oder nur befriedigt, die Ausscheidungen über die Niere absolut quantitativ zu verfolgen. Jedoch findet man den mit den Sulfiten eingeführten Schwefel quantitativ in Harn und Kot wieder (4)[1].

Die schweflige Säure verläßt den Organismus als Sulfat, und eine Exhalation über die Lungen findet (bei Kaninchen und Hund) nicht statt. Die Resorption von SO_2 über das Respirationssystem ist wiederholt Gegenstand experimenteller Untersuchungen gewesen. Die Ergebnisse haben in diesem Zusammenhang nur eine Hilfsfunktion zu erfüllen. Die Exposition in 500 mg SO_2-Gas pro kg führt bei Hunden zu entzündlichen Veränderungen der Schleimhaut der Bronchien und erhöht den Lungenwiderstand sowie die Elastizität (19). Die SO_2-Resorption über die Atemwege fällt mit abnehmender Konzentration in der Luft (20).

Daß SO_2 nach Inhalation resorbiert wird, zeigen auch die von Lee (21) bestimmten Parameter, wonach beim Meerschweinchen eine einstündige Exposition in SO_2-haltiger Atemluft (7 bis 310 mg pro kg) zu einem Anstieg des Hämoglobingehaltes im Blut führt und auch der Gehalt an anorganischem Schwefel im Blut bei Konzentrationen von mehr als 19 mg/kg ansteigt. Die Resorption erfolgt schon von der Nasenschleimhaut aus. Nach Einatmungskonzentrationen von 16,1 mg SO_2 pro kg wird beim Menschen die gesamte Menge resorbiert, jedoch tritt keine Sättigung ein, und bei der Ausatmung finden sich nur 15 % der eingeatmeten Konzentration wieder (22).

4) Stoffwechselwirkung

Nach der Resorption verwellt Sulfit nicht im Organismus. Rost (4) bezeichnet die Salzwirkung auf den Stoff- und Flüssigkeitswechsel als vorübergehend. Das infolge schneller Oxidation entstehende Oxidationsprodukt ist als indifferent anzusehen.

Den Stoffwechsel beeinflußt Sulfit nur im Sinne einer Salzwirkung, und beim Menschen ist auch nach größeren Gaben von Sulfit keine Änderung des Stoffwechsels zu beobachten. Lediglich eine örtlich ausgelöste Reizung führt bisweilen zur Minderung der Resorption stickstoffhaltiger Substanzen. 1 g Sulfit, täglich verabfolgt, ändert beim Menschen weder die Kohlendioxid- noch die Wasserstoffabgabe (4). Hunde, die tägliche Gaben von 1 bis 1,2 g Sulfit pro kg über 25 Tage erhielten (415 g) und vertrugen, erlitten keine Stoffwechselbelastungen und zeigten keine anderen Wirkungen also solche, die auch von anderen Neutralsalzen ausgelöst werden.

Eichholtz (23) bezeichnet Schwefeldioxid und die Salze der schwefligen Säure schlechthin als Fermentgifte.

0,125 % SO_2-Lösung hemmt die Fermente Ptyalin und Trypsin, aber nicht Pepsin. Demgegenüber bleibt die Sulfitwirkung aus oder gering (4). Die Labgerinnung wird nach Weitzel durch 0,006 % Sulfit bereits merklich verzögert (zit. in (4)).

5) Pharmakologische Wirkung

Die Wirkung von SO_2 und den Salzen der schwefligen Säure ist auch auf die schweflige Säure selbst zurückzuführen; eine Wirkung hält nur so lange an, als schweflige Säure vorhanden ist. Es bedarf hoher Dosen. Eine Vergiftung beruht einmal auf einer „Salzwirkung" und zum anderen auf der Säurewirkung (schweflige Säure). Hierzu gesellt sich eventuell eine mehr oder weniger spezifische Sulfitwirkung, die jedoch nicht allein auf den Sauerstoffentzug zurückzuführen ist.

Über zentrale Lähmung des Gefäß- und Atemzentrums nach vorübergehender Steigerung der Reflexe — jedoch unter Ausbleiben von Krämpfen — kommt es schnell zum Exitus. Pathologisch sind dann am Lungengewebe entzündliche Veränderungen mit Blutungen zu erkennen; eine entspre-

[1] Nach intravenöser Injektion beim Hund fällt die Reaktion auf SO_2 bereits nach 2 bis 6 Minuten negativ aus.

