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Methemoglobinemia From Eating Meat With High Nitrite Content

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TEN CASES of methemoglobinemia occurred near New Orleans, La., in October 1955. From epidemiological and laboratory investigations, we determined that they were due to nitrite poisoning which resulted from eating wieners and bologna containing large amounts of the chemical.

Before detailing the circumstances of the outbreak, we shall review briefly the medical aspects of methemoglobinemia and consider the use of nitrites in meat.

Methemoglobin in the Blood

Methemoglobinemia is a condition in which a part of the hemoglobin has been oxidized to methemoglobin with a consequent reduction in the oxygen-combining and oxygen-carrying power of the blood. The oxidation may be caused by absorption, inhalation, or ingestion of large doses of certain chemicals, particularly the nitrites, nitrates, sulfonamides, and aniline derivatives.

Onset of symptoms is sudden, usually within 1 or 2 hours after contact with, or ingestion of, the poison. The symptoms include nausea, vomiting, profuse sweating, and an intense cyanosis of the fingertips, nose, and ears.

Mild cases of methemoglobinemia result when less than 50 percent of the hemoglobin is oxidized to methemoglobin. After 70 to 80 percent is oxidized, there is a progression of ataxia, dyspnea, excessive salivation, prostration, and unconsciousness. Oxidation of 90 percent or more is fatal unless immediate treatment is given (*1*).

A presumptive diagnosis of methemoglobinemia should be made if there is a history of rapid onset of cyanosis without physical findings of cardiac, pulmonary, or intracranial disorder. A history of ingestion of a substance containing nitrites or nitrates within the past few hours aids in establishing the diagnosis. If cyanosis is unrelieved by oxygen therapy and if blood withdrawn from a vein shows the characteristic chocolate-brown coloration, the diagnosis is almost certain (*2*).

A definitive diagnosis of methemoglobinemia can be made with the laboratory procedure reported by Evelyn and Malloy (*3*). This procedure, which uses a single sample of blood from the finger, is an accurate photoelectric method of determining the oxyhemoglobin, methemoglobin, and sulfhemoglobin levels of the blood.

Methylene blue is an effective antidote for toxic methemoglobinemia. The recommended dosages are 1 mg. of methylene blue per kilogram of body weight for adults and 2 mg. of methylene blue per kilogram of body weight for children. These amounts should be injected

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intravenously over a 5-minute period. Dosages should not exceed 7 mg. per kilogram since toxic effects such as dyspnea, precardial pain, restlessness, a sense of oppression, apprehension, and fibrillar tremors may occur (*1*).

Numerous cases of methemoglobinemia have been traced to the consumption of well water containing large amounts of nitrates (*4–7*), contact with aniline dyes used in crayons (*8–11*) and laundry marking ink (*12*, *13*), accidental ingestion of leather dyes and furniture polish (*2*), and ingestion or absorption of varied medications containing hemoglobin oxidizing components (*14–16*). Only a few outbreaks have been reported in which the causative agent was contained in food. One of these was an outbreak in New York City in which 11 aged men who had eaten breakfast at the same cafeteria were affected. Investigation showed that sodium nitrite was used in place of table salt in the seasoning of the oatmeal they had eaten (*17*). Another was an outbreak of acute toxic methemoglobinemia among a group of people who had eaten wheatcakes and "maple sirup" at a diner in Syracuse, N. Y. In this outbreak, investigation revealed that a commercially prepared corning extract containing 8 percent nitrites, 4 percent nitrates, and sucrose was inadvertently served as maple sirup to the affected individuals (*18*).

Nitrites in Meat

Nitrites and nitrates are used in curing meats mainly because of their color-fixing qualities, although these substances also have some preservative properties. When added to meat, sodium nitrate (Chile saltpeter) or potassium nitrate (saltpeter) is reduced to the corresponding nitrite by the action of certain bacteria that occur naturally in the meat and on processing equipment. The nitrites are further broken down by hydrolysis to form nitric oxide. This simple compound combines with the muscle hemoglobin (myoglobin) of the meat to form a more permanent pink substance, nitric oxide myoglobin. This pigment is more stable than the natural pigment found in blood and muscle fibers and is also more resistant to the action of air, heat, and bacteria (*19*).

Federal meat regulations and most of the State food and drug regulations prohibit the use of more than 200 p.p.m. of nitrite in meat products for human consumption. These regulations also require that products containing nitrates and nitrites be labeled to that effect.

The amount of nitrite that can be utilized by meat is limited by the amount of myoglobin originally in the muscle tissues. The addition of excessive amounts of nitrites will not increase or intensify the color, but it may have an adverse effect on the meat.

Circumstances of the Outbreak

The Louisiana State Department of Health first learned of the outbreak of methemoglobinemia in the New Orleans area from a report of two cases from a practicing pediatrician.

The first patient was a 2-year-old girl who became ill about 2 hours after eating 2 wieners. Symptoms, as observed by the child's mother, included profuse sweating, vomiting, and a bluish-gray cyanosis around the lips and nails. The family physician was called immediately, and the child was hospitalized with a presumptive diagnosis of methemoglobinemia. The child responded well to an intravenous injection of methylene blue and was discharged from the hospital the following day.

Specimens of the vomitus, which contained particles of wieners, and samples of wieners obtained from the same package from which the meal was prepared were submitted to the chemical-toxicological laboratory of the State health department for determination of the nitrite content. Chemical analysis showed that the vomitus contained 11.8 p.p.m. of nitrite and that the wieners contained 5,420 p.p.m. of nitrite.

The following day the second case of methemoglobinemia was reported in a 3-year-old boy. Speech disturbance and cyanosis of the lips and nails were observed about 2 hours after the child had eaten 2 wieners manufactured by the same meat plant that prepared the ones eaten by the first patient. The child recovered rapidly after hospitalization and treatment with methylene blue injected intravenously. Samples of the wieners obtained from the same package as those eaten by this child contained 5,420 p.p.m. of nitrite.

The remaining 8 patients exhibited symptoms similar to the first 2, and all responded well to treatment with methylene blue. Seven had eaten wieners and one had eaten bologna, all manufactured at the same plant.

Of the 10 persons ill in this outbreak, 8 were between the ages of 1½ and 5 years. For this group the interval between eating the meat and onset of symptoms was 2 hours. The other 2 patients were 10-year-olds, and the time lapse between eating the meat and onset of symptoms was about 6 hours.

Inspection of the Plant

After the report of the third case and the finding of excessive quantities of nitrite in the wieners, State and local health department sanitarians were notified to place under seizure all retail stocks of wieners manufactured by the meat plant in question. Later, when the case in which bologna appeared to be the cause was reported, some retail stocks of bologna manufactured by this plant were also seized. One or more samples from each lot of meat placed under seizure were sent to the State chemical-toxicological laboratory for nitrite determinations.

In 131 samples of the assorted meat products, nitrite concentrations varied from 3.3 p.p.m. to 6,570 p.p.m. Seventeen of the samples (14 of wieners and 3 of bologna) contained nitrites in excess of the maximum allowable of 200 p.p.m. The lots from which these samples were taken were destroyed by burning.

In an effort to determine the conditions responsible for the excessive amounts of nitrites in these meat products, we subsequently made an inspectional tour of the manufacturing plant. (This plant does not use Federal, State, or local meat inspection services.) The general manager and the sausage production manager of the plant explained each operation in the wiener and bologna manufacturing process, with particular emphasis on the mixing and blending operations.

In preparing wieners or bologna, approximately 400 pounds of coarsely ground meat and meat products are added to a silent cutter, along with ice for cooling, a prepackaged spice preparation, and 1.5 pounds of a commercially prepared powder containing nitrite and nitrate. This powder is composed of approximately 6 percent sodium nitrite, 4 percent sodium nitrate, 87 percent sodium chloride, and 3 percent moisture. After 12 to 20 minutes in the silent cutter, the material is conveyed to the stuffing room, where the artificial casings are filled. Samples of the artificial casing contained only a trace of nitrite.

During our inspection of the spice storage room, we noticed an unopened container of "pure" sodium nitrate powder. On close questioning concerning the use of this powder, the sausage production manager stated that this material was just recently obtained. The commercially prepared nitrite-nitrate powder produced too fast a "cure" on some products, he explained, and he had ordered the "pure" sodium nitrate to correct this difficulty. He knew of no "pure" sodium nitrate compounds ever having been used in the plant.

Since the commercially prepared powder contained only 10 percent sodium nitrite and sodium nitrate, about 36 to 40 pounds of this material would have to be added to a 400-pound batch of meat to provide the concentration of 6,570 p.p.m. found in one sample of wieners. The amount of sodium chloride contained in 36 to 40 pounds of the commercial preparation would have made the wieners very distasteful. Hence, it would seem that a product containing a larger concentration of nitrate or nitrite (or both) than the powder would have had to be used to result in the quantities of nitrite reported by the laboratory.

After our tour of the plant, we obtained information regarding personnel changes during recent months, disposition of broken and imperfect wieners, records of nitrite and nitrate inventories, and routine laboratory controls on the meat products. Investigations of these items, however, did not help us determine how the excessive quantity of nitrite got into the wieners and bologna.

Many safeguards were taken to insure the accuracy of the chemical analyses. Two laboratories in addition to the State chemical-toxicological laboratory analyzed samples of the meat products, and all found similar nitrite concentrations. Laboratory examination of the commercially prepared powder used at the

plant indicated that it did not contain more than the 10 percent sodium nitrite and sodium nitrate indicated on the label. The salt content of the wieners manufactured by the plant was found to be within normal limits.

Laboratory analysis of samples of wieners manufactured by other plants using the same commercial nitrite-nitrate preparation did not show the excessive amounts of nitrites found in the products of the plant under suspicion.

Bacteriological examination of samples of the suspected meat products did not reveal any organisms of the food-poisoning type.

Although we were unable to determine exactly how the additives were applied to the meat products to result in the excessive concentrations of nitrite, it seems most likely that a product containing a large quantity of nitrate or nitrite (or both) was mistakenly added to one or more batches of the wiener and bologna mixtures. Since the inventory controls of the additives were haphazard and the code dating of the meat products was unreliable, we were unable to learn the date that the incident occurred. Had this information been available, we could have directed our investigations to the events of the day in question.

Summary and Conclusion

In October 1955, an outbreak of 10 cases of methemoglobinemia occurred in the New Orleans, La., area. Early evidence pointed to the ingestion of large amounts of nitrite in wieners and bologna as the cause.

Investigation revealed that 9 victims had eaten wieners and 1 had eaten bologna manufactured by the same plant. Laboratory analysis of 131 samples from retail stocks of meat products manufactured by this plant indicated that 17 samples contained nitrite concentrations in excess of 200 p.p.m., the maximum amount permissible under Federal and State regulations. The concentrations ranged up to 6,570 p.p.m. A commercially prepared powder containing 10 percent sodium nitrite and sodium nitrate was used routinely in preparing the wieners and bologna.

Exactly how the excessive quantity of nitrite got into the meat products could not be determined. It seems likely, however, that a product containing a larger quantity of nitrate or nitrite (or both) than the commercially prepared powder had been added by mistake to one or more batches of the wiener and bologna mixtures.

This outbreak demonstrates the need for effective meat inspection services in all meat packing plants. An official meat inspection program should be the aim and eventual goal of every State and municipality. In the meantime, all owners and employees of meat plants should be made aware of the dangers of careless handling of nitrates, nitrites, and other chemical additives.

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Since the occurrence of this outbreak, a similar incident was reported in Florida. Two children, a 4-year-old boy and a 15-month-old girl, from the same household became ill after eating uncooked wieners. The subsequent death of the 4-year-old child has been attributed to eating the uncooked wieners.

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techniques

Soda Fountain School

Employees of Cleveland's 600 drug stores will be trained at the School of Soda Fountain Sanitation operated in the city hall by the Cleveland Division of Health. Graduates will receive a food-handler's button to wear on their uniforms.

Designed to reduce the need for enforcement of sanitary regulations, the school's short course combines audience participation, group discussion, and role playing in four 2-hour sessions. Students are told and shown why a clean, colorful fountain will attract new customers, establish a regular clientele, increase sales for the drug store, and add pennies to their own pockets.

Classes began January 2, 1957. They are open to fountain employees of independent and chain stores throughout the Cleveland area in first-come, first-served order. Owner-manager representatives of the drug stores are encouraged to attend at times not used by their employees. Eventually, the course will be offered to applicants attending high school.

The classroom seats 35. To one side is a fully equipped luncheonette, complete with mechanical dishwashing, refrigeration, and cooking facilities. All of the equipment has been donated by manufacturers and associations. Teaching aides include motion pictures, slides, flip charts, bulletin boards, pamphlets, checklists, and self-inventory sheets. Three regular and two guest instructors conduct the classes and give everyone individual attention when needed.

On completing the course, each graduate receives a permanent number, a card, and a button. Some of the stores have agreed not to employ new help until they have earned the food-handler's button.

Every store sending all of its employees through the course receives a certificate for public display if it also has had no sanitary violations. The certificate is revokable when violations are found.

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THE EFFECTS OF NO_2 AND SALTS OF NO_2 UPON ESTABLISHED CELL LINES[1]

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Abstract

The effects of several concentrations of NO_2, $NaNO_3$, and $NaNO_2$ respectively, upon strain L, mouse liver cells, and HeLa cells, were studied and a modified system designed to permit continuous exposure of cells to air pollutants is described. In NCTC medium 109 containing serum, cells tolerate concentrations of NO_2 up to 4100 p.p.m. and some may even tolerate 8600 p.p.m. Removal of the serum lowers the lethal concentration of NO_2 to less than 100 p.p.m. If the cells were covered only by a thin film of BSS (balanced salt solution) medium, a concentration of 100 p.p.m. NO_2 proved toxic within ½ hour. If, however, the NO_2 concentration was reduced to 5 or 10 p.p.m., cells survived a daily 8-hour exposure but many, if not most, of the cells were dead after several days. The presence of as little as 25 mg% $NaNO_2$ retarded proliferation. On the other hand, $NaNO_3$ was tolerated well in the three cell lines tested; HeLa cells seemed to be the most sensitive of the cell strains with respect to these salts.

Introduction

The great increase in air pollution over the past half century has aroused considerable concern as to its possible harmful effects upon animals and plants. Consequently, extensive studies have been conducted covering many aspects of this problem. Many of the detrimental pollutants found in air are now known (1, 2). They differ widely in kind, number, and quantity from one community to another, depending upon kinds of local industry, type of fuel commonly used, number of automobiles, etc.

Many investigations have been made upon various species or organisms to ascertain their reaction to air pollutants. These include nitrogen dioxide, the gas chosen for this study. NO_2, along with nitric oxide, occurs in oxidant "smog". It plays an important part in photochemical oxidation; in the presence of sunlight it dissociates into ozone and nitric oxide. Very little is known concerning repeated low-grade exposures to NO_2 and, as far as is known, nothing has been reported in regard to the effects of low concentrations on cells cultivated in vitro. Since it is only through organized study at the cellular level that we may hope to gain fundamental knowledge concerning the effects of pollutants, singly or in combination, and since we now have available numerous cloned cell types which are suitable for such studies, the following investigations were made.

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Contribution from the Institute for Cellular Research, Department of Physiology, University of Nebraska, Lincoln, Nebraska. This investigation was supported by grants (RG-6439) from the Division of General Medical Sciences, U.S.P.H.S., and the Cooper Foundation, Lincoln, Nebraska, through the University of Nebraska Foundation.

Materials and Methods

The cell lines employed consisted of strain L (NCTC Clone 929), mouse liver cells (NCTC Clone 1469), and HeLa cells.

Two different culture media were used: one, a "biological medium" containing 40% balanced salt solution, 40% horse serum, and 20% chick embryo extract (1:1); the other, NCTC No. 109 medium supplemented with 10% horse serum (3). This latter medium proved the more desirable for growth and was used exclusively in later experiments.

Replicate cell cultures were prepared as described by Pace and Aftonomos (4). At first, Carrel flasks and, later, modified T-60 flasks were used as culture vessels. To each Carrel flask, 1.0 ml of cell suspension was added; to each T-60 flask, 10.0 ml was added. In each experiment, following a 48-hour adjustment period, the cultures were divided into test and control groups.

Initial and final cell numbers were ascertained either by cell nuclei enumeration as described by Sanford *et al.* (5) or by means of a Coulter counter (6).

Results

(A) Effects of Sodium Nitrate and Sodium Nitrite

Since under ordinary circumstances nitrates or nitrites may be formed when NO_2 is passed through a water solution, the effects of the sodium salts added to the nutrient medium also were observed.

Solutions of $NaNO_3$ and $NaNO_2$ were prepared in the culture medium (NCTC No. 109 plus 10% horse serum) prior to filtration. Concentrations of 200, 100, 50, and 10 mg% were employed. Following the initial adjustment period, the culture media were changed. However, those of the test cultures were replaced with medium containing a known concentration of $NaNO_3$ or $NaNO_2$. The cultures were observed microscopically every 24 hours. Cell counts using the nuclei enumeration procedure were made every 48 hours throughout each experiment. The results are summarized in Table I.

These experiments reveal that proliferation of mouse liver cells exposed to $NaNO_3$ is not adversely affected at concentrations of 200 mg%, or lower. However, with as little as 10 mg% $NaNO_3$, there was some retardation of proliferation in one experiment (in which strain L was used) and in HeLa cells. Growth in all cell lines was reduced at all concentrations of $NaNO_2$ tested. With 100 mg% $NaNO_2$, almost complete inhibition of growth occurred in all cultures of mouse liver and HeLa cells, and in 200 mg%, these cells died within 2 to 5 days. Growth of strain L cells was also retarded considerably in 100 mg%. Little, if any, growth occurred in most cultures of strain L cells at 200 mg%. As might be expected, the lower concentrations, 10 mg% and 50 mg%, evidenced lesser effects on cell multiplication for all cell lines exposed.

(B) Exposure of Cells in "Biological Medium" to NO_2

In preliminary tests it was found that cells cultured in "biological media" could tolerate rather high concentrations of NO_2. Nitrogen dioxide was mixed

TABLE I

A comparison of the effects of various concentrations of sodium nitrate and sodium nitrite on proliferation of strain L, mouse liver, and HeLa cells

Cell type	Length of experiment in days	Initial cell No. × 10^3	Concentration in mg% 0	10	50	100	200
			Final cell number / Initial cell number (growth ratio)				
			Sodium nitrate				
Strain L	7	290	6.2	3.1	5.5	4.5	4.1
Strain L	7	320	5.2	5.2	3.4	3.1	4.2
Mouse liver	7	170	5.4	4.4	5.2	5.0	4.8
Mouse liver	7	250	5.1	4.8	7.8	5.7	6.9
HeLa	6	420	4.5	3.2	3.9	4.0	2.9
HeLa	6	440	4.6	2.6	3.3	3.5	2.9
			Sodium nitrite				
Strain L	7	290	6.2	3.3	2.8	2.4	1.1
Strain L	7	330	4.9	3.3	2.5	2.0	1.2
Strain L	7	320	5.2	4.2	4.2	3.2	3.0
Mouse liver	7	170	5.4	3.4	2.4	1.8	Dead 2nd day
Mouse liver	7	250	5.1	3.5	2.9	0.8	Dead 5th day
HeLa	6	420	4.5	3.4	2.0	0.7	Dead 4th day
HeLa	6	440	4.6	2.3	0.1	Dead 4th day	Dead 2nd day

NOTE: The results are expressed as the ratio of the final cell count compared with the initial cell count. The nutrient medium was Earle's 109 plus 10% horse serum. Each figure represents the average for 10 cultures.

with 5% CO_2 in air to obtain the various desired concentrations. There was surprisingly very little change in pH except at the highest concentrations. Every 48 hours, when the nutrient medium was changed, the cultures (Carrel flasks) were "flushed" for 30 seconds with the gas mixture, or, in the case of the controls, with 5% CO_2 – air. The vessels were then stoppered and placed in the incubator for 48 hours, at which time the procedure was repeated. Immediately prior to each medium change, cultures were randomly selected for cell counts. All cultures were observed microscopically every 24 hours. The results are presented in Table II.

Proliferation was retarded in 8600 p.p.m. and 4100 p.p.m. NO_2, although not as much in the latter concentration as in the former. When exposed to 2400 p.p.m., proliferation was retarded in most of the cultures, although in three experiments with strain L there appears to be an actual stimulation. The same was true in one experiment with mouse liver cells. In still lower concentrations (1500 and 800 p.p.m.) there were no deleterious effects; in fact, there was, at times, an increase in proliferation. In one experiment with strain L and one with mouse liver cells, the synthetic medium (NCTC No. 109 plus 10% horse serum) was used. The results suggest a stimulation of growth at these concentrations when the cells were cultivated in this medium. At least there is no evidence of a retarding action.

TABLE II

The effects of high concentrations of nitrogen dioxide on growth of strain L and mouse liver cells in "biological medium" (40:40:20)

Length of experiment in days	Initial cell No. × 10^3	Concentration of NO_2 in p.p.m. 0	8600	4100	2400	1500	800
		Final cell number / Initial cell number (growth ratio)					
		Strain L cells (NCTC No. 929)					
9	600	9.6	5.4	5.1	5.1		
8	600	7.9	3.6	5.0	8.3		
8	550	7.4	5.4	6.7	10.1		
8	2000	2.9	1.6	1.5	2.6		
8	1800	2.7	1.2	1.8	3.1		
8	1400	3.6	2.8	2.5	3.2		
12	450	7.5				7.4	8.0
12	340	3.4				5.6	3.0
10*	500	5.5				7.8	7.8
		Mouse liver cells (NCTC No. 1469)					
6	430	6.7	5.6	5.2	7.3		
6	410	12.2	4.6	4.9	11.6		
6	500	10.6	3.6	8.8	9.6		
8	450	11.0	1.6	5.5	9.8		
9*	210	8.3				9.6	8.6

NOTE: The results are expressed as the ratio of the final cell count compared with the initial cell count. Each figure represents the average for 10 cultures.
*Cells in medium NCTC No. 109 plus 10% horse serum.

The cells appeared to be "protected" from the effects of NO_2 by the various substances present in horse serum and embryo extract as well as the amino acids in NCTC No. 109, all of which undoubtedly reacted with the NO_2. The layer of nutrient medium covering the cells was sufficiently deep to require relatively considerable quantities of NO_2 before a toxic action was noticed.

(C) Direct Exposure of Cells to NO_2

Since the results of the preceding experiments indicate that there was a "detoxifying" action by actual combination of the gas with the constituents of the nutrient medium, the experimental procedure was altered. Following an initial adjustment period of 48 hours, when the cells had begun to proliferate in the flasks, the nutrient medium was replaced with balanced salt solution and the cells then exposed to the gas.

The T-60 flasks used in these experiments were modified as illustrated in Fig. 1. At the juncture with the upper glass surface and the conical base of the vessel, a small glass tube was attached. Through this tube, by means of a hypodermic needle, liquids could be added or removed aseptically. The neck of the culture vessel was modified into a standard taper, ground-glass joint which permitted an airtight seal with the glass conducting head. The gassing head consisted of a gas inlet tube surrounded by a ground-glass sleeve joint. An outlet tube through which the gas passed from the culture flask was attached at right angles to this outer sleeve.

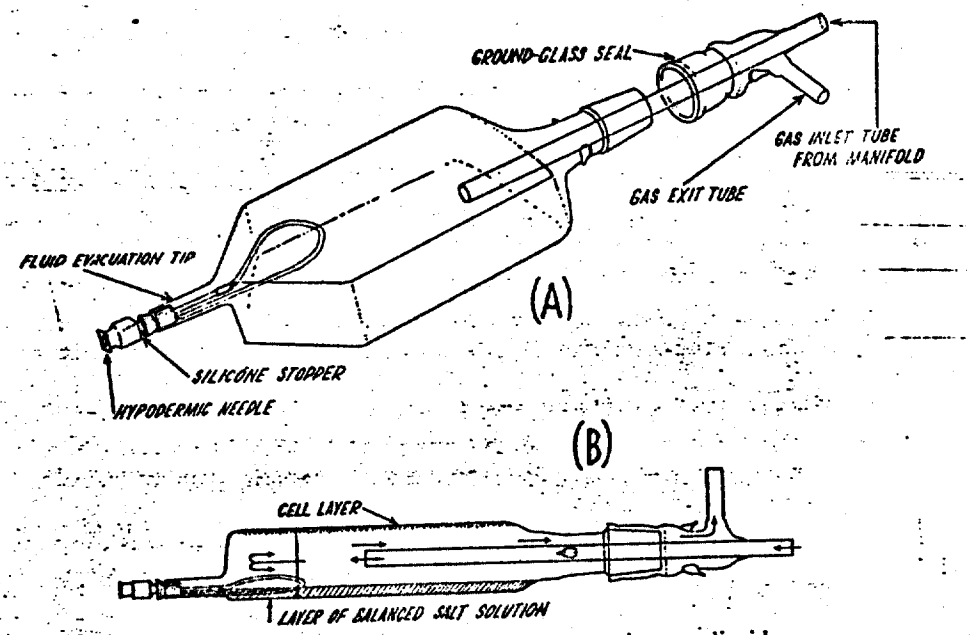

FIG. 1. Modified T-60 flask used in exposing cells to nitrogen dioxide.

The flask became filled with the gas rather rapidly (a volume equal to that of the flask passed through every minute). The cells in the culture flasks were exposed to concentrations of NO_2 ranging from 1000 p.p.m. to 5 p.p.m. The duration and frequency of the exposure varied with each concentration. Control cultures were treated identically, except that 5% CO_2 – air was used instead of the NO_2 mixtures.

Just prior to the exposure period the nutrient medium was removed from the flasks and replaced with sterile balanced salt solution (BSS). The culture vessel could then be inverted so that the cells were covered only by a very thin film of BSS. The BSS, however, remained in the flask and served to maintain a suitable humidity during the gassing interval. Following each exposure period, the cells were rinsed with the BSS.

In order to ascertain the effect of this gaseous pollutant on growth, cell numbers of test and control cultures were compared at the end of each experiment; the number of detached cells and the viability of the cells were noted at the end of each exposure period (7).

Photomicrographs were made of strain L cells exposed to 100 p.p.m. NO_2. The cells were exposed for 2 hours without interruption in the manner described above, after which the BSS was replaced with nutrient medium. Photos of these cells, as compared to cells exposed to CO_2 – air only are shown in Figs. 2A and 2B. These photos were taken about 2 hours after the cells were returned to the nutrient medium. In experiments in which higher concentrations of NO_2 were used, the cells appeared to become fixed and, with both erythrosin B and time-lapse photography, evidence indicated that all the cells were dead.

Similar results were obtained with ½-hour exposures to 100 p.p.m. NO_2, indicating a much greater toxic activity than some of the earlier results (in biological medium) had displayed. Obviously, much lower concentrations of NO_2 were indicated in order to avoid an immediate toxic effect.

Therefore, in the following experiments, the concentrations of NO_2 were reduced to 10 and 5 p.p.m., respectively. The gas mixture flowed through each flask at a rate of 100 to 125 ml per minute. The same was done with control cultures, using CO_2–air mixture.

In the first series of tests the cultures were exposed after a 48-hour adjustment period to 10 p.p.m. NO_2 for a single 8-hour interval. The cells were bathed intermittently every hour by simply inverting the flask for a few seconds. This was done to prevent drying of the thin film of solution covering the cells, although 10 ml BSS was always present in the flask as a further precaution. The results are presented in Table III. Each count is the average value for 15 cultures. The number of dead cells in the test cultures exceeded by far those in control cultures.

In the second series of tests, the cultures were exposed to NO_2 for 8 hours on each of 2 successive days. During the 16 hours between exposures, the BSS solution in the flasks was replaced with nutrient medium.

In the third series, the cells were exposed to NO_2 for 8 hours on 3 successive days with a 16-hour period between each exposure when the cells were covered with nutrient medium.

These experiments evidenced several interesting phenomena. In all the experiments, the final cell population in the experimental groups to some degree exceeded those of control groups. However, at the same time, the total number of dead cells in the experimental groups was 35 to 40 times greater. Perhaps NO_2 had a slight stimulating effect concomitant with a cytotoxic effect. This reduced the number of viable cells in the cultures exposed to 10 p.p.m. NO_2 to below that found in control cultures. The net effect, therefore, was to inhibit cell growth at this concentration and under these conditions. The degree of this inhibition increased with increased length of exposure.

In another group of experiments, the cells were exposed to 5 p.p.m. NO_2. The same procedure was employed as in the preceding; only the concentration of the pollutant was changed. The results of these experiments are also summarized in Table III.

In the first series of tests, the final cell population in the controls slightly exceeded those exposed to 5 p.p.m. NO_2. It is doubtful whether the difference was great enough to be of statistical significance. However, difference in the total number of dead cells was of considerable significance. There were about 25 times more dead cells in the test cultures receiving 5 p.p.m. NO_2 than in control cultures.

In the two series of tests in which cells were exposed continuously to 5 p.p.m. NO_2 for 8 hours for 5 days in succession, there was very little difference in the final cell population counts, but when allowance was made for the number of

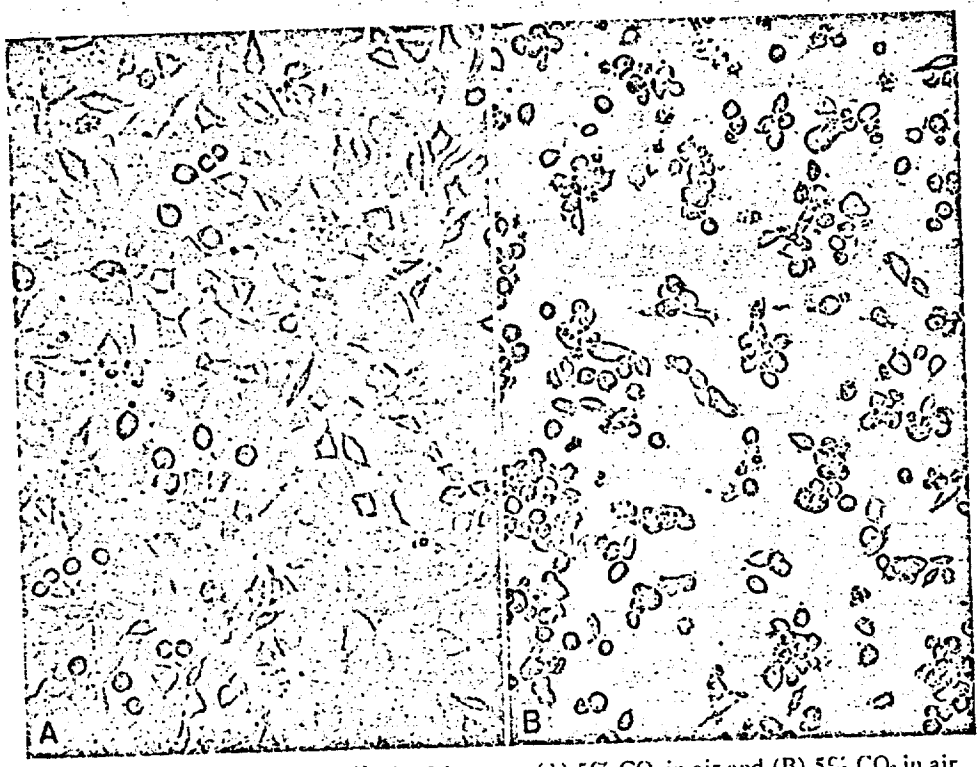

FIG. 2. Strain L cells exposed for 2 hours to (A) 5% CO_2 in air and (B) 5% CO_2 in air plus NO_2, 100 p.p.m. The photographs were taken 2 hours after the NO_2 exposed cells had been returned to the 5% CO_2–air mixture.

TABLE III

Effect of 10 p.p.m. and 5 p.p.m. NO_2, respectively, on strain L cells

Experimental conditions	Number of cells per ml	
	Controls	10 p.p.m. NO_2
1. Exposed 8 hours (1 day)		
Inoculum size	237,500	237,500
Final cell number	346,650	368,820
Number of dead cells	1,500	66,560
Number of living cells	345,150	302,260
2. Exposed 8 hours (2 days in succession)		
Inoculum size	223,650	223,650
Final cell number	503,370	718,290
Number of dead cells	10,160	402,930
Number of living cells	493,210	315,360
3. Exposed 8 hours (3 days in succession)		
Inoculum size	253,420	253,420
Final cell number	651,730	779,370
Number of dead cells	14,640	542,280
Number of living cells	637,090	237,090

Experimental conditions	Number of cells per ml	
	Controls	15 p.p.m. NO_2
1. Exposed 8 hours (2 days in succession)		
Inoculum size	246,350	246,350
Final cell number	425,870	401,620
Number of dead cells	9,120	247,150
Number of living cells	416,750	154,470
2. Exposed 8 hours (5 days in succession)		
Inoculum size	274,220	274,220
Final cell number	1,053,980	1,161,060
Number of dead cells	28,580	665,800
Number of living cells	1,025,400	495,260
3. Exposed 8 hours (5 days in succession)		
Inoculum	281,300	281,300
Final cell number	1,114,580	1,226,150
Number of dead cells	36,400	748,200
Number of living cells	1,078,180	477,950

NOTE: These cells were exposed continuously for 8 hours and then returned to control culture conditions for 16 hours before termination of experiment or re-exposure to the test condition. The numbers below represent the number of cells per ml and each is an average of 15 separate cultures.

dead cells, considerable effect was observed. There were more than 20 times the number of dead cells in cultures exposed to 5 p.p.m. NO_2 than in control cultures. Hence, the total number of viable cells in control cultures was more than twice as great as that observed in cells exposed to NO_2.

Discussion

The primary and almost sole source of NO_2 as found in polluted air is combustion of fuels. It is known to be produced in rather high concentrations in automotive exhausts, especially during acceleration (8). Its role in air pollution is not completely understood. Although it is found in concentrations of only 0.4 p.p.m. in heavy smog, Haagen-Smit believes that it speeds the oxidation of organic material in the presence of sunlight, thus abetting the production of ozone and nitro-ozonides which are considered serious hazards in the Los

Angeles type smog (9). The toxic level of NO_2 for humans is given as 20 p.p.m. The maximum allowable concentration is given as 5 p.p.m. (10).

In investigations in which gases are passed over or through media containing organic substances, especially protein and amino acids, one might expect a reaction or union to take place between some of the constituents and NO_2. However, the degree to which the medium "protected" the cells from the gas was much greater than anticipated. When administered at 48-hour intervals, 8600 p.p.m. NO_2 caused a reduction in proliferation, but 4100 p.p.m. inhibited growth only slightly. At 2400 p.p.m. NO_2 had little, if any, deleterious effect upon proliferation. When the cells were killed by the NO_2, it appeared to "fix" them. It is very doubtful whether changes in pH were directly responsible for the death of the cells except in the highest concentration (8600 p.p.m.), since no serious pH shifts were indicated. The readings were never below pH 7.0. This may be due in part to the relatively high buffering capacity of the medium.

The opinion that serum might serve as a protecting agent has been fairly well substantiated by the results observed in experiments involving direct exposure of the cells. When the serum-containing medium is temporarily removed and the cell sheet washed free of serum with BSS, much lower concentrations of NO_2 affect the cells; for example, a single 30-minute exposure to 100 p.p.m. kills the cells and apparently "fixes" them.

The modified T-60 flask (Fig. 1) proved effective in overcoming some of the difficulties which were apparent with the standard flask in studies on air pollutants. The very thin layer of BSS which covers the cells when the flasks are inverted permits more direct action of the pollutant upon the cells. Spectrophotometric determination suggests that very little reaction occurs between the pollutant gas and the BSS. This is in agreement with the fact that much lower concentrations of NO_2 were effective upon the tissue cells. We believe that this type of experimental procedure is very useful in determining the effects of various gaseous substances on tissue cells. It more nearly simulates the conditions as they exist in the lung passages.

In light of the results obtained in the direct exposure method, it may be assumed that 800, 1500, and perhaps as much as 2400 p.p.m. were ineffective because of a nullification of their action by the presence of serum. This may suggest a mechanism whereby the epithelial lining of the respiratory system is protected from more drastic effects by gases of this type.

Since nitrates and nitrites are probably produced on passing NO_2 through a medium, studies on these salts were undertaken in order to compare with those obtained with the NO_2 gas. As expected, nitrite proved to be much more toxic than nitrate. In fact, liver cells seemed to be very resistant to any ill effects that might be produced by sodium nitrate, although they were as sensitive as other cells to the action of nitrite.

This report does not include studies on cells exposed to hydrocarbons, or to hydrocarbons in the presence of NO_2, or ozone, although such investigations

are to be made. It is felt that before these complex mixtures are evaluated, it was necessary that single components be assayed, in order that proper evaluation of synergistic or antagonistic effects be made.

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THREE FATAL CASES OF SODIUM NITRITE POISONING

LOUIS R. PADBERG, M.D., AND THOMAS MARTIN, M.D.
Coroner and Autopsy Physician, Respectively
ST. LOUIS

Sodium nitrite and other inorganic nitrites are used mainly in the manufacture of dyestuffs and more recently as anti-corrosives in automobile anti-freeze mixtures. Inorganic nitrites are rarely encountered as the cause of human poisoning. It is therefore considered of value to report the following cases:

Three white men, J. N., aged 55, T. H., aged 65, and A. K., aged 70, were inmates of a cheap lodging house near the river front, the location of several wholesale chemical companies, one of which is a large distributor of sodium nitrite. Oct. 27, 1938, at about noon these three men were observed preparing a meal consisting of "beef stew" in the room of J. N. Four hours later the landlady of the house saw A. K. staggering down the stairs; when he reached the bottom he collapsed without making a statement. The landlady stated that the man's face was very pale and that his lips and the areas around the lips were purplish. The City Hospital ambulance was called, and all three men were pronounced dead on arrival at the hospital.

The bodies were immediately taken to the city morgue, where autopsies were performed.

SUMMARY OF AUTOPSIES

Gross examination of the three bodies showed the following conditions in each body:

The skin was very cyanotic, about the color of slate; the blood was thin and did not coagulate and was a dark brown, simulating prune juice.

Specifically the results of the examination in each are recorded as follows:

J. N. was 5 feet 8 inches (173 cm.) tall and weighed 140 pounds (63.5 Kg.). The hair was white and the eyes were blue; the left forearm had been amputated below the elbow. Dense fibrotic adhesions were seen in the right pleural cavity and old fibrosis was present throughout the right lung, particularly in the apex. The capsule of the kidney was adherent and did not strip readily. Other than this there were no gross abnormalities.

T. H. was 5 feet 11 inches (180 cm.) tall and weighed 175 pounds (79.4 Kg.). The hair was white and the eyes were blue. There was edema of the brain and lungs, hypertrophy of the heart, moderate sclerosis of the vessels and degenerative changes within the kidney.

A. K. was 5 feet 10 inches (178 cm.) tall and weighed 180 pounds (81.6 Kg.). The hair was white and the eyes were blue. There was edema of the brain and lungs, hypertrophy of the heart with atheromatous changes in the aorta and sclerosis of the coronary. The arteries were moderately sclerosed and the kidney showed some parenchymatous changes. Other than these abnormalities the gross examination of all viscera except the stomach was negative.

The three deaths occurring almost simultaneously after a meal might have been suggestive of botulism except that the time factor was too short and the food eaten was known to have been cooked. The viscera and blood samples of the three men together with a portion of the "stew," a box of salt and pepper and an ice cream carton containing a yellowish white crystalline substance were submitted to the coroner's toxicologist for chemical examination. Within forty-eight hours the following report was received:

On acid steam distillation of 100 Gm. portions of each portion of stomach and contents there was chemically detected in the dilute sodium hydroxide of the receiver the presence of a nitrite. A portion of this nitrite was subsequently isolated as silver nitrite and so identified. A quantitative analysis was performed on steam distillates from weighed portions of stomach and contents by the method of Lunge. Approximate amounts of nitrite, calculated as sodium nitrite and based on total weights of stomach and contents, are as follows: in J. N., 0.5 Gm. of sodium nitrite; in T. H., 0.7 Gm., and in A. K., 0.1 Gm.

Samples of the blood of the three men were submitted to spectroscopic examination for the presence of methemoglobin: A strong absorption band of methemoglobin (630) was observed at 1:100 dilution, *pH* 7.1, in all three samples.

A portion of food containing meat and tomatoes was submitted to acid steam distillation and the presence of a nitrite was detected in the distillate.

The sample of salt and pepper was examined and found to be sodium chloride and finely ground black pepper.

Seventy-five Gm. of a yellowish white crystalline substance contained in an ice cream carton was submitted to analysis and found to be almost chemically pure sodium nitrite.

A thorough investigation was made as to the motive, but up to the present time these cases are unsolved, i.e. as to whether they were accidental, suicidal or homicidal.

1300 Clark Avenue.

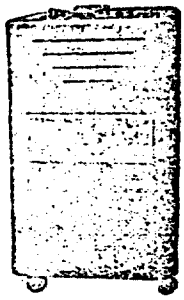
Council on Physical Therapy

THE COUNCIL ON PHYSICAL THERAPY HAS AUTHORIZED PUBLICATION OF THE FOLLOWING REPORTS. HOWARD A. CARTER, SECRETARY.

POLYTHERM SHORT WAVE APPARATUS ACCEPTABLE

Manufacturer: Polytherm, Incorporated, 707 Broadway, Paterson, N. J.

The Polytherm Short Wave Apparatus considered in this report includes the "Advance," "Standard Cabinet" and "Portable" models. These units are identical in circuit and component parts. They are designed for use in medical and minor surgical diathermy. Standard equipment includes pad electrodes, felt spacers and surgical accessories.



Polytherm Short Wave Apparatus.

Two tubes (211-B) are employed in a typical tuned-plate, tuned-grid circuit, operating at approximately 14 meters wavelength. There is a ventilator to dissipate heat from the tubes by convection. The bipolar surgery which is optional on any unit is the introduction of one terminal which is a tap on the output or pick-up coil.

The Council tests showed that with an input of 610 watts and an electromotive force of 120 volts, the maximum output obtainable was 245 watts. After two hours of operation at a maximum input of 610 watts the Council tests demonstrated a final temperature in the outside windings of the transformer which stayed within allowable limits.

The firm submitted evidence to substantiate the efficacy of the machine in producing a temperature rise in the human thigh

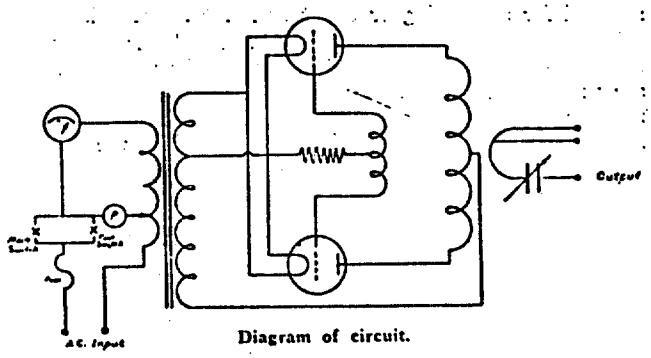


Diagram of circuit.

with the cuff technic. A reliable investigator was secured by the firm to perform these tests. Six tests were carried out and three normal male subjects were used. Cuffs measuring 17¾ by 4¾ inches were wrapped about the thigh, 7½ inches apart. The average spacing was 1⅛ inches for the right and 1¼ inches for the left. Temperature measurements were taken by thermocouples before and after twenty minute treatments. The thermocouples were inserted into the subcutaneous and intramuscular

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INVESTIGATION INTO THE MUTAGENIC EFFECT OF SEVERAL NITROAMINE AND NITROSAMIDE COMPOUNDS (Untersuchungen über die mutagene Wirkung verschiedener Nitrosamin- und Nitrosamid-Verbindungen)

Author: Luise Pasternak

Source: Arzenmittelforschung Vol 14, No. 7, P. 802-804 (1964).

The nitrosamines - which are relatively simple chemical compounds - have become the object of detailed investigations, on account of their special biological activity. Since in 1956, Magee und Barnes (Reference 1), and in 1959 Schmähl and Preussmann (Ref. 2) proved experimentally that different nitrosamines (N.A.) are carcinogenic in rats, studies regarding the mechanism of decomposition in vivo of these substances have been conducted in order to identify the active constituents involved. Dutton and Heath (Ref. 3) detected that the N.A. are first dealkylated, and that the carcinogenic action is produced through products of decomposition. Systematic investigations by Druckrey and Collaborators (Ref. 4) about the chemical constitution and carcinogenic action of these compounds led to the assumption that N.A. can become cancerogenically active only if they have a mobile H-atom in the α-C, so that they can be decomposed into diazoalkane after oxidation of the element in question. Finally a highly reactive carbenium ion develops from the diazoalkane.

The assumption of the described path of decomposition of the N.A. in vivo into an alkylating agent suggested as interesting the investigation of the N.A. with respect to possible mutagenic effects which, for the so far known alkylating substances, belog to the characteristic features involved.

The following N.A. were investigated in Drosophila melanogaster, with respect to a mutagenic action:

1. Dimethylnitrosamine (DMNA)
2. Diethylnitrosamine (DENA). (The two substances exist in the liquid state.

3. Nitrosomethyl urea (NMH), a light yellow substance consisting of fine crystals)

4. Nitrosomethyl urethane (NMU), a slightly pink colored liquid.

The four compounds mentioned possess carcinogenic effect.

5. Ethyl-tertiary-butyl-nitrosamine (E-tert. BNA), a yellowish liquid that is not cancerogenic.

6. Methyl-benzyl-nitrosamine (MBNA), also a yellowish liquid; the substance is cancerogenic.

7. Methyl-vinyl-nitrosamine (MVNA), a red-brown, easily volatile liquid, the vapors of which already possess a carcinogenic action.

8. Methyl-nitroso-piperazine (MNP), a yellowish liquid; the cancerogenicity of this substances is not definitely established.

These compounds, in 5% sugared water, and in concentrations of 0.002% to 0.01% solutions (in accordance with the previously determined compatibility of the substance for Drosophila), were administered in the diet. In order to determine the mutation rate, the Muller-5-test was used in ascertaining the rates of recessively lethal mutations of the X-chromosome of Drosophila.

Male flies of the Berlin-wild-strain were fed for three days on glass filter crucibles according to the method of Lüers (Reference 5) with the substance to be tested. After interruption of the treatment, the males were paired at three days' intervals each time with a new virginal female of the Muller-5-strain. The offsprings of the female in question are called series. The F_2-generations, in which the presence of a recessive lethal factor was evident, were bred through other generations in order to detect the genetic character of the factor.

Results:

A survey of the recessive-sex conditioned mutation rate in Drosophila after treatment with different nitroso compounds is provided in Table 1.

Table 1: Determination of the recessive-sex conditioned mutation rate in Drosophila after treatment with different nitroso compounds.

a. Tested compound b. Concentration % * (* Concentration of the tested substance, administered in the food, in 5% sugared water)
c. Average mutation rate in % of the 1st - 4th series ± 3 m
d. Mutagenic c. Carcinogenic

1. Dimethylnitrosamine was administered as 0.005% solution; higher doses are toxic. In several tests, 3550 X-chromosomes were investigated; they exhibited 181 recessive lethal mutations. The total rate of the mutations of the 1st - 4th series is about 5%. With respect to the controls, this rate is statistically safely established through the triple average error. The mutation rate for the controls, which was determined along with the testing of the 8 substances, is given at the end of Table 1. Altogether 9295 X-chromosomes were evaluated as controls; the number of the mutations observed was 27, the mutation rate is nearly 0.29%.

2. Diethylnitrosamine was fed as 0.01% solution. Among altogether 2911 investigated X-chromosomes of the 1st - 4th series, 135 exhibited regressively lethal mutations, that is, the average mutation rate is 4.6%. The statistical certainty with respect to the controls is satisfactory. In the later series (3rd series) a further increase of the mutation rate up to 8% was observed.

3. Nitrosomethyl urea (a substance which can already liberate diazomethane spontaneously) was administered as 0.01% solution; with 9.1%, it gave the highest mutation rate of the compounds tested. In the concentration given, the dissolved substance possessed a high sterilizing action. As a result, only two series were assessable. The number of the offsprings was considerable reduced. Among the 384 X-chromosomes investigated, 35 mutations appeared. The results are well established statistically.

4. Nitrosomethyl urethane, administered as 0.006% solution, exhibited a low mutation rate. Among 2135 investigated X-chromosomes, 12 mutations were found. The mutation rate thus is 0.56%; through the triple mean error, the result cannot be established with certainty as compared with the controls; certainty can be determined only through the simple average error. Thus the result in this case is not quite sure. Tests are repeated.

5. Ethyl-tertiary-butyl-nitrosamine, a nitrosamine without any carcinogenic effect (probably this substance is not decomposed in vivo) exhibited, after administration as 0.006% solution, no mutagenic properties either. Altogether 2739 X-chromosomes were investigated. In this test, it was possible to determine only 5 mutations. As compared with the controls, the result cannot be determined through error statistics, that is, there is a definite lack of mutagenic action.

6. Methyl-benzyl-nitrosamine was also fed as 0.006% solution. In 819 investigated X-chromosomes, 26 mutations were observed. Thus the mutation rate during the first three series is 3.2%. The determination through error statistics exist.

7. Methyl-vinyl-nitrosamine was fed as 0.0002% solution. Among 1095 investigated X-chromosomes, 37 mutations were counted. The mean mutation rate, thus, is 3.34%. The results if determined by error statistics with respect to the controls.

8. Methyl-nitroso-piperazine was administered as 0.005% solution. 1901 X- chromosomes were investigated and 77 mutations were found. The mutation rate for the three series observed is 3.04%. With respect to the controls, the result is well established by error statistics.

Discussion:

Since the first tests (Pasternak 1962, Ref. 6) indicated a marked mutagenic action of dimethylnitrosamine (DMNA) in Drosophila, 7 other N.A. or nitrosamine-like substances were tested. According to Table 1, up to the ethyl-tertiary-butyl-nitrosamine, all the compounds are mutagenic. On the basis of the mutagenic effect of differently built N.A. with the same fudamental body it could be concluded that this effect is caused by an identical product of decomposition.

For the decomposition of DMNA in vivo, Magee and Hultin (Ref. 7) as well as Magee and Farber (Ref. 8) were able to conclude that after administration to rats, an enzymatic, oxidative demethylation occurs in the lever. Since the mononitrosamine formed, however, is instable, diazomethane and possible other products of decomposition formed must be of decisive importance for the carcinogenesis. The striking parallism, visible in the Table, between mutagenic carcinogenic effects raises the problem of the role of diazolkanes developed from the N.A. for the mutagenesis.

Already in 1948, Rapoport (Ref. 8) reported about mutagenic properties of diazomethane in Drosophila. Diazomethane is connected with the mutagenic substances that exert their effect through alkylating reactions.

The mutagenicity testing of other substances (NMH. NMU), which without any enzyme participation, gave diazomethane already under a slightly alkaline condition, was also positive, as is shown by the tests described. The pronounced mutagenicity observed for NMH is supported by tests conducted by Rapoport (Ref. 10), who through nitrosoethyl urea released also a very high mutation rate in Drosophila. It must be assumed that niazomethane develops from the nitroso urea compounds through decomposition in vivo. For nitrosomethyl urethane, the same conditions seem to exist also. In 1948 already, Rapoport (Ref. 9) observed in Drosophila after treatment with NMU 1% recessive sex conditioned lethal mutations. Zelterberg (Ref. 11) found that the substance is mutagenic for Ophistoma, and Kihlmann (Ref. 12) produced in Vicia faba root tips radiomimetic effects. Mutagenic effects are produced in saccharomyces not only by NMU and NMH, but also by other alky-acyl-nitrosamides, as was observed by Marquardt and Collaborators (Ref. 13) Nevertheless, for nitrosamines, which do not hydrolize, but are no doubt decomposed enzymatically, it was not possible to determine any mutations in saccharomyces.

The necessity of the decomposition of the N.A. is demonstrated also by the results obtained with the noncancerogenic ethyl-tertiary-butyl-nitrosamine. In the opinion of Druckrey and Collaborators (Ref. 4), this compound cannot be decomposed. The lack of mutagenic action confirms the presumption that the differently built nitrosamines act through definite joint products of decomposition. Hence it must be concluded that in the case of the Drosophila there must be a mechanism of decomposition for the N.A., taking place through an oxidative demethylation. This process would very much resemble the one occurring in the rat liver. According to Magee and Farber (Ref. 8) and according to Heath (Ref. 14), the actual carcinogenic factor is assumed to be an alkylating agent, the carbenium-ion, which develops from the diazoalkane. In the case of the mutation ~~solution~~ release through nitrosamines - after their decomposition in vivo - it must be concluded that an alkylatin product of decomposition is formed. The literature contains a large number of biochemical investigation concerning the mechanism of mutations through different alkylating agents. Lawley (Ref. 15) was able

through UV spectroscopy and ion exchange chromatography to detect methylations of the nucleinic acids, for instance through N-mustard gas compounds in the N-7-atom of guanine. Magee and Farber (Ref. 8) showed that after an injection of ^{14}C labeled DMNA in rats the isolated nucleinic acids exhibit predominantly a labeling of the 7-methyl guanine. According to Lawley and Brookes (Ref. 16) N-7-methyl guanine possesses the property to split a H atom of the six-membered ring, which makes possible a new bonding with the opposite base. The additional new and free bonding is the occasion of a false base conjugation, that is instead of a cytosine, a thymine is added by the methylated guanine. In consequence of a further DNS doubling, a G-C pair is replaced by a A-T- pair.

In addition to the hypothesis just described involving a mutation release through base methylation, the triester formation of the phosphate (Ross, Ref. 17) in the nucleinic acid is discussed with respect to alkylating agents. The triesters formed are unstable. Through hydrolysis, fractions (breaks) can appear in the sugar phosphate chain; under certain conditions, this can cause a loss of definite genetical data (Röhrborn, Ref. 8). For the products of decomposition of nitrosamines no reaction with the phosphate groups has been detected.

In addition to the investigations of nitroso compounds, described in this study, other substances are known to possess a sumultaneously mutagenic (Röhrborn, Ref 18) and cancerogenic (Badger, Ref. 19) action. What is involved in this case, except for the latter substances, are compounds that are regarded as belonging to the known groups of the alkylating agents, hence S- and N-mustard gas, ethylenimines, epoxides, as well as β-propiolactone, for which Roberts and Warwick (Ref. 20) observed reactions with the N-7-atom in guanine.

For alkaloids also, the fundamental bodies of which consist of a pyrrolizidine, a parallelism of mutagenic and cancerogenic action is present. For instance, Senecio-alkaloids exert a mutagenic action in Drosophila, according to Clark (Ref. 21). After administration to mammals, liver tumors develop by the same alkaloids. Culvenor and others (Ref. 22) assume also for these substances in the case of liver damage an alkylation mechanism.

Carcinogenicity and mutagenicity are also known in the case of 4-nitro-quinoline oxid. Nakao and Morioka (Ref. 23) report the mutagenic action of the substance in Drosophila, for which, since 1957, carcinogenicity has been known through the study of Nakahara and others (Ref. 24).

Regarding a general parallelism of carcinogenic and mutagenic action it remains to be seen whether other carcinogenic substances, for instance compounds from the hydrocarbon series, for which so far no mutagenicity has been confirmed, exert a mutagenic action in the proper object of experimentation and with the use of suitable methods. However, the N.A. in this respect could form an exception, since their decomposition leads to the development of alkylating compounds, for which carcinogenic as well as mutagenic actions are known.

Arzneimittelforschung 14: 802-804 (1964)

Aus dem Institut für experimentelle Krebsforschung (Direktor: Professor Dr. A. Graffi). Abteilung für Genetik (Komm. Leiter: Dr. E. Geißler) der Deutschen Akademie der Wissenschaften zu Berlin, Berlin-Buch

Untersuchungen über die mutagene Wirkung verschiedener Nitrosamin- und Nitrosamid-Verbindungen

Herrn Professor Dr. H. Druckrey zum 60. Geburtstag gewidmet

Von Luise Pasternak

Die Nitrosamine, die chemisch relativ einfache Verbindungen darstellen, sind auf Grund ihrer besonderen biologischen Wirksamkeit zum Gegenstand eingehender Untersuchungen geworden. Nachdem 1956 von Magee u. Barnes [1] sowie 1959 von Schmähl u. Preussmann [2] der experimentelle Beweis erbracht wurde, daß verschiedene Nitrosamine (N. A.) bei Ratten carcinogen sind, wurden zu Identifizierung der wirksamen Komponenten Arbeiten über den in vivo Abbaumechanismus dieser Substanzen durchgeführt. Dutton u. Heath [3] wiesen nach, daß die N. A. zunächst dealkyliert werden und die carcinogene Wirkung durch Abbauprodukte zustande kommt. Systematische Untersuchungen von Druckrey u. Mitarb. [4] über die chemische Konstitution und carcinogene Wirkung dieser Verbindungen führten zu der Hypothese, nach der nur solche N. A. carcinogen wirksam werden können, die über ein bewegliches H-Atom am α-Kohlenstoff verfügen und dadurch nach Oxydation desselben zum Diazoalkan abgebaut werden können. Aus dem Diazoalkan entsteht schließlich ein hochreaktives Carbenium-Ion.

Die Annahme des beschriebenen Abbauweges der N. A. in vivo zu einem alkylierenden Agens, ließ es interessant erscheinen, die N. A. auf etwaige mutagene Wirkungen zu untersuchen, die für die bisher bekannten alkylierenden Substanzen zu den charakteristischen Merkmalen gehören.

Folgende N. A. wurden an *Drosophila melanogaster* auf mutagene Wirkung untersucht:

1. das Dimethylnitrosamin (DMNA),
2. das Diäthylnitrosamin (DENA),
   beide Substanzen kommen in flüssigem Zustand vor.
3. der Nitrosomethylharnstoff (NMH),
   eine hellgelbe, aus feinen Kristallen bestehende Substanz.
4. Das Nitrosomethylurethan (NMU), eine leicht rosa gefärbte Flüssigkeit.

Sämtliche vier angeführten Verbindungen besitzen carcinogene Wirkung.

5. Das Äthyl-tertiär-butyl-nitrosamin (Æ-tert. BNA),
   eine gelbliche Flüssigkeit, die nicht carcinogen ist.
6. Das Methyl-benzyl-nitrosamin (MBNA),
   ebenfalls eine gelbliche Flüssigkeit; die Substanz ist carcinogen.
7. Das Methyl-vinyl-nitrosamin (MVNA),
   eine rotbraune, leicht flüchtige Flüssigkeit, deren Dämpfe bereits carcinogene Wirksamkeit besitzen.
8. Das Methyl-nitroso-piperazin (MNP),
   eine gelbliche Flüssigkeit, die Carcinogenität dieser Substanz ist nicht sicher erwiesen.*)

Sämtliche Verbindungen wurden in Konzentrationen von 0,002%igen bis 0,01%igen Lösungen, entsprechend der vorher bestimmten Verträglichkeit der Substanz für *Drosophila* in 5%igem Zuckerwasser verfüttert. Zur Bestimmung der Mutationsrate wurde der Muller-5-Test angewendet, durch den die Raten rezessiv letaler Mutationen des X-Chromosoms von *Drosophila* ermittelt werden.

Männliche Fliegen des Berlin-wild-Stammes wurden für die Dauer von drei Tagen auf Glasfiltertiegeln nach der Methode von Lüers [5] mit der zu testenden Substanz gefüttert. Nach Absetzen der Behandlung wurden die Männchen in dreitägigen Abständen mit jeweils einem neuen virginen Weibchen des Muller-5-Stammes gepaart. Die Nachkommen der betreffenden Weibchen werden als Serien bezeichnet. Die F_1-Generationen, bei denen das Vorhandensein eines rezessiven Letalfaktors angenscheinlich war, wurden über weitere Generationen gezüchtet, um den genetischen Charakter des Faktors nachzuweisen.

*) Herrn Professor Dr. H. Druckrey, der uns die unter 5–8 aufgeführten Substanzen freundlicherweise zur Verfügung stellte, möchten wir an dieser Stelle herzlich danken.

Ergebnisse

Die Übersicht über die rezessiv-geschlechtsgebundene Mutationsrate bei *Drosophila* nach Behandlung mit verschiedenen Nitrosoverbindungen ist in Tab. 1 dargestellt.

1. Das Dimethylnitrosamin wurde als 0,005%ige Lösung verabfolgt; höhere Dosen wirkten toxisch. In mehreren Versuchen wurden 3330 X-Chromosomen untersucht, die 181 rezessive Letalmutationen zeigten. Die Gesamtrate der Mutationen der 1.–4. Serie beträgt ca. 5%. Sie ist statistisch gegenüber den Kontrollen durch den 3fachen mittleren Fehler gut gesichert. Die Mutationsrate für die Kontrollen, die parallel zur Testung der 8 Substanzen durchgeführt wurde, ist am Schluß der Tab. 1 zusammengefaßt dargestellt. Insgesamt wurden 9295 X-Chromosomen als Kontrollen ausgewertet, die Zahl der beobachteten Mutationen betrug 27, die Mutationsrate liegt bei 0,29%.

2. Das Diäthylnitrosamin wurde als 0,01%ige Lösung verfüttert. Von insgesamt 2911 untersuchten X-Chromosomen der 1.–4. Serie zeigten 135 rezessiv le-

Tab. 1: Bestimmung der rezessiv-geschlechtsgebundenen Mutationsrate bei *Drosophila* nach Behandlung mit verschiedenen Nitroso-Verbindungen.

a gestestete Verbindung	b Konz.*) %	c durchschnittl. Mutationsrate in % der 1.–4. Serie ± 3 m	d Mutagen	e Carcinogen
Dimethylnitrosamin $O{=}N{-}N(CH_3)_2$	0,005	5,09 ± 1,08 (1.–4. S.)	+ +	+ +
Diäthylnitrosamin $O{=}N{-}N(C_2H_5)_2$	0,01	4,63 ± 1,17 (1.–4. S.)	+ +	+ +
Nitrosomethylharnstoff $O{=}N{-}N(CH_3){-}C(=O){-}NH_2$	0,01	9,11 ± 4,50 (1.–2. S.)	+ +	+ +
Nitrosomethylurethan $O{=}N{-}N(CH_3){-}O{-}C(=O){-}C_2H_5$	0,006	0,562 ± 0,34 (1.–4. S.)	+	+ +
Äthyl-tertiär-butyl-nitrosamin $O{=}N{-}N(C_2H_5){-}C(CH_3)_3$	0,006	0,18 ± 0,24 (1.–4. S.)	–	–
Methyl-benzyl-nitrosamin $O{=}N{-}N(CH_3){-}CH_2{-}C_6H_5$	0,006	3,18 ± 1,84 (1.–3. S.)	+ +	+ +
Methyl-vinyl-nitrosamin $O{=}N{-}N(CH_3){-}CH{=}CH_2$	0,0002	3,38 ± 1,64 (1.–3. S.)	+ +	+ +
N-Methyl-N'-nitrosopiperazin	0,005	4,04 ± 1,56 (1.–3. S.)	+ +	?
Kontrollen		0,29 ± 0,03		

*) Konzentration der verfütterten getesteten Substanz in 5% Zuckerlösung.

tale Mutationen, d. h. die durchschnittliche Mutationsrate beträgt 4,6%. Die statisische Sicherung gegenüber den Kontrollen ist gut. In den späteren Serien (5. Serie) wurde ein weiterer Anstieg der Mutationsrate bis auf 8% beobachtet.

3. Der Nitrosomethylharnstoff (eine Substanz, die bereits spontan Diazomethan freisetzen kann) wurde als 0,01%ige Lösung verabfolgt; sie ergab mit 9,1% die höchste Mutationsrate der getesteten Verbindungen. Bei der gegebenen Konzentration besaß die gelöste Substanz eine starke sterilisierende Wirkung. Als Folge konnten nur zwei Serien ausgewertet werden. Die Zahl der Nachkommen war stark reduziert. Unter den 384 untersuchten X-Chromosomen traten 35 Mutationen auf. Die Ergebnisse sind statistisch gut gesichert.

4. Das Nitrosomethylurethan, als 0,006%ige Lösung verabfolgt, zeigte eine niedrige Mutationsrate. Unter 2133 untersuchten X-Chromosomen wurden 12 Mutationen gefunden. Die Mutationsrate beträgt danach 0,56%, durch den 3fachen mittleren Fehler ist das Ergebnis gegenüber den Kontrollen nicht abzusichern, die Sicherung ist nur durch den einfachen mittleren Fehler möglich. Demnach ist das vorliegende Ergebnis nicht eindeutig. Wiederholungsexperimente werden durchgeführt.

5. Das Äthyl-tertiär-butyl-nitrosamin, ein Nitrosamin ohne carcinogene Wirkung (wahrscheinlich wird diese Substanz in vivo nicht abgebaut) zeigte nach Verfütterung als 0,006%ige Lösung auch keine mutagenen Eigenschaften. Es wurden insgesamt 2739 X-Chromosomen untersucht. Bei diesem Versuch konnten nur 5 Mutationen festgestellt werden. Gegenüber den Kontrollen ist das Ergebnis fehlerstatistisch nicht zu sichern, d. h. das Fehlen der mutagenen Wirkung ist eindeutig.

6. Das Methyl-benzyl-nitrosamin wurde ebenfalls als 0,006%ige Lösung verfüttert. Bei 819 untersuchten X-Chromosomen wurden 26 Mutationen beobachtet. Danach beträgt die Mutationsrate während der ersten drei Serien 3,2%. Die fehlerstatistische Sicherung ist vorhanden.

7. Das Methyl-vinyl-nitrosamin wurde als 0,0002%ige Lösung verfüttert. Unter 1095 untersuchten X-Chromosomen wurden 37 Mutationen gezählt. Die durchschnittliche Mutationsrate beträgt demnach 3,34%. Das Ergebnis ist fehlerstatistisch gegenüber den Kontrollen gesichert.

8. Das Methyl-nitroso-piperazin wurde als 0,005%ige Lösung verabfolgt. 1906 X-Chromosomen wurden untersucht und 77 Mutationen gefunden. Die Mutationsrate für die beobachteten drei Serien beträgt 3,04%. Gegenüber den Konrollen ist das Ergebnis fehlerstatistisch gut gesichert.

Diskussion

Nachdem die ersten Versuche (Pasternak 1962 [6]) eine ausgeprägte mutagene Wirkung des Dimethylnitrosamins (DMNA) bei *Drosophila* ergaben, wurden 7 weitere N. A. bzw. nitrosaminähnliche Substanzen getestet. Aus Tab. 1 geht hervor, daß bis auf das Äthyl-tertiär-butyl-nitrosamin alle Verbindungen mutagen sind. Auf Grund des mutagenen Effektes verschieden gebauter N. A. mit gleichem Grundkörper könnte gefolgert werden, daß diese Wirkung durch ein identisches Abbauprodukt hervorgerufen wird.

Für den Abbau des DMNA in vivo konnten Magee u. Hultin [7] sowie Magee u. Farber [8] folgern, daß nach Verfütterung an Ratten eine enzymatische, oxydative Demethylierung in der Leber stattfindet. Da das entstehende Mononitrosamin aber instabil ist, müssen Diazomethan und möglicherweise weiterhin entstehende Zerfallsprodukte von entscheidender Bedeutung für die Carcinogenese sein. Die aus der Tabelle ersichtliche, auffällige Parallelität zwischen mutagener carcinogener Wirkung läßt nach der Rolle entstehender Diazolkane aus den N. A. für die Mutagenese fragen.

1948 wurde bereits von Rapoport [9] über mutagene Eigenschaften des Diazomethans bei *Drosophila* berichtet. Das Diazomethan wird jenen mutagenen Substanzen zugeordnet, die ihre Wirkung über alkylierende Reaktionen ausüben.

Die Mutagenitätsprüfung anderer, ohne Enzymbeteiligung bereits unter schwach alkalischer Bedingung Diazomethan liefernder Substanzen (NMH, NMU) verlief — wie aus den beschriebenen Versuchen hervorgeht — ebenfalls positiv. Die beobachtete starke Mutagenität des NMH ist durch Versuche von Rapoport [10] gestützt, der durch Nitrosoäthylharnstoff auch eine sehr hohe Mutationsrate bei *Drosophila* auslöste. Es ist anzunehmen, daß aus den Nitrosoharnstoffverbindungen durch Zerfall in vivo Diazomethan entsteht. Auch für Nitrosomethylurethan scheinen die gleichen Verhältnisse vorzuliegen. Bereits 1948 beobachtete Rapoport [9] bei *Drosophila* nach Behandlung mit NMU 1% rezessiv-geschlechtsgebundene Letalmutationen. Zelterberg [11] fand, daß die Substanz für *Ophistoma* mutagen ist und Kihlmann [12] erzeugte an *Vicia faba* Wurzelspitzen radiomimetische Effekte. Bei Saccharomyces wirken neben NMU und NMH noch weitere Alkyl-acyl-nitrosamide nach Beobachtungen von Marquardt u. Mitarb. [13] mutagen. Jedoch für Nitrosamine, die nicht hydrolysieren sondern enzymatisch abgebaut werden müssen, konnten bei Saccharomyces keine Mutationen festgestellt werden.

Für die Notwendigkeit des Abbaues der N. A. sprechen nicht zuletzt die Ergebnisse, die mit dem nichtcancerogenen Äthyl-tertiär-butyl-nitrosamin erhalten wurden. Diese Substanz kann nach Ansicht von Druckrey u. Mitarb. [4] nicht abgebaut werden. Das Fehlen der mutagenen Wirkung bestätigt die Vermutung, daß die verschieden gebauten Nitrosamine über bestimmte gemeinsame Abbauprodukte wirken. Also ist zu folgern, daß bei der *Drosophila* ein Abbaumechanismus für die N.A. vorliegen muß, der über eine oxydative Demethylierung erfolgt. Dieser Prozeß wäre demjenigen in der Rattenleber sehr ähnlich. Nach Magee u. Farber [8] sowie nach Heath [14] wird als eigentliche carcinogene Wirkform ein alkylierendes Agens, das Carbenium-Ion, welches aus dem Diazoalkan entsteht, angenommen. Auch bei der Mutationslösung durch Nitrosamine — nach deren Abbau in vivo — darf gefolgert werden, daß ein alkylierend wirkendes Abbauprodukt gebildet wird. In der Literatur sind zahlreiche biochemische Untersuchungen über den Mechanismus von Mutationen durch verschiedene alkylierende Agentien zu finden. Lawley [15] konnte durch UV-Spektroskopie und Ionenaustauschchromatographie Methylierungen der Nucleinsäuren z. B. durch N-Lostverbindungen am N-7-Atom des Guanins nachweisen. Magee u. Farber [8] zeigten, daß nach Injektion von ^{14}C-markiertem DMNA in Ratten die isolierten Nucleinsäuren überwiegend eine Markierung des 7-Methylguanins aufweisen. Nach Lawley u. Brookes [16] besitzt N-7-Methylguanin die Eigenschaft ein H-Atom des Sechserringes abzuspalten, wodurch eine neue Bindungsmöglichkeit zur gegenüberliegenden Base ausgebildet wird. Die zusätzliche neue freie Bindung gibt zu einer falschen Basenpaarung Anlaß, d. h. statt eines Cytosins wird vom methylierten Guanin ein Thymin angelagert. Als Folge einer weiteren DNS-Verdoppelung wird ein G-C-Paar durch ein A-T-Paar ersetzt.

Außer der soeben beschriebenen Hypothese einer Mutationsauslösung durch Basenmethylierung wird für alkylierende Agentien noch die Triesterbildung des Phosphats (Ross [17]) in den Nucleinsäuren diskutiert. Die entstandenen Triester sind instabil; durch Hydrolyse können Brüche in der Zuckerphosphatkette auftreten, wodurch es unter Umständen zum Verlust bestimmter genetischer Informationen (Röhrborn [18]) kommen kann. Für die Abbauprodukte

der Nitrosamine ist jedoch eine Reaktion mit den Phosphatgruppen nicht nachgewiesen worden.

Neben den in der vorliegenen Arbeit beschriebenen Untersuchungen an Nitrosoverbindungen sind weitere Stoffe mit gleichzeitig mutagener (Röhrborn [18]) und cancerogener (Badger [19]) Wirkung bekannt. Es handelt sich dabei mit Ausnahme der letzten Substanzen um Verbindungen, die zu den bekannten Gruppen der alkylierenden Agentien gerechnet werden, also S- und N-Lost, Äthylenimine, Epoxyde sowie um das β-Propiolacton, für das Roberts u. Warwick [20] Reaktionen mit dem N-7-Atom am Guanin beobachteten.

Aber auch für Alkaloide deren Grundkörper aus einem Pyrrolizidin besteht, ist eine Parallelität von mutagener und cancerogener Wirkung vorhanden. Zum Beispiel wirken *Senecio*-Alkaloide bei *Drosophila* nach Clark [21] mutagen. Nach Verfütterung an Säugetiere entstehen durch die gleichen Alkaloide Lebertumoren. Culvenor u.a. [22] nehmen auch für diese Substanzen bei der Leberschädigung einen Alkylierungsmechanismus an.

Carcinogenität und Mutagenität ist ebenfalls für 4-Nitro-chinolinoxyd bekannt. Nakao u. Morioka [23] berichten über die mutagene Wirkung der Substanz bei *Drosophila*, für die bereits seit 1957 durch Nakahara u.a. [24] die Carcinogenität bekannt ist.

Bezüglich einer allgemeinen Parallelität von carcinogener und mutagener Wirkung bleibt abzuwarten, ob auch andere carcinogene Substanzen, z. B. Verbindungen aus der Reihe der Kohlenwasserstoffe, für die bisher keine Mutagenität festgestellt wurde, im geeigneten Versuchsobjekt und bei Anwendung entsprechender Methoden mutagen wirken. Jedoch könnten die N. A. in dieser Hinsicht eine Ausnahme bilden, da ihr Abbau zur Entstehung alkylierender Verbindungen führt, für die sowohl carcinogene als auch mutagene Wirkungen bekannt sind.

Zusammenfassung

1. Die carcinogenen Verbindungen Dimethylnitrosamin und Diäthylnitrosamin induzierten nach Verfütterung an Männchen des Berlin-wild-Stammes von *Drosophila melanogaster* eine Erhöhung der rezessiv-geschlechtsgebundenen Mutationsrate von 0,2% auf etwa 5%. Es wird angenommen, daß die Nitrosamine die mutagene Wirkung nach enzymatischem Abbau in vivo über einen Alkylierungsmechanismus ausüben.
2. Unter gleichen methodischen Bedingungen konnte auch für Nitrosomethylharnstoff sowie Nitrosomethylurethan, die als Carcinogene bekannt sind, mutagene Wirkung festgestellt werden. Beide Substanzen setzen spontan Diazomethan frei, das als alkylierende Verbindung Mutationen auslösen kann.
3. Das nicht carcinogene Äthyl-tertiär-butyl-nitrosamin zeigte im Mutationsversuch keinen Effekt. Die Substanz wird in vivo wahrscheinlich nicht abgebaut.
4. Drei weitere getestete Verbindungen, das Methylbenzyl-nitrosamin, das Methyl-vinyl-nitrosamin und das N-Methyl-N'-nitrosopiperazin, erwiesen sich nach Verfütterung an *Drosophila* ebenfalls als mutagen. Unter leicht alkalischen Bedingungen in vivo zerfallen diese Nitrosamide unter Bildung von Diazomethan bzw. Carbeniumionen.
5. Aus den Versuchen wird gefolgert, daß die Nitrosamine nach Abbau in vivo ihre mutagene Wirkung über alkylierende Abbauprodukte ausüben. Es wird auf die Parallelität von mutagener und carcinogener Wirkung bei den Nitrosaminen und Nitrosamiden hingewiesen. Die Mechanismen der Mutagenese durch alkylierende Verbindungen sowie der Krebsentstehung durch Nitrosamine werden diskutiert.

Die Möglichkeit eines gleichen molekularen Mechanismus für beide Prozesse wird für die Nitrosamine angenommen.

Summary

Investigation into the Mutagenic Effect of Several Nitrosamine and Nitrosamide Compounds

1. The carcinogenic compounds dimethylnitrosamine and diethylnitrosamine, after having been fed to males of the Berlin-wild strain of *Drosophila melanogaster*, induced an increase in the mendelian recessive mutation rate of males from 0,2% to approx. 5%. It is assumed that the nitrosamines exert their mutagenic effect after enzymatic degradation in vivo via an alkylation mechanism.
2. Under identical methodological conditions, mutagenic effects could be demonstrated in regard to nitrosomethylurea and nitrosomethylurethane — both of which are known carcinogens. Both these substances are subject to spontaneous liberation of diazomethane, which, as an alkylating compound, may trigger mutations.
3. Ethyl-tertiary-butylnitrosamine — which has no carcinogenic action — did not have any effect in mutation experiments. Probably this substance is not broken down in vivo.
4. Three more substances subjected to this test — methyl-benzyl-nitrosamine, methyl-vinyl-nitrosamine and N-methyl-N'-nitroso-piperazine — also proved to have mutagenic effects after having been fed to *Drosophila*. Under slightly alkaline conditions in vivo, these nitrosamides disintegrate under formation of diazomethane or carbenium ions.
5. From the results of these investigations it is concluded that the nitrosamines — after breakdown in vivo — exert their mutagenic effect via alkylating breakdown products. The parallelism between mutagenic and carcinogenic effects of nitrosamines and nitrosamides is pointed out. The mechanism of nitrosamines is discussed.

The possibility of an identical molecular mechanism for both processes is considered feasible in regard to nitrosamines.

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ACUTE METHEMOGLOBINEMIA INDUCED IN AN ADULT THROUGH POTASSIUM NITRATE INGESTION

Comments on an Observation (Méthémoglovinémie aiguë de l'adulte par ingestion de nitrate de potasse. A propos d'une observation)

Authors: J. Paty, J.-C. Favarel-Garrigues, D. Michon, I. Fénelon and L. Arné

Source: Bordeaux Médical, No. 5, May 1970, P.1293-1301.

Cases of toxic methemoglobinemia through the ingestion of nitro derivatives are known especially in children (impurities in drinking water, fertilizer residues, some food additives, some drugs, etc.). They are much rarer in adults. In this connection, the observation conducted in the case of Mrs. Tour is especially interesting.

Observation:

Mrs. Annette Tour, 31 years old, a dairy worker, is hospitalized in Professor Arné's ward on September 4, 1968 because of a coma with intense cyanosis, toward 7:00 PM.

For several months, she had felt tired and was subject to accesses of "dizziness". She dozed off after the noon meal. A few hours later, people around her began feeling uneasy about her deep sleep and the coloration exhibited.

This is the case of a young woman in a coma vigil state. There is intense and generalized cyanosis which, in this sunburnt person, assumes a slate-grey shade. The lips, ears and nails are very blue.

The examination reveals no respiratory insufficiency.

The coma is not very deep and is without any respiratory troubles. On the other hand, no digital hippocratism is noted. The 100 tachycardia and a slight mesocardiac systolic souffle, only slightly irradiating, do not involve any cardiopathy as to an explanation of the existing state.

No signs of phebities exist either.

At the neurological level, the vertebral column is flexible; the osteo-tendon reflexes are quick and diffused, with the presence of osteoperiostitis in the lower members.

No further information is provided by the rest of the examination.

To sum up: Coma and intense cyanosis without any respiratory distress or cardiac attack.

The first aid, in this case, consists in the watching, usual with patients of this type, and in the administration of O through a mask. The patient wakes up rapidly.

An interrogation is then started for the possible absorption of toxic substances.

It is learnt that for the last two years the patient has been taking K nitrate tablets for the purpose of "treating" a premenstrual syndrome (?). The relative ineffectiveness of the product induced the patient to take up to 50 tablets per month (ingested in 3 to 4 days).

On the day of her hospitalization, the patient had just started a new "treatment" by taking 3 g of K nitrate. Since the morning, the only food she had taken was about one litre of milk (1'76 pints). A few hours later, after an intense asthenia had developed, she lost consciousness.

Finally, it is reported to us that about two weeks before people around the patient had been alarmed by a brief cyanosis occurrence, and that this dairy worker was in the habit of absorbing daily from one to two litres of raw milk.

No member of her family exhibits any abnormal coloration of the teguments or of the mucous membranes.

After finding that the blood of the veins is of a brownish shade that is not modified by contact with O, one diagnoses a toxic methemoglobinemia through ingestion of nitrates.

The spectrophotometric quantative determination of methemoglobin by the Kplan method confirm the diagnosis: 50% of the total hemoglobin are transformed (Figure 1).

The administration of vitamin C (3 g intraveinously) and of cytochrome causes the abnormal coloration to disappear within one hour.

The other biological examinations revealed nothing abnormal: blood count, alkaline reserve, renal balance, glycemia, kaliemia, pH of the aerated blood, $PaCo_2$, PaO_2, SaO_2, etc.

Later on, the evolution of the case was rapid and very favorable:

- 12% only of methemoglobin at the end of 24 hours, then, after a complementary treatment with methylene blue, a normal rate was found on the following days.

- Disappearance of every abnormal clinical signs, inclusive of the slight systolic souffle found at the time of the hospitalization.

- Normal electroencephalogram on the following day.

- Normal blood count, reticulocyte rate and bilirubinemia on the fifth day.

- No modification of the electrophoresis of the proteins, of the induced hypergly cemia test, of the kidney and liver examinations.

A more thorough erythrocyte and hemoglobin study was undertake a little later (Professor Bentégeat's Laboratory) as well as an investigation of enzymatic anomalies. Through these examinations it was possible to rule out the existence of a congenital methemoglobinemia.

- Investigation of M hemoglobin: negative.

- Diaphoresis I rate: normal (34 units in the patient, 40 in the control.

- Normal resistance of the blood cells to hypotonic solutions.

- Glucose-6-phosphate-dehydrogenase with 315 units for 10^9 blood cells (no enzymatic defficiency).

- Definitely normal hemoglobin electrophoresis (especially, no elevation of the fetal hemoglobin).

It was not possible to study the patient's disgestive flora, before she left on September 12, after recovery.

Some unusual features of this observation should be emphasized:

- Nitrate poisoning rarely results in so marked methemoglobinemia in adults.
- The acute attack described here occurred in a patient who was in the habit of ingesting K nitrate.
- The milk diet played probably an important part; we shal come back to this point.

Before explaining the physiopathology and the methods of diagnosis relative to nitrate poisonings, we would like to make a brief survey of the symptomatology involved in methemoglobinemias.

The Symptoms of Methemoglobinemias:

These diseases are characterized by cyanosis. In toxic methemoglobinemias, it appears suddenly in the lips, ears and extremities when 10% of the total hemoglobin have been transformed. The greyish or slate-bluish shade is very different from that of true cyanoses.

The signs of visceral pain are connected with hypoxie, since the methemoglobin is unable to transport the O:

- Tiredness and dizziness from 20%;
- Cardiovascular signs for higher rates. Tachycardia, increase of the cardiac rate (output) (Bodawsky, Lenègre), transitory systolic souffle, anomalies in the electrocardiogram (Scotti);
- Consciousness troubles from 50%.

Although heart or brain injuries during methemoglobinemias are rare and are normally tolerated even by aged patients (Goulon), death can occur through anoxia. (Lethal rate between 70 and 85% in animal and man according to authors).

Diagnosis of Methemoglobinemias:

In every case of cyanosis of undetermined origin, one should think of this diagnosis. When a venous puncture is performed, if the methemoglobin rate is sufficient, the blood obtained is chocolate-brown, but the serum is clear if there is no associated hemolysis.

If the blood, brought into contact with O (Nelson's method) fails to recover its color, the diagnosis of methemoglobinemia is quite probable.

Through spectrophotometry, it is possible to reveal and to quantitatively determine methemoglobin in the blood (aerated or venous blood). This is the Kaplan technique used at the laboratory of the Ressucitation Center of Bordeaux (Professor Néverre, A. Brachet-Liermain and D. Michon).

Different techniques are involved in the methods used in identifying and in quantitatively determining methemoglobin:

- Through the electrophoresis at pH 9.2, it is possible to separate the hemoglobin from the methemoglobin. Since this method is relatively time consuming, it is not used in toxic methemoglobinemia cases. Furthermore, the quantitative determination of methemoglobin by this technique lacks precision.

- The reference method is represented by the quantative determination of methemoglobin by the gasometric technique using the Van Slyke device. Being time consuming and exacting, this method is not widely used.

- The techniques based on the spectrum properties of hemoglobin and methemoglobin are much more utilized.

At pH 6.60 the spectra of these two pigments assume the following appearance:
The oxygenated hemoglobin exhibits two peaks at 540 and 575 mμ and a minimum at 560.
The methemoglobin exhibits two peaks at 500 and 635 mμ.
Finally, for the same total hemoglobin concentration, the two spectra intersect at two points called "isobestic" at 525 and 595 mμ.

According to the methemoglobin percentage, the spectrum will assume an intermediate aspect.

Whereas, for very high methemoglobin rates, the trend of this spectrum is characteristic, it can be modified for rates lower than 20%.

It will be always useful, however, to obtain the spectrum in order to eliminate:

- Either another pigment derived from the hemoglobin, such as the sulfhemoglobin, a compound exhibiting an absorption maximum at 620 mμ, unchanged by cyanide addition;

- Or a methemoglobinemia with abnormal hemoglobin (Hb M), exhibiting an absorption maximum at 600 mμ, especially visible if the total amount of hemoglobin has been transformed into methemoglobin. In this case, one obtains a mixture of methemoglobin A and of methemoglobin M.

Starting with this spectrum, many methods have been proposed:

. The method of Evelyn and Malloy uses the absorption at 634 mμ.

. We prefer to use the Kaplan technique, based on a two wavelength measurement (575 and 525). The value of the ratio of these two wavelengths (575/525) is proportional to the methemoglobin per cent. Hence, it suffices to refer to a standardization curve, obtained for several control bloods.

If it has been impossible to find a toxic cause, it will be wise to examine the blood cell enzymes responsible for the methemoglobin reduction, by performing a quantative determination of diaphorase I, or a methemoglobin reversion test in the presence of different substrates.

<u>The Physiopathology of Methemoglobinemias:</u>

Methemoglobin derives from hemoglobin through the <u>oxidation</u> of Fe contained in the heme. Because of this Fe(III), hemoglobin is unable to transport O.

Methemoglobin, although constantly formed in the organism, never excees 0.20 g for 100 ml of blood (1.50 %) under normal conditions, since it undergoes constant reduction through H-providing enzymatic systems.

The main system consists of the two (oxidized and reduced) forms of methemoglobin reductase or diaphorase I, which draws its H from the $NADH_2$ formed during anaerobic glycolysis. The absence of diaphorase I is responsible for the congenital methemoglobinemias (Coudounis; Leng-Lévy and collaborators).

An accessory system, that is not functional in the physiological state, is started by the introduction into the organism of a electron carrier such as methylene blue. This system draws its H from the $NADPH_2$ provided by the aerobic glycolysis (pentose path). The leukoderivative formed through reduction of the methylene blue acts on a methemoglobin reductase II which itself reduces the methemoglobin.

Finally, some reducing agents, such as ascorbic acid or reduced glutathion, can act directly on methemoglobin.

Some poisons which produce methemoglobin disturb the enxymatic reactions of glycolysis. Other oxidizers act directly on the methemoglobin by exceeding the physiologic reducing system.

The action of the nitrites might be double:

- Direct oxidation of hemoglobin with methemoglobin formation through the nitrohemoglobin (Jung and collaborators);
- Inactivation of myoglobin through the formation of methemyoglobin and nitroso myoglobin (Musil and collaborators).

Factors Facilitating Nitrate Poisoning:

Several medicamental nitrated compounds can start a methemoglobinemia (Ag nitrate, Bi nitrate and subnitrate, Ammonium nitrate, Na nitrate, Na nitrites and K nitrites, etc).

The preliminary transformation of nitrates into nitrites is necessary for the formation of methemoglobin (Cornblath and Hartmann). This is what happens in methemoglobinemia through drinking water containing nitrates (well-water, some city water) (Comly; Verger and collaborators; Aussanaire and collaborators), or through the absoprtion of nitrogenized fertilizer residues. Now the action of

nitrifying flora is possible only at a pH exceeding 5. The development of this flora in the high part of alimentary tube is possible only if there is a deficiency of gastric acidity or a trouble in the passage involved, so that the nitrates are not re-absorbed before being transformed into nitrites.

Several facilitating factors must be considered:

- Presence of fetal hemoglobin, more sensitive to oxidation (Richard, 1963). Such a mechanism seems already to be impossible in children (Verger and Mullon).
- Presence of hypoglycemia. Although a glucosed serum perfusion lowers the nitritemia (increase of glycolysis or reducing action of glucose), such a mechanism seems to be secondary in nitrate poisoning.
- Immaturity of the physiological enzymatic systems which reduce the methemoglobin. Such a mechanism, sometimes alluded to in the case of infants, seems to be quite inconstant.

On the other hand, two other factors appear to play an important part in the formation of methemoglobin through nitrate absorption: the insufficiency of gastric acidity and the intervention of certain germs.

Experimentally, Cornblath and Hartmann have showed that only children with insufficient gastric acidity can develop a methomoglobinemia based on nitrates. Now, Bouissou and Claverie have showed that between two feeding times, the infant's stomach does not have the time to contain free HCl which would oppose microbial pullulation in the high part of the alimentary tube. Feeding with cow milk tends also to alkalinize the medium. We should remember that our patient used to ingest daily a large amount of milk (dilution of the gastric secretions and alkalinization of the alimentary bolus).

The effects exerted by some germs is now better known: sulfhemoglobinemia of septicemias with perfringens, sulf and methemoglobinemia of certain intestinal colibacilloses (Fandre and collaborators; Ferrant and collaborators; Zanesco; J.-P. Bergel; Aussanaire and collaborators). Many intestinal germs reduce the nitrates into nitrites; salmonellas, brucellas and shigellas, enterococci, proteus, klebsiella aerobacter, etc). One recently insisted on the role of

which are currently present in the form of spores in milk and can develop, even in acid medium (Saxl; Knotek and collaborators; M. Merlio; Verger and collaborators).

Thus it is certain that a milk diet can facilitate poisoning through nitrates owing to Subtil bacilla. This might have happen in the case of our patient who, because of disgestive troubles, had ingested only milk on the day when poisoning occurred. This would explain the development of methemoglobinemia in a person accustomed to intake K nitrate without any trouble.

Summary P. 1302.

N° 5. — Mai 1970.

MÉTHÉMOGLOBINÉMIE AIGUË DE L'ADULTE PAR INGESTION DE NITRATE DE POTASSE

A PROPOS D'UNE OBSERVATION

par MM. J. PATY, J.-C. FAVAREL-GARRIGUES, D. MICHON, I. FÉNELON et L. ARNÉ *

Les méthémoglobinémies toxiques par ingestion de dérivés nitrés sont surtout connues chez l'enfant (impuretés dans l'eau de boisson, résidus d'engrais, certains additifs alimentaires, certaines drogues, etc.). Elles sont beaucoup plus rares chez l'adulte et, à ce titre, l'observation de Mme Tour... est particulièrement intéressante.

OBSERVATION

Mme Tour... Annette, trente et un ans, laitière, est hospitalisée dans le service de M. le Professeur Arné, le 4 septembre 1968, pour coma avec cyanose intense, vers 19 heures.

Fatiguée depuis plusieurs mois, sujette à des « vertiges », elle s'était assoupie après le repas de midi. Quelques heures plus tard, l'entourage s'inquiétait de la profondeur du sommeil et de la coloration de la malade.

On se trouve en présence d'une femme jeune en coma vigil. On est surtout frappé par une cyanose intense, généralisée, qui, chez cette personne hâlée, donne une teinte gris ardoise. Les lèvres, les oreilles et les ongles sont très bleus.

L'examen ne montre aucun signe d'insuffisance respiratoire.

Le coma est peu profond, sans troubles respiratoires. Par ailleurs, on ne note pas d'hippocratisme digital et, s'il existe une tachycardie à 100 et un petit souffle systolique mésocardiaque de tonalité douce, irradiant peu, il ne paraît pas qu'une cardiopathie rende compte de cet état.

Il n'existe pas non plus de signes de phlébite.

Sur le plan neurologique, le rachis est souple, les réflexes ostéo-tendineux vifs sont diffusés, avec présence d'ostéopériostés aux membres inférieurs.

Le reste de l'examen n'apporte aucun autre renseignement.

En résumé : Coma et cyanose intense sans détresse respiratoire ni atteinte cardiaque.

Le traitement d'urgence comporte simplement, en plus de la surveillance habituelle de tels malades, l'administration d'oxygène au masque. On assiste à un réveil rapide.

On peut alors rechercher par l'interrogatoire une éventuelle absorption de toxiques.

On apprend alors que, depuis deux ans, la malade absorbe des comprimés de nitrate de potassium, pour « traiter » un syndrome prémenstruel (?). L'inefficacité relative du produit l'a amenée à prendre jusqu'à 50 comprimés par mois (pris en 3 à 4 jours).

Le jour de son hospitalisation, la malade venait de commencer un nouveau « traitement » par la prise de 3 g de nitrate de potassium. Pour toute alimentation, elle avait absorbé, depuis le matin, environ un litre de lait. Quelques heures plus tard, après l'installation d'une asthénie intense, elle perdait conscience.

Enfin, on nous rapporte qu'un bref épisode de cyanose avait alarmé l'entourage environ quinze jours plus tôt et que cette laitière avait l'habitude d'absorber, tous les jours, un à deux litres de lait cru.

Aucun membre de sa famille ne présente de coloration anormale des téguments et des muqueuses.

Après avoir constaté que le sang veineux présente une teinte brunâtre non modifiée par contact avec l'oxygène, on porte un diagnostic de méthémoglobinémie toxique par ingestion de nitrates.

Le dosage spectrophotométrique de la méthémoglobine par la méthode de Kaplan confirme le diagnostic : 50 % de l'hémoglobine totale sont transformés (*fig. 1*).

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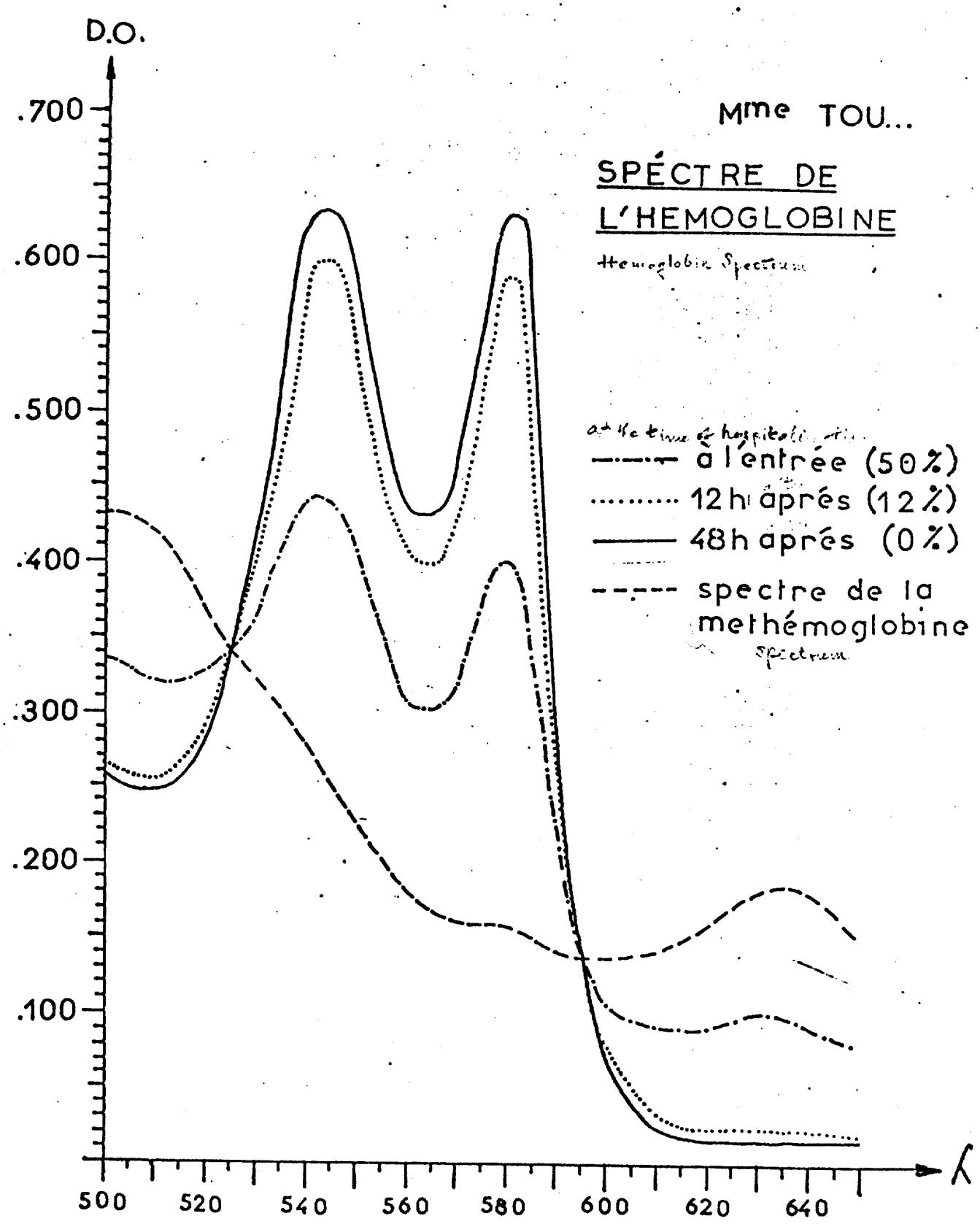
* Communication présentée à la Société de médecine et de chirurgie de Bordeaux, séance du 21 janvier 1970.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Mme TOU...
SPÉCTRE DE L'HEMOGLOBINE
Hemoglobin Spectrum
à l'entrée (50%)
12h aprés (12%)
48h aprés (0%)
spectre de la methémoglobine
spectrum

L'administration de vitamine C (3 g intraveineux) et de cytochrome entraîne la disparition de la couleur anormale en une heure.

Les autres examens biologiques n'ont rien montré d'anormal : numération globulaire, réserve alcaline, bilan rénal, glycémie, kaliémie, *p*H du sang artériel, PaCo2, PaO2, SaO2, etc.

L'évolution fut, par la suite, rapide et très favorable :

— 12 % seulement de méthémoglobine au bout de 24 heures, puis, après traitement complémentaire au bleu de méthylène, un taux normal les jours suivants.

— Disparition de tous les signes cliniques anormaux, y compris le petit souffle systolique signalé à l'entrée.

— Electroencéphalogramme normal le lendemain.

— Numération globulaire, taux de réticulocytes, bilirubinémie normaux au cinquième jour.

— Pas de modification de l'électrophorèse des protéines, de l'épreuve d'hyperglycémie provoquée, des examens rénaux ou hépatiques.

Une étude plus complète du globule rouge et de l'hémoglobine fut entreprise un peu plus tard (laboratoire du Pr Bentégeat) ainsi que la recherche d'anomalies enzymatiques. Ces examens permirent d'éliminer une méthémoglobinémie congénitale.

— Recherche d'hémoglobine M négative.

— Taux de la diaphorase I normal (34 unités chez le malade, 40 chez le témoin).

— Volume globulaire normal.

— Résistance normale des hématies aux solutions hypotoniques.

— Glucose-6-phosphate-déshydrogénase à 315 unités pour 10^9 hématies (pas de tare enzymatique).

— Electrophorèse de l'hémoglobine strictement normale (en particulier, pas d'élévation de l'hémoglobine fœtale).

Il n'a pas été possible d'étudier en détail la flore digestive de cette malade avant son départ le 12 septembre, guérie.

Soulignons quelques traits inhabituels de cette observation :

— L'intoxication par les nitrates conduit rarement à une méthémoglobinémie aussi importante chez l'adulte.

— L'accident aigu décrit ici est survenu chez une malade absorbant habituellement du nitrate de potasse.

— L'alimentation lactée a probablement joué un rôle important sur lequel nous reviendrons.

Avant d'exposer la physiopathologie et les méthodes de diagnostic des intoxications par les nitrates, nous rappellerons brièvement la symptomatologie des méthémoglobinémies.

SYMPTOMES DES METHEMOGLOBINEMIES

Ces malades sont *cyanosés*. Cette cyanose, qui apparaît brutalement, dans les méthémoglobinémies toxiques, aux lèvres, aux oreilles et aux extrémités, lorsque 10 % de l'hémoglobine totale ont été transformés, a une tonalité grisâtre ou ardoisée très différente de celle des cyanoses vraies.

Les signes de souffrance viscérale sont liés à l'hypoxie, car la méthémoglobine ne peut transporter d'oxygène :

— Fatigue et vertiges à partir de 20 % ;

— Signes cardio-vasculaires pour des taux supérieurs. Tachycardie, augmentation du débit cardiaque (Bodawsky, Lenègre), souffle systolique transitoire, anomalies de l'électrocardiogramme (Scotti) ;

— Troubles de la conscience à partir de 50 %.

Bien qu'il y ait rarement de lésions cardiaques ou cérébrales au cours des méthémoglobinémies, qui sont bien supportées, même par des personnes âgées (Goulon), la mort peut survenir par anoxie. (Taux léthal compris entre 70 et 85 % chez l'animal et chez l'homme selon les auteurs).

DIAGNOSTIC DES METHEMOGLOBINEMIES

Il faut penser à ce diagnostic devant toute cyanose d'origine indéterminée. A la ponction veineuse, si le taux de méthémoglobine est suffisant, on recueille un sang brun chocolat, mais le sérum est clair en l'absence d'hémolyse associée.

L'absence de recoloration du sang mis en contact avec de l'oxygène (méthode de Nelson) est très en faveur du diagnostic de méthémoglobinémie.

Par *spectrophotométrie*, il est possible de mettre en évidence et de doser la méthémoglobine dans le sang (artériel ou veineux). C'est la technique de Kaplan, utilisée au laboratoire du Centre de réanimation respiratoire de Bordeaux (Pr Néverre, A. Brachet-Liermain et D. Michon).

Les méthodes d'identification et de dosage de la méthémoglobine font appel à différentes techniques :

— L'électrophorèse à *p*H 9,2 permet de séparer l'hémoglobine de la méthémoglobine. Cette méthode est relativement longue et, de ce fait, peu adoptée en cas de méthémoglobinémie toxique. De plus, le dosage de la méthémoglobine par cette technique est imprécis.

— Le dosage de la méthémoglobine par la technique gazométrique utilisant l'appareil de Van Slyke représente la méthode de référence. Mais elle est longue, délicate et, par conséquent, d'un usage peu courant.

— Beaucoup plus utilisées sont les techniques reposant sur les propriétés spectrales de l'hémoglobine et de la méthémoglobine.

A *p*H 6,60, les spectres de ces deux pigments se présentent de la façon suivante :

L'hémoglobine oxygénée présente deux pics à 540 et 575 mμ et un minimum à 560.

La méthémoglobine présente deux pics à 500 et 635 mμ.

Enfin, pour une même concentration en hémoglobine totale, les deux spectres se coupent en deux points appelés points isobestiques à 525 et 595 mμ.

Suivant le pourcentage de méthémoglobine, le spectre montrera un aspect intermédiaire.

Si, pour des taux très importants de méthémoglobine, l'allure de ce spectre est caractéristique, il est peu modifié pour des taux inférieurs à 20 %.

Cependant, il sera toujours utile de le réaliser pour éliminer :

— soit un autre pigment dérivé de l'hémoglobine, sulfhémoglobine par exemple, composé qui montre un maximum d'absorption à 620 mμ, inchangé par l'addition de cyanure ;

— soit une méthémoglobinémie à hémoglobine anormale (Hb M), qui présente un maximum d'absorption à 600 mμ, surtout visible si l'on a transformé la totalité de l'hémoglobine en méthémoglobine. On obtient alors un mélange de méthémoglobine A et de méthémoglobine M.

A partir de ce spectre, de nombreuses méthodes ont été proposées :

⊙ La méthode de Evelyn et Malloy utilise l'absorption à 635 mμ.

⊙ Nous préférons utiliser la technique de Kaplan, basée sur la mesure à deux longueurs d'onde (575 et 525). La valeur du rapport de ces deux longueurs d'onde (575/525) est proportionnelle au pourcentage de méthémoglobine. Il suffit donc de se reporter à une courbe d'étalonnage réalisée sur plusieurs sangs témoins.

Si aucune cause toxique n'a pu être mise en évidence, il conviendra d'explorer les enzymes globulaires responsables de la réduction de la méthémoglobine en effectuant un dosage de diaphorase I ou une épreuve de réversion de la méthémoglobine en présence de différents substrats.

PHYSIOPATHOLOGIE DES METHEMOGLOBINEMIES

La méthémoglobine dérive de l'hémoglobine par *oxydation* du fer contenu dans l'hème. Ce fer ferrique rend l'hémoglobine impropre au transport de l'oxygène.

La méthémoglobine est formée de manière constante dans l'organisme, mais, à l'état normal, il n'y en a jamais plus de 0,20 g pour 100 ml de sang (1,50 %). En effet, la méthémoglobine est réduite au fur et à mesure de sa formation par des systèmes enzymatiques donneurs d'hydrogène.

Le système principal est constitué par les deux formes, oxydée et réduite, de la méthémoglobine réductase ou diaphorase I, qui tire son hydrogène du $NADH_2$ formé au cours de la glycolyse anaérobie. L'absence de diaphorase I est responsable des méthémoglobinémies congénitales (Coudounis ; Leng-Lévy et coll.).

Un système accessoire, non fonctionnel à l'état physiologique, est mis en route par l'introduction dans l'organisme d'un transporteur d'électrons comme le bleu de méthylène. Ce système prend son hydrogène au $NADPH_2$ fourni par la glycolyse aérobie (voie des pentoses). Le leuco-dérivé formé par réduction du bleu de méthylène agit sur une méthémoglobine réductase II qui réduit elle-même la méthémoglobine.

Enfin, certains corps réducteurs, comme l'acide ascorbique ou le glutathion réduit, peuvent agir directement sur la méthémoglobine.

Certains poisons méthémoglobinisants perturbent les réactions enzymatiques de la glycolyse. D'autres oxydants agissent directement sur la méthémoglobine en débordant le système réducteur physiologique.

L'action des nitrites serait double :

— Oxydation directe de l'hémoglobine avec formation de méthémoglobine par l'intermédiaire de nitro-hémoglobine (Jung et coll.) ;

— Inactivation de la myoglobine par formation de méthémyoglobine et de nitrosomyoglobine (Musil et coll.).

FACTEURS FAVORISANT L'INTOXICATION PAR LES NITRATES

Divers composés nitrés médicamenteux peuvent entraîner une méthémoglobinémie (nitrate d'argent, nitrate et sous-nitrate de bismuth, nitrate d'ammonium, nitrate de soude, nitrites de sodium et de potassium, etc.).

La transformation préalable des nitrates en nitrites est indispensable à la formation de méthémo-

globine (Cornblath et Hartmann). C'est ce qui se passe dans la méthémoglobinémie par eau de boisson chargée en nitrates (eau de puits, certaines canalisations urbaines) [Comly ; Verger et coll. ; Aussanaire et coll.] ou par absorption de résidus d'engrais azotés avec l'alimentation. Or, l'action de la flore nitrifiante n'est possible qu'à un pH supérieur à 5. Le développement de cette flore dans la partie haute du tube digestif n'est possible que s'il y a une déficience de l'acidité gastrique ou un trouble du transit empêchant la réabsorption des nitrates avant leur transformation en nitrites.

Divers facteurs favorisants doivent être envisagés :

— Présence d'hémoglobine fœtale, plus sensible à l'oxydation (Richard, 1963). Un tel mécanisme paraît déjà inacceptable chez l'enfant (Verger et Mullon).

— Présence d'une hypoglycémie. Bien qu'une perfusion de sérum glucosé fasse baisser la nitrémie (augmentation de la glycolyse ou action réductrice du glucose), un tel mécanisme paraît accessoire dans l'intoxication par les nitrates.

— Immaturité des systèmes enzymatiques physiologiques réducteurs de la méthémoglobine. Un tel mécanisme, parfois invoqué chez le nourrisson, paraît très inconstant.

Par contre, deux autres facteurs semblent jouer un grand rôle dans la formation de méthémoglobine par absorption de nitrates : l'insuffisance de l'acidité gastrique et l'intervention de certains germes.

Expérimentalement, Cornblath et Hartmann ont montré que, seuls, les enfants dont l'acidité gastrique est insuffisante peuvent développer une méthémoglobinémie à partir des nitrates. Or, Bouisson et Claverie ont montré qu'entre deux tétées, l'estomac du nourrisson n'a pas le temps de contenir l'HCl libre qui s'opposerait à la pullulation microbienne dans la partie haute du tube digestif. L'alimentation au lait de vache tend encore à alcaliniser le milieu. Remarquons que notre malade ingérait quotidiennement une grande quantité de lait (dilution des sécrétions gastriques et alcalinisation du bol alimentaire).

L'influence de certains germes est maintenant mieux connue : sulfhémoglobinémie des septicémies à *perfringens*, sulf et méthémoglobinémie de certaines colibacilloses intestinales (Fandre et coll.; Ferrant et coll. ; Zanesco ; J.-P. Bergel ; Aussanaire et coll.). De nombreux germes intestinaux réduisent les nitrates en nitrites : salmonelles, brucelles et shigelles, entérocoques, *proteus*, *klebsiella aerobacter*, etc.). On a récemment insisté sur le rôle du bacille subtil qui se trouve couramment dans le lait à l'état de spores et peut se développer, même en milieu acide (Saxl ; Knoteckz et coll. ; M. Merlio ; Verger et coll.).

Il est donc certain que l'alimentation lactée peut favoriser l'intoxication par les nitrates grâce aux *bacillus subtilis*. C'est peut-être ce qui s'est passé chez notre malade qui, à cause de troubles digestifs mineurs, n'avait absorbé que du lait le jour de son intoxication. On expliquerait ainsi la survenue d'une méthémoglobinémie chez une personne habituée à prendre du nitrate de potasse sans ennuis.

RÉSUMÉ

Les auteurs rapportent une observation de méthémoglobinémie sévère chez une laitière de trente et un ans. Cette intoxication était liée à l'absorption dans un but thérapeutique de nitrate de potasse.

Il faut insister sur les circonstances qui favorisent la transformation des nitrates en nitrites et, en particulier, un déficit de l'acidité gastrique, la présence de certains germes dans le tube digestif et l'action du bacille Subtil, hôte fréquent du lait cru.

*

Die Autoren berichten über eine Beobachtung von schwerer Methämoglobinämie bei einer 31 jährigen Milchhändlerin. Diese Vergiftung stand mit der Absorption von Kaliumnitrat zu therapeutischen Zwecken im Zusammenhang.

Man muss auf die Umstände, die die Umwandlung der Nitrate in Nitrite begünstigen, besonders hinweisen, und zwar besonders ein Defizit der Magenazidität, die Anwesenheit gewisser Keime im Verdaungstrakt und die Wirkung des Subtilisbazillus, häufiger Gast der rohen Milch.

*

Los autores aportan una observación de metahemoglobinemia severa en una lechera de 31 años. Esta intoxicación estaría ligada a la absorción con propósito terapéutico de nitrato de potasio.

Es necesario insistir sobre las circunstancias que favorecen la transformación de los nitratos en nitritos y en particular un déficit de la acidez gástrica, la presencia de determinados gérmenes en el tubo digestivo y la acción del bacilo Subtilis, huésped frecuente de la leche cruda.

*

Gli Autori riferiscono un'osservazione di metemoglobinemia severa di una lattaia di anni 31. Questa intossicazione è legata all'assorbimento con scopo terapeutico di nitrato di potassa.

Bisogna insistere sulle circostanze che favoriscono la trasformazione in nitriti ed in modo particolare un deficit dell'acidità gastrica, la presenza di certi germi nel tubo digerente e l'azione del bacillo Subtil, ospite frequente del latte crudo.

*

The authors reported a case of severe methemoglobinemia in a 31 year old female dairy worker. This poisoning was caused by taking nitrate of potassium therapeutically.

The conditions which transform nitrates into nitrites were pointed out, especially a deficiency of gastric acidity, the presence of certain germs in the digestive tract and the action of the Subtil bacilla, a frequent host of raw milk.

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acids, though the balance between the essential amino acids is much better than in wheat. Similarly, opaque-2 is superior to normal corn. In agreement with feeding tests of Sure (1955), the chemical amino acid assays indicate that buckwheat has a better balance and better potential than cereal grains of supplementing foods which are low in lysine.

There is presumptive evidence that concentrations of the essential amino acids of egg protein are higher than concentrations required by man (FAO/WHO/UNICEF, 1970). Isoleucine and methionine are particularly high, and the use of egg as a reference may overestimate the extent to which those amino acids are limiting and may underestimate the quality of a protein for human use. Consequently, the nutritional value of opaque-2 corn, oat, and buckwheat proteins is probably higher than indicated in the data given in Table VI. On the other hand, amino acid analyses do not measure one of the most important parameters that determine nutritive value of a food, its digestibility, and "chemical scores" should be considered primarily as a powerful and convenient screening tool.

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Spectral and Gas Chromatographic Characteristics of Some *N*-Nitrosamines

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The mass spectral, infrared spectral, and gas–liquid chromatographic retention time data of 25 *N*-nitrosamines are reported, together with general procedures for the synthesis of *N*-nitrosamines not commercially available.

Since the discovery of the toxic and later the carcinogenic properties of dimethylnitrosamine (Barnes and Magee, 1954; Magee and Barnes, 1956) many investigators have been working with nitrosamines. The most extensive review on the properties of *N*-nitroso compounds has been reported by Druckrey *et al.* (1967).

The report of dimethylnitrosamine and its causal relationship to liver damage in sheep (Ender *et al.*, 1964) has raised the question of the occurrence of various nitrosamines in the food supply. The only confirmed reports of a nitrosamine in food products have been those of dimethylnitrosamine in herring fish meal (Ender *et al.*, 1964), in a South African Bantu food plant (DuPlessis *et al.*, 1969), and in various fish products (Fazio *et al.*, 1971). However, there have been other reports of the presence of nitrosamines in foods: wheat meal (Kroeller, 1967; Marquardt and Hedler, 1966), cheese (Freimuth and Gläser, 1970; Kroeller, 1967; Marquardt and Hedler, 1966), milk (Marquardt and Hedler, 1966), mushrooms (Ender and Ceh, 1968), African alcoholic beverage (McGlashan *et al.*, 1968), meat products (Ender and Ceh, 1968; Freimuth and Gläser, 1970), and fish products (Ender and Ceh, 1968; [illegible]). The accuracy of the

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forming the identity of the nitrosamines were not utilized and a number of other food components are known to appear as artifacts in the analyses.

Analytical procedures for nitrosamines in foods must include unambiguous confirmation of identity. The best analytical technique known at present is mass spectrometry, which is a major technique in structural elucidation because of its sensitivity and capability in analysis of compounds in mixtures.

Although N-nitrosamines are not uncommon compounds, very little correlated mass spectral, infrared, and glc information is available in the literature. Collin (1954) reported the mass spectra of four symmetrical dialkylnitrosamines ranging from dimethyl through dibutyl. Schroll *et al.* (1967) investigated these same compounds together with several substituted N-nitrosoanilines and alicyclic nitrosamines. Ultraviolet data and some other physical data were listed for many nitrosamines by Druckrey *et al.* (1957). However, data are not available for many nitrosamines which are not commercially obtainable.

Therefore, in view of our interest and that of others in the study of these compounds in foods, this paper is a convenient compilation for reference of some of the physical properties of nitrosamines such as boiling points, gas–liquid chromatographic, infrared, and mass spectral data, most of which are not available in the literature.

EXPERIMENTAL

Synthesis of Nitrosamines. Precautions should be taken in the handling of these compounds to prevent inhalation and exposure to the skin, since they have potentially toxic properties.

N-Nitrosamines were prepared from the appropriate secondary amine and either nitrosyl tetrafluoroborate or sodium nitrite.

NITROSYL TETRAFLUOROBORATE. N-Nitroso-N-methylbutylamine, N-nitroso-N-methylbenzylamine, N-nitroso-N-ethylbenzylamine, and N-nitroso-N-butylaniline were prepared according to the procedure described by Olah *et al.* (1956).

To an absolute ether solution of 1 mol of the secondary amine is added 0.5 mol of nitrosyl tetrafluoroborate in small portions with vigorous stirring and cooling with ice. After addition is completed, the mixture is stirred for an additional 10 min. Two layers appear. The ethereal yellow upper layer contains the nitrosamine. The layers are separated and the ether solution is dried over anhydrous sodium sulfate, filtered, concentrated under a stream of nitrogen, and then fractionally distilled *in vacuo*.

SODIUM NITRITE. N-Nitrosodiallylamine, N-nitroso-N-methylethylamine, N-nitroso-N-methylpropylamine, N-nitroso-N-ethylpropylamine, N-nitroso-N-ethylbutylamine, N-nitroso-N-propylbutylamine, N-nitrosodiamylamine, N-nitroso-N-ethylaniline, N-nitroso-N-propylaniline, and N-nitrosomorpholine were prepared as follows. An equimolar secondary amine–hydrochloric acid solution is cooled with ice. To this, a twofold excess of an aqueous solution of sodium nitrite is added. After addition has been completed, the reaction mixture is heated at 60°C for 1 hr and then cooled. The nitrosamine, separating as a yellow oil, is extracted three times with ether and the combined extracts are dried over anhydrous sodium sulfate, filtered, concentrated under a stream of nitrogen, and fractionally distilled *in vacuo*.

N-Nitroso-N-methylethanolamine was prepared as reported.

The other N-nitrosamines used in this study were obtained from commercial sources.

All samples were checked for purity by gas chromatography prior to analysis and, when necessary, pure material was collected by preparative gas chromatographic procedures.

Gas–Liquid Chromatography. A Perkin-Elmer model 800 dual column gas chromatograph equipped with flame ionization detectors was used. The detector and injector port temperatures were 250 and 195°C, respectively. A 10-ft × 1/8-in. o.d. stainless steel column containing 5% Carbowax 20M-TPA on 100–120 mesh Supelcoport was used with a helium carrier flow of 60 cm^3 per min. The air and hydrogen flows were 430 and 24 cm^3 per min, respectively. Temperature program: 5 min isothermal at 100°C, then 100 to 185°C at 5°C per min. A 10-ft × 1/8-in. o.d. stainless steel column containing 5% OV-1 on 35 to 50 mesh Anakrom ABS was used with a helium carrier flow of 118 cm^3 per min. The air and hydrogen flows were 430 and 40 cm^3 per min, respectively. Temperature program: 70 to 185°C at 5°C per min. The retention times for even C_6–C_{16} methyl esters of straight chain acids are given to indicate where the nitrosamines would elute under other conditions.

Infrared Spectra. The nitrosamines were dissolved in carbon tetrachloride and the spectra recorded in a 0.5-mm path-length NaCl cell with a Perkin-Elmer Model 421 infrared spectrophotometer. Due to its lack of solubility in a suitable solvent and poor spectra as a film or pellet, no ir is presented for N-nitroso-N-methylethanolamine.

Mass Spectra. Mass spectra were obtained with a Du Pont model 21-492 mass spectrometer at an ionizing voltage of 70 eV and an ion source temperature of 210°C. The volatile liquids were introduced *via* the bath inlet heated at 150°C. The relatively nonvolatile liquids and solids were introduced *via* the solid probe at the lowest temperature required to produce volatility.

RESULTS AND DISCUSSION

N-Nitrosamines were prepared from secondary amines by two methods—reaction with sodium nitrite or nitrosyl tetrafluoroborate. The latter method is preferred because, with ether-soluble amines, this simple reaction proceeds without the formation of secondary reaction products.

The gas chromatographic retention times for the N-nitrosamines are listed in Table I, together with their boiling or melting points. The simple aliphatic compounds were readily separated on a Carbowax 20M column, but difficulty was experienced with aromatic nitrosamines under the same conditions.

There was significant decomposition of the phenyl-substituted compounds, methyl through butyl, on the Carbowax 20M column with the formation of the corresponding aniline and asymmetrical hydrazines. This decomposition occurred to a lesser extent on the OV-1 column, which, with modification of the temperature program, permitted satisfactory separations. A retention value could not be obtained for N-nitrosophenylbenzylamine since there were indications that it is more labile than the other alkyl- and benzyl-substituted nitrosamines, possibly because nitric oxide is split off thermally as reported by Welzel (1971) for diaryl nitrosamines.

The gas chromatographic data were obtained with 1/8-in. packed columns and the resolution was compared favorably with the separations reported by Heyns and Roper (1970) of other nitrosamines using an alkaline UCON LB 550X capillary column.

Table I. Gas Chromatographic Data and Boiling or Melting Points

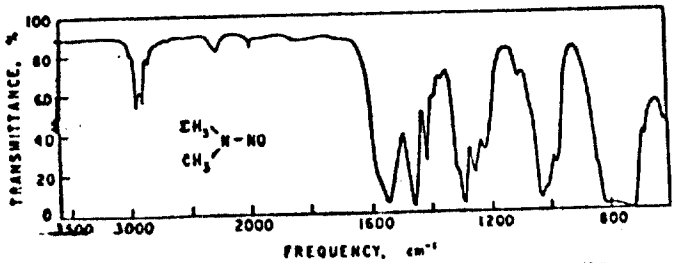
$RR'N{-}N{=}O$

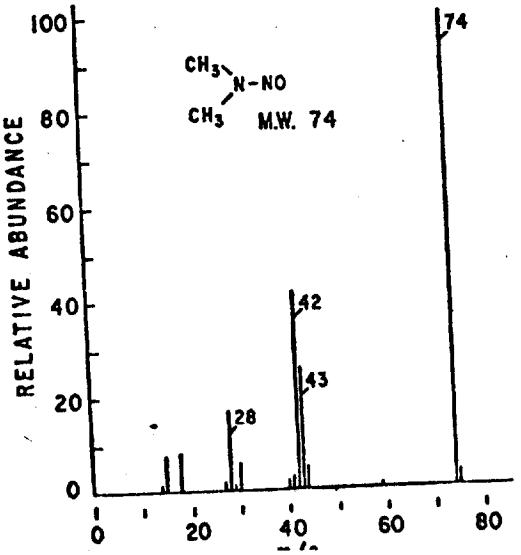
R	R′	Molecular formula	Mol wt	bp or mp	CW20M-TPA RT, min	OV-1 RT, min
Me	Me	$C_2H_6N_2O$	74	151°C/760 mm	3.0	
Me	Et	$C_3H_8N_2O$	88	161°C/760 mm	3.7	
Me	Pr	$C_4H_{10}N_2O$	102	90°C/40 mm	5.4	
Me	Bu	$C_5H_{12}N_2O$	116	198°C/760 mm	7.8	
$HOCH_2CH_2$	Me	$C_3H_8N_2O_2$	104	110°C/1 mm	22.0	
Et	Et	$C_4H_{10}N_2O$	102	64°C/17 mm	4.3	
Et	Pr	$C_5H_{12}N_2O$	116	104°C/44 mm	6.0	
Et	Bu	$C_6H_{14}N_2O$	130	95°C/14 mm	8.4	
Pr	Pr	$C_6H_{14}N_2O$	130	81°C/5 mm	7.8	
Pr	Bu	$C_7H_{16}N_2O$	144	103°C/13 mm	10.0	
Bu	Bu	$C_8H_{18}N_2O$	158	116°C/14 mm	12.3	
Am	Am	$C_{10}H_{20}N_2O$	186	124°C/5 mm	16.4	
Allyl	Allyl	$C_6H_{10}N_2O$	126	41°C/1 mm	8.0	
$-(CH_2)_5-$		$C_6H_{10}N_2O$	114	100°C/14 mm	12.6	
$-(CH_2)_4-$		$C_4H_8N_2O$	100	98°C/12 mm	13.3	
$-(CH_2)_2O(CH_2)_2-$		$C_4H_8N_2O_2$	116	96°C/6 mm	14.5	
$-(CH_2)N(CH_2)(CH_2)_2-$		$C_5H_{11}N_3O$	129	83°C/4 mm	14.8	
Ph	Me	$C_7H_8N_2O$	136	128°C/19 mm	18.2	6.4
Ph	Et	$C_8H_{10}N_2O$	150	131°C/20 mm	18.3	7.6
Ph	Pr	$C_9H_{12}N_2O$	164	109°C/3.5 mm	19.6	9.1
Ph	Bu	$C_{10}H_{14}N_2O$	178	139°C/8.5 mm	21.3	11.5
Ph	$PhCH_2$	$C_{13}H_{12}N_2O$	212	mp 57–58°C	...	...
$PhCH_2$	Me	$C_8H_{10}N_2O$	150	158°C/26 mm	21.2	8.2
$PhCH_2$	Et	$C_9H_{12}N_2O$	164	162°C/23 mm	21.6	9.6
$PhCH_2$	$PhCH_2$	$C_{14}H_{14}N_2O$	226	mp 58–59°C	...	20.0
			Markers			
C_6	Methyl hexanoate				1.7	
C_8	Methyl octanoate				4.0	4.2
C_{10}	Methyl decanoate				9.2	8.5
C_{12}	Methyl dodecanoate				14.1	12.8
C_{14}	Methyl tetradecanoate				18.5	17.1
C_{16}	Methyl hexadecanoate				22.5	21.0

characterization, the infrared spectra may be helpful. Figure 1 shows the infrared spectrum of *N*-nitrosodimethylamine in carbon tetrachloride. Spectra of other *N*-nitrosamines have been deposited with the ACS Microfilm Depository Service. The most distinctive band is the strong $\gamma_{N=O}$ stretch band from 1435 to 1485 cm^{-1}. The alicyclic nitrosamines absorb in the 1440 cm^{-1} region, simple disubstituted aliphatic and benzyl–allyl nitrosamines absorb in the 1460 cm^{-1} region, and phenyl–alkyl nitrosamines absorb in the 1480 cm^{-1} region. This is in substantial agreement with the data reported by Williams *et al.* (1964).

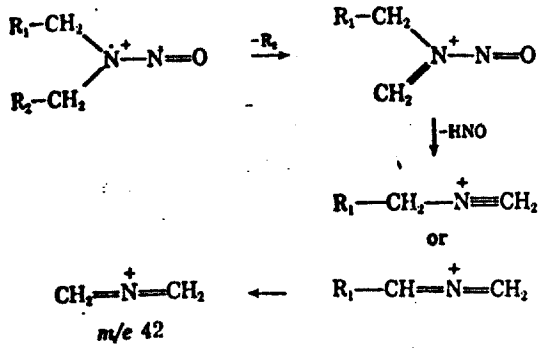
For *N*-nitrosamines present in low concentrations, in complex mixtures, gas–liquid chromatography is the most potent tool for separation and isolation and mass spectrometry for unequivocal confirmation of identity. The mass spectrum of *N*-nitrosodimethylamine is listed in Figure 2 in the form of a bar graph. Spectra of other *N*-nitrosodimethylamines have been deposited with the ACS Microfilm Depository Service.

In general, the fragmentation process of the aliphatic nitrosamines follows the scheme described by Budzikiewicz *et al.*





(1967) with the important ions being *m/e* 30, 42, a relatively large parent ion and peak due to α-cleavage of the alkyl side chain, with subsequent loss of NOH, as well as a peak at P-17 due to the loss of the hydroxyl radical. An important pathway can be described in the following scheme for the *m/e* 42, except in the case of dimethylnitrosamine, which bypasses the α-cleavage step.



The phenyl-substituted nitrosamines all have an appreciable parent peak and a strong P-30 peak. The benzyl-substituted nitrosamines have a strong parent peak and a strong *m/e* 91 peak, which in some cases is the base peak of the spectrum. The *m/e* 91 is analogous to the splitting of the bond β to the phenyl ring, as described by Grubb and Meyerson (1963).

Currently, a more specific and detailed study is in progress utilizing both high-resolution mass spectrum and the metastable spectrum of each compound. This will allow us to more fully explain the fragmentations that occur.

The determination of *N*-nitrosamines in food products and other natural substances is complicated by the presence of large numbers of interfering compounds. Therefore, adequate isolation and cleanup procedures are necessary to permit sampling of the nitrosamines so that the spectra described in this paper can be utilized.

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Processing of Cauliflower Leaf Waste for Poultry and Animal Feed

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Leaf waste from commercially grown cauliflower was dehydrated in a pilot scale alfalfa dehydrator to give dried meals suitable for poultry and cattle feeds. Separation of the poultry and cattle meal fractions was accomplished *via* air classification of the dried plant material. The poultry meal fraction contained 375 to 620 mg/kg of xanthophyll and 26 to 31% protein, while the cattle meal contained 17 to 21% protein. The xanthophylls in the poultry meal were as effective in pigmenting broiler skin as the xanthophylls in dehydrated alfalfa meal. No undesirable flavor was imparted to poultry meat by cauliflower meal. Pressing of cauliflower leaf prior to dehydration increased its solids content and lessened the quantity of water to be evaporated per pound of dried product by nearly 30%. The pressed and dehydrated meals were almost equal in quality to the unpressed dehydrated meals.

Commercial production of vegetables in the United States results in more than 4 million tons of fresh vegetable wastes annually (Willaman and Eskew, 1948; U.S. Dept. Agr. Stat., 1969). A large portion of these wastes is green leaf plant materials that are removed at the packing shed. An estimated 60,000 tons of cauliflower leaf wastes are produced annually in the California Salinas Valley. Presently, following removal of the flowers for the fresh and frozen vegetable markets, leaf and stem portions are chopped and returned to the fields where they constitute an odor problem as they decompose to form a green manure.

Prior studies at this laboratory have been concerned with

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Inhibition of clostridia by nitrite

J. A. PERIGO* AND T. A. ROBERTS†

Summary. An earlier report that sodium nitrite heated in a laboratory medium is highly inhibitory against *Clostridium sporogenes* has been extended to thirty clostridial strains including *Cl. botulinum* types A, B, E and F (fourteen strains) and *Cl. welchii* (eight strains).

Introduction

Microbiological investigations on the inhibitory effect of nitrite in laboratory media do not fully explain the stability of lightly processed cured meat products (Roberts & Ingram, 1966). To inhibit spores of *Clostridium sporogenes* in an agar medium it was necessary to add 800–1200 ppm sodium nitrite at pH 7·5 and 50–100 ppm at pH 6·5. Subsequent investigations using vegetative cells of *Cl. sporogenes* in a different system (Perigo, Whiting & Bashford, 1967) produced essentially the same order of inhibitory concentrations: 500 ppm at pH 7·5 and 160 ppm at pH 6·5. The latter authors also heated sodium nitrite in the growth medium before inoculation and found that heating at 90–130°C reduced the concentrations of sodium nitrite required to inhibit growth to as low as 3·5 ppm. This level was virtually undetectable chemically after the heat process, and suggested the formation of a potent growth inhibitor.

It was clearly of interest to determine whether this phenomenon was generally applicable to a wide range of clostridial species, particularly *Cl. botulinum* and *Cl. perfringens* (*welchii*).

Experimental

Vegetative inocula were obtained by subculturing in cooked meat medium, TPG (Roberts, 1965) or Reinforced Clostridial Medium (Oxo Ltd, London, S.E.1). Subcultures seen, by phase contrast microscopy, to contain spores were rejected. *Cl. botulinum* type E was incubated at 30°C; all other subcultures were incubated at 37°C. Five drops of vegetative cell culture, 18–42 hr old, were inoculated into a duplicate series of 1 fluid oz screwcap bottles containing the basal medium (Perigo *et al.*,

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1967) and increasing concentrations of sodium nitrite at pH 6 and 7, heated at 121°C for 20 min or unheated, as indicated in Table 1. Cultures were incubated for 7 days at 37°C (*Cl. botulinum* type E, 30°C) although in no case was inhibition delayed longer than 72 hr.

TABLE 1. Concentrations of sodium nitrite (ppm) used against clostridial species

pH	Treatment*	Concentration (ppm)									
7	Unheated	0	10	20	40	80	160	320	640	1280	2560
	Heated	0	1·25	2·5	5	10	20	40	80	160	320
6	Unheated	0	10	20	40	60	80	120	160	200	240
	Heated	0	1·25	2·5	5·0	7·5	10	15	20	40	60

* Treatment: Unheated = basal medium + filter sterilized nitrite solution; Heated = basal medium + nitrite solution heated at 121°C for 20 min.

Organisms used

The sources of most of the organisms used (Table 2) may be obtained by reference to Roberts (1967). *Cl. botulinum* G2A was isolated by and received from Dr D. Gimenez, Institute of Microbiology, Universidad Nacional de Cuyo, Mendoza, Argentina. *Cl. sporogenes* PA-R and 93-R were received from J. Baltzer, The Danish Meat Research Institute, Roskilde, Denmark.

Results and discussion

Results are summarized in Table 2, the tabulated nitrite concentrations being the highest concentration at which growth was observed in either of the duplicate bottles.

Unheated nitrite

At pH 7 the inhibitory concentrations were of the order which might have been anticipated from previous publications (Roberts & Ingram, 1966; Perigo *et al.*, 1967). The three strains of *Cl. botulinum* type E were among the most sensitive, and *Cl. perfringens* was most resistant, although one strain of *Cl. botulinum* type A, *Cl. botulinum* type F ('Langeland') and *Cl. bifermentans* were as resistant.

At pH 6, *Cl. perfringens* was again most resistant. Three strains of *Cl. botulinum* (62A, 7272A and 4318/63) failed to grow, even in the absence of nitrite.

TABLE 2. Range within which the inhibitory concentration (ppm) of unheated and heated nitrite at pH 6 and pH 7 falls

Species		Unheated†		Heated‡	
		pH 6	pH 7	pH 6	pH 7
Cl. sporogenes	532*	160–200	640–1280	5–7·5	10–20
	PA 3679	60–80	320–640	5–7·5	5–10
	PA-R	80–120	160–320	5–7·5	5–10
	93-R	80–120	320–640	5–7·5	10–20
Cl. botulinum Type A	62 A	0–10	640–1280	2·5–5·0	20–40
	1192 A	120–160	320–640	5–7·5	10–20
	3805* A	10–20	640–1280	0–1·25	10–20
	7272* A	0–10	1280–2560	0–1·25	10–20
	G2 A	20–40	320–640	15–20	10–20
Type B	751* B	80–120	640–1280	7·5–10	10–20
	213 B	10–20	640–1280	15–20	40–80
	3807* B	120–160	640–1280	7·5–10	10–20
(Non-proteolytic)	17 B	60–80	320–640	10–15	10–20
Type E	16/63	60–80	160–320	5–7·5	5–10
	1537/62	10–20	160–320	7·5–10	10–20
	4318/63	0–10	80–160	5–7·5	10–20
Type F	Langeland	60–80	1280–2560	5–7·5	40–80
(Non-proteolytic)	202F	80–120	320–640	7·5–10	10–20
Cl. perfringens (*welchii*) Classical	8237*	Above 240†	640–1280	10–15	10–20
	3181*	160–200	1280–2560	7·5–10	10–20
	8084*	Above 240†	1280–2560	7·5–10	10–20
	8503*	120–160	640–1280	5–7·5	10–20
	8081*	200–240	640–1280	10–15	5–10
'Food-poisoning'	8239*	Above 240†	1280–2560	5–7·5	10–20
	8797*	60–80	640–1280	7·5–10	10–20
	10240*	Above 240†	640–1280	7·5–10	10–20
Cl. septicum	547*	80–120	640–1280	2·5–5·0	5–10
Cl. tetani	5411*	40–60	160–320	7·5–10	5–10
Cl. bifermentans	506*	160–200	1280–2560	15–20	10–20
Cl. histolyticum	503*	160–200	640–1280	2·5–5·0	10–20

* N.C.T.C. catalogue number.
† Growth occurred in the highest concentration of nitrite used.
‡ Unheated and heated, see Table 1.

Heated nitrite

At both pH 7 and pH 6 growth was generally inhibited at an initial nitrite level of 5–10 ppm, only six cultures of the sixty tested showing growth at a higher level, the highest being 40 ppm (*Cl. botulinum* 213B and F 'Langeland'). Inhibitory levels at pH 6 were slightly lower than at pH 7.

The inhibitory levels tabulated for PA 3679 are consistent with those previously published (Perigo *et al.*, 1967).

Preliminary results with spores of *Cl. sporogenes* and *Cl. botulinum* type B indicate that the inhibitory concentrations of both unheated and heated nitrite are of the same order as demonstrated above for vegetative inocula.

Conclusion

The enhancement of the inhibitory effect of nitrite after heating in laboratory media is amply confirmed, and appears to be general to a wide selection of clostridia, and not restricted solely to *Cl. sporogenes*. It is, therefore, a matter of even greater interest to identify the inhibitory substance with a view to its possible use as a food additive.

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Observations on the inhibition of vegetative cells of *Clostridium sporogenes* by nitrite which has been autoclaved in a laboratory medium, discussed in the context of sub-lethally processed cured meats

J. A. PERIGO, E. WHITING AND T. E. BASHFORD

Summary. The ability of sodium nitrite incorporated in a laboratory medium to inhibit an inoculum of vegetative *Clostridium sporogenes* is greatly influenced by the temperature at which the nitrite containing medium is held prior to inoculation. At pH values of 6·0 and above, the inhibitory effect of a given concentration of nitrite is substantially enhanced when it is first autoclaved in the medium. It is believed that the nitrite, on heating, reacts with some component of the medium producing an unknown substance which is extremely inhibitory to the vegetative growth of *Cl. sporogenes*. The inhibitory activity of this unknown substance is very different from the inhibitory activity of sodium nitrite. The possible significance of these observations in relation to the stability of sublethally processed cured meats is discussed.

Introduction

Canned cured meat products are frequently subjected to a minimal heat treatment in order to maintain a satisfactory appearance and texture. This heat treatment is commonly so low that it would exert a barely significant lethal effect on certain spores considered in terms of their heat resistance in uncured media. Such sub-lethally processed cured meats, however, enjoy an excellent commercial and public health record; the majority of them are completely stable when stored for long periods at ambient temperatures.

A number of factors have been demonstrated to contribute to this stability. The relevant literature has been comprehensively reviewed by Spencer (1966) and Riemann (1963), and no extensive reappraisal will be attempted in this paper. Because the incidence of spores in the raw product is usually low (Gross, 1954) and because these spores may be much less heat resistant than similar spores grown in traditional laboratory media (Vinton, Martin & Gross, 1947), Riemann (1963) concludes that few

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spores may survive the traditional cured meat heat process. Considering other evidence (Yesair & Cameron, 1942; Stumbo, Gross & Vinton, 1945; Silliker, Greenberg & Schack, 1958), Riemann (1963) also concludes that whilst the curing salts do not directly reduce the heat resistance of the spores, they do appear to inhibit the subsequent germination and outgrowth of those spores which survive the heat process. The surviving spores appear to have been damaged by the heat treatment and are consequently unable to germinate and grow in the presence of the curing salts. They will, however, germinate and grow if subcultured into salt-free media. Spencer (1966) concludes that in practice a stable product may be expected to result from a heat process of $F_0 = 0{\cdot}1$–$0{\cdot}4$ with a sodium chloride concentration of 3·5–4·0% in the water phase, an initial nitrite concentration of 75–150 ppm falling to a post process concentration of about 20 ppm and an incidence of less than 1 clostridial spore/g, but that this stability cannot be accounted for by any one of these factors in isolation and must be due to some interaction between the spores, the heat treatment and the curing salts.

Roberts & Ingram (1966) heated aerobic and anaerobic spores to various degrees, roughly from $F_0 = 0{\cdot}0015$ to $F_0 = 1{\cdot}5$, in the absence of curing salts and then transferred them to nutrient media containing these salts in various concentrations. In this way, they demonstrated that the more severe heat treatments are capable of rendering surviving spores considerably more sensitive to subsequent inhibition by salt concentrations of the order found in cured meats. These findings undoubtedly represent a major advance in understanding, illuminating a mechanism which may account for some of the anomalous results obtained by previous workers. Roberts & Ingram (1966) claim with justification that their findings for the first time point a clear way to a quantitatively satisfying explanation of the surprising effectiveness of modest heat treatments in preventing the development of bacterial spores in cured meat products; and yet these findings, especially concerning the role of nitrite, are not wholly satisfying.

Nitrite is a notoriously unstable salt, and this instability has, we believe, given rise to great confusion in the literature. Jensen & Hess (1941), when considering the stability of canned cured meat products, state: 'Nitrites react with protein when heated and are destroyed, thus leaving the meat in much the same state as fresh-cooked uncured meat'. The first half of this statement implies a reasonable assumption: it is common knowledge that a significant proportion of the inorganic nitrite added to these raw products is no longer detectable by the relevant analytical procedure after the product has been heated. This disappearance of inorganic nitrite is much greater when the nitrite is heated in meat than when it is heated to the same degree in water, and it is thus not unreasonable to assume that the meat proteins are reacting with and reducing the nitrite during the heat process. The second half of the statement, however, is not an essential nor even rational corollary to the first. It implies that the nitrite which disappears ceases to exert any inhibitory activity in the finished product. This assumption appears to be widely and uncritically accepted. Most authors appear

to be specifically concerned with the inhibitory activity of residual inorganic nitrite and many entirely evade the issue by adding unheated sterile filtered inorganic nitrite solutions to their previously heated experimental system.

In the experiments described in this paper we have directly challenged this assumption. Various quantities of nitrite have been heated at various temperatures and pH, in a basic medium which induces substantial nitrite disappearance. The ability of the resulting substrates to inhibit the vegetative growth of *Clostridium sporogenes* has then been determined by challenging each substrate with a standard, vigorously growing vegetative inoculum of this organism and observing whether this inoculum grows or is inhibited on subsequent incubation. In this way, we have investigated the interactions between nitrite, substrate, pH and processing temperature in terms of inhibitory activity without introducing the confounding interaction between the test organism and processing temperature.

Materials and methods

The basic medium used in this investigation was chosen because it supported vigorous growth of the test organism and also because substantial disappearance of nitrite resulted when this salt was autoclaved in the medium. Nitrite was determined by the absorptiometric estimation of the orange di-azo dye which is formed under standard conditions with sulphanilic acid and α naphthol.

The basic medium consisted of tryptone (Oxoid) 20 g, peptone (Oxoid) 10 g, Lab-lemco (Oxoid) 10 g, yeast extract (Oxoid) 5 g, NaCl (B.D.H. Analar) 5 g, K_2HPO_4 (B.D.H. Lab. Reagent) 2·5 g, glucose (B.D.H. Analar) 2 g, soluble starch (B.D.H. Analar) 1 g, sodium thioglycollate (B.D.H. Lab. Reagent) 1 g, bromocresolpurple (B.D.H.) 5 ml of a 0·4% solution, water 1000 ml. The pH of the medium was adjusted as required with HCl or NaOH (B.D.H. Analar) prior to the medium being dispensed in 20 ml quantities in 1-oz McCartney bottles. These bottles were closed with wadless polypropylene screw caps and then sterilized by autoclaving for 20 min at a steam pressure of 15 lb/in^2.

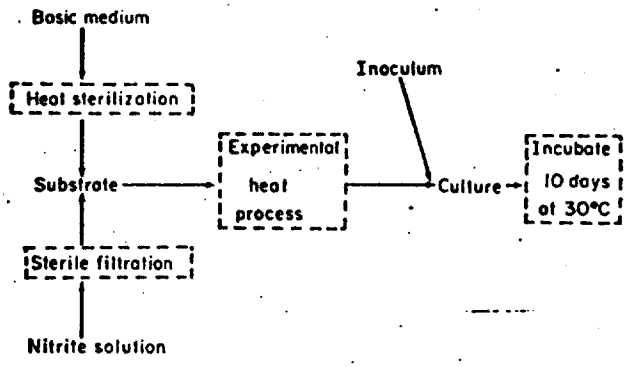
On completion of this sterilization and when the bottles had cooled, appropriate quantities of sterile filtered sodium nitrite (B.D.H. Analar) solutions were aseptically added. The concentration of the nitrite solutions used were such that the required dilutions could be obtained by adding not less than 0·05 ml and not more than 1·5 ml to each 1-oz bottle of medium.

The experimental heat processes were then accomplished by heating the McCartney bottles for 20 min at one of various temperatures. Temperatures up to 90°C were attained in a water bath, temperatures of 100°C in a steamer and temperatures above 100°C in an autoclave. With the object of simulating the canning process and in order to minimize the possible loss of volatile components, the McCartney bottle caps were tightly screwed down during the experimental heat process. In certain cases when the experimental heat process was a sterilizing process in its own right, the prior sterilization

of the basic medium and nitrite solution was omitted. Subsidiary experiments demonstrated that this omission did not significantly influence the final result.

The resulting bottles of substrate were subsequently inoculated with the test organism, putrefactive anerobe 3679, strain 8053 from the National Collection of Industrial Bacteria. Each McCartney bottle was inoculated with approximately 8×10^6 vigorously growing vegetative cells. The inoculum consisted of five drops from a 24-hr culture incubated in the basic medium at 30°C. After inoculation, each bottle of substrate is referred to as a culture.

The experimental procedure described above may be schematically represented as follows:



The work described in this paper may logically and conveniently be separated into three major experiments, although chronologically the work consisted of many smaller exercises performed over a period of approximately 1 year. The three major experiments were as follows:

(1) *The influence of pH on the inhibitory activity of unheated nitrite in the basic medium*

Various concentrations of nitrite incorporated in basic medium at various pH and not subjected to any experimental heat process were challenged with the test organism.

(2) *The influence of pH on the inhibitory activity of nitrite heated in the basic medium*

Various concentrations of nitrite were incorporated in the basic medium at various pH and heated for 20 min at 121°C prior to being challenged with the test organism.

(3) *The influence of heating temperature on the inhibitory activity of nitrite heated in the basic medium at constant pH*

Various concentrations of nitrite were incorporated in the basic medium at pH 6·0 and heated for 20 min at various temperatures prior to being challenged with the test organism.

Results and discussion

(1) *The influence of pH on the inhibitory activity of unheated nitrite in the basic medium*

The results of this experiment are set out as fractions in the body of Table 1. Each fraction refers to a unique substrate with a defined pH in the range pH 5·4–8·0 and with a defined nitrite content in the range 0–1500 ppm. The denominator of each fraction specifies the number of replicate cultures prepared at that locus, i.e. the number of bottles of unique substrate challenged with the test inoculum, and the numerator of each fraction specifies the number of these replicate cultures which failed to grow on subsequent incubation. These results are based on the preparation and observation of 1833 individual cultures.

At each pH a number of independent test inocula have been exposed to a nitrite treatment at various concentrations. At each of these nitrite levels a proportion r of the n inocula are observed to be inhibited. The interpretation of these quantal dose responses is facilitated if a model is established which relates the nitrite concentration x to the probability p of r cultures being inhibited out of n cultures tested. Such a model can be established if p is transformed so that the relationship between $y(p)$ and x is linear. The transformation most commonly employed to this end is the Probit transformation (Finney, 1952) where y is the standardized normal deviate exceeded with probability $1 - p$ (5 is commonly added in hand computation but this convention is not adopted here). Accordingly, probit regression lines have been fitted to the data in Table 1. Nitrite concentrations were expressed arithmetically as ppm and also logarithmically as $\log_{10}$ ppm. This analysis was accomplished with the aid of an ICT Orion computer using a maximum likelihood programme (Orion 25) developed by G. J. S. Ross of the Rothamsted Experimental Station. On completion of the analysis the following values are obtained at each pH level.

(i) The probability of inhibition corresponding to each experimental level of nitrite.

(ii) The 50% effective dose (ED_{50}), i.e. the concentration of nitrite most likely to inhibit 50% of the inocula.

(iii) The slope of the regression line.

(iv) Chi-squared, which provides an estimate of the probability of the deviations of observed responses from the computed regression line being due to chance variation alone.

On consideration of the results of this analysis it was immediately apparent that the best fit was obtained when the probit of inhibition was regressed on the logarithm of nitrite concentration. Thus the results of Experiment (1) are illustrated on this basis by the series of graphs in Fig. 1.

At each pH the computed regression is drawn as a solid line and the experimentally observed responses are plotted as open circles. It should be noted that the statistical weight carried by each point in Fig. 1 is dependent on the number of replicate cultures tested at that locus, which may be established by consulting the denominator of the

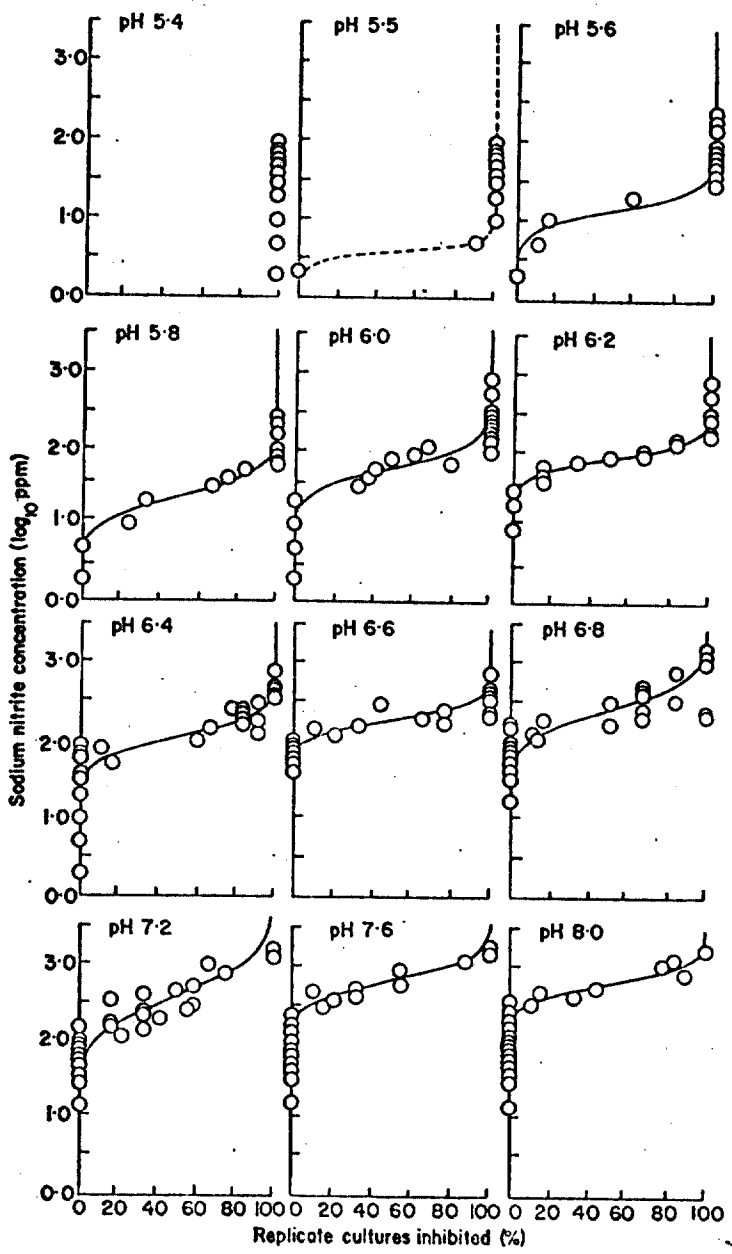


FIG. 1. The influence of unheated sodium nitrite concentration on the probability of vegetative cultures of *Cl. sporogenes* PA 3679 being inhibited at various pH values. Continuous curves established by probit analysis. The broken curve is a free-hand estimate.

TABLE 1. The influence of pH on the inhibitory activity of unheated sodium nitrite in the basic medium (No. of cultures inhibited/No. of cultures tested)

TABLE 1. The influence of pH on the inhibitory activity of unheated sodium nitrite in the basic medium (No. of cultures inhibited/No. of cultures tested)

pH	Nitrite concentration (ppm)																																
	0	2	5	10	15	20	30	40	50	60	70	80	90	100	120	140	150	160	180	200	220	240	250	300	350	400	450	500	750	1000	1200	1500
5·4	1/9	9/9	9/9	9/9	–	9/9	9/9	9/9	9/9	9/9	9/9	9/9	–	9/9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5·5	0/10	0/9	9/10	10/10	–	10/10	9/9	10/10	9/9	10/10	9/9	10/10	–	10/10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5·6	0/12	0/9	1/9	2/12	–	7/12	12/12	12/12	12/12	12/12	12/12	12/12	–	12/12	–	–	3/3	–	–	3/3	–	–	3/3	–	–	–	–	–	–	–	–	–
5·8	0/12	0/9	0/9	3/12	–	4/12	8/12	9/12	10/12	12/12	9/9	12/12	–	12/12	–	–	3/3	–	–	3/3	–	–	3/3	–	–	–	–	–	–	–	–	–
6·0	0/16	0/3	0/4	0/7	–	0/10	3/9	5/13	5/12	8/10	3/6	8/13	3/3	11/16	9/9	9/9	3/3	6/6	6/6	9/9	3/3	3/3	6/6	6/6	–	–	–	3/3	3/3	–	–	–
6·2	0/9	–	–	0/3	–	0/6	0/6	1/6	1/6	1/6	1/3	3/6	2/3	6/9	5/6	5/6	3/3	3/3	3/3	6/6	–	–	6/6	3/3	–	–	–	3/3	3/3	–	–	–
6·4	0/15	0/3	0/3	0/3	–	0/3	0/3	0/9	2/12	0/6	0/6	1/9	0/3	9/15	11/12	8/12	–	10/12	11/12	10/12	5/6	5/6	7/9	11/12	3/3	3/3	3/3	9/9	6/6	–	–	–
6·6	0/9	–	–	–	–	–	–	0/3	0/9	0/3	0/3	0/3	0/3	0/9	2/9	1/9	–	3/9	7/9	6/9	3/3	3/3	7/9	4/9	3/3	3/3	3/3	9/9	6/6	–	–	–
6·8	0/15	–	–	–	0/3	–	0/3	0/3	0/6	0/3	0/3	0/6	0/3	2/15	1/9	0/6	0/6	3/6	1/6	8/12	3/3	2/2	6/9	6/12	5/6	6/9	4/6	8/12	10/12	9/9	6/6	6/6
7·2	0/15	–	–	–	0/3	–	0/3	0/3	0/6	0/3	0/3	0/6	0/3	0/15	2/9	2/6	0/6	1/6	1/6	5/12	1/3	1/3	5/9	7/12	1/6	3/9	3/6	7/12	9/12	6/9	6/6	6/6
7·6	0/12	–	–	–	0/3	–	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/12	0/3	–	0/9	–	–	0/9	–	–	1/6	2/9	2/6	1/9	2/6	5/9	5/9	8/9	6/6	6/6
8·0	0/12	–	–	–	0/3	–	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/12	0/3	–	0/9	–	–	0/9	–	–	0/6	1/9	0/6	3/9	1/6	4/9	8/9	7/9	5/6	6/6

corresponding fraction in Table 1. Because a logarithmic nitrite scale is used, the nitrite free controls cannot be represented in Fig. 1. As can be seen from Table 1, however, of a total of 146 nitrite-free controls inoculated, only one failed to grow and this occurred at pH 5·4. Because insufficient data were obtained at pH 5·4 and pH 5·5, the computer was unable to establish a regression line. A broken curve has been fitted by hand at pH 5·5 on the basis of the data available and taking into account the curves established at higher pH.

The unheated nitrite ED_{50} values obtained by this analysis are plotted against pH as open circles in Fig. 4. These values are computed from the experimental data in Table 1. On consideration of Fig. 4, it is at once apparent that the inhibitory effect of unheated nitrite is highly pH dependent. It is widely contended that the effective inhibitory agent in nitrite solutions is undissociated nitrous acid, and that on this basis a unit decrease in pH value would result in a ten-fold increase in the inhibitory effect of nitrite. Roberts & Ingram (1966) observed that for PA 3679 spores heated at 80°C for 20 min, the concentrations of nitrite needed to reduce the percentage survival to 1% over the range pH 6·5–7·5 were reasonably consistent with the 1 : 10 concentration ratio expected on the basis of the above-described theory. From Fig. 4 it will be seen that for vegetative cells of PA 3679, the ED_{50} values which we have observed are consistent with this theory over the range pH 5·8 (22 ppm) to pH 6·8 (250 ppm), but at pH values outside this range the relationship breaks down, the ED_{50} values being less than expected. The deviation from theory at pH values below pH 5·8 is probably due to the growth limiting effect of pH in its own right. The lower limit for vegetative growth of PA 3679 in the nitrite-free basic medium has been established to be between pH 5·3 and 5·4. The deviation from theory at pH values above pH 6·8 is not as easily explained, for although the upper pH growth limit has not been established, it is certainly higher than pH 8·0, which is the highest pH investigated in these experiments.