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chende Nierenschädigung nach schwefliger Säure fehlt, und ebenso findet sich im Urin auch kein Eiweiß.

Speziell hinsichtlich des Kreislaufs kommt es nach intravenöser Injektion von Sulfit zur Lähmung der Gefäße und auch des Herzmuskels (24). Die Wirkung in der Peripherie zieht Blutdrucksenkung mit Vergrößerung der Pulse, dann Verlangsamung des Pulses sowie Arrhythmie, Lähmung des Herzmuskels und Herzstillstand in der Diastole nach sich.

Eine Kapillarwirkung seitens der schwefligen Säure scheint nur gering zu sein; vielleicht kommt es zu einer leichten Gefäßerweiterung.

H_2SO_3 und Sulfite sind keine Blutgifte (4). Die Wirkungen, die auf die Koagulation von Eiweiß zurückzuführen wären, setzen in vivo eine Resorption großer Mengen voraus. Eine Gefährdung ist gering, weil eine schnelle Oxidation der schwefligen Säure im Blut erfolgt. Nach der Aufnahme von Komplexsalzen hängt eine Beeinflussung von der Größe des Komplexzerfalles und somit vom Freiwerden von Hydrogensulfit ab, wobei Methämoglobin bzw. Hämatin entsteht und auch Hämolyse auftreten kann. Die Blutkatalase wird durch Sulfit nicht beeinflußt. Braun (11) berichtet über das Auftreten (nach Inhalation) von Polyzytaemie und nach größeren Dosen von Knochenmarksatrophie.

8) Atmung

Oral verabfolgtes Natriumsulfit ändert Atmungstiefe und -frequenz beim Kaninchen nicht (4). Lee (21) fand bei Versuchen an Meerschweinchen bei Inhalation von SO_2 nach einstündiger Beatmung mit 19 mg/kg einen Anstieg des Atemvolumens und einen Abfall der Atemfrequenz; 7 bis 17 mg/kg führten zum Abfall des Atemvolumens und zum Anstieg der Atemfrequenz.

VI. Sulfit beim Wiederkäuer

Die Auswirkungen von schwefliger Säure und ihren Salzen beim Wiederkäuer sind überraschend wenig untersucht. Ursache hierfür mag sein, daß einmal die Räudebehandlung mit SO_2-Gas bei dieser Tierart mühsam war und selten geübt wurde, und zum anderen, daß Zwischenfälle durch oral aufgenommene Sulfite — sei es als H_2SO_3, sei es als Kontaminationsprodukt des Futters oder als Silierhilfsmittel — wohl zu den Seltenheiten zählen dürften.

Die Verträglichkeitsspanne ist offensichtlich sehr groß. Luedke (25) verabfolgte Disulfit (vertritet Metabisulfit) bzw. SO_2-Gas in den Pansen von Rindern. Anorexie wurde bei täglichen Gaben von 80 bis 160 g Disulfit bzw. SO_2-Gas ausgelöst. Diese Substanzen repräsentieren sich als Hemmer von Bakterien und Fermentsystemen. Ähnliche Hemmeffekte beschreibt auch Nuvel (26), der in vivo wie in vitro eine Minderung der Methanbildung nach Natriumsulfit infolge direkten Einflusses auf die Bakterien sah. In vivo tritt dazu eine gleichzeitige Steigerung der Propionsäurekonzentration. Allerdings sind die Dosen erheblich hoch gewählt, nämlich für 8 Tage täglich 2 × 5 g Natriumsulfit für ein Schaf bzw. in Stoffwechselversuchen 8 g Sulfit pro Tag.

Bei einer Aufnahme von 100 lb mit Disulfit bereiteter Silage erhielten die Rinder etwa 45 g Sulfit/Tag (23).

Bei 600 Kühen des Versuchsanstellers blieb eine solche Fütterung über 5 Jahre schadlos. Selbst die doppelte Menge hatte in 180 Tagen keine negative Auswirkung. Jedoch bedingt die Gabe von 80, 120 und 160 g, die wöchentlich gesteigert wurde, Anorexie. Außer Anorexie sind weitere allgemeine Vergiftungszeichen Verstopfung, Durchfall, Depression und Dysenterie.