(2) *The influence of pH on the inhibitory activity of nitrite heated in the basic medium*

The results of this experiment are set out in Table 2 using the same conventions as were used for Table 1. These results are based on the preparation and observation of 784 individual cultures. On subjecting these data to probit analysis, it was again found that the best fit was obtained when the nitrite concentration was expressed logarithmically. These results are presented as a series of graphs in Fig. 2, using the same conventions as in Fig. 1.

At pH 5·5 the computed regression line was an extremely improbable fit. The probability of the deviation of the data from the computed curve being due to chance was less than 0·001 based on the calculated value of Chi-squared. Thus we are forced to conclude that substantial experimental error has occurred in this instance and have rejected the computed curve and replaced it with a free-hand estimate (broken curve). At pH 8·0 the data was not sufficient to enable a curve to be computed and again a

TABLE 2. The influence of pH on the inhibitory activity of sodium nitrite which has been heated for 20 min in the basic medium at 121°C (No. of cultures inhibited/No. of cultures tested)

pH	Nitrite concentration (ppm)													
	0	3	4	5	6	7	8	9	10	11	12	15	20	25
5·4	2/6	1/6	0/3	2/6	1/3	0/3	5/6	6/6	6/6	6/6	6/6	6/6	3/3	3/3
5·5	1/7	2/6	0/4	3/6	0/3	1/3	3/7	6/6	6/6	6/6	7/7	4/4	3/3	3/3
5·6	0/6	0/6	0/3	0/6	0/3	0/3	3/6	3/6	5/6	6/6	6/6	6/6	3/3	3/3
5·8	0/6	0/6	0/3	0/6	0/3	2/3	5/6	6/6	6/6	6/6	6/6	6/6	3/3	3/3
6·0	0/27	0/10	0/14	0/21	1/16	2/11	12/17	8/11	19/23	6/6	9/9	8/8	16/16	8/8
6·4	0/6	0/6	0/3	0/6	0/3	0/3	2/6	3/6	3/6	3/6	3/6	4/6	3/3	3/3
6·8	0/6	0/6	0/3	0/6	0/3	2/3	3/6	5/6	6/6	6/6	6/6	6/6	3/3	3/3
7·2	0/6	0/6	0/3	0/6	0/3	0/3	0/6	1/6	4/6	2/6	5/6	6/6	3/3	3/3
7·6	0/6	0/6	0/3	0/6	0/3	0/3	0/6	0/6	0/6	1/6	2/6	5/6	3/3	3/3
8·0	0/3	0/3	—	0/3	—	—	0/3	0/3	0/3	0/3	0/3	0/3	1/3	3/3

broken free-hand estimate is indicated. In both these cases the estimated curve is based on the data available and on the curves computed at other pH levels. Again the nitrite-free controls cannot be represented, but from Table 2 it is apparent that of seventy-nine such controls only three were inhibited, two at pH 5·4 and one at pH 5·5.

The ED_{50} values for heated nitrite are also plotted against pH as solid circles in Fig. 4. These ED_{50} values are computed from the data in Table 2. It is immediately apparent that the inhibitory effect of heated nitrite is very different from that of unheated nitrite, being only slightly pH dependent. The ED_{50} of heated nitrite rises from about 6 ppm at pH 5·5 to about 18 ppm at pH 8·0, whereas for unheated nitrite the corresponding ED_{50} values are approximately 3 ppm at pH 5·5 and 590 ppm at pH 8·0. Consequently, at higher pH values, nitrite which has been heated in the basic medium is very much more inhibitory than the same quantity of nitrite incorporated in the basic medium without heating. At pH 7·0 approximately 300 ppm of unheated nitrite are observed to inhibit 50% of the inocula, whereas 12 ppm nitrite

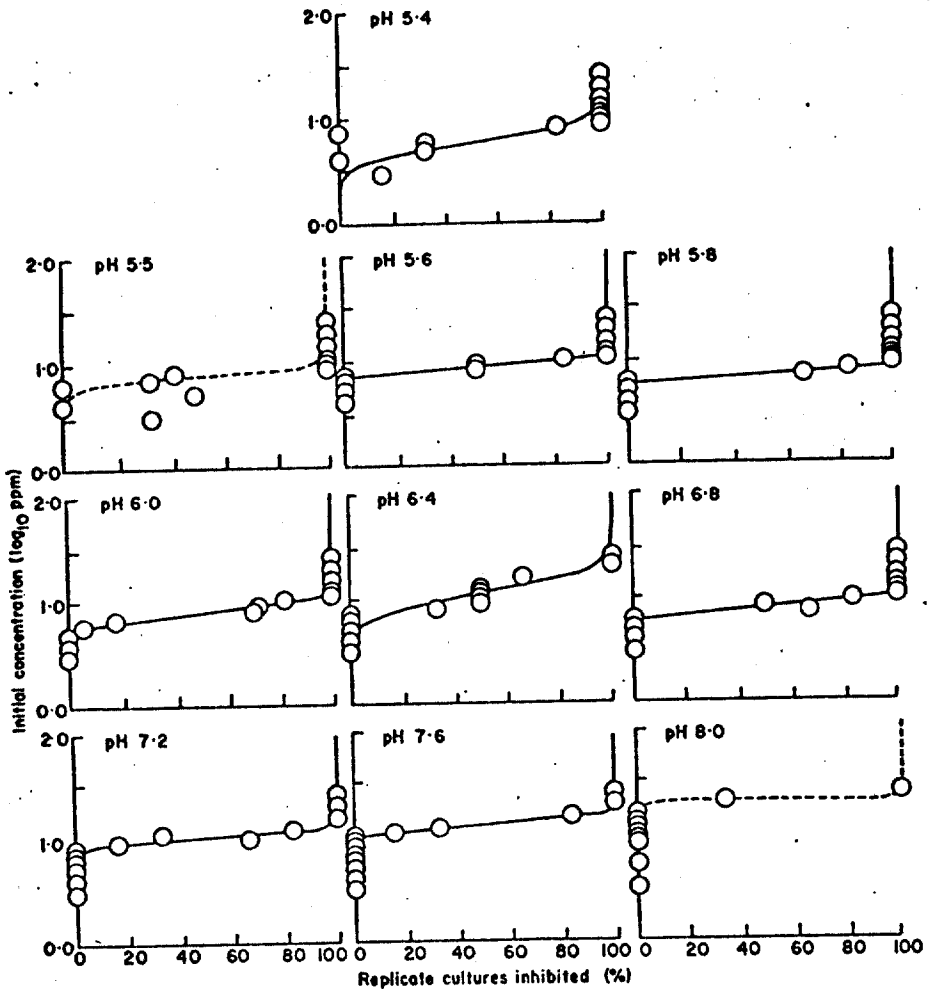


FIG. 2. The influence of sodium nitrite concentration, after heating in basic medium for 20 min at 121°C, on the probability of vegetative cultures of *Cl. sporogenes* PA 3679 being inhibited at various pH values. Continuous curves established by probit analysis. The broken curves are free-hand estimates.

are equally inhibitory after heating. At pH 6·0, which is more appropriate in the context of canned cured meats, the corresponding ED_{50} values are 52 ppm for unheated nitrite and 8 ppm for heated nitrite.

(3) *The influence of heating temperature on the inhibitory activity of nitrite in the basic medium at constant pH*

The results of this experiment are set out in Table 3 using the same conventions as before. The results are based on the preparation and observation of 1180 individual

TABLE 3. The influence of heating temperature on the inhibitory activity of sodium nitrite heated for 20 min in the basic medium at pH 6·0 (No. of cultures inhibited/No. of cultures tested)

TABLE 3. The influence of heating temperature on the inhibitory activity of sodium nitrite heated for 20 min in the basic medium at pH 6·0 (No. of cultures inhibited/No. of cultures tested)

Heating temperature (°C)	Nitrite concentration (ppm)																																
	0	1	2	3	4	5	6	7	8	9	10	11	12	14	15	16	18	20	25	30	35	40	45	50	55	60	65	70	75	80	90	100
Unheated	0/12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0/3	–	1/6	0/3	2/9	4/9	5/12	5/11	9/12	6/9	9/9	3/3	3/3	3/3	3/3
50°	0/9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1/6	0/3	3/6	0/3	4/6	2/6	3/9	4/9	4/9	3/3	4/6	3/3	3/3	3/3	3/3
60°	0/12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2/6	0/3	2/9	1/6	3/12	7/12	5/12	10/12	10/12	5/6	9/9	3/3	2/3	3/3	2/3
70°	0/12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0/6	0/3	3/6	0/3	3/12	3/12	6/12	6/12	12/12	5/6	9/9	3/3	3/3	3/3	3/3
80°	0/12	–	–	–	–	0/3	–	–	–	–	2/3	–	–	–	3/3	–	–	3/6	4/6	3/6	0/3	4/9	0/6	6/12	3/9	7/12	5/6	8/9	3/3	3/3	–	–
90°	0/14	–	–	–	–	0/3	–	–	–	–	2/3	–	–	–	3/3	–	–	3/6	3/3	6/9	0/3	9/12	7/9	13/15	11/12	15/15	11/12	15/15	3/3	3/3	–	–
95°	0/12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0/3	–	0/6	0/3	4/8	6/9	9/12	10/12	10/12	12/12	12/12	3/3	–	–	–
100°	0/6	–	0/3	–	0/3	1/3	0/3	–	1/3	–	3/6	–	–	–	5/6	–	–	6/6	6/6	6/6	–	3/3	–	–	–	–	–	–	–	–	–	–
109°	0/4	0/3	0/4	0/3	3/4	3/3	4/4	3/3	4/4	3/3	1/1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
115°	0/7	–	0/4	0/3	0/4	0/6	3/7	2/3	4/4	3/3	4/4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
121°	0/27	0/1	1/5	0/10	0/14	0/21	1/16	2/11	12/17	8/11	19/23	6/6	9/9	2/2	8/8	3/3	2/2	16/16	8/8	6/6	–	–	–	–	–	–	–	–	–	–	–	–
126°	0/4	–	0/1	–	0/4	0/3	0/3	0/3	0/4	–	0/4	–	1/4	3/4	–	4/4	1/1	1/1	–	–	–	–	–	–	–	–	–	–	–	–	–	–

cultures. Again, on probit analysis, the logarithm of nitrite concentration was found to give the best fit and the results are accordingly presented as a series of graphs in Fig. 3. The curves computed for 80° and 90°C were highly improbable on the basis of Chi-squared and have consequently been rejected and dashed free-hand estimates substituted. As will be seen from Table 3, none of the 131 nitrite-free controls was inhibited in this experiment.

The ED_{50} values for nitrite heated in the basic medium at various temperatures

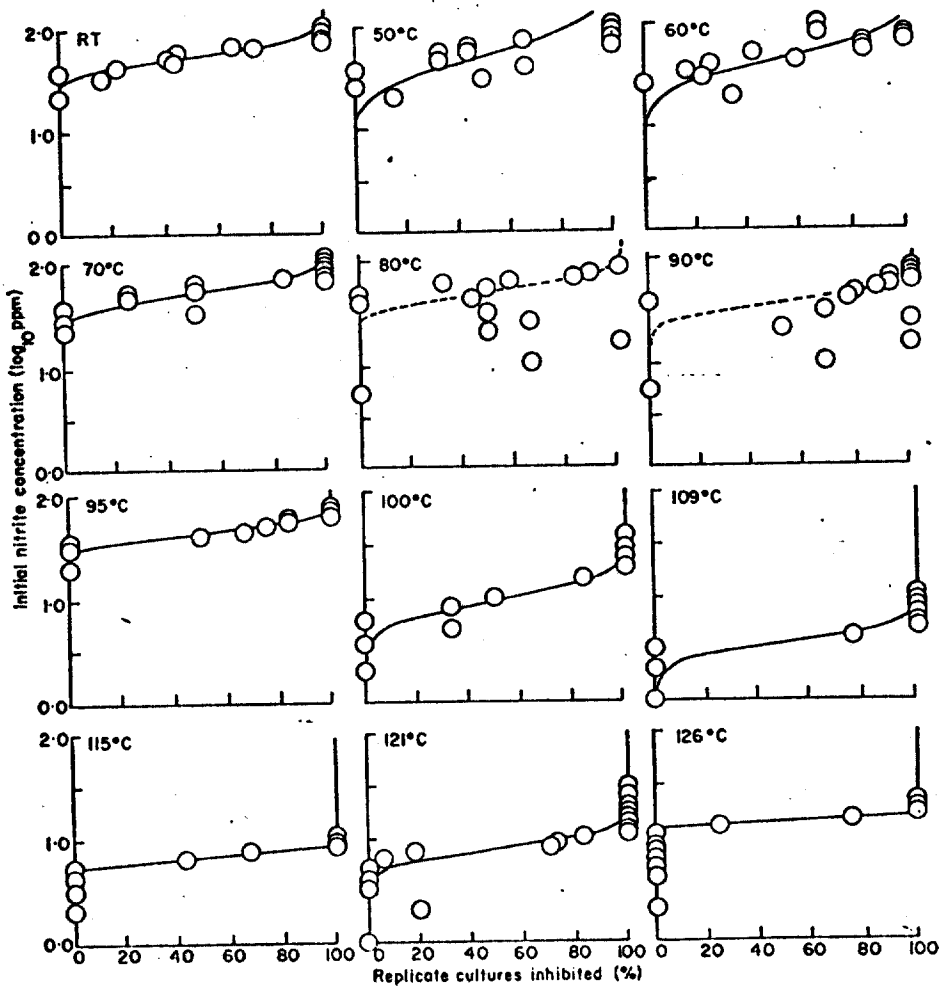


FIG. 3. The influence of sodium nitrite concentration, after heating in basic medium for 20 min at various temperatures, on the probability of vegetative cultures of *Cl. sporogenes* PA 3679 being inhibited at pH 6·0. Continuous curves established by probit analysis. The broken curves are free-hand estimates.

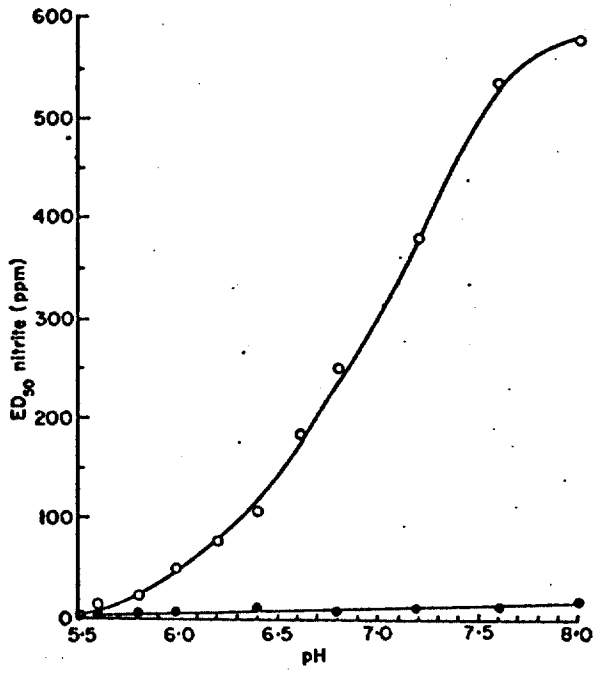


Fig. 4. The influence of pH on the ED_{50} values of nitrite incorporated in the basic medium without heating and of nitrite heated in the basic medium. o, Unheated nitrite; •, nitrite heated in the basic medium for 20 min at 121°C.

are plotted as open circles in Fig. 5. These ED_{50} values are computed from the data in Table 3. In each instance the heating time was 20 min and the substrate pH 6·0. The computed ED_{50} values corresponding to heating temperatures of 80° and 90°C are rejected as previously described and estimated values, represented by open squares, have been substituted in Fig. 5.

The ED_{50} value determined for the unheated nitrite controls in this experiment is 50 ppm. This value is in close agreement with the value of 52 ppm found for unheated nitrite at pH 6·0 in Experiment (1). As the heating temperature is increased from 50° to 95°C, the ED_{50} remains virtually constant and is very similar to that of unheated nitrite. As the temperature exceeds 100°C, however, the ED_{50} falls dramatically until at a temperature of 109°C it reaches a minimum value of 3·5 ppm. As the heating temperature is increased beyond 109°C the ED_{50} rises again, reaching 13 ppm at 126°C. It is of interest to note that in this experiment at 121°C the ED_{50} was found to be 7·6 ppm, which is very close to the ED_{50} value of 7·9 found at pH 6·0 in Experiment (2).

Thus it is apparent that the inhibitory activity of a given concentration of nitrite is increased if it is heated to an appropriate degree in the basic medium. This increase may be expressed quantitatively if the ED_{50} observed after 20 min heating at a par-

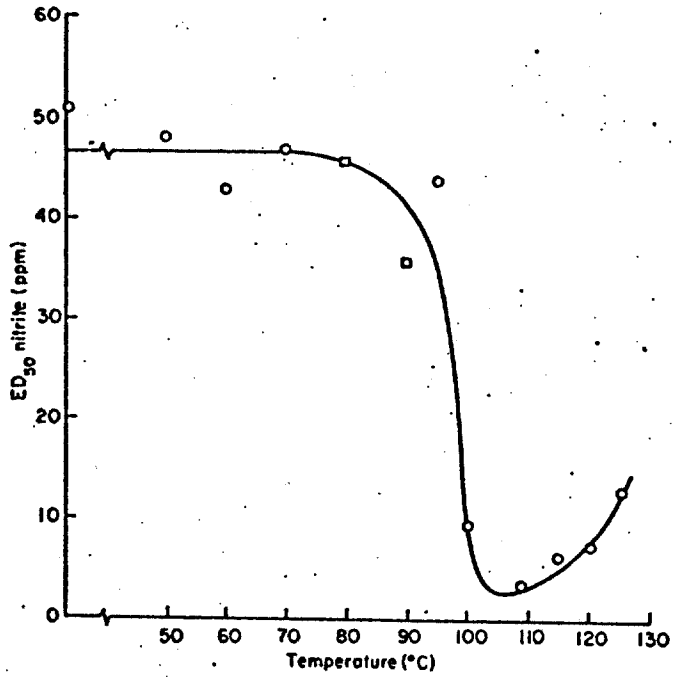


FIG. 5. The influence of heating temperature on the ED_{50} values of nitrite heated for 20 min in the basic medium at pH 6·0. ○, Values calculated by probit analysis; □, values transcribed from free-hand curves in Fig. 3.

ticular temperature is referred to the ED_{50} of unheated nitrite when an enhancement coefficient may be derived, i.e.:

$$\text{Enhancement coefficient} = ED_{50} \text{ unheated nitrite}/ED_{50} \text{ heated nitrite.}$$

In Fig. 6 the results of Experiment (3) are represented in terms of this enhancement coefficient. The graph is obtained by transposing the values represented by the curve in Fig. 5. It is apparent that the enhancement coefficient is very close to unity at heating temperatures between 50° and 90°C, i.e. the inhibitory activity of nitrite heated for 20 min at temperatures not exceeding 90°C is virtually identical with that of unheated nitrite. As the heating temperature is increased beyond 100°C, however, the enhancement coefficient increases dramatically until at about 109°C heated nitrite is more than fifteen times as inhibitory as unheated nitrite. As the heating temperature is increased beyond 109°C, the enhancement coefficient declines, but even at 126°C heated nitrite is still approximately three and a half times as inhibitory as the same concentration of unheated nitrite.

A subsidiary experiment was performed to establish the relationship between heating temperature and nitrite disappearance. Twelve aliquots each consisting of 20 ml basal medium at pH 6·0 and containing 50 ppm nitrite were dispensed into 1-oz

screw capped bottles. Eleven of these bottles were then heated for 20 min at one of the eleven temperatures investigated in Experiment (3). The remaining bottle served as an unheated control. Subsequently the residual nitrite concentration was determined in each bottle by the absorptiometric method previously described. The results of this

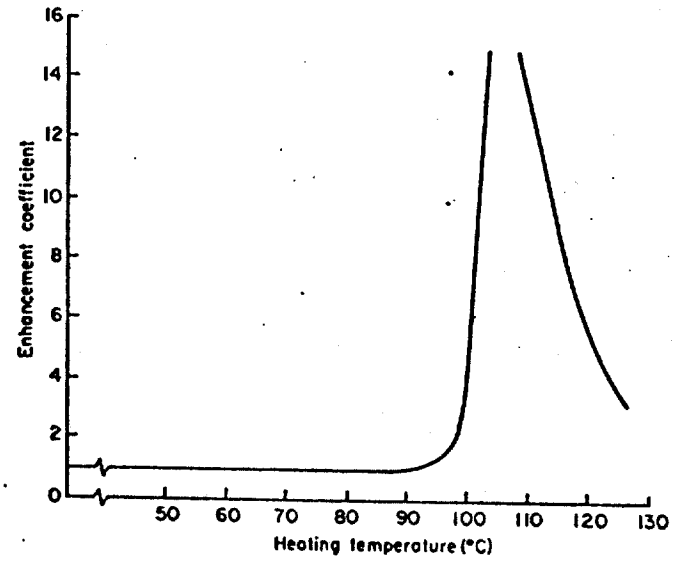


Fig. 6. The influence of heating temperature on the enhancement coefficient.

$$\text{Enhancement coefficient} = \frac{ED_{50} \text{ unheated nitrite at pH } 6{\cdot}0}{ED_{50} \text{ nitrite after 20 min heating at pH } 6{\cdot}0}.$$

investigation are illustrated in Fig. 7, in which percentage nitrite destroyed is plotted against heating temperature. It is apparent that when the initial nitrite concentration is 50 ppm at pH 6·0, perceptible nitrite destruction commences at a temperature of about 70°C when 2% (1 ppm) of the initial nitrite is destroyed in 20 min. As the heating temperature is increased the rate of nitrite disappearance increases in an exponential fashion, doubling with each 8°C rise of temperature, until at 115°C approximately 84% (42 ppm) of the initial nitrite is destroyed in 20 min. At higher temperatures the curve deviates from exponential; perhaps the reaction rate declines as the residual nitrite concentration approximates to zero, until at 126°C the curve is virtually horizontal. At this temperature some 93% (46·5 ppm) of the initial nitrite is destroyed in 20 min, the remaining 7% (3·5 ppm) apparently being very resistant to destruction.

If Figs. 6 and 7 are now compared, a correlation is apparent between the enhancement of nitrite inhibition and its disappearance in the analytical sense. At heating temperatures up to 90°C enhancement of inhibition is virtually absent (Fig. 6) and disappearance of nitrite is only slight (Fig. 7). As the heating temperature exceeds 100°C, however, marked enhancement of inhibition occurs (Fig. 6) and similarly nitrite disappearance becomes increasingly apparent.

The observations reported above are consistent with the hypothesis that, when nitrite is heated in the basic medium beyond a critical time and temperature, some reaction occurs which results in the substrate becoming more inhibitory to vegetative cells of PA 3679. This reaction might take one of two forms.

(1) The nitrite may react on heating with some component of the basic medium to yield an unknown substance which is actively inhibitory to the vegetative growth of PA 3679.

(2) The nitrite, on heating, may react with and destroy some essential nutrient in the basic medium, thereby rendering it incapable of supporting the vegetative growth of PA 3679.

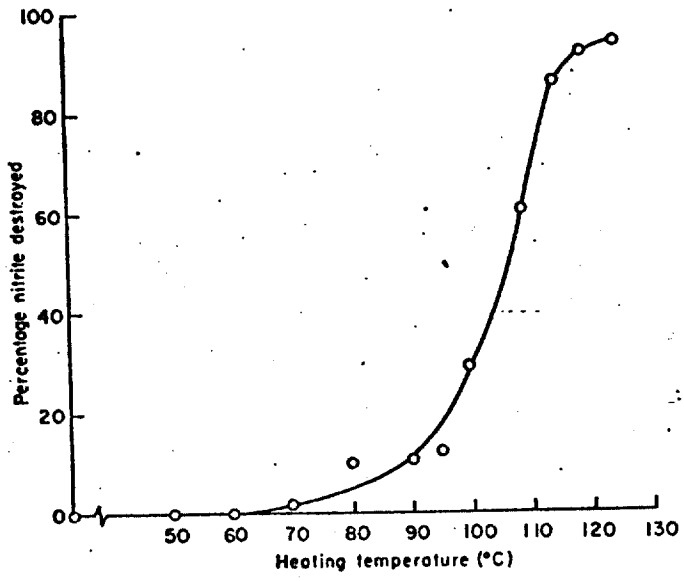


Fig. 7. The influence of heating temperature on the proportion of nitrite which disappears when a 50 ppm solution of nitrite in basic medium is heated for 20 min at pH 6·0.

Of these two alternatives, the first is considered to be the more probable on the following grounds. Inhibitory substrate cannot be rendered non-inhibitory simply by re-fortification with any of the basic medium components, but only by dilution to a degree consistent with the original concentration of autoclaved nitrite involved. If, for example, basic medium is autoclaved for 20 min at 121°C with 30 ppm nitrite, which is four times the ED_{50} under these process conditions, it can only be rendered non-inhibitory (supporting the growth of more than 50% of the inocula) by the addition of at least three volumes of sterile nitrite-free basic medium.

Moreover, the inhibition of nitrite is progressively enhanced as the heating temperature is increased from 90°C to about 105°C, but this enhancement declines as the

heating temperature is raised above 110°C (Fig. 6). This decline at high temperatures is consistent with the theory that an unknown actively inhibitory agent is formed when nitrite and basic medium are heated to a temperature in excess of 90°C and that this unknown agent is itself destroyed at temperatures exceeding 110°C. The decline of inhibitory activity at high temperature is not so easily reconciled with the alternative theory of essential nutrient destruction.

Conclusions

(1) *The inhibitory activity of unheated nitrite*

In Experiment (1) it was observed that when nitrite was incorporated, without heating, in the basic medium at each of the pH levels investigated, the probability of survival of an inoculum of 8×10^6 vegetative PA 3679 cells, when expressed in units of probit, was directly proportional to the log of the nitrite concentration. The slope of the computed regression at pH values of 5·6, 5·8, 6·0, 6·2, 6·4, 6·6, 6·8, 7·2 and 8·0 was 4·5, 3·5, 3·8, 5·3, 4·0, 5·3, 3·0, 2·4, 4·3 and 4·8, respectively. These values of slope do not appear to vary systematically with changing pH and, therefore, an average value of 4·1 is assumed for the true slope of the regression line over the pH range investigated. Thus we conclude that at any pH in the range 5·6–8·0, a ten-fold (one log cycle) change of unheated nitrite concentration will result in a change in the probability of inhibition equivalent to 4·1 units of probit.

Assuming a common slope of 4·1 and accepting the upper curve in Fig. 4 as the best estimate of variation of ED_{50} with changing pH, a synoptic figure may be derived which relates the probability of inhibition to the nitrite concentration at any pH in the range pH 5·6–8·0. In Fig. 8, which has been derived on this basis, nitrite concentration is represented on a log–linear ordinate and the percentage probability of inhibition is represented on a probit–linear abscissa. Fig. 8 is, of course, restricted specifically to the probability of inhibition of an actively growing inoculum of 8×10^6 vegetative cells. If smaller inocula or single cells are considered, then the probability of inhibition at any defined pH and nitrite concentration will be increased accordingly.

As noted previously, the inhibitory effect of unheated nitrite is highly pH dependent. As the pH is increased over the range pH 5·8–6·8, a ten-fold increase in inhibitory activity is apparent, which is consistent with the assumption that the effective inhibitory agent is undissociated nitrous acid. At pH values outside this range, however, the situation becomes more involved.

(2) *The enhancement of the inhibitory activity of nitrite which has been heated in the basic medium*

When nitrite is heated in the basic medium for 20 min at temperatures above 90°C a decline occurs in the concentration of nitrite which is detectable by the relevant analytical procedure and at the same time the inhibitory effect of the substrate is increased. The nitrite apparently reacts on heating with some component of the

basic medium to yield an unknown substance which is actively inhibitory to the vegetative growth of PA 3679. The rate at which this unknown substance is produced is maximal at a temperature of about 110°C. At temperatures exceeding 110°C the unknown substance appears to breakdown or react in such a way that its inhibitory activity declines.

Any quantitative consideration of the inhibitory activity of this unknown substance is complicated because its concentration cannot be determined. It may be that after a defined heat process in basic medium at constant pH, the concentration of unknown

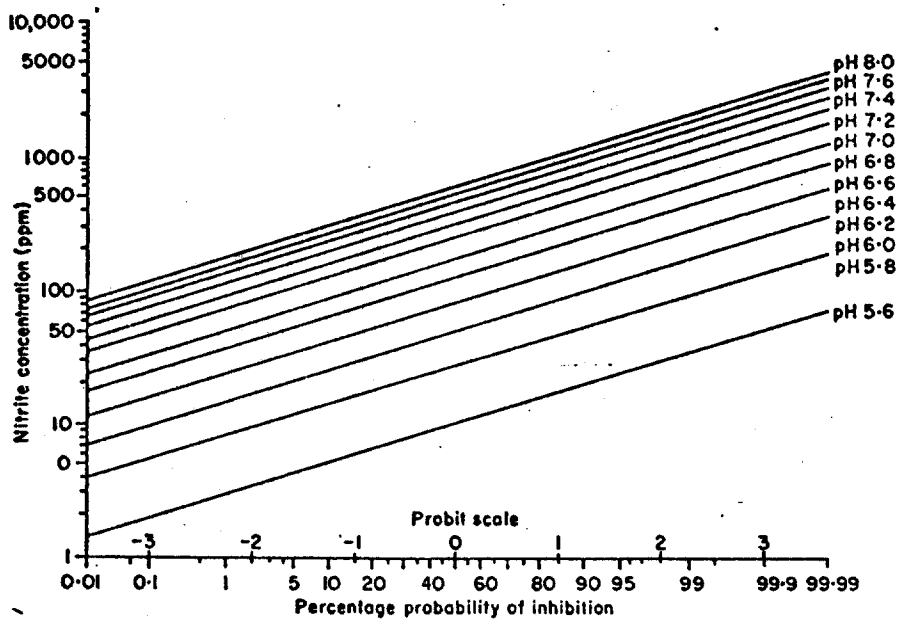


Fig. 8. The influence of unheated sodium nitrite concentration on the probability of an inoculum of 8×10^6 vegetative cells of *Cl. sporogenes* PA 3679 being inhibited in basic medium at various pH values.

which is formed is proportional to the concentration of nitrite initially present. If this is the case, then an examination of the relationship between initial nitrite concentration and post process inhibitory activity may reflect the relationship between the resulting concentration of unknown and its inhibitory effect.

In Experiment (2) it was observed that when nitrite was heated in the basic medium for 20 min at 121°C, at each pH the probability of survival of the test inoculum when expressed in units of probit was directly proportional to the log of the initial nitrite concentration. The slope of the computed regression at pH values of 5·4, 5·6, 5·8, 6·0, 6·4, 6·8, 7·2 and 7·6 was 6·5, 16·7, 22·7, 12·3, 5·8, 15·1, 14·8 and 16·6, respectively.

These values of slope do not appear to vary systematically with pH but are somewhat inconsistent, varying from 5·8 to 22·7. In the circumstances, the average of 13·8 is

the best estimate which can be made of the true slope of the regression line over the pH range investigated but this average can only be accepted with extreme caution. If the lower curve in Fig. 4 is accepted as the best estimate of variation of ED_{50} with pH for nitrite heated in basic medium at 121°C for 20 min, and assuming a slope of 13·8, a synoptic figure may be derived which relates the probability of inhibition to the heated nitrite concentrations. In Fig. 9, which has been derived on this basis, heated nitrite concentration is represented on a log–linear ordinate and the percentage probability of inhibition is represented on a probit linear abscissa. As postulated above,

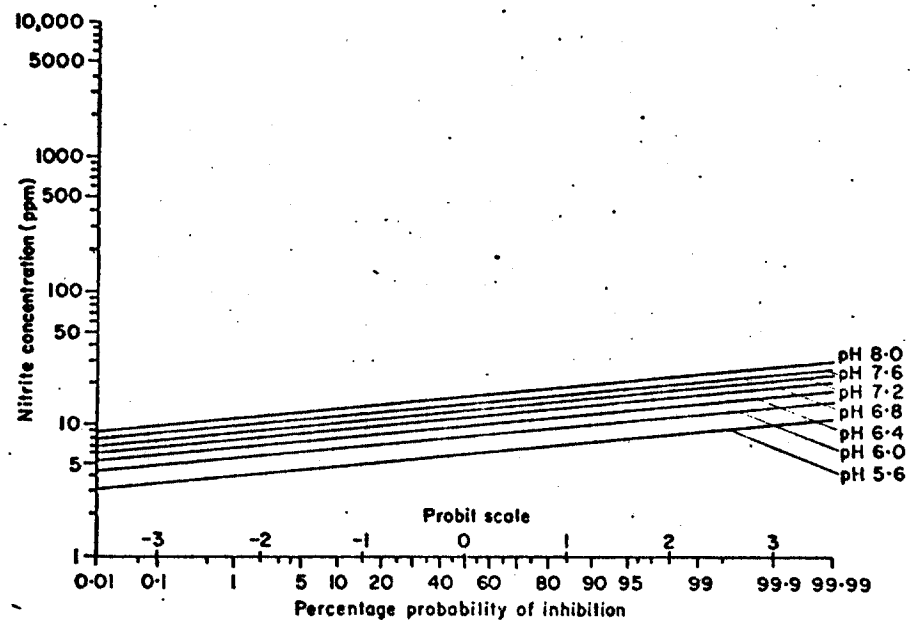


FIG. 9. The influence of sodium nitrite concentration, after heating in basic medium for 20 min at 121°C, on the probability of an inoculum of 8×10^6 vegetative cells of *Cl. sporogenes* PA 3679, being inhibited at various pH values.

it is possible that each value of initial nitrite concentration on the ordinate of Fig. 9 may correspond to some specific but indeterminate concentration of unknown inhibitor. Fig. 9, like Fig. 8, is specifically applicable only to an inoculum of 8×10^6 vegetative PA 3679 cells. Because of the doubt as to the slope of the regression lines however, Fig. 9 cannot be accepted with the same degree of confidence as Fig. 8.

Thus, in summary, our thesis is that when nitrite is sufficiently heated in the basic medium, it is involved in a reaction resulting in the production of some unknown inhibitory substance. This unknown substance differs from inorganic nitrite in three important respects. First, its inhibitory activity is only slightly pH dependent, whereas the activity of inorganic nitrite is highly pH dependent (Fig. 4). Secondly, it elicits

a much less variable response from the test inoculum. For example, as can be seen from Figs. 8 and 9, to increase the probability of inhibition from 5 to 95% (i.e. 3·3 probit units) necessitates a 1·7-fold (0·24 log cycle) increase of heated nitrite concentration, whereas to obtain the same increase in the probability of inhibition using unheated nitrite necessitates a 6·3-fold (0·8 log cycle) increase of concentration. Thirdly, it is apparently an extremely potent inhibitor. For example, sufficient unknown inhibitor is produced by autoclaving only 3·5 ppm nitrite for 20 min in basic medium at 109°C to inhibit the test inoculum in 50% of trials. Moreover, it is unlikely that more than a small proportion of this initial 3·5 ppm of nitrite may be usefully employed in producing the unknown inhibitor.

Turning now to the practical implications of this thesis: whilst it may have been demonstrated in this investigation that an unknown inhibitory agent is formed when nitrite is heat processed in a synthetic laboratory medium, it has not to date been established that the same agent is formed when nitrite is processed in commercially canned cured meat products. It is known, however, that substantial disappearance of nitrite does occur during the processing of these products and it seems not improbable that, in some circumstances at least, part of this nitrite may be involved in reactions leading to the production of such an inhibitory agent. If this is the case, then two interesting points arise. First, the commercial heat process must be considered not only in terms of its microbial lethal effect, but also in terms of its nitrite degrading effect. Secondly, Roberts & Ingram (1966) conclude that small variations in pH may markedly affect the stability of cured meats. This conclusion is probably justified in the case of commodities such as large canned hams and ox tongues in which the temperature of a substantial part of the pack may not exceed 90°C during processing and in which the effective inhibitory agent may consequently be the pH dependent undissociated nitrous acid. The conclusion may not be justified, however, in the case of smaller ham, tongue and pork luncheon meat packs, where substantially higher centre temperatures are attained and in which the effective inhibitor may be the unknown pH independent agent.

It would be naïve to suppose that the mechanism described above could wholly account for the remarkable stability of sublethally processed cured meats, but it may well play an important complementary role to the spore sensitizing mechanism described by Roberts & Ingram (1966). Certainly there is sufficient justification for the mounting of a serious attempt at isolating and identifying this agent in the synthetic system and searching for it in commercial products.

Acknowledgments

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programme, which was made available to the Metal Box Company by kind permission of Dr Frank Yates, C.B.E., Head of the Department of Statistics at Rothamsted, Mr D. H. Rees providing copies of the appropriate tapes. We also thank H. W. Gearing and the staff of the Metal Box Company Finance Department for the provision of computer facilities.

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EFFECT OF DIETARY NITRITE ON THE LIVER STORAGE OF VITAMIN A IN THE RAT[1]

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Abstract

When rats were fed 1% potassium nitrite in diets containing carotene or vitamin A, their livers contained less vitamin A than those of control rats. After prolonged feeding of potassium nitrite the total amount of vitamin A stored in the liver after a single dose of carotene or vitamin A was reduced, whereas there was no reduction after the administration of vitamin A aldehyde. Ingestion of nitrite did not affect the ability of rats to store vitamin A in the liver nor alter the rate of disappearance of the vitamin from the liver. It is suggested that dietary nitrite degrades carotene and vitamin A in the digestive tract before their absorption, and does not affect the conversion of carotene, the transport of the vitamin, nor the function of the liver.

Introduction

Decreased utilization of β-carotene and symptoms of vitamin E deficiency resulted when O'Dell *et al.* (1) fed nitrite to rats. Microorganisms in the rumen of sheep (2) and cattle (3) convert nitrate to nitrite, and methemoglobin occurs in the blood concurrently.

The prevalence, during the last decade, of vitamin A deficiency in cattle has been ascribed in some instances to increased nitrate in feedstuffs, which is presumed to lead to disturbed metabolism of the vitamin. Smith *et al.* (4) found liver and plasma vitamin A levels unaffected when nitrate was fed to steers for 56 days or to sheep for 30 days. Goodrich *et al.* (5) likewise found the plasma vitamin A of sheep unaffected, but the liver stores of the vitamin were reduced. Bruggemann and Tiews (6) suggested that nitrate interfered with the absorption of vitamin A in chicks and prevented its accumulation in the liver.

Several studies (7–11) have shown conclusively that carotene and vitamin A can be destroyed in vitro by nitrite, but not by nitrate. Destruction is most rapid

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at pH 1–3, falls off as the pH is brought to 6–7, and is minimal in neutral or alkaline media. The β-apo-8′- and β-apo-12′-carotenals are less severely attacked than β-carotene. The data of Roberts and Sell (11) suggest that vitamin A is inactivated in the presence of nitrite in the proventriculas of the chick, at approximately pH 4.

The hydrogen ion concentration of the digestive tract of cattle normally changes progressively from about pH 8 in the rumen to pH 2–4 in the abomasum. The foregoing brief review indicates that nitrite could effect destruction, as in vitro, of vitamin A and carotene before they are absorbed by the intestinal route. In an attempt to determine whether nitrite acts before or after absorption, we fed rats a diet containing a minimal amount of vitamin A and recorded the effect of dietary nitrite ingestion on levels of vitamin A in the liver when carotene or vitamin A were administered by stomach tube or intraperitoneally.

Materials and Methods

Random-bred Sprague–Dawley female rats from the colony at this institute were weaned at 21 days of age and fed, unless otherwise stated, a semipurified vitamin A-free diet (12). Stabilized vitamin A was added to it in amounts equivalent to 180 μg of vitamin A alcohol per kg, which is known to be adequate for growth but not for liver storage. This is the basal diet referred to later.

In experiment 1 the basal diet was supplemented with 6.0 mg of β-carotene per kg, and for one group also with 1% potassium nitrite. In experiment 2, 3.0 mg of vitamin A alcohol was substituted for the carotene. In experiment 3, vitamin A alcohol or vitamin A aldehyde was administered intraperitoneally.

For oral administration, crystalline β-carotene or vitamin A was dissolved in cottonseed oil. For parenteral administration the vitamin was dissolved in a minimum volume of ether containing Tween 80. Ether was removed under reduced pressure and water was added to give a final Tween:water ratio of 12:88.

Rats were killed at specified times, and the vitamin A in their livers was determined (13). Appropriate statistical analyses were performed on the data.

Experimental

The effect of concomitant feeding of potassium nitrite and 6 mg of carotene or 3 mg of vitamin A alcohol on liver storage of the vitamin was determined in two experiments. Ten rats were killed at 21 days of age as zero-day controls for each of experiments 1 (carotene) and 2 (vitamin A alcohol). Ten rats from each group were killed after 5, 10, and 20 days.

Table I shows the effect of dietary nitrite when either carotene or preformed vitamin A was fed. In experiment 1 the concentration of vitamin A in the liver showed no statistically significant difference when the overall effect of treatment and time were analyzed. A highly significant ($P = 0.01$) interaction between time and nitrite treatment was observed, however, and further analyses demonstrated that the linear trend of vitamin A concentration with

TABLE I

Vitamin A in rat livers after concomitant feeding of 1% potassium nitrite and carotene or vitamin A

KNO_2 in diet (%)	Days on diet	Animals per group	Experiment 1 (carotene)* (μg vitamin A ± S.E.‡)		Experiment 2 (vitamin A)† (μg vitamin A)	
			Per g liver	Per liver	Per g liver	Per liver
0	0	10	11.3±0.7	24±2	12.3±1.1	23±2
0	5	10	8.6±0.7	22±2	16.4±1.1	43±5
0	10	10	9.7±0.8	25±2	27.8±2.0	77±7
0	20	10	11.8±0.4	40±3	32.2±1.8	118±6
1	5	10	9.7±1.0	20±2	10.7±1.2	22±3
1	10	10	9.10±0.6	21±3	10.0±1.1	22±3
1	20	10	7.5±0.9	23±3	8.3±1.7	24±5

*β-Carotene, 6 mg per kg of diet.
†Vitamin A alcohol, 3 mg per kg of diet.
‡S.E. = standard error of the mean.

time was different ($P = 0.01$) when no nitrite was fed than when 1% nitrite was fed. Liver concentrations of vitamin A increased with time in the absence but not in the presence of nitrite. Over the 20-day feeding period the inclusion of 1% potassium nitrite in the diet significantly ($P = 0.01$) decreased the total amount of vitamin A stored in the liver, the trend being linear with time. In experiment 2, in which vitamin A was fed in the absence and presence of 1% potassium nitrite, highly significant effects ($P = 0.01$) of nitrite were observed. In the absence of dietary nitrite both the concentration and total amount of the vitamin stored in the liver increased with time, whereas no increase was observed when nitrite was fed concomitantly. The data do not indicate the point at which nitrite acted.

This was checked in experiment 3 with rats fed the basal diet with or without nitrite. After 30 days, 471 μg of vitamin A alcohol or 817 μg of vitamin A aldehyde were administered intraperitoneally. All animals were killed 24 hours later and the livers were excised for analysis. Table II shows that although dietary nitrite reduced growth (body weight) and liver mass ($P = 0.01$), it did not reduce the concentration of vitamin A in hepatic tissue. The total amount of vitamin A per liver decreased by 21% (vitamin A alcohol, $P = 0.01$) and 9% (vitamin A aldehyde, $P = 0.05$) although liver weights were about 37 and 29% less, respectively, than those of the control animals.

An objective of experiment 4 was to determine whether the consumption of nitrite affected the rate of disappearance of vitamin A from the liver. Approximately 40 rats were fed the basal alone, or with 0.5% or 1% potassium nitrite. On day 30 all animals were given 349 μg of vitamin A acetate by mouth and the basal diet was replaced by one that contained no vitamin A. On days 30 + 2 and 30 + 22 representative animals were killed and their livers were analyzed. Table III shows that consumption of up to 1% potassium nitrite in the diet did not alter the rate of decrease of liver vitamin A over a 20-day period.

TABLE II

Vitamin A in livers of control and nitrite-fed rats given vitamin A alcohol or aldehyde intraperitoneally (experiment 3)

Group*	Material administered	Nitrite in diet (%)	Initial body weight (g)	Final body weight (g)	Liver weight (g)	μg vitamin A	
						Per g liver	Per liver
1	Vitamin A alcohol†	0	55	134±4	6.81±0.23	32.6±1.4	220±8
2	Vitamin A alcohol†	1	54	90±2	4.25±0.14	41.6±2.6	174±8
3	Vitamin A aldehyde‡	0	55	118±8	5.94±0.14	63.1±2.4	372±11
4	Vitamin A aldehyde‡	1	54	88±3	4.20±0.13	82.0±3.3	340±10

*Fifteen animals per group.
†471 μg vitamin A alcohol injected intraperitoneally per rat.
‡817 μg vitamin A aldehyde injected intraperitoneally per rat.

TABLE III

Effect of nitrite feeding on rate of decrease of liver vitamin A stores (experiment 4)

Group	No. of rats per group	KNO_2 in diet (%)	Days after dosing	Final body weight (g)*	Liver weight (g)	μg vitamin A		Vitamin A† (% of original)
						per g liver	per liver	
A	17	0	2	130	6.80±0.21	12.4±0.8	84±5	
A	18	0	22	186	8.84±0.25	6.5±0.6	56±5	67
B	20	0.5	2	118	6.70±0.19	12.6±0.7	83±4	
B	16	0.5	22	166	8.54±0.23	6.4±0.7	53±5	64
C	19	1.0	2	91	5.43±0.26	14.6±1.2	75±3	
C	16	1.0	22	143	6.48±0.21	7.9±0.9	50±6	67

*Initial body weight = 67 g.
†Original vitamin A is total amount in liver 48 hours after 349 μg vitamin A acetate orally.

*Initial body weight = 67 g.
†Original vitamin A is total amount in liver 48 hours after 310 μg vitamin A ac[illegible] orally.

TABLE IV

Effect of ingestion of nitrite on utilization of orally administered vitamin A alcohol, vitamin A aldehyde, and carotene

Group*	Nitrite in diet %	Material administered orally	Final body weight (g)	Liver weight (g)	μg vitamin A		Reduction† (%)
					Per g liver	Per liver	
A	0	311 μg vitamin A alcohol	87	4.32	51.9±2.5	224±10	
B	0	984 μg vitamin A aldehyde	92	4.80	30.0±1.7	142±6	
C	0	1255 μg β-carotene	89	4.39	21.0±1.5	91±6	
D	1	311 μg vitamin A alcohol	73	3.64	48.1±2.9	173±9	23
E	1	984 μg vitamin A aldehyde	70	3.76	34.9±1.4	130±5	4
F	1	1255 μg β-carotene	71	3.50	18.8±1.2	65±4	29

*Each group contained 15 rats.
†Reduction in the μg vitamin A per liver.

Experiment 5 was similar to experiment 3, but the vitamin and provitamin were administered by mouth instead of parenterally. The basal diet alone or with 1% potassium nitrite was fed for 30 days, then 311 μg of vitamin A alcohol, 984 μg of vitamin A aldehyde, or 1255 μg of β-carotene were administered orally to representative animals, which were killed 24 hours later and their livers analyzed.

Table IV confirms the depression in growth (body weight) and liver weight that follows the ingestion of nitrite. Contrary to the results shown in Table II, where parenteral injection increased the concentration of vitamin A in hepatic tissue, the ingestion of nitrite produced no significant changes in the concentration of vitamin A in the liver when vitamin A alcohol, vitamin A aldehyde, or carotene was administered in a single oral dose. Of prime importance was the observation that the total amount of vitamin A stored in the liver was significantly ($P = 0.01$) reduced following the administration of vitamin A alcohol or of carotene to animals fed nitrite. Nitrite feeding had no effect on the total amount of vitamin A in the liver when vitamin A aldehyde was administered.

Discussion

The effect of a dietary constituent which modifies the amount of vitamin A in the liver when either carotene or vitamin A is fed can be the resultant of many factors. Decreased liver vitamin A may occur as a result of direct oxidation of the vitamin or provitamin in the digestive tract before absorption, or in body fluids after absorption; decreased absorption; enzymatic processes associated with conversion of carotene to vitamin A; or direct action on the liver, modifying either the uptake or release of vitamin A.

It was apparent from the experiments described here that the feeding of 1% potassium nitrite concomitantly with either carotene or vitamin A decreased the amount of vitamin A in the liver (Table I). This did not result from inability of the liver to store vitamin A, since the intraperitoneal injection of vitamin A after the feeding of nitrite resulted in increased concentrations of it in the liver (Table II). This confirms observations of Emerick and Olson (14), who injected vitamin A subcutaneously into rats fed nitrite. Since the rate of decrease of liver vitamin A was unaffected by the feeding of nitrite over a 20-day period (Table III), it can be concluded that the lower levels of liver vitamin A that occur after the feeding of nitrite (Table IV) are not a direct effect of nitrite on the liver or on hormonal mechanisms which modify the release of vitamin A from the liver.

The enzymatic steps in the conversion of carotene to vitamin A have not been elucidated, but whether degradation of carotene takes place by central fission or by terminal oxidation, vitamin A aldehyde is an intermediate (15). Our experiments demonstrated that the prolonged feeding of nitrite followed by the oral administration of either carotene, vitamin A alcohol, or vitamin A aldehyde reduces the storage of vitamin A in the liver only when carotene or vitamin A alcohol is administered but has little effect on the aldehyde. Since

this intermediate in carotene metabolism is unaffected, it is probable that absorption and transport mechanisms are also unaffected by nitrite. If enzymatic processes in the conversion of carotene to vitamin A were directly affected by nitrite, the decrease in liver storage of vitamin A would be much greater when carotene rather than when vitamin A was administered, since storage would reflect the resultant of the effect of nitrite on vitamin A per se and the decreased conversion of carotene to vitamin A. The similar degree to which nitrite affected storage of the vitamin from carotene and from vitamin A demonstrates that the conversion of carotene is not directly influenced.

It was pointed out in the introduction that carotene and vitamin A are oxidized by nitrite in vitro and the carotenals are less severely attacked. The experimental data presented here are compatible with the view that in the rat, reduction of liver storage of vitamin A from carotene or the preformed vitamin is a direct effect of oxidation by nitrite on the vitamin or provitamin, and does not involve interference with absorption, enzymatic processes associated with conversion of carotene, or liver function.

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Nitrate Content of Foods — Public Health Implications

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Introduction

Public concern has been aroused over the possible health hazard of high levels of nitrates found in some foods, and to the use of these foods in infant diets.

Comly (1945) demonstrated that some cases of methaemoglobinemia observed in babies were caused by high nitrate content of well water. Since then many surveys have been made in various parts of the world on the incidence of methaemoglobinemia and the nitrate content of the local water supply. Methaemoglobinemia is not new but has been recognized by the agricultural and medical sciences for many years. Interest has been aroused in recent years by the changing practices of agriculture. The most significant being the increased use of nitrogen containing fertilizers which, in general, results in increased nitrate content of plant material. Although the agricultural sciences have extensively studied the effects of nitrate ingestion in many species, little has been reported on the effects to humans of nitrate in foods. The World Health Organization (1962) has briefly reviewed the toxicity of nitrates and nitrites and proposed acceptable daily intakes for man. The comment was made that in establishing tolerances for added nitrate it is important to take into account the nitrate present in other foods. The sensitivity of normal babies and apparently dyspeptic babies made it impossible for the experts working under the aegis of WHO to make an estimate of an acceptable dose level for babies of six months of age or less on the basis of animal experimentation or clinical experience.

It is the purpose of the present report to examine the scientific data dealing with nitrates in foods in an attempt to clarify certain aspects of this question which have aroused concern and at the same time to provide guidance to those who are interested in this matter.

While it is necessary to introduce brief statements on the chemistry of methaemoglobin, biochemical relationships and its physiological significance, the reader is referred to a review by Bodansky (1951) for greater detail.

Methaemoglobinemia

Methaemoglobinemia is a condition in which the iron, normally in the ferrous form, is oxidized to ferric. This change is reversible and usually is not accompanied by any red cell damage or destruction (Stolman, 1961). The methaemoglobin is incapable of transporting oxygen and is considered to be a temporarily inactive respiratory pigment. In this condition the tissues are liable to anoxemia, due not only to the loss of O_2 capacity of the blood but also to increased difficulty in unloading O_2 which is available. In a mixture of methaemoglobin and oxyhaemoglobin the dissociation curve $HbO_2 \rightleftharpoons Hb + O_2$ is shifted to the left making less oxygen available to the tissue.

Normally about 1% of the total haemoglobin is present as methaemoglobin (Lane, 1952). Reducing enzymes in the red cells keep all but this small percentage of the total haemoglobin active unless the formation of methaemoglobin is abnormally high. With amounts of methaemoglobin under 5% no clinical symptoms are observed in children while, with greater than 5%, light cyanosis is obvious (Knotex and Schmidt, (1964). Death from asphxia due to methaemoglobinemia will occur when its concentration in the blood exceeds 70% of the total blood pigment present, provided there is no anaemia initially (Locket, 1957). In the presence of anaemia, lower concentrations will be fatal since the deciding factor is the amount of oxygen-carrying pigment available to the body

Many substances are known which directly or indirectly oxidize haemoglobin to methaemoglobin. This review will be limited to nitrite which is known to form methaemoglobin while nitrate does not. "Nitrate poisoning" might more aptly be called "nitrite poisoning" since the nitrate must be converted to nitrite prior to or during the ingestion and absorption of the nitrate. Nitrates themselves are toxic but at levels higher than are encountered in foods. High intakes produce irritation of the gastrointestinal tract, nausea, vomiting and acute gastroenteritis, muscular weakness, bloody stools, irregular pulse, convulsions, collapse and albuminous and possible bloody urine (Sollman, 1957; Wirth and Hecht, 1951; Goodman and Gilman, 1955). For a review of compounds other than nitrate-nitrite which produce methaemoglobinemia the reader is referred to Steyn (1960).

Microbiology of the Human Alimentary Tract

Since nitrate must be reduced to nitrite either prior to, or during the ingestion and absorption of foods then it is appropriate to briefly discuss the microbial population of the human alimetary tract as reviewed by Cuthbertson and Hobson (1962) and relate significant observations to nitrate metabolism.

The intestine at birth is relatively sterile but infection occurs rapidly. The intestinal flora of the breast-fed infant is mainly *Lactobacillus bifidus* which may constitute 99% of the total organisms in the faeces in the early weeks. A few enterococci and coliform bacilli have been reported. With weaning, the coliform

and enterococci increase. Clostridia and members of the Fusiformis group become characteristic of the faecal flora. Staphylococci of both the albus and aureus species are often present being probably derived from the infant's nose. In the bottle-fed baby *L. bifidus* is uncommon and *L. acidophilus* is usually present in large numbers.

Cuthbertson and Hobson (1962) state that although there is no really satisfying investigation concerning the localization of this flora in the breast-fed baby it appears in general that the duodenum was relatively sterile but bacteria, mainly enterococci, were found in the jejunum and in increasing numbers towards the ileum. There was a marked bacterial proliferation in the caecum and distal to it. The pH reached a peak of 7.0 to 8.0 in the caecum and tended to fall in the distal portions of the colon.

The adult empty stomach is generally sterile but after a meal it contains numerous organisms which have been ingested with the food. These, with the exception of acid resistant bacilli and sporing bacteria, appear to be killed off rapidly.

From the above review a number of items related to nitrate-nitrite metabolism become apparent. Lactobacillus organisms including streptococci and enterococci do not produce nitrite from nitrate (Breed, Murray and Smith, 1957) although coliforms will, thus in the normal infant, where the predominant organisms are Lactobacillus, there is little possibility of nitrate being converted to nitrite. The relative sterility of the duodenum further tends to minimize the effect of nitrate ingestion. Kubler (1958) observed that when children, aged 3½ - 8 months, were fed vegetables with a high nitrate content, nitrate was excreted rapidly in the urine and dropped during lower nitrate intake. This suggests that nitrate was absorbed rapidly in the upper part of the small intestine where relatively little or no reduction of nitrate would take place. *B. aerogenes, B. freundii* and *B. coli.* which normally inhabit the lower regions of the bowel (Ewing and Mayon-White, 1951) may invade the upper parts of the intestine. These organisms possess the property of rapidly reducing nitrates to nitrites. Under conditions of achlorhydria the upper part of the gastrointestinal tract is inhabited by these organisms. In one study (Ewing and Mayon-White, 1951) methaemoglobinemia following the intake of nitrate containing water occurred only in infants whose gastric juice had a pH greater than 4.

Expression of the Nitrate and Nitrite Content of Foods and Water

Considerable confusion and erroneous statements have been made due to the misinterpretation of the form in which the nitrate or nitrite was expressed. The most common fethods are to express the nitrate content in terms of the potassium or sodium equivalent (KNO_3 or $Na\ NO_3$), the nitrate ion (NO_3-) or as nitrate-nitrogen (NO_3-N). Due to the differences in molecular weights, 1 ppm KNO_3 is equivalent to 0.61 ppm NO_3 or 0.14 ppm NO_3-N. Conversely, the mathematical transformation from nitrate-N to nitrate requires multiplication by a factor of 4.4 and 6.1 if converting to sodium nitrate. A standard method of reporting nitrate values would be beneficial. This author prefers nitrate or nitrate-nitrogen.

Nitrate Toxicity in Relation to Water Supply

It has been well documented (Knotex and Schmidt, 1964; Steyn, 1960; Werner, Thal and Wuttke, 1965; Anon, 1951 a, b) that high nitrate content of the water supply has been responsible for many infant deaths and constitutes the major human health hazard of nitrate toxicity. The seriousness and worldwide importance of the problem is emphasized by the study conducted by the World Health Organization (1949). Many surveys have been made relating the incidence of methaemoglobinemia to nitrate concentration in the water and need not be repeated here except for selected studies which provide data for significant interpretation to other aspects of nitrate toxicity.

Simon Mazke, Kay and Mrowitz (1964) have recently studied the 745 cases of infant methaemoglobinemia due to nitrate in water reported in Germany from 1956 to 1964. Water from private wells was associated with 97.3% of the cases while 2.7% were associated with municipal supplies. Nitrate concentration were over 100 mg/1 in 83.8% of the cases. Nitrite was found in 10.4% of the water samples, 97.7% of the infants were not over three months of age, 53% had diarrhea and 8.6% died. Infant formulas in the metheamoglominemia cases were powdered milk in one-fifth of the cases, and diluted cow's milk or diets for diarrhea in the remaining infants. Healthy infants aged one, two or 3 months had different concentrations of methaemoglobin in the blood due to the drinking water. Infants older than three months had low values of methaemoglobin independent of the nitrate content of the drinking water. This report confirms and emphasizes previous observations that infants under three months of age are susceptible to nitrate toxicity while older infants and adults can tolerate large quantities. These facts are probably due to the following factors. Fetal haemoglobin which can be more readily oxidized is present in children under 3 months (Betke and Rau, 1952), difference in the capacity of the oxidation — reduction system in different individuals (Gibson, 1948), and the susceptibility of young infants with gastroenteritis in which intestinal bacteria ascend to the duodenum and reduce nitrates to nitrites (Gross, 1961). Burden (1961) considers that the high fluid intake in proportion to body weight is a significant contributing factor in infants. Infants of an assumed body weight of 3 kg consume approximately 0.5 l. of water per day while a 60 kg adult consumes only 1 l. per day. Burden (1961) recommends that the permissible limit of nitrate-nitrogen in water be 24 ppm if given to infants but may go as high as 240 ppm if for adult use. This recommendation is similar to that of the South African Bureau of Standards (1951) which specifies a limit of 20 ppm nitrate-nitrogen in drink-

ing water but higher than the 10 ppm set in some states of the United States (Anon. 1951) and recommended by the W.H.O. (1949) and the Committee of the Division of Medical Science of the National Research Council (Maxcy, 1950).

It should be further noted that most of the above cases of methaemoglobinemia resulted from the consumption of water from private wells as opposed to municipal sources; this would imply that bacterial contamination may also be a contributing factor. Hanway, Herrick, Willrich, Bennett and McCall (1963) however, consider that water containing more than 45 ppm nitrate (10 ppm nitrate-nitrogen) is unsafe for infant feeding, including formula, regardless of the bacterial quality of the water. Bacterially impure water may be sterilized by boiling, but the boiling and resulting evaporation concentrates the nitrate in the remaining water and therby increases the possibility of cyanosis. It is apparent, therefore, that water for infants should always be obtained from a source which has been tested safe according to both bacterial and nitrate limitations.

Nitrate Content of Foods

Nitrates are natural constituents of plant material since they are the main source of nitrogen required for growth. Even though legumes are not dependent on nitrates as a source of nitrogen they do take up nitrate and decrease the amount of nitrogen fixed by the nodule bacteria as the soil nitrate increases. Nitrites are rarely found in soils or plants in significant amounts unless under very unusual conditions (Hanway *et al*, 1963).

The agricultural sciences have recognized many disease conditions in domestic animals associated with ingestion of plant material having a high nitrate content. These conditions have included oat-hay poisoning (Bradley, Eppson and Beath, 1939), cornstalk disease, borna disease of horses and grass tetany of cattle. From the awareness of these conditions in domestic animals, Wilson (1949) made an extensive survey of foods for human consumption. More recently, Kamm, McKeown and Smith (1965) examined the nitrate content of Canadian baby foods.

Wilson (1949) found a number of vegetables grown in New York State which contained over 1000 ppm nitrate. These included beets (1,333), broccoli (1,552-3,816), cabbage (2,222), cauliflower (2,000), rhubarb (1,000-5,454). Tomatoes were the only vegetable containing no nitrate while intermediate values were found for carrots (442), cucumber (121), curly lettuce (500) and muskmelon (433). As would be expected from the above results the juices from green vegetables and quick frozen foods were high in nitrate; cabbage (1,861-1,200), carrots (181-325), rhubarb (1,818-4,545) and spinach (300-3,636). It is obvious that the nitrate content varies considerably not only between type of vegetable but within a given species. This may reflect part varieties, available nitrate in the soil and other environmental factors such as shading, moisture content of the soil, etc. In respect to fertilization, Schuppan (1965) found 450 mg nitrate/100 g dry weight in a sample of spinach from a field under normal cultivation but 3482 mg nitrate/100 g dry weight from a field treated with four times the normal amount of nitrogenous fertilizer.

Wilson (1949) also studied the nitrate content of strained baby foods. These are shown in Table I with selected data from Canadian baby foods determined by Kamm *et al* (1965). The concentration of nitrate in Canadian foods was higher than those reported for New York State. This may reflect in part differences in methodology used by Kamm *et al* (1965), changes in agricultural practice in the intervening years between surveys, or ecological factors.

Table 1. Nitrate Content of Prepared Infant Foods.

Food	Nitrate (ppm)	
	Wilson (1949)	Kamm et al* (1965)
Beets	333-750	634-2165
Spinach	616-833	1074-1668
Wax beans	—	321-568
Green beans	37	71-312
Squash	0	44-409
Carrots	0-3	66-167
Peas	0	—
Mixed vegetables	—	92-106
Precooked cereals	—	18-211
Meats	—	4-48
Dairy Products	—	0-31
Fruits	—	0-31

* Values converted from reported nitrate-nitrogen to nitrate.

Wilson (1949) stated that if it is assumed that 1 g of potassium nitrate ingested at a single meal is toxic, a basis is available for evaluating the effect of nitrate on human adults. From theoretical considerations he claimed a toxic quantity would be ingested from only 3.9 oz. of rhubarb or about 2.3 oz. of spinach, turnips or certain other vegetables. The basic assumption of the toxicity of one gram of potassium nitrate must be critically examined in relation to the ingestion of nitrate in foodstuffs. It is continually repeated that nitrates *per se* are relatively non-toxic (Garner, 1957); however, from clinical cases of accidental poisoning Smith and Simpson (1957) consider the normal fatal dose of potassium nitrate to be 15 to 30 g for an adult, although they quote a report (Peterson, Haines and Webster, 1961) of the death of an adult having taken 8 g. Sollman (1957) states that a total oral dose in excess of 4 g or a single dose of more than one gram though not lethal, may result in poisoning. From the above data it is apparent that Wilson's (1949) assumption that 1 g of potassium nitrate ingested in a single oral dose may be toxic is valid; however, the extrapolation that a similar amount ingested at a single meal from foods would similarly be toxic is unfounded. Assuming the average adult to be 70 kg, then the dose becomes 14.3 mg potassium nitrate per kg body weight or 8.7 mg nitrate. There is a paucity of data on direct clinical studies on the effects of feeding nitrate containing foods to humans; however, one study has been conducted by Kubler (1958) where seven infants (3½ to 8 months of age) were fed spinach which provided nitrate at an intake of up to 21 mg nitrate

per kg body weight. Of the seven infants, six showed no increase in methaemoglobin whatsoever and only one showed a very slight increase which was not sufficient to cause any symptoms of methaemoglobinemia. This latter infant showed elevated levels of methaemoglobin prior to the feeding of the nitrate rich spinach. Age is one of the most important consideration in evaluating nitrate toxicity. The above studies were conducted on infants which are much more susceptible to nitrate poisoning than adults and after feeding over twice the level considered as dangerous by Wilson (1949) no evidence of toxicity was apparent. Thus, the extrapolation by Wilson that the ingestion of 3.9 oz. of rhubarb or 2.3 oz. of spinach would supply a toxic quantity of nitrate is completely unjustified. Further since the quantity of nitrate fed by Kubler (1958) produced no toxic effect, then the value of 21 mg nitrate per kg body weight must be considered a minimum since no studies have been conducted in which larger quantities produced an effect on methaemoglobin levels.

The nitrate content of the baby foods reported in Table I are of interest in relation to the age factor which is of importance in methaemoglobinemia. As previously stated in relation to drinking water, Simon *et al* (1964) found that infants older than three monts of age had low methaemoglobin levels independent of the nitrate content of the water. Recommended feeding practices (Dept. of National Health and Welfare, 1953) suggest approximately this same age for the first feeding of solids. Dairy products contained the lowest amount of nitrate. Prepared, precooked cereals are fed beginning with one teaspoonful at 2½ months of age and increasing to 3 tablespoons by 5 months of age. The nitrate content of Canadian precooked cereals was not excessively high, the average amount being 57 ppm, which is not significantly different from the tolerance level of 44-50 ppm recommended for water. Further, the total intake of solids is low relative to the high fluid intake. Fruits are added to the infant diet at 3-4 months of age. This class of food does not appear to present any problem as relatively low amounts of nitrates are accumulated. The food class having the highest nitrate content are the vegetables which are not added to the diet until the fifth or sixth month of age. At this age normal infants would tolerate a high nitrate intake. It would appear advisable, however, to be selective in the choice of foods if the infant shows symptoms of a gastrointestinal disorder which may produce conditions conducive to the conversion of nitrate to nitrite.

Two cases of methaemoglobinemia have been reported (Holscher and Natzchka, 1964) in young infants aged 2 and 3.5 months shortly after eating spinach puree. Spinach from the same source was found to contain only traces of nitrate but 218 mg nitrite per hundred grams wet weight (2,180 ppm). Since this vegetable is known to store nitrate it was suggested that this particular batch contained a high concentration of nitrate which was somehow reduced during storage. Schuphan (1965) recently confirmed the above hypothesis. Spinach grown on a field treated with four times the normal amount of nitrogenous fertilizer contained as much as 3182 mg nitrate per 100 g dry weight but no appreciable amounts of nitrite. Following four days storage the nitrite content increased from 3 mg per 100 g at harvesting to as much as 355 mg. Schuphan attributes this change to nitrate taking on the role of an oxygen donor under anaerobic conditions. A flavin nucleotide nitrate reductase has been isolated and purified from spinach (Paneque, Del Campo, Ramirez and Losada, 1965). It was also shown (Schuphan, 1965) that conversion of nitrate to nitrite occurred when deep frozen spinach was allowed to warm up for 24-48 hours and this was thought to be due to bacterial reduction of nitrate. The above studies were on fresh spinach and more recently Simon, Kay and Mrowetz (1965) examined 82 baby food spinaches and found 82-1210 mg nitrate per kg but no nitrite or bacteria.

More recently, 14 further cases of methaemoglobinemia in infants aged 2 to 10 months have been reported from Germany (Sinios and Wodsak, 1965) after eating spinach. In nearly all cases the spinach was purchased as a fresh vegetable. Although clinical symptoms were not apparent in 12 cases when the food was ingested soon after preparation, it did become toxic after storage for 24 to 48 hours even in some cases under refrigeration. As in the previously reported cases the nitrate was converted to nitrite prior to ingestion.

From the above discussion on the nitrate content of foods and further extensive surveys of the literature, this author and others (Steyn, 1960; Anon. 1950) found evidence lacking for the toxicity to man of nitrates in foods. Nitrates may be considered as the index or precursor to the amount of nitrite which may be formed. If a food contains large amounts of nitrate then it is a potential hazard if conditions during storage or processing are such as to be conducive for conversion to nitrite. This has been demonstrated for spinach but not for other foods.

Recently, studies were conducted on fresh and processed spinach as purchased on the Canadian retail market to determine the nitrite—N content under conditions of storage and cooking which would be used by the consumer (Phillips, 1968). Selected samples of fresh spinach chosen from the Canadian market contained little or no nitrite when purchased. The processing procedures used in the production of the canned, pureed baby food, and frozen spinach did not give rise to appreciable quantities of nitrite. The nitrate present in the fresh leafy vegetable was reduced to nitrite under conditions of storage. The reduction was accelerated when the vegetable was stored at room temperature as compared with refrigeration. The nitrite, however, was in a dynamic state and decreased at room temperature so that high levels did not accumulate. High levels of nitrite did accumulate, in one case, when the fresh vegetable was stored under refrigeration. This may have been the result of the length of time the spinach had been stored prior to purchase and will require further study. Nitrite did not accumulate when frozen spinach was allowed to thaw for periods of up to 15 hours but did if an excessive period of 39 hours was permitted. Nitrite, therefore, would not be expected to increase in frozen spinach following recommended practices for

the use of this vegetable. Cooking of fresh spinach decreased the total nitrate— and nitrite—N contents of the edible material to approximately 20% of the initial value. The nitrite concentrations found in samples of pureed baby foods, canned spinach, or frozen spinach, even after long periods of storage, suggest they could be eaten with impunity with little or no hazard from nitrite toxicity. In most cases, fresh spinach presents no hazard. However, storage under refrigeration can produce conditions such that nitrite may accumulate to toxic amounts.

From a standpoint of nitrite toxicity due to the presence of nitrite prior to ingestion, frozen, canned, or baby food spinaches present little or no hazard. Fresh spinach may accumulate nitrite. These studies have not been extended to measure the conversion of nitrate to nitrite by the intestinal microflora in humans. In agreement with Simon (1966), it is recommended that unprocessed spinach be stored a minimum length of time and that recommended infant feeding practices be adhered to in regard to the age of the child at the time of introduction of vegetables to the diet. Spinach should not be introduced prior to 3 months of age.

Protective Factors in Foods

In considering the difference in effect of nitrate in well water and the lack of effect of nitrate in vegetables, Kilgore, Stasch and Barrentine, (1964), considered that a protective agent may be present. Since ascorbic acid has been used therapeutically to treat methaemoglobinemia in man and animals then its presence may influence nitrate toxicity. Normal or scorbutic guinea pigs dosed orally with nitrite showed no difference in the formation of methaemoglobin. Similarly, the content of human blood treated with nitrite (after withdrawal) showed no difference in methaemoglobin levels whether ascorbic acid was added or not. If a protective agent does exist in foods it is not ascorbic acid. No other data is available on protective agents in foods with the exception of a report by Knotex and Schmidt, (1964). When dried milk powders were mixed with water containing nitrates the amount of nitrites in the blood of suckling rats increased. Neither reduction of nitrate to nitrite nor methaemoglobinemia occurred when the milk powder was replaced by dried butter-milk. The authors claim that dried milks contain spores of the *B. subtilis* group which intensively reduce nitrate to nitrite. Only the microflora of the milk products need be considered as buttermilk differs from the other types of milk only in this regard. This phenomena as yet cannot be explained.

Aspects of Nitrate Metabolism other than Methaemoglobinemia

Bloomfield and Welsch (1961) have assessed the functioning of the thyroid gland following the feeding of nitrates to rats and sheep. This study was initiated by the demonstration (Wyngaarden, Wright and Ways, 1952) that several monovalent anions, including nitrate, when injected into the animal, can interfere with normal iodine metabolism of the thyroid gland. Iodine metabolism of the thyroid gland was affected when 0.31 and 0.92% potassium nitrate was consumed by rats or sheep (Bloomfield and Welsch, 1961). This effect would appear to be due to nitrate *per se* since the authors found no difference in methaemoglobin levels between treatment groups. The interference with iodine metabolism of the thyroid gland can be reversed by increasing the iodine intake from the diet (Wyngaarden *et al*, 1952). The nitrate content of a typical Canadian meal consisting of meat, salad, potatoes (neglected in calculation), a green vegetable, carrots and a dessert from figures for average portions (Dept. of National Health and Welfare, 1964) of each food and nitrate values from Wilson (1949) and Kamm *et al* (1965) was calculated. An evening meal was chosen as probably containing the highest nitrate intake. The total intake of 700 g of diet would contain 313 mg of nitrate which is equivalent to 0.045% nitrate on a fresh weight basis or an intake for a 70 kg adult of 4.5 mg per kg of body weight. This intake would not produce a significant effect on thyroid metabolism in rats. Since iodine reverses the effect of nitrate then the iodine intake in a normal diet would further nullify the effect. thus suggesting that the levels of nitrate found in human foods do not present a hazard in regard to thyroid function.

Since the above level would not adversely affect thyroid function then it is improbable that the conversion of carotene to vitamin A would be impaired. A functioning thyroid gland is necessary for this conversion (Johnson and Bauman, 1947). Nitrate-nitrite ingestion has been implicated in the increased incidence of vitamin A deficiency in ruminants in North America. The feeding of up to 1% potassium nitrate in the diet of rats does not impair vitamin A metabolism in the rat; however, potassium nitrite does. The ingestion of potassium nitrite decreases the amount of vitamin A in the liver when the source of the vitamin is either carotene or vitamin A. Nitrite does not affect the ability of the liver to store the vitamin or alter hormonal mechanisms which modify the release of vitamin A from the liver. It would appear, therefore, that reduction of liver storage of vitamin A is a direct effect of oxidation by nitrite on the vitamin or provitamin (Phillips, 1966). From the above it can be concluded that the presence of nitrates in foods should not adversely affect vitamin A metabolism in humans. If, however, nitrate is converted to nitrite during storage as has been demonstrated with spinach, then destruction of the vitamin or provitamin may occur. Foods having high nitrite content must be avoided but since nitrate *per se* can be ingested with impunity then in regards to vitamin A metabolism no direct health hazard exists in normal healthy individuals. Cases of dyspepsia or prolonged diarrhea in young infants may present a hazard since liver stores of the vitamin may be low. Prophylactic or therapeutic administration of vitamin A may be advisable under these conditions; however, no research has been conducted to definitely establish a relationship.

Summary and Recommendations

Nitrates *per se* are relatively non-toxic constituents of foods. Since nitrites are the toxic principle at lower intake levels it follows that the nitrate content of water or food is an index of the amount of nitrite which may be formed and thus represent the potential hazard. No evidence has been found of cases of nitrate toxicity in humans from foods even though the nitrate content is greater than that found in water which would be toxic.

On the basis of present knowledge the nitrate content of Canadian foods used as recommended (Dept. of National Health and Welfare, 1953) should not present a health hazard to normal human subjects. As previously emphasized, children under 3 months of age and age groups up to one year suffering from specified conditions, require separate considerations.

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Changes in the Nitrate and Nitrite Contents of Fresh and Processed Spinach during Storage

1671

W. E. J. Phillips

The influence of storage on the nitrate- and nitrite-N contents of fresh and processed spinach was studied. Under conditions applicable to the consumer, nitrite-N accumulated in some samples of fresh spinach, but not in canned, baby food, or frozen spinach. Nitrite-N accumulated if frozen spinach was allowed to thaw at room temperatures for excessively long periods. Cooking reduced the total nitrate- and nitrite-N contents of the edible portions of fresh spinach by extraction into the cooking water. The nutritional significance is discussed.

The possible production of methemoglobinemia in infants due to ingestion of foods containing large amounts of nitrates has been discussed from time to time. Comly (1945) demonstrated that some cases of methemoglobinemia observed in babies were caused by a high nitrate content of well water. It has been well documented (Knoteck and Schmidt, 1964; Steyn, 1960; Werner, 1965; World Health Organization, 1949; Simon *et al.*, 1964) that a high nitrate content in the water supply has resulted in illness and infant deaths and constitutes the major health hazard in regards to nitrate toxicity. In few cases was the contribution of the nitrate content of the food determined. Age of the child is one of the most important considerations in evaluating nitrate toxicity. Infants under 3 months of age are more susceptible than older infants, probably owing to the presence of fetal hemoglobin (Betke and Rau, 1952) and the reduction of nitrates to nitrites by intestinal bacteria which ascend to the duodenum in infants with gastroenteritis. Burden (1961) considers that the high fluid intake in proportion to body weight is a significant contributing factor in infants. There is a paucity of data from direct clinical studies of effects from feeding nitrate-containing foods to humans. One study, however, was conducted by Kubler (1958). Seven infants (3½ to 8 months of age) were fed spinach containing nitrate at an intake of up to 21 mg. of nitrate per kg. of body weight. Of the seven infants, six showed no increase in methemoglobin, but one showed a very slight increase which was not sufficient to cause any symptoms of methemoglobinemia. The latter infant exhibited elevated levels of methemoglobin prior to the feeding of the nitrate-rich spinach. Previously Wilson (1949) claimed from theoretical considerations that a toxic quantity of nitrate for adults would be ingested from 3.9 oz. of rhubarb or about 2.3 oz. of spinach, turnips, or certain other vegetables. Assuming the average adult to weigh 70 kg., the dose becomes 14.3 mg. of nitrate per kg. of body weight. The studies by Kubler (1958) were conducted on infants, and they are more susceptible to nitrate poisoning than adults. Even so, after feeding nearly twice the level considered dangerous by Wilson (1949), no evidence of toxicity was apparent. More recently the *British Medical Journal* (1966) discussed the findings of Sinios and Wodsak (1965) with the heading "Spinach—A Risk to Babies." The authors described children, age 2 to 10 months, that developed methemoglobinemia (including one death) after eating spinach. In nearly all cases, the spinach was purchased as a frozen vegetable. Although clinical symptoms were not apparent in 12 cases when the food was ingested soon after preparation, it became toxic after storage for 24 to 48 hours, even when under refrigeration in some cases. It was concluded that nitrate was not the toxic principle but, rather, that nitrite had been formed during storage. Our laboratory and others (Steyn, 1960; *Nut. Rev.*, 1950) found evidence lacking for the toxicity of nitrates per se in foods. Nitrites are the toxic principle which may be formed prior to ingestion or during digestion and absorption of food. Nitrate may be considered as the index or precursor to the amount of nitrite which may be formed. If a food contains large amounts of nitrate, it then is a potential hazard if conditions during storage or processing are conducive to conversion to nitrite.

Studies were conducted on fresh and processed spinach as purchased on the Canadian retail market to determine the nitrite-N content under conditions of storage and cooking which would be used by the consumer. The work described deals only with the formation of nitrite-N prior to ingestion and does not deal with the possible conversion of nitrate in foods to nitrite by intestinal microflora.

MATERIALS AND METHODS

During 1966 and 1967 unprocessed fresh spinach and three types of processed spinach (baby food, canned, and frozen) were purchased in local retail outlets and analyzed for nitrate- and nitrite-nitrogen by the method of Kamm *et al.* (1965). Samples for series 1, 2, and 3 were purchased in August and November 1966 and January 1967, respectively. For each study, three different samples were divided into two portions and stored either at room temperature in the dark or under refrigeration. Spinach leaves and stalks were left intact during storage to simulate ordinary conditions of use by the consumer. At specified periods, 10-gram samples of spinach were removed, mechanically homogenized in water, and the nitrate- and nitrite-N contents were determined as cited above. This yielded variable nitrate- and nitrite-N levels. Canned and baby food spinaches were opened, sampled, exposed briefly to the air, resealed, stored under refrigeration, and

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the sampling procedure repeated after various time intervals. Frozen spinach was sampled while frozen and stored for periods up to 5 months. In one series, frozen spinach was allowed to thaw at room temperature and the nitrate- and nitrite-N contents were determined. The pH of samples was not determined.

The distribution and changes in the nitrate- and nitrite-N contents of the vegetable were determined after cooking. The spinach was cooked under practical conditions by adding 30 grams of fresh spinach to 200 ml. of boiling water. Samples were cooked for approximately 10 minutes and the spinach was separated from the cooking water. Analyses were conducted on each fraction.

RESULTS

Fresh Spinach. Spinach used in the first series was purchased during the summer and stored at room temperature. This series was sampled after 4, 6, and 8 days' storage. Nitrate-N decreased extremely rapidly (Figure 1). During the first 4 days of storage, nitrate-N was reduced to approximately 30% of its initial level and was practically absent by the eighth day. An accumulation of nitrite-N was not apparent during this sampling. To study the changes occurring during the first 4 days of storage, a second series of samples was studied during the winter months. Room temperature was similar to the previous study (70° F.). The concentration of nitrite-N began increasing immediately on storage at room temperature while nitrate-N decreased. This indicated conversion of nitrate to nitrite occurred during the first 4 days followed by a decomposition of nitrite during the next 3 days.

Under refrigeration (Table I), the reduction of nitrate was delayed. The nitrate-N content of the spinach was extremely variable between sampling days. In series 2 and 3, no significant conversion of nitrate to nitrite took place within the first 14 or 22 days, respectively. In only one case (series 1) did nitrite-N accumulate rapidly within 8 days of storage. The nitrite-N formed did not decrease as observed when spinach was stored at room temperature.

Cooking of fresh spinach demonstrated that the total nitrate- and nitrite-N content was reduced to approximately 20 to 25% of the initial value. The decrease was accounted for by extraction into the cooking water. For example, when a quantity of spinach was cooked, the total nitrate and nitrite-N contents decreased from 4.5 to 0.97 mg. and 3.4 mg. was found in the cooking water.

Baby Foods. Three jars of baby food (one pureed spinach and two jars of mixed vegetables containing spinach) were opened and sampled for periods up to 35 days. The nitrate-N content of the mixed vegetables (Table II) was lower than fresh spinach. In both cases, the nitrite-N content was less than 1 p.p.m. For 15 days after opening the jars and with subsequent storage under refrigeration, there was no increase in the nitrite-N content. By day 35 the nitrite-N content of spinach had increased to 3 p.p.m., but no increase was observed in the mixed vegetables. The nitrate-N content did not decrease further over the storage period, indicating no major conversion of nitrate.

Frozen Spinach. The nitrate-N content of frozen spinach varied from 97 to 556 p.p.m., depending on the source. However, the nitrite-N content (Table III) was approximately 1 p.p.m. or less. There was no significant increase in the nitrite-N content with periods of storage

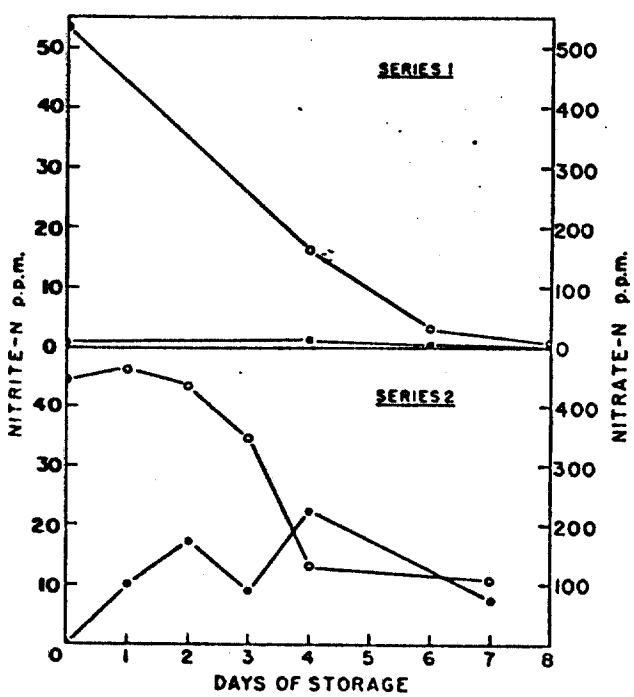

Figure 1. Effect of storage at room temperature on the nitrate- and nitrite-N contents of fresh spinach

●——● Nitrite-N content
O——O Nitrate-N content

Table I. Summary of Changes of Nitrate-N and Nitrite-N Contents of Fresh Spinach Stored under Refrigeration

		Duration of Storage, (Days)					
		0	8–11	14	21–22	28	39
Series 1	Nitrite-N[a]	0.4	32	210	543	439	...
	Nitrate-N	526	685	430	33	12	...
Series 2	Nitrite-N	0.4	0.2	0.3	26	27	...
	Nitrate-N	445	479	337	412	326	...
Series 3	Nitrite-N	0	0.3	...	0.4	17	18
	Nitrate-N	339	374	...	304	553	453

[a] Values in p.p.m.

Table II. Effect of Storage on Nitrate- and Nitrite-N Contents of Baby Food Samples

		Time, Days					
Sample		0	2	6	9	15	35
Spinach	Nitrite-N[a]	0.4	0.1	0.2	0.3	0.1	3.1
	Nitrate-N	180	192	214	197	208	214
Mixed vegetables	Nitrite-N	0.5	0.2	0.4	0.3	0.2	0.04
	Nitrate-N	42	37	43	42	44	46

[a] Values in p.p.m.

Table III. Effect of Storage and Thawing on Nitrite-N Content of Frozen Spinach

Storage time, months	0	1	3	4	5	5	5
Thawing time, hours	0	0	0	0	0	15	39
	NITRITE-N, P.P.M.						
Sample 7	0.4	0.4	0	0.1	1.0	0	30
Sample 8	0.5	0.3	0	0.3	0.6	0	19
Sample 9	0.3	0.5	0	0.4	1.2	0	74

in the frozen state of up to 5 months. The length of time samples were allowed to thaw did influence the nitrite-N content. Thawing up to 15 hours at room temperature did not increase the nitrite-N content, but a longer period of 39 hours did.

Canned Spinach. Three different brands of canned spinach were studied for a period of up to 7 days after opening (Table IV). The nitrate-N content was lower than the fresh vegetables studied and varied from 114 to 324 p.p.m. with an average of 200 p.p.m. The nitrite-N content was less than 1 p.p.m. when opened and did not increase significantly over the 7-day period.

DISCUSSION

The data presented above demonstrate that the selected samples of fresh spinach chosen from the Canadian market contained little or no nitrite when purchased. The processing procedures used in the production of the canned, pureed baby food, and frozen spinach did not give rise to appreciable quantities of nitrite. The nitrate present in the fresh leafy vegetable was reduced to nitrite under conditions of storage. The reduction was accelerated when the vegetable was stored at room temperature as compared with refrigeration. The nitrite, however, was in a dynamic state and decreased at room temperature so that high levels did not accumulate. High levels of nitrite did accumulate, in one case, when the fresh vegetable was stored under refrigeration. This may have been the result of the length of time the spinach had been stored prior to purchase and will require further study. Nitrite did not accumulate when frozen spinach was allowed to thaw for periods of up to 15 hours but did if an excessive period of 39 hours was permitted. Nitrite, therefore, would not be expected to increase in frozen spinach following recommended practices for the use of this vegetable. Cooking of fresh spinach decreased the total nitrate- and nitrite-N contents of the edible material to approximately 20% of the initial value. The findings reported above are similar to and in agreement with those observed in Germany by Sinios and Wodsak (1965).

The establishment of tolerance levels for nitrate and nitrite is extremely difficult. The World Health Organization (1949) has reviewed the toxicity of nitrate and nitrites and has stated that the sensitivity of normal babies and apparently dyspeptic babies makes it impossible to establish an acceptable dose level for babies of 6 months of age or less on the basis of animal experimentation or clinical experience. For nitrite, however, the problem can be simplified in regard to the feeding of vegetables. In the newborn, fetal hemoglobin accounts for 60 to 80% of the total hemoglobin. This is reduced to approximately 30% by 3 months of age. Fetal hemoglobin (Betke and 1952) is more readily oxidized. Thus, children u months of age are more susceptible to methemoglobi It is not until after this age that recommended f practices ("Canadian Mother and Child," 1953) sugge introduction of vegetables to the diet. In considerin toxicity of nitrite in older children and adults, the am of nitrite required to produce methemoglobinemia c estimated. About 1% of the total hemoglobin nor is present as methemoglobin (Lane, 1952). With am of methemoglobin under 5%, no clinical symptom observed in children, while light cyanosis is ob (Knoteck, 1964) with greater than 5%. Death from phyxia due to methemoglobinemia occurs when the centration in the blood exceeds 70% of the total b pigment present, provided there is no anemia init (Locket, 1957). In the presence of anemia, lower con trations are fatal, since the deciding factor is the amo of oxygen-carrying pigment available in the body. though both methemoglobin-forming and methemoglo reducing reactions take place concurrently (Bodan 1951), only the former reactions will be considered for purpose of calculation. The data were calculated fo 1-year old child having a body weight of 22.5 lb. (Falkn 1962) with a blood volume of 9% of body weight (Lo 1961). Assuming a hemoglobin content of 17 grams p 100 ml. of blood, this is equivalent to 1.118 mmoles hemoglobin. Greenberg *et al.* (1943) have shown that o molecule of nitrite reacts with two molecules of hem globin to form two molecules of methemoglobin. There fore, 1 μMole or 14 μg. of nitrite-N could convert 2 μmole or 30.4 mg. of hemoglobin. The amounts of nitrite-N and the equivalent amounts of spinach which would nee to be ingested to produce methemoglobinemia of varying degrees are shown in Table V. These figures cannot be exact and provide a safety factor because the reconversion of methemoglobin to hemoglobin has been neglected. A further four fold increase in the amount of spinach which could be safely ingested would be introduced during cooking by extraction of the nitrate and nitrite into the cooking water. The nitrite concentrations found in the present investigation in samples of pureed baby foods, canned spinach, or frozen spinach, even after long periods of storage, suggest they could be eaten with impunity with little or no hazard from nitrite toxicity. In most cases, fresh spinach presents no hazard. However,

Table IV. Effect of Storage under Refrigeration on the Nitrate-N and Nitrite-N Contents of Canned Spinach[a]

	Storage Time, Days			
	0	1	4	7
Nitrite-N	0.2	0.5	0.4	0.7
Nitrate-N	200.0	210.1	190.4	176.3

[a] Each value represents the average of three brands. Values in p.p.m.

Table V. Postulated Relationship of Ingested Nitrite-N to Methemoglobinemia

Age	Weight, Lb.	Total Blood Vol., Ml.	% Methemoglobin	NO_2-N Required, Mg.	Spinach Required Containing 10 P.P.M. of NO_2-N,[a] Lb.
1 year	22.5	900	5	3.52	0.78
1 year	22.5	900	30	21.13	4.65
1 year	22.5	900	70	49.30	10.86
Adult	148	6000	5	23.45	5.17
Adult	148	6000	30	140.84	31.02
Adult	148	6000	70	328.72	72.41

[a] = 4.54 mg. NO_2-N/lb.

storage under refrigeration can produce conditions such that nitrite may accumulate to toxic amounts. From the data observed in series 1 (Table II), storage of spinach for 14 days resulted in 210 p.p.m. nitrite-N which would require ingestion (if eaten uncooked) of only 0.037 lbs. to yield sufficient nitrite for a detectable cyanosis in a 1-year old child. The cause of the variability of nitrite accumulation may be related to the length of storage prior to purchase or bacterial contamination. Further research is needed to establish the incidence of spinach samples which will accumulate nitrite on storage.

From a standpoint of nitrite toxicity due to the presence of nitrite prior to ingestion, frozen, canned, or baby food spinaches present little or no hazard. Fresh spinach may accumulate nitrite. As stated previously, these studies have not been extended to measure the conversion of nitrate to nitrite by the intestinal microflora in humans. In agreement with Simon (1966), it is recommended that unprocessed spinach be stored a minimum length of time and that recommended infant feeding practices be adhered to in regard to the age of the child at the time of introduction of vegetables to the diet. Spinach should not be introduced prior to 3 months of age.

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Lack of Nitrite Accumulation in partially Consumed Jars of Baby Food

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Abstract

Partially consumed jars of commercial baby food, representing ten food classes having various levels of naturally occurring nitrate, were stored under refrigeration for seven days. Under the conditions used in the present experiment nitrite did not accumulate to levels which would present a health hazard in regard to methaemoglobinemia.

Résumé

Des aliments pour bébés, sur le marché et représentant dix sortes d'aliments avec différent taux de nitrate naturel, ont été partiellement employé et réfrigéré durant sept jours. Dans ces conditions, il n'y a pas eu d'accumulation de nitrite à un taux suffisant pour qu'un danger à la santé soit causé par la methaemoglobinemie.

Introduction

Recent reports (Commoner 1968a, b) have aroused public concern over the use of foods containing high le of nitrates for infant feeding. The public health implications of the nitrate content of foods has been reviewed (Phillips, 1968) and it was concluded the nitrate content of Canadian foods should not present a hazard to normal infants if used as recommended. Nitrates *per se* are relatively non-toxic constituents of foods and serve only as an index of the amount of nitrite which might be formed, and thus represent the potential hazard in regards to infant methaemoglobinemia. To present a direct hazard nitrate must be reduced to nitrite either prior to, or during the ingestion and absorption of foods.

Cases of methaemoglobinemia have been reported from Germany (Sinios & Wodsak, 1965) in infants aged 2 to 10 months as a result of consumption of spinach. In nearly all cases the spinach was purchased as a fresh vegetable and cooked in the home. Although clinical symptoms were not apparent in 85% of the cases when the food was ingested soon after preparation, it did become toxic after storage for 24 48 hours due to the conversion of nitrate to nitrite prior to ingestion. Brown and Smith (1967) consider that the same possibility exists with commercial baby food and have stated "there is no ready solution to this problem other than discarding partially consumed jars of baby food".

The following studies were conducted to determine whether significant amounts of nitrite accumulated in jars of partially-consumed commercially prepared baby foods under conditions applicable to the Canadian consumer.

Experimental Methods.

Ten classes of baby foods containing various levels af naturally occurring nitrate were purchased on the Canadian market. Cooperation of parents with infants between the ages of 2 to 10 months was obtained. Since baby food may be used in geriatric nutrition, adult volunteers were used as a limited number of subjects. Instructions were given that jars were to be opened in the home and the subject fed with a spoon directly from the jar. In some cases the food was heated in the open jar prior to serving. Half the contents of the jar was fed at one time, the jar recapped and placed in the home refrigerator. Samples were returned to the laboratory the day following feeding. Samples were stored under refrigeration for a further 7 days and were analyzed for nitrate-nitrogen (NO_3-N) and nitrite-nitrogen (NO_2-N) on days 0, 1, 2 and 7 by the method of Kamm *et al* (1965). One jar from each food class was kept in the laboratory as a control sample. Exaggeration of the storage effect was accomplished by storing all of the creamed spinach and beets under refrigeration for periods of from 7 - 52 days followed by 7 days storage at room temperature.

Results and Discussion

The nitrate-nitrogen content of the infant foods shown in Tables 1 and 2, are with one exception within the ranges reported by Kamm *et al* (1965) the exception being creamed spinach which contains approximately one-quarter of the amount of nitrate nitrogen previously observed. Applesauce, custard and meat products contained low levels of nitrate nitrogen while the strained vegetables were relatively high. Infants up to three months of age are most susceptible to methaemoglobinemia and it is interesting to observe that the food classes recommended for infant feeding at early ages are lower than the vegetable classes which would normally be introduced later.

Of prime importance is the observation that nitrite-nitrogen did not accumulate in any of the food samples during storage under refrigeration for periods up to seven days. Lack of conversion was further demonstrated by the unchanged nitrate-nitrogen levels throughout the course of the experimental period

Table I

Effect of storage under refrigeration on the nitrate and nitrite - nitrogen content of partially consumed baby foods

	Number of Samples	Age range	Control or exposed	Nitrate - nitrogen (ppm) Day 0	Day 1	Day 2	Day 7	Nitrite - nitrogen (ppm) D. 0	D. 1	D. 2	D. 7
1. Junior Apple-sauce	8	2-10 months	C	0.4	1.6	1.4	1.5	0.0	0.0	0.0	0.0
			E		1.4±0.63*	2.6± 0.85	1.6± 0.48		0.0	0.0	0.0
2. Strained chocolate custard	5	9 months - adult	C	8.4	10.0	9.4	2.7	0.0	0.0	0.0	0.0
			E		2.6±0.76	3.7± 1.37	2.7± 0.62		0.0	0.0	0.0
3. Junior mixed vegetables	4	9-10 months	C	9.4	8.9	15.7	15.6	0.0	0.0	0.0	0.0
			E		14.9	9.1± 3.13	8.8± 3.56		0.0	0.0	0.0
4. Junior Dinner Eggs and bacon with vegetables	5	3-10 months	C	10.5	7.5	7.9	7.0	0.0	0.0	0.0	0.0
			E		7.7±1.20	7.6± 1.15	7.2± 0.95		0.0	0.0	0.0
5. Junior Meat Ham with broth	9	2 months adult	C	13.1	9.4	10.2	8.8	0.0	0.0	0.0	0.0
			E		8.4±0.60	8.7± 0.68	9.2± 0.86		0.0	0.0	0.0
6. Strained Dinner Eggs and bacon with vegetables		4- 9 months	C	13.5	7.8	9.2	7.4	0.0	0.0	0.0	0.0
			E		4.9±0.50	6.4± 1.55	11.3± 6.50		0.0	0.0	0.0
7. Strained garden vegetables	4	2-10 months	C	55.2	51.2	52.8	53.0	0.0	0.0	0.0	0.0
			E		51.6±2.52	55.6± 0.55	50.6± 3.26		0.0	0.0	0.0
8. Strained creamed spinach	9	2 months - adult	C	64.9	58.0	69.0	70.2	0.0	0.0	0.0	0.0
			E		69.3±4.75	71.0± 2.91	66.3± 2.35		0.0	0.0	0.0
9. Strained golden wax beans	4	2- 9 months	C	89.5	83.4	90.2	87.1	0.0	0.0	0.0	0.0
			E		109.3±2.30	116.4± 2.45	111.9± 6.18		0.0	0.0	0.0
10. Strained beets	7	3 months - adult	C	117.0	117.0	122.5	100.5	0.0	0.0	0.0	0.0
					120.3±8.85	106.7±11.67	129.0±12.39		0.0	0.0	0.0

* ± Standard error of the means.

Table 2

Effect of storage at room temperature following prolonged refrigeration storage on the nitrate and nitrite - nitrogen content of partially consumed baby foods.

	Number of Samples	Day 0 at room temperature: Sample Age	NO_3-N ppm	NO_2-N ppm	Day 7 at room temperature: Sample Age	NO_3-N ppm	NO_2-N ppm
1. Strained creamed spinach	9	7-56 days	66.6±1.74	0.0	14-63 days	33.3±7.21	0.73±0.72
2. Strained beets	7	7-52 days	127.4±7.70	0.0	14-59 days	113.6±9.40	0.10±0.10

Storage tests were exaggerated for strained creamed spinach and beets (Table 2). The samples remaining after feeding were retained for periods of from 7 to 56 days under refrigeration. In the case of spinach, nitrate-nitrogen decreased, without however a significant accumulation of nitrite-nitrogen. There were no changes in either the nitrate or nitrite-nitrogen content of strained beets.

Many millions of jars of commercially prepared baby foods have been consumed in North America over the past 20 years yet not attributable cases of methaemoglobinemia have been reported. The present data suggests that the advice to discard partially used containers of commercially prepared baby foods the same day they are opened is unnecessarily conservative. Under the normal home conditions used in the present experiment, nitrite did not accumulate to levels which would present a hazard in regard to methaemoglobinemia.

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Naturally Occurring Nitrate and Nitrite in Foods in Relation to Infant Methaemoglobinaemia*

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SUMMARY

The nitrate and nitrite content of vegetables and commercially prepared baby food has been reviewed in the light of literature reports of infant methaemoglobinaemia. Methaemoglobinaemia results from absorption of the nitrite ion formed by conversion of nitrate. Documented evidence indicates conversion prior to ingestion as the primary health hazard and European experience implicates only nitrate-rich unprocessed spinach. Unprocessed spinach should be stored for a minimum length of time under proper conditions, and home-prepared spinach should never be stored for subsequent feeding. No cases of toxicity resulting from nitrates in foods have been reported in North America, but in view of the apparent sensitivity of young infants, prudence would dictate that foods, such as spinach and beets, containing high levels of nitrate should not be introduced into the diet of children below 3 months of age. Selectivity should be used in the choice of foods given during infancy if signs of gastro-intestinal disorder become apparent.

There is no evidence that the nitrate content of commercially prepared foods available on the Canadian market constitutes a hazard to human health.

Introduction

Time and experience have shown us that technological advances often create new problems in our ecosystem. The intrusion of man into the nitrogen cycle is but another example resulting in changes in our environment. This threat to the integrity of the nitrogen cycle has recently been reviewed by Commoner (1968), who emphasized that any appreciable elevation in the normally low concentrations of oxidized forms of nitrogen must be regarded as a serious threat to our environment. The threat to man through pollution of surface waters by excessive amounts of nitrate, resulting in 'algal blooms' and subsequent depletion of the oxygen content of the water, can be appreciated. The direct health hazard to man of ingestion of nitrate or nitrite in water is well documented, but the effect of nitrate ingestion in foods is more difficult to evaluate and resolve. This review will be limited to an examination of the naturally occurring nitrate and nitrite content of foods, primarily in relation to

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the development of methaemoglobinaemia. It is appreciated that there are other considerations of nitrate ingestion which are beyond the scope of the present discussion, such as the formation of nitrosohaemoglobin (Jung, Rein, Ristau & Kupfer, 1967; Smith, 1967), thyroid function (Bloomfield, Welsch, Garner & Muhrer, 1961) and vitamin A and carotenoid metabolism (Phillips, 1966).

Chemistry and physiology of methaemoglobin

Before discussing the nitrate content of foods it is wise to review briefly some aspects of the chemistry of methaemoglobin and its physiological significance. Methaemoglobinaemia is a condition in which the iron normally in the ferrous form is oxidized to ferric. Many substances ranging from aniline (furniture polish, marking inks) to phenacetin and even bee stings will directly or indirectly oxidize haemoglobin to methaemoglobin. The methaemoglobin formed is incapable of transporting oxygen and is considered to be a temporarily inactive respiratory pigment. About 1% of the total haemoglobin is present as methaemoglobin (Lane, 1952) in the healthy adult, while newborn or premature infants average about 2%, with some values up to 5% (Kravitz, Elegant, Kaiser & Kagan, 1956). Reducing enzymes in the red cell keep all but this small percentage of the total haemoglobin active unless the formation of methaemoglobin is abnormally high. No clinical symptoms are observed in children with methaemoglobin levels up to 5%, but above this level cyanosis becomes obvious (Knotek & Schmidt, 1964). Death from asphyxia will occur when the concentration of methaemoglobin exceeds 70% of the total blood pigments (Locket, 1957). Lower concentrations would be fatal if associated with anaemia since the total amount of oxygen-carrying pigment is the deciding factor.

Gastro-intestinal reduction of nitrate

Nitrates are toxic but only at levels higher than are encountered in foods. They do not produce methaemoglobin directly but first must be reduced to nitrite either prior to or during the digestion of the food. In the reduction of nitrate in the gastro-intestinal tract, the microflora are of paramount significance. Lactobacillus organisms including Streptococci and Enterococci do not produce nitrite from nitrate (Breed, Murray & Smith, 1957) although coliforms will. In the normal infant, where the predominant organisms are Lactobacilli, there is therefore little possibility of conversion. The ability of some strains of *Escherichia coli* to induce methaemoglobinaemia was observed in infants during an epidemic of infantile gastro-enteritis in France (Fandre, Coffin, Dropsy & Bergel, 1962) where nine deaths were recorded. In these cases the children were 15–21 days of age and had methaemoglobin levels up to 59%. The mere presence of bacteria capable of reducing nitrate is not sufficient for the production of the nitrite ion, however. Kübler (1958) fed vegetables with a high nitrate content to infants aged 3·5–8 months and obtained results suggesting that nitrate was absorbed rapidly in the upper part of the small intestine, where relatively little or no reduction of nitrate would take place, and was then rapidly excreted in the urine. The conversion of nitrate to nitrite in the upper part of the small intestine would require conditions that would lead to the invasion of the upper part of the gastro-intestinal tract by the organisms that normally inhabit the lower bowel. This may happen in cases of achlorhydria. In one study of infants ingesting water containing nitrate, only those whose gastric juice had a pH greater than 4 developed methaemoglobinaemia (Ewing & Mayon-White, 1951). It is apparent then that a number of conditions must exist simultaneously for the conversion of nitrate to nitrite in the gastro-intestinal tract.

Nitrates in the water supply

Although the present discussion concerns primarily the nitrate content of foods, a brief review of some aspects of the toxicity of nitrates in the water supply provides data for significant interpretation in relation to foodstuffs. A high nitrate level in the water supply has been responsible for many infant deaths and probably constitutes the major human health hazard of nitrate toxicity. Many surveys have been reported relating the incidence of methaemoglobinaemia to nitrate concentrations in the water. One selected survey (Simon, Manzke, Kay & Mrowetz, 1964) summarizes very well 745 cases of infant methaemoglobinaemia due to nitrate in water, reported in Germany over an 8-year period ending in 1964. Water from private wells was associated with 97·3% of the cases while municipal sources were associated with 2·7%. Nitrate concentrations were over 100 mg/litre in 84% of the cases and nitrite was found in 10%. Of the infants involved, 98% were under 3 months old, 53% had diarrhoea and 8·6% died. Infants older than 3 months had low methaemoglobin levels in the blood independent of the nitrate content of the water. The data from this survey confirm previous observations that infants under 3 months of age are particularly susceptible to nitrate toxicity, while older infants and adults can tolerate large quantities. The differences in susceptibility in relation to age are probably due to three factors: foetal haemoglobin, which is present in children under 3 months of age (Betke & Rau, 1952), can be more readily oxidized, there are age differences in the oxidation–reduction systems (Gibson, 1948), and the achlorhydria or low gastric acidity associated with the post-natal period makes the young infant susceptible to gastro-enteritis in which intestinal bacteria ascend to the duodenum and reduce nitrates to nitrites (Gross, 1964). Burden (1961) considers that the high fluid intake in proportion to body weight is also a significant contributory factor in infants; for example an infant with an assumed body weight of 3 kg would consume 0·5 litre water/day while a 60-kg adult would consume only 1 litre/day. On this basis he recommends that the permissible limit of nitrate-nitrogen should be 24 ppm in water given to infants but could go up to 240 ppm in water for adult use. The most generally accepted standard for water for all feeding practices is 10 ppm nitrate-nitrogen.

Nitrate and nitrite levels in foods

Having established an upper limit for nitrate-nitrogen in water, it is now pertinent to discuss the nitrate levels in foods, since in a number of cases they exceed that recommended above for water. Nitrates are natural constituents of plant material, being the main source of nitrogen required for growth. Nitrites are rarely found in soils or plants in significant amounts except under very unusual conditions. Listed in Table 1 are the nitrate-nitrogen contents of various classes of vegetable determined by different workers at two periods separated by some 60 years. The data have been converted and are expressed throughout in terms of nitrate-nitrogen. Data are reported in the literature in terms of potassium or sodium nitrate, (KNO_3 or $NaNO_3$), the nitrate ion (NO_3^-) or as nitrate-nitrogen (NO_3-N). Unfortunately this multiplicity of forms leads to errors in the interpretation and comparison of data.

The vegetables analysed by Richardson (1907) were grown during an era when fertilization was effected primarily with natural manure and only negligible quantities of chemical fertilizers were in use, while the converse was true for those samples analysed by Jackson, Steel & Boswell (1967). Although some have attempted to use data of this type to show time trends in nitrate content, surveys of this nature cannot be used to determine differences

Table 1. *Nitrate-nitrogen content of fresh vegetables*

Vegetable	Mean (and range of) levels (ppm) of nitrate-nitrogen in samples analysed in 1907*	1964†
Radishes	413 (120–690)	343 (288–399)
Beets	587 (210–1820)	275 (154–380)
Lettuce	387 (90–800)	153 (112–205)
Spinach	434 (70–860)	121 (55–170)
Cabbage	46 (8–110)	72 (36–108)
String beans	100 (10–150)	53 (34–70)
Potatoes	18 (9–24)	24 (8–143)
Carrots	15 (9–20)	23 (4–45)
Tomatoes	12 (6–20)	16 (11–25)
Peas	12 (10–13)	11 (6–14)

*Richardson (1907).
†Jackson *et al.* (1967).

between the nitrate content of present-day vegetables and those produced more than half a century ago. The data do demonstrate clearly, however, that the accumulation of nitrate is dependent on the type of vegetable, with such species as beets and spinach accumulating relatively large amounts. It is obvious that the nitrate content varies not only between species but very widely within a given species. The latter variation may be accounted for by factors affecting the accumulation of nitrate, such as temperature, sunlight, soil moisture and the level of available nitrogen in the soil (US Department of Agriculture, 1965). Concerning fertilization, Brown & Smith (1966, 1967) observed that nitrogen fertilizer applied to a variety of vegetables tended to increase the nitrate content of the edible portion of the vegetables, except in the case of carrots. With beets, the data indicated that nitrate accumulation in the roots was not affected to any large extent by nitrogen fertilization

Table 2. *Nitrate-nitrogen and nitrite-nitrogen contents of prepared infant foods sold in Canada**

Food	Levels of Nitrate-nitrogen (ppm) Range	Mean	Nitrite-nitrogen (ppm) Range	Mean
Spinach	224–379	312	0·3–1	0·5
Beets	144–492	222	0–2	0·8
Wax beans	73–129	101	1–2	2
Squash	10–93	64	0–1	0·7
Garden vegetables	19–62	41	0–2	0·7
Carrots	15–38	23	0–2	0·7
Pre-cooked cereals	0·4–48	13	2–8	4
Meats	1–11	4	0–2	0·9
Dairy products	0–7	3	0·4–4	2
Fruits	0–7	2	0–2	0·5

*Kamm *et al.* (1965).

while the nitrate content of beet tops was. The relationship of fertilization to the nitrate content of spinach will be discussed below.

Since methaemoglobinaemia is particularly relevant to infancy, the nitrate content of commercially prepared infant foods sold on the Canadian market was surveyed by Kamm, McKeown & Smith (1965) and a summary of their findings is shown in Table 2. In general the values for nitrate-nitrogen were higher than those reported earlier from the USA by Wilson (1949). As would be expected from the data on fresh vegetables, spinach and beets were very high in nitrate-nitrogen, while low values were found in dairy products, fruits and cereals, those foods which would normally be introduced first into the infant's diet. Nitrite-nitrogen was relatively low in all cases.

Toxicology of dietary nitrate

Having established the ranges of the levels of nitrate and nitrite found in foods, it becomes more difficult to assess their toxicological significance. Although the Joint FAO/WHO Expert Committee on Food Additives (1962) applied the standard approach of a 'no-effect' level in animals multiplied by a safety factor of 100, for the addition of nitrate and nitrite to foodstuffs for a large proportion of the population, it appreciated that the sensitivity of normal babies and the apparent sensitivity of dyspeptic babies made it impossible to estimate an acceptable dose level for babies under 6 months of age. One must, therefore, apply the limited amount of clinical evidence available. There is a paucity of data from clinical studies on the feeding of nitrate-containing foods to man: however, Kübler (1958) has reported that of seven infants fed spinach to provide an intake of up to 4·8 mg nitrate-nitrogen (or 21 mg nitrate)/kg body weight/day, six showed no increase in methaemoglobin and the other showed a very slight increase which was not sufficient to cause any symptoms of methaemoglobinaemia. The latter infant showed elevated levels of methaemoglobin prior to the feeding of the nitrate-rich spinach. Cornblath & Hartmann (1948) reported that one 10-day-old infant was fed up to 23 mg nitrate-nitrogen (100 mg nitrate)/kg body weight/day for 8 days without developing symptoms of cyanosis. These data demonstrate, as in the case of nitrate in drinking water, that the normal healthy, even young infant can tolerate relatively large quantities of dietary nitrate.

Although we have discussed the theoretical or potential conversion of dietary nitrates to nitrites in the gastro-intestinal tract, our reviews of the literature (Phillips, 1968a) and those of others (Steyn, 1960; *Nutrition Reviews*, 1950) revealed a lack of evidence for the toxicity to man resulting from the ingestion of nitrate in foods. More direct evidence was suggested by the survey of Ciavaglia & Thompson (1969), who reported on the absence of infant methaemoglobinaemia in urban areas of New York and in the province of Ontario in Canada where the concentration of nitrate in the drinking water is extremely low. Records from 19 hospitals over a 10-yr period (1958–1967) listed only three suspected cases and in these instances the cause was unknown. The authors considered these a negligible number in the very large population area of New York City and the other urban areas as compared with the large number of cases reported from relatively small areas having excessive nitrates in the drinking water (the report of Rosenfield & Huston (1950), for example, cited 146 cases during an 8-yr period in Minnesota). Since cases of methaemoglobinaemia are only recorded when cyanosis is clinically apparent, further research is needed to determine the extent, if any, of sub-clinical methaemoglobinaemia in infancy in relation to dietary habits. Kübler & Simon (1967) consider it astonishing that no one has so far described a case of methaemoglobinaemia triggered solely by nitrate after eating spinach. Obviously the

statement of Fassett (1966), that it is difficult to imagine any situation in which adults might be affected and that it would seem to require unusual circumstances for methaemoglobinaemia to be produced in infants from this source, appears to have considerable support.

Reduction of nitrates in foods

Having discussed the conversion of nitrate to nitrite after ingestion of a food there is another serious aspect to the problem. Cases of methaemoglobinaemia, some resulting in death, have been observed following the consumption of spinach. Hölscher & Natzschka (1964) reported two cases in young infants (aged 2 and 3·5 months) who had eaten spinach purée. Spinach from the same source contained only traces of nitrate-nitrogen but 661 ppm nitrite-nitrogen. Since this vegetable was known to accumulate nitrate it was suggested that this particular batch contained a high concentration of nitrate which was reduced during storage. Fourteen further cases of methaemoglobinaemia in infants (aged 2–10 months) were reported from Germany (Sinios & Wodsak, 1965). Unprocessed spinach was used almost exclusively in the preparation of the meals and preparation took place at least 24 hours before the mealtime. Since in most cases some of the same spinach had been eaten 24 and 48 hours before without causing illness the authors assume that the nitrite was formed within the final 24 hours of storage. Information in seven cases showed that the mother tasted the spinach before feeding the child and no change in taste was apparent. None of the children refused the meal which led to the poisoning. The possibility that conversion of nitrate to nitrite could take place in fresh spinach was demonstrated by Schuphan (1965) who observed that spinach grown on a field treated with four times the normal amount of nitrogenous fertilizer contained no appreciable nitrite but high amounts of nitrate. After storage for 4 days the nitrite content had increased from 30 ppm dry matter at harvesting to 3550 ppm. Schuphan (1965) explained this change on the grounds that nitrate took on the role of an oxygen donor under anaerobic conditions. More recently, during 1966 and 1967, unprocessed fresh spinach was purchased on the Canadian market (Phillips, 1968b) and analysed for nitrate- and nitrite-nitrogen. For each study, three different samples were divided into two portions and stored either at room temperature in the dark or under refrigeration. The room-temperature studies demonstrated that nitrate-nitrogen was reduced during the first 4 days of storage to approximately 30% of its initial value and was practically absent by day 8. The conversion of nitrate to nitrite occurred during the first 4 days followed by a decomposition of nitrite during the next 3 days. These studies definitely confirmed the observation of Schuphan (1965) that the nitrate in 'fresh' unprocessed spinach could be converted to nitrite. Under refrigeration, although the reduction of nitrate was delayed and varied widely between samples, nitrite-nitrogen levels did accumulate (in one case to extremely high levels) and the nitrite formed did not decrease as it did when the spinach was stored at room temperature. In similar studies on frozen spinach, the nitrate-nitrogen content varied from 97 to 556 ppm depending on the source, but the nitrite-nitrogen content was approximately 1 ppm or less. There was no significant increase in the nitrite-nitrogen content during storage in the frozen state for periods of up to 5 months. The length of time samples were allowed to thaw did influence the nitrite-nitrogen content, however. Thawing for up to 15 hours at room temperature did not increase the nitrite-nitrogen content but a period of 39 hours did. Sinios & Wodsak (1965) concluded from their studies that the increase in nitrite when frozen spinach was thawed was directly related to the numbers of micro-organisms present.

A very limited study was conducted in a similar manner on three types of commercially prepared baby foods (one puréed spinach and two jars of mixed vegetables containing spinach). The jars were opened and sampled at intervals for up to 35 days. For 15 days after the jars were opened and stored there was no increase in the nitrite-nitrogen content, but by day 35 the nitrite-nitrogen content of the spinach had increased to 3 ppm. The nitrate-nitrogen content did not decrease significantly during the storage period, indicating no major conversion of nitrate. Since this storage study was conducted under ideal laboratory conditions, further investigations were undertaken to determine whether significant amounts of nitrite accumulated in jars of partially-consumed commercially prepared baby foods under conditions applicable to the Canadian consumer (Phillips, 1969). Ten classes of baby food (4–9 samples for each class of food) containing various levels of naturally occurring nitrate were distributed to a small number of parents with infants between the ages of 2 and 10 months, and a few samples were given to adult volunteers. Instructions were given that jars were to be opened in the home, and the subject was to be fed with a spoon directly from the jar. Half the contents of the jar was fed at one time, and the jar was recapped and placed in the home refrigerator. Samples were returned to the laboratory the day following feeding, stored under refrigeration and sampled periodically for a further 7 days. Nitrite-nitrogen did not accumulate in any of the food samples under refrigeration. These data can only imply relative safety under conditions applicable to the consumer since Sinios & Wodsak (1965) were able to demonstrate accumulation of nitrite in commercially processed baby foods that were inoculated and incubated with *E. coli*. Cornblath & Hartmann (1948) isolated aerophilic Streptococci, *Aerobacter aerogenes*, *Staphylococcus aureus* and *E. coli*, from the mouths and upper gastro-intestinal tract of two infants who were cyanotic from well-water, and demonstrated that these organisms were capable of reducing nitrate to nitrite. Again, however, no cases of methaemoglobinaemia have been reported following the feeding of commercially prepared baby foods. An industry estimate of 350 million jars of beets and spinach consumed over the past 20 years in North America without any associated cases of methaemoglobinaemia provides further indirect evidence of safety.

Nitrate levels in relation to soil fertilization and spinach processing

Since documented evidence suggested that the direct health hazard was related to unprocessed spinach, further studies were undertaken. During the past year Canadian Canners Ltd. have collaborated with the Food and Drug Directorate (J. J. B. Powell, T. G. Stevens & W. E. J. Phillips, unpublished data 1970) in a study of the accumulation of nitrates in spinach grown on plots receiving various levels of nitrogen fertilizer under Canadian climatic conditions, since Schuphan (1967) was able to show a relationship between the accumulation of nitrate-nitrogen in spinach at various levels of fertilization between 0 and 240 kg nitrogen/hectare. The effects of storage conditions and commercial processing on the nitrate-nitrite content of the spinach were also studied. Fertilizer was applied twice, in accordance with standard practice, to eight plots, comprising two four-plot replicates. A pre-plant treatment with ammonium phosphates, sulphates and nitrate (ranging from 0 to 120 lb nitrogen/acre) was followed by a side dressing of ammonium nitrate (ranging from 0 to 67 lb) when the plants had achieved 2–3 in. of growth. Following both the pre-plant and side-dressing applications, all plots showed an increase in the nitrate content of the soil. Fertilization had a significant effect on growth of the spinach. The amount harvested from the control plots (no fertilization) was 15–22% of the yield from plots receiving 93 lb nitrogen/acre. Higher yields were observed from plots fertilized with 185 lb nitrogen/

acre (i.e. twice the normal rate). Fertilization increased the nitrate-nitrogen content of the fresh spinach ($P = 0{\cdot}08$), but there was no significant difference between the nitrate-nitrogen content of the spinach from the three plots treated with different levels of fertilizer. These observations were similar to those reported by Brown & Smith (1967) under conditions in the United States where, although fertilizer treatment increased the nitrate-nitrogen content of spinach, there were no differences within the range of 50–200 lb nitrogen/acre. However, there was an increase at 400 lb nitrogen/acre. After harvest the fresh spinach was promptly stored either at ambient temperature (70°F) or under refrigeration (40°F). Samples stored at ambient temperature were analysed daily. The moisture content of fresh spinach (90–92%) decreased to a minimum of 77% in the most heavily fertilized spinach after storage for 3 days. Conversion of nitrate to nitrite was significant after storage for 2 days. Higher levels of nitrite occurred in the spinach from the fertilized plots. Considered in conjunction with data from other workers, this suggests that excessive fertilization should be avoided.

Samples under refrigeration contained small quantities of nitrite-nitrogen after storage for 1 wk, but the concentrations did not rise greatly during the subsequent 3-wk period. One of the major objectives of this experiment was to determine whether there existed in spinach a threshold level of nitrate above which conversion to nitrite occurred readily and below which little or no conversion would take place. Since relatively little conversion took place in any samples, no information could be obtained on this factor.

It is apparent from these and the previous studies discussed that nitrite can accumulate to toxic amounts in unprocessed spinach, but that the conversion and accumulation is variable and unpredictable. It may be the result of a number of factors, notably the level of the plant nitrate, the activity of plant enzymes, storage conditions (aerobic or anaerobic, for example) and microbial contamination. All of these factors should be investigated further to resolve and eliminate the problem.

The effect of commercial processing on the nitrate and nitrite content of fresh spinach was studied using the plant material obtained from the four fertilizer treatments previously discussed. After harvesting, spinach was placed on rotating cylindrical screens (which removed stones, soil and other undesirable matter), conveyed to washing tanks and then blanched. Part of the produce was puréed in a blending machine. After the filling and seaming processes, the puréed and blanched leafy spinach was thermally processed. Samples were taken at various stages during the processing, and samples of water were removed from the wash tank and blancher. The concentration of nitrate-nitrogen in the spinach emerging from the wash tanks and blancher tended to be lower than the concentration in the fresh leafy spinach. Confirmation of removal of nitrate was evident from the concentration of nitrate-nitrogen in the water from the blancher. Of particular interest was the fact that although the nitrate-nitrogen of fresh leafy spinach showed no direct correlation with the level of fertilization, the final commercially-packed leafy spinach did. At no point during the commercial processing was nitrite observed.

Conclusion

To summarize, nitrate toxicity results from the formation of the nitrite ion, and the nitrate content of a food may thus be considered as the index or precursor of the amount of nitrite which may be formed. Although conversion in the gastro-intestinal tract of man is possible, documented evidence indicates that conversion prior to ingestion is the primary health hazard. Present evidence implicates only nitrate-rich unprocessed spinach. Although

our only concern must be to estimate potential human health hazards and apply existing knowledge to eliminate the possibility of diet-induced infant methaemoglobinaemia, we must resist the temptation to resort to measures which may be more extreme than the evidence warrants. As Kübler & Simon (1967) have stated, it would be regrettable if the practice of feeding leaf vegetables to infants were discredited, for these plants are extremely important dietary sources of vitamins and trace elements. Since concentrations of methaemoglobin are not increased in the blood of mature infants who have eaten spinach, there is no need to stop feeding this vegetable (Kübler, 1958 & 1959).

We have recommended previously (Phillips, 1968a,b), and in this are in agreement with some of the European recommendations (Simon, 1966; Kübler & Simon, 1967), that to minimize the possibility of infant methaemoglobinaemia, foods containing high levels of nitrate (namely spinach and beets) should not be introduced to the diet of infants younger than 3 months. Moreover, prudence would dictate selectivity in the choice of foods during infancy if symptoms of gastro-intestinal disorder were apparent. The recommendation to withhold introduction of these vegetables would not be contrary to nutritional recommendations, since the Committee on Nutrition, American Academy of Pediatrics (1958) has stated that clinical studies have not offered substantial proof of superior nutritional status when solid foods are fed prior to 3 months of age.

Unprocessed spinach should be stored for a minimum length of time under proper conditions and cooked unprocessed spinach should never be stored for subsequent feeding.

Although infant methaemoglobinaemia from foods containing nitrate does not appear to be a problem in North America at this time, it is a potential threat, which could be reduced by a programme of education encouraging adherence to the above recommendations.

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LXX. THE ACTION OF NITROUS ACID ON *p*-CRESOL AND TYROSINE

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In connexion with an investigation of the inactivation of pepsin by nitrous acid, the results of which appear in the next paper, we have been led to examine the action of nitrous acid on tyrosine. It seems to be generally supposed [Gibbs, 1926–27] that the yellow product obtained when nitrous acid acts on dilute aqueous tyrosine or *p*-cresol is an *o*-nitroso compound. We have now obtained evidence that although this compound is first formed, it does not accumulate, but at once undergoes further reaction. The usual product is chiefly a diazo compound accompanied by a nitro compound and photolysis products from the diazo compound (possibly also condensation products), the proportions varying with the conditions. We have also proved that substances belonging to the class of *o*-nitrosophenols are formed in the Millon reaction, but that they differ from the simple derivatives.

Reaction scheme for p*-cresol and tyrosine*

The scheme in Fig. 1 summarizes the reactions which we have studied. It applies to both tyrosine and *p*-cresol, which differ only kinetically, apart from the deamination of the tyrosine; and it permits a wide choice of reactions according to conditions. The evidence for the scheme is based partly on conditions of formation and partly on the properties of the products, and is therefore diffused throughout the paper without being given separate consideration. Tyrosine and *p*-cresol will be referred to collectively as "phenols", since other monohydric phenols probably conform.

The chief possibilities are as follows:

(1) With dilute nitrite and phenols in acetate buffer plus $CuSO_4$ at temperatures from 0 to 40° the nitroso compounds are trapped as the very stable Cu derivatives.

(2) Under similar conditions in absence of $CuSO_4$ little or no nitroso compound can be detected at any stage of the reaction by adding $CuSO_4$ since it is rapidly transformed further. If, however, very dilute nitrite is added to a large excess of *p*-cresol, considerable amounts of nitroso compound are formed.

(3) The products of further reaction depend on the concentrations of HNO_2 and phenols, and on the temperature. If the ratio [HNO_2]/[phenol] is low, red substances are obtained. Their structure and origin are at present unknown, but the product from *p*-cresol sometimes crystallizes directly from the reaction mixture. If the concentration of HNO_2 is increased and the concentration of phenol is not too high the chief product in the dark is diazo compound, unless air or nitrogen is bubbled through the solution. (Such treatment removes nitric oxide as explained below.) At still higher concentrations of nitrous acid, the yield of

diazo compound falls off, probably in favour of mono- or di-nitro compound. In the case of *p*-cresol these three regions are telescoped, while with tyrosine the middle one is much the most extensive.

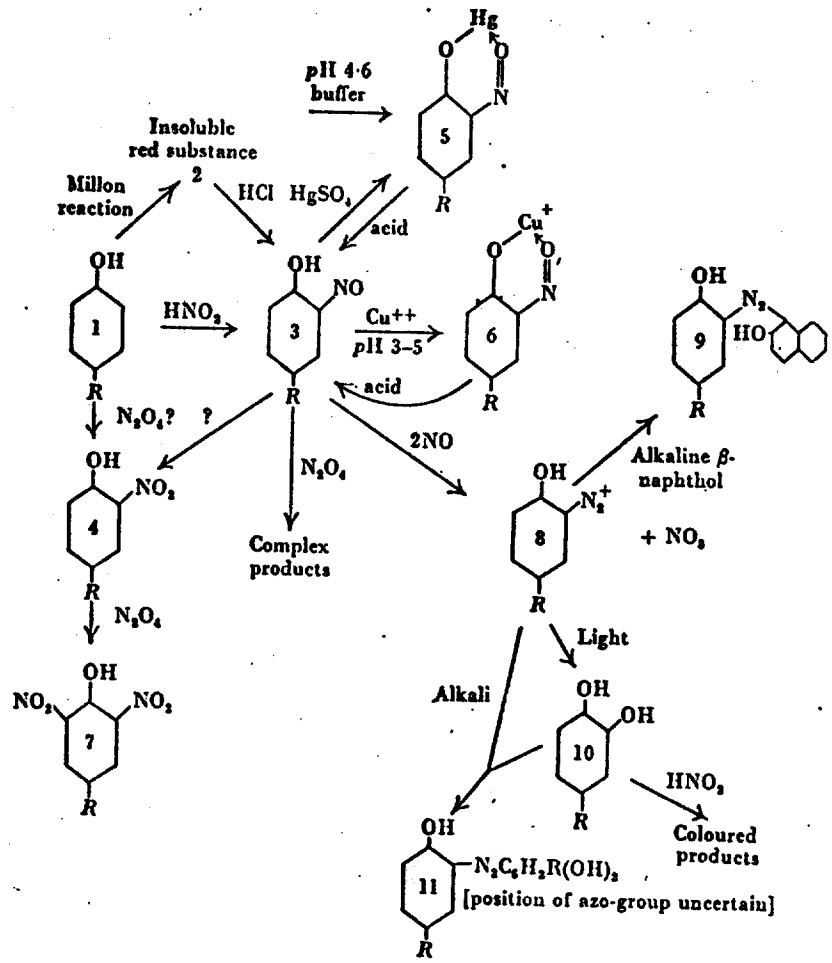

Fig. 1. $R = CH_3$, $CH_2.CH(NH_2)CO_2H$ or $CH_2.CHOH.CO_2H$.
Substances 3, 4, 6, 7, and 9 have been obtained crystalline.

The decomposition equilibrium of nitrous acid

The above conditions can be partly explained by consideration of the decomposition equilibrium of nitrous acid.

The reactions

$$4HNO_2 \overset{1}{\rightleftharpoons} 2N_2O_3 + 2H_2O \overset{2}{\rightleftharpoons} N_2O_4 + 2NO + 2H_2O \overset{3}{\rightleftharpoons} HNO_3 + HNO_2 + 2NO + H_2O$$

have been described by Abel *et al.* [1930].

The equilibrium point of reaction 3 normally lies well towards the right, but it is established much more slowly than the other two equilibria, which maintain the curious relationship:

$$\frac{[NO]^2[N_2O_4]}{[HNO_2]^4} = \text{const.}$$

Explanation of above-mentioned conditions. (1) Dilute HNO_2 is used to avoid the action of N_2O_4 on the phenols. Acetate buffer is better than mineral acid, because it allows the HNO_2 to be continuously replenished from an internal reserve of NO_2. The *p*H of HNO_2 is about 3·3; with the concentrations of nitrite commonly used by us, e.g. $N/16$, the concentration of HNO_2 ranges from about 0·02 N at *p*H 3·7 to about 0·002 N at *p*H 4·7. A further reason for using this region of *p*H is that the Cu derivative is more stable than at lower *p*H values.

(2) The rate of formation of nitroso compound has been shown to be proportional to $[HNO_2] \times [\text{phenol}]$ by kinetic experiments which will be published later, while its further transformation into diazo compound will probably depend on $[\text{nitroso compound}] \times [NO]^2$. Hence low values of $[HNO_2]$ and high concentrations of phenols favour accumulation of nitroso compound.

(3) The unknown red substances are obtained whenever there is not enough NO or NO_2 or Cu^{++} present to react with the nitroso compound as fast as it is formed. We think we have excluded the possibility of their formation from the nitroso compound, either by further reaction with the HNO_2 system, or by condensation with itself or anything else. (Autocondensation does occur, but quite slowly and to a different product.) We are almost driven to postulate a precursor of the nitroso compound, e.g. an addition product of the phenol and HNO_2, as postulated by Veibel [1930, 1, 2]. This precursor could undergo side reactions to give the red substance unless it were rapidly removed in other ways.

Preparations and properties of products

Nitroso compounds. The purple Cu chelated derivatives of the *o*-nitrosophenols are very stable between *p*H 3 and 8, particularly in presence of an excess of cupric salts. They are therefore formed under these conditions preferentially to any other derivatives. The similar derivative of phenol itself has been described by Baudisch [1918] though the preparation here described seems to be new. There are two derivatives from *p*-cresol. One, formed in presence of one equivalent of Cu is insoluble in water, but soluble in many organic solvents, while the other, formed in presence of excess Cu, is soluble in water and not extracted from aqueous solution by organic solvents. These are presumably the di- and mono-derivatives respectively; a supposition which agrees with the analysis of the supposed Cu di-derivative, given below. The corresponding derivatives from tyrosine are both water-soluble and not easily separated.

Preparation of Cu di-o-nitroso-p-cresol, and o-nitroso-p-cresol

10·8 g. *p*-cresol (0·1 mol.) were dissolved in 400 ml. of acetate buffer of *p*H 4·6, and 50 ml. of M $CuSO_4$ were added. 59·3 ml. of 4·22 N $NaNO_2$ were added and the solution made up to 1 l. and left 2 days at room temperature. Black crystals separated and were sucked off; the yield was 22 g. of damp cake. It was recrystallized by dissolving in hot alcohol and precipitating with water, or better, by evaporating a solution in a 2:3-alcohol:chloroform mixture. (Found: Cu 19·15 %. $C_{14}H_{12}N_2O_4Cu$ requires Cu 18·95 %.) The substance can conveniently be kept as an $M/10$ solution in the alcohol-chloroform mixture. It is also readily soluble in dioxane.

To obtain free *o*-nitroso-*p*-cresol for any experiment, the alcohol-chloroform solution may be shaken twice with N H_2SO_4, to decompose the Cu complex, and washed twice with water, a chloroform solution of the nitroso compound resulting. The nitroso compound may be obtained solid by extraction of such a solution with a small amount of N NaOH, and acidifying at 0°, when orange

crystals separate. They may be sublimed at 38°/20 mm. pressure, M.P. 58·5° (uncorr.). Evaporation of the chloroform solution gives nearly colourless crystals, but much is lost through volatilization.

Properties. The Cu di-*o*-nitroso-*p*-cresol is very stable, but decomposes explosively on heating. It is readily interconvertible with the water-soluble mono-derivative, according as nitroso-*p*-cresol or Cu is in excess.

It may be mentioned here that ordinary phenol also gives Cu *o*-nitrosophenols under conditions such as described above, whereas in ordinary nitrosation it gives very little *o*-derivative.

The free *o*-nitroso-*p*-cresol is fairly stable in dilute aqueous solution, but solutions stronger than $N/10$ apparently undergo condensation fairly rapidly in either water or organic solvents. It is acted on very rapidly by 50 % conc. HCl. A solution of the orange-red Na salt, made by shaking $N/10$ NaOH with an excess of the chloroform solution, is rather more stable, but seems to oxidize in the air in the course of some weeks. The compound gives the Liebermann nitroso reaction, and forms bright red nickel and mercuric derivatives. These properties, and the smell and volatility, agree with those of *o*-nitrosophenol as described by Baudisch [1918].

The purple colour of the Cu mononitroso-derivative may be used for its colorimetric estimation. Since the Cu solutions used are coloured green before any purple nitroso compound is present, monochromatic (sodium) light was used to avoid difficulties of colour matching. Colour intensities were measured in terms of glass filters as permanent standards. A solution was assigned a value of 100 when it matched a certain filter at a setting of 10 mm. The increase in colour units was taken as proportional to the amount of nitroso compound formed. By this means the yield of Cu *o*-nitroso-*p*-cresol was measured under various conditions, for which purpose a solution prepared by weighing out the solid di-derivative, and dissolving in an excess of buffered $CuSO_4$, thus forming the mono-derivative, was used as standard. The yield was found to be 95 % ± about 2 % in experiments at 38°, room temperature and 0°.

Experiments were also made to determine the effect of bubbling N_2 through the solution on the yield of the derivative from tyrosine. A solution was made up, at *p*H 3·7, containing 2 mg. tyrosine per ml., and $NaNO_2$ at a concentration of $N/8$. This was split into two parts, and each was heated at 38° for 6 hr., till the reaction was nearly complete, one being gassed with N_2 and the other not. Portions were removed at intervals, diluted 1 : 6 with buffer and their colour intensities determined. The final intensities were for the gassed solution 130 units, for the untreated 122 units; the slight excess in the former is attributable to evaporation. There is no significant difference; that is, neither NO nor NO_2 attacks the Cu compound with appreciable velocity, a conclusion which is supported by various qualitative experiments.

Tryptophan gives no coloured complex with HNO_2 in presence of Cu.

Nitrosophenols and the Millon reaction

We have examined briefly the relation between the formation of *o*-nitrosophenols and the Millon reaction. *o*-Nitroso-*p*-cresol forms an intensely red mercuric derivative, at *p*H about 3–4, and may be prepared from *p*-cresol using Hg instead of Cu. This complex is not identical with the red substance obtained in the Millon reaction, since it is chloroform-soluble and instantly dissociated in $2N$ H_2SO_4. The red precipitate, obtained by performing the modified Millon reaction [Cole, 1933] on *p*-cresol, is not soluble in chloroform, and is fairly stable in $2N$ H_2SO_4 acid, but is converted by shaking with acetate buffer of *p*H 4·6 into

a chloroform-soluble substance, apparently identical with the mercuric complex of *o*-nitroso-*p*-cresol. On shaking the red chloroform solution with 2*N* H_2SO_4 even in presence of much $HgSO_4$, a yellow chloroform solution is obtained, which, when shaken with buffered $CuSO_4$ at *p*H 4·6, gives a purple aqueous layer; the behaviour is identical with that of the mercuric complex of *o*-nitroso-*p*-cresol.

If an excess of NaCl is added to the mercurial phenol obtained in the modified Millon test by heating *p*-cresol with a solution of $HgSO_4$ in 2*N* H_2SO_4, the solution so obtained fails to give the Millon test on addition of $NaNO_2$ as is well known. If not too much nitrite is added, a yellow substance is obtained soluble in water but not in chloroform, which gives a red colour with excess of $HgSO_4$, or a purple solution with $CuSO_4$, even in 2*N* H_2SO_4. It is therefore an *o*-nitrosophenol, though not *o*-nitroso-*p*-cresol itself, since the latter is soluble in chloroform and does not form complexes at so low a *p*H. If an excess of nitrite is used in the above experiment, diazo compound can be detected in the resulting yellow solution.

This behaviour can be explained on the assumption that the products insoluble in chloroform are mercurated in the nucleus, so that the red substance responsible for the Millon reaction would be a mercurated mercury complex of an *o*-nitrosophenol. The red substance obtained under similar conditions from tryptophan must be different in nature, and when it is decolored with NaCl, the product does not develop a colour with cupric salts.

Lugg [1937] has been led to postulate two classes of *o*-nitrosophenols, one of which gives red mercuric complexes, while the other, obtained from tyrosine and other phenols in absence of mercuric salts, does not. It is clear that the latter derivatives are what we have shown to be diazo compounds. There are, as we have shown above, two classes of nitroso compound, but both give red mercuric complexes under appropriate conditions.

The distinction drawn by Lugg between *o*-quinone monoximes and *o*-nitrosophenols seems now to be unnecessary; we have found no trace of such tautomerism when dealing with free *o*-nitroso-*p*-cresol, and the chelated metallic derivatives have a structure which is a resonance hybrid of the two types.

Diazo compounds

Diazo derivatives are formed with great ease by the action of HNO_2 on tyrosine and *p*-cresol. The one formed from tyrosine is much the more stable against further action of nitrous acid, and is formed under almost all conditions when tyrosine and HNO_2 react between 0 and 40°. Even if only one equivalent of HNO_2 is present, some diazo compound is formed from tyrosine, so that the reaction can compete with the deamination.

We have not isolated the diazo compounds, but they show characteristic colour reactions, by which they may be detected and estimated. They give red or purple coupling products in alkaline solution with such phenols as β-naphthol, resorcinol or phloroglucinol, and, if exposed to light in alkaline solution, form a red substance, which is photosensitive itself, so that the colour shows a maximum with time. This colour has been shown to be due to the coupling of unchanged diazo compound with a catechol derivative formed on irradiation by loss of nitrogen. Such a reaction of *o*-diazophenols has already been observed by Schmidt & Meier [1931]. If irradiated in sufficiently acid solution, these compounds give no colour, but if the irradiated solution is made alkaline, it will couple with fresh diazo compound. Though catechol does not readily couple, homocatechol, and more readily, "dopa" (3:4-dihydroxyphenylalanine) will couple, in alkaline solution, with diazodeaminotyrosine.

The diazo compounds do not couple with monohydric phenols, or with aniline, benzidine or dimethylaniline. They do, however, couple with α-naphthylamine, slowly in the cold, more rapidly on warming, at *p*H about 3.

The yield of diazo compound from tyrosine, in presence of various concentrations of HNO_2 has been investigated at 0 and 38°; a maximum is found at intermediate concentrations. The following figures give the amount of diazo compound formed in arbitrary units, measured colorimetrically by coupling with resorcinol, in presence of the respective concentrations of $NaNO_2$, at *p*H 3·7. One set of experiments at 0° was carried out with a layer of paraffin floated on the solution (as explained above this increases the NO concentration). The times before sampling were inversely proportional to the nitrite concentrations, so that an equal fraction of the reaction had occurred in each case (about 90%, as deduced from kinetic measurements).

Final conc. $NaNO_2$	0°	0° paraffin	38°
2*N*	90	—	73
N	95	99	81
N/2	97	99	83
N/4	102	107	81
N/8	100	104	65
N/16	94	95	53
N/32	85	—	—

The results indicate a definite optimal concentration of HNO_2.

The reaction mechanism by which diazo compounds are formed in this reaction has been determined. Kinetic data, to be given in detail in a later paper, indicate that the nitroso compound is a precursor of the diazo compound. It is known that "nitrous fumes" can convert other nitroso compounds into diazo compounds in ethereal solution, the reaction being

$$R.NO + 2NO \rightarrow R.N_2.NO_3.$$

We have shown that *o*-diazo compounds are formed from ethereal or chloroform solutions of the *o*-nitrosophenols in the same way, and the above-mentioned effect of bubbling N_2 supports the view that the reaction is the same in aqueous solution. Ethereal N_2O_3 or NO_2 give no *o*-diazo compounds.

These diazo compounds are extremely stable in absence of excess HNO_2; their solutions may be boiled without decomposition, and they do not undergo the Sandmeyer reaction even with HI except at 100° and then slowly. They react rather slowly with excess HNO_2, forming products whose nature has not been investigated. They may be reduced, presumably to amino or hydrazino derivatives, by the action of $TiCl_3$ or $Na_2S_2O_4$. A solution of diazopepsin titrated with $TiCl_3$ was found to require 3·8 atoms of H per diazo group corresponding with a reduction $R.N_2OH \rightarrow R.NH.NH_3 + H_2O$.

The coupling product of diazocresol and β-naphthol has been isolated. The preparation is as follows.

40 ml. of 0·2 *M* *p*-cresol in water (0·86 g.) and 10 ml. conc. HCl were made up to 1 litre with water, and 10 ml. 4 *N* $NaNO_2$ added. After 1 hr. the bright yellow solution was made strongly alkaline with 10 *N* NaOH, and 2 g. solid β-naphthol added. After 1 hr. more the solution was extracted with ethyl acetate, the extract washed with dil. NaOH to remove β-naphthol, and then twice with water, and the ethyl acetate dried with $CaCl_2$, filtered and evaporated on a water bath; the residual liquid was allowed to evaporate at room temperature; 1·3 g. of crystals

were obtained, this being a yield of 60%. The product can be purified by sublimation at low pressure, or better by recrystallization from ethyl acetate, M.P. 109° (uncorr.). The substance is generally similar to the phenolazo-β-naphthol described by Niementowski [1902], though of a lower M.P. (phenolazo-β-naphthol melts at 193°). The yield of 60% shows that, at least at one stage of the reaction between *p*-cresol and HNO_2, the amount of diazo compound present must be large.

Nitro compounds and condensation products

The study of the other products has been greatly hindered by lack of colour reactions for their detection and estimation. It was thought at first that the product obtained by the action of HNO_2 gassed with N_2 on *p*-cresol might be a nitro compound; but though the product of the reaction was extractable by ether, no definite compound could be isolated. By shaking a dilute ethereal solution of *p*-cresol with an aqueous mixture of acetate buffer and moderately concentrated $NaNO_2$ and evaporating the separated ether layer a good crop of crystals was obtained. These were recrystallized from ether plus light petroleum and proved to be dinitro-*p*-cresol, M.P. 81°. The same compound was got by the action of mixtures of $NaNO_2$ and 60–90% conc. HNO_3, both from cresol and nitrosocresol. The mononitro compound, M.P. 33°, was obtained by the action of moderately concentrated nitrite and *p*H 3·7 buffer on very dilute *p*-cresol, 0·5 mg./ml., along with some diazo compound. We think, but cannot prove, that these are all cases of direct nitration by N_2O_4.

Various attempts were made to produce the nitro compound from the nitroso compound under gentle conditions in acid solution by oxidation, but without success. Titration of a solution of nitroso-*p*-cresol in *p*H 3·7 buffer with $KMnO_4$ showed that each mol. took up 6O, which presumably indicated the opening of the benzene ring.

Application to pepsin. Some, but not all, of the tyrosine groups in pepsin can be converted into diazo derivatives or Cu nitroso derivatives, without denaturing the protein or completely inactivating it. This will be described in the succeeding paper.

Summary

1. The first recognizable product of the action of nitrous acid on monophenols is a nitrosophenol.

2. The *o*-nitrosophenol, however, rapidly undergoes further reaction, chiefly with nitric oxide to form the *o*-diazophenol.

3. The *o*-nitrosophenol can be trapped and isolated, via the very stable copper complex.

4. The red product of the Millon reaction is not the simple mercury-*o*-nitrosophenol, but is readily converted into it. The reverse change has not proved possible.

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Note added March 1938. The irradiation products of the diazophenols differ from "Dopa" and other catechol derivatives in that they couple much more rapidly with unchanged diazophenols at *p*H 10·4, and give less blue colour with Folin's "phenol" reagent at *p*H 5 and 100°, and practically no blue colour with arsenomolybdate at *p*H 3·7 and 100°. In these respects they resemble resorcinol very closely indeed. Attempts to obtain a fluorescein reaction with them were unsuccessful, possibly owing to insufficient purification; nevertheless it seems possible that they are 1:3- instead of 1:2-hydroxy derivatives.

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LXXI. THE ACTION OF NITROUS ACID ON PEPSIN

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In the search for the source of the specific activity of a protein such as pepsin one of the few available methods is to act on the protein with reagents which are known to attack certain types of group and to see whether the activity changes. Thus Herriott & Northrop [1934] and Herriott [1935–36] have acetylated pepsin with ketene, and Herriott [1937] has iodinated it. The conclusion from both researches was that tyrosine groups played a prominent part in the activity of pepsin.

The work here described was started some time ago when the conclusions would have been more novel. Nevertheless it provides further evidence of the participation of tyrosine in the activity of pepsin, and modifies the frequently quoted claim of Hugounenq & Loiseleur [1925] that the activity of pepsin is unaffected by nitrous acid. It also confirms other evidence that only a portion of the tyrosine groups in pepsin can react, at least at rates comparable with that of free tyrosine.

Preliminary experiments

The earliest experiments were done in collaboration with Miss W. Hessey and Mr B. Mainwaring.

(1) *Action of $NaNO_2$ and HCl.* It was found that 1·7 *N* $NaNO_2$ plus 1·7 *N* HCl added to a solution of crystalline pepsin (about 1 %) at 25° caused rapid formation of a pale yellow precipitate. Activity determinations were uncertain owing to the insolubility of the precipitate, but very little activity could be detected. Hence the statement made by Hugounenq & Loiseleur [1925] that pepsin is not inactivated by nitrous acid "in a medium of hydrochloric acid" does not hold for pure pepsin though it may have held for the crude preparations available at that time.

(2) *Action of $NaNO_2$ in acetate buffer solutions.* It was found that provided that the *p*H was kept above 3·8 by acetate buffer no precipitate was formed, but the solution slowly became bright yellow and the activity fell off at a measurable rate to about half its original value, with a very much slower subsequent fall to zero. The yellow colour was accompanied by general absorption of light rising continuously from the blue to the short ultra-violet, and quantitative measurement showed that the band at 350–60 mμ, due to NO_2^-, was not perceptibly diminished in intensity, while that at 275 mμ, due chiefly to tyrosine groups in the pepsin, was diminished, although the total absorptions at both wavelengths were greatly increased by the yellow substance. This yellow substance is now known to be a diazo compound, since it shows all the colour reactions (coupling in alkaline solution with phenols, production of a red colour on irradiating in alkaline solution, colourless on irradiation in acid solution) characteristic of *o*-diazo-*p*-cresol and *o*-diazodeaminotyrosine, which we have

described in the preceding paper [1938]; some of these colour reactions have been used for its colorimetric estimation.

(3) *Liberation of N_2.* Over the *p*H range mentioned in the previous paragraph the concentration of undissociated HNO_2 was low, even in presence of *N* nitrite. Hence the decomposition to NO was slow enough to permit measurement of the rate of N_2 evolution from amino groups in a Barcroft or Warburg apparatus. The small amount of NO evolved was absorbed by permanganate dissolved in dilute phosphoric acid. It was essential to displace O_2 completely by purified N_2. The final technique was still scarcely quantitative, but it showed that under the conditions used most of the N_2 was liberated in half an hour, by which time the fall in activity and production of diazo compound had hardly started. This supports the contention of Hugounenq & Loiseleur that the peptic activity does not depend on the presence of amino groups, and it also agrees with the finding of Herriott & Northrop [1934] that the amino groups can be acetylated without change of activity. The experiments on N_2 evolution were done first in a Barcroft apparatus in collaboration with (Mrs) F. J. Philpot and later in a Warburg apparatus in collaboration with Dr E. Chain.

(4) *Action of $NaNO_2$ plus acetate buffer on amino-acids.* In presence of sodium nitrite at *p*H about 4 (the conditions finally chosen for pepsin) a yellow colour was given slowly by tyrosine, very much more rapidly by tryptophan, but not by cystine, leucine, aspartic acid, phenylalanine, histidine or arginine.

Of the amino-acids shown by Calvery *et al.* [1936] to be present in pepsin, the only ones not tried were lysine and glutamic acid, neither of which is likely to give a colour. The yellow product from pepsin gives the colour reactions of diazotyrosine as described in the preceding paper, and can therefore be considered as due to tyrosine, and possibly in part to tryptophan, though we have no definite evidence on the latter point.

Final experiments

The preliminary experiments and the work of Herriott and Northrop on other reactions suggested that the partial inactivation of pepsin by HNO_2 was due to the action on tyrosine groups in the molecule. The final experiments were designed to prove this as rigidly as was practicable by showing not only that the kinetics of the partial inactivation of pepsin were identical with those of colour development from pepsin and free tyrosine, but that other amino-acids (tryptophan) and even other *p*-substituted phenols (*p*-cresol) react at quite different rates.

This is important for two reasons. First, even though tyrosine is concerned in the peptic activity of the protein, it is conceivable that this activity might depend on the presence of intact groups of both tyrosine and some other amino-acid, e.g. tryptophan. Since tryptophan is attacked by HNO_2 much faster than pepsin is inactivated, we conclude that it is not concerned in the activity of pepsin. Secondly, in order to exclude the possibility that the activity is due to some other *p*-substituted phenol, not yet isolated from pepsin, it is necessary to show that the velocity constants obtained are highly specific.

Runs were done with the following *p*-substituted phenols: *p*-cresol, *p*-hydroxyphenylacetic acid, tyrosol and α-bromopropionyltyrosine, to investigate the effect of the side chain on the reaction rate.

Materials

Pepsin was prepared by the following modification of the Northrop method: 100 g. Parke Davis 1 : 10,000 pepsin were dissolved in 100 ml. H_2O and 100 ml. N H_2SO_4 were added. The solution was filtered through Whatman No. 44 filter paper and 200 ml. saturated $MgSO_4$ were then dropped in from a capillary with mechanical stirring over a period of ½ hr. The finely granular precipitate was centrifuged and redissolved in 70 ml. acetate buffer of *p*H 4·6 (AcONa, 0·5 N; AcOH, 0·548 N). The solution was dialysed with gentle stirring at 1 atm. excess pressure in cellophane, reinforced with netting, against a solution containing 0·3 N NaCl, 0·0144 N AcONa and 0·186 N AcOH, *p*H about 3·5. Crystallization was complete after 5 days at room temperature, yield 18 g. of moist crystals. The product was recrystallized by dissolving in the *p*H 4·6 acetate buffer and repeating the dialysis; yield 60 %.

The specimens used were generally twice crystallized. For storage the crystals were dissolved as before, pressure-dialysed against distilled water, and the concentrated nearly salt-free solution was left in a vacuum desiccator. The product consisted of brownish glassy flakes resembling the starting material in appearance. It was easily denatured by grinding, but otherwise would dissolve to a clear solution which possessed undiminished activity, was homogeneous in the ultracentrifuge, and as far as could be found contained no more non-protein material than the original solution of crystals. Great difficulty was experienced in determining the activity in absolute units, owing to variations in sensitivity in different preparations of haemoglobin.

As a result of earlier fruitless attempts to crystallize by dialysis it was observed that crystallization only occurred within a critical range of salt concentration, which was more restricted the lower the *p*H. Outside the critical range, only amorphous precipitates could be obtained. Below about *p*H 3·3 no salt concentration would yield crystals, while above *p*H 4·0 the pepsin became too soluble and crystallization was very slow and incomplete. When the salt concentration was at the limit of the critical range the edges of the crystals were rounded.

The amino-acids and phenols were the purest available, but no special precautions were taken as to their purity, since it was not likely to affect any of the conclusions of this paper.

Technique

Peptic activity was measured by the method of Anson & Mirsky [1932–33]. Control experiments showed that the method was unaffected by small amounts of HNO_2 added to the haemoglobin, and that the lower apparent activity of the pepsin derivative was not due to its instability under the conditions of the activity determination.

The light absorption of the yellow products was measured by means of a small Hilger spectrograph (not the standard model) used in conjunction with a rotating logarithmic sector and a Dobson microphotometer in a manner similar to that described by Dobson *et al.* [1926]; in experiments on tryptophan, and some on tyrosine, the increase in absorption at 411·5 mμ, a wave-length at which neither unchanged amino-acid nor nitrite absorbs appreciably, was taken as a measure of the extent of reaction. This method was unfortunately the only one available for use with tryptophan.

In some experiments on pepsin and tyrosine the amount of the yellow diazo compound produced was estimated colorimetrically by one of the colour reactions mentioned in the preceding paper [1938], that with β-naphthol in alkaline

solution being found most convenient: although the colour produced with resorcinol was more intense, the excess resorcinol oxidized in air after some time, so that the intensity of colour was not constant. In a kinetic run, samples were taken out at intervals and made alkaline, sufficient alkali being added to give a final concentration of N NaOH: finally 1 ml. of a 1 % solution of β-naphthol in dilute alkali was added, and the colour estimated. The intensity of the red colour developed on illumination of the alkaline solution was also used to follow the reaction, but was much less satisfactory. All solutions of diazo compounds were kept in the dark.