Extreme Dosierungen führen zum Tod, und pathologisch treten Gewebeschäden im Anfangsteil des Verdauungstraktes, im Larynx und im ventralen Teil der Trachea auf. Wechselnde Blutbilder und fehlender Einfluß auf den Blutglucosegehalt stehen dem Abfall des Hämatoffspiegels gegenüber (25).

Luedke (25) bemißt die tödliche Gesamtmenge an SO_2 in 16 bzw. 21 Tagen auf 1995 bzw. 1944 g. Umgerechnet entspricht das einer täglichen Aufnahmemenge von 125 bis 92,6 g SO_2 (Einzelangaben zu toxischen Schäden der Gewebe nach hoher SO_2-Gabe siehe (25)).

Ein weiterer — nicht publizierter — Versuch der Staatlichen Versuchs- und Lehranstalt für Viehhaltung in Aulendorf (27) besagt, daß Kühe, die in einem 4-Wochen-Versuch für 3 Wochen Hauptversuchsperiode täglich mit 2,5 kg Trockenschnitzeln mit 408, 1490 und 3000 mg SO_2/kg gefüttert wurden, keine Intoxikationserscheinungen zu erkennen waren. Milchleistung und -zusammensetzung blieben unverändert. Die täglich aufgenommene SO_2-Menge betrug mithin 1,02, 3,73 bzw. 7,73 g/Tier = ca. 2,04, 7,46 bzw. 15,4 mg/kg Körpergewicht.

Nimmt man überschlagsweise die vorliegenden Versuchsdaten zu Hilfe, so stehen die in Tabelle 4 angegebenen Relationen zur Diskussion.

Es ist weiterhin bekannt, daß es im Zusammenhang mit der Heufermentation durch Hydrogensulfit zu Störungen der Äthanolbildung kommt. Bei der Untersuchung möglicherweise vorhandener gleichsinniger Effekte im Pansen konnte Alhassan (23) nach Gaben von Natriumsulfit eine Zunahme von Propionat und eine Abnahme von Acetat beobachten. Selbst eine längerfristige Verabfolgung von 137 bis 160 g Natriumsulfit im Rahmen anderer Versuche führte zu keiner signifikanten Veränderung der Gesamtfettsäurekonzentration im Pansen, jedoch stieg auch hier der Propionatanteil, indes die Acetatbildung nachließ. Die Milchproduktion, die Milchfettkonzentration, das Milcheiweiß usw. wurden nicht negativ beeinflußt. Gesundheitsschäden traten bei Kühen nicht auf (29).

VII. Diskussion

SO_2 entzieht anderen Substraten sehr leicht den Sauerstoff. Es ist also leicht oxidabel. Ferner können verschiedene Stoffe wie Aldehyde und Ketone mit SO_2 Additionsverbindungen bilden. Die Fähigkeit, mit Wasser schweflige Säure zu bilden, und das Entstehen von Sulfiten, Hydrogensulfiten oder auch komplexen Salzen, etwa mit Glucose o. ä., braucht bei der oralen Verabfolgung an Wiederkäuer nicht unbedingt nachteilig ausgelegt zu werden. Auch bei monogastrischen Tieren beobachtete Antithiaminwirkungen — d. h. die Toxizitätssteigerung von SO_2 bei Thiaminmangel — wird beim Wiederkäuer bei ungestörter Pansensymbiose und somit gesicherter Vitamin-B-Synthese eine mildere Beurteilung erfahren müssen. Schließlich bleibt beachtenswert, daß schweflige Säure sofort mit dem im Pansen vorhandenen Dicarbonat und anderen Puffersubstanzen konfrontiert wird.

Tabelle 4
Umrechnungstabelle

Art	Gewicht	SO_2	mg pro kg Körpergewicht	lt. Pansen-Inhalt	mg/Liter	Autor
Rind	ca. 500 kg	80 g	160	ca. 120	0,68	Luedke (25)
Schaf	ca. 45 kg	8 g	177	ca. 25	0,32	Nuvel (26) (27)
Rind (Schnitzel — Übliche Normalmenge)	ca. 500 kg	(max.) 7,73 g				

Die im Pansen ablaufenden Umsetzungsprozesse, die sich als Reduktionsprozesse manifestieren, können dagegen im Rahmen der Wiederkäuerernährung geradezu als willkommenes Adjuvans bei der Schwefelversorgung angesehen werden, zumal die Pansenflora in der Lage ist, Sulfatschwefel und Sulfit zu verwerten, d. h. den Schwefel in Thioaminosäuren einzubauen.

Für eine Überschlagsberechnung im Lichte der Praxisverhältnisse ergibt sich folgendes Bild:

Rübenschnitzel enthalten durchschnittlich 1000 mg SO_2 pro kg als Sulfit, meist jedoch weniger. Ein Rind nimmt somit bei einer Schnitzelgabe von 2,5 kg pro Tier und Tag ca. 2,5 g Sulfit auf. Das entspricht bei einem angenommenen mittleren Gewicht von 500 kg einer oralen Gabe von 5 mg/kg Körpergewicht.

Die Aufnahme von 2,5 kg SO_2 entspricht der Aufnahme von 1,25 g Schwefel.

Aus den vorliegenden Literaturangaben und Versuchsergebnissen ist unschwer zu folgern, daß die Sulfitmenge, die durch Trockenschnitzel Wiederkäuern im Rahmen der Fütterung zugeführt wird — auch bei Dauergabe —, praktisch als unschädlich anzusehen ist. Die absolute aufgenommene Sulfitmenge, gleichgültig in welcher Form, bleibt weit hinter den als akut oder chronisch toxisch zu bezeichnenden Quantitäten zurück. Außerdem ist die quantitative Bemessung möglicher Schädigungsgrenzwerte in Relation zu monogastrischen Tieren physiologisch nicht vertretbar. Während bei solchen Tieren stets eine mehr oder weniger direkte Kontaktmöglichkeit mit den Schleimhäuten des Verdauungstraktes in erheblichem Umfange besteht, trifft SO_2 (Sulfit) beim Wiederkäuer auf die Vorlage des Pansensaftes mit mehr als 100 Litern beim Rind und bis ca. 25 Liter beim Schaf. Dies ist gleichbedeutend mit der Aufnahme in dieses Substrat, d. h. es setzt primär ein Verdünnungseffekt ein. Außerdem erfolgt sofort eine Auseinandersetzung mit den Panseninhaltsstoffen.

Zusammenfassung

An Hand der Angaben im Schrifttum zur Verträglichkeit von Sulfiten (SO_2 usw.) ergibt sich, daß

1. überwiegend die Inhalationstoxizität und nachfolgend die Schleimhautaffektionen Gegenstand der Untersuchungen waren,
2. die Bedingungen an monogastrischen Tieren und Menschen nicht mit denen beim Wiederkäuer gleichgesetzt werden können,
3. der Wiederkäuer wesentlich größere Mengen von Sulfiten toleriert als der Mensch bzw. monogastrische Tiere,
4. das Vormagensystem der Wiederkäuer als Verdünnungs- und Pufferesystem wirkt,
5. im Rahmen einer länger andauernden Zufuhr sulfithaltiger Trockenschnitzel, die üblicherweise ca. 1000 mg SO_2 pro kg enthalten, keine Störungen auftreten, die zu Bedenken Anlaß geben, da gefährdende Grenzwerte ca. 30mal höher liegen.

Summary

From data in the literature on tolerance to sulphites (SO_2, etc.) it is found that

1. Inhalation toxicity has been the dominant theme of investigations, followed by mucous membrane infections,
2. Conditions with monogastric animals and humans cannot be equated with those for ruminants,
3. The ruminant tolerates considerably larger amounts of sulphites than do man and monogastric animals,
4. The pre-rumen system in ruminants acts as a dilution and buffer system,
5. Within the framework of prolonged feeding of sulphite-containing dry pulp, which usually holds about 1000 ppm SO_2, no disorders occur which could give rise to doubts since danger levels are about 30 times higher.

Résumé

Les données bibliographiques au sujet de l'accomodation envers les sulfites (SO_2, etc.) montrent que:

1. La plupart des études avaient comme objet la toxicité pendant l'inhalation et donc les affections de la muqueuse;
2. Les conditions valables pour les animaux monogastriques ou les êtres humains ne peuvent pas être comparées directement à celles s'appliquant aux ruminants;
3. Les ruminants supportent des quantités de sulfite nettement plus élevées que l'homme ou l'animal monogastrique;
4. La panse des ruminants peut opérer comme système de dilution et comme tampon;
5. L'addition à longue durée des pulpes séchées contenant environ 1000 ppm de sulfite, ne provoque pas de perturbations sérieuses parce que les valeurs limites dangereuses se situent environ à des niveaux 30 fois plus élevés.