The rate of formation of the Cu complex of the *o*-nitroso derivative from tyrosine (see previous paper) was also studied, the amount of the compound produced being estimated colorimetrically, using sodium light. The reaction was also followed in a very few instances by using Folin's reagent. The intensity of blue colour at the end of the reaction with tyrosine was about 10 % of its initial value; the decrease in blue colour was taken as proportional to the extent of the reaction.

When the various methods had been shown to lead to the same velocity for tyrosine, and it had been established that they were measuring the velocity of the same reactions (i.e. $R.C_6H_4.OH + HNO_2 \rightarrow R.C_6H_3.(NO).OH + H_2O$, the rate-determining step which also controls the production of diazo compound) it was found most convenient to use the Cu nitroso compound, since it is the simplest, most convenient and most accurate.

The concentrations generally used for activity measurements were about 2·5 mg. pepsin per ml., and samples were diluted 1:50 with distilled water for activity measurements. The concentration of tyrosine was about 1 mg./ml. for measurement as diazo compound or nitroso compound. When pepsin was used for these two latter methods, its concentration was chosen so that the amount of tyrosine reacting was about the same.

The final concentrations of $NaNO_2$ used were $N/16$ at 38° and N at 0°. The two temperatures used, 38 and 0°, were obtained respectively by a gas thermostat, and by immersing in ice and distilled water kept in the cold room. Both were correct to 0·05°.

The pH was controlled by means of acetate buffers of ionic strength 0·5. Their composition was calculated from data given by Clark [1928]. Corrections were made for the buffering effect of the nitrite in calculating the concentration of free HNO_2. The buffering effect of the pepsin was negligible. The pH of buffers was occasionally checked by means of a glass electrode (the pH of 0·5 N sodium acetate plus 0·548 N acetic acid being taken as 4·6) and agreed within 0·05 unit. Systematic errors in pH were probably the limiting factor in the accuracy of the velocity constants finally obtained.

The concentration of free HNO_2 in equilibrium with nitrite at a given pH was calculated using a value for the dissociation constant extrapolated for 38° from the data of Klemenč & Hayek [1929] for 0, 12·5 and 30°. The values so obtained are given in Table 1 below.

Below pH 3·8 the product from pepsin precipitated even from quite dilute solutions, causing technical inconveniences, while above pH 5 the rate of alkaline inactivation of pepsin was comparable with that of the reaction under investigation. The final experiments were therefore confined to pH 3·8–4·6. Control experiments showed that within this range the inactivation of pepsin in absence of nitrite was negligible during the time of the experiment.

Table I. *Concentrations of HNO_2 in buffer solutions*

$NaNO_2 = 0{\cdot}0625$, Na acetate $= 0{\cdot}25$, 38°

Buffer *p*H	Final *p*H	$[HNO_2]$
3·7	3·727	0·0137
3·75	3·775	0·0126
3·8	3·823	0·0116
3·9	3·920	0·00965
4·0	4·017	0·00810
4·1	4·115	0·00689
4·2	4·213	0·00567
4·3	4·311	0·00462
4·4	4·410	0·00372
4·5	4·509	0·00299
4·6	4·608	0·00239
5·0	5·004	0·000970

"Buffer *p*H" is the *p*H of the buffer at 18° before addition of nitrite.
"Final *p*H" is the *p*H at 18 after addition of nitrite, calculated allowing for the buffering effect of the nitrite.
$[HNO_2]$ is the concentration of free nitrous acid at 38 or 0 respectively.

$NaNO_2 = 1{\cdot}0$, Na acetate $= 0{\cdot}25$, 0°

3·7	4·002	0·198
3·8	4·079	0·171
3·9	4·158	0·147
4·0	4·236	0·127
4·2	4·397	0·0944

Table II. *Velocity constants at 38°*

(*a*) $NaNO_2 = 0{\cdot}0625$; ionic strength $= 0{\cdot}313$

Method A. As diazo compound by photo-reaction.
" B. As diazo compound by coupling with phenols.
" C. As nitroso compound in presence of copper.
" D. By decrease in blue colour with Folin's reagent.
" P. By peptic activity. Method S. Spectrographically.

Substance	Method	*p*H	K_u	K_b
Pepsin	P	3·8	0·00226	0·00748
	P	3·8	0·00248	0·00821
	P	3·8	0·00246	0·00815*
	P	4·0	0·00190	0·00875
	P	4·0	0·00186	0·00855†
	B	4·1	0·00161	0·00895
	P	4·1	0·00160	0·00890
	P	4·2	0·00120	0·00815
	A	4·2	0·00119	0·00809
	P	4·2	0·00120	0·00815
	P	4·3	0·00098	0·00815
	P	4·3	0·00100	0·00823
	P	4·4	0·00078	0·00808
	P	4·5	0·00073	0·00936
	P	4·5	0·00067	0·00860
			Mean	0·00839
Tyrosine	A	3·8	0·00270	0·00895
	C	3·8	0·00285	0·00943
	D	3·8	0·00223	0·00739
	C	3·8	0·00228	0·00755
	B	4·0	0·00173	0·00796
	C	4·0	0·00178	0·00821
	C	4·0	0·00178	0·00821
	A	4·2	0·00115	0·00780
	B	4·2	0·00126	0·00855
	B	4·2	0·00120	0·00815
	B	4·2	0·00115	0·00780
	C	4·2	0·00119	0·00807
	D	4·2	0·00115	0·00780
	C	4·4	0·00076	0·00792
	C	4·6	0·00050	0·00805
	D	4·6	0·00051	0·00820
	C	3·8	0·00251	0·00832
	C	3·9	0·00212	0·00844
	C	4·0	0·00178	0·00821
	C	4·1	0·00147	0·00816
			Mean	0·00816

Table II (*cont.*)

Substance	Method	*p*H	K_u	K_b
α-Bromopropionyl-tyrosine	C	3·75	0·00260	0·00780
	C	3·8	0·00270	0·00895
			Mean	0·00838
Tyrosol	C	4·0	0·00285	0·0131
	S	4·2	0·00150	0·0115
	S	4·6	0·00070	0·0113
			Mean	0·0120
p-Cresol	C	3·75	0·00625	0·0190
	C	3·8	0·00540	0·0179
	C	4·0	0·00416	0·0191
			Mean	0·0187
p-Hydroxyphenyl-acetic acid	C	3·8	0·00268	0·00890
	C	3·9	0·00222	0·00885
	C	4·0	0·00202	0·00925
	C	4·1	0·00164	0·00912
			Mean	0·00903
Tryptophan	S	4·2	0·019	0·15‡

* Illuminated Hg vapour lamp. † In presence Cu; final activity = 30%.
‡ Only approx.

(*b*) Effect of varying salt concentration. Tyrosine, *p*H 4·4, $NaNO_2$ = 0·0625, copper nitroso method

Ionic strength μ	K_u	Salt added
0·425	0·0082	$CuSO_4$
0·81	0·0085	$Na_2SO_4 + CuSO_4$
0·875	0·0085	$NaCl + CuSO_4$
1·31	0·00875	$NaCl + CuSO_4$

Table III. *Velocity constants at 0°*

(*a*) $NaNO_2$ = 1·0; total salt concentration = 1·25

Substance	Method	*p*H	K_u	K_b	Remarks
Pepsin	P	3·7	0·0077	0·00150	
	B	—	0·0069	0·00134	
	P	3·8	0·0045	0·00101	
			Mean	0·00128	Methods as in Table I
Tyrosine	B	3·7	0·0066	0·00128	
	C	—	0·0065	0·00126	
	C	3·8	0·0054	0·00121	
			Mean	0·00125	

(*b*) Effect of varying nitrite. Tyrosine, *p*H 3·8, method C

Nitrite	Ionic strength	K_u	K_u' $\mu = 1·25$	$[HNO_2]$	K_b
0·5	0·75	0·0025	0·0026	0·103	0·00097
1·0	1·25	0·0054	0·0054	0·171	0·00121
1·5	1·75	0·0086	0·0083	0·225	0·00142

K_u' is K_u corrected for salt effect, assumed the same as at 38°.
If $V = k_{b_0}$ (tyrosine) (HNO_2) $(1 + a[NO_2^-])$, $a = 0·66$ and $K_{b_0} = 0·000775$.
Mean velocity of pepsin and tyrosine at 0°, corrected for salt effect and nitrite effect, i.e. calculated for $\mu = 0·3125$ and total nitrite = 0·0625 as used at 38°, is 0·00078.

Results

(1) Under the conditions used, there being a very large excess of nitrite, all the reactions are pseudo-unimolecular. This is illustrated by the logarithmic plots for four runs in Fig. 1. A curve of peptic activity against time is also given, to illustrate that the final activity is 50%, the mean value found for the series being 50 ± 1%. This final activity is fairly stable, but further diminishes at a

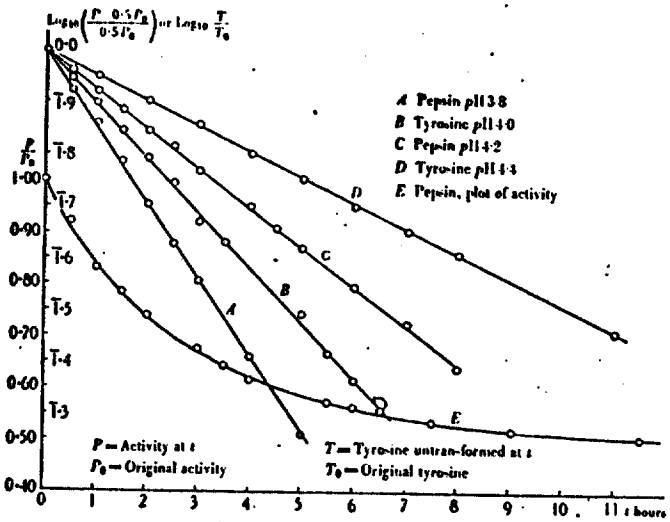

Fig. 1.

very low rate, which is comparable with the rate at which HNO_2 attacks diazo compound. If the HNO_2 is removed with urea or by dialysis no further loss of activity occurs.

(2) The pseudo-unimolecular constants for the inactivation of pepsin, for the production of diazo compound from tyrosine or pepsin, and the production of copper nitroso compound from tyrosine or α-bromopropionyltyrosine are almost identical under similar conditions, and with a constant $[NO_2^-]$ are proportional to the $[HNO_2]$.

p-Cresol and tyrosol react at quite different rates from pepsin or tyrosine, but the rate of *p*-hydroxyphenylacetic acid is not very different. However, application of the "Student" *t*-test to the rates of pepsin and *p*-hydroxyphenylacetic acid shows that the probability that the figures obtained are all members of the same set is only 0·02. To eliminate the possibility that differences in technique might upset this conclusion, four runs (the four last of the tyrosine series in Table II) were done by the same method which was used for *p*-hydroxyphenylacetic acid (the copper method), two before and two after the set of experiments on the latter substance, and they agreed excellently with earlier results. By application of the *t*-test, it was shown that the probability of the rates of tyrosine and *p*-hydroxyphenylacetic acid being members of the same set is only 0·01.

The rates of reaction of different *p*-substituted phenols are therefore sufficiently specific for conclusions to be drawn from them. There is, however, one factor which is difficult to eliminate, that is the influence of the formation of peptide or similar linkages on the rate of reaction. Two runs were done with

α-bromopropionyltyrosine, and the rates were found to agree very well with the rate of reaction of tyrosine. It is perhaps rather surprising, in view of the effect of different side chains on the rate of reaction, that the groups

$$-CH_2.CHOH.CO_2H \quad \text{and} \quad -CH_2.\underset{\underset{CO_2H}{|}}{CH}.NH.OC.CHBr.CH_3$$

in deaminated tyrosine and α-bromopropionyltyrosine respectively should have the same effect; but it appears from this that peptide formation does not appreciably influence the rate of reaction of the benzene nucleus. A further rather surprising fact is that the velocity constants of deaminated tyrosine and *p*-hydroxyphenylacetic acid do not appear to vary with *p*H, though the *p*H of the experiments is in the region of their *p*K, and hence ionization of side chains can have little effect.

Exposure to light during the reaction does not alter the reaction rate, as is shown by the run quoted and one or two others.

In Table II $K_u = \frac{-d \log_{10} (\text{activity or tyrosine})}{dt_{\text{min.}}}$, $K_b = \frac{K_u}{[HNO_2]} \times 0{\cdot}0384$, and is the apparent bi-molecular constant in litres/mol. sec.; K_b is not, however, independent of nitrite concentration, as Table III (*b*) shows, but is proportional to $(1 + a[NO_2^-])$. This may be interpreted with the aid of the reaction complex of phenol and HNO_2 assumed by Veibel [1930] to be in equilibrium with its components. The concentration of this complex will be proportional to [tyrosine] × $[HNO_2]$, its rate of breakdown to *o*-nitroso compounds gives the independent term and a reaction with NO_2^- to give the same product gives the term proportional to NO_2^-. This effect of NO_2^- is perhaps analogous to that found by Taylor [1929] in the reaction between HNO_2 and various amines. The salt effect is very small, which is consistent with the view that one of the species reacting is uncharged, e.g. HNO_2.

The identity of the rates of production of copper nitroso compound and diazo compound from tyrosine support the views put forward in our previous paper that the nitroso compound is a precursor of the diazo compound and is rapidly transformed into it.

(3) Tryptophan reacts nearly 20 times as fast as tyrosine. Several runs were done, but in most cases no good value could be obtained for the velocity constant, owing to the technical difficulties of the spectrographic method. One figure is quoted, to give the order of magnitude of the velocity constant.

(4) Table III gives velocity constants for pepsin and tyrosine at 0°. The substantial identity of the velocity constants at this temperature also very much increases the likelihood that the two reactions are really the same. Since the kinetics are not quite simple, the value of 10,400 calories which is calculated for the heat of activation for both these reactions depends on the exact magnitude of several correction factors applied to the velocity constant at 0°, and must be regarded only as giving the order of magnitude; moreover, the mechanism may, as suggested above to account for the effect of NO_2^-, be complex. However, the abnormally low value of the temperature coefficient is of interest, as it can be explained by the greater dissociation at the higher temperature of the intermediate complex mentioned above.

(5) The properties of the product from pepsin are being further investigated and will be described in a later paper. It has been shown to be homogeneous in the ultra-centrifuge if prepared at 0° and to have approximately the same sedimentation constant as pepsin, which supports the conclusion derived from the kinetic data, that the diminution in peptic activity is due to a specific

effect of the introduction of substituents into the tyrosine group rather than to general damage of the molecule.

The activity of the diazo compound is not changed when it is acted on by light, either in presence or in the absence of HNO_2. Reduction with $Na_2S_2O_4$ does not change the activity by more than 5%. Hence the diazo, hydroxy and several other derivatives have equal peptic activities.

(6) The purple colour developed from pepsin in presence of Cu and HNO_2 is approximately 45% of that expected from its tyrosine content. If the pepsin is broken down by autolysis at *p*H 2 and 38°, or hydrolysis with strong alkali at 100°, it then develops the same amount of colour as a tyrosine solution of equivalent concentration. This suggests that only a portion of the tyrosine in native pepsin is free to react, and recalls the observation of Herriott [1935 36] that several proteins, including pepsin, only give about 60% of the theoretical colour intensity with Folin's reagent at *p*H 8.

Discussion

The chief result of the present work is to add nitrous acid to the brief list of reagents which can act in a known manner on a native protein without damaging the molecule as a whole. A more special result is to provide further evidence in support of the claim of Northrop and Herriott that tyrosine groups, or at least some phenolic groups, play a part in peptic activity. While Herriott's isolation of diiodotyrosine from iodinated pepsin showed conclusively that tyrosine groups in pepsin react with iodine, it did not exclude the possibility that the fall in peptic activity was due to one or two other phenolic groups reacting at the same rate as tyrosine. Our finding that *p*-cresol, tyrosol and *p*-hydroxyphenylacetic acid react at measurably different rates from tyrosine makes it increasingly probable that the phenolic group concerned with peptic activity is that of tyrosine itself. The very rapid reaction of tryptophan also excludes the possibility that unsubstituted tryptophan groups are essential for peptic activity.

The action of nitrous acid on pepsin seems to be simpler kinetically, as far as peptic activity is concerned, than that of either ketene or iodine. The unimolecular approach to a 50% active product, with only a very slow subsequent change, is unprecedented. That the 50% active product also possesses about 50% of its tyrosine groups intact is probably mere coincidence; but it is possible that those tyrosine groups which are protected from nitrous acid are nevertheless somehow able to participate in peptic activity.

In any case it is probable that those groups which do react with nitrous acid become quite inactive enzymically, since they can undergo subsequent changes, e.g. by irradiation, without causing any change in total peptic activity. The relationship between our 50% active product and the several products yielded by ketene and iodine is obscure, and can only be elucidated by further work, e.g. by successive action of the different reagents.

Summary

1. The reaction between pepsin and nitrous acid has been studied up to *p*H 4·5 and at 0 and 38°.

2. The rate is proportional to the concentration of unchanged pepsin and of undissociated nitrous acid, and depends slightly upon the nitrite concentration.

3. The product is a yellow photosensitive diazo compound and has 50% of the original activity. Subsequent changes are very slow.

4. Comparisons with the action of nitrous acid on amino-acids and other substances support the view that the fall in activity is due to an action on the tyrosine groups in pepsin.

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Factors Affecting the Safety of Canned, Cured, Shelf-stable Luncheon Meat Inoculated with Clostridium botulinum

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Abstract

Ground pork was inoculated with spores of Clostridium botulinum types A and B and thermally processed. Factors which affected toxinogenesis were the number of spores, the concentrations of sodium chloride (salt) and sodium nitrite, and the amount of thermal processing. Meat inoculated with 1 spore per g and processed to Fo = 0.6 became toxic if salt and nitrite were omitted. In contrast, meat inoculated with 10^6 spores per g remained non-toxic after the same thermal process provided sufficient salt and nitrite were present, i.e., with a brine (% salt in the aqueous phase) of 6.1% and no added nitrite, with a brine of 4.6% and 300 ppm of added nitrite, or with intermediate concentrations of salt and nitrite.

The amount of thermal processing required to prevent toxinogenesis in meat with 146 ppm of nitrite and 5.5% brine depended on the concentration of spores. An Fo of 0.15 did not prevent toxinogenesis in meat inoculated with 10^4 spores per g; an Fo of 0.3 prevented toxinogenesis with 10^4, but not with 10^6 spores per g; and an Fo of 0.6 prevented toxinogenesis in meat inoculated with 10^6 spores per g. However, when the brine was decreased to 4.8%, meat with 146 ppm of nitrite and 10^6 spores per g became toxic even after an Fo of 0.68.

Viable spores of C. botulinum were recovered 18 months after processing although the meat remained non-toxic, and normal in appearance and odour during this period.

It is probable that some shelf-stable cured meats are safe because of the scarcity of C. botulinum in the raw product.

Résumé

Du porc haché est inoculé avec des spores de Clostridium botulinum types A et B et traité à la chaleur. Les facteurs qui affectent la toxinogénèse sont le nombre de spores, les concentrations en chlorure de sodium (sel) et en nitrite de sodium, et le degré de traitement thermique. La viande inoculée au taux d'une spore par gramme et chauffée jusqu'à un F_0 de 0.6 devient toxique en l'absence de sel et de nitrite. Par contre, la viande inoculée à raison de 10^6 spores au gramme ne devient pas toxique après le même traitement thermique si l'on a pris soin d'ajouter suffisamment de sel et de nitrite, soit une saumure (% de sel dans la phase aqueuse) de 6.1% et 0 nitrite, soit une saumure de 4.6% et 300 ppm de nitrite, ou soit toute autre concentration intermédiaire de sel et nitrite.

Le degré de traitement thermique nécessaire à la prévention de la toxinogénèse dans la viande contenant 146 ppm de nitrite et 5.5% de saumure dépend de la concentration en spores. Ainsi, un F_0 de 0.15 ne prévient pas la toxinogénèse dans la viande inoculée au taux de 10^4 spores au gramme; un F_0 de 0.3 prévient la toxinogénèse avec une inoculation de 10^4, mais pas de 10^6 spores au gramme; cependant, un F_0 de 0.6 prévient la toxinogénèse dans la viande inoculée à raison de 10^6 spores au gramme. Toutefois, si le niveau de saumure est à 4.8%, la viande contenant 146 ppm de nitrite et inoculée à raison de 10^6 spores au gramme devient toxique même avec un traitement atteignant un F_0 de 0.68.

Des spores de C. botulinum sont trouvées viables 18 m après le conditionnement même si la viande ne devient toxique et conserve une apparence et une odeur normales.

Il est probable que certaines viandes affinées qui conservées sans réfrigération doivent leur salubrité à la rare de C. botulinum dans le produit cru.

Introduction

Canned, cured, shelf-stable meat products have with rare exceptions (Reed *et al*, 1965; Meyer and Eddie, 1965), an enviable safety record with respect to botulism. Comminuted cured pork luncheon meat has never been reported to be a cause of botulism although spoilage of this product by putrefactive anaerobes does occasionally occur. The similarity of growth requirements of such spore-forming putrefactive anaerobic bacteria to those of *Clostridium botulinum* leads to the assumption that a product which at some time undergoes spoilage would, if sufficiently contaminated by *C. botulinum*, become toxic. Cured meat products, inoculated with *C. botulinum*, have been shown to form toxin prior to the occurrence of organoleptically detectable spoilage (Pivnick and Bird, 1965; Greenberg *et al*, 1959). For this reason an understanding of the role of curing salts in the safety of such products with respect to botulism is desirable.

With the exception of cured meat products, the basis for safety of all hermetically sealed, shelf-stable low acid foods is a thermal process designed to destroy at least 10^{11} heat resistant spores of *C. botulinum*, i.e. fulfill the classical 12D concept. To obtain this destruction in phosphate buffer at pH 7.0, a thermal process of Fo = 2.78 is required: in low acid foods a greater thermal process is usually necessary. Many cured meat products would, however, be organoleptically unacceptable if heated to Fo = 2.78. Processes of Fo = 0.4 – 0.6 are most commonly used for cured luncheon meats but some manufacturers have employed much lower processes. Processes of less than Fo = 0.01 have been described to obtain shelf-stable cured meats (Shack and Taylor, 1963), but they use much higher nitrite concentrations than are commonly employed in Canada or are permitted in the United States.

Reviews by Riemann (1963) and Silliker (1966) indicate that the usual commercial process is adequate for the following reasons: (1) the inhibitory activity of sodium chloride to *C. botulinum*, especially when heat damaged; (2) the inhibitory activity of nitrite and, especially, (3) the occurrence of only low numbers

This report is based on work that was initiated while the senior author was a member of Connaught Medical Research Laboratories, University of Toronto, and was completed at the above address.

of spores of *C. botulinum* and other anaerobic bacteria in raw meat. Many reports (*e.g.*, Silliker *et al*, 1958) indicate that some of the commonly used thermal processes and concentrations of curing salts (78 – 156 ppm of sodium nitrite and 2.0 – 2.50% sodium chloride) would be inadequate if large numbers of anaerobic spores were present (*e.g.*, 10^2 – 10^4 per g). Evidently the same standards of thermal processing do not apply to cured meats as apply to other low-acid foods.

Although the safety of the product depends, to a large extent, on the factors mentioned above, firm guide lines do not exist with respect to amounts of sodium chloride and nitrite, and the extent of thermal processing. Can manufacturers, however, do suggest the following: (1) 35 lb of salt per 1000 lb of meat which would give a brine (salt in the aqueous phase of the meat) of over 6%; (2) 1.25 – 2.50 oz of sodium nitrite per 1000 lb of meat which would give 78 – 156 ppm before processing; and (3) time-temperature relations for processing to obtain Fo values of 0.7 – 1.0 (Anonymous). These suggestions are rarely followed in their entirety. For example, we have examined numerous samples of canned pork luncheon meat from many manufacturers and have never found any that contained as much as 3.5% sodium chloride. Also, there is only limited evidence that 78 ppm of sodium nitrite (Silliker *et al*, 1958) is as effective as 156 ppm. And, as mentioned previously, some manufacturers use thermal processes considerably lower than those suggested. For these reasons we have attempted to determine how much the suggested procedures can be modified with respect to thermal process, salt, and nitrite, and still obtain a safe product. This report presents the results of our completed studies. A report of some preliminary data has been published (Pivnick and Thatcher, 1968).

Materials and Methods

Spore suspensions. Five type A strains of *C. botulinum* (1, 17, 62, 109 and T) and 5 type B strains (52, 53, 54, 113 and 139S3) were allowed to sporulate, harvested, and washed as described by Schmidt *et al*, (1962). The heat resistance of the 10 individual spore suspensions in M/15 phosphate buffer, pH 7.0, was determined by sealing 23 ml of each suspension (107 spores per ml) in a 30-ml vaccine vial, and autoclaving at 6.5 psi. The heating and cooling of the suspensions were monitored with thermocouples during processes that obtained Fo values of 0.23 and 0.64. Spores were enumerated using a 3-tube MPN technique in 4 media: pork-pea medium without agar (Andersen, 1951); thioglycollate broth (Difco) containing 0.1% starch (Olsen and Scott, 1950); cooked meat medium (Difco); and peptone milk (Riemann, 1957), all layered with vaspar and incubated 1 mo at 30C. The 4 media were used because we felt that a single medium might be inadequate for all strains after heating.

When required as an inoculum for meat equal numbers of spores of each strain were mixed. For each experiment, spores in the mixture were enumerated in pork-pea agar (Andersen, 1951).

Preparation and inoculation of meat. Meat was prepared in the processing department of a meat packing company. In most instances a single uniform lot was prepared for a single experiment by grinding frozen 75% lean pork trim through a ⅛" plate, adding ingredients common to all variables of the experiment, and mixing for 5 min. Unless otherwise stated the common ingredients were 1 lb of sucrose (cane sugar) and 3 lb of ice. Sodium chloride (salt) was added for some experiments (Tables 2, 6, 8 and 9) at 2.75 lb per 100 lb of meat. For other experiments (Tables 3 and 5) salt was added in the processing department at 1 lb per 100 lb of meat, and additional salt was added as described below. After mixing, the lot was subdivided into batches and held at –18C. All subsequent procedures were carried out in a laboratory separated from the meat packing company to prevent introduction of spores of *C. botulinum* into the plant.

As required, meat was warmed to 0–4C and introduced to a meat mixer that had been precooled with solid carbon dioxide. While mixing, spore suspensions, nitrite solutions and, when required, additional salt were added. The suspensions and solutions contributed 1 lb of additional water per 100 lb of meat. After adding the required ingredients, mixing was continued for 9 min under 23 in of vacuum. This amount of mixing evenly distributed a suspension of *Serratia marcescens* used to test the efficacy of the procedure.

Canning. The mixed meat was canned in a 4C cold room. Unless otherwise stated, 340 ± 5 g of meat mixture was stuffed into each 300 x 308 can (3 in diameter x 3 8/16 in high) and the cans were sealed in a semi-

Table 1. Effect of sodium nitrite on toxinogenesis in canned luncheon meat[a] during 18 months of incubation

Lot	Brine (%)	Sodium nitrite (ppm) Added	Found after process	Found after 18 mo	No. cans toxic / No. cans incubated	Weeks for swelling[c]
A	0.4	0	—		20/20	≤1
B	5.0	0	—		43/46	15(10-22)
C	5.3	146	45	0	3/44	29(21-33)
D[b]	5.5	146	59	0	1/45	41

[a] 10^6 spores per g; in all experiments a mixture of 5 strains of type A and 5 of type B were used. Processed to Fo = 0.57.
[b] Lot D also contained 30 ppm of sodium nitrate.
[c] Mean and range.

Table 2. Effect of varying salt and nitrite on toxinogenesis in canned meat[a]

Brine (%)	Cans with toxin/Cans incubated — Sodium nitrite added (ppm) 75	150	300
3.6	6/7	8/10	2/10
4.1	9/10	7/10	2/10
4.6	8/10	6/10	0/10
5.2	7/10	5/10	0/10
5.4	5/10	0/10	0/9

[a] 10^6 spores per g; 70 g meat per 211 x 105 can; processed to Fo = 0.61; incubated 5 mo.

automatic machine under 23 in of vacuum, heated in steam at 108.9 – 109.4C, and cooled in water to below 43C. The thermal process was followed by distributing 4 to 6 cans of meat fitted with thermocouples throughout the load and reading temperatures at intervals of 1 min. Thermocouples, in the geometric centre of the cans, were attached to a Leeds and Northrop Model 8693 potentiometer modified to obtain 10 leads. The thermal process (Fo) was calculated from lethality tables using all temperatures above 93.4C (200 F), and assuming a Z value of 18.

Examination of cans. Cans were incubated at 30C and examined for swelling at weekly or shorter intervals during the first 2 mo, approximately twice a mo between 2 and 8 mo, and about every 2 mo thereafter. Swollen cans were removed when found, and stored at —20C pending examination for odour and toxin. These cans were opened while frozen thus preventing the spraying of liquids that usually follows the opening of unfrozen swollen cans. With rare exceptions that are specifically mentioned, all swollen cans contained putrid, toxic meat.

After 18 mo of incubation, all cans remaining unswollen were examined for odor and toxin. Some of these were also examined for nitrite and viable spores.

In several experiments in which few or no swollen cans were obtained after 4 mo of incubation, 5 unswollen cans were removed at that time. These were examined only for odor and toxin.

Chemical analyses. Water, sodium chloride, and sodium nitrite were determined as described previously (Pivnick and Bird, 1965). The AOAC method for nitrite was used for samples taken immediately before and after processing. However, because of low levels of nitrite after 18 mo at 30C, nitrite was then determined by the method of Kamm *et al,* (1965). The AOAC (1960) and Kamm methods, tested with several canned cured meat products containing sodium nitrite in the 15-25 ppm range, were equally suitable. However, the AOAC method was of little value in determining nitrite at less than 5 ppm while the Kamm method enabled quantitative recovery of as little as 2 ppm of sodium nitrite added to canned cured meat that had no detectable nitrite. In this report, nitrite and sodium nitrite are used synonymously, sodium chloride (salt) is stated as per cent w/w, and brine % is calculated by the formula:

$$\frac{\text{g salt}}{\text{g salt} + \text{g water}} \times 100$$

Tests for toxin. The meat in each can was homogenized with an equal volume of water, and the supernatant was tested for toxin by injection into mice as described previously (Pivnick and Bird, 1965).

Detection of viable spores in canned meat. Cans were disinfected by immersion for 20-30 min in M/20 phosphate, pH 6.2, to which was added immediately before use 10 ml of commercial hypochlorite bleach per liter (Ito *et al,* 1967). After drying under an ultraviolet lamp the cans were opened aseptically, and the total content was homogenized with an equal volume of water in a Waring Blendor. Two media, Reinforced Clostridial Medium (RCM) (Oxoid), and Wynne's Fluid Medium (Difco) with 0.1% of filter-sterilized sodium bicarbonate, were used in an attempt to detect viable spores. Four tubes of RCM and 4 tubes of Wynne's medium, each containing 18 ml of medium, were inoculated with 8 ml of homogenate. Thus, each tube contained 4 g of meat, and a total of 32 g of meat from each can was cultured.

Two inoculated tubes of each medium were deaerated by heating in boiling water for 10 min (5.5 min for content of tube to reach 100C), sealed with vaspar (60% vaseline – 40% paraffin), and incubated in air. The remaining two tubes of each medium were placed in an anaerobic incubator which was evacuated and flushed twice with 90% hydrogen – 10% carbon dioxide, and incubated for 4-5 weeks under the same gas mixture at 3 in of vacuum. All tubes wer incubated at 30C.

Our criteria for growth in these media were any one of the following: gas production, formation of malodourous compounds, or digestion of meat. We did not examine the contents microscopically because, in our experience, cultures of the strains of *C. botulinum* used would show one or more of the signs listed. Only those tubes giving evidence of growth, according to our criteria, were tested for toxin. Tubes showing growth without toxin were subcultured in RCM and these subcultures were tested for toxin after 1 week. We

Table 3. Effect of varying salt and nitrite on toxinogenesis in canned meat processed to Fo = 0.57[a]

Brine (%)	Cans with toxin/cans incubated — Sodium nitrite added (ppm) 0	75	150	300	Weeks for swelling[c] Mean	Range
4.9	10/10	9/10	0/10	0/10	9	6-32
5.5	8/10	6/10	0/10	0/10	14	3-19
5.8	4/10	0/10	0/10	0/10	26	23-32
6.1	0/10	0/10	0/10	ND[b]		

[a] 10^6 spores per g; incubated 18 mo.
[b] Not done.
[c] Values for meat with 0 and 75 ppm of nitrite are combined because nitrite did not appear to influence time for swelling. See text.

Table 4. Effect of concentration of sodium nitrite and spores on toxinogenesis during 18 months at 30 C[a]

Sodium nitrite (ppm)[b]			Cans toxic/cans incubated[c]					
Added	Found after process	Found after 18 mo	Spores per g 0	10^0	10^2	10^4	10^6	Weeks for swelling
0	—	—	0/45	0/45	0/47	1/45	29/46	30(18-56)[d]
15	—	—	0/45	0/45	0/44	0/45	26/45	28(14-49)
75	20.2 ± 0.8	0.6 ± 0.5	0/45	0/45	0/45	0/45	11/45	33(24-56)
150	24.3 ± 4.9	0.8 ± 0.6	ND	0/46	0/45	0/45	1/45	51
300	69.3 ± 1.9	4.1 ± 0.5	ND	ND	0/49	0/45	0/45	

[a] Brine 5.16%; Fo = 0.60 — 0.64.
[b] Mean and standard deviation of 6 — 10 cans.
[c] All cans containing toxic meat were swollen, and the meat was putrid.
[d] Mean and range of weeks of incubation for swelling to occur in cans containing meat inoculated with 10^6 spores per g.

felt that this step was necessary because of the possibility of toxin being formed and degraded during the 4-5 week incubation of the first culture.

Results

Heat resistance of spores in M/15 phosphate buffer, pH 7.0. The 10 suspensions, before heating, each contained 1 x 10^7 viable spores per ml. Heating to Fo = 0.23 caused a 1-2 $\log_{10}$ decrease in 6 of the 10 spore suspensions and 3-6 $\log_{10}$ decreases in the other 4. After heating to Fo = 0.64, two of the suspensions were sterile and the remaining 8 contained 10^0—10^2 viable spores per ml. The mean number of viable spores for the 10 suspensions heated to Fo = 0.64 was 21 per ml indicating an average reduction of about 6 $\log_{10}$. Thus, the spores we used for inoculating meat were not unusually heat resistant in neutral phosphate buffer. Calculations from the data of Ito *et al* (1967) indicate that their most heat resistant spores of *C. botulinum* would not be decreased more than 3-4 $\log_{10}$ by an Fo of 0.64 in pH 7.0 buffer.

Although the 4 media we used were almost equally suitable for enumerating unheated spores by the MPN method, there was considerable variation in the ability of these media to support the growth of the heated spores. In addition, some media appeared to be superior for growing heated spores of some strains but not for others. The numbers of spores surviving the heating is based on maximum recovery in any of the 4 media used.

Toxinogenesis in meat without curing salts. The effect of thermal processing on toxinogenesis in meat devoid of curing salts and sugar was determined by mixing spores with meat in a mixer previously sterilized with formaldehyde and steam at 5 PSI. Spore suspensions were added to obtain 0, 1, 100, 10,000, or 1,000,000 spores per g of meat. The weight of water in each suspension was 4.5% of the weight of the meat. Ten to eleven cans were prepared with each inoculum and the cans were processed to Fo = 0.62.

This process did not stabilize the uncured meat. With only 1 spore per g (340 per can), 1 of 11 cans became toxic; with 100 spores per g, meat in 8 of 11 cans became toxic; and with 10,000 or more spores per g, meat in all cans became toxic. All cans containing toxic meat were swollen within 15 days, and the meat was putrid. Non-toxic meat was found in 2 cans that swelled after 40 and 114 days, and in these 2 cans the meat had an off-odour that was not putrid. Twenty-one cans remaining unswollen after 18 mo contained non-toxic meat. Twelve of these 21 cans were cultured and found to be sterile.

Effect of sodium chloride and sodium nitrite on toxinogenesis in commercial-type formulations. Four lots of meat were inoculated with 10^6 spores per g and processed to Fo = 0.57. Lot A was devoid of curing salts and sugar; the 3 remaining lots, B, C and D, contained a commercial formulation of 2.75 lb salt, 1 lb of sugar, and 3 lb of ice per 100 lb of meat. Lot B contained no sodium nitrite, but nitrite was added to lots C and D at 0.25 oz per 100 lb of meat, a commonly used commercial concentration. This amount would supply 156 ppm of sodium nitrite in the meat, or 146 ppm in the mixture of meat, sodium chloride, sucrose, and ice. Lot D also contained sodium nitrate at 30 ppm.

Table 1 shows the composition of the 4 lots of meat and toxinogenesis during 18 months of incubation. The nitrite concentration was decreased by about two-thirds during the processing and nitrite was not detectable after 18 mo. Meat devoid of both salt and nitrite (Lot A) was toxic within 1 week. With 5.0% brine but no nitrite (Lot B), 43 of 46 cans swelled and became toxic, but the minimum time for detectable

Table 5. Toxin and odour in unswollen cans after 4 months at 30 C[a]

Nitrite added (ppm)	No. cans examined	No. cans with toxin	No. toxic cans with odour of spoilage	No. toxic cans without odour of spoilage
0	5	2	1	1
15	5	3	3	0
75	5	1	0	1
150	5	0		
300	5	0		

[a] Brine = 5.16%; 10^6 spores per g; Fo = 0.60 — 0.64.

Table 6. Effect of spore number and thermal process on toxinogenesis

Fo	Sodium nitrite (ppm)[a]		Maximum temperature at centre (C°)	10^4 spores per g		10^6 spores per g	
	After processing	After 18 mo at 30 C		Cans toxic[b] / Cans incubated	Weeks for swelling	Cans toxic[b] / Cans incubated	Weeks for swelling
0	101		4.5				
0.15	64	1.8	101.2	9/52	14(9-48)	58/58	7(4-48)
0.29	51	1.3	103.0	0/44		3/46	32(9-41)
0.61	49	1.3	105.7	0/45		0/45	
1.27	27	1.5	107.3	0/45		0/45	
2.36	17	3.8	108.5	0/45		0/45	
3.70	15	4.2	109.0	0/45		0/45	

[a] 146 ppm added; values after processing and after 18 mo are means of 2 and 4 cans respectively.
[b] 5.5% brine.

swelling was 10 weeks. The addition of both salt and nitrite (Lots C and D) resulted in a great decrease in the number of cans which became toxic and an increased time for detectable swelling of the cans.

The brine concentrations in the 3 lots of meat were slightly different because of the presence of a higher fat content in lots C and D and, correspondingly, less water. As will be shown later (Fig. 1), these small differences in brine might significantly influence toxinogenesis in meat with 10^6 spores, 146 ppm of nitrite, and processed to Fo = 0.60.

The presence of 30 ppm of sodium nitrate in lot D is probably of little consequence in determining whether or not toxinogenesis will occur. Even much higher concentrations have not inhibited growth of *C. botulinum* (Tanner and Evans, 1933).

We examined 5 unswollen cans of lot C and 5 of lot D for toxin after 4 months at 30C: none was found. Viable spores were not found after 18 months of incubation in the 3 remaining cans of lot B nor in 5 cans from each of lots C and D.

Effect of varying sodium chloride and nitrite on toxinogenesis. The interaction of different concentrations of sodium chloride and sodium nitrite was tested in 3 similar experiments, each using 10^6 spores per g of meat and processing to approximately Fo = 0.6.

For the first experiment we used 7 concentrations of sodium chloride ranging from 0 – 3.5% w/w and 5 concentrations of sodium nitrite from 0 – 300 ppm. In all, 35 lots, each containing 10 vials, were prepared. The meat was dispensed in 6 ml vaccine vials with a caulking gun, stoppered, sealed, processed to Fo = 0.50, and incubated at 30C. This experiment showed that with as little as 2 % w/w of sodium chloride present toxinogenesis was decreased as the concentration of nitrite was increased. However, in the absence of sodium chloride, even 300 ppm of nitrite did not prevent toxinogenesis.

In the second experiment (Table 2) we used 5 concentrations of salt (2.0 – 3.25% w/w which gave brines of 3.6 – 5.4%) and 3 concentrations of nitrite (75, 150 and 300 ppm). For this experiment, we canned 70 g of meat mixture per 211 x 105 can. The cans were sealed at atmospheric pressure, processed to Fo = 0.64, and incubated at 30C until swollen, or for 5 months. All swollen cans contained toxic meat. All cans remaining non-swollen at the end of the incubation contained non-toxic meat. Table 2 shows that both sodium

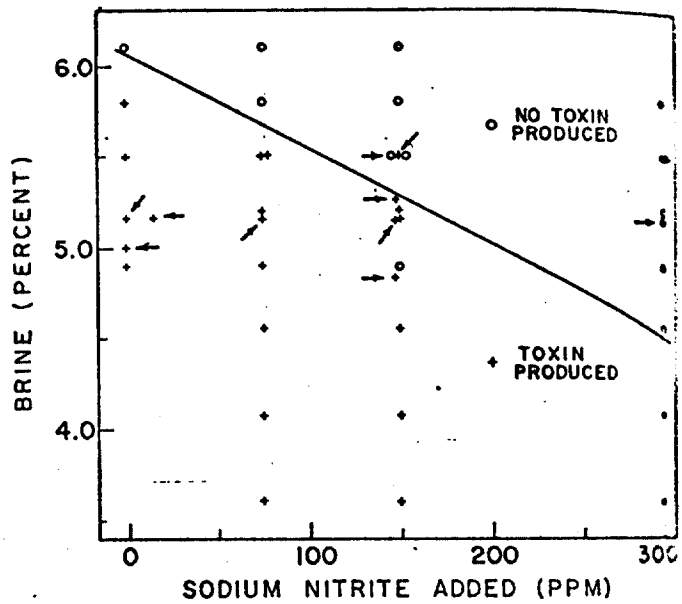

Fig. 1. Interrelationship of soduim chloride and sodium nitrite in preventing toxinogenesis in canned pork luncheon meat inoculated with 1 x 10^6 spores per g and processed to Fo = 0.6 ± 0.05. "+" indicates that at least one can in lot became toxic; "O" indicates that none of cans became toxic. Points marked with arrows represent 45-50 cans; the remaining points usually represent 10 cans.

chloride and sodium nitrite are important in determining whether or not toxinogenesis will occur: as the brine concentration was increased, the concentration of nitrite needed to prevent toxinogenesis was decreased. However, even 300 ppm of added nitrite did not prevent toxinogenesis when the brine concentration was 3.6 – 4.1%. In contrast, as little as 150 ppm of nitrite in conjunction with a brine of 5.4% prevented toxinogenesis in all 10 cans. At 75 ppm of nitrite, none of the concentrations of brine was completely effective although there is a trend which shows that as the brine was increased, toxinogenesis was progressively inhibited. A similar trend is apparent with 150 ppm of nitrite.

In contrast to the first and second experiments the third experiment (Table 3) simulated industrial practice: we used 300 x 308 cans and sealed them under 23 in of vacuum. These were processed to Fo = 0.5. In this experiment, besides studying toxinogenesis, we also observed the stability of nitrite: 80 – 90% of the nitrite disappeared during processing and, after 18 mo, only about 1% of the added nitrite remained.

The brine concentrations (Table 3) used in the third experiment were higher than most of those employed in the second experiment (Table 2) and, as a result, toxinogenesis was more severely inhibited. Table 3 shows that even without nitrite a brine of 6.1% prevented toxinogenesis in all 10 cans, and that as little as 75 ppm of nitrite prevented toxinogenesis when the brine was 5.8%. With 150 ppm of nitrite toxinogenesis was prevented by even lower concentrations of salt.

The time required for noticeable swelling of cans increased with increased salt concentration and, with the exception of the highest brine concentration, most of the cans that swelled did so within 5 mo (Table 3). Seperate calculations, not shown in Table 3, indicated that nitrite played no role in delaying swelling, and this observation was substantiated in a subsequent experiment (Table 4).

All cans remaining unswollen for 18 mo contained meat that was non-toxic and had a normal smell. Forty-five of these unswollen cans (all cans remaining from lots that had produced some swollen cans during incubation and 3 - 5 cans from each lot that had produced no swollen cans during incubation) were cultured for spores but none were found.

Graphic presentation of salt-nitrite interaction in preventing toxinogenesis. The interrelationship between salt and nitrite in preventing toxinogenesis is shown graphically in Fig. 1. This figure is based on the data in Tables 2 and 3, and represents 30 individual nitrite-salt combinations with a total of 296 cans, all containing 10^6 spores per g of meat, and all processed to approximately Fo = 0.6. To prepare this figure we plotted a "O" for each lot in which all cans remained non-toxic and a "+" for each lot that contained one or more cans of toxic meat. With one exception (4.9% brine, 150 ppm of sodium nitrite) the line in Fig. 1 separates the lots in which all cans remained non-toxic from those in which some of the cans contained toxic meat.

Subsequently we used this line to predict whether or not various concentrations of salts and nitrite would prevent toxinogenesis in 300 x 308 cans inoculated with 10^6 spores per g, processed to Fo = 0.60 ± 0.05, and incubated 18 mo. From 4 experiments we had data for 155 cans divided among 10 lots, each of which contained 15 - 50 cans. The points for these lots are indicated by arrows in Fig. 1. Of the 10 lots, 9 gave predicted results: 7 lots which, by virtue of their salt-nitrite concentrations, were below the line all contained some cans with meat that became toxic while in 2 of 3 lots above the line all cans remained non-toxic. However, in one lot (5.5% brine, 150 ppm of nitrite) which was above the line, one of 45 cans became swollen and toxic after 10 mo of incubation. This exception may have been anticipated because the line was established on the results from lots of 10 cans while each point marked with an arrow represents 45 - 50 cans. It may also be anticipated that, had we established the salt-nitrite relationship on the basis of larger lots with the same number (10^6) of spores per g, or used 10 cans per lot but increased the spore concentrations to 10^7 per g, the position of the line in Fig. 1 would be displaced vertically.

Effect of varying nitrite concentrations and spore numbers. Having established an interrelationship between salt and nitrite in the saftety of meat inoculated with 10^6 spores and processed at Fo = 0.6 we next determined the interrelationship of nitrite and spore numbers. A single brine concentration (5.16%) was used. This brine concentration was obtained with 2.90% w/w of salt, a high but organoleptically acceptable level. The thermal process was Fo = 0.60 – 0.64.

Table 4 shows the nitrite remaining after processing and storage, and the number of cans becoming toxic within 18 mo. None of the 546 cans containing 10^2 or fewer spores per g swelled or became toxic. Meat inoculated with 10^4 spores per g yielded only 1 toxic can, and this was from meat devoid of nitrite. However, with 10^6 spores per g over 50% of the cans containing 0 or 15 ppm of nitrite became toxic; with 75 ppm, 11 of 45 cans became toxic; and with 150 ppm, only 1 of 45 cans became toxic. None of the cans which remained unswollen for 18 mo contained malodourous or toxic meat.

The prevention of toxinogenesis by 75 ppm of nitrite compared with 0 nitrite is significant ($P = < 0.001$). The superiority of 150 ppm over 75 ppm is also significant ($P = < 0.01$).

In addition to the results shown in Table 4, we examined 110 unswollen cans (5 from each lot) after 4 mo. None of the meat inoculated with 10^4 or fewer spores per g was malodorous or toxic. However, with 10^6 spores per g, meat in 6 of these unswollen cans did become toxic, and in two of these the meat was without concomitant malodour (Table 5). It is probable that these cans would have become swollen and the meat malodorous after a longer period of storage.

No viable spores were found in 25 unswollen cans incubated for 18 mo (5 from each lot inoculated with 10^6 spores).

Effect of varying thermal process and spore numbers on toxinogenesis. In an attempt to determine the effect of the thermal process on safety, meat containing 5.5% brine and 146 ppm of sodium nitrite was inoculated with 10^4 or 10^6 spores per g and processed to obtain Fo values of 0.15 – 3.70. Table 6 shows that with increasing thermal process the temperature obtained at the centre of the can increased while the residual nitrite decreased. After 18 mo, 1 - 3% of the added nitrite remained. This table also shows that an Fo of 0.15 was inadequate when the meat contained 10^4 spores per g but an Fo of 0.61 prevented toxinogenesis despite an inoculum of 10^6 spores per g.

However, processing to Fo = 0.68 did not prevent toxinogenesis in similarly inoculated meat when the brine concentration was lower. In Table 7 comparison is made between some of the results from Table 6 (Experiment B) with those obtained from a similar but much smaller experiment (A). Although both lots of meat contained 2.9% w/w of salt, the brine content in experiment A was 0.7% lower than that in experiment

Table 7. Effect of concentration of salt and amount of thermal process on toxinogenesis in canned luncheon meat containing 10^6 spores per g[a]

Experiment A (4.8% brine)		Experiment B (5.5% brine)	
Fo	Cans toxic / Cans incubated	Fo	Cans toxic / Cans incubated
0.13	4/4	0.15	58/58
0.24	4/4	0.29	3/46
0.68	2/4	0.61	0/45
1.13	0/4	1.27	0/45
2.38	0/4	2.36	0/45
3.54	0/4	3.70	0/45

[a] In both experiments 0.25 oz of sodium nitrite and 2.75 lb of salt were added per 100 lb of meat, and both lots, or analysis, were found to contain 2.9% sodium chloride w/w. Experiment A contained 25% fat and 57% water; experiment B contained 34% fat and 50% water, hence the difference in brine % in the 2 lots.

B. This difference resulted from a lower fat and, consequently, higher water concentration in experiment A.

Non-toxic meat from five unswollen cans from several of the lots shown in Table 6 was cultured after 18 mo to determine if viable spores remained. Table 8 shows that viable spores of *C. botulinum* were recovered from 3 cans which contained meat inoculated with 10^6 spores per g and processed to Fo of 0.29 or 0.61. In addition, 5 cans yielded non-toxinogenic spore-forming organisms which were not further identified. RCM was superior for recovering viable spores as all viable spores which grew did so in RCM: some of these cultures, but not all, also grew in Wynne's medium. Only media which were deaerated by heating in a boiling water bath after inoculation yielded cultures. Although the heating undoubtedly killed some spores, this method was superior to incubating unheated cultures in an anaerobic chamber. Replicate tubes, unheated after inoculation and incubated under nitrogen-carbon dioxide, did not yield growth, possibly because oxygen was incorporated with the inoculum. As described under Methods, we had inoculated 18 ml of medium with 8 ml of meat emulsion taken a few minutes after blending, and this inoculum would be saturated with air.

In addition to the results shown in Table 6, 55 unswollen cans (5 from each of 11 lots) were examined for odour and toxin after 4 mo at 30C. All appeared normal and were non-toxic.

Discussion

Our work shows the interrelationship of salt, nitrite, spore numbers, and degree of thermal process on the safety of shelf-stable pork luncheon meat. The data substantiate the reports of previous workers that salt is the most important curing agent in stabilizing such products (Jensen, L. B., quoted by Halvorson, 1955; Jensen, 1954; Dack, 1949; Stumbo *et al*, 1950) and indicate that 156 ppm of added sodium nitrite will provide a greater margin of safety than 78 ppm (cf. Silliker *et al*, 1958). Our work also shows that the safety depends on the number of spores and the amount of thermal process.

However, our findings must be considered in relative rather than in absolute terms because our spore suspensions were not unusually heat resistant. If they been as heat resistant as some of those described by Ito *et al*, (1967) it is quite possible that more of the meat inoculated with 10^4 spores (Tables 4 and 6) would have become toxic.

The severity of a thermal process and, hence, the degree of safety provided has been largely based on experience. Outbreaks of botulism from commercially prepared low-acid vegetables and other foods resulted in the imposition of thermal processes which are usually greater than Fo = 2.78. Four decades of subsequent practice show that these processes yield safe products (Meyer and Eddie, 1965).

Until recently, cured meat products have also been considered safe. However, an outbreak of botulism due to "underprocessed" liver paste, a cured product (53% water, 1.8% salt, 3.28% brine, 10 ppm of residual nitrite, pH 5.5), did occur in 1963 (Erdman and Klaziak, 1965) and, in part, stimulated our investigation. The question facing both industry and regulatory agencies is "What constitutes a safe process for cured meats, and what constitutes underprocessing?" Undoubtedly, the safety of a thermal process as applied to cured meats is inextricably related to the concentrations of added salt and of nitrite, and to the number of spores present in the meat (Riemann, 1963).

Numerous studies (Gross and Ogilvy, 1953; Silliker *et al*, 1958; Brown and Vinton, 1964) have indicated that low thermal processes (Fo = 0.05 – 0.1) may produce a shelf-stable product if the spore population in the raw meat is low. However, most of these studies

Table 8. Recovery of viable spores from non-toxic meat[a] in unswollen cans after 18 months of incubation

Fo	No. spores of C. botulinum per g before processing	No. cans examined	No. cans yielding: C. botulinum	No. cans yielding: Non-toxinogenic spore-forming cultures
0.15	10^4	5	0	4
	[b]			
0.29	10^4	5	0	1
	10^6	5	2	0
0.61	14^4	5	0	0
	10^6	5	1	0
1.27	10^4	5	0	0
	10^6	5	0	0

[a] 5.5% brine and 146 ppm of nitrite.
[b] All 58 cans containing meat inoculated with 10^6 spores per g and processed to Fo = 0.15 swelled within 12 mo (See Table 6).

used meat that contained about 3.5% salt, and this would give a brine of over 6.0%. Although such concentrations of salt were commonly used 25 years ago, (Yesair and Cameron, 1942; Vinton *et al*, 1947a, 1947b; Jensen, 1954) they are rarely found in contemporary practice, and yet low thermal processes are still used by some manufacturers. Low thermal processes in conjunction with low concentrations of salt have probably been safe because of the low numbers of spores of *C. botulinum* in meat (Greenberg *et al*, 1967). However, this set of circumstances can not be relied upon to ensure safety. It is possible that a low thermal process and a low concentration of salt could be applied to a meat mixture which contains a sufficient number of spores of *C. botulinum* to survive the thermal process and grow. It was such conditions which, after at least 2 years of practice, resulted in the above mentioned outbreak of botulism due to liver paste. To prevent occurrence of additional incidents it seems desirable that a group of experts should define a realistic spore challenge that any shelf-stable cured meat product must withstand. Our work indicates that there is ample opportunity for a manufacturer to vary the nitrite, salt, and thermal process within broad limits and still obtain an organoleptically acceptable product which is safer than some that are currently produced. It also warns of the severe hazards to be anticipated if a manufacturer either accidentally or purposely reduces one or more of these 3 factors without consideration of the others.

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Effect of Nitrite on Destruction and Germination of Clostridium Botulinum and Putrefactive Anaerobes 3679and 3679h in Meat and in Buffer

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Abstract

Spores of Clostridium botulinum strains 62A and 13983B and Putrefactive Anaerobe (PA) strains 3679 and 3679h were heated in meat or in phosphate buffer, pH 6.2, with increasing concentrations of sodium nitrite. Thereafter, they were incubated at 30 C. Nitrite at commercially used levels (200 ppm) neither enhanced destruction during heating nor germination during incubation. As the nitrite concentration was increased to 400 - 1600 ppm, there was a trend to destruction of more spores by heat, and more germination of spores during post-heating incubation. However, with C. botulinum, nirite-induced germination was insignificant compared to germination by meat alone: 99.0-99.9% of the spores germinated during 1 day of post-heating incubation in meat without nirite.

PA3679 spores were destroyed little, or not at all, by heating to Fo=0.8 with up to 400 ppm of nitrite. However, when nitrite was increased to 800 - 1600 ppm there was a trend that showed destruction of more spores during heating, and germination of more spores during post-heating incubation. Spores of PA3679 did not germinate in meat to the same extent as spores of C. botulinum and PA 3679h.

PA 3679h spores were much more heat sensitive and germinated to a greater extent than spores of the other cultures. In buffer, germination during incubation was directly proportional to the pre-incubation heat treatment and was affected only slightly or not at all by up to 800 ppm of sodium nitrite.

The main value of nitrite in stabilizing canned, cured shelf-stable meats appears to reside in its ability to aid in the prevention of growth from spores that survive heat processing and germinate during post-processing storage.

Résumé

Des spores de Clostridium botulinum, lignées 62A et 13983B et d'anaérobies putréfactives (PA), lignées 3679 et 3679h, inoculées dans de la viande ou dans du tampon ou phosphate de pH 6.2, ont été chauffées en présence de nitrite de sodium à des concentrations de plus en plus fortes. Elles ont été ensuite incubées à 30 C. Aux niveaux utilisés dans la pratique (200 ppm), le nitrite a été sans influence sur la destruction par le chauffage et sur la germination à l'incubation. Aux concentrations de 400 à 1600 ppm, le nitrite a aidé la destruction des spores par la chaleur et la germination des spores durant l'incubation après le chauffage. Toutefois, avec C. botulinum, la germination due au nitrite a été négligeable par rapport à celle due à la viande seule où 99.0-99.9% des spores ont germé dès le premier jour d'incubation après le chauffage.

Les spores de PA 3679 ont résisté au chauffage à Fo=0.8 avec du nitrite jusqu'à 400 ppm. Toutefois, avec du nitrite aux concentrations de 800-1600 ppm la destruction des spores au chauffage et leur germination à l'incubation après le chauffage sont favorisées. Ces spores n'ont pas germé dans la viande autant que les spores de PA 3679h et les spores de C. botulinum.

Les spores de PA 3679h étaient beaucoup plus sensibles à la chaleur et ont germé beaucoup plus que celles des autres cultures. Dans le tampon, la germination à l'incubation était directement proportionnelle au traitement thermique préalable t n'était affectée que très peu par le nitrite de sodium jusqu'à une concentration de 800 ppm.

Le principal mérite du nitrite dans la stabilisation des viandes en conserve, mainées et stables sans refrigération semble résider dans son aptitude à ralentir la croissance des spores qui survivent au chauffage et qui germent au cours de l'entreposage subséquent.

Introduction

Nitrite is added to some canned meat products for three reasons: (a) to form a stable pink product by reacting with myoglobin: (b) to obtain a typical cured flavour; and (c) to aid in stabilizing the meat against spoilage or toxinogenic microorganisms. For colour and flavour development, about 15 ppm of nitrite is adequate (Barnett *et al.*, 1965); for prevention of growth from bacterial spores, much higher concentration are necessary (Perigo and Roberts, 1968).

The sequence of events that occurs when spores are present in a non-inhibitory medium such as meat is as follows:

Spore → germinated spore → vegetative cell → dividing cell → growing culture → spores

Only the spore is heat resistant; germinated spores and vegetative cells are rapidly destroyed within a few minutes at 80 C. Vegetative cells do not divide in the presence of adequate concentrations of nitrite (Duncan and Foster, 1968c).

At the concentrations used commercially, however, nitrite by itself does not prevent spores from progressing to a growing culture in meat. To produce a stable canned, cured meat, nitrite must be used in conjunction with salt and thermal processing. In addition, these 3 factors are inextricably interconnected with pH (Baird-Parker and Freame, 1967; Koelensmid and van Rhee, 1968) and spore load of the meat in determining stability and safety (Pivnick *et al.*, 1969).

Nitrite is lost during thermal processing and subsequent storage (Nordin, 1969), and it is common to find canned, shelf-stable meat products on the market with little or no nitrite present. It is also common for spores to survive the thermal process, and yet they do not grow. Part of the reason for this failure to grow is that heat-damaged spores are more readily inhibited by sodium chloride than are unheated spores (Roberts and Ingram, 1966; Duncan and Foster, 1968a; Pivnick and Thacker, 1970, 1970a; Cook and Gilbert, 1969). However, the low salt concentrations and thermal processes used for some products indicate that inhibition of heat-damaged spores by salt can be only part of the reason for the safety of canned, shelf-stable, cured meats.

Attempts to explain the paradoxical situation resulting from spores surviving the thermal process, but not growing despite the decrease in nitrite during processing and storage, have been the subject of a recent study by Perigo *et al.* (1967). They found that nitrite heated with a bacteriological medium produced a potent, pH-independent, stable antimicrobial agent.

studies in our laboratory (Johnston *et al.*, 1969) confirmed Perigo's results, but did not indicate that the inhibitor was important in explaining the stability of canned meat: the inhibitor was inactivated when added to meat.

An especially attractive concept concerning the role of nitrite has been reported recently by Duncan and Foster (1968a,b,c). They found that nitrite, at suitable concentrations, increased the rate of destruction of Putrefactive Anaerobe (PA) 3679h during heating in phosphate buffer at 98 C; increased its germination rate during incubation; and prevented division of vegetative cells that emerged from spores. Nitrite-induced germination approached a maximum rate during heating at 80 C. All studies on the potentiating effect of nitrite on killing during heating were done at 98 C. If these observations pertained to meat, then an explanation for the stabilizing effect of nitrite might include any or all of the following: (a) nitrite induces germination during the heating process, and the germinated spores are readily killed by heat; (b) nitrite potentiates direct killing by heat, *i.e.*, without prior germination; (c) nitrite increases the germination rate of spores that survive processing; (d) these rapidly germinated spores are then prevented from growing by the nitrite present after processing, and they die before the nitrite has decreased appreciably (the half-life of nitrite in cured meat, pH 6.0, at 23.9 C is 7.8 days; Nordin, 1969).

To test some of these possible roles of nitrite, spores of 4 cultures were heated in phosphate buffer or in meat, each containing nitrite. Buffer has been used for extensive studies by Duncan and Foster (1968a, 1968b, 1968c), and comparison was desirable; but an explanation for the stability of canned meat required that meat be used also. Spores were heated at 80 C and at 100 C; 80 C approximates the temperature obtained in pasteurized canned meats while 100 C approaches the temperature obtained at the centre of canned meat processed to Fo=0.2 (Pivnick *et al.*, 1969). This is a commonly used process for shelf-stable cured meats (Greenberg *et al.*, 1965).

Materials and Methods

Spore suspensions

The 4 cultures used in these experiments were *Clostridium botulinum* type A and type B, PA 3679, and PA 3679h. *C. botulinum* type A (strain 62A) and type B (strain 13983B) were obtained from K. Ito and F. Schmidt, respectively. Growth, sporulation, and washing of spores were done by the method of Schmidt *et al.*, (1962). Putrefactive Anaerobe 3679, commonly used for testing the efficacy of thermal processing, was obtained from W. Bohrer. PA 3679h, a mutant of PA 3679, was obtained from C. Duncan. Spores of these cultures were obtained following growth in the medium of Uchara *et al.* (1965) using the methods described by Duncan and Foster (1968a). The washed spores were, however, not cleaned with Carbowax as described by Duncan and Foster (1968a).

Preparation of meat

In order to simulate industrial conditions raw meat was used in all experiments; but to avoid problems that might arise from indigenous microbial contaminants, the meat was first sterilized by gamma irradiation. Irradiation does not appear to alter the meat; and it does not make it inhibitory to *C. botulinum* (Anellis *et al.*, 1965; Greenberg *et al.*, 1965). Pork (75% lean) ground through a ⅛ in plate was sealed in 300x308 cans, frozen, and irradiated in a Gamma Cell 220 (Atomic Energy of Canada Ltd., Ottawa, Can.) at 1.69 megarads per hr for a total of 2 megarads. All bacteria were destroyed by this dosage, which indicated no more than a moderate concentration of spores in this meat. After irradiation, the cans were held in a dry ice chest until used. As required, the cans of meat were allowed to defrost overnight at 4 C, and the contents were ground 5 min with an equal weight of water at about 40 C in a Waring Blendor. Then the mixture was apportioned, as desired, into several sterile Blendor jars, and frozen at −20 C for a maximum of 5 days. This meat-water (50% w/w) mixture is hereafter called meat. It had a pH of 6.2.

Heating spores in meat

Blendor jars containing meat were defrosted overnight at 4 C, spores and nitrite were added, and the Blendor was operated at a high speed for 30 sec. The volume of added spore suspension and nitrite solution was less than 2% of the volume of the meat. Inoculated meat was immediately dispensed in 5 ± 0.1 g quantities into pre-chilled 15 x 100 mm plastic tubes. While dispensing, the Blendor jar was held at 0 C. Filled tubes were capped with stainless caps and held up to 1 hr at 0 C until heated.

Heating was carried out in covered water baths with tubes immersed to a depth of about 2.5 cm. The air above the water was 1-2 C cooler than the water. After heating, tubes were cooled in an ice-water bath. In order to calculate Fo values, assuming z=18, temperatures were measured at the centre of the meat during heating. At 100 C, Fo=0.1 was obtained after 18.5 min, Fo=0.2 after 31 min, and Fo=0.8 after 106 min.

Heating spores in buffer

Phosphate buffer, M/15, pH 6.2, was heated to the desired temperature in 100 ml amounts, and then nitrite and spores were added. At intervals samples of 10-15 ml were withdrawn to chilled tubes.

Enumeration of spores and vegetative cells

Spores were enumerated after heating, or after various periods of incubation at 30 C. If incubated, the meat or buffer was heated 20 min at 80 C to destroy germinated spores and vegetative cells. When enumeration of all viable units (spores, germinated spores, and vegetative cells) in an incubated preparation was desired, heating for 20 min at 80 C was omitted.

Meat or buffer that contained bacteria was diluted in 10-fold steps with Butterfield's (1932) diluent. The first dilution of meat was carried out by adding 5 g of meat and about 10 g of glass beads to 45 ml of diluent and shaking vigorously to disperse the meat.

Spores in buffer were enumerated in Wynne Agar

(Difco) using triplicate Prickett tubes for each dilution. Spores in meat were enumerated in Wynne Fluid Medium (Difco) by a 3-tube Most Probable Number (MPN) determination (Pivnick and Thacker, 1970). Both media contained 0.1% of filter-sterilized sodium bicarbonate. All data for spores in meat represent the average of MPN determinations on at least 3 (usually 5) separate samples of meat.

Nitrite determination

In this report nitrite and sodium nitrite are used synonymously with all values expressed as ppm of sodium nitrite. Nitrite in buffer was assayed by the AOAC (1960) method and in meat, by the method of Kamm *et al.* (1965). All of the nitrite added to buffer was recovered, even after heating to Fo=0.8. In the meat (50% meat - 50% water), 77-89% of added nitrite was found before heating and 48-66% after heating to Fo=0.8.

Results

The results of heat treatment and incubation are presented separately for each of the 4 cultures. Not all organisms were subjected to the same treatment because of the very great differences in their heat resistance.

C. botulinum strain 62A

The effect of nitrite in meat during heat shock (23 min at 80 C), and during subsequent incubation is shown in Table 1. Nitrite did not influence destruction of spores during heating, nor did it influence the extent of germination during incubation. During 1 week at 30 C, approximately 3 $\log_{10}$ spores germinated; but 1600 ppm of nitrite did not cause more germination than 200 ppm. Meat without nitrite permitted growth and sporulation. Meat with 200 ppm of nitrite did not permit growth.

To test the effect of more prolonged heating at 80 C, spores were heated for 5 hr in buffer without nitrite, and in meat with 0 and 200 ppm of nitrite. Fig. 1 shows that heating in buffer did not destroy spores, but that heating in meat destroyed about 1.3 $\log_{10}$ of spores. However, the presence of 200 ppm of nitrite in meat did not cause more destruction than 0 ppm. Germination during post-heating incubation occurred equally in meat with and without nitrite: almost 2 $\log_{10}$ of spores germinated within 1 hr, and an additional $\log_{10}$ germinated within 1 day. As in the previous experiment (Table 1) meat devoid of nitrite permitted growth and sporulation while meat with 200 ppm of nitrite prevented growth of the germinated spores: 200 ppm of nitrite did not, however, cause death of the germinated spores within 2 days at 30 C. Additional experiments, not detailed in this report, showed that spores of strain 62A, germinated in 20% cooked meat medium, pH 6.0, with 128 ppm of nitrite, died slowly during continued incubation (10 days for 1 $\log_{10}$ reduction).

The effect of nitrite during heating in buffer at 100 C is shown in Table 2. Heating with up to 1600 ppm of nitrite did not destroy more spores than heating without nitrite, and there is little indication that nitrite was more effective than buffer alone in causing spores to germinate during post-heating incubation.

Table 1 Effect of sodium nitrite during heat shock and subsequent incubation on spores of Clostridium botulinum 62A in meat[a]

Sodium nitrite (ppm)	$\log_{10}$ spores per g: After heating[b]	After 1 week at 30C
0	3.70	8.40
200	3.42	0.99
400	3.89	0.99
800	3.99	0.89
1600	3:55	0.77

[a] Meat means 50% meat - 50% water in this and all subsequent tables.
[b] Heated at 80C for 23 min.

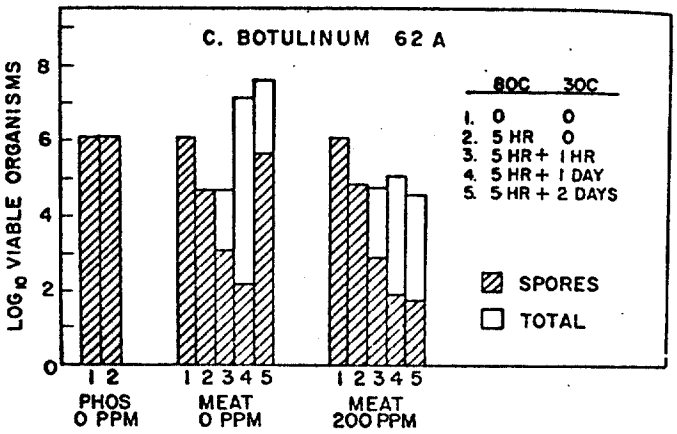

Fig. 1 Effect of nitrite on spores of C. botulinum 62A in meat and buffer during heating and subsequent incubation. Spores are those viable units that were not destroyed when heated at 80C, initially for 5 hr. or, after incubation, for 20 min; "total" includes heat-resistant spores, germinated (heat-sensitive) spores and vegetative cells. Throughout this report "meat" means a mixture of 50% raw pork and 50% water.

C. botulinum strain 13983B

Strain 13983B was heated for 5 hr at 80 C in meat and in buffer. Subsequently, both were incubated at 30 C. Fig. 2 shows that heating in meat caused a small decrease of spores of 13983B compared to 62A (Fig. 1), and this decrease was not influenced by up to 2000 ppm of nitrite. During incubation for 1 day, meat devoid of nitrite permitted germination of more spores than meat with 200 and 2000 ppm. With longer

Table 2 Effect of sodium nitrite on destruction during heating and on germination during subsequent incubation of spores of Clostridium botulinum 62A in phosphate buffer[a].

Sodium nitrite (ppm)	$\log_{10}$ spores per ml: Fo = 0.002 After heating	Fo = 0.002 After 1 week at 30C	Fo = 0.1 After heating	Fo = 0.1 After 1 week at 30C
0	4.69	4.78	2.61	2.50
200	4.75	4.74	2.70	2.54
400	4.78	4.74	2.65	2.47
800	4.77	4.69	2.46	2.23
1600	4.75	4.31	2.50	2.23

[a] Heated in buffer, pH 6.2, at 100C for 15 sec. (Fo = 0.002) and 12.5 min. (Fo = 0.1).
[b] After heating, spores were incubated in the same solution for 1 week at 30C.

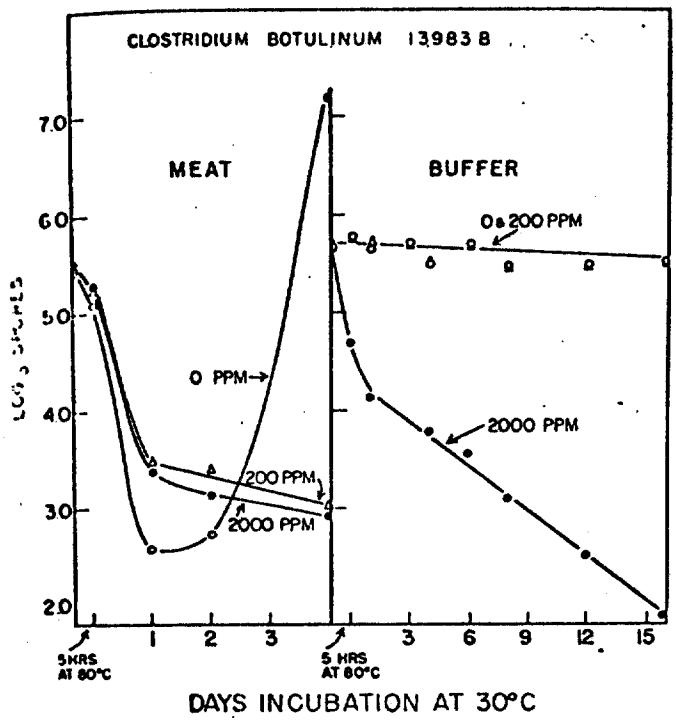

Fig. 2 Effect of nitrite on germination of C. botulinum 13983B heated and incubated in meat and in M/15 phosphate buffer, both at pH 6.2. Heating was at 80C for 5 hr.; incubation was at 30C for time shown.

incubation, the meat devoid of nitrite allowed growth and sporulation while that containing 200 and 2000 ppm of nitrite experienced an almost identical decrease in spores. Fig. 2 shows also that heating spores of 13983B in buffer containing 0 and 200 ppm of nitrite caused no destruction; however, heating with 2000 ppm of nitrite caused destruction of about 1 $\log_{10}$ of spores. Post heating incubation with 200 ppm of nitrite (193 ppm still present after 16 days) did not result in germination of more spores than incubation in buffer without nitrite. Incubation with 2000 ppm of nitrite caused a steady decline in spores between 1 and 16 days.

Strain 13983B was also heated at 100 C in meat and buffer. In meat (Fig. 3), slightly more spores were destroyed by heat when nitrite was present than when it was absent, the amount of destruction depending on the severity of the process and the concentration of nitrite. However, 200 ppm of nitrite, which approximates the concentration used in commerce, caused only a slight increase in destruction, even with a process of Fo=0.4. With 800 ppm of nitrite, a process of Fo=0.4 destroyed about 1.2 $\log_{10}$ of spores more than when nitrite was absent.

Meat heated to Fo=0.1 and Fo=0.4 was incubated 1 week and spores were then enumerated. Table 3 shows the result for meat heated to Fo=0.1. Without nitrite, growth and sporulation occurred, and the spore population increased to $\log_{10} = 8.3$. With nitrite, spore populations decreased to the same extent (2 $\log_{10}$) regardless of the nitrite concentration. Meat heated with nitrite to Fo=0.4 contained 1.6-2 $\log_{10}$ of spores per g after heating, but all of these

Table 3 Decrease in spores of Clostridium botulinum 13983B in meat[a] containing nitrite during post-process incubation.

Sodium nitrite (ppm)	$\log_{10}$ spores per g After Fo = 0.1	After 1 week at 30C
0	4.43	8.30
200	4.18	2.05
400	4.08	1.89
800	4.10	2.00

[a] Meat inoculated with 145,000 spores per g and processed at 100C to Fo = 0.1.

germinated during 1 week of post-heating incubation.

Table 4 shows the effect of nitrite during heating in buffer at 100 C and after 1 week of post-heating incubation at 30 C. Data for heating to Fo=0.002 have been omitted because up to 800 ppm of nitrite did not influence destruction during heating or germination during incubation. During more severe heating (Fo=0.1-0.4) and post-heating incubation, 200 ppm of nitrite had no more effect than 0 ppm. With 400 and 800 ppm, destruction during heating and germination during incubation increased with the increased severity of the heat process.

PA 3679

PA 3679 is so heat resistant (19.3 min at 110 C to destroy 1 $\log_{10}$ of spores; Lund *et al.*, 1957) that no studies were done at 80 C. All heating was carried out at 100 C.

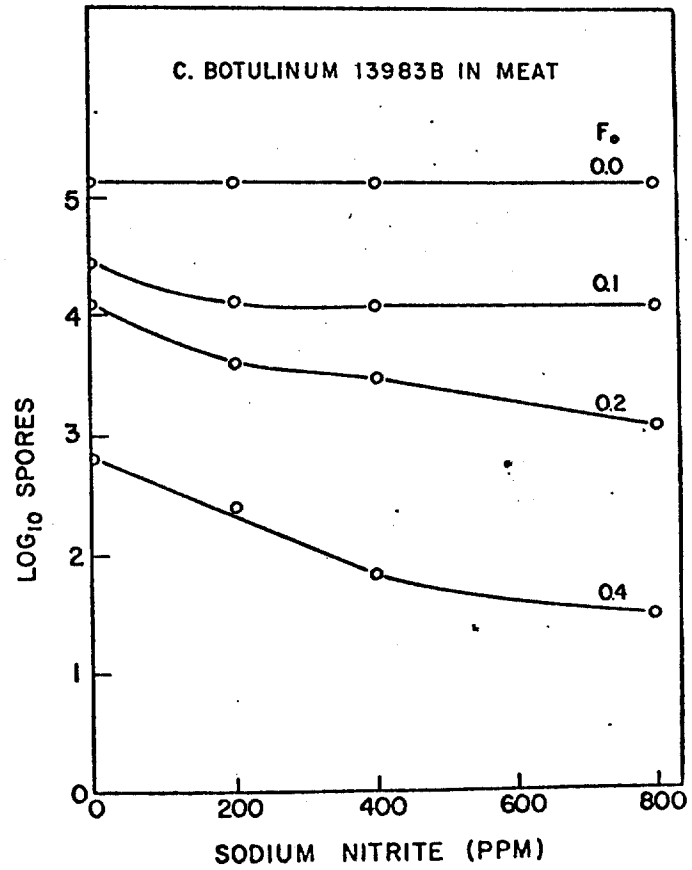

Fig. 3 Effect of nitrite on destruction of C. botulinum 13983B during heating. Spores were added to meat at 0C and the meat was processed at 100C to obtain Fo values indicated.