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食品添加物の消長について（第1報）乾果物中の亜硫酸

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衛生試験所報告　第81号　別刷

1963年10月

Reprinted from Bulletin of National Institute of Hygienic Sciences

No. 81　1963

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考察

諸外国での食用許可色素は毎年変更があるものであり，これらについては浜田[2]，Castang[3]，Banfield[4]らによって報告されている．以上の結果から試料のなかにはわが国で許可されていない着色料が検出されている．輸入業者はこれらの国々の法規についてよく知る必要があると思われる．

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（昭和38年5月31日受付）

食品添加物の消長について（第1報）乾果物中の亜硫酸

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現今わが国には相当多量の乾燥果実類が輸入されている．なかでもアメリカより乾燥あんず，乾燥桃乾燥ブドーの輸入は特に多く，これらの中には添加使用されている亜硫酸の検出量がわが国の法定量をはるかに超えるものがかなりある．

食品添加物としての亜硫酸は乾燥果実類に対してその防腐防酸効果を期待するほか品質保持，すなわち褐変あるいは黒変等の変色防止の目的に用いられ，また製糖工業における砂糖の漂白など広く利用されているものである．わが国の食品衛生法においては，亜硫酸の使用量は乾燥あんずについてはその1kg中2.0gまで，砂糖および乾燥ブドーはその他の食品とともに1kg中0.03g以下に規定されている．

輸入乾燥果実類は諸外国において製造され船舶輸送などの日程を経て消費者に行きわたるまでには諸種の条件を異にする環境下で相当長期間保存を必要とするものである．したがって乾燥果実類の品質保持の面から現行亜硫酸の使用量について検討の必要を生じ実験を行なったのでその結果を報告する．

試料　アメリカ大使館および厚生省食品衛生課の協力を得て，カリフォルニア乾果物協会より，乾燥あんず，乾燥桃の2種について，それぞれ亜硫酸としてお3,000 ppm，2,000 ppm，および1,000 ppm含有するごとく添加し製造したものであって，それらの約200gずつをセロファン袋詰とし，なおこれをボール箱入として船便により輸送されたものである．本試料について試験開始の時期は米国において試料製造後約6カ月目である．

試験方法　各種試料を室温ならびに温度35°，湿度約55%の恒温槽および温度2°の冷蔵庫中にそれぞれ保存し，保存中の外観，色調，水分，亜硫酸含有量などの変化について試験を行なった．

A　色調の試験：細切した試料15gを乳鉢中にとり，50%アルコール200 ml中の一部を加えてよくすりつぶし，300 mlの共栓三角フラスコ中に入れ，50%アルコールの全量を加え，ときどき振り混ぜながら20～22°で24時間放置した後ろ過し検液とする．検液の着色度を光電比色計を用いて430 mμ，1 cmの液層において比色定量することによって着色の度合を測定した．

B　亜硫酸：薬学会協定衛生試験法による

試験成績　試験当初における試料についての外観，臭味などの異状を認めなかった．とくに色相は亜硫酸添加量の高い順に淡色であった．ただし乾燥桃は乾燥あんずに比して着色度高く，なお亜硫酸添加3種の間に色相の差があまり見られなかった．

A　色相の変化　乾燥あんずについて吸光度を測定した結果はFig. 1のごとくである．すなわち室温保存の場合3種の試料とも若干の着色の増加がみられたが大なる変化はなかった．ただし試料1,000 ppmの

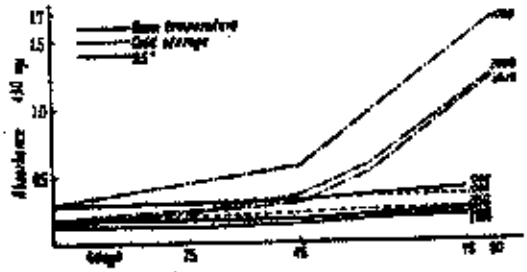

Fig. 1.　Color changes of dried apricots

Iwao Kawashiro, Kohei Kawada, Fumi Fukuzawa, Yutaro Hasegai, Ryuji Amano and Kazuko Takemi
: Studies on Vicissitudes of Food Additives. 1. Determination of Sulfur Dioxide in Dried Fruits.