Table 4 Effect of Sodium nitrite on destruction during heating and on germination during subsequent incubation of spores of Clostridium botulinum 13983B in phosphate buffer[a]

	Log₁₀ spores per ml					
	Fo = 0.1		Fo = 0.2		Fo = 0.4	
Sodium nitrite (ppm)	After heating	After incubation	After heating	After incubation	After heating	After incubation
0	4.62	4.49	4.00	3.86	2.20	1.95
200	4.61	4.58	4.14	3.88	2.11	1.90
400	4.57	4.38	3.70	3.17	0.70	0.00
800	4.58	4.25	3.84	3.28	0.60	<1.00

[a] Spores and nitrites were added to buffer, pH 6.2, preheated to 100C. After heating to Fo = 0.1, 0.2, and 0.4 (12.5, 25 and 50 min, respectively), suspensions were incubated 1 week at 30C.

There was little or no destruction of spores during heating up to Fo=0.8 in buffer or meat containing 0 - 400 ppm of nitrite, and there was little or no germination (≦0.3 $\log_{10}$) in either material during 1 week of incubation with the following exceptions: spores heated to Fo=0.8 with 200 and 400 ppm germinated to the extent of about 0.5 $\log_{10}$ during incubation. The results of heating and incubating spores in buffer and meat with 800 and 1600 ppm of nitrite did, however, show a greater effect in some cases. Table 5 shows that heating with 800 ppm of nitrite to Fo=0.8 caused only slightly greater destruction than heating to Fo=0.1, but that heating with 1600 ppm of nitrite to Fo=0.8 destroyed 2.7 $\log_{10}$ more spores in buffer and 1.2 $\log_{10}$ more spores in meat than heating to Fo=0.1. Germination of spores during incubation tended to increase with increased heat treatment and increased nitrite. However, germination of PA 3679 in meat during incubation was always considerably less than germination of the other cultures studied.

PA 3679h

Preliminary experiments showed that PA 3679h spores were readily destroyed by heating at 100 C. In M/15 phosphate, pH 7.0, a decrease of 1 $\log_{10}$ occurred in 8.7 min. This was in sharp contrast to the heat resistance of spores of PA 3679 which did not decrease in number during 100 min at 100 C.

Because of their modest resistance to heat, spores of PA 3679h were not heated severely. Meat containing spores and nitrite was heated 23 min at 80 C and then incubated. Table 6 shows that heating with up to 1600 ppm of nitrite did not cause more destruction than 0 ppm. Incubation at 30 C for 1 week caused 4 $\log_{10}$ spores to germinate, but the extent of germination was not affected by up to 1600 ppm of nitrite. In meat with 200 ppm of added nitrite (164 ppm still present after heating) there were more spores present after 1 week than with 400 ppm. Separate experiments conducted to explain this difference, found that PA 3679h could grow and sporulate in meat which contained 200 ppm of nitrite before heating.

Table 5 Effect of sodium nitrite on destruction during heating and on germination during subsequent incubation of spores of PA 3679 in phosphate buffer and in meat.

Sodium nitrite (ppm)		Log₁₀ spores per ml or per g in:					
		Buffer			Meat		
	Heated at 100C to Fo =	0.1	0.4	0.8	0.1	0.4	0.8
	After heating	5.85	5.83	5.52	5.98	5.74	5.78
800	After incubation[a]	5.83	5.38	4.88	5.75	5.49	5.28
	After heating	5.80	5.80	3.08	5.56	5.88	4.32
1600	After incubation	5.78	4.61	2.01	4.66	4.66	3.64

[a] After heating, buffer and meat were incubated 1 week at 30C.

Table 6 Effect of sodium nitrite on destruction during heating and on germination during subsequent incubation of spores of PA 3679h in meat.

	Log₁₀ spores per g	
Sodium nitrite (ppm)	After heating[a]	1 week at 30C
0	6.10	7.68
200	6.20	2.67
400	6.25	2.16
800	6.45	1.96
1600	6.07	2.16

[a] Heated 23 min at 80C.

Heating in buffer with 1600 ppm of nitrite for 15 sec at 100 C (Fo=0.002) did not cause more destruction of spores than heating with 0 ppm; and post-heating incubation for 1 week at 30 C caused no germination of spores even with 1600 ppm of nitrite. Table 7 shows that more severe heating (Fo=0.1) also did not cause any more destruction in the presence of 1600 ppm of nitrite than in its absence; but in contrast to spores heated to Fo=0.002, post-heating incubation for 1 and 2 weeks at 30 C did result in germination. However, germination was increased only slightly more by 800 ppm of nitrite than it was by buffer alone. With 1600 ppm of nitrite in the buffer no viable spores were found after 2 weeks.

Experiments comparable to those shown in Table 7 were done to compare nitrite-induced germination at 35 C with germination at 30 C during 1 and 2 weeks of incubation; there was no difference due to temperature of incubation.

Table 7 Effect of sodium nitrite on destruction during heating and on germination during subsequent incubation of spores of PA 3679h in buffer[a, b]

Sodium nitrite (ppm)	Log₁₀ spores and per cent decrease				
	After heating	After 1 week at 30C		After 2 weeks at 30C	
	Log₁₀	Log₁₀	% decrease	Log₁₀	% decrease
0	2.83	1.63	94	0.97	98.6
200	2.83	1.50	95	0.54	99.6
400	2.78	1.34	96	0.67	99.2
800	2.72	1.40	95	0.35	99.7
1600	2.76	0.77	99	<1.00	>99.9

[a] Spores and nitrite were added to pH 6.2 buffer preheated to 100C. After heating 12.5 min (Fo = 0.1), suspensions were incubated at 30C.
[b] 6.6 $\log_{10}$ added per ml.

Discussion

The work reported herein indicated that, at the [illegible] used commercially (about 150 - 300 ppm), nit- [illegible] neither enhanced destruction of bacterial spores [illegible]ing heating nor increased the number of spores [illegible]minating during post-heating incubation. How- [illegible], when used at 2 to 8 times the commercial levels, [illegible]rite did enhance destruction during heating, and [illegible]mination after heating, of some organisms: the [illegible]ount of enhancement increased with increased nit- [illegible] concentration and severity of heating.

The chief value of nitrite in stabilizing cured [illegible]at appears to be its ability to prevent growth from [illegible]minated spores; this is shown in Fig. 1. In the [illegible]ence of nitrite, the germinated spores became active- [illegible] growing vegetative cells; and the vegetative cells [illegible]ulated. In the presence of nitrite, the germinated [illegible] did not divide; eventually they die (Duncan [illegible] Foster, 1968c; unpublished research by the auth- [illegible].

Futher discussion of the effect of nitrite on spores [illegible] be divided into the 4 following areas: (1) destruc- [illegible]n during heating in buffer; (2) destruction during [illegible]ating in meat; (3) germination during incubation [illegible] buffer; and (4) germination during incubation in [illegible]at.

1. *Destruction in buffer.* Enhanced destruction of [illegible]es of PA 3679h during heating in nitrite-contain- [illegible] buffer has been reported by Duncan and Foster [illegible]a). They found that heating at 98 C in buffer [illegible]hout nitrite destroyed 1 $\log_{10}$ of spores in 13 min [illegible] pH 7.0 and 9 min at pH 6.0. With 100 ppm of nitrite, [illegible] of spores was destroyed in about 7 min at each [illegible]H, but increasing the nitrite up to 900 ppm did not [illegible]ase the destruction. In our work with PA 3679h [illegible] did not find that 200 - 1600 ppm of nitrite was [illegible] effective than 0 ppm in enhancing the destruction [illegible] spores heated at 100 C to Fo=0.1 in buffer at pH [illegible]. Similarly, up to 1600 ppm of nitrite failed to en- [illegible]ce destruction of *C. botulinum* strains 62A and [illegible]3B, and PA 3679 during heating to Fo=0.1. More [illegible] heating of 139B3B to Fo=0.4 with 400 ppm of [illegible]rite, and PA 3679 to Fo=0.8 with 1600 ppm of [illegible]rite did cause greater destruction (1 - 2 $\log_{10}$) [illegible] the same heating with less nitrite.

Additional experiments, not detailed in this re- [illegible], showed that 200 ppm of nitrite did not enhance [illegible]ction of spores of *C. botulinum* 62A heated at [illegible] to Fo=0.2 in M/15 buffer at pH 5.7, 6.0, 6.5 [illegible] 7.0.

2. *Destruction in meat.* Heat shock of *C. botulinum* [illegible] and PA3679h at 80 C for 23 min did not destroy [illegible] spores with 1600 ppm of nitrite than with 0 ppm. [illegible]ged heating, 5 hr at 80 C, with 2000 ppm of [illegible] did not destroy more spores of 139B3B than [illegible] same heating without nitrite. More severe heating, [illegible] 100 C, showed that nitrite enhanced destruction [illegible]ly or not at all when the process was Fo=0.1. [illegible]er, when the process was increased to Fo=0.4 [illegible] strain 139B3B in 400 ppm of nitrite and to Fo= [illegible] with PA 3679 in 1600 ppm of nitrite, destruction [illegible] increased about 1 $\log_{10}$ over that obtained in the absence of nitrite.

3. *Germination in buffer.* Nitrite did not enhance germination during post-heating incubation unless the concentrations were high, the heat process was severe, or both. For example, strain 139B3B was not germinated by 200 ppm during 16 days at 30 C, but it was germinated to the extent of 3 $\log_{10}$ with 2000 ppm during the same time. Strain 62A, PA 3679, and PA 3679h showed little evidence of nitrite-induced germination following heating to Fo=0.1 and incubation for 2 weeks in buffer with 800 ppm of nitrite. Most interesting was the germination of 98.6% of PA3679h spores surviving Fo=0.1 (but not those surviving Fo=0.002) during 2 weeks at 30 C in buffer devoid of nitrite. It occurred to us that vegetative cells of PA 3679h, collected with the spores by centrifugation, may have lysed during storage of the spore preparation, and thus provided sufficient nutrients for triggering germination in the more severely heated spores. However, calculations indicated that the inoculum could have provided no more than 10 micrograms of total organic material (spores, vegetative cells, lysate, etc) per ml of buffer. Microscopic examination indicated that most of this was spores.

4. *Germination in meat.* Meat devoid of nitrite provided excellent conditions for germination of spores of the two strains of *C. botulinum*, and for PA 3679h. Preliminary experiments, not reported here, and Fig.'s 1 and 2 showed that maximum germination usually occurred within one day after heating. Nitrite up to 1600 ppm did not result in increased germination of these 3 organisms during 1 week of incubation.

Spores of PA 3679 did not germinate in meat to the same extent as the other 3 organisms. Under severe conditions (800 - 1600 ppm of nitrite and heating to Fo=0.4 or 0.8), however, about 1 $\log_{10}$ of spores germinated. The reasons for failure of PA 3679 to germinate to the same extent as the other 3 organisms is not readily explained. The pH of the meat, 6.2, was considered in view of the optimum of pH 8.5 for L-alanine-induced germination of PA 3679h (Uehara and Frank, 1965). However, in unreported experiments, we heat shocked PA 3679 in meat at pH 6.2-8.3 and incubated the meat at 30 C; pH in this range did not affect germination. Riemann (1957) has reported wide differences in the germination rate of PA 3679; these differences depended on the spore preparation and the medium used for germination.

The rapid germination of spores of *C. botulinum* in meat, and the failure of nitrite at 2 to 4 times the concentration used commercially to increase either the rate or extent of germination mediates against the concept that nitrite influences germination of spores in canned meat under industrial conditions. Reports by Hyatt and Levinson (1961), Black (1964), Bester *et al.*, (1967), and Duncan and Foster (1968b) that nitrite induced germination of spores of aerobic and anaerobic bacteria are based on experiments that used high concentrations (1000 - 150,000 ppm) of nitrite in aqueous solutions. It may be reasoned (although these authors did not do so) that high concentrations of nitrite might cause, in a short period of time, an effect that a low concentration would bring

about in a long period of time: thus, their observations could be pertinent to an explanation of the role of nitrite in the safety of canned meat. However, the rapid decrease in nitrite in canned meat (Nordin, 1969) during storage negates this type of reasoning.

That nitrite is important in stabilizing canned, shelf-stable meats against growth of sporogenic anaerobes is unquestionable (Silliker *et al.*, 1958; Pivnick *et al.*, 1969). Several reports indicate that pH influences the activity of nitrite. Duncan and Foster (1968a) and Perigo and Roberts (1968) have shown in bacteriological media that nitrite is about 10 times more inhibitory to a wide variety of anaerobes at pH 6.0 than it is at pH 7.0. Perigo and Roberts (1968) showed that at pH 6.0, 3 strains of *C. botulinum* type A and 3 of type B were inhibited by 10-160 ppm of nitrite, but 5 of 8 strains of *Clostridium perfringens* were not inhibited by 240 ppm. At pH 7.0, the inhibitory concentrations of nitrite for these organisms were in the range of 640-2560 ppm. Although experimentation with nitrite in bacteriological media is convenient, and does provide some useful information, conclusions should be drawn with caution concerning the relevance of data thus obtained to the role of nitrite in stabilizing canned meat (Johnston *et al.*, 1969). Moreover, too much emphasis should not be placed on the low concentrations of nitrite required to inhibit anaerobes at pH 6.0: many canned hams have a pH 6.5, or even higher, while few contain as much as 160 ppm of nitrite. In fact, we have found that many canned cured meats on the market contain little or no nitrite when purchased although they undoubtedly did at time of processing.

The most plausible explanation for the stabilizing effect of nitrite is that it acts in conjunction with sodium chloride in preventing the growth of clostridia in meat (Riemann, 1963). Pivnick *et al.* (1969) have observed a linear interrelationship between salt and nitrite in preventing the growth of *C. botulinum* in meat, pH 6.1-6.2, processed to Fo=0.6. However, a more comprehensive investigation using meat in the range of pH 6.0-7.0, nitrite at permissible levels, and a series of heat treatments in the range of Fo=0.1-0.6 is required in order to establish limits for safe processing.

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Americans in the preparation of "tortillas." One part of corn is placed in two parts of lime solution (approximately 1% CaO), heated at about 80° for 20 min, and allowed to stand overnight. The corn is ground finely and sold as "masa" in markets (Nicholls, 1961).

This method, similar to the Nigerian way of preparing bean balls ("akara") is worth emulating. First of all, the lime greatly increases the amount of calcium. The increase in iron probably comes from the hot plate. Another method of protein supplementation is by introducing soya or groundnut flour into the ogi. This will boost the protein content tremendously and will not necessarily change the taste. It will be recalled that ogi is the post-weaning diet in Nigeria, and this may be responsible for the malnutrition of the children.

A very cheap way of increasing the protein content of the maize gruel is by adding leaf protein. This could supply as much as 8 g of good-quality protein. However, the Yorubas are accustomed to adding greenish-black concoctions given by native doctors to maize gruel, and there will be a tendency to regard leaf protein as a "medicine" rather than as an item of food. Hence, this procedure has not been pursued.

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Effect of Sodium Nitrite and Temperature on Toxinogenesis by *Clostridium botulinum* in Perishable Cooked Meats Vacuum-Packed in Air-Impermeable Plastic Pouches

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SUMMARY

Sliced processed meats were made with increased concentrations of sodium nitrite and inoculated with spores of *Clostridium botulinum* types A and B. After vacuum-packaging in air-impermeable plastic pouches the meats were stored at 30, 25, or 20°C, and at intervals samples were tested for toxin and spoilage.

At any given temperature, delay in toxinogenesis and spoilage was directly related to the concentration of nitrite. The concentration of nitrite did not influence the acceptability of the product on the first day of incubation that toxin was found.

Apparently heat injury of spores was not necessary for sodium nitrite to inhibit toxinogenesis.

INTRODUCTION

Previous publications (Pivnick and Bird, 1965; Pivnick and Barnett, 1965; Steinke and Foster, 1951; Christiansen and Foster, 1965) have shown that *C. botulinum* types A and B may grow in cured cooked luncheon meats. Toxinogenesis is affected by the size of inoculum, temperature of incubation, pH, and sodium chloride concentration of the product. Toxinogenesis is not affected by packaging because luncheon meats become toxic whether air is allowed to enter the package or is rigidly excluded. However, packaging *in vacuo* in air-impermeable plastic pouches does delay spoilage more than in pouches which allow entry of air.

Although many hundreds of millions of pounds of such products packed in air-impermeable plastic pouches have been consumed, there has only been one reported, non-fatal outbreak of suspected botulism due to eating such luncheon meat (Mather and Thompson, 1965). The material suspected of causing the alleged outbreak was not available for examination and the etiological agent was, therefore, not determined.

Cooked meats, *e.g.*, bologna, ham, liver sausage, *etc.*, are sometimes subjected to abusive storage temperatures both by the distributor and by the consumer. For this reason we have explored methods of making such products more resistant to toxinogenesis by *C. botulinum*. Previous work

(Pivnick and Barnett, 1965) has shown that moderate increases of sodium chloride delayed toxinogenesis types A and B, sometimes to a remarkable extent. Toxinogenesis by type E was prevented by salt concentrations of 2.7% (3.5% in the aqueous phase) of a product which also contained 62 ppm of sodium nitrite.

In further investigations we have studied the effect of increased concentrations of sodium nitrite on toxinogenesis in cooked meats. This report presents some of our results.

MATERIALS AND METHODS

Bacteriological, chemical, and toxin tests, and packaging have been described previously (Pivnick and Bird, 1965). Preparation and inoculation of cooked sliced ham and jellied tongue was identical with the methods of Pivnick and Barnett (1965). Meats were not incubated at temperatures less than 20°C because previous studies (Pivnick and Barnett, 1965) showed that cured meats inoculated with spores of types A and B prior to cooking were unlikely to become toxic at 15°C.

A total of 348 packages of inoculated ham and 230 packages of inoculated jellied pork tongue was examined for odor and toxin. Samples were examined at intervals until the odor of the meat was extremely offensive or until samples were exhausted. Preliminary work with *C. botulinum* types A and B indicated that inoculated meat incubated at 30°C and 20°C might become toxic within 1 day and 10 days respectively. Also, increased nitrite was expected to delay toxinogenesis. For these reasons, sampling was started earlier and conducted more frequently with product which was incubated at 30°C and contained less nitrite. Conversely, as the incubation temperature was decreased and nitrite was increased, sampling was begun later and was less frequent.

At each interval of time and for each variable of nitrite or temperature, packages were examined for odor and toxin. The odor was examined by a panel which usually contained the same 5 people, and the product was scored as acceptable or unacceptable. The percentage of scores considering the product acceptable on the first day toxin was found, and the number of days of incubation when 90% of the scores rated the meat as unacceptable are presented in the tables.

RESULTS

Nitrite is not completely stable during cooking and subsequent storage of the sliced meat in air-impermeable plastic pouches. Although added nitrite was recovered quantitatively from comminuted ham immediately after mixing, Table 1 shows that subsequent holding of the comminuted ham for a few hours at 4°C and cooking to an internal temperature of 70°C resulted in losses of one-third to one-half of the nitrite. Storage of the cooked, sliced ham for one week resulted in even greater losses. Temperature of storage was important, and losses of nitrite were from 2 to 35 times greater at 30°C than at 20°C.

Table 1. Effect of temperature on stability of sodium nitrite in cooked sliced ham.[a]

Sodium nitrite (ppm)			
	Found after cooking[b]	Found after 1 week	
Added		Temp. (C°)	nitrite (ppm)
0	0	30	0
		25	0
		20	0
50	37	30	9
		25	9
		20	23
100	67	30	9
		25	14
		20	48
200	114	30	9
		25	35
		20	111
300	160	30	15
		25	42
		20	130

[a] After cooking the ham contained 3.0% salt and 70% water (4.1% brine).
[b] Cooked in an oven at 76°C until the center was 70°C, then cooled, sliced, and vacuum-packed in air-impermeable plastic pouches.

The nitrite which remained after cooking may have influenced the onset of toxinogenesis. Table 2 shows that at any given temperature of storage, the days of incubation required for toxin to appear were proportional to the nitrite added before cooking. For example, the addition of 0, 50, 100, 200 or 300 ppm of nitrite to ham which was incubated at 25°C after cooking resulted in toxinogenesis after 3, 4, 5, 8, and 25 days respectively. Unfortunately, sufficient samples were not prepared to determine the periods of incubation for toxinogenesis at 20°C at all levels of nitrite. However, it is obvious that the trend obtained at 20°C is similar to that obtained at 25°C.

The possibility of a toxic product being consumed depends on the degree of spoilage of the product. If a product is offensive it will be rejected. This concept can be applied, (a) to the acceptability of a product on the first day it becomes toxic and (b), to the lapse of time after toxinogenesis for spoilage to progress to the stage that would cause most consumers to reject the product. On the first day toxin was found, some of the ham was considered acceptable by all of those who examined it while in one instance (300 ppm of nitrite, 25°C incubation) the ham was rejected by all members of the panel (Table 2). On the average 55% of the scores considered the ham acceptable on the first day that toxin was detected.

How long does it take after toxinogenesis for ham to spoil to the extent that 90% of the scores considered it unacceptable? Do temperature and nitrite influence this relationship? Table 2 shows no trend which would indicate that either temperature or nitrite influenced time necessary for toxic ham to become unacceptable. In two separate experiments no trends

Table 2. Effect of nitrite and temperature on toxinogenesis and acceptability of cooked sliced ham inoculated with *C. botulinum*.[a,b]

Nitrite added (ppm)	Temp. of incubation (C°)	Per cent acceptability on first day meat was toxic	Days to become toxic	Days to become unacceptable[c]
0	30	50	2	3
	25	70	3	6
	20	40	15	18
50	30	60	3	5
	25	100	4	8
	20	10	25	25
100	30	90	3	6
	25	87	5	7
	20	....	>25	>25
200	30	20	8	9
	25	100	8	15
	20	30	30	35
300	30	60	8	14
	25	0	25	22
	20	....	>60	>60

[a] Inoculated before cooking with 10,000 spores per g (5 strains of type A and 5 strains of type B).
[b] Same foot notes as Table 1.
[c] Ninety % or more of scores considered ham unacceptable.

Table 3. Effect of nitrite and temperature on toxinogenesis and acceptability of jellied pork tongue inoculated with *C. botulinum*.[a,b]

Nitrite added (ppm)	After cooking		Temp. (C°)	Percent acceptability on first day meat was toxic	Days to become toxic	Days to become unacceptable
	Nitrite (ppm)	Brine (%)				
			30	90	3	4
0	0	3.8	25	80	4	6
			20	90	15	25
			30	100	6	> 6
75	35	4.3	25	63	10	12
			20	60	30	35
			30	100	5	7
150	60	4.3	25	75	10	13
			20	100	20	35
			30	100	8	10
300	188	4.2	25	100	15	27
			20	100	30	>45
			30	100	10	18
450	396	4.0	25	50	30	33
			20	100	45	>60

[a] Inoculated between slices to obtain 10,000 spores per g (5 strains of type A and 5 strains of type B).
[b] After inoculation 225 g of sliced meat was vacuum-packed in each air-impermeable plastic pouch.

were established which would relate temperature and nitrite concentration to toxinogenesis and acceptability, either on the first day that toxin was found or thereafter.

Table 3 shows the results of an experiment with jellied pork tongue. The methodology in this experiment was identical to that used for ham with the following exception—the minced ham was inoculated by mixing with spores prior to cooking, while the tongue was cooked, sliced, and then inoculated between slices to provide 10,000 spores per g. Both were sealed *in vacuuo* in air-impermeable plastic pouches.

The nitrite concentration decreased during the cooking process (Table 3). The sodium chloride and water content of the 5 lots were very similar, and the slight differences should not influence toxinogenesis.

With minor exceptions, nitrite concentration and temperature influenced toxinogenesis in tongue in a manner similar to toxinogenesis in ham. Increased nitrite and decreased temperatures delayed toxinogenesis. However, the tongue was much more resistant to spoilage than ham. On the first day that toxin was detected, 50 to 100% of the scores for odor rated the tongue acceptable: the average was 87% compared to 55% for ham. In addition the tongue remained acceptable for longer periods after toxinogenesis than did the ham.

This increased duration of acceptability was especially noticeable when tongue made with 300 and 450 ppm of added nitrite was stored at 20°C: when freshly opened packages of this material were examined 15 days after the onset of toxinogenesis, all 5 members of the panel considered them acceptable on the basis of odor. Thus, tongue, if it became toxic due to *C. botulinum*, would more likely be eaten than toxic ham.

DISCUSSION

This study and previous investigations (Pivnick and Bird, 1965; Pivnick and Barnett, 1965) were undertaken to determine if vacuum packaging of semi-perishable luncheon meats in plastic pouches constituted a hazard with respect to botulism. The studies were stimulated by the apparent contributions of such packaging to fatal outbreaks of botulism following ingestion of smoked fish. Our results indicate that luncheon meats do not constitute a hazard equivalent to that obtained from smoked fish. Indeed, they are very safe foods.

Although vacuum packaging does definitely allow toxinogenesis without spoilage, this phenomenon is important mainly to the smoked fish industry: semi-preserved meats are unlikely to cause botulism. There are some well-studied reasons for the difference in potential hazard of the two types of food.

Fresh fish from many sources contains *C. botulinum* type E. This organism grows well at refrigerator temperature, does not produce offensive odors, and is not destroyed by the cold smoking process used for smoking some fish. Although the organism is inhibited by 4–5% of sodium chloride in the aqueous phase (brine %), the salt content of a smoked fish probably varies considerably depending on the portion of the fish that is examined. Also nitrite is not used in fish.

In contrast, *C. botulinum* is probably rare in meat (Greenberg, 1966) although there are exceptions to this idea (Prevot and Brygoo, 1952); types A and B, associated with meat borne outbreaks, do not grow at refrigerator temperature; the heat processing of luncheon meats would destroy vegetative cells of *C. botulinum* if they existed; and many luncheon meats are prepared from comminuted products with even distribution of sodium chloride. Also, luncheon meats contain sodium nitrite.

Steinke and Foster (1951) showed that nitrite was important in delaying toxinogenesis by *C. botulinum* in liver sausage and our results with other products confirm their observation. The mechanism of inhibition by nitrite is unknown, but Shank *et al.* (1962) believe that conversion to nitrous acid is necessary. Heat injury of spores in the presence of nitrite may be important in the stability of canned luncheon meats which are normally processed to Fo of 0.1 or more, and may be of some importance in semi-perishable luncheon meats. However, heat injury is not necessary for inhibition of toxinogenesis: we inoculated jellied tongue after heat processing and obtained inhibition by nitrite. The specific role of nitrite as an inhibitor of toxinogenesis remains unknown.

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EFFECTS OF NITRATES AND NITRITES ON FOOD ASSIMILATION (Vliyaniye nitratov i nitritov na usvoyayemost' pishchevykh veshchestv)

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In recent years in the Soviet Union and abroad, studies appeared, shedding light on the toxicological significance of drinking water.

Foreign authors describe cases of methemoglobinemia involving individual lethal results in infants at different stages of babyhood, who had been fed with mixtures prepared with well-water containing from 50 to 300 mg/l of nitrates (Keleti and Kamensky, Thal and Co-authors).

According to Soviet scientists, methemoglobinemia, without any other marked evidences of disease, is observed also in nursery children, preschool and school children, after they had used well-water with a high content of nitrates (F. N. Subbotin, Sh. Kh. Kapanadze).

The material mentioned in this article consists of a fragment of study about the possible nocive effects of nitrates and nitrites when these are used in manufacturing food products, and when they are present in potable water. ~~We intend in this article to elucidate their effects on food assimilation (absorption).~~

In the accessible literature, we have been unable to find anything on this subject.

As a basis for the experimental part we used the procedure of food assimilation study, utilized at the Food Institute (Moscow) and described by D.I. Romashov. The experimentation was performed on 10 adult male rats of a weight ranging from 210 to 230 g.

Before experimentation, the animals were placed for 7 days under the test situation so that they might become accustomed to the ration and upkeep conditions.

The experimental work was conducted on the same rats as follows: 1st control period, experimental and 2nd control periods; duration of each period: 7 days.

Between the experimental and the 2nd control periods, the animals were transferred for 5 days to their usual cages (for rest and possible liberation from remnants of introduced nitrates and nitrites).

The animals were fed with dry briquetted food concentrate and dry carrot, previously comminuted, carfully mixed, moistened with water and then rolled into pellets in order to lessen the food loss. The animals received the entire daily food amount in the morning after defecation (? "liberation" = "posle osvobozhdeniya") and following a careful cleaning of the cages from any feces and food remnants from the preceding day.

In order to separate from one another the periods, we added carmine (0.04 g) to the feed given to the animals the day before the first day of each period and on the day following its termination. In this way, the uncolored feces, limited on both sides by "stoppers" (portions of colored feces), corresponded to the eaten food of the given period. Only uncolored fecal materials were collected and investigated. The portions of carmine-colored feces were not studied, and were entirely disposed of.

During the experimental period, we added 4.4 mg of K nitrate (from a calculation of 20 mg/kg) and 0.33 mg of K nitrite (1.5 mg/kg) to the food in 24-hour periods. During the entire test period, the animals received altogether 30.8 mg of K nitrate and 2.31 mg K nitrite.

The doses of nitrates and nitrites were selected on the basis of their maximum possible amount introducible into the organism of a human adult with well-water and a daily normal amount of meat, fish and milk products in which nitrates and nitrites were used.

From these premises it follow that as a whole with the ration and water collected, man can receive 399 mg of nitrates and 30 mg of nitrites in 24-hour periods, or 6.65 and 0.5 mg for 1 kg of human body weight (average man's weight: 60 kg).

A triple aggravation was assumed and daily doses of nitrates of 20 mg/kg, and of nitrites of 1.5 mg/kg were arranged in the experimentation.

For the purpose of calculating the uneaten food and the feces produced, the animals were placed in separate cages of special construction, described in the work of F.E. Budagyan and L.I. Smirnova. The cage arrangement enables the remnants of food and feces to be collected separately and prevents coprophagy.

During the experimental period and the two control periods, feces and food remnants of each rat were daily collected and slightly dried on a water bath until air-dry conditions were obtained, in order to prevent disintegration (? decomposition ?, putrefaction ?).

Food remnants, collected during one week, were dried up to constant weight; the amount of actually eaten food and of fats, albumins, ashes and C was calculated on the basis of the absolutely dry material.

The feces of the entire 7 days period were comminuted, and dried up to constant weight and were subjected to a determination of their contents in fat by the Sokclet method (5 g of weighed sample), common N by the Keldahl (?) method (0.9 g of weighed sample), ash (0.9 g); the carbohydrates were determined on the basis of the difference between the dry material and the sum of fats, albumins and ash.

One calculated also the amount of unassimilated food produced with the feces during the entire period (control or experimental periods) on the basis of the absolutely dry material. Then one determined the amount of assimilated food during each period in per cents.

The same methods were used in determining the moisture and food content, in a preliminary process, in the dry food concentrate and in dry carrot.

The materials, obtained as a result of the test, were subjected to a statistic calculation by the method of I.A. Oyvin. The probability level $P < 0.05$ was selected; one considered as reliable the difference of the arithmetic means,

the substantiality index of which (t) corresponds to $P < 0.05$. The amounts of unassimilated dry food in the 1st control period, in the experimental period and in the 2nd control period averaged respectively to 15.47 17.32 and 17.11%.

The statistic treatment showed that the decrease of the assimilation of the dry materials in the experimental period as compared with the first control period was reliable (substantiality index = 3.28). The difference between the assimilation in the 1st and 2nd control periods was reliable also (t = 5.52). The assimilation of dry materials in the 2nd control period did not essentially differ from the level of assimilati n in the experimental period (t = 0.49).

The amount of unassimilated fat in the 1st control period, in the experimental period and in the 2nd control period were 11.93 24.12 and 8.37%.

The assimilation of fat under the effect of nitrates and nitrites decreased in the experimental period as compared with the 1st control period by 12.19%. The reliability of this change is confirmed statistically (t = 8.38). In the second control period the fat assimilation was higher than in the experimental period by 15.75% and somewhat raised the assimilation level in the 1st control period.

The difference between the fat assimilation in the 2nd control period and the experimental period, in the 1st and 2nd control periods was confirmed statistically (substantiality indices 10 48 and 4.69).

The amount of unassimilated albumin of the fool in the 1st control period, in the experimental period and in the 2nd control period was 20.11 22.28 21.13%. The decrease of the assimilation of albumin in the experimental period as compared with the 1st control period by 2.17% was statistically confirmed (t = 3.38). The difference between the assimilation of albumin in the 1st and in the 2nd control periods (1.02%) during statistic treatment was not reliable (substantiality index 1.52). There was some increase of the albumin assimilation (1.15%) in the 2nd control period as compare with the experimental period, but this increase was insignificant (t = 2.10).

The average amounts of unassimilated ash elements in the 1st control, experimental and 2nd control periods were 58.03 63.32 and 66.27%.

The decrease of assimilation of the ash elements in the experimental period as compared with the 1st control period was 5.29%.

This change in the assimilation was statistically confirmed (t = 2.64). In the and control period the assimilation of the ash elements was lower than the assimilation in the experimental period by 2.95%, which is unreliable statistically (t = 2.06). The difference between the assimilation of ash elements in the 1st and in the 2nd control periods (8.24%) was statistically reliable (t = 3.53).

The amount of unassimilated hydrocarbons (by difference) in the 1st control period, in the experimental period and in the 2nd control period was equal to 9.94 10.51 and 1.42%, that is the hydrocarbon assimilation was practically the same in all the test periods. This conclusion is confirmed also by the statistically treatment of the data obtained: the substantiality index of the difference (0.57%) of assimilation of the hydrocarbons in the experimental period and in the 1st control period is equal to 1.10; in comparing the experimental period and the 2nd control period (difference 0.91%) one finds t = 109, and a comparison of the assimilation of the 1st and 2nd periods (difference 1.48%) gives a reliability limit of 2.71.

The external appearance and the behavior of the animals in all the test periods were usual, without any particular features. During testing all the animals increased in weight from 30 to 50 g.

The tests showed the influence of nitrates and nitrites on the assimilation of some food. Let us note the extreme decrease of the assimilation of fats, the somewhat lesser decrease of albumins and ash elements; the assimilation of hydrocarbons did not change practically. It is not excluded that this change for some food is connected with the action of the nitrates and nitrites on the disgestive ferments.

The level of assimilati n of food after stopping the ingestion of nitrates and nitrites into the organism is ununiformly restored: the assimilation of fats

and albumins was restored entirely in the 2nd control period; the assimilation of ash elements in the 2nd control period decreased still more than in the experimental period, and reached a lowest value.

Attention is drawn to the incomplete restoration of the assimilation of ash elements during our experimentation. In the 2nd control period the action of the nitrates and nitrites was still felt irrespective of the 5 day gap between it and the previous experimental period. The caused involveld in this case is not quite clear to us.

In connection with the test results obtained it is advisable to study the possible action of the nitrates and nitrites on some disgestive ferments such as lipase, protease and others.

МИНИСТЕРСТВО ЗДРАВООХРАНЕНИЯ СОЮЗА ССР

ГИГИЕНА И САНИТАРИЯ

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МАРТ

ИЗДАТЕЛЬСТВО «МЕДИЦИНА»
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substance was introduced intratracheally in an amount of 50 mg in 0.6 ml physiological saline. The test lasted 12 months. A definite increase of the lung weight coefficient was produced by all the three substances but it proved highest in the case of SiO_2.

Morphological lesions that developed in the lungs were of different character: those caused by Si were the least significant. Si and Si_3N_4 especially affected the bronchial tree but the lesions produced by Si_3N_4 were far more pronounced. Certain shifts were noted in the blood serum protein components. Their extent increased consecutively from Si to Si_3N_4 and to SiO_2. It may be supposed that the maximal permissible concentrations of Si and Si_3N_4 in the air could be slightly higher than those of SiO_2.

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ВЛИЯНИЕ НИТРАТОВ И НИТРИТОВ НА УСВОЯЕМОСТЬ ПИЩЕВЫХ ВЕЩЕСТВ

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В отечественной и зарубежной литературе за последние годы появились работы, освещающие токсикологическое значение нитратов питьевой воды.

Зарубежные авторы описывают случаи метгемоглобинемии с отдельными летальными исходами у детей раннего грудного возраста, получающих питательные смеси, приготовленные на колодезной воде с содержанием нитратов от 50 до 300 *мг/л* (Келети и Каменски, Thal и соавторы).

По сообщениям отечественных ученых, метгемоглобинемия без других выраженных симптомов болезни наблюдается также у детей ясельного, дошкольного и школьного возраста после употребления ими колодезной воды с повышенным содержанием нитратов (Ф. Н. Субботин, Ш. Х. Капанадзе).

Сообщаемые в данной статье материалы являются фрагментом работы по изучению вопроса о возможном вредном влиянии нитратов и нитритов в связи с их применением в производстве пищевых продуктов и наличием в питьевой воде. В этой работе мы поставили целью выяснить их влияние на усвояемость (всасываемость) пищевых веществ.

В доступной литературе каких-либо материалов по этому вопросу нам найти не удалось.

При постановке эксперимента за основу была принята методика изучения усвояемости пищевых веществ, принятая в Институте питания (Москва) и описанная Д. И. Ромашовым. Эксперимент проводили на 10 взрослых крысах-самцах весом 210—230 *г*.

Перед экспериментом животные были поставлены на 7 дней в условия опыта для привыкания к рациону и режиму содержания.

Экспериментальная работа была проведена на одних и тех же крысах по следующей схеме: 1-й контрольный период, опытный и 2-й контрольный периоды; продолжительность каждого периода 7 дней.

Между опытным и 2-м контрольным периодами животных пересаживали на 5 дней в обычные клетки (для отдыха и возможного освобождения от остатков введенных нитратов и нитритов).

Животным скармливали стандартный брикетированный сухой кормовой концентрат и сухую морковь, предварительно измельченные, тщательно перемешанные, смоченные водой и скатанные затем в шарики для того, чтобы уменьшить потери корма. Всю суточную норму корма животные получали утром после освобождения и тщательной очистки клеток от кала и остатков пищи за предыдущий день.

Для отделения периодов друг от друга в корм животным накануне 1-го дня каждого периода и на следующий день после его окончания добавляли кармин (по 0,04 *г*). Таким образом, неокрашенный кал, ограниченный с двух сторон «пробками» (порциями окрашенного кала), соответствовал съеденному корму данного периода. Собирали и исследовали лишь неокрашенные каловые массы. Порции окрашенного кармином кала не исследовали и целиком отбрасывали.

В опытный период к корму добавляли по 4,4 *мг* нитрата калия (из расчета 20 *мг/кг*) и 0,33 *мг* нитрита калия (1,5 *мг/кг*) в сутки. Всего за время опыта животные получили по 30,8 *мг* нитрата калия и 2,31 *мг* нитрита калия.

Дозы нитратов и нитритов были выбраны исходя из максимально возможного их количества, которое может быть введено в организм взрослого человека с колодезной водой и суточной нормой мясных, рыбных и молочных продуктов, изготовляемых с применением нитратов и нитритов.

Из этих предпосылок следует, что в целом с подобранным рационом и водой человек может получить за сутки 399 *мг* нитратов и 30 *мг* нитритов или по расчету на 1 *кг* веса тела человека — соответственно 6,65 и 0,5 *мг* (считается, что средний вес человека 60 *кг*).

В опыте была принята агравация в 3 раза и установлены суточные дозы нитратов 20 *мг/кг* и нитритов 1,5 *мг/кг*.

Для учета количества несъеденного корма и выделенного кала животных поместили в отдельные клетки специальной конструкции, описанные в работе Ф. Е. Будагяна и Л. И. Смирновой. Устройство клеток позволяет раздельно собирать остатки корма и кал, а также предупреждает копрофагию.

В течение опытного и 2 контрольных периодов кал и остатки корма от каждой крысы ежедневно собирали и подсушивали на водяной бане до воздушно-сухого состояния с целью предупреждения разложения.

Собранные за неделю остатки корма высушивали до постоянного веса, рассчитывали количество фактически съеденного корма и содержащихся в нем жиров, белков, золы и углеводов в пересчете на абсолютно сухое вещество.

Кал за весь 7-дневный период измельчали, высушивали до постоянного веса и в нем определяли жир по Сокслету (из навески 5 *г*), общий азот по методу Кьельдаля (из 0,9 *г*), зольность (из 0,9 *г*); углеводы определяли по разности между сухим веществом и суммой жиров, белков и золы.

Далее рассчитывали количества выделенных с калом неусвоенных пищевых веществ за весь период (контрольный или опытный) в пересчете на абсолютно сухое вещество. Затем определяли количество усвоенных пищевых веществ за каждый период в процентах.

Сухой кормовой концентрат и сухую морковь предварительно исследовали на содержание влаги и пищевых веществ теми же методами.

Материалы, полученные в результате опыта, были подвергнуты статистической обработке по методу И. А. Ойвина. В данной работе избран уровень вероятности $P<0{,}05$; достоверной считается разность средних арифметических, показатель существенности которой (t) соответствует $P<0{,}05$. Неусвоенные сухие вещества корма в 1-м контрольном, опытном и 2-м контрольном периодах составили в среднем соответственно 15,47, 17,32, 17,11%.

Статистическая обработка показала, что уменьшение усвояемости сухих веществ в опытном периоде по сравнению с 1-м контрольным достоверно (показатель существенности равен 3,28). Разница между усвояемостью в 1-м и 2-м контрольных периодах также достоверна

($t = 5{,}52$). Усвояемость сухих веществ во 2-м контрольном периоде существенно не отличается от уровня ее в опытном периоде ($t = 0{,}49$).

Количество неусвоенного жира в 1-м контрольном, опытном и 2-м контрольном периодах составляло 11,93, 24,12, 8,37%.

Усвояемость жира под действием нитратов и нитритов снизилась в опытном периоде по сравнению с 1-м контрольным на 12,19%. Достоверность этого изменения подтверждается статистически ($t = 8{,}38$). Во 2-м контрольном периоде усвояемость жира была выше, чем в опытном, на 15,75% и несколько превысила уровень усвояемости в 1-м контрольном периоде.

Разница между усвояемостью жира во 2-м контрольном и опытном периодах, в 1-м и 2-м контрольных периодах статистически достоверна (показатели существенности 10, 48 и 4,69).

Количество неусвоенного белка корма в 1-м контрольном, опытном и 2-м контрольном периодах: 20,11, 22,28, 21,13%. Уменьшение усвояемости белка в опытном периоде по сравнению с 1-м контрольным на 2,17% статистически достоверно ($t=3{,}38$). Разница между усвояемостью белка в 1-м и 2-м контрольных периодах (1,02%) при статистической обработке оказалась недостоверной (показатель существенности 1,52). Некоторое повышение усвояемости белка (1,15%) во 2-м контрольном периоде по сравнению с опытным несущественно ($t = 2{,}10$).

Количество неусвоенных зольных элементов в 1-м контрольном, опытном и 2-м контрольном периодах составило в среднем 58,03, 63,32, 66,27%.

Снижение усвояемости зольных элементов в опытном периоде по сравнению с 1-м контрольным составило 5,29%.

Это изменение усвояемости статистически достоверно ($t = 2{,}64$). Во 2-м контрольном периоде усвояемость зольных элементов была ниже уровня усвояемости в опытном периоде на 2,95%, что является статистически недостоверным ($t = 2{,}06$). Разница между усвояемостью зольных элементов в 1-м и 2-м контрольных периодах (8,24%) статистически достоверна ($t = 3{,}53$).

Количество неусвоенных углеводов (по разности) в 1-м контрольном, опытном и 2-м контрольном периодах было равно 9,94, 10,51, 11,42%, т. е. усвояемость углеводов была практически одинаковой во всех периодах эксперимента. Этот вывод подтверждается и статистической обработкой полученных данных: показатель существенности разницы (0,57%) усвояемости углеводов в опытном и 1-м контрольном периодах равен 1,10; при сравнении опытного и 2-го контрольного периодов (разница 0,91%) $t = 1{,}09$ и сравнение усвояемости 1-го и 2-го контрольного периодов (разница 1,48%) дает предел достоверности 2,71.

Внешний вид и поведение животных были во все периоды эксперимента обычными, без каких-либо особенностей. За время опыта все животные прибавили в весе от 30 до 50 *г*.

Эксперименты на животных показали влияние нитратов и нитритов на усвояемость некоторых пищевых веществ. Отмечено наибольшее снижение усвояемости жиров, несколько меньшее белков и зольных элементов; усвояемость углеводов практически не изменилась. Не исключено, что это изменение для некоторых пищевых веществ связано с действием нитратов и нитритов на пищеварительные ферменты.

Уровень усвояемости пищевых веществ после прекращения введения в организм нитратов и нитритов восстанавливался неравномерно: усвояемость жиров и белков полностью восстановилась во 2-м контрольном периоде; усвояемость зольных элементов во 2-м контрольном периоде снизилась еще больше, чем в опытном, и достигла минимального значения.

Обращает внимание неполное восстановление усвояемости зольных элементов в течение срока нашего эксперимента. Во 2-м контрольном

периоде действие нитратов и нитритов еще проявлялось, несмотря на 5-дневный разрыв между ним и предшествующим опытным периодом. Причина этого нам не совсем ясна.

В связи с полученными результатами опыта целесообразно изучить возможное действие нитратов и нитритов на некоторые пищеварительные ферменты, как-то: липазу, протеазу и др.

Выводы

1. Наибольшее влияние нитраты и нитриты оказывают на усвояемость жиров, несколько меньшее — на усвояемость белков и зольных элементов.

2. Не выявлено влияния нитратов и нитритов на усвояемость углеводов.

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THE EFFECT OF NITRATES AND NITRITES ON THE FOOD ASSIMILATION

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With referrence to the published data concerning instances of methemoglobinemia among children, receiving food prepared in water containing considerable amounts of nitrates (50 and more mg/l of water), the present work was undertaken with a view to clarifying any possible pathological effect of nitrates and nitrites on the digestive tract functions.

Experiments performed on the male rats weighing 210 to 230 g, showed that a daily addition of both potassium nitrate (20 mg/kg body weight) and potassium nitrite (1.5 mg/kg body weight) food ration for a period of seven days decreased the fat assimilation by 12 to 15% and that of protein — by 1.15 to 2.17%, as compared with the two control periods before and after the experiment. The carbohydrate assimilation remained unaffected.

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ВЛИЯНИЕ ЗИМНЕГО ПЛАВАНИЯ В ОТКРЫТОМ БАССЕЙНЕ НА ЗАКАЛИВАНИЕ ДЕТЕЙ МЛАДШЕГО ШКОЛЬНОГО ВОЗРАСТА

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В общем комплексе средств закаливания наиболее эффективны водные процедуры, особенно купание. Но нередко возможности его проведения ограничены (отсутствие природного водоема, время года, суровые климатические условия и т. п.). В связи с этим повышается значение закрытых и открытых искусственных купально-плавательных бассейнов.

В последнее время в Москве получило широкое распространение купание детей школьного возраста в открытых плавательных бассейнах в течение круглого года. Однако научно обоснованные рекомендации

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CAROTENE DEGRADATION

Reaction of Carotene with Nitrite Solutions

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Nitrite solutions of low pH, which would include any chemical changes in nitrite resulting from acidic conditions, are shown to cause destruction of carotene. Vitamin A alcohol is also readily destroyed, while β-apo-8′- and β-apo-12′-carotenal are more resistant to attack by these solutions. Since carotene is a major source of vitamin A for cattle, it may be destroyed by nitrite before absorption and conversion can take place. The recent apparent increase in vitamin A requirement for cattle fed forages high in nitrate may conceivably be explained on this basis.

In 1945, Wilson (*9*) demonstrated the disappearance of the yellow color from carotene solutions when they were shaken with nitrous acid. He had earlier reviewed the literature relating to the occurence of nitrite or nitrous acid in nature (*8*). He also determined the nitrate content of the expressed sap of a number of genera and species of plants, finding amounts ranging from a trace to 10,000 p.p.m. (*10*). Flynn *et al.* (*3*) found high levels of nitrate in corn plants, particularly after nitrogen fertilization and drouth conditions.

Using artificial rumens, Barnett and Bowman (*1*) have shown that nitrate can be reduced to nitrite by rumen liquor. Lewis (*6*) found nitrite accumulation in vivo under certain conditions of nitrate feeding. Case (*2*) has discussed the deleterious effects of both of these substances on animals, and investigation by O'Dell *et al.* (*7*) showed that dietary nitrite enhanced vitamin A deficiency in rats and reduced vitamin A storage when carotene was fed. Garner (*4*) reported an increase in the vitamin A requirement of cattle when nitrate-containing rations were being fed.

The following in vitro experiments were run to investigate further the effect of both nitrite and hydrogen ion concentration on the destruction of carotene, since nitrate has been found nondestructive at pH 1 to 7 by this laboratory.

Procedure

A Latin square experimental design was used in these experiments. The variables involved were the molar ratio of nitrite to carotenoid pigment and the pH of the solution. In the first series of experiments, nitrite to carotene ratios were varied from 1:1 to 100:1 and were used second series of experiments, β-carotene, β-apo-8′-carotenal, β-apo-12′-carotenal, and vitamin A alcohol were subjected to similar conditions.

Test tubes measuring 13 × 100 mm. were used in these experiments. The total volume per tube was 7 ml.—2 ml. of stock carotenoid solution, 2 ml. of the appropriate sulfuric acid solution, and 3 ml. of an appropriate dilution of the stock KNO_2 solution.

The reagents were mixed at room temperature, then incubated 4 hours at 37° C. The absorbance was determined by the use of a Beckman Model DU spectrophotometer. The blank was composed of the original reagent mixture with double-distilled water replacing the KNO_2 portion of the solution. β-carotene, β-apo-8′-carotenal, and β-apo-12′-carotenal were read at 450 mμ while vitamin A alcohol was read at 325 mμ.

Stock Solutions. Potassium nitrite stock solution was prepared to contain 126.48 μg. of KNO_2 per ml. After drying overnight, 7.925 grams of KNO_2 were weighed into a 500-ml. volumetric flask, and made to volume with double-distilled water. An aliquot of 7.98 ml. of this solution was diluted to 1 liter to give the final desired concentration (126.48 μg. per ml. or 148.8 × 10^{-6} moles per ml.).

The carotenoid (or vitamin A alcohol) stock solutions were prepared as an aqueous dispersion with 1% Tween 80 (Atlas Powder Co., Wilmington, Del.). Crystalline β-carotene (Nutritional Biochemicals Corp.), β-apo-8′-carotenal, β-apo-12′-carotenal (Hoffmann-La Roche, Inc.), and vitamin A alcohol (Distillation Products Industries) were used. Each solution was prepared to contain a final concentration of 3.72 × 10^{-5} moles of dissolved reactant per ml. The crystal- mg. of β-apo-8′-carotenal, 3.2 mg. of β-apo-12′-carotenal, 2.1 mg. of vitamin A alcohol) was dissolved in approximately 40 ml. of absolute ethanol by warming. The ethanolic solution was added slowly, with mixing, to 100 ml. of warm 1% Tween 80 solution. Additional 1% Tween 80 solution was used to bring the solution to volume in a 200 ml. volumetric flask.

A series of sulfuric acid solutions was prepared, ranging in pH from 0.5 to 5.5. Fresh, double-distilled water was used for the solution of pH 7. The pH of the sulfuric acid solutions was checked with a pH meter and adjusted when necessary. When diluted to the final concentration in the reaction, these solutions provided a calculated pH of 1 through 7.

Results and Discussion

Figure 1 shows the results of triplicate experiments on the destruction of β-carotene by nitrite. The pH of the solution was not a factor in the destruction of carotene in the control series, while destruction by nitrite depended markedly on the pH of the system. The amount of destruction at pH 5 to 7 at any given nitrite to carotene ratio was much less than at lower pH values. The destruction of carotene was greatest at pH 1 to 2 with a tendency toward a plateau at pH 2 to 3. At high nitrite to carotene ratios the amount of destruction between pH 2 and 3 tended to equal that at pH 1. An intermediate amount of destruction occurred at pH 4.

Previous work (*5*) on degradation of carotenoids would indicate that a possible pathway of degradation is through the apo-carotenals. Figure 2 shows the results of duplicate experiments comparing the destruction

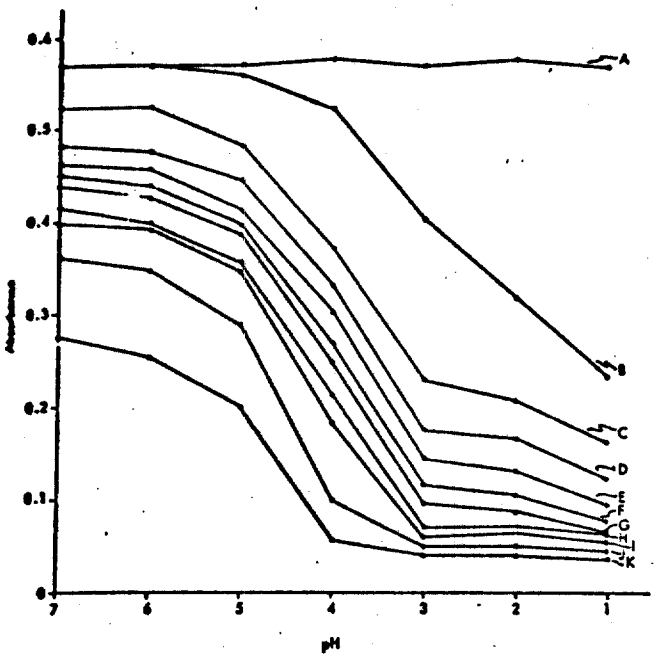

Figure 1. Destruction of β-carotene (4 hours at 37° C.) as a function of pH and nitrite concentration

Molar nitrite to carotene ratios: A-control, B-1:1, C-4:1, D-6:1, E-8:1, F-10:1, G-12:1, H-16:1, I-20:1, J-40:1, and K-100:1

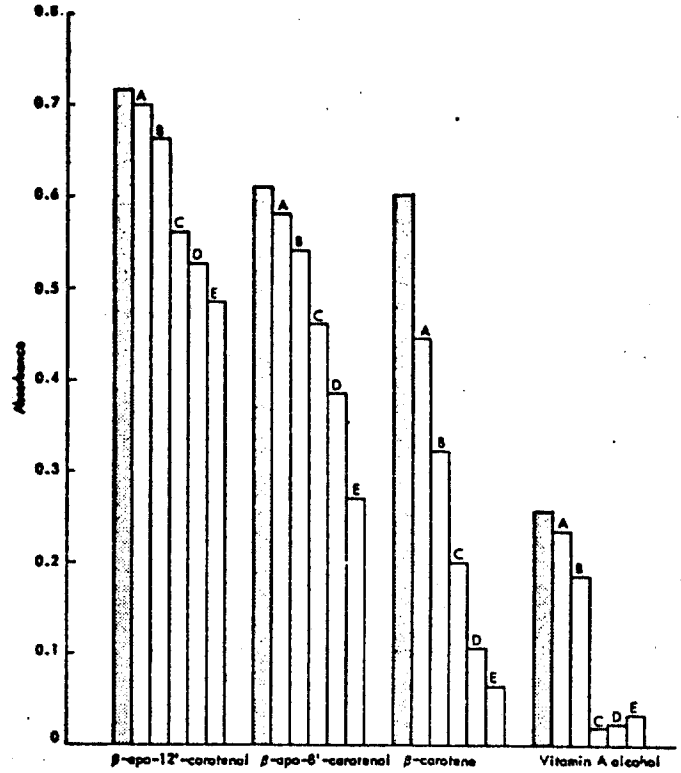

Figure 2. Comparison of the destruction of three provitamin A compounds and vitamin A alcohol by nitrite at pH 2

Molar nitrite to carotenoid ratios: solid bar-control, A-0.5:1, B-1:1, C-4:1, D-10:1, and E-20:1

12′-carotenal, and vitamin A alcohol. This figure compares results at pH 2 only. The two carotenals are more stable in the presence of nitrite than is β-carotene, β-apo-12′-carotenal being more stable than β-apo-8′-carotenal. Vitamin A alcohol was more susceptible to degradation by nitrite than the other three substances.

In silage, a pH of 4 is normal. Any nitrite formed at this pH would readily destroy carotene because of an extended reaction period and elevated temperatures. In the true stomach of animals (pH 2 to 3), the presence of nitrate along with dietary carotene could lead to a low vitamin A status. These data, although collected using pure compounds and relatively simple reaction mixtures, suggest a possible reason for the apparent increase in vitamin A requirement of cattle fed forages containing a high level of nitrate.

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Formation and Stability of Nitric Oxide Myoglobin

I. Studies with Model Systems

SUMMARY—The rate and extent of the formation of nitric oxide myoglobin (NOMb) were studied in aqueous model solutions containing 1–1.5 mg Mb per ml, to which various substances were added to study their effect. Conversion of Mb to NOMb was optimum when 1 mol sodium nitrite per mol Mb was present, provided that the solution contained optimum sodium ascorbate (200 mols). Results were the same with sodium erythorbate, ascorbic acid, and erythorbic acid.

Cysteine also furthers the formation of NOMb; its optimum concentration was 100–200 mol per mol Mb, but even then the NOMb formation was appreciably lower than that found with ascorbate. The optimum nitrite concentration in the presence of cysteine was not less than 3 mols nitrite per mol Mb; this could be attributed to the chemical side reactions between nitrite and cysteine. Glutathion showed very little effect on the reaction between nitrite and Mb. The color intensity of NOMb was less in sodium chloride solution than in water. Sodium chloride also had an adverse effect on the formation of NOMb, but sodium nitrate and several sodium polyphosphates had no effect.

Ferrous sulfate, on the other hand, accelerated the formation and produced a higher final concentration of nitric oxide myoglobin. Higher temperature and lower pH during the formation of NOMb both markedly accelerated its velocity, but the optimum quantities of NOMb formed were about the same. Investigations of the stability of NOMb in model solutions on exposure to light while in contact with air showed that the ratio between the concentrations of Mb, nitrite, and ascorbate and pH are the decisive factors, and that cysteine, glutathion, potassium nitrate, and polyphosphates had no effect in this respect.

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INTRODUCTION

THE COLOR OF RAW PIG MUSCLE tissue is due to the dark red pigment, myoglobin (Mb); the cherry red pigment oxymyoglobin (O_2Mb); and the brown pigment, metmyoglobin (MMb). It is well known that these pigments are not stable when the muscle tissue is heated. To obtain a more stable red pigment in heated commercial meat products, nitrite is added before heating, resulting in the formation of the bright-red pigment nitric oxide myoglobin (NOMb). When, thereupon, the meat product is heated the protein part of NOMb is denatured and a rather stable red pigment is formed, named nitric oxide myochromogen (DNOMb) or nitric oxide pigment. The light absorption curves of these pigments are given in Figs. 1 and 2.

Myoglobin, though closely related to hemoglobin (Hb), differs from it in many respects so that it is not admissible to apply to Mb the observations made concerning Hb

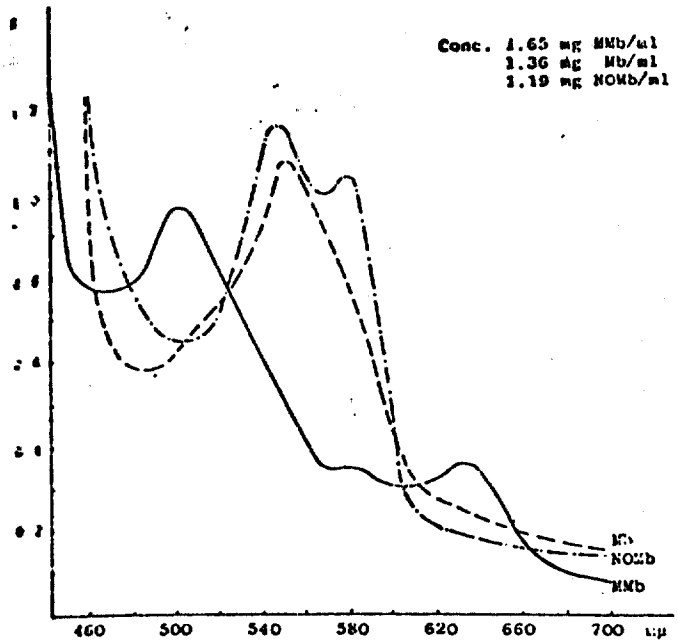

Fig. 1. Absorption spectra of Mb, MMb, and NOMb.

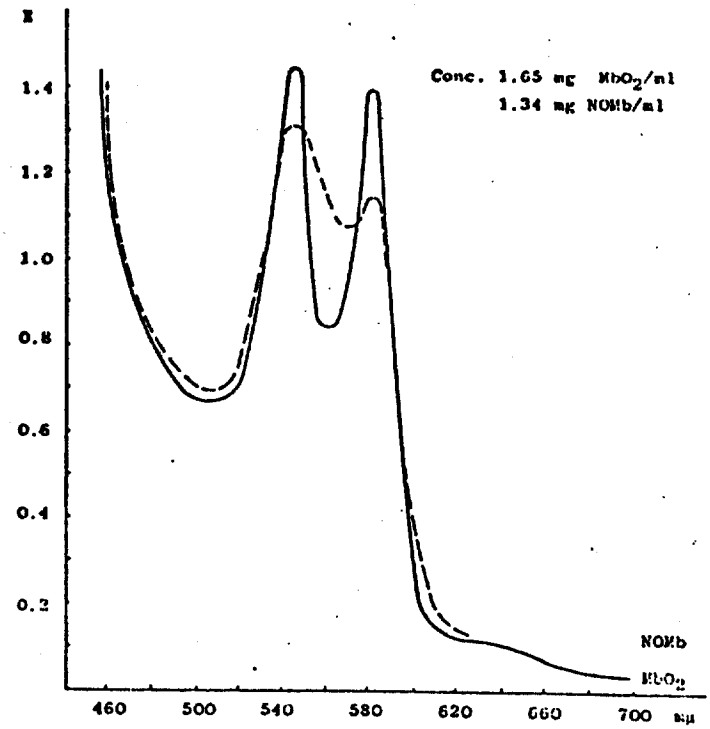

Fig. 2. Absorption spectra of NOMb and O_2Mb.

without verification. Several factors are known or supposed to have influence on the formation and stability of DNOMb; however, they have never been studied under the same experimental conditions, allowing a comparison of their importance. These two aspects led to the study of formation and stability of NOMb in model-solutions.

DNOMb is known to discolor fairly rapidly by oxidation in air and by exposure to light. The oxidation to MMb may also occur if much more nitrite is added than necessary for optimal NOMb-formation. It has been observed that a low concentration of nitrite also caused conversion of NOMb (Siedler and Schweigert, 1959). The oxidation may be inhibited by substances containing SH groups, especially cysteine and glutathion, normally present in meat (Hornsey, 1959). Several investigators (Walsh and Rose, [illegible]; Hornsey, 1957; Dudley, 1961) observed that oxidation is accelerated by light.

Inhibition of oxidation of DNOMb in meat products has been tried by addition of reducing agents, exclusion of air (Tauber and Simon, 1963), exclusion from light, adjustment of pH (Bailey *et al.*, 1964), and addition of phosphates. These factors have been studied.

Ascorbic acid is generally regarded as the most efficient reducing agent in this respect (Souci, 1958; Grau and [illegible], 1955, 1958; Pohja and Niinivaara, 1957; Bailey [illegible] 1964). Mullins (1957), however, did not share this opinion. Addition of ascorbic acid has the advantage that considerably less nitrite is needed for optimum pigment formation (Grau and Böhm, 1955, 1958; Möhler, [illegible] 1958; Souci, 1958).

The changes which Mb can undergo through the action of various additives can be investigated in the meat itself, in the natural medium of myoglobin. However, since the composition of samples of meat differs, the results of experiments cannot be fully compared. Therefore, great importance should be attached to "model" tests with pure solutions of exactly known composition, by adding the various auxiliary substances used in the processing of meat, enabling comparison of their efficacy and value. These tests are described.

EXPERIMENTAL METHODS

Preparation of myoglobin stock solution

For the isolation of Mb from beef, the method of Theorell (1932) as modified by Morgan (1936) and by Ginger *et al.* (1954) was applied with slight alterations. The resulting purified MMb solutions, containing 8–12 mg/ml, were filtered and stored in a sealed bottle at 0°C.

Determination of total Mb content of stock solutions was according to Ginger *et al.* (1954). Dilute 2 ml of the Mb-solutions to 5 ml with water, add 10 ml potassium iron(III)hexacyanide ($K_3Fe(CN)_6$)-solution (0.6m*M*/100 ml), and 10 ml potassium cyanide solution (0.8m*M*/100 ml), and filter. Measure the optical density of the solution at 540 nm in a 1-cm cell (Unicam SP600 spectrophotometer was used). The concentration of Mb expressed as mg Mb per ml stock solution was calculated with the formula.

$$c = \frac{\text{optical density}}{11{,}500} \cdot 16{,}500 \times 12.5$$

molecular weight of Mb = 16,500
molar extinction coefficient of CNMHb = 11,500, according to Drabkin and Austin (1935).

Experiments with model solutions of myoglobin

Before using the stock solution in experiments reduce the MMb to Mb by keeping under nitrogen and adding 2000 ppm sodium ascorbate. Check the reduction by measuring the absorption spectrum, which shows that the MMb is converted first to O_2Mb and then to Mb. After reduction remove the excess of sodium ascorbate by dialysis. Adjust pH of the dialyzed solution to 6.0 by adding 1/10 of its volume of McIlvaine's buffer solution (pH = 6.0; mix 36.85 ml 0.1*M* citric acid solution and 63.15 ml

0.2*M* Na_2HPO_4 solution), and dilute the solution by adding water to a concentration of 1 mg Mb per ml. As a control, measure the iron content of the Mb solution and calculate the Mb concentration from this (mol. wt. of Mb = 16,500; iron content (Theorell) = 0.32%). The value thus calculated must agree with that found according to Ginger *et al.* (1954).

Pipet 25 ml of the Mb solution thus prepared into each of two glass cylinders. Use one solution for comparison. Add the reacting substances (ascorbate, nitrite, etc.) to the other solution in the same sequence as given in the tables indicating the molar ratios. Pass nitrogen through both solutions at the same speed. Add after 5 min the auxiliary substances needed for each test. Determine the spectra of the solutions between 440 and 700 mμ in 1-cm cells with a Unicam SP600 spectrophotometer after 5 hr.

To determine the color stability transfer the solutions immediately after reading the spectra to glass Petri dishes; cover with glass lids. Expose them to the light of an electric bulb of 1200 lux cooling at the same time with a table fan. Read extinctions at 545 and 630 mμ after 1, 2, and 3 hr.

Carry out the experiments at about 20°C. For studying the effect of temperature on reaction velocity, however, use thermostats.

RESULTS AND DISCUSSION

When preparing the myoglobin solution (see "Experimental Methods") Mb is converted during dialysis to O_2Mb. This is of no consequence since the same happens in practice during meat processing.

The concentration about 1 mg Mb per ml in model solutions was chosen because according to our analysis Dutch pig muscle contains 0.6-2.0 (av. 1.0) mg Mb per g.

When describing our results few figures only are represented (Figs. 3–7); many other figures are to be found in the original Dutch publication (Szakály, 1963).

Effect of varying sodium nitrite and constant sodium ascorbate

The solutions were treated as indicated under "Experimental Methods." In this case it was not necessary to remove the 2000-ppm sodium ascorbate added for reduction.

By adding 0.5% sodium nitrite solution, the following molar ratios were obtained:

Mb-sodium ascorbate-nitrite	
1:200	:½
1:200	:1
1:200	:2
1:200	:5

The spectra showed that the ratio of 1 Mb to 1 $NaNO_2$ produced optimum NOMb formation, as shown in Figs. 3 and 4.

Our result makes probable the following reaction in the presence of ascorbate:

$$HNO_2 + Mb + H \longrightarrow NOMb + H_2O$$

The spectra also showed that the presence of more nitrite than in the ratio 1:1 did not affect the color content.

The stability of NOMb, however, was most favorable at the ratio 1:1, and larger quantities of nitrite accelerated its decomposition.

Effect of varying sodium ascorbate and erythorbate and constant sodium nitrite

The molar ratios tested in these experiments were:

Mb-sodium ascorbate-nitrite		
1:	20	: 2
1:	50	: 2
1:	100	: 2
1:	200	: 2
1:	500	: 2

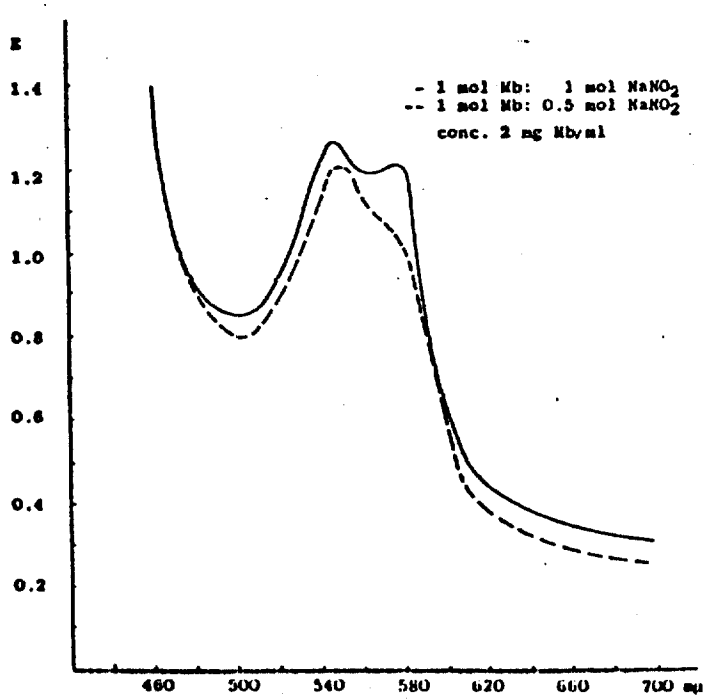

Fig. 3. Influence of different nitrite concentrations (constant ascorbate content).

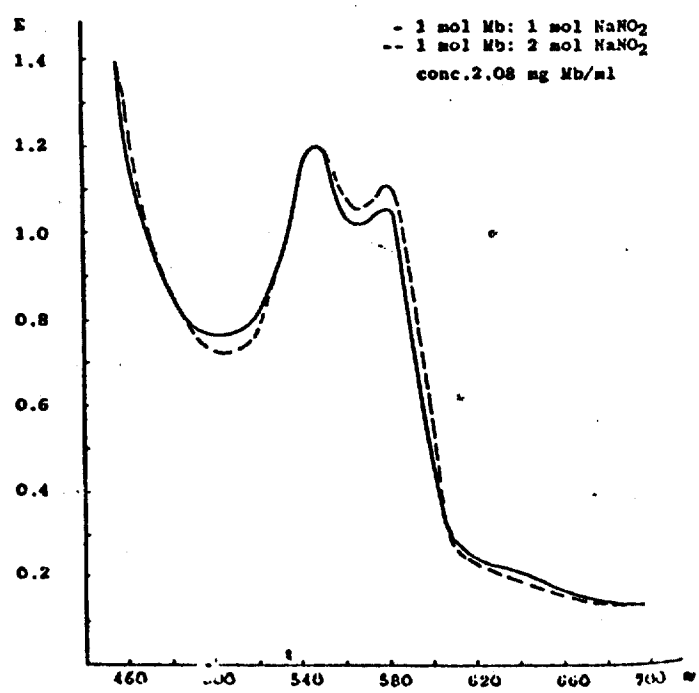

Fig. 4. Influence of different nitrite concentrations (constant ascorbate content).

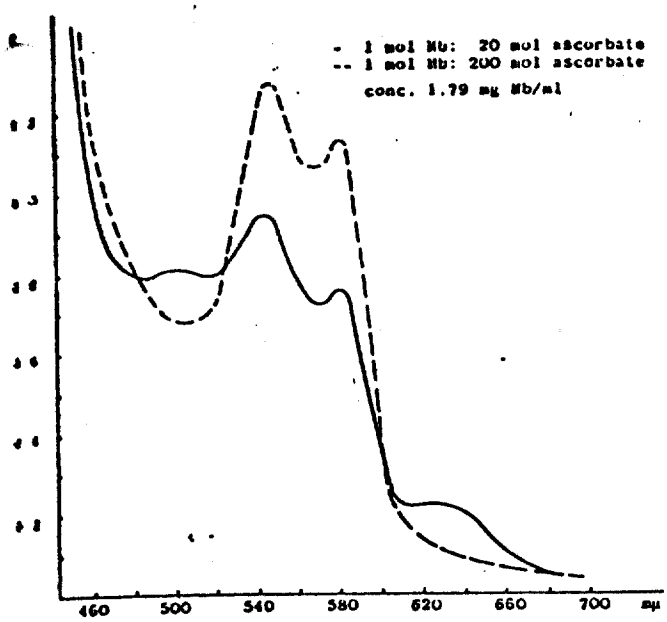

Fig. 5. Influence of different ascorbate concentrations (constant nitrite content).

Fig. 5 shows that some MMb (small peak at 500 mμ) accompanied NOMb in model solutions containing lower ascorbate concentrations (molar ratio Mb-ascorbate = 1:20). When higher ascorbate concentrations had been added, the MMb peak was absent. There was a marked increase of NOMb concentration up to a ratio 1:200, but not above this ratio (Fig. 6).

We have checked that even the highest ascorbate addition did not affect the pH of the model solution.

Observations were the same when ascorbate was replaced by erythorbate in the experiments mentioned. Both sodium salts were compared in the following test. A nitrite-containing Mb-solution was divided into two parts:

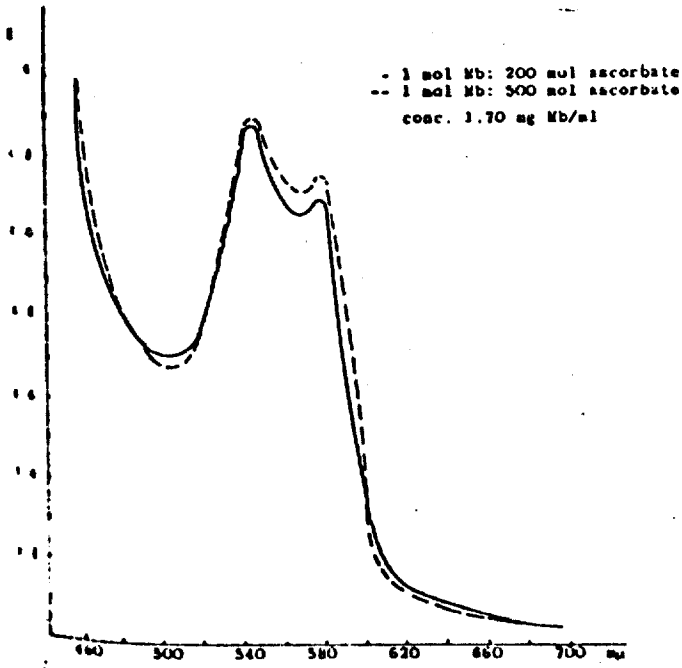

Fig. 6. Influence of different ascorbate concentrations (constant nitrite content).

sodium ascorbate was added to one part and sodium erythorbate to the other, both in molar ratio Mb-sodium salt = 1:100. No difference in effects was observed.

Sodium ascorbate improved color stability during exposure to light, with the breakdown of NOMb slower than in experiments without ascorbate, though the higher ratios 1:200 and 1:500 were not as favorable as the lower ratios.

Results on color stability in light were the same with sodium erythorbate. It is interesting to mention that sodium ascorbate caused a better color stability in light than sodium erythorbate in Borenstein's experiments (Borenstein, 1963).

Experiments with the free acids, ascorbic and erythorbic acids, have proved that they had the same effects on NOMb formation and its stability, even though they lowered the pH of the only slightly buffered model solutions. The same effects were also found when both acids were compared in one experiment as described.

Effect of varying sodium nitrite and constant cysteine or glutathion

Since pork averages 100 mg Mb and 200 mg cysteine per 100 g (Schweigert, 1952, 1956), the molar ratio of Mb-cysteine is about 1:200. The influence of cystein on NOMb formation was investigated in model solutions freed from ascorbate by dialysis and containing the following molar ratios:

Mb-sodium	ascorbate-cysteine-nitrite	
1:0	: 200	: 1
1:0	: 200	: 2
1:0	: 200	: 3
1:0	: 200	: 5

The results showed that, for attaining optimum NOMb formation, sodium nitrite must be added in a molar ratio Mb-$NaNO_2$ = 1: at least 3, being a much higher nitrite concentration than was necessary in former experiments. How is this to be explained?

It was probable that two reactions with nitrite are involved here: the oxidation of cysteine to insoluble cystine, and the reaction with the amino-group of cysteine, producing the OH group and N_2. The results of the following test were clarifying. The pH values of solutions of 0.5 mM/ml sodium nitrite were adjusted to 4.5, 6.0, and 7.0 by means of citric acid and disodium hydrogenphosphate. Half of each solution was used as a control, and cysteine-HCl was added to the other half to obtain 10 mM/ml. Nitrogen was passed through both liquids for 3 hr. The solutions containing cysteine produced the smell of NO_2, and showed an increasing white turbidity. At the end of 3 hr the nitrite contents (in ppm) of the solutions were as follows:

	pH 4.5	pH 6.0	pH 7.0
Nitrite solution (control)	33	38	32
Nitrite solution + cysteine	3.4	3	18

The nitrite in the control solutions remained unchanged. In the solutions containing cysteine-HCl, nitrite was broken down, especially at pH 4.5 and 6.0, and to an appreciable extent also at pH 7.0, accompanied by the

formation of cystine. One has to expect therefore that more nitrite will be necessary in cysteine-containing meat than in our model solutions for optimal color formation.

Experiments showed that glutathion has very little influence on the formation of NOMb.

Influence of sodium chloride

The model solutions contained the following molar ratios:

Mb-ascorbate-NaCl-nitrite		
1:100	: 0	:3
1:100	:3000	:3

Spectrophotometric measurement showed that the color intensity of the solution with sodium chloride was 92% of that of the control. Is the adverse effect of sodium chloride caused by the sodium ion or the chloride ion? We compared the effects of sodium chloride with those of ammonium chloride and sodium sulfate, keeping the molar ratios Mb-Na^+ and Mb-Cl^- the same as in the described test with sodium chloride. We observed that the color intensity after 5 hr of the solutions containing the salts mentioned was about 85% respectively, 94% of that of the control (Fig. 7).