ものは他の二者に比して色相外観やや不良であった．冷蔵庫中に保存されたものもほぼ同じような結果が得られた．35° に恒温保存の場合は 45 日目頃から急激に変色現象を生じ，著しい着色を示した．なお試料 2,000 ppm と 3,000 ppm との色調の差はほとんど見られなかったが，わずかに試料 3,000 ppm の方が良好であった．

乾燥桃について吸光度を測定した結果は Fig. 2 に示すごとくである．すなわち 35° 恒温保存で 3 種の試料の吸光度はいずれも増加するが三者の間にその色相の差はほとんどみられなかった．乾燥桃の場合は乾燥あんずに比してむしろ試験当初より三者とも著しく着色していた．

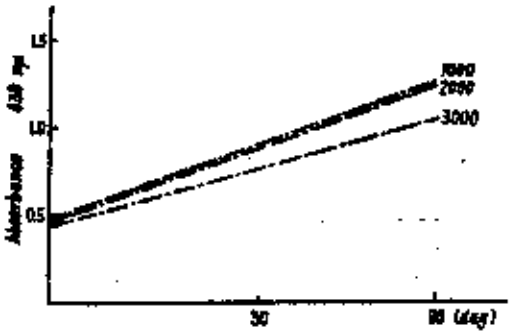

Fig. 2. Color changes of dried peaches

また乾燥あんずの色相検液について吸光度を分光光度計を用いて測定した結果は Fig. 3 のごとくであってその色相は試料 1,000 ppm のものが最も濃厚であった．

B. 亜硫酸定量値　試験当初試料について亜硫酸の定量を行なった結果乾燥あんずの試料 1,000 ppm から亜硫酸として 119 ppm，試料 2,000 ppm 中から 718 ppm，試料 3,000 ppm の中から 1,383 ppm を検出した．

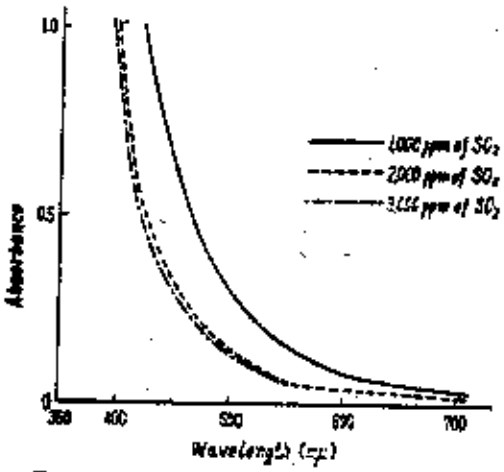

Fig. 3. Absorption spectra of the extract of dried apricots

乾燥桃については同様にして 126, 250 および 470 ppm を検出した．

乾燥あんずの保存中亜硫酸の定量成績は Fig. 4 のごとくであって，室温保存 70 日間においては試料 950 ppm まで減少した

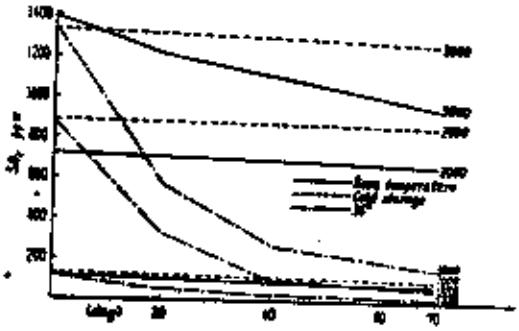

Fig. 4. Sulfur dioxide changes in apricots

35° に保存の試験は 2 回実施した．第 1 回試験の成績は Fig. 4 のごとく 70 日間に試料中の亜硫酸量は 31 ppm, 78 ppm および 156 ppm まで減少した．第 2 回試験の成績もほぼ同様で，40 日間においてそれぞれ 48 ppm, 99 ppm および 210 ppm まで減少した．冷蔵庫中に保存したものも Fig. 4 に示すごとく試料 3 種とも亜硫酸定量値はいずれも減少するが僅少であった．

C. 水分定量値　常法により試料について水分の定量を行なった結果，乾燥あんずの試料 3 種はいずれも水分約 32.5% 付近であり，35 日間室温に保存した場合は 3～5% の減少をみた．

結　語

以上の試験結果を総合すると試料に添加された亜硫酸は保存中減少する傾向があり，保存温度の高いほどその減少は著しい．また乾燥果実は保存中変色しやすく，亜硫酸の添加量の大なるほどその防止に効果的であった．また添加された亜硫酸の減少とともに着色は濃くなる傾向がみられた．

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(昭和38年5月31日受付)