The following test gives more insight on the sodium chloride effect. NOMb solution prepared without sodium chloride was divided into two parts. Half was used as a control; to the other half sodium chloride was added in a molar ratio of Mb-NaCl = 1:3000. The color intensity of the second solution was appreciably lower than that of the control. So, apart from a possible influence which sodium chloride may exert on the formation of NOMb, it was demonstrated that sodium chloride lowers the light absorption of this substance.

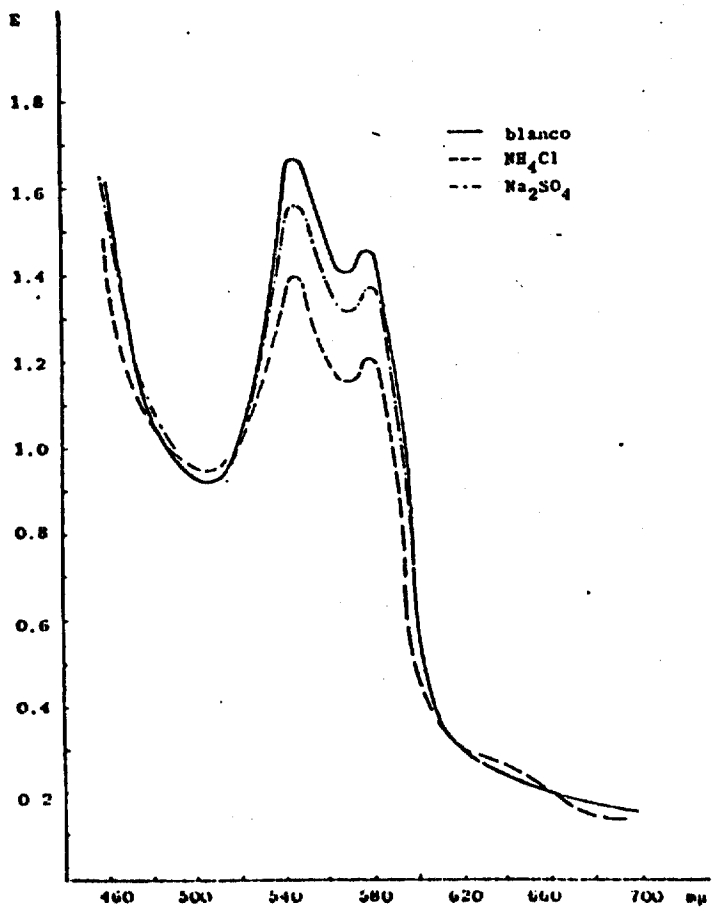

Fig. 7. Influence of salts.

Influence of potassium nitrate

Has potassium nitrate (saltpetre) any effect on the NOMb-formation or its stability, if it is not transformed to nitrite by microbial action? To answer this question, model solutions were prepared containing the following molar ratios:

Mb-ascorbate-KNO_3-nitrite		
1:100	: 0	: 3
1:100	: 50	: 3

In these experiments, potassium nitrate had no effect on color formation and stability.

Influence of polyphosphates

Tests were carried out with the following four polyphosphates most used in the meat industry, added separately: $Na_4P_2O_7 \cdot 10\ H_2O$, $Na_2H_2P_2O_7$, $Na_5P_3O_{10}$, and Graham's sodium polyphosphate (20–3000 phosphorus atoms).

To an Mb solution were added sodium ascorbate and nitrite in molar ratios Mb–ascorbate–$NaNO_2$ = 1:100:3, and the mixture was divided into several parts. One of the polyphosphates was added to each portion, resulting in an Mb:P_2O_5 ratio of 1:200. The absorption curves were not significantly different from the control solution. It appears that these four phosphates have no effect upon the formation or stability of NOMb.

Influence of ferrous sulphate

It has been claimed that ferrous sulfate promotes the formation of NOMb. To study its effect model Mb-solutions were prepared, containing the following molar ratios:

	Mb:ascorbate:ferrous sulfate:nitrite			max NOMb formed after
a	1:300	: 0	:3	4 hr
b	1:300	:10	:3	1 hr
c	1:100	:10	:3	1 hr

The color intensity of solution *a* was about 10% less than that of solutions *b* and *c*. Thus, ferrous sulfate improved both the speed and degree of conversion to NOMb.

The stability of NOMb was the same in all three solutions.

Influence of temperature

We examined the influence of temperature on the formation and stability of NOMb at 10, 30, 40, and 50°C. One-half of a model MMb solution was kept at 20°C, while the other half was exposed to a temperature mentioned. NOMb was formed more as temperature increased. The reaction between 1 mol Mb and 3 mol sodium nitrite was optimum at:

10°C after 14 hr
20°C after 5 hr
30°C after 2 hr
40°C after 40 min
50°C after 20 min

The optimum quantity of NOMb formed at 20, 30, 40, and 50°C was the same. Tauber and Simon (1963) observed that the extent of color formation depended on temperature.

In these solutions the stability of NOMb in light did vary at the temperatures mentioned.

Influence of pH

In all the model experiments so far, a buffer solution with a pH of 6.0 was added to prevent fluctuations in pH. The influence of pH on the formation and stability of NOMb was tested in model solutions containing the following molar ratios:

Mb–ascorbate–nitrite
1:100 : 3
at pH 5.0, 5.5, 6.0, 6.5, or 7.0

pH was adjusted with citric acid and disodium hydrogenphosphate.

The results showed that at the pH 6.5 and 7.0 the nitrite takes effect more gradually and causes less MMb to form than at lower pH. The MMb peaks (at 500 and 630 mμ) are smaller than at pH 6.0; the solutions at pH 6.5 and 7.0 became less brown than the solution at pH 6.0.

The reaction between Mb and sodium nitrite was much more rapid at pH 5.0 or 5.5 than at pH 6.0. At pH 5.0 the brown color of MMb appeared within a few minutes of nitrite addition. The return of the red color as a result of formation of NOMb was also quicker.

Although the velocity of color formation depended to a large extent on pH, no significant difference was found between the solutions in final color intensity.

With higher pH values NOMb was much more stable in light than with low values. At pH 7.0 no discoloration was observed at the end of 3 hr, whereas at pH 5.0 and 5.5 there was marked discoloration. We suppose that at low pH values free nitrous acid formed from excess nitrite oxidized Mb to MMb. At pH 7.0 the nitrous acid concentration in balance with nitrite is extremely low.

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1761

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Formation and Stability of Nitric Oxide Myoglob[illegible]

II. Studies on Me[illegible]

SUMMARY—Results in model Mb solutions were confirmed by tests on pork samples treated with the mentioned additives and heated. The content of nitric oxide chromogen (DNOMb) in the samples was determined according to Hornsey. It was found that optimum DNOMb formation required not less than 5 mols nitrite per mol Mb; this may be due partly to side reactions with cysteine and other components of meat. In the presence of sufficient sodium ascorbate (200 mols per mol Mb), 3 mols nitrite per mol Mb was optimum. A large excess of nitrite (500 mols per mol Mb) induced the formation of green pigments, and appreciably less DNOMb was found.

Reheating meat containing denatured Mb with nitrite converted Mb to a red pigment, but the color intensity so obtained was 40–60% of that obtained with normal nitrite treatment before heating.

Potassium nitrate did not influence the DNOMb formation.

The higher the temperature to which meat is heated, the lower its DNOMb content.

Exclusion of oxygen by means of carbon dioxide during the treatment of meat improved the final DNOMb content.

Irradiation by light of meat containing DNOMb showed that the concentration of nitrite necessary for optimal DNOMb formation in the absence of ascorbate (5 mols nitrite to 1 mol Mb) also caused optimum stability to irradiation in air.

Potassium nitrate did not affect this stability. The breakdown of DNOMb increased with the light intensity. In a carbon dioxide atmosphere the breakdown of DNOMb is much slower than in air and it is independent of the light intensity.

INTRODUCTION

WITH THE AID OF MODEL SOLUTIONS the formation and stability of NOMb were studied. It should be borne in mind, however, that while the experiments with model solutions supply valuable indications, they cannot give any decisive information on the optimum conditions for meat processing by industry. Conditions in meat differ from those in the model solutions. In the meat, Mb is incorporated together with many other substances in the cell structure, whereas in our model solutions it was dissolved in water accompanied by only a few other substances. Important in meat processing are not only the formation and stability of NOMb, but also the denaturing of this substance to DNOMb by heating of the meat product, a process which cannot be copied in model solutions. Thus, there are sufficient reasons why tests on the formation and stability of nitric oxide pigment in meat should be carried out.

EXPERIMENTAL METHOD

MINCE LEAN PORK IN A CUTTER from Krämer and Grebe. Add ice-water, sodium chloride, sodium [illegible] and (if indicated) reducing compounds during [illegible] Fill the mass immediately after mincing in square [illegible] portions of 340 g, seal in vacuum, and heat 90 min [illegible] 100 or 110°, and cool to 5°C.

Determine the DNOMb content according to H[illegible] (1956): mince the content of the can in a mincing-m[illegible] (perforations 2 mm). Shake a mixture of 10 g [illegible] meat, 40 ml of acetone and 3 ml of water in a flas[illegible] 5 min. Filter the mixture through a folding-filter [illegible] measure the optical density of the filtrate (containi[illegible] acetone-nitric oxide-heme-complex) at 540 nm in a [illegible] cell in a Unicam Sp600 spectrophotometer.

To determine the color stability on exposure to [illegible] weigh 10-g portions of minced and heated meat. S[illegible] the material consisting of very thin threads as a thin [illegible] in a Petri dish, diameter 10 cm, and expose to incandes[illegible] light from a 100W bulb at a distance of 60 cm. [illegible] determine the DNOMb content of the meat after 1, 2, [illegible] 3 hr of exposure.

Influence of sodium nitrite

Meat samples of the composition as indicated in T[illegible] were, according to the description in "Experim[illegible] Method," tinned, sealed, and pasteurized for 2 hr at [illegible] The nitric oxide pigment content and its stability on [illegible] sure to light were investigated, with results as indi[illegible] in Table 1.

The ratio of 1:1 was insufficient. At 1:2 and 1:3 [illegible] formation of pigment was much better, but at 1:5 or [illegible] maximum intensity was obtained. At 1:500, howev[illegible] green discoloration and a low DNOMb content [illegible] observed.

Addition of more nitrite than necessary for opti[illegible] DNOMb formation did not seem to have any influen[illegible] the velocity of decomposition in light.

Table 1. Influence of sodium nitrite concentration.

% NaCl—Mb—$NaNO_2$ (mols)	Extinction at 540 nm after exposure to light for: 0 hr	1 hr	2 hr	[illegible]
2:1: 1	0.036	0.030	0.028	[illegible]
2:1: 2	0.156	0.096	0.068	[illegible]
2:1: 3	0.170	0.122	0.060	[illegible]
2:1: 5	0.201	0.135	0.045	[illegible]
2:1: 25	0.205	0.132	0.047	[illegible]
2:1:100	0.208	0.147	0.055	[illegible]
2:1:200	0.206	0.146	0.058	[illegible]
2:1:500	0.165	0.088	0.065	[illegible]

Table 2. Influence of sodium ascorbate.

[illegible] ascorbate (mols)	Extinction at 540 nm after exposure to light for: 0 hr	1 hr	2 hr	3 hr
: 1 50	0.128	0.052	0.030	0.022
: 2 50	0.147	0.094	0.048	0.035
: 3 50	0.225	0.121	0.055	0.034
: 4 50	0.230	0.132	0.058	0.040
: 5 50	0.228	0.135	0.056	0.042

[illegible] of potassium nitrate

[illegible] minced meat sample was divided into three parts. [illegible] part was mixed with potassium nitrate (240 [illegible] second with sodium nitrite (100 ppm), and the [illegible] both potassium nitrate (240 ppm) and sodium [illegible] ppm).

[illegible] first part (without nitrite) showed no color; at [illegible] the extinction of the acetone-water extract was [illegible] The second and third parts both showed the same [illegible] We conclude that potassium nitrate does not [illegible] influence on the color of meat products treated with [illegible] and heated. The same negative results have been [illegible] with nitrate in model myoglobin solutions.

[illegible] of sodium ascorbate and erythorbate

[illegible] the first series of tests a constant quantity of sodium [illegible] in combination with different quantities of so[illegible] nitrite was added (see Table 2). The results show [illegible] addition of ascorbate produces higher DNOMb con[illegible] than have been obtained in the experiments without [illegible] The molecular ratio 1 Mb:3 nitrite was suffi[illegible] to arrive at the maximum content. The application of [illegible] thus reduces the required quantity of nitrite 1:5 [illegible] in experiments mentioned in absence of ascorbate.

[illegible]upon the influence of higher ascorbate additions [illegible] (see Table 3). The results show that the [illegible] intensity both before and after exposure to light [illegible] from the addition of more sodium ascorbate than [illegible] 1:50 applied in the previous tests. Increase of [illegible] over 1:200, however, does not further improve [illegible] intensity and stability. These results correspond [illegible] obtained in model solutions.

[illegible] compare the effect of ascorbate and erythorbate, [illegible] minced pork of the following composition were [illegible]:

[illegible]:Mb (mols) : reducing salt (mols) : nitrite [illegible]

[illegible] : 1: 100 sodium ascorbate : 5

[illegible] : 1: 100 sodium erythorbate : 5

[illegible] mixtures were canned and heated at 100°C as described under "Experimental Method." No difference in DNOMb content was observed.

Table 3. Influence of sodium ascorbate.

Mb—$NaNO_2$—ascorbate (mols)	Extinction at 540 nm after exposure to light for: 0 hr	1 hr	2 hr	3 hr
1:3: 50	0.225	0.121	0.055	0.034
1:3:100	0.269	0.166	0.062	0.041
1:3:200	0.285	0.174	0.110	0.058
1:3:500	0.280	0.168	0.104	0.048

Influence of sodium nitrite on denatured pigments

Nitrite is always added to the meat simultaneously with sodium chloride, before heating. The following tests show that adding nitrite after heating leads to some formation of DNOMb.

One part of a minced meat sample was mixed with 100 ppm sodium nitrite, tinned, and heated at 100°C for 90 min. The other part of the meat was tinned without addition of nitrite, taken out of the tins, and divided into two. Added to one-half was 100 ppm nitrite, and to the other half 100 ppm nitrite and 500 ppm sodium ascorbate. Thereupon, both mixtures were tinned again, and the tins were once more divided into two groups. One part was stored immediately at 5°C, and the other was first heated at 100°C for 90 min and after this stored at 5°C.

The DNOMb content of each is represented in Table 4. We conclude that a reaction between denatured pigment and sodium nitrite indeed occurs. Heating, addition of sodium ascorbate or both combined have a favorable influence on the color formation. The intensity of the color obtained in this way comes to about 40–60% of that obtained by nitrite addition before heating.

Influence of temperature

In industry, meat products may be heated to temperatures varying from 100 to 115°C. What influence has temperature on the intensity and stability of the color? To answer this question, meat was treated as described in "Experimental Method," adding 100 ppm nitrite. Tins filled with parts of the treated sample were heated to 100, 105, 110, and 115°C and cooled.

These tests (see Table 5) showed that the visually Mb-rich muscle tissue furnished products richer in DNOMb than the visually Mb-poor muscle tissues.

Nitric oxide pigment decomposition increased in all samples with heating temperature.

Ascorbate had a very favorable influence on the formation of DNOMb in these tests. Exposure to light caused

Table 4. Influence of nitrite added after heating.

$NaNO_2$ added before or after 1st heating	Ascorbic acid (ppm) after 1st heating	Treatment after being tinned for the second time	Extinction at 540 nm after exposure to light for: 0 hr	1 hr	2 hr	3 hr
Before	0	not heated	0.264	0.148	0.122	0.091
After	0	not heated	0.104	0.062	0.035	0.025
After	0	heated	0.141	0.095	0.076	0.049
After	500	not heated	0.127	0.080	0.053	0.034
After	500	heated	0.157	0.122	0.090	0.063

Table 5. Effect of temperature.

Type of meat	Sterilization temperature (°C)	Extinction at 540 nm after exposure to light for: 0 hr	1 hr	2 hr	3 hr	5 hr
Light-colored muscle tissues	100	0.106	0.073	0.056	0.042	0.033
	105	0.098	0.049	0.042	0.032	0.022
	110	0.096	0.047	0.043	0.031	0.019
	115	0.075	0.044	0.035	0.027	0.016
Dark-colored muscle tissues	100	0.168	0.105	0.076	0.065	0.044
	105	0.138	0.088	0.063	0.053	0.033
	110	0.135	0.084	0.062	0.051	0.028
	115	0.017	0.065	0.041	0.034	0.020
Dark-colored muscle tissues with 500 ppm ascorbate	100	0.265	0.185	0.150	0.136	0.100
	105	0.225	0.142	0.125	0.106	0.086
	110	0.224	0.140	0.124	0.104	0.068
	115	0.197	0.116	0.098	0.089	0.057

a considerable decrease of color in all cases. After 5 hr of exposure the favorable influence of ascorbate was still perceptible.

Influence of carbon dioxide

Oxygen oxidizes DNOMb; we investigated therefore the influence of replacing air by carbon dioxide.

The sample of meat was divided into two parts. One part was minced in the usual way in contact with air. After flushing the cutting machine with CO_2 the other part of the meat was minced in the CO_2 atmosphere. Sodium nitrite (100 ppm) and sodium chloride (2%) were added to both parts during mincing. Thereupon they were tinned, heated 90 min at 110°C and cooled. The DNOMb contents of three samples of meat (Table 6) show that samples treated in CO_2 atmosphere had a higher color intensity than the corresponding samples treated in contact with air. However, the exclusion of air during the formation of pigment had no significant influence on its content after exposure to light.

Moreover, we have studied the effect of air exclusion during exposure to light. A meat sample was mixed with 100 ppm sodium nitrite and 2% sodium chloride in the usual way in contact with air and heated. Thereupon one part of this material was exposed to light in contact with air and the other part in CO_2 atmosphere. The following results were obtained:

Extinction at 540 nm after exposure to light for:

	0 hr	1 hr	2 hr	3 hr
In air	0.144	0.076	0.056	0.033
In CO_2	0.147	0.109	0.102	0.096

Exclusion of air during the exposure to light proved to be favorable to the stability of DNOMb. The slight decrease in DNOMb content in the CO_2 series may be due to oxidation by air during preparation of the acetone-extract.

Other experiments were carried out in which CO_2 was substituted for air, both during the formation of NOMb and during exposure to light. The DNOMb content was higher than in previous experiments.

Influence of irradiation

Parts of a minced meat sample treated with nitrite and sodium chloride were exposed to light intensities varying from 0 to 1500 lux. The results are repre[illegible] Table 7. They show that increase of light intensi[illegible] increase of DNOMb decomposition.

The same test carried out in a CO_2 atmosphere [illegible] 8) indicates that illuminated DNOMb is more [illegible] CO_2 atmosphere than in air. They suggest that the [illegible] position of DNOMb is independent of light inten[illegible] is replaced by CO_2.

Table 6. Effect of carbon dioxide.

Treatment	Extinction at 540 nm after exposure to light for: 0 hr	1 hr	2 hr
Test I in air	0.108	0.065	0.049
Test I in CO_2	0.154	0.090	0.060
Test II in air	0,135	0.078	0.056
Test II in CO_2	0.169	0.078	0.056
Test III in air	0.172	0.104	0.073
Test III in CO_2	0.200	0.106	0.076

Table 7. Influence of light intensity. Samples in a[illegible]

Illumination (lux)	Extinction at 540 nm after exposure to [illegible] 0 hr	1 hr	2 hr
0	0.145	0.103	0.102
90	0.146	0.096	0.080
240	0.146	0.092	0.073
450	0.144	0.076	0.056
720	0.148	0.055	0.041
1500	0.144	0.045	0.025

Table 8. Influence of light intensity. Samples in CO_2 atm[illegible]

Illumination (lux)	Extinction at 540 nm after exposure to [illegible] 0 hr	1 hr	2 hr
0	0.144	0.118	0.114
450	0.147	0.109	0.102
720	0.148	0.111	0.107
1500	0.148	0.116	0.114

REFERENCE

Hornsey, H. C. 1956. The colour of cooked cured pork [illegible] mation of the nitric oxide-haem pigments. J. Sci. [illegible] 7, 534.

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Gas Chromatography and Selective Detection of N-Nitrosamines

1765

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Abstract

The pyrolysis of N-nitrosamines after GC separation results in the formation of ammonia and provides the basis for the selective detection of the N-nitrosamines in neutral extracts of cigarette tars.

Introduction

It has been well established that many N-nitrosamines are highly carcinogenic. Since the suggestion first made by Druckrey and Preussman (1), that the conditions exist in cigarette smoke for the production of N-nitrosamines, there has been considerable interest in the examination of cigarette tar for the presence of N-nitrosamine compounds (2).

In order to develop a GC method to accomplish this objective, four primary steps are necessary:

1. Collection of smoke as a condensate,
2. Preparation of the condensate for chromatography,
3. Resolution of the N-nitrosamines, and
4. Detection of the N-nitrosamines using a highly sensitive and selective system.

This paper reports efficient GC conditions and a detection system which is selective for the N-nitrosamines. This detection system is based on the use of the Liquid Conductivity Detector developed by Coulson (3).

The Coulson Liquid Conductivity Detector (Tracor, Inc.) system, when used in the normal reductive mode, selectively detects all organic nitrogen compounds by converting the organic nitrogen to ammonia which is absorbed in a small continuous flow of deionized water. It is the change in conductivity of this flowing liquid which is detected. Removal of acid gases is required to obtain selectivity for ammonia. This is usually accomplished by the use of an appropriate subtractor material, e.g., strontium hydroxide or potassium carbonate. To convert the organic nitrogen to ammonia effectively, nickel wire or shot is required as a catalyst in the quartz hydrogenation tube which is heated to about 850°C. Hydrogen gas is mixed with the column outlet gas prior to entering the reactor tube so that essentially complete conversion of organic nitrogen to ammonia is obtained. The entire system is then very selective for the detection of nitrogen-containing organic compounds.

The principle involved in the selective detection of amines and N-nitrosamines is to alter conditions so that pyrolysis, rather than hydrogenation, occurs in the heated zone and ammonia is detected as thermal degradation product. By controlled pyrolysis, it is possible to detect amine-type compounds in the presence of relatively large amounts of other nitrogen-containing materials.

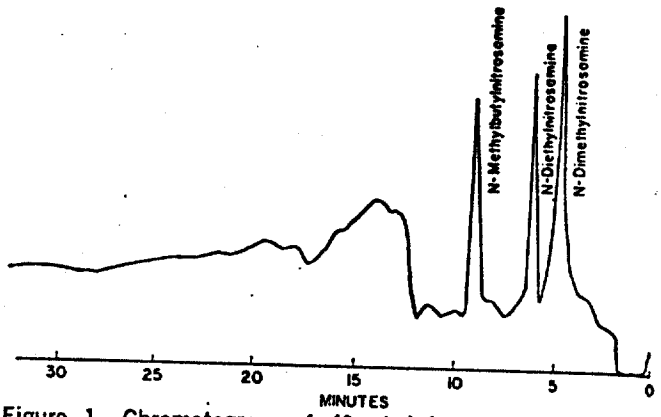

Figure 1. Chromatogram of 40 μl injection of extract of spiked cigarette tar using thermal degradation mode of detection.

Experimental

Detection of N-nitrosamines

The Coulson Liquid Conductivity system is readily converted to an amine detector through alteration of the operating parameters. An inert gas, such as argon, is used as the carrier gas, and an empty quartz tube is substituted for the hydrogenation catalyst. The furnace is operated in the range of 400°-600°C. In this temperature range ammonia is obtained as a degradation product of many amines and N-nitrosamines, whereas other types of organic nitrogen compounds produce little or no ammonia.

The usefulness of the detector system may be demonstrated as follows: The tar collected from 20 cigarettes was spiked with 1 μg each of N-dimethylnitrosamine, N-diethylnitrosamine and N-methylbutylnitrosamine. A neutral volatile fraction of the spiked tar was obtained in 0.25 ml of dichloromethane. The chromatogram in Figure 1 shows the results obtained

1. Druckrey, J., and Preussmann, R., Naturwissenschaften 49, 498 (1962).
2. National Cancer Institute Monograph 28, "Toward a Less Harmful Cigarette," June 1968.
3. Coulson, D. M., "Advances in Chromatography," Vol. 3, J. C. Giddings and R. A. Keller, eds., 1966, p. 197.

from a 40 μl injection when the detector was used in the thermal degradation mode for selective detection of amine-type compounds.

Figure 2 shows the chromatogram obtained from a 1 μl injection when the detector was used in the normal reduction mode for the detection of all nitrogen-containing compounds.

Both chromatograms were obtained on the same 4 ft column of 10% Carbowax 1540 on 80/100 mesh Gas Chrom Q, temperature programmed from 70-150°C at 5°/min, and using argon carrier gas saturated with water vapor at room temperature. The water vapor was added to reduce tailing of the N-dimethylnitrosamine.

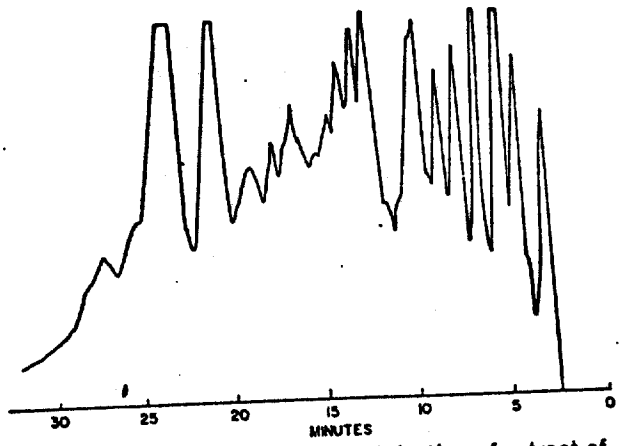

Figure 2. Chromatogram of 1 μl injection of extract of spiked cigarette tar using reductive mode of detection.

Discussion

A comparison of the two chromatograms shows the advantage of the thermal degradation mode for the detection of N-nitrosamines. As illustrated in Figure 1, selectivity for the N-nitrosamines in the sample permits large injections due to detector rejection of other nitrogen-containing compounds. The presence of other nitrogen compounds, in much larger amounts than the added N-nitrosamines, is readily apparent when the detector is used in the reductive mode for the detection of all nitrogen compounds as shown in Figure 2. In this instance, it was necessary to limit the injection of 1 μl in order to keep the large peaks due to nitrogen compounds on the chart paper. With this small injection the added N-nitrosamines produce peaks only a few chart units in height, and they are completely masked by the other nitrogen compounds. From the results obtained it would appear that the GC determination of these N-nitrosamines in cigarette tar would be impossible without the help of the thermal degradation mode of detection. A hydrogen FID would be useless for examining the neutral fraction of the tar.

The selectivity of the detection system reported here permits the analysis of N-nitrosamines at the nanogram level with a minimum of sample preparation. To utilize other detector systems which have little or no selectivity for N-nitrosamines would require collection of tar from considerably more than 20 cigarettes and a thorough cleanup of the sample.

The N-nitrosamine peaks in Figure 1 represent 50 ng/cigarette of each of the added N-nitrosamines; however, it is likely that the limit of sensitivity with the present system will permit the analysis of 1 ng/cigarette of the N-nitrosamines. The reported method for the GC separation and detection of N-nitrosamines appears to have adequate sensitivity and selectivity for the determination of selected N-nitrosamines in cigarette tar. The method that was used for the collection of cigarette tar was developed in these laboratories and previously reported (4). Methods for the preparation of samples prior to GC analysis are currently under investigation. Successful completion of this phase will then permit the analysis of cigarette tars obtained under different smoking conditions and other condensate collection methods and thus clarify the problem of N-nitrosamines in cigarette smoke. The procedure should be useful for application to other samples such as various foods and beverages.

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Examination of Nonfat Dry Milk for the Presence of Nitrosamines[1]

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Abstract

Nonfat dry milk prepared using indirect steam heat and direct gas firing was examined for detectable levels of nitrosamines. The addition of ten nanograms of nitrosamine per gram of nonfat dry milk could be detected by oxidation of the extracted nitrosamine to nitramines and detection by electron capture gas chromatography. No volatile nitrosamines were found in freshly prepared or stored (30 days) dry milk dried by indirect steam heat or by direct gas firing.

Introduction

Since Magee and Barnes (4) discovered the carcinogenicity of dimethylnitrosamine, publications have appeared concerning the toxic, carcinogenic, and mutagenic properties of various nitrosamines (1,6). Nitrosamines are carcinogenic to several species (9) and induce tumors at different locations in the body. Some nitrosamines cause cancer after only one dose (5). This class of compounds is one of the most potent carcinogens known to man.

Newell and Sisken (8) reported finding 1.9 ppb of a naturally occurring compound in fluid milk that co-chromatographed with dimethylnitrosamine by gas chromatography. They did not identify this unknown and could not state that it was dimethylnitrosamine. Nitrosamines have not been found in unprocessed foods but may be formed during certain processing operations (2). Nitrosamines may be formed from biogenic amines, certain amino acids, and proteins in the presence of nitrite at high temperatures. Manning and Coulter (7) reported up to 2 ppm nitrite and 3.8 ppm nitrate in spray dried milks with fossil fuels as a heat source. Therefore, the potential exists for the reaction of nitrite with milk constituents to form nitrosamines at normal drying temperatures. This study was conducted to determine if nonfat dry milk contains nitrosamines.

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[1]Scientific journal series paper 7993, Minnesota Agricultural Experiment Station, St. Paul.

Experimental Procedures

Source of chemicals. Dimethylnitrosamine, diethylnitrosamine, dipropylnitrosamine and dibutylnitrosamine standards and trifluoracetic acid were purchased from Eastman Chemical Company (Rochester, New York). Nanograde dichloromethane and hexane were obtained from Mallinckrodt (St. Louis, Missouri).

Sample preparation. Nonfat dry milk (NFDM) samples were prepared from commercial condensed skimmilk (30% TS) with a pilot model of the Coulter spray drier. This drier uses either indirect steam heat or direct gas firing (natural gas) as heat for the drying air. Inlet air at 163 C and an exit air of 82 C were used for the steam heat trial, and 260 C and 163 C inlet air and 110 C and 82 C exit air were used for the gas fired trials. Samples were analyzed for nitrosamines immediately after drying and after 30 days at 21 to 25 C.

Isolation by vacuum steam distillation. In this and subsequent procedures, glassware was washed with dilute HCl and thoroughly rinsed with deionized water followed by glass distilled water to insure freedom from contamination.

One hundred grams of NFDM were reconstituted with 400 ml of deionized water. After adding 5 g of K_2CO_3, the sample was transferred to a 3-liter round bottomed boiling flask and 3 drops of antifoam B (Dow-Corning) were added. The solution was then steam distilled under vacuum (28 C) until approximately 200 ml of distillate were collected. Twenty grams of K_2CO_3 were added to the distillate which was then extracted three times with 50 ml of nanograde dichloromethane. The dichloromethane fraction was dried with anhydrous magnesium sulfate, filtered, and concentrated to .5 ml under a stream of dry N_2 for subsequent oxidation.

Oxidation of nitrosamines to nitramines. Oxidation by adding 3 ml of trifluoracetic acid and 4 ml of 50% hydrogen peroxide to the dichloromethane extract proceeded for 12 to 15 hr (11). The solution was then poured over 10 to 15 g cracked ice and carefully neutralized with about 40 ml of 20% K_2CO_3. This solution was extracted two times with 50 ml nanograde

dichloromethane and the extract was dried with anhydrous magnesium sulfate, filtered, and concentrated to about 5 ml under a stream of dry nitrogen. The concentrate was transferred to a 15 ml standard taper graduated centrifuge tube and 1 ml of nanograde hexane was added. The solution was then concentrated to about .2 ml for analysis.

Gas chromatographic analysis. The concentrated extract was analyzed for nitramines with a Hewlett-Packard Model 5750 gas chromatograph equipped with a Nickel 63 electron capture detector. A 1.83 m × .32 cm stainless steel column packed with 6% Rheoplex 400 on AWDMCS treated Chromosorb W (60/80 mesh) was operated isothermally at 175 C to separate the nitramines. Five percent methane: argon was the carrier gas at 35 ml per minute. The injection port and detector were at 210 and 280 C.

Results and Discussion

Initially, a TLC procedure by Sen et al. (10) was used for the detection of nitrosamines extracted from NFDM. This method consisted of chromatography of the dichloromethane extract on silica gel G-HR and detection with specific spray reagents. This method lacked sensitivity to detect nitrosamines in NFDM at less than .5 ppm. Flame ionization gas chromatography was unsuccessful due to interfering compounds. Therefore, a procedure was used whereby nitrosamines are oxidized to nitramines and detected by electron capture gas chromatography (11). This method with electron capture detection increased sensitivity by factors of 100 to 1,000 over the flame ionization method. The response specificity of electron capture helped to eliminate interfering substances co-chromatographing with nitramines (Fig. 1, A). Distillation under reduced pressure instead of atmospheric pressure also helped to eliminate interfering substances. It was possible to isolate and detect the addition of 50 ng nitrosamine per gram of NFDM. However, tentative detection could be made by adding 10 ng per gram NFDM (Fig. 1).

No nitrosamines were detected in indirect steam or direct gas fired NFDM immediately following drying or after 30 days storage (Fig. 1). A peak appeared in high temperature direct gas fired NFDM chromatogram that co-chromatographed with the dimethylnitramine (DMN) standard (Fig. 1, B). This same peak also occurred in the low temperature gas fired sample but in lower concentration. It was impossible to identify the unknown peak by mass

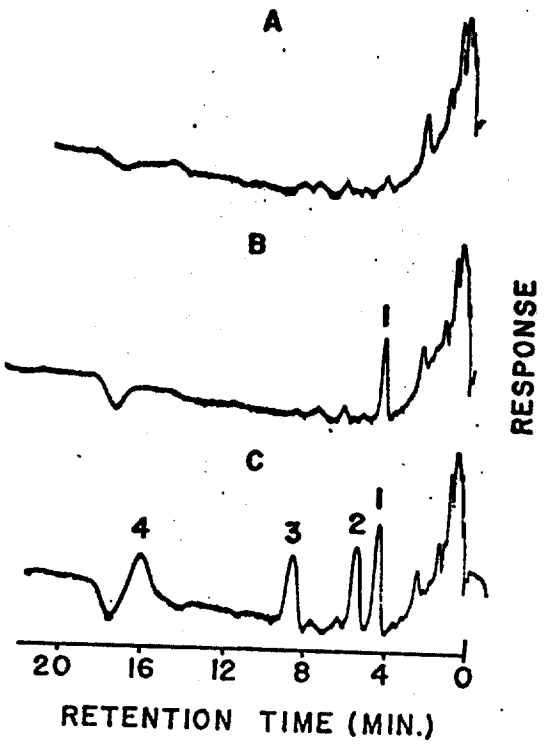

FIG. 1. Chromatograms of fractions that would contain nitramines isolated from (A), steam heated NFDM (163 C inlet, 82 C exit), (B) direct gas fired NFDM (260 C inlet, 163 C exit), and (C) (260 C inlet, 110 C exit) steam heated NFDM plus 50 ng/g NFDM added dimethyl (1), diethyl (2), dipropyl (3), and dibutyl (4) nitramines.

spectrometry due to massive contamination that appeared using the total ion detector of the mass spectrometer. Gas chromatographic columns of different polarity determined if the unknown would co-chromatograph over a range of column polarities. The contaminant co-chromatographed with DMN on both a 3.05 m × .32 cm od stainless steel column packed with 10% DEGA and on a 15.25 m × .2 mm id SCOT column coated with Carbowax 20 M. With a 1.83 m × .32 cm od 3% OV-1 column, the contaminant could not be located on the chromatogram because components that normally ran with the solvent front on the polar columns ran with the unknown. The fraction containing the unknown compound was isolated by trapping from a 3.05 m × .64 cm od DEGA column. It was then possible to get a single peak on the Rheoplex 400 column and on the OV-1 column. The unknown contaminant again co-chromatographed with the DMN on Rheoplex 400, but there was about 6 min difference in retention times on the OV-1. Dimethylnitramine was subjected to the same

GLC isolation procedure and was used as a standard for retention times to ensure against degradation during isolation. Therefore, it was concluded that the unknown contaminant peak was not dimethylnitramine and its identification was not pursued. It is conceivable that the extraneous peak was a product of the browning reaction. The higher drying temperatures used in this study are considerably above those conventionally used in the manufacture of NFDM. While these temperatures were chosen to maximize the probability of nitrosamine formation, they also maximize the probability of artifact formation. The extraneous peak may be a product of the browning reaction such as a furfural or pyrazine. Havre and Ender (3) have shown that products of the browning reaction (e.g. 2,5-dimethylpyrazine) may be incorrectly identified as nitrosamines if care is not taken in identification.

In conclusion, our methodology permitted the isolation and detection of nitrosamines added to NFDM at 10 ng/g (10 ppb). An investigation of NFDM samples showed there are no steam distillable nitrosamines in NFDM at 10 ppb and that the heat source (direct gas fired versus indirect steam) is of no consequence.

Acknowledgements

The authors wish to acknowledge the help of Peggy Bakkum for sample analysis and Don Peterson for sample preparation.

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W. H. EMERSON.

INDUSTRY, UNITED STATES DEPART-
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ATS OF RENARD'S TEST FOR
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lard, and the same lard with 10 per cent. peanut oil admixed. Determinations made on pure peanut oil are added for comparison.

	% solid acids	% crystallizing from 90% alcohol	% remaining in solution	Beginning of crystallization	Iodine number of crystals	Melting point of crystals
Peanut oil	13.73	4.24	9.49	37°	0.7	71.5
Lard	39.30	5.93	33.37	20°	7.4	54.3
Lard plus 10% peanut oil	37.35	1.62	35.73	19°	4.2	55.0
Oleostearin	36.31	15.18	20.98	25°	4.9	61.5

The amount of solid acids in oleostearin and lard crystallizing at 15° from 90 per cent. alcohol is far greater than in peanut oil, although the temperature at which crystallization begins is far lower. The cause of the precipitation, while primarily, of course, the same as that of arachidic acid, *viz.* the supersaturation of the solution, differs in this, that the arachidic acid precipitates because of its very slight solubility in 90 per cent. alcohol, while the other acids come down because of the great quantity of them in the solution, as shown by the amount remaining in solution after crystallization.

As shown by the table, the melting points of the crystals from the pure lard and those from the lard containing 10 per cent. of peanut oil were about the same and hence not conclusive. The crystals were therefore redissolved in 90 per cent. alcohol and allowed to recrystallize. The melting points were again determined and found to be, pure lard 55°, lard with 10 per cent. peanut oil 77°, showing that nearly pure arachidic acid had been obtained in the latter case.

These results show that the addition of fats containing large percentages of solid fatty acids to cottonseed oil or other oils interferes materially with the detection of peanut oil by this method, and in case the presence of peanut oil is suspected in such mixtures, some modification of the method will be necessary to detect it; either to increase the proportion of 90 per cent. alcohol used, which would be objectionable, or to recrystallize the "arachidic acid" until a melting point higher than that of stearic acid is obtained, when the presence of arachidic acid is established.

SOUTH OMAHA, NEB.,
Aug. 9, 1907.

THE OCCURRENCE OF NITRATES IN VEGETABLE FOODS, IN CURED MEATS AND ELSEWHERE.

1768

BY W. D. RICHARDSON.
Received October 9, 1907.

For the purpose of this paper the term "vegetable foods" is used so as to include fruits.

The presence and amount of nitrates in vegetable foods has been noted and determined by Frühling and Grouven, by Sutter and Alwens, by E. Schultze and H. Schultze and others[1]. Frühling and Grouven state

[1] Koenig, Nahrungs und Genussmittel, Vol. 2, p. 94-95.

that nitric acid is not present in ripe seeds, but in green plants of the Gramineae and Leguminosae they found as much as 0.10 per cent. Sutter and Alwens working on beets found quantities up to 3.49 per cent. nitric acid in the dry substance. E. Schultze and H. Schultze report in beet juice from 0.13 per cent. to 0.285 per cent. nitric acid; E. Schultze and A. Ultrich in beets 0.41 to 0.407 per cent. The same authors report, that of the nitrogen in beets, 32.51 per cent., or nearly one-third the total, exists in the form of nitric acid. Sugar beets contain according to Zoller, 0.324 to 0 926 per cent. nitric acid.[1] E. Egger states that grapes contain no nitrates[2]. Hence the deduction has been made that the presence of nitrates in wine indicates watering of this product. On the other hand Spica[3] finds nitrates occurring naturally in certain musts, and hence concludes that their presence does not necessarily indicate adulteration. Schönbein reports nitric acid present in beer and wine up to 4 to 10 mg. per liter.

The results of the investigations carried out in this laboratory and detailed below, indicate a more general distribution and the presence of larger quantities of nitric nitrogen in vegetable foods than has been commonly supposed. Inasmuch as nearly all of the nitrogen demanded by plants for their growth is taken from the soil in the form of nitrates —a smaller quantity being absorbed possibly as ammonium salts—it is logical to infer that, especially in the earlier stages of growth, significant amounts of nitrates should be found in the plant organism. It would seem reasonable too, to conclude that as the plant develops and approaches maturity, elaborating the nitrate nitrogen into the form of protein bodies, smaller quantities should be found. In other words, as would be expected of a substance which enters at once into metabolic process and is at once changed thereby, the quantities of nitrates found should vary greatly, and the smallest quantities should occur in the mature plants. This seems generally to be the case. The surprising fact is, however, that in certain crops, particularly beets, considerable quantities occur in the fully matured plants. Even in ripe seeds and mature fruits the presence of nitrates has been established qualitatively and quantitatively in practically all samples analyzed. This is the more astonishing in the case of fruits inasmuch as during the ripening process the nitrates must be in contact with many reducing substances.

The presence of nitrites has also been established in a number of vegetable foods, and it seems that here as elsewhere in nature where nitrates occur, nitrites may reasonably be expected. In celery, lettuce, turnips, radishes and beets, quantitative determinations of nitrites were

[1] Koenig, Nahrungs und Genussmittel, 4th Ed., Vol. 2, p. 94-95.
[2] Chem. Centr., 1885, 71-72.
[3] Gazz. chim. ital., 1907, 37, ii. 17-22.

made by a colorimetric method employing naphthylamine and sulphanilic acid in acetic results:

Sample.	Moisture.	Nitrites in Vegetable Foods. Nitrite Nitrogen.	Equivalent to Potassium Nitrite
Celery,	94.3%	0.001 %	0.0044 %
Lettuce,	76.4	0.0023	0.0109
Turnips,	86.4	0.00013	0.00058
Radish,	93.6	0.0023	0.0109
Beets,	92.1	0.0018	0.0088

The question whether the nitrites contained in as such from the soil or whether they are produ duction processes occurring in the plant was no eral occurrence of nitrites in soils and surface possibility of their entering the plant from th has observed that beef cut in small cubes and tables such as string beans, tomatoes, potatoes color of nitrosohemochromogen, thus indicatin in the same way as when fresh beef is boiled wi or a dilute solution of nitrite itself.

In the following investigations the vegetabl purchased in the open market during the mo being obtained from wholesale and some from vegetables in the Chicago market in some instan erable distance, it was not possible in every ca fresh condition as desirable and this might le analyses were made as promptly as possible a sample at the laboratory, inasmuch as it is pr present would gradually be reduced as the samp ered.

The Schloesing-Wagner method was used for grams being the quantity of substance used. W medium except in those cases where the presence starch precluded its use and then a mixture of e hol and water was used. When working on 100 cc. of nitric oxide is the equivalent of about 0. 0.004 per cent. and the method will detect as litt of this quantity or 0.0004 per cent. to 0.0008 per fully small quantities were checked qualitatively amine and sulphuric acid working on an extrac stance, or with potassium iodide and starch so nitric oxide liberated in the determination, by su through the test solution contained in a test tube

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[1] ussmittel, 4th Ed., Vol. 2, p. 94-95.

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made by a colorimetric method employing Griess' solution of alpha naphthylamine and sulphanilic acid in acetic acid, with the following results:

Sample.	Moisture.	Nitrites in Vegetable Foods. Nitrite Nitrogen.	Equivalent to Potassium Nitrite.	Dry Basis Nitrite Nitrogen.	Equivalent to Potassium Nitrate.
Celery,	94.3%	0.001 %	0.0044 %	0.0173%	0.0772 %
Lettuce,	76.4	0.0023	0.0109	0.0097	0.0461
Turnips,	86.4	0.00013	0.00058	0.00095	0.0004
Radish,	93.6	0.0023	0.0109	0.035	0.170
Beets,	92.1	0.0018	0.0088	0.0227	0.114

The question whether the nitrites contained in vegetables are absorbed as such from the soil or whether they are produced from nitrates by reduction processes occurring in the plant was not entered into. The general occurrence of nitrites in soils and surface waters would indicate the possibility of their entering the plant from these sources. The writer has observed that beef cut in small cubes and stewed with fresh vegetables such as string beans, tomatoes, potatoes and onions, assumes the color of nitrosohemochromogen, thus indicating the presence of nitrites in the same way as when fresh beef is boiled with saltpeter-cured bacon or a dilute solution of nitrite itself.

In the following investigations the vegetables and fruits used were purchased in the open market during the month of September, some being obtained from wholesale and some from retail dealers. Because vegetables in the Chicago market in some instances come from a considerable distance, it was not possible in every case to obtain them in as fresh condition as desirable and this might lead to low results. The analyses were made as promptly as possible after the arrival of the sample at the laboratory, inasmuch as it is probable that the nitrates present would gradually be reduced as the sample grew stale and withered.

The Schloesing-Wagner method was used for all determinations, 100 grams being the quantity of substance used. Water was the extracting medium except in those cases where the presence of large quantities of starch precluded its use and then a mixture of equal quantities of alcohol and water was used. When working on 100 grams of material one cc. of nitric oxide is the equivalent of about 0.004 grams saltpeter or 0.004 per cent. and the method will detect as little as one or two-tenths of this quantity or 0.0004 per cent. to 0.0008 per cent. Nearly all doubtfully small quantities were checked qualitatively by a test with diphenylamine and sulphuric acid working on an extract of the original substance, or with potassium iodide and starch solution working on the nitric oxide liberated in the determination, by sucking the nitric oxide through the test solution contained in a test tube fitted with long inlet-

and short outlet-tubes and closed by a doubly perforated rubber stopper. This method was found to be very sensitive.

For the analytical work shown in the following tables the writer is indebted to Messrs. Scherubel, Morgan, Hansen, Lehnert, Berry, Winkley, Heath and Hale and Miss Wishart of this laboratory.

TABLE A.

NITRATES IN VEGETABLE FOODS ARRANGED IN THE ORDER OF MAXIMUM QUANTITIES FOUND.

Food	Sample No.	Moisture %	Nitrate Nitrogen %	Equivalent to Saltpeter %	Dry Basis: Nitrate Nitrogen %	Dry Basis: Saltpeter %
Beet	1	75.1	0.182	1.327	0.731	5.33
	2	90.4	0.067	0.469	0.767	5.45
	3	76.4	0.059	0.431	0.230	1.66
	4	74.3	0.034	0.249	0.137	0.99
	5	77.3	0.027	0.200	0.119	0.97
	6	65.3	0.021	0.170	0.061	0.49
	7	92.1	0.021	0.170	0.263	1.91
Egg Plant	1	92.2	0.100	0.801	1.287	10.27
	2	93.5	0.015	0.102	0.228	1.57
	3	92.9	0.011	0.080	0.155	1.13
	4	94.0	0.008	0.062	0.142	1.03
Spinach	1	90.3	0.086	0.624	0.893	6.49
	2	90.2	0.049	0.360	0.557	4.09
	3	90.1	0.042	0.307	0.427	3.13
	4	90.3	0.033	0.240	0.341	2.48
	5	89.7	0.007	0.051	0.068	0.49
Lettuce	1	92.2	0.080	0.580	1.025	7.44
	2	76.4	0.048	0.347	0.187	1.35
	3	93.0	0.028	0.210	0.400	3.00
	4	74.7	0.024	0.190	0.102	0.75
	5	92.3	0.009	0.071	0.143	1.05
Radish	1	93.2	0.069	0.500	1.020	7.41
	2	95.3	0.050	0.369	1.066	7.85
	3	95.2	0.041	0.300	0.861	6.31
	4	93.9	0.039	0.289	0.635	4.71
	5	95.4	0.037	0.266	0.798	5.79
	6	93.2	0.012	0.103	0.179	1.31
Celery	1	95.8	0.065	0.476	1.559	11.32
	2	94.5	0.034	0.245	0.617	4.48
	3	91.2	0.029	[illegible]	[illegible]	[illegible]
	4	90.6	0.024	0.180	[illegible]	[illegible]
	5	94.3	0.018	0.129	[illegible]	[illegible]
Turnip	1	91.0	0.065	0.470	[illegible]	[illegible]
	2	79.2	0.035	0.256	[illegible]	[illegible]
	3	91.7	0.011	[illegible]	[illegible]	[illegible]
	4	85.9	0.005	[illegible]	[illegible]	[illegible]
	5	86.1	0.002	0.017	[illegible]	[illegible]
Canned Spinach	1	91.3	0.044	0.318	[illegible]	[illegible]
	2	91.4	0.030	0.218	[illegible]	[illegible]

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Nitrate Nitrogen %	Equivalent to Saltpeter %	Dry Basis: Nitrate Nitrogen %	Dry Basis: Saltpeter %
0.182	1.327	0.731	5.33
0.067	0.469	0.767	5.45
0.059	0.431	0.230	1.66
0.034	0.249	0.137	0.99
0.027	0.200	0.119	0.97
0.021	0.170	0.061	0.49
0.021	0.170	0.263	1.91
0.100	0.801	1.287	10.27
0.015	0.102	0.228	1.57
[illegible]1	0.080	0.155	1.13
[illegible]08	0.062	0.142	1.03
0.086	0.624	0.890	6.46
0.049	0.360	0.557	4.09
0.042	0.307	0.427	3.13
0.033	0.240	0.341	2.48
0.007	0.051	0.068	0.49
0.080	0.580	1.025	7.44
0.048	0.347	0.187	1.36
0.028	0.210	0.400	3.00
0.024	0.190	0.102	0.75
0.009	0.071	0.143	1.05
0.069	0.500	1.020	7.41
0.050	0.369	1.006	7.85
0.041	0.300	0.861	6.31
0.039	0.289	0.638	4.74
0.037	0.266	0.798	5.78
0.012	0.103	0.178	1.51
0.065	0.476	1.559	11.32
0.034	0.245	0.637	4.62
0.029	0.200	0.509	3.51
0.024	0.180	0.307	1.91
0.018	0.129	0.481	3.50
0.065	0.470	0.721	5.21
0.035	0.256	0.168	1.23
0.011	0.090	0.134	1.08
0.005	0.039	0.038	0.28
0.002	0.017	0.017	0.13
0.044	0.322	0.487	3.70
0.030	0.218	0.350	2.54

Food	Sample No.	Moisture %	Nitrate Nitrogen %	Equivalent to Saltpeter %	Dry Basis: Nitrate Nitrogen %	Dry Basis: Saltpeter %
Canned Spinach	3	92.9	0.025	0.179	0.348	2.51
	4	91.7	0.024	0.176	0.290	2.15
	5	92.3	0.006	0.048	0.074	0.63
Parsley	1	64.1	0.032	0.241	0.125	0.93
	2	82.7	0.017	0.123	0.098	0.71
Summer Squash	1	96.7	0.031	0.225	0.939	6.82
	2	94.6	0.015	0.106	0.270	1.96
	3	96.7	0.010	0.072	0.303	2.18
	4	94.6	0.007	0.052	0.131	0.96
Onion	1	90.7	0.019	0.139	0.205	1.50
	2	86.9	0.0008	0.006	0.006	0.046
	3	76.4	0.0005	0.0038	0.002	0.016
	4	73.0	0.0004	0.003	0.001	0.011
String Beans	1	92.2	0.015	0.109	0.192	1.40
	2	90.6	0.014	0.104	0.169	1.23
	3	91.5	0.010	0.079	0.117	0.93
	4	91.1	0.001	0.009	0.013	0.10
Cucumbers	1	95.0	0.012	0.088	0.240	1.76
	2	97.3	0.002	0.017	0.085	0.63
	3	96.3	0.002	0.014	0.051	0.38
	4	96.1	0.001	0.013	0.035	0.32
	5	96.7	0.001	0.007	0.027	0.21
Leek	1	89.1	0.011	0.080	0.102	0.73
	2	87.7	0.009	0.064	0.075	0.52
Cabbage	1	93.0	0.011	0.078	0.153	1.11
	2	81.6	0.005	0.036	0.031	0.22
	3	91.6	0.004	0.029	0.046	0.34
	4	82.3	0.002	0.014	0.010	0.75
	5	93.1	0.0008	0.006	0.016	0.09
Cauliflower	1	90.2	0.009	0.064	0.090	0.65
	2	89.9	0.0006	0.004	0.006	0.036
	3	88.9	0.0006	0.004	0.006	0.036
Beans (Navy)	1	12.9	0.002	0.016	0.002	0.018
	2	13.5	0.0011	0.008	0.0014	0.010
" Kidney	3	20.0	0.0004	0.003	0.0005	0.004
" Lima	4	17.2	0.007	0.048	0.0084	0.058
Canned Pumpkin	1	91.2	0.006	0.042	0.066	0.478
German Celery	1	90.0	0.005	0.036	0.049	0.360
	2	89.9	0.004	0.030	0.041	0.297
Canned Kidney Beans	1	76.0	0.004	0.030	0.016	0.119
	2	74.4	0.0008	0.006	0.003	0.023
Bread	1	32.6	0.0009	0.007	0.001	0.010
	2	33.2	0.0006	0.005	0.0009	0.016
Tomato	1	93.8	0.002	0.0147	0.034	0.237
	2	94.2	0.0017	0.0128	0.025	0.188
	3	94.3	0.0009	0.007	0.016	0.123
	4	94.5	0.0009	0.007	0.016	0.123
	5	95.5	0.0006	0.005	0.013	0.111

Food	Sample No.	Moisture %	Nitrate Nitrogen %	Equivalent to Saltpeter %	Dry Basis: Nitrate Nitrogen %	Dry Basis: Saltpeter %
Potato	1	84.3	0.0024	0.015	0.0152	0.097
	2	80.4	0.002	0.014	0.010	0.071
	3	82.4	0.002	0.014	0.007	0.047
	4	83.4	0.0014	0.0108	0.0084	0.065
	5	80.0	0.0009	0.007	0.0045	0.035
Carrots	1	85.5	0.002	0.016	0.014	0.114
	2	85.5	0.0016	0.012	0.010	0.083
	3	93.2	0.0009	0.007	0.013	0.102
Corn Meal	1	13.3	0.0019	0.014	0.002	0.016
	2	12.9	0.0015	0.012	0.0017	0.013
Sweet Potato	1	70.0	0.0029	0.014	0.0096	0.047
	2	74.5	0.0029	0.014	0.0114	0.055
	3	73.2	0.0009	0.007	0.0033	0.026
	4	73.4	0.0009	0.007	0.0033	0.026
	5	73.8	0.0008	0.006	0.0030	0.022
	6	74.3	0.0006	0.004	0.0023	0.017
Canned Tomatoes	1	94.2	0.0017	0.013	0.029	0.220
	2	95.5	0.0004	0.003	0.009	0.067
Plums	1	86.7	0.0013	0.011	0.010	0.081
	2	77.0	0.0009	0.006	0.004	0.027
	3	88.0	0.0006	0.004	0.003	0.018
	4	75.9	0.0006	0.004	0.002	0.017
Ban.	1	73.8	0.0015	0.011	0.006	0.042
	2	70.9	0.0014	0.0106	0.005	0.035
Apples.	1	92.3	0.0013	0.0100	0.017	0.130
	2	90.2	0.0012	0.0089	0.012	0.091
	3	92.8	0.0004	0.003	0.006	0.042
	4	83.2	0.0003	0.002	0.002	0.012
Canned Peas	1	75.6	0.0013	0.0095	0.0053	0.039
	2	73.9	0.0010	0.0072	0.0038	0.028
	3	92.9	none	none	none	none
	4	90.5	"	"	"	"
Sweet Corn	1	79.2	0.001	0.009	0.005	0.043
	2	67.6	0.0007	0.005	0.003	0.020
Rice.	1	12.3	0.0013	0.009	0.0015	0.0102
	2	11.9	0.0004	0.0031	0.0005	0.0035
Canteloupe	1	90.6	0.0011	0.008	0.012	0.085
	2	93.1	0.0006	0.0045	0.009	0.065
Parsnip	1	80.0	0.001	0.008	0.005	0.039
	2	78.0	0.0008	0.006	0.0036	0.027
	3	80.1	0.0004	0.003	0.0019	0.015
Orange	1	91.4	0.0009	0.0071	0.0100	0.081
Barley	1	11.2	0.0009	0.0071	0.0010	0.008
"Cream of Wheat"	1	11.9	0.0010	0.0072	0.0013	0.009
Watermelon	1	91.5	0.0009	0.0060	0.0106	0.071
Canned Corn	1	76.1	0.0008	0.006	0.0035	0.025
	2	74.7	none	none	none	none
	3	76.3	"	"	"	"

Food	Sample No.	Moisture %	Nitrate Nitrogen %	Equi- to S- peter
Peach	1	81.0	0.0007	0.0
	2	93.5	0.0005	0.0
Oat Meal	1	11.1	0.0005	0.0
	2	11.4	0.0004	0.0
Pear.	1	80.3	0.0004	0.0
	2	83.4	none	no
Canned Asparagus	1	94.8	0.0006	0.0
	2	...	none	no
Crab Apples	1	86.1	0.0004	0.0
	2	85.3	0.0004	0.0
Lemon	1	83.3	0.0004	0.0
Oats	1	12.3	0.0004	0.0
Grapes	1	75.6	0.0004	0.0
	2	...	none	no
Grape Nuts	1	...	"	"
	2	...	"	"
Wheat	1	43.8	"	"

In the tables the nitrates are calculated as pot reasons: first, it was desired to have the figures re in vegetables and those in meats on a comparati potassium is the principal metallic constituent of analyses were made on the edible portions only.

In order to have a comparison of the quantitie in vegetables and in cured meats a number of bacons of various brands were analyzed and the following tables. It is to be noted that the qua order—although the maximum is not so great—as th

Schönbein and others have found that nitrates a ties in human urine, and they conclude that the drinking water and vegetable food (since fresh mea According to Weyl and Citron the quantity of nit a meat diet and greatest with an exclusively vege age amount is about 42.5 mg. per liter. In severa nitrate content was determined and the general con tigators confirmed. Nitrates were found normall as would be expected of herbivorous animals. I impossible, although desirable, from determinati ascertain the total quantities of nitrates ingested w as a large proportion is undoubtedly reduced first nitrogen in the digestive tract through the action according to the equations:

$$(1)\ MNO_3 \rightarrow MNO_2 + O \quad \text{and}$$
$$(2)\ NH_4NO_3 \rightarrow 2H_2O + N_2$$

Nitrate Nitrogen %	Equivalent to Saltpeter %	Dry Basis: Nitrate Nitrogen %	Dry Basis: Saltpeter %
0.0024	0.015	0.0152	0.097
0.002	0.014	0.010	0.071
0.002	0.014	0.007	0.047
0.0014	0.0108	0.0084	0.065
0.0009	0.007	0.0045	0.035
0.002	0.016	0.014	0.114
0.0016	0.012	0.010	0.083
0.0009	0.007	0.013	0.102
0.0019	0.014	0.002	0.016
0.0015	0.012	0.0017	0.013
0.0029	0.014	0.0096	0.047
0.0029	0.014	0.0114	0.055
0.0009	0.007	0.0033	0.026
0.0009	0.007	0.0033	0.026
0.0008	0.006	0.0030	0.022
0.0006	0.004	0.0023	0.017
0.0017	0.013	0.029	0.220
0.0004	0.003	0.009	0.067
[illegible] 3	0.011	0.010	0.081
0.0009	0.006	0.004	0.027
0.0006	0.004	0.003	0.018
0.0006	0.004	0.002	0.017
0.0015	0.011	0.006	0.042
0.0014	0.0106	0.005	0.035
0.0013	0.0100	0.017	0.130
0.0012	0.0089	0.012	0.091
0.0004	0.003	0.006	0.042
0.0003	0.002	0.002	0.012
0.0013	0.0095	0.0053	0.039
0.0010	0.0072	0.0038	0.028
none	none	none	none
"	"	"	"
0.001	0.009	0.005	0.043
0.0007	0.005	0.003	0.020
0.0013	0.009	0.0015	0.0102
0.0004	0.0031	0.0005	0.0035
0.0011	0.008	0.012	0.085
0.0006	0.0045	0.009	0.065
0.001	0.008	0.005	0.039
0.0008	0.006	0.0036	0.027
0.0004	0.003	0.0019	0.015
0.0009	0.0071	0.0100	0.083
0.0009	0.0071	0.0010	0.008
0.0010	0.0072	0.0013	0.008
0.0009	0.0060	0.0106	0.071
0.0008	0.006	0.0035	0.025
none	none	none	none
	"	"	"

Food	Sample No.	Moisture %	Nitrate Nitrogen %	Equivalent to Saltpeter %	Dry Basis: Nitrate Nitrogen %	Dry Basis: Saltpeter %
Peach	1	81.0	0.0007	0.005	0.004	0.026
	2	93.5	0.0005	0.003	0.008	0.046
Oat Meal	1	11.1	0.0005	0.004	0.0006	0.005
	2	11.4	0.0004	0.003	0.0004	0.003
Pear	1	80.3	0.0004	0.003	0.002	0.018
	2	83.4	none	none	none	none
Canned Asparagus	1	94.8	0.0006	0.004	0.0115	0.077
	2	...	none	none	none	none
Crab Apples	1	86.1	0.0004	0.003	0.0028	0.022
	2	85.3	0.0004	0.003	0.0027	0.020
Lemon	1	83.3	0.0004	0.003	0.0028	0.0203
Oats	1	12.3	0.0004	0.003	0.0005	0.004
Grapes	1	75.6	0.0004	0.003	0.002	0.012
	2	...	none	none	none	none
Grape Nuts	1	...	"	"	"	"
	2	...	"	"	"	"
Wheat	1	43.8	"	"	"	"

In the tables the nitrates are calculated as potassium nitrate for two reasons: first, it was desired to have the figures representing the nitrates in vegetables and those in meats on a comparative basis, and second, potassium is the principal metallic constituent of the ash of plants. All analyses were made on the edible portions only.

In order to have a comparison of the quantities of nitrates occurring in vegetables and in cured meats a number of samples of hams and bacons of various brands were analyzed and the results appear in the following tables. It is to be noted that the quantities are of the same order—although the maximum is not so great—as that found in vegetables.

Schönbein and others have found that nitrates appear in small quantities in human urine, and they conclude that their origin is probably drinking water and vegetable food (since fresh meats contain no nitrates) According to Weyl and Citron the quantity of nitrates is smallest with a meat diet and greatest with an exclusively vegetable diet. The average amount is about 42.5 mg. per liter. In several samples of urine the nitrate content was determined and the general conclusions of other investigators confirmed. Nitrates were found normally in cattle urine also, as would be expected of herbivorous animals. It would be of course impossible, although desirable, from determinations made on urine, to ascertain the total quantities of nitrates ingested with the food inasmuch as a large proportion is undoubtedly reduced first to nitrites and then to nitrogen in the digestive tract through the action of denitrifying bacteria according to the equations:

$$(1)\ MNO_3 \rightarrow MNO_2 + O \quad \text{and}$$

$$(2)\ NH_4NO_3 \rightarrow 2H_2O + N_2$$

TABLE B.

Saltpeter in Cured Meats of Various Brands as Purchased in Open Market.

Product.	Maker	Moisture per cent.	Nitrate Nitrogen per cent.	Equivalent to Saltpeter per cent.	Dry Basis. Nitrate Nitrogen per cent.	Dry Basis. Equivalent to Saltpeter per cent.
Ham	A	66.6	0.014	0.10	0.042	0.30
"	A	67.7	0.001	0.01	0.004	0.03
"	B	65.7	0.003	0.02	0.009	0.06
"	B	66.2	0.003	0.02	0.009	0.06
"	C	59.8	0.024	0.17	0.060	0.42
"	C	66.2	0.009	0.07	0.027	0.21
"	D	63.8	0.032	0.23	0.088	0.64
"	D	63.3	0.032	0.23	0.087	0.63
"	E	64.4	0.032	0.23	0.090	0.65
"	E	65.1	0.038	0.28	0.109	0.80
"	F	65.6	0.014	0.10	0.041	0.29
"	F	66.1	0.007	0.05	0.021	0.15
"	G	63.9	0.005	0.04	0.014	0.11
"	G	62.7	0.009	0.07	0.024	0.19
"	G	64.6	0.003	0.02	0.008	0.06
"	G	58.8	0.017	0.12	....	....
"	H	66.8	0.048	0.34	0.144	1.02
"	H	65.2	0.048	0.34	0.138	1.00
"	H	65.1	0.024	0.17	0.069	0.49
"	H	65.9	0.022	0.16	0.066	0.47
Bacon	A	22.7	0.008	0.06	0.010	0.078
"	B	24.9	0.0007	0.005	0.0009	0.007
"	B	19.8	0.004	0.034	0.005	0.037
"	C	12.0	0.015	0.11	0.017	0.125
"	C	25.0	0.014	0.10	0.019	0.133
"	D	21.2	0.017	0.13	0.022	0.165
"	D	17.2	0.008	0.06	0.010	0.073
"	E	17.7	0.042	0.30	0.051	0.36
"	E	19.2	0.003	0.02	0.004	0.025
"	F	12.2	0.001	0.01	0.002	0.011
"	F	18.3	0.005	0.04	0.006	0.049
"	G	19.8	0.002	0.014	0.002	0.017
"	G	21.8	0.001	0.010	0.0018	0.013
"	G	20.2	0.001	0.01	0.0018	0.013
"	G	20.5	0.001	0.01	0.0018	0.013
"	H	14.4	0.015	0.11	0.018	0.13
"	H	18.2	0.007	0.05	0.009	0.061
"	H	14.9	0.018	0.13	0.021	0.15
"	H	19.8	0.005	0.04	0.006	0.05
Dried Beef	A	62.8	0.010	0.075	0.053	0.20
"	A	59.0	0.021	0.157	0.046	0.35
"	B	52.9	0.034	0.246	0.072	0.52
"	B	56.7	0.030	0.223	0.070	0.51
"	C	61.9	0.020	0.150	0.052	0.39
"	C	56.7	0.028	0.207	0.064	0.48
"	D	60.2	0.027	0.200	0.069	0.50
"	D	65.9	0.020	0.150	0.059	0.43
"	E	66.2	0.024	0.174	0.071	0.51
"	E	63.8	0.025	0.182	0.069	0.50
"	F	54.1	0.017	0.120	0.037	0.26
"	F	60.5	0.009	0.070	0.023	0.18

ANDS AS PURCHASED IN OPEN

	Dry Basis.	
ent to peter cent.	Nitrate Nitrogen per cent.	Equivalent to Saltpeter per cent.
10	0.042	0.30
.01	0.004	0.03
.02	0.009	0.06
0.02	0.009	0.06
17	0.060	0.42
07	0.027	0.21
23	0.088	0.64
0.23	0.087	0.63
0.23	0.090	0.65
28	0.109	0.80
10	0.041	0.29
.05	0.021	0.15
0.04	0.014	0.11
07	0.024	0.19
.02	0.008	0.06
12	....	....
0.34	0.144	1.02
34	0.138	1.00
.17	0.069	0.49
16	0.066	0.47
0.06	0.010	0.078
005	0.0009	0.007
034	0.005	0.037
.11	0.017	0.125
0.10	0.019	0.133
0.13	0.022	0.165
.06	0.010	0.073
30	0.051	0.36
.02	0.004	0.025
0.01	0.002	0.011
04	0.006	0.049
014	0.002	0.017
.010	0.0018	0.013
0.01	0.0018	0.013
0.01	0.0018	0.013
.11	0.018	0.13
.05	0.009	0.061
.13	0.021	0.15
0.04	0.006	0.05
.075	0.053	0.20
.157	0.046	0.35
0.246	0.072	0.52
0.223	0.070	0.51
.150	0.052	0.39
.207	0.064	0.48
.200	0.069	0.50
0.150	0.059	0.43
.174	0.071	0.51
.182	0.069	0.50
.120	0.037	0.26
0.070	0.023	0.18

In the second equation the presence of ammonium salts is assumed. In spite of our inability to determine from the nitrates of the urine the total quantity ingested, it is interesting to know that nitrates are regularly taken into the system with the food and regularly eliminated by the kidneys, and when we consider that there is present of nitrates in various fresh vegetables, including beets, squash, parsley, turnips, radishes, celery, cabbage. lettuce, cucumbers, spinash, string beans, and egg plant the equivalent or more than the equivalent of the quantity of saltpeter ordinarily present in cured meats, and considering also the relatively small quantity of meat and particularly of salt meat included in the average diet, we must conclude that the average person obtains a larger quantity of nitrates from his vegetable than from his meat foods.

A diet consisting largely of fresh vegetables is not considered injurious and has not been known to cause injury. Yet, it is possible that on such a diet a person, as a vegetarian, might easily consume the equivalent of one to two grams and possibly more, of saltpeter daily.

In the following table a few calculations are made of the quantities of nitrates figured as saltpeter which would be consumed by a person eating the kinds and quantities specified. All of the percentages are taken from the tables. Those samples containing the largest quantities of saltpeter are purposely selected.

Vegetable	Weight consumed grams	Saltpeter %	Weight Saltpeter consumed grams
	EXAMPLE No. 1		
Beets	100	1.327	1.327
	EXAMPLE No. 2		
Egg Plant	100	0.801	2.168
Spinach	200	0.624	
Celery	25	0.476	
	EXAMPLE No. 3		
Radish	25	0.500	1.645
Turnips	200	0.470	
Lettuce	100	0.580	

Even after making a liberal allowance for nitrates extracted by boiling and pouring off the water during the preparation for the table of some of the foods shown, it will be seen that from one to two grams of nitrates figured as saltpeter could easily be consumed. In passing, it should be noted that the quantities of foods assumed to be eaten per 24 hours are moderate for a person on a mixed diet and extremely moderate for a vegetarian.

No case is known of injury traceable to the consumption of saltpeter contained in cured meats and it is known that persons may eat considerable quantities of such meats even for long periods, without injury. In the light of these facts it would seem that saltpeter as used in cured meats must be classed as a harmless substance.

It has been persistently reported[1] that the sole object in using saltpeter in the curing of meats is for the purpose of maintaining a red color. This is only one of several objects gained in using the substance. That it is an essential curing agent and that a large percentage of sour meats (in the trade sense) would result if its use were discontinued, can not be successfully controverted. While it must be admitted that saltpeter is not an antiseptic in the sense ordinarily understood by this term it appears to protect in some way the nitrogenous tissue against bacterial invasions.

Possibly the most beneficent rôle of saltpeter in the curing of meats is its transformation of what would otherwise, be anaerobic conditions, into aerobic ones in the bacteriologic sense, a full discussion of which is reserved for a later paper. I need only say here that it has been shown that aerobic bacteria will develop in the absence of air if saltpeter is present in the culture medium and that anaerobes refuse to grow in the absence of air and presence of saltpeter[2]. The fact that typical putrefaction (faülnis) is an anaerobic process[3] and that this process can be transformed into an aerobic one by the presence of saltpeter, is a most important point in the curing of meat.

The presence of nitrates in one other food product is of interest. It was anticipated that inasmuch as nitrates are normally ingested and eliminated there was a possibility of their being present in various bodily etions particularly milk, although it has been reported in the case of milk as in the case of musts and wines that the presence of nitrates indicated watering. We found nitrates present qualitatively and quantitatively in three out of four samples examined, as follows:

Sample No.	Nitrate Nitrogen %	Equivalent to Saltpeter %
1	0.0016	0.012
2	0.0011	0.007
3	0.0009	0.005
4	none	none

Summary.

1. Nitrates are of general occurrence in plants in all stages of growth, and particularly in the early stages.

2. In the mature parts of plants such as ripe seeds and fruits only small quantities of nitrates are found.

[1] Leach, Food Inspection and Analysis p. 173. Rideal, Disinfection and Preservation of Food, p. 148, 409, 417. Mitchell, Flesh Food, p. 107. Ostertag-Wilcox, Handbook of Meat Inspection, p. 803. Wiley, Foods and Their Adulteration, p. 50. Thresh and Porter, Preservatives in Food and Food Examination, p. 166. König, Chemie der menschlichen Nahrungs und Genussmittel, II, p. 518 and 443.

[2] Pakes and Jollyman, J. Chem. Soc. Trans., 1901, 322, 386, 459 and Frankland, Ibid., 1888, 373.

[3] Flugge, Die Mikroorganismen, I, 259. Lehmann and Neumann, Bacteriology, II, p. 88. Kolle und Wasserman, Pathogenen Mikroorganismen I, p. 110. Lafar, Technical Mycology, I, p. 291. Lafar, Handbuch der Technischen Mykologie, Vol. 3, p. 86.

3. In some cases notable quantities of nitrates r
plant: instance ripe beets and turnips.

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CHEMICAL LABORATORY OF SWIFT AND CO.
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CORRECTION.

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I did in the parallel experiment with bromine
of the preceding page.

NEW BOOKS.

THE ELEMENTS OF PHYSICAL CHEMISTRY. BY HA
Revised and Enlarged. New York, The MacMillan

The new edition of this excellent and well k
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the appearance, five years since, of the ear
fairly within the scope of the book has been i
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3. In some cases notable quantities of nitrates remain in the mature plant: instance ripe beets and turnips.

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5. A person on a diet consisting of fresh vegetables, wholly or largely, would consume more nitrates than one on a mixed diet consisting in part of cured meats.

6. As much as the equivalent of from one to two grams of saltpeter daily could be consumed by a person eating fresh vegetables.

7. Inasmuch as a fresh vegetable diet is entirely harmless and as no case of injury from saltpeter in cured meats is on record, saltpeter in the quantities used in cured meats must be classed as a harmless substance.

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Chicago, Ill.

CORRECTION.

On page 573, lines 5, 6 and 7, of the current volume of the Journal, is found the sentence, "3 cc. of nitric oxide gas mixed with three liters of air will efficiently bleach a kilo of flour." In writing the sentence, I intended to use the word "distinctly" instead of "efficiently," just as I did in the parallel experiment with bromine vapor given at the bottom of the preceding page.

S. AVERY.

NEW BOOKS.

THE ELEMENTS OF PHYSICAL CHEMISTRY. BY HARRY C. JONES. Third Edition, Revised and Enlarged. New York, The MacMillan Company, 1907. Price $4, net.

The new edition of this excellent and well known work has been carefully revised. Of the abundant and important material published since the appearance, five years since, of the earlier edition, whatever was fairly within the scope of the book has been incorporated in it, with the result that it has been enlarged by about a seventh part. A goodly number of references to original papers will enable the student to follow a given discussion further than the limits of the text. Naturally, the chapters on Solutions and on Electrochemistry contain the most of the new material, but half of the other chapters show additions.

The new edition well deserves the same commendation and the same welcome as that with which the earlier one was received.

EDWARD W. MORLEY.

ENTWICKELUNGSGESCHICHTE DER CHEMIE. VON A. LADENBURG. Braunschweig, Friederich Vieweg und Sohn, 1907. Price, unbound, 12 marks.

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J. Physiol. (1954) 126, 155–168

THE EFFECT OF NITRATE ON THE ACTIVE STATE OF MUSCLE

By J. M. RITCHIE

From the National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 14 *May* 1954)

The inherent response of the contractile material of muscle to a stimulus, the active state, is abrupt and reaches its full extent immediately at the end of the latent period; at this time the contractile material is capable of exerting the same tension in response to a single stimulus as it does in a tetanus (Hill, 1949). But the development of tension by whole muscle in an isometric contraction is gradual, because there is a series elastic component which must be stretched while tension is being developed and the rate of this stretching is limited by the relation between force and velocity of shortening of the contractile material. For a tetanus, where the successive elements of the stimulus maintain the active state at its full extent for the period of stimulation, Hill (1949) has shown that the form of the isometric myogram depends only on the stress-strain relation of the series elasticity and on the characteristic relation between force and velocity of shortening. In a single maximal twitch a third factor is involved, for the active state begins to decay very soon after it has been established (Hill, 1949, 1953*a*; Macpherson & Wilkie, 1954; Ritchie, 1954). Thus, the myogram in a twitch quickly diverges from that in a tetanus (Macpherson & Wilkie, 1954) and the tension developed is usually only a fraction of that in a tetanus.

Any change in the maximal twitch response of a muscle must be a reflexion of a change in one or more of these three factors determining the shape of the myogram. Adrenaline, for example, causes an increase in the twitch tension of unfatigued mammalian muscle. Goffart & Ritchie (1952) concluded that the most plausible explanation was that adrenaline caused a prolongation of the time course of the active state and suggested that such a prolongation might also account for the effects of other means by which the single maximal twitch can be increased: e.g. addition of caffeine or of potassium, previous activity, or the application of high pressure. Their evidence was of necessity indirect, because they could not use the quick-stretch technique employed by Hill (1949) on frog's muscle at 0° C to obtain the form of the active state curve, as

this procedure is difficult to apply to the more rapid mammalian muscle (Hill, 1951).

In the present experiments the effects of the nitrate ion and of caffeine on the shape of the active state curve have been studied. Both are known to augment the contraction of single maximal twitches (Kahn & Sandow, 1950; Goffart & Ritchie, 1952). Two series of experiments were made. In the one, the earliest time after the last stimulus in a tetanus at which a fall in tension could be detected was measured; this time can be taken to be that at which decay set in of the active state elicited by a single stimulus (Ritchie, 1954). In the other, the time course of the active state during its phase of decay was determined by applying quick releases to an isometrically contracting muscle at various times after a single stimulus and studying the subsequent redevelopment of tension. It will be shown later (see p. 163) that the peaks of the family of tension/time curves so obtained lie on the active state curve. The use of these two types of experiment allowed the active state curve to be studied over the greater part of its time course. Both the nitrate ion and caffeine were found to prolong the active state in much the same way as was postulated for adrenaline.

METHODS

All experiments were done at 0° C on the frog's sartorius muscle mounted on a multi-electrode stimulating assembly (Hill, 1949), by means of which the muscle could be directly stimulated at many points on its surface simultaneously. The muscle was bathed throughout the experiment in Ringer's solution (NaCl 0·675 %, KCl 0·015 %, $CaCl_2$ 0·040 %, w/v) except when this was changed for a solution in which all the NaCl had been replaced by $NaNO_3$ (0·982 %, w/v). Both solutions contained 1/50,000 tubocurarine chloride (w/v). The solutions were brought to the same pH, about 7·0 as far as could be measured with a phenol-red indicator, by the addition of sodium phosphate buffer (0·008 g P/100 ml.). The muscle was connected by a fine, straight copper wire to a tension recorder, a piezo-electric crystal (Ritchie, 1954) whose time constant was sufficiently large for its effect in the records to be ignored; in some experiments the crystal was replaced by an RCA 5734 mechano-electronic transducer valve. The voltage output from the recorder was fed to an RC or to a directly coupled amplifier, depending on the experiment, displayed on a cathode-ray oscillograph, and photographed.

In the quick-release experiments, where the redevelopment of tension after quick releases at various phases of the twitch was studied, the copper wire attached to the muscle was connected to the end of a light isotonic lever: this in turn was joined by a short flexible connexion to the tension recorder. The lever, and the wire hanging from it, were balanced by a weight close to the fulcrum: the equivalent mass of the whole system at the point of attachment was about 125 mg. A stiff brass arm, carried on the armature of a magnetic relay, was engaged with the end of the lever. The muscle was held at a length about 10 % longer than its standard length (the greatest in the body) and there was an equal amount of slack in the connexion between the end of the lever and the tension recorder. During an experiment the muscle was stimulated on the opening of a key by a Keith Lucas pendulum and the muscle contracted isometrically against the stop on the lever. At a variable time afterwards, the lever was released on the opening of a second key by the pendulum. The muscle then shortened isotonically under zero load until the slack between the lever and tension recorder was taken up, after which isometric tension developed and was recorded.

The frequency of stimulation at which fusion of the mechanical response is judged to occur depends on the sensitivity of the tension recorder. In the experiments to be described in the first

section of the results, where it was possible to detect a fall in tension of less than 2 mg in a contraction of about 80 g, i.e. about 0·002% of the total developed tension, fusion did not occur until the frequency of stimulation was more than 40–50 shocks/sec. However, in the experiments of subsequent sections of the results, where the whole of the tetanic tension curve was recorded and the sensitivity of the recorder was low, 12 shocks/sec were sufficient to give a maximum, apparently fused response.

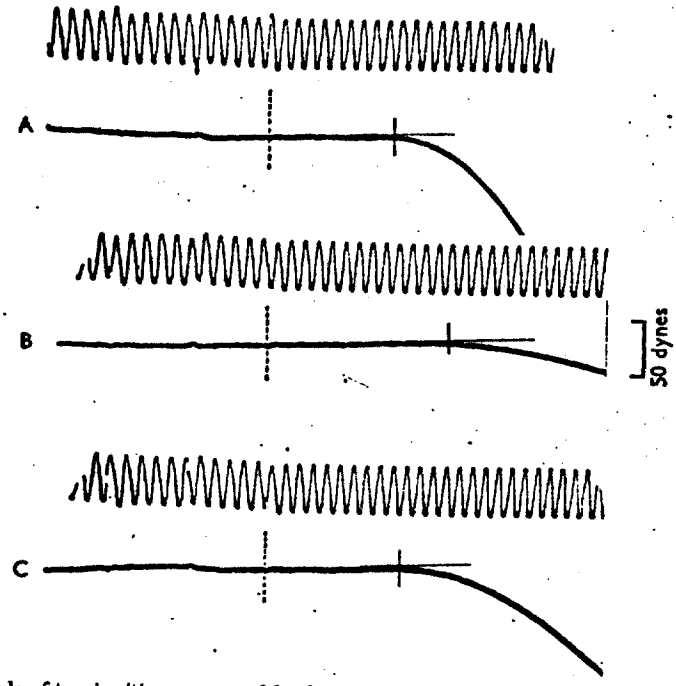

Fig. 1. Records of tension/time curves of frog's sartorius at 0° C during and after terminal stages of tetanus. A: in Ringer's solution; B: after soaking for 15 min in nitrate solution; C: after a further 15 min in Ringer's solution. In each record the interrupted vertical line marks the position of the last stimulus and the small vertical bar indicates the earliest time at which a fall in tension is detected. Upper trace in each record: 300 c/s time with pips to indicate the stimuli. Tension calibration: 50 dynes.

RESULTS

The effect of nitrate on the onset of decay of the active state

In the first series of experiments the muscle (at 0° C) was connected directly to the crystal: the amplifier had a time constant of about 100 msec and its gain was such that a change in tension of a few mg could easily be detected. Tetani of just over 1 sec duration at a frequency of about 55 shocks/sec were given at 15 min intervals. Photographs were made of the tension/time curve during and after the terminal stages of the tetani: the time after the last stimulus of the tetanus at which tension first began to fall was then measured. Two such tetani were given to allow the muscle to reach a steady state. After the second tetanus, the bathing fluid was changed and the next record obtained is shown in Fig. 1A. The Ringer's solution was then replaced by one where all the sodium

chloride had been replaced by an equivalent amount of sodium nitrate. Fig. 1B shows the record obtained after the muscle had been soaked in the nitrate solution for just under 15 min. The solution was again replaced by Ringer's solution and, after a further 15 min, the record shown in Fig. 1C was made.

These records show that the time at which a fall in tension can be detected after cessation of stimulation is increased while the muscle is soaking in nitrate solution and that the effect is reversible. In the experiment of Fig. 1, the times after the last stimulus at which relaxation occurred (marked by vertical bars) were 32 msec at the beginning of the experiment, 45 msec while soaking in nitrate, and 33 msec 15 min after the nitrate had been replaced by Ringer's solution. For five muscles the corresponding mean times were 32, 43 and 37 msec, the standard errors of the means being about 1 msec. These times can be taken to indicate the moments at which decay in the active state occurred (Ritchie, 1954).

The effect of nitrate on the falling phase of the active state

In the quick release experiments a directly coupled amplifier was used and the tension recorder was the RCA 5734. The experimental procedure adopted was as follows. Beginning soon after the muscle was mounted, it was stimulated throughout the experiment with single maximal shocks at a rate of 1/min. After about an hour, whilst stimulation continued, quick releases were given 10, 40, 60, 100, 175, 250 and, occasionally, 400 msec after consecutive stimuli. A family of five records so obtained, together with a record of the ordinary isometric twitch of the muscle at its standard length (top record), is shown in Fig. 2. Ten min after the series of quick releases had begun, a short tetanus at about 12 shocks/sec was given and the tension recorded. The whole experiment was then repeated once or twice before the Ringer's solution was replaced by the nitrate solution. A new series of records was then made, beginning 15 min after soaking in the new solution, and finally two further series were usually made when the nitrate solution had again been replaced by Ringer's solution. The timing in these experiments was accurate so as to allow a steady state to be maintained; the beginning of any individual series followed the beginning of the preceding by 25 min and the latest tetanus by 15 min.

The peak tension developed in each record of a series was then plotted against the time it took for this tension to develop. In Fig. 2 these points are indicated by vertical bars. It will be shown (see p. 163) that these points must be on the curve of the active state. A curve drawn through these points thus gives the tail of the active state curve. The full extent of this curve is, of course, equal to the maximum tetanic tension which was always determined. When two or three successive series of quick releases experiments were performed in Ringer's solution and the results plotted in this way, all points were found to be on the same curve.

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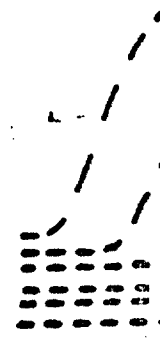

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Fig. 3 shows two such active state curves from the same muscle. Curve *A* was obtained whilst the muscle was in Ringer's solution and curve *B* whilst it was treated with nitrate. The isometric myograms of the single twitches at the standard length are shown for both curves. As seen from the figure, the twitch tension developed in the nitrate solution was about 10% larger than that in Ringer's solution and there was an increase of about 22% in the contraction time. Experiments were done on nine different muscles. In all, the twitch tension and contraction time, after soaking in nitrate solution, were increased

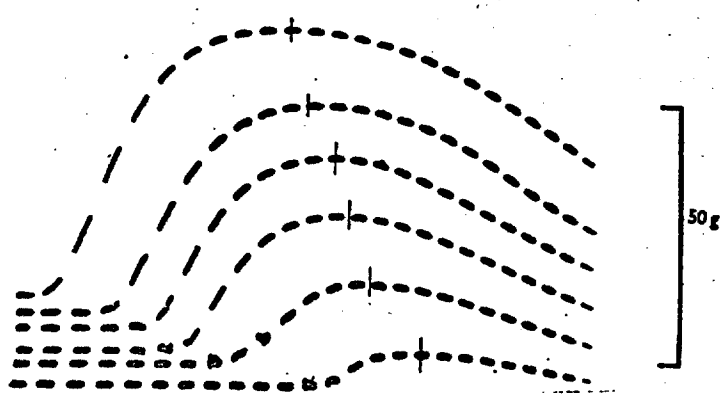

Fig. 2. Records of tension/time curves of frog's sartorius at 0° C. Standard length: 37 mm; weight: 129 mg. The stimulus begins the time base and is locked to the oscillator producing the brightness modulation of the trace (at 50 c/s). Top record is ordinary isometric twitch at standard length. Subsequent records have been preceded by quick releases of about 3 mm to the standard length timed at about 10, 40, 60, 100 and 175 msec after the stimulus: a record with a quick release at 250 msec after the stimulus and completing the series was taken on another film and is not shown here. The vertical bars mark the peaks of the contraction curves. Tension calibration: 50 g. The tetanic tension was 75 g. The blobs at the beginning of some of the records are the result of mechanical vibrations following the quick release.

by an average of 5 and 18% respectively, and the whole of the active state was prolonged. After the nitrate had again been replaced by Ringer's solution, there was always large, almost complete, recovery.

When the muscle was kept in the nitrate solution for longer than 15 min, there was little further prolongation in the active state curve. This result is consistent with the finding that the increase in twitch tension caused by the nitrate solution is maximal within a few minutes. When records of the twitch were taken every 1 or 2 min, it was found that the onset of the nitrate effect was rapid and the full extent reached by about 5 min. This is shown in Fig. 4, for the effects on the maximal twitch tension and on the contraction time. A similar result was found by Kahn & Sandow (1950) in experiments at 25° C.

In all experiments the stimulus was of about 2 msec duration and there was little change in the threshold in Ringer's solution throughout the experiment. The stimulus strength used was almost always about 10 times this threshold;

when it was doubled or reduced slightly, there was no detectable change in the active state curves obtained in Ringer's solution or in nitrate solution. The stimulus used could therefore be considered as maximal.

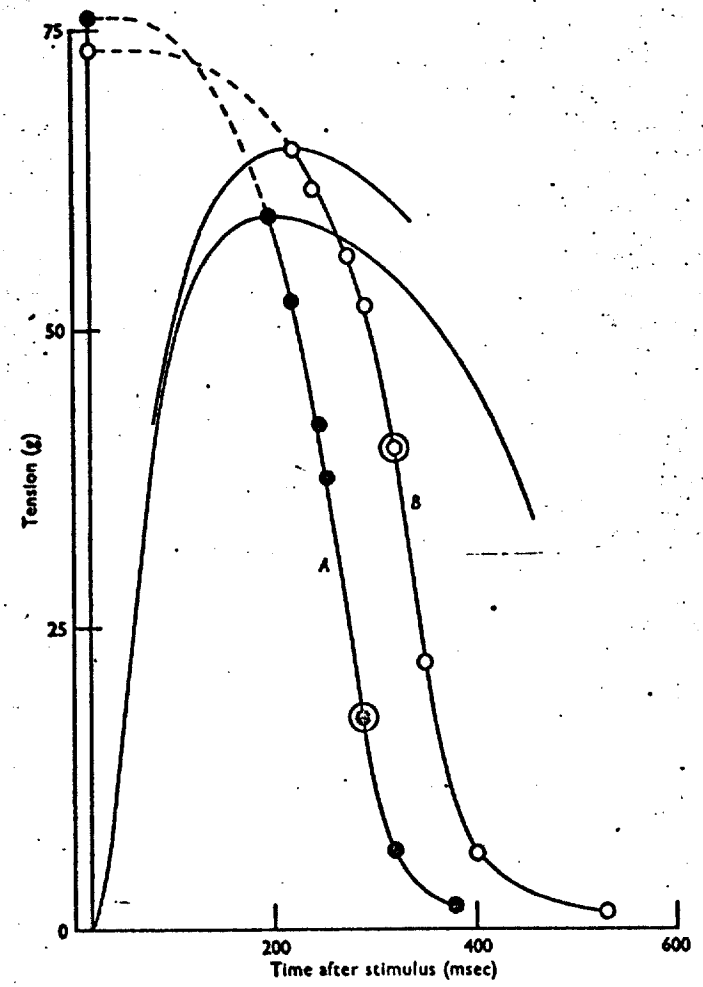

Fig. 3. Active state curves of frog's sartorius at 0° C at standard length, 34 mm. Weight of muscle: 127 mg. Curve *A*: in Ringer's solution; curve *B*: after soaking for 15 min in nitrate solution. The interrupted parts of the lines mark the region of curves not obtained experimentally. The ordinary isometric twitches at the standard length are shown both for the muscle in Ringer's and in nitrate solution. The ringed points in both curves represent the peaks of the twitch contractions which had quick releases applied at 100 msec after the stimulus.

The effect of nitrate on tetanic tension

The effect of nitrate on tetanic tension was difficult to ascertain because, even in Ringer's solution, there was a gradual, slight decrease in the tension

developed in successive tetani. However, the decrease became definitely greater when the muscle was soaked in nitrate solution. In order to give a quantitative measure to the nitrate effect, three (occasionally two) successive tetani were made with the muscle in Ringer's solution; the decline in tension gave a falling base-line to which the tension developed during a tetanus in nitrate solution was referred. The nitrate effect could then be measured, and amounted to a decrease in tension varying in thirteen experiments between 3 and 15% (mean value 7%).

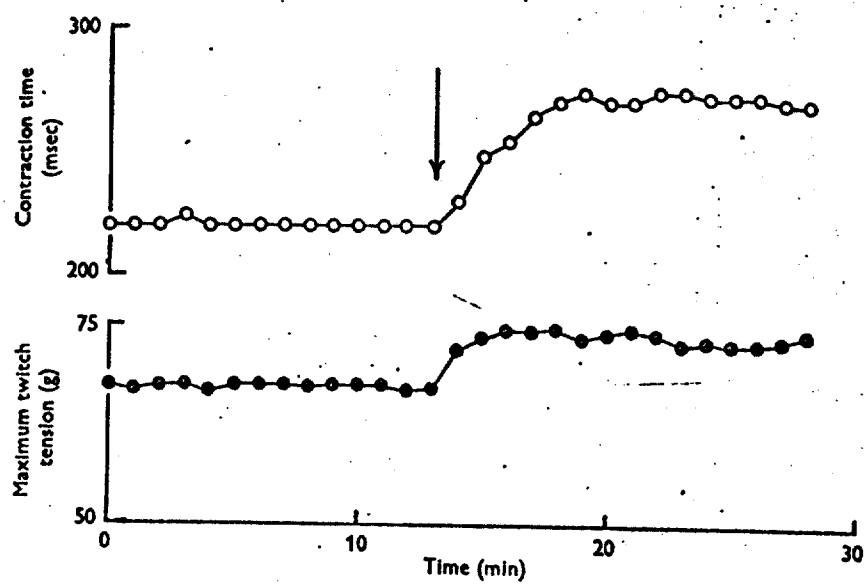

Fig. 4. The effect of nitrate on the time courses of the maximum twitch tension (solid circles) and the time after the stimulus at which this tension is reached (open circles) in a frog's sartorius at 0° C. Records of the twitch were made every minute. At the arrow the Ringer's solution bathing the muscle was replaced by nitrate solution.

The effect of caffeine on the active state

In the few experiments in which caffeine (final concentration, 6×10^{-5}, w/v) was added to the bath, the ordinary isometric twitch was found to be augmented, the active state curve prolonged, and the duration of the plateau of the active state increased. The effect of caffeine differed from that of nitrate in that recovery was much slower and less complete when the caffeine was removed, and in that a definite reduction of tetanus tension occurred only in two of the six experiments: in the others it remained unchanged. The irregular reduction of tetanic tension with caffeine had already been observed by Hartree & Hill (1924).

The effect of muscle length on the active state

The length of the muscle in all experiments so far described was the standard length, i.e. the greatest in the body. Although the length of the whole muscle was kept constant, a variable amount of shortening of the contractile component against the series elasticity must have occurred during the isometric twitches; the greater the tension developed, the greater this shortening. For

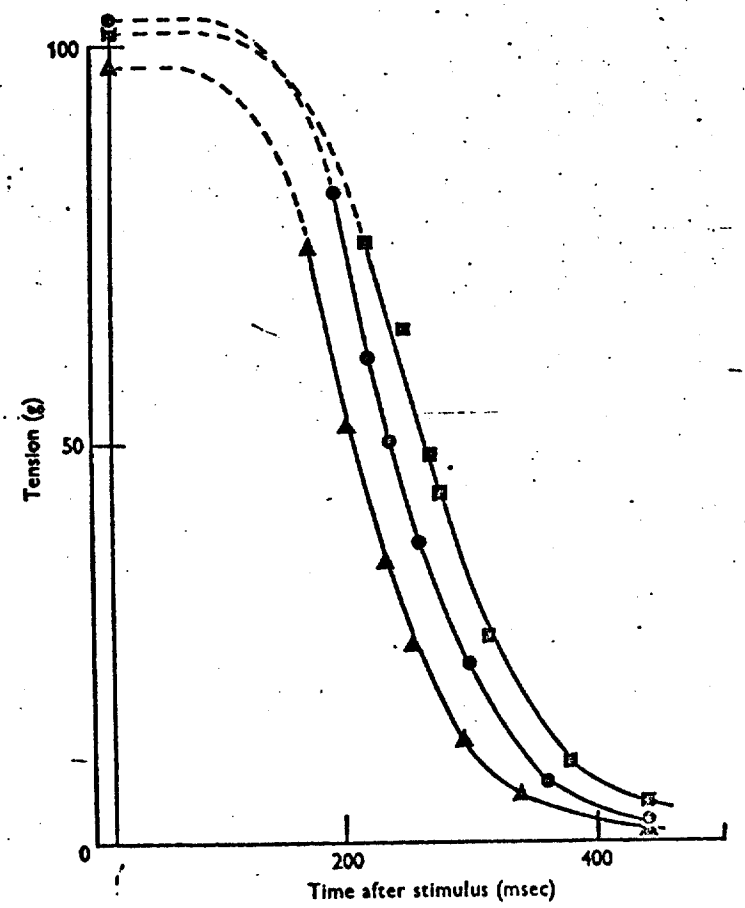

Fig. 5. Active state curves of frog's sartorius at 0° C. Standard length: 35 mm; weight: 162 mg. Circles: curve taken at standard length; triangles: at 3 mm less than standard length; squares: at 3 mm greater than standard length. Interrupted lines as in Fig. 3.

example, in the experiment of Fig. 2, the greatest amount must have occurred in the top record and the least in the bottom. The curves plotted in Fig. 3, thus, do not represent the active state at a fixed length of the contractile material.

These considerations led to experiments where active state curves were obtained not only at the standard length but also at muscle lengths about

3 mm greater and about 3 mm less. These curves are shown in Fig. 5. It is clear that the duration of the active state was dependent on the muscle length. Five such experiments were done which all gave a similar result: the shorter the muscle, the smaller was the duration of the active state. In three of these experiments the effect of nitrate on the active state was determined at the standard length and at the short length. Nitrate prolonged the active state at both lengths and increased the ordinary isometric twitch, but at the shorter length this increase was more than twice that at the standard length.

DISCUSSION

The method used in the present experiments to determine the active state curve is based on the assumption that this curve crosses the isometric myogram when the latter has reached its peak. The reasons for making this assumption are as follows. The intensity of the active state can be measured at any moment by the tension which the contractile material can maintain at that moment when neither shortening nor lengthening. In the early stages of contraction, when the intensity of the active state is high, the tension in the whole muscle rises towards it as fast as the force/velocity relation of the contractile material and the stress/strain characteristic of the series elastic component will permit. During the later stages of the contraction in a twitch, the active state has begun to decay and the rate of development of muscle tension falls below that found at a corresponding time in a tetanus; and when the intensity of the active state in the twitch has fallen to a value equal to the tension developed by the muscle, there can be no further increase in tension. After this time the active state continues to decay and muscle tension decreases, but at a slower rate than the active state because the elastic component is now stretching the contractile material. The moment at which the tension curve stops rising and begins to fall is, thus, the time at which the active state curve crosses it. It must be remembered, however, that the active state curves obtained on these grounds (e.g. Figs. 3 and 5) do not relate to a fixed length of the contractile material because no correction has been made for the variable amount of internal shortening of this material against the series compliance occurring in the quick release experiments.

The fact that the active state decays much more rapidly than muscle tension falls was first shown by Hill (1949). The present experiments give quantitative information about these changes. In the experiment illustrated in Fig. 3*A*, the peak of the tension curve occurred at about 200 msec after the stimulus; at this time the intensity of the active state was about 80% of its maximum. During the next 120 msec the intensity of the active state fell to less than 10% of its maximum, whereas the twitch tension had scarcely fallen. Similar experiments on 13 muscles showed that, at an average time of 340 msec after the stimulus, when the intensity of the active state had fallen to 5% of its

maximum, the tension by the muscle was, on the average, still 85% of the maximum twitch tension. The rapid decay of the active state, and its virtual disappearance about 350 msec after the stimulus, do seem to be related to the heat production of muscle. Up to the time that the maximum twitch tension is developed heat is produced by the muscle, rapidly at first and at a gradually decreasing rate in the latest stages. But after about 300–400 msec from the stimulus, no heat whatever is produced (Hill, 1953*b*). Such a correspondence in the time courses of the heat production and the active state occurs presumably because both are signs of the chemical reactions underlying a muscular contraction.

Both the nitrate ion and caffeine prolong the duration of the active state of muscle. This was shown to be true for the first part of the curve by the experiments where the beginning of fall in tension after a tetanus was measured, and for the last part of the curve by the quick release experiments. To obtain the intermediate part of the active state curve, i.e. the interrupted lines in Figs. 3 and 5, the quick stretch technique of Hill (1949) would have to be used. It is unlikely that this method would have yielded additional information of any great usefulness, because the present methods, when used together, allow the greater part of the active state curve to be studied. In the intermediate regions the two curves of internal activity are probably closer together than in the later stages, as indicated in Fig. 3, and therefore it might be difficult to resolve any difference in the two curves by the quick stretch technique.

A prolongation of the active state must augment the twitch tension because of the longer time allowed for the contraction to develop. But this might not be the only factor involved in the augmentation of the twitch seen in a muscle treated with nitrate solution or caffeine, because the form of the twitch is also dependent on the relation between force and the velocity of shortening of the contractile material which, according to Sandow & Mauriello (1953), is affected by nitrate ions. They analysed their results in terms of the characteristic equation, determining the relation between the force P which a muscle exerts and the velocity v at which it shortens: in this equation, $(P+a)(v+b)=\text{constant}$, P_0 is the maximum isometric tension and a and b both constants. They found that the nitrate ion caused an increase of about 50% in both a/P_0 and b, and that there was little change in P_0 and in the maximum velocity of shortening. This means that for any tension in the muscle between zero and P_0 the velocity of shortening, against an isotonic lever or against the series elastic component, would be greater in nitrate than in Ringer's solution. The rate of development of tension in a twitch would thus be larger in the treated muscle and the twitch tension would increase. It might be unwise to compare the results of their experiments too rigorously with the present ones because they found no increase in the twitch contractions at 0° C, whereas in the present experiments these contractions were always augmented in the nitrate solution. In addition,

they mention that the nitrate ion has been found to increase slightly the tetanic tension of a muscle at 25° C (Kahn & Sandow, 1950), but it is not clear whether or not the same effect was found in their own experiments at 0° C. Although a change in the force/velocity relation such as they describe would increase the maximum twitch tension, this tension would be reached at an earlier time, whereas in the present results the contraction time was always found to be increased. Such an effect on the force/velocity relation cannot therefore account for the increased twitch tension in nitrate solution.

The effect of nitrate occurs within a few minutes. This time would be insufficient for the nitrate ions to penetrate the muscle cells. In the present experiments the full effect of nitrate on both contraction time and twitch tension was reached in about 5 min, as shown in Fig. 4. According to Levi & Ussing (1949), the half renewal times of the chloride ions in the intercellular space and inside the cell are 2 and 10 min respectively for the frog's sartorius. These times should be longer for the hydrated nitrate ion, for it is larger than the hydrated chloride ion and the muscle membrane seems to be less permeable to it than to the chloride ion (Conway & Moore, 1945). It is thus unlikely that in 5 min the nitrate ion has had time to penetrate further than the intercellular spaces to any large extent. It was therefore rightly argued by Kahn & Sandow (1950) that the nitrate ion acts on the muscle membrane. The conclusion that the effect of nitrate on muscular contraction is not one directly on the contractile material, but is mediated by a change produced by nitrate on the membrane, raises the interesting problem of the relation between the electrical and mechanical response of muscle. If changes in the membrane can affect the active state of a muscle, it may be that membrane changes as recorded in the action potential, for example the after-potential, reflect the active state. The nitrate ion is known to produce one change at the membrane, a lowering of its threshold to electrical excitation (Chao, 1935; Kahn & Sandow, 1950); but it is unlikely that this accounts for the augmenting effect of nitrate on the twitch response either by recruitment of new fibres, because the effect is obtained with maximal stimuli, or by repetitive firing as there is no sign of this in records of the action potential obtained by Kahn & Sandow (1950).

A clue to the relation between the heat production and the mechanical response of a muscle might be obtained from a comparison of the effects of nitrate or caffeine on both of them. Hartree & Hill (1924) found that caffeine, which has been shown in the present experiments to prolong the duration of the active state, increases the heat production—by about 25 % for the aerobic initial heat; but they used a concentration of caffeine about 10 times as high as that used in the present experiments, and with these large concentrations contracture of the muscle occurs sooner or later. Unfortunately the effect of nitrate on heat production is unknown.

At higher temperatures nitrate has a much greater effect on the twitch

contraction than at 0° C and may cause a severalfold increase in tension (Kahn & Sandow, 1950; Hill & Macpherson, 1954). This pronounced effect of nitrate may well be explained, on the assumption that nitrate causes a prolongation of the active state at the higher temperatures similar to that shown in Fig. 3 for a muscle at 0° C but with an appropriate alteration in the time scale. The tension in a muscle in Ringer's solution reaches a maximum which is smaller than the maximum tetanic tension, because the velocity of shortening of the contractile material is low enough for the development of tension to be curtailed by the decay of the active state which begins soon after stimulation; this limitation of tension development is greater the higher the temperature, because the temperature coefficient of the velocity of shortening seems to be substantially less than that of the decay of the active state (Hill, 1951). Thus, in a muscle at 0° C, the active state elicited by a single stimulus does not decay much during the time that twitch tension develops, as shown in Fig. 3*A*, whereas, say, at 20° C the active state decays rapidly throughout a large period of tension development: this is reflected in the large twitch/tetanus ratio found at 0° C, and the small ratio at 20° C, 0·68 and 0·22 respectively (Hill, 1951). Therefore, because the decay of the active state is a major factor in determining the form of the single isometric twitch at a high temperature and a relatively minor factor at 0° C, nitrate, whose main action is to impede this decay of the active state, has a large effect on the twitch of a muscle at a high temperature and only a small effect at 0° C.

The effect of nitrate on maximum twitch tension at 0° C was found to be greater at a short muscle length than at the standard length. This influence of length on the effect of nitrate may be accounted for in the same way as for the influence of temperature, because although the duration of the plateau of the active state is independent of length (Macpherson & Wilkie, 1954; Ritchie, 1954) the rate of decay of the active state seems to be larger, the shorter the length of the muscle, as illustrated in Fig. 5. Nitrate is thus more likely to have a larger effect at short than at long muscle lengths.

An effect similar to that produced by an increase in temperature is obtained when the effect of nitrate on the development of tension is examined at 0° C after a quick release. In Fig. 3 the two ringed points on the active state curves represent the maximum tension obtained from records of single isometric twitches each of which was preceded by a quick release; both records were made under identical experimental conditions except that one was obtained while the muscle was in Ringer's solution (ringed solid circle) and the other while in nitrate solution (ringed open circle). It can be seen from the figure that nitrate has produced a much greater increase in the maximum twitch tension than in the ordinary isometric twitch. The explanation of this is that both records were obtained after quick releases had been applied to the muscle at 100 msec after the stimulus, and in consequence tension development did

not begin until about 150 msec after the stimulus; therefore, during the entire time course of each twitch, the active state was decaying and continuously modifying the development of tension. Under these conditions the decay of the active state was one of the main factors determining the form of the myogram, and because at all times during the contraction the instantaneous intensities of the active state were much higher in nitrate than in Ringer's solution, as can be seen in Fig. 3, nitrate more than doubled the maximum tension developed, although it caused only a 10% increase in the ordinary isometric twitch. The quick releases in such experiments at 0° C thus have an influence on the effect of nitrate which is analogous to that of temperature on the ordinary isometric twitch.

The effect of nitrate and caffeine on the active state curve determined directly in the present experiments is remarkably similar to the effect postulated by Goffart & Ritchie (1952) for adrenaline and other drugs. Little is known at the present time about the underlying chemical reactions which eventually give rise to the development of the active state. But the fact that not only a wide range of drugs such as nitrate, caffeine, adrenaline, potassium, but also treatments such as lowering the temperature, application of high pressure, prolong its duration accompanied by a general tendency for the tetanic tension to fall, might indicate that they all exert their effect by an action on the same system.

SUMMARY

1. The effect of nitrate and of caffeine on the active state of the frog's sartorius at 0° C was examined. The intensity of the active state was determined by applying a quick release at varying times during the muscle twitch and studying the subsequent redevelopment of tension. The duration of the plateau of full activity was determined by measuring the earliest time after the last stimulus in a tetanus at which a fall in tension could be detected.

2. The duration of the active state depends on the initial length of the muscle: the shorter the muscle, the smaller is the duration of the active state.

3. Nitrate and caffeine both prolong the active state of muscle and the plateau of full activity. This accounts for the increase produced by those substances in the twitch tension.

4. The tetanic tension is reduced with nitrate. This effect is not regularly obtained with caffeine.

Note added in proof. Since this paper has gone to press, a communication on the same subject has been made by Hill & Macpherson (1954) to the Physiological Society. In addition, I was privileged to be shown the results of experiments which they will present in a paper which is now in press and will appear in the Proceedings of the Royal Society. These authors have

recently investigated the action of the bromide and iodide ions as well as the nitrate ion on muscular contraction. Their conclusion is similar to that reached in the present paper and is that the increase in twitch tension produced by these substances is caused by a prolongation of the active state.

They found that nitrate increased not only the tension but also the heat production in a single twitch, whereas in a tetanus neither was affected. A close relationship thus exists between the time courses of heat production and the active state: both begin and end together, and substances such as caffeine and nitrate which prolong one, prolong the other.

The fact that nitrate decreased tetanic tension in the present experiments but had no effect in those of Hill & Macpherson may probably be explained by a difference in experimental technique. For example, because nitrate prolongs the active state and in consequence lowers the fusion frequency, Hill & Macpherson used smaller frequencies for tetanic stimulation of a muscle in nitrate than in Ringer's solution: in the present experiments the frequency was the same in both solutions.

The argument put forward in the present paper that the effect of nitrate is caused by a prolongation of the active state and cannot be accounted for by a change in the force/velocity relation is strengthened by their observation that in any case nitrate has no effect whatsoever on this relation.

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The effect of sodium chloride, potassium nitrate and sodium nitrite on the recovery of heated bacterial spores*

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Summary. The ability of aerobic and anaerobic spores, after various degrees of heating, to produce macrocolonies in media containing different concentrations of sodium chloride, potassium nitrate and sodium nitrite has been examined. The effect of different heat treatments, roughly from F_0 = 0·0015 to F_0 = 1·5, on the inhibitory levels of these curing ingredients, showed that the more severe treatments were capable of rendering surviving spores considerably more sensitive to subsequent inhibition by concentrations of the order found in cured meats. To produce this effect, less heating was needed with two *Bacillus* strains than with three of *Clostridium*. Sodium chloride and potassium nitrate had quantitatively similar effects. The inhibitory effect of sodium nitrite increased roughly ten-fold from pH 7 to pH 6.

Introduction

To maintain a product of good appearance and texture, the heat treatment given to canned cured meats is minimized, and is frequently so low as to have, in itself, only a small lethal effect on bacterial spores. Although viable bacterial spores, both aerobic and anaerobic, may in consequence often be isolated from such products, as is well known, their excellent record as regards unrefrigerated shelf stability and public health indicates that the spores present are incapable of normal development while they remain in the product. However, numerous papers describing the stabilizing effects of curing ingredients have, in general, thrown little light on the exact conditions and mechanism which prevent the outgrowth of such bacterial spores; although it is generally agreed that: (i) with the heat treatments commonly used, the spores will develop if the meat is without curing salts, and (ii) unprocessed spores introduced from outside are likely to grow in the cured meat; from which it has long been plain that control depends on some combined effect of heating and the salts.

Earlier workers had investigated the effect of the presence of salts on the direct effect of heating, but this influence of salts must be small. Jensen & Hess (1941) suggested that bacterial spores were more sensitive to heat in the presence of curing ingredients. More comprehensive, later, studies led to the conclusion that this was incorrect (Yesair & Cameron, 1942; Stumbo, Gross & Vinton, 1945a, b; Silliker,

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Greenberg & Schack, 1958). Similarly, unpublished results from this laboratory have shown conclusively that up to 6% (w/v) sodium chloride alone is without effect on the heat resistance of spores of the strain of *Cl. sporogenes* used below.

In the present experiments, accordingly, spores were heated to various degrees in the absence of salts, and then transferred to nutrient media containing the relevant salt in different concentrations to see how far the effect of the salts might lie specifically in hindering the subsequent development of a previously heated spore.

Materials and methods

Organisms and preparation of spore suspensions

The organisms used were *Clostridium sporogenes* PA 3679 (NCTC 8594); a strain of *Clostridium* resembling *Cl. oedematiens*, isolated from a blown can of luncheon meat (*Clostridium* I); an unidentified species of *Clostridium* isolated from bacon (*Clostridium* II), *Bacillus subtilis* and a strain of *Bacillus* (referred to as *Bacillus* F) isolated from a blown can of lightly processed bacon and known to be unusually resistant to sodium chloride and nitrite (Eddy & Ingram, 1956).

Spore suspensions of the anaerobes were prepared, using the method of the National Canners' Association (Reed, Bohrer & Cameron, 1951), in a medium comprising pork extract, peptone, tryptone, dextrose, and K_2HPO_4. After incubation for 1 week at 37°C, followed by 2 weeks at 30°C, spores were harvested by centrifugation, washed three times with distilled water, then suspended in distilled water and heated at 80°C for 30 min to kill vegetative cells. All the heated spore suspensions were stored at 1°C.

Spore suspensions of the aerobes were obtained by washing the surface growth from Hartley's digest agar contained in Roux bottles, after incubation at 37°C for 7 days. Spores were harvested in distilled water, shaken vigorously to break up clumps, then washed in distilled water three times, heated at 80°C for 30 min, and stored at 1°C, as for the anaerobes.

Heat treatment of the spores

Prior to plating in the different media, the approximate heat resistance of each spore suspension was determined, in order to arrive at a suitable level of inactivation for the purpose of the experiment.

Heat resistance was determined by total immersion of small volumes of spore suspension (0·01–0·1 ml) contained in ampoules in a thermostatically controlled water bath; or, for temperatures exceeding 100°C, in either an oil-bath or a small pressure vessel which had a rapid come-up time and could be rapidly cooled by spraying with water. Large volumes, up to 50 ml, used during the main experiments, were heated at temperatures up to 100°C by total immersion in a water-bath, and at temperatures exceeding 100°C in the small pressure vessel. The design of these experiments was not critically dependent on exact times of heating.

To permit rough comparison of the various heat treatments, they have been approximately calculated as arbitrary F_0 values, on the common assumed basis of $z = 18°F$.

Viable counts

With the anaerobic spores, decimal dilutions were made in quarter-strength Ringer's solution + 0·1% sodium thioglycollate. Replicate 1-ml samples were placed in test tubes containing 10 ml Reinforced Clostridial Agar (RCA) (Oxo Ltd, London) containing varying amounts of sodium chloride, potassium nitrate or sodium nitrite. A black glass rod was added to facilitate subsequent counting of colonies (Ingram & Barnes, 1956) and, after solidification, the contents were sealed with a deep layer of agar containing the appropriate concentration of the salt. Incubation at 37°C was continued up to 14 days.

With aerobic spores, duplicate 1-ml samples of decimal dilutions in quarter-strength Ringer's solution were pour plated in Hartley's digest agar containing varying concentrations of the curing salts. Plates were overpoured with a thin layer of aqueous agar plus the appropriate salt concentration to prevent colonies spreading. Incubation at 37°C was continued for at least 48 hr, and up to 14 days where growth was slowed by the higher concentrations of the curing salts.

In both counting methods, sodium chloride and potassium nitrate were added to the agar before the final autoclaving. Sodium nitrite was added in solution just before the medium was used (at 50°C).

Results

The effect of incubation on nitrite concentration was checked by examining plates containing initial concentrations of 0, 100, 200, 400, 800, 1200 and 1600 ppm (i.e. mg $NaNO_2$/l—this basis is used throughout). Freezing at −20°C broke the gel and the released liquid was filtered off after thawing. Nitrite was determined colorimetrically (Eddy, 1958) in duplicate plates at pH 5·6, 6·6 and 7·6. A control series, frozen and thawed immediately after pouring, was compared with plates incubated for 48 hr at 37°C before freezing. No fall in nitrite concentration occurred in this 48 hr; except at pH 5·6, when a fall of 20% occurred.

pH (checked by capillators) was little affected by incubation.

False low counts were occasionally obtained upon plating a ×10 dilution of the heated suspension. This was overcome by placing an inoculum of 1 ml in 100 ml of counting medium instead of the usual 10 ml. Washing the heated spore suspension in quarter-strength Ringer's solution failed to remove the inhibitory factors.

The effect of sodium chloride on recovery of heated anaerobic spores

Table 1 gives the recoveries of viable spores of *Cl. sporogenes* PA 3679 in RCA containing up to 10% (w/v) added sodium chloride, after heating at 80°C for 20 min

Table 1. The effect of sodium chloride on recovery of heated spores of *Cl. sporogenes* P.A. 3679

% NaCl added to RCA medium	No. ($\log_{10}$) of spores recovered/ml in medium indicated after heating at:			
	80°C for 20 min	100°C for 20 min	115°C for 5 min	115°C for 6 min
0	7·18	5·48	4·67	2·91
1·0	7·11	5·36	3·87	2·83
1·5	—	—	3·71	1·00
2·0	7·20	5·23	3·58	0·70
2·5	—	—	3·58	—
3·0	—	—	3·08	—
3·5	—	—	2·48	—
4·0	6·72	4·48	0·70	—
5·0	6·48	5·04	—	—
6·0	6·28	5·08	—	—
6·5	4·48	3·15	—	—
6·75	3·10	2·36	—	—
7·0	2·48	<1·00	—	—
10·0	<1·00	—	—	—

(F_0 = 0·0015), 100°C for 20 min (F_0 = 0·17), 115°C for 5 min (F_0 = 1·3), or 115°C for 6 min (F_0 = 1·6).

These recoveries are compared on a percentage basis in Fig. 1. In this and subsequent Figures spores given a particular heat treatment are regarded together, the number, developing as macroscopically visible colonies on medium with particular salt concentrations, being represented as percentages of the number in absence of salt.

Table 2. The effect of potassium nitrate on recovery of heated spores of *Cl. sporogenes* P.A. 3679

% KNO_3 added to RCA medium	No. ($\log_{10}$) of spores recovered/ml in medium indicated after heating at:				
	80°C for 20 min	100°C for 20 min	108°C for 15 min	115°C for 5 min	115°C for 6 min
0	6·86	5·75	5·30	4·61	2·91
0·5	6·92	5·81	—	—	—
1·0	6·99	5·77	—	4·28	3·46
1·8	—	—	5·54	—	—
2·0	6·90	5·75	—	4·64	3·15
3·0	—	—	5·43	4·00	2·23
3·5	6·69	5·43	—	—	—
4·0	—	—	5·04	3·40	1·18
5·0	5·74	4·34	2·00	<1·00	<1·00
6·0	—	—	<1·00	—	—
6·5	<2·00	<2·00	—	—	—

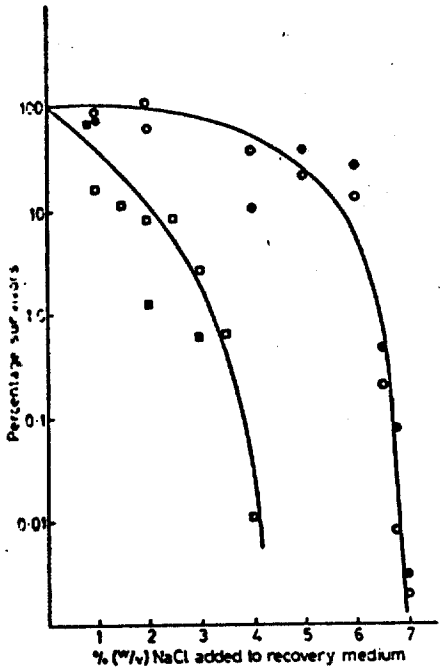

FIG. 1

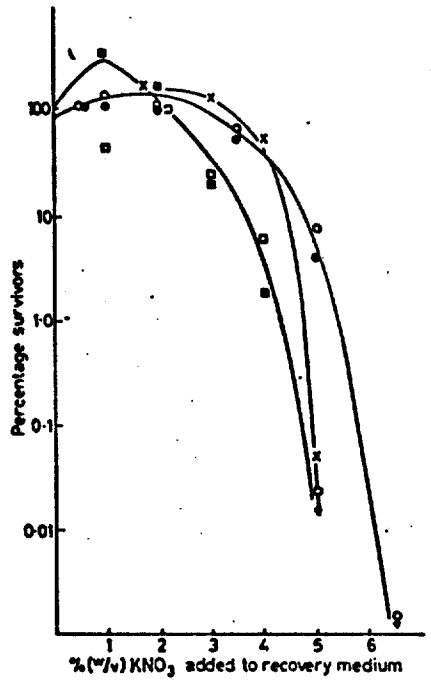

FIG. 2

FIG. 1. The effect of sodium chloride on recovery of heated spores of *Cl. sporogenes* P.A. 3679. ○, Heated at 80°C for 20 min; ●, 100°C for 20 min; □, 115° C for 5 min; ■, 115°C for 6 min.

FIG. 2. The effect of potassium nitrate on recovery of heated spores of *Cl. sporogenes* P.A. 3679. ○, Heated at 80°C for 20 min; ●, 100°C for 20 min; ×, 108°C for 15 min; □, 115°C for 5 min; ■, 115°C for 6 min.

Spores of PA 3679 which had been subjected to 80°C for 20 min (to kill only vegetative cells, i.e. nominal 100% survival), or to 100°C for 20 min (equivalent to 2·0% survival), possessed a similar tolerance to sodium chloride in the recovery medium: (w/v) depressing the recovery to 10–35% and 6½% (w/v) to 0·2–0·5%, of that in RCA. However, heating at 115°C for 5 min (0·31% survival) or 6 min (0·0055% survival) substantially increased the susceptibility of the surviving spores to subsequent inhibition in the plating medium. After these treatments, recovery in medium containing 3% (w/v) sodium chloride was reduced to about 2·5% of that in absence of salt; whereas with spores subjected to the milder heat treatments (80°C/20 min; 100°C/20 min) 3% (w/v) sodium chloride barely depressed the count significantly.

The effect of potassium nitrate on recovery of heated anaerobic spores

Table 2 gives recoveries of viable spores of *Cl. sporogenes* PA 3679 in RCA containing

up to 6·5 % (w/v) added potassium nitrate, after heating at 80°C for 20 min ($F_0 = 0{\cdot}0015$), 100°C for 20 min ($F_0 = 0{\cdot}17$), 108°C for 15 min ($F_0 = 0{\cdot}8$), 115°C for 5 min ($F_0 = 1{\cdot}3$) or 115°C for 6 min ($F_0 = 1{\cdot}6$). These recoveries are compared on a percentage basis in Fig. 2.

Spores heated at 80°C for 20 min (i.e. 100% survival) or at 100°C for 20 min (7·7% survival) possessed a similar tolerance to potassium nitrate in the recovery medium, 4% (w/v) being slightly inhibitory and higher concentrations markedly so. After heating at 108°C for 15 min (2·7% survival) there was no change in the tolerance of surviving spores to concentrations of potassium nitrate up to 4% (w/v) but survivors were more sensitive to higher concentrations. In the presence of 5% (w/v) potassium nitrate, recovery was only 0·05% of that in RCA without nitrate, compared with 4–7·5% after the two lighter heat treatments.

More drastic heating at 115°C for 5 min (0·56% survival) and 115°C for 6 min (0·011% survival) slightly increased the sensitivity to potassium nitrate in the plating medium but the overall sensitization was considerably less than that caused by sodium chloride.

There was repeated evidence of a small stimulation of recovery of PA 3679 by concentrations of potassium nitrate up to 2% (w/v) after heating at 80°C for 20 min and 100°C for 20 min, a more marked effect of 2 and 3% (w/v) after heating at 108°C for 15 min, and a very marked stimulation by 1% (w/v) after heating at 115°C for 6 min (Fig. 2).

There was no such stimulation with *Clostridia* I and II, results for which are presented in Tables 7 and 8. In other respects, their behaviour was similar to that of *Cl. sporogenes* PA 3679.

The effect of sodium nitrite on recovery of heated anaerobic spores

Table 3 summarizes the recoveries of spores of PA 3679 in RCA containing up to 1200 ppm of sodium nitrite at pH values between 5·6 and 7·5; data compared on a percentage basis in Fig. 3. Whether heated at 80°C for 20 min ($F_0 = 0{\cdot}0015$) or 100°C for 20 min ($F_0 = 0{\cdot}17$), the increase in sensitivity with decreasing pH is very marked. After 80°C for 20 min, recovery at pH 7·5 is depressed to about 1% of that in RCA by 300–400 ppm sodium nitrite. Reducing the pH to 7·0 results in a similar depression of recovery by 125–200 ppm, and at pH 6·5 a slightly lower concentration is equally effective. After heating at 115°C for 5 min ($F_0 = 1{\cdot}3$) growth of the surviving spores (0·9%) was inhibited rather more readily at pH 6·8 and 6·2, about 50 ppm being sufficient to reduce recovery to 1% of that in RCA.

Similar results were obtained with *Clostridium* I after heating at 80°C for 20 min or 108°C for 20 min ($F_0 = 1{\cdot}0$), recovery at pH 6·8 equivalent to 1% of that in nitrite-free RCA being obtained using 350–400 ppm sodium nitrite in the former instance, and 75 ppm in the latter.

TABLE 3. The effect of sodium nitrite on recovery of heated spores of *Cl. sporogenes*

pH	ppm $NaNO_2$ added to RCA medium	No. (log_{10}) of spores recovered/ml in medium indicated after heating at:		
		80°C for 20 min	100°C for 20 min	115°C for 5 min
7·5	0	6·65	5·95	—
	200	6·38	4·64	—
	400	5·56	4·08	—
	800	3·40	2·78	—
	1200	<2·00	<2·00	—
7·0	0	6·72	5·78	—
	100	5·85	4·04	—
	200	4·79	3·91	—
	400	<2·00	<2·00	—
6·8	0	—	—	4·61
	10	—	—	3·60
	20	—	—	3·40
	30	—	—	3·30
	40	—	—	2·30
	50	—	—	1·78
	60	—	—	1·30
	70	—	—	<1·00
6·5	0	6·75	5·52	—
	50	4·42	4·94	—
	100	<2·00	<2·00	—
6·2	0	—	—	4·61
	10	—	—	3·57
	20	—	—	2·95
	30	—	—	2·46
6·0	0	6·42	4·23	—
	50	<2·00	<2·00	—
	100	<2·00	<2·00	—
5·6	0	—	—	2·48
	10	—	—	<1·00
	20	—	—	<1·00

The effect of sodium chloride on the recovery of heated aerobic spores

Table 4 summarizes the effects of including up to 16% sodium chloride in the recovery medium for *B. subtilis*, heated at 80°C for 20 min ($F_0 = 0{\cdot}0015$) or at 95°C for 45 min ($F_0 = 0{\cdot}12$); and for *Bacillus* F after 80°C for 20 min or 90°C for 30 min ($F_0 = 0{\cdot}025$). Comparison on a percentage basis is made in Fig. 4. The *Bacilli* were considerably more tolerant than the *Clostridia* studied to the inclusion of sodium chloride in

TABLE 4(a). The effect of sodium chloride on recovery of heated spores of *Bacillus subtilis*

% NaCl added to medium	No. ($\log_{10}$) of spores recovered/ml in medium indicated after heating at:	
	80°C for 30 min	95°C for 45 min
0	8·83	6·34
1	8·96	5·46
2	8·96	5·63
4	8·78	5·20
8	8·11	4·49
12	4·30	<1·00
16	<1·00	<1·00

TABLE 4(b). The effect of sodium chloride on recovery of heated spores of *Bacillus* F

% NaCl added to medium	No. ($\log_{10}$) of spores recovered/ml in medium indicated after heating at:	
	80°C for 30 min	90°C for 30 min
0	8·95	6·56
2·5	8·89	5·76
5·0	8·79	4·46
10·0	7·91	3·11
15·0	7·17	2·20

TABLE. 5. The effect of sodium nitrite and pH on the recovery of heated spores of *Bacillus subtilis*

ppm $NaNO_2$ added to medium	No. ($\log_{10}$) of spores recovered/ml in medium indicated after heating at:							
	80°C for 30 min				95°C for 45 min			
	pH 7·6	pH 6·6	pH 6·2	pH 5·6	pH 7·6	pH 6·6	pH 6·2	pH 5·6
0	8·87	9·00	8·89	8·83	6·30	6·55	6·04	6·23
100	8·85	8·64	—	8·76	6·25	6·11	—	5·65
200	8·87	8·55	8·91	5·00	5·55	6·08	5·74	<1·00
400	8·49	8·59	8·74	<1·00	5·47	6·42	5·54	—
600	—	—	3·71	—	—	—	1·83	—
800	8·46	8·43	<1·00	—	5·17	5·55	<1·00	—
1600	8·74	8·43	—	—	5·47	4·88	—	—

TABLE 6. The effect of sodium nitrite and pH on recovery of heated spores of *Bacillus* F

ppm $NaNO_2$ added to medium	No. (log_{10}) of spores recovered/ml in medium indicated after heating at:											
	80°C for 30 min				90°C for 30 min				100°C for 35 min			
	pH 7·6	pH 6·6	pH 6·2	pH 5·6	pH 7·6	pH 6·6	pH 6·2	pH 5·6	pH 7·6	pH 6·6	pH 6·2	pH 5·6
0	8·81	8·59	8·89	9·92	6·52	5·92	5·76	5·23	3·11	2·90	2·74	2·11
100	8·83	8·30	—	8·76	6·17	6·00	—	4·74	—	—	—	1·00
200	8·76	8·58	8·80	—	5·97	6·17	5·73	—	—	—	2·14	—
400	8·70	8·63	8·71	—	6·04	5·89	5·81	—	3·28	3·06	<1·00	—
600	—	—	2·56	—	—	—	—	—	—	—	—	—
800	8·53	8·63	1·54	—	5·85	6·14	—	—	3·23	3·11	—	—
1200	—	—	—	—	—	5·50	—	—	3·23	2·78	—	—
1600	8·53	8·76	—	—	6·07	—	—	—	3·14	2·40	—	—

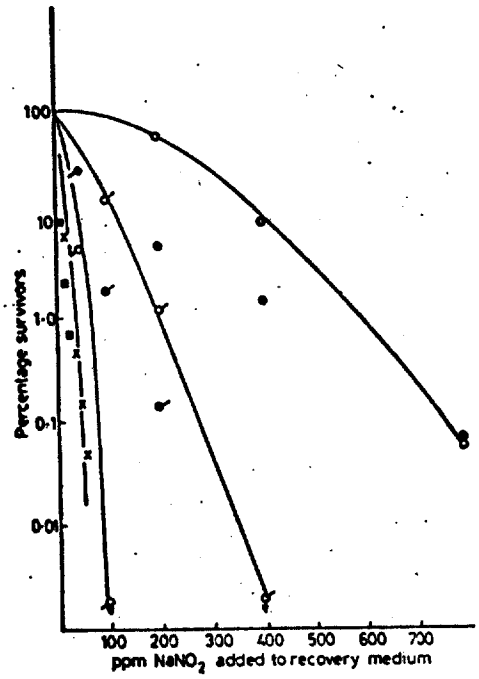

FIG. 3

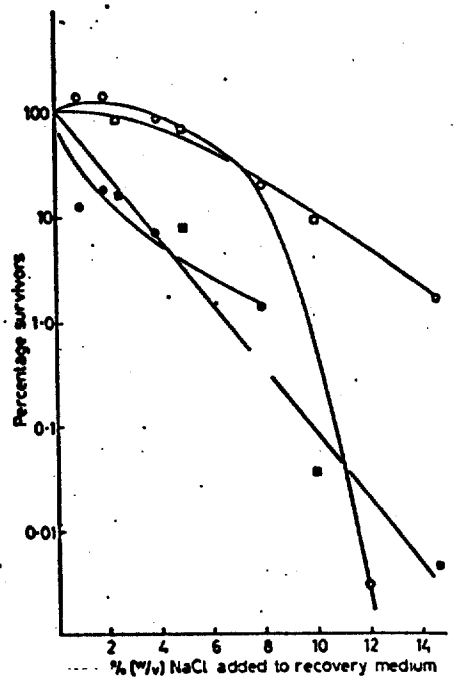

FIG. 4

FIG. 3. The effect of sodium nitrite on recovery of heated spores of *Cl. sporogenes* P.A. 3679. ○, Heated at 80°C for 20 min; ●, 100°C for 20 min: no tag, pH 7·5; right-hand tag, pH 7·0; left-hand tag, pH 6·5; ×, 115°C for 5 min (pH 6·8); ■, 115°C for 5 min (pH 6·2).

FIG. 4. The effect of sodium chloride on recovery of heated spores of *B. subtilis* and *Bacillus* F. *B. subtilis*: ○, Heated at 80°C for 30 min; ●, 95°C for 45 min. *Bacillus* F: □, Heated at 80°C for 30 min; ■, 90°C for 30 min.

the recovery medium; but after heating, a reduction in this tolerance was observed, similar to that observed with the *Clostridia*. The inclusion of up to 4% sodium chloride in the recovery medium was without effect on the recovery of *B. subtilis* spores subjected to a treatment equivalent to $F_0 = 0{\cdot}0015$; but after heating to $F_0 = 0{\cdot}12$, recovery in a medium containing 1, 2 or 4% sodium chloride was reduced to about 10% of that in RCA.

A similar reduction in salt tolerance was evident with *Bacillus* F after heating at only $F_0 = 0{\cdot}025$ (Fig. 4).

The effect of sodium nitrite on recovery of heated aerobic spores

Tables 5 and 6 summarize data on the recovery of spores of *Bacillus subtilis* and *Bacillus* F, in the presence of concentrations of sodium nitrite up to 1600 ppm at pH values within the range 5·6–7·6, after different heat treatments.

At pH 7·6, after heating at 80°C for 30 min (F_0 = 0·0024), spores of *B. subtilis* were able to produce colonies in the presence of nitrite concentrations as great as 1600 ppm (Fig. 5), and this also occurred at pH 6·6. Reduction of the pH to 6·2 had a remarkable effect on the ability of spores surviving the heat treatment to produce colonies. Recovery was similar at concentrations up to 400 ppm, but 600 ppm permitted less than 0·001% of the surviving spores to produce colonies. At pH 5·6 the inhibitory effect of sodium nitrite was even more marked, and in the presence of 200 ppm sodium nitrite, recovery was only 0·016% of that in the absence of nitrite. Heating at 95°C for 45 min (F_0 = 0·12) did not greatly affect this recovery pattern (Fig. 6) except that recovery was slightly lower at pH 7·6 and 6·6 than after the lighter heat treatment.

Similar trends were observed with *Bacillus* F after heating at 80°C for 30 min (F_0 = 0·0024), 90°C for 30 min (F_0 = 0·025), or 100°C for 35 min (F_0 = 0·27). After F_0 = 0·0024 (Fig. 7) concentrations of sodium nitrite up to 1600 ppm did not greatly depress

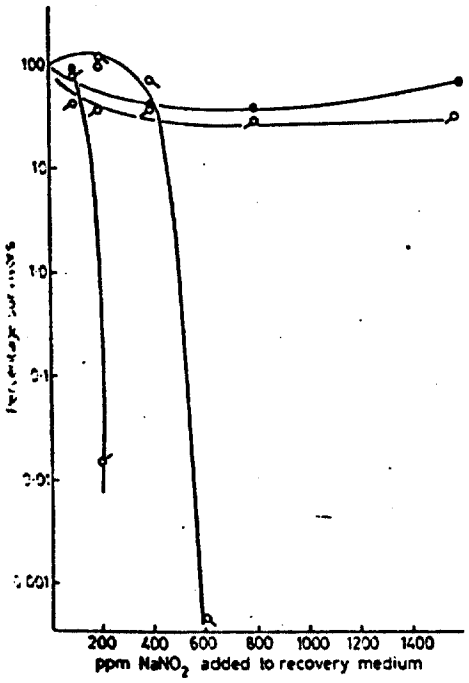

FIG. 5

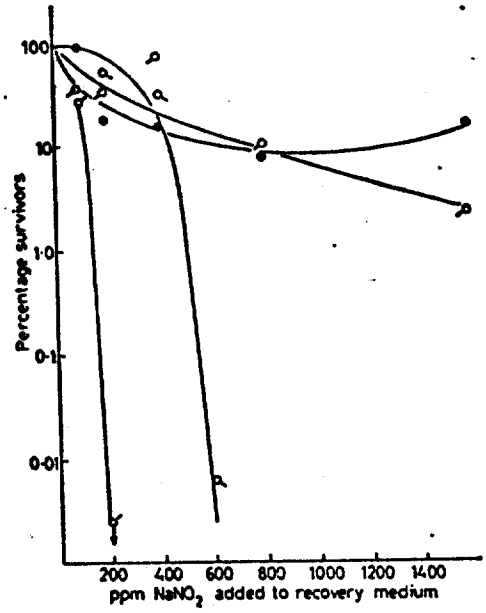

FIG. 6

FIG. 5. The effect of sodium nitrite on recovery of spores of *B. subtilis* heated 80°C for 30 min. ●, pH 7·6; ○: tagged bottom left, pH 6·6; tagged bottom right, pH 6·2; tagged top right, pH 5·6.

FIG. 6. The effect of sodium nitrite on recovery of spores of *B. subtilis* heated at 95°C for 45 min. ●, pH 7·6; ○: tagged bottom left, pH 6·6; tagged bottom right, pH 6·2; tagged top right, pH 5·6..

Table 7. The effect of sodium chloride, potassium nitrate and sodium nitrate on recovery of heated spores of *Clostridium* I [No. ($\log_{10}$) of spores recovered/ml in medium indicated after heating for 20 min at 80°C (A) and 108°C (B)]

% NaCl added to RCA medium	A	B	% KNO_3	A	B	ppm $NaNO_3$ (pH 6·8)	A	B
0	7·62	3·25	0	7·62	2·57	0	7·62	3·25
0·25	—	3·23	1	7·46	2·54	10	—	2·30
0·50	—	2·98	2	—	2·15	20	—	2·17
0·75	—	2·77	3	7·07	1·54	30	—	1·70
1·00	7·36	2·47	4	—	<1·00	40	—	1·70
1·50	—	1·81	5	6·77		50	7·59	<1·00
2·0	—	1·00	7	2·30		100	7·11	
3·0	7·11	—	8	1·40		150	6·44	
5·0	6·79	—	9	1·47		200	5·34	
7·0	5·97	—	10	2·44(?)		250	3·34	
8·0	<1·00	—				300	3·38	
						350	3·17	
						400	2·66	
						450	2·38	
						500	2·23	
						600	2·14	

Table 8. The effect of sodium chloride, potassium nitrate and sodium nitrite on recovery of heated spores of *Clostridium* II

% NaCl added to RCA medium	Spores*	% KNO_3 added to RCA medium	Spores*	pH	ppm $NaNO_2$ added to RCA medium	Spores*
0	3·61	0	4·36	6·8	0	4·11
					50	3·47
1	3·14	1	4·30		100	2·30
					150	1·00
3	3·42	3	4·30			
				6·0	0	3·00
5	2·59	5	2·30		50	<1·00
6	2·20	6	<1·00	5·8	0	2·70
					50	<1·00

* No. ($\log_{10}$) of spores recovered/ml in medium indicated after heating at 115°C for 5 min.

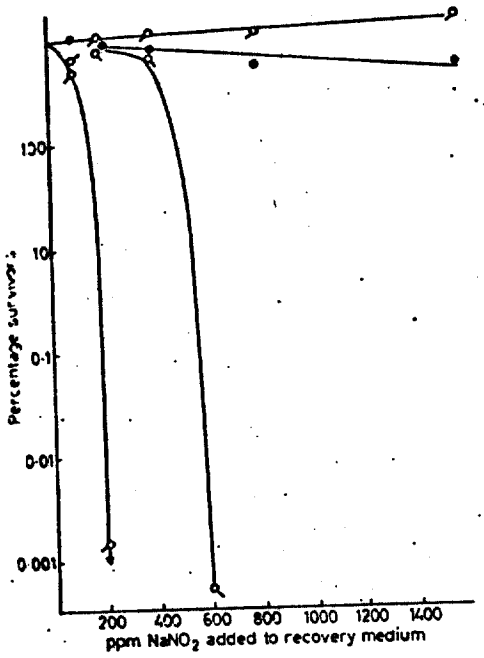

FIG. 7

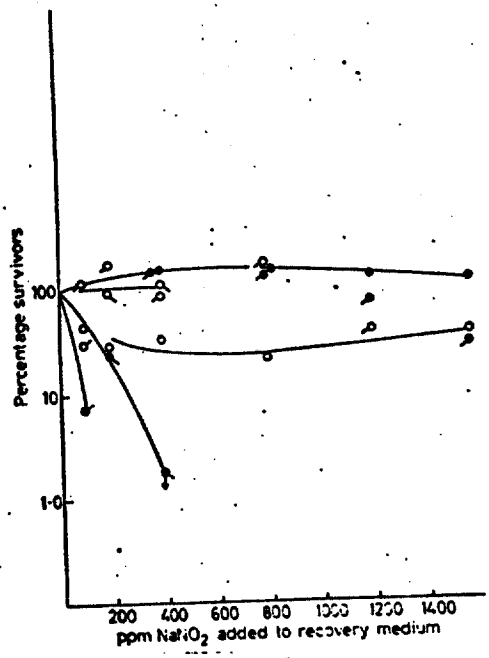

FIG. 8

FIG. 7. The effect of sodium nitrite on recovery of spores of *Bacillus* F heated at 80°C for 30 min. ●, pH 7·6; ○: tagged bottom left, pH 6·6; tagged bottom right, pH 6·2; tagged top right, pH 5·6.

FIG. 8. The effect of sodium nitrite on recovery of heated spores of *Bacillus* F. ○, Heated at 90°C for 30 min; ●, 100°C for 35 min; no tag, pH 7·6; tagged bottom left, pH 6·6; tagged bottom right, pH 6·2; tagged top right, pH 5·6.

the recovery of surviving spores at pH 7·6 and 6·6 However, a reduction in pH to 6·2 resulted in virtually all the surviving spores becoming sensitive to concentrations of sodium nitrite over 400 ppm, yet remaining unaffected by concentrations up to 600 ppm. A further drop in pH to 5·6 resulted in no growth in concentrations exceeding 100 ppm. After more severe heating, $F_0 = 0{\cdot}27$ (Fig. 8), *Bacillus* F still gave good recovery at pH 7·6 and 6·6 even in the presence of 1600 ppm sodium nitrite. The effects of sodium nitrite at pH 6·2 and 5·6 were similar to those on *Bacillus subtilis*.

Discussion

The processing of canned cured meats, in relation to bacterial spores, has been reviewed by Riemann (1963). Both aerobic and anaerobic spores are present in cured meats (e.g. Brown, Vinton & Gross, 1960); and although numbers of the latter may be as low as 10^{-2} to 10^{-3} per g (Riemann, 1963; Steinkraus & Ayres, 1964), it appears from the relative ease with which *Bacilli* and *Clostridia* may be isolated from some products

(Ingram, 1952; Ingram & Hobbs, 1954), that the incidence of contamination is at times relatively high. Spores of *Bacilli* are reported to be inactivated by approximately $F_0 = 0{\cdot}1$–$0{\cdot}6$ and those of *Clostridia* by $F_0 = 0{\cdot}6$–$1{\cdot}0$ (Brown *et al.*, 1960). The lighter heat treatments used for cured meats are therefore unlikely to have much effect on the load of *Clostridium* spores, and significant numbers of viable spores might be expected to survive in the finished product as is actually observed.

It seems clear, from the observations in this paper, that an important factor in the stability of such moderately heated cured meats arises from the ability of curing salts to interfere with some stage in the germination and development of surviving heated spores, at concentration levels which would be ineffective with unheated spores. It is reasonable to suppose that the phenomenon is a general one, since it has been observed with distinctly different strains of *Bacillus*, and several of *Clostridium*. It would seem worth while to verify that it occurs, in particular, with *Cl. botulinum* and *Cl. perfringens*.

It is equally clear that the details may differ in different cases; for example, the effective concentrations obviously depend on the organism in question, while we have seen mild activation phenomena with one strain of *Clostridium* only. A broad comparison of Figs. 1 and 4 suggests that *Bacilli* are rendered more sensitive to sodium chloride by lower heat treatments than are *Clostridia*. Whereas $F_0 = 0{\cdot}12$ (*B. subtilis*) and $F_0 = 0{\cdot}025$ (*Bacillus* F) caused a considerable reduction in salt tolerance (Fig. 4), $F_0 = 0{\cdot}17$ did not produce a detectable change in the salt tolerance of *Cl. sporogenes* (Fig. 1). Thus, although initially *Bacilli* are often present in greater numbers than *Clostridia*, their spores are generally more sensitive to heat and their tolerance to salt is considerably reduced by comparatively mild heating. It is interesting that this heat sensitization was most pronounced with the organism (*Bacillus* F) otherwise most resistant to sodium chloride. Obviously, organisms with both a high inherent sensitivity and, in addition, high degree of sensitization by heat, are not likely to be recovered from heated cured products.

The above observations could explain the fact, well attested in practice, that a process which controls small numbers of spores is likely to fail with larger numbers. Any particular combination of heating and curing ingredients will, apparently, permit only a certain minute proportion of the spores to develop. If the initial numbers are so small that this minute proportion of them is a negligible number, the process will be satisfactory; but as the initial numbers rise, so will the importance of this fixed proportion, until the absolute number of failures becomes appreciable and the process is deemed to fail.

The findings of Shipp (1962) that mildly heated spores of *Cl. sporogenes* germinated in samples containing 3% sodium chloride, or 2·7% sodium chloride + 0·5% sodium nitrate, but failed to grow in a residual concentration of 167 ppm sodium nitrite at pH 5·7 can be explained entirely by reference to the present study.

In the case of sodium chloride Gould (1964) has shown, with lightly heated spores

several species of *Bacillus*, that salt concentrations of about 15% are needed to prevent germination, while 4–7% (depending on the species) suffice to prevent out-growth. Similarly with spores of *Clostridium*, circumstantial evidence suggests that the inhibition occurs at the outgrowth of the germinated spore. Mundt, Mayhew & Stewart (1954) observed germination of 90% of the spores of *Cl. sporogenes* in the presence of 8% sodium chloride, and at pH values down to 5·3; but this germination was not followed by vegetative growth. Riemann (1957) found that spores which have lost their heat resistance remain resistant to sodium chloride until outgrowth occurs. Vegetative cells are much more sensitive to sodium chloride than are spores (Mundt & Kitchen, 1951). All this suggests that the effectiveness of sodium chloride, in combination with heat as with radiation (Roberts, Ditchett & Ingram, 1965), lies in preventing outgrowth of sensitized spores after they have germinated, because the concentrations used in the present study appear too low to have inhibited germination, though this point awaits direct confirmation.

From the broad similarity between the effects of sodium chloride and potassium nitrate, it seems possible that their influence is in some way osmotic, and perhaps depends fundamentally on the activity of water in the system. This point also deserves more careful verification. In any case, the concentrations of potassium nitrate in cured meats are much below (*ca.* one-tenth) those of sodium chloride, hence it appears that its separate effect must be negligible in practice. Roughly, its effect might be regarded as that of an equal concentration of sodium chloride; as has previously been indicated by others, though on rather different evidence (Bullman & Ayres, 1952).

The effect of sodium nitrite has certainly a different basis; not only are the concentrations involved much smaller, but they are very much dependent on pH. Where we were fortunate to obtain a suitable range of observations, for example in Fig. 3, they appeared consistent with the view that the effective agent is undissociated nitrous acid. For example, Fig. 3 indicates that for spores heated at 80°C for 20 min, the respective concentrations of nitrite needed to reduce the percentage survival to 1%, at pH 7·5, 7·0 and 6·5 respectively, were about 575, 200 and 50 ppm; which is not far from the concentration ratio $10 : \sqrt{10} : 1$ which would be expected on that basis. Similar, though less well defined, relations were indicated with other organisms, for example in Fig. 8 for a *Bacillus*. Gould (1964) has reported a similar pH-dependent effect of nitrite (0·03% $NaNO_2$ at pH 6) in inhibiting outgrowth, and at slightly higher concentrations germination, of *Bacillus* spores. It appears fortunate that the pH of canned meats is normally not far from 6, a degree of acidity sufficient to make nitrite highly effective, but not sufficient to make it decompose rapidly; quite small differences in pH, arising for example from additions of phosphate, might apparently have an important effect on the bacteriological stability of cured meats.

Combinations of these curing salts might conceivably have effects greater than the sum of those indicated here for the individual substances. It also seems that the effect

of salt itself might well bear some relation to pH, though Gould (1964) did not observe this, and that it might be influenced by incubation temperature (Schmidt & Segner, 1964). These matters too deserve more extensive investigation. The present experiments have altogether neglected the effect of temperature, having been conducted for convenience entirely at 37°C, near the optimum for the bacteria tested. It may be that storage temperature is not, at least in the U.K., a factor obviously contributing towards the good commercial and public health record of most canned cured products, because refrigerated storage is never enforced, and seldom even recommended. But it seems probable that the sensitization here reported might be affected, at least in magnitude, by the temperature prevailing while the damaged spore is struggling against the inhibitory salts; because such phenomena are known with both heated (Wheaton & Pratt, 1961) or irradiated (Wheaton, Pratt & Jackson, 1959; Freeman & Bridges, 1959) bacteria on ordinary media.

Nevertheless, though various supporting investigations remain to be made, the phenomena described here seem, for the first time within our knowledge, to offer a quantitatively satisfying explanation of the surprising effectiveness of modest heat treatments in preventing the development of bacterial spores in cured meat products.

Acknowledgments

This work was largely carried out in 1958–60 by Miss J. M. H. Stapp, working under the supervision of the late Dr B. P. Eddy, the project having been suggested by M.I. The data have recently been completed, analysed and prepared for publication by T.A.R.

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VITAMIN A DESTRUCTION BY NITRITE *IN VITRO* AND *IN VIVO*[1]

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DIETARY nitrite has been shown to reduce liver vitamin A storage in chicks (Sell and Roberts, 1963) and in rats (O'Dell *et al.*, 1960; Smith *et al.*, 1961; Emerick and Olson, 1962). However, in cattle and sheep Smith *et al.* (1962) and in sheep Cline *et al.* (1962) reported no significant change in liver vitamin A level when nitrates were fed. Conversely, Goodrich *et al.* (1962) reported a significant lowering of liver vitamin A stores in sheep receiving $NaNO_3$.

Pugh *et al.* (1962), using an *in vitro* technique, presented evidence that vitamin A or carotene destruction in the presence of nitrite is dependent on pH. At pH 1–3 destruction was very rapid and at pH 5–7 relatively little destruction occurred. Furthermore, Olsen *et al.* (1963) reported that the addition of nitrite to abomasum juice (pH 3) caused almost complete destruction of B-carotene and suggested that this nitrite effect might occur in monogastric animals when carotene and nitrites are administered orally.

The present studies were conducted to investigate the effect of nitrite on vitamin A destruction, both *in vitro* and *in vivo*, at physiological conditions encountered in sheep and chickens.

Experimental

Experiment I. Effects of Nitrite on the Destruction of Vitamin A In Vitro. Rumen and abomasum contents were collected from two sheep fitted with either a rumen or abomasum cannula. Shortly after collection the ingesta was strained through cheese cloth and pH of the fluid was determined. Crop, ventriculus and intestine contents were obtained from broilers which were slaughtered 2 hours after they had been fed a standard broiler growing ration. The ingesta from each gut segment was collected from six birds and pooled. Equal quantities of ingesta and water (W/V) were homogenized, and the homogenates were strained through cheese cloth. The pH values of the resultant fluids were determined.

Duplicate 26 ml. samples of fluid from each source (table 1) plus 1 ml. of an aqueous suspension of vitamin A were pipetted into 125 ml. Erlenmeyer flasks. After shaking briefly, samples were taken from each flask for vitamin A analysis. To one flask containing fluid from each source, 0.5 ml. of KNO_2 solution (110 mg. KNO_2/ml.) was added, and after shaking briefly samples were removed from all flasks for nitrite analysis. The flasks were then shaken in a water bath at 39° C. for 60 minutes. Subsequent samples were pipetted from each flask at 15 and 60 minutes for vitamin A analysis and at 60 minutes for nitrite analysis. The pH was also determined on the remaining fluids after 60 minutes.

Rate of vitamin A destruction in the presence of nitrite, at a relatively low pH, was investigated by taking additional samples for vitamin A analysis from the flasks containing ventriculus fluid at 0.5, 1, 2, 4, 8 and 10 minutes after adding the nitrite.

Experiment II. Effects of Nitrite upon Vitamin A Destruction in Sheep. Six mature fistulated wethers were divided into two groups consisting of two sheep with abomasum fistulae and one sheep with a rumen fistula per group. Each group received one of the two experimental rations shown in table 2 for a 5-day period, and then the rations were switched and fed for a subsequent 5-day period. The sheep were individually fed and received 3 lb. of ration daily. On the final day of each period, ingesta was collected from the abomasum and rumen of the animals at approximately 90 minutes after feeding. The samples were strained through cheese cloth, and the fluids analyzed for vitamin A, carotenoids and nitrite. The pH of each sample was measured.

Experiment III. Effects of Nitrite upon Vitamin A Destruction in Broilers. Twelve 8-week-old broilers were randomly allotted to two pens of six birds each. Feed was withheld from the birds for 16 hours and they were then fed rations similar to these used by Sell

[1] Appreciation is expressed to Pfizer of Canada and Hoffman La Roche, Limited for kindly supplying vitamin A used in these studies.
[2] Department of Animal Science.

TABLE 1. EFFECTS OF ADDED KNO_2, *IN VITRO*, UPON VITAMIN A AND NITRITE CONCENTRATION AND pH OF RUMEN AND ABOMASUM FLUIDS OF SHEEP, AND CROP, VENTRICULUS AND INTESTINE FLUIDS OF BROILERS AT VARIOUS TIMES OF INCUBATION

	Treatment														
	Control							Control+KNO_2							
	Vitamin A (mcg./ml.)			KNO_2 (mg./ml.)		pH		Vitamin A (mcg./ml.)			KNO_2 (mg./ml.)		pH		
Item	0 min.	15 min.	60 min.	0 min.	60 min.	0 min.	60 min.	0 min.	15 min.	60 min.	0 min.	60 min.	0 min.	60 min.	
Rumen fluid	18.8	18.0	17.1	0	0	6.08	5.81	18.4	16.7	15.0	2.14	1.65	6.08	6.14	
Abomasum fluid	17.5	16.7	18.0	0	0	2.51	2.60	18.0	0.6	0.4	1.32	0.67	2.51	3.55	
Crop fluid	17.1	16.7	16.7	0	0	6.30	6.18	18.4	16.3	16.7	1.98	1.94	6.30	6.19	
Ventriculus fluid	18.0	17.5	15.4	0	0	3.82	3.96	17.5	1.4	0.5	1.75	1.30	3.82	4.58	
Intestine fluid	18.0	17.5	18.8	0	0	7.06	6.96	18.0	16.3	17.1	2.04	2.04	7.06	6.96	

and Roberts (1963), except that both contained 200,000 I.U. of vitamin A per lb. of feed, and in addition one contained 0.74% KNO_2. Two hours after feeding, the birds were killed and the gut contents from the crop, ventriculus and intestine were obtained. The contents from each gut segment of six birds receiving each treatment were obtained and the contents of two birds were pooled (three samples per treatment) and then homogenized. Sufficient water was added to the samples before homogenizing to produce fairly liquid homogenates. Subsamples of the homogenates were used for vitamin A and nitrite analyses and pH measurements. Neither feed consumption during the two hour feeding period nor dry matter of the gut contents were determined.

The samples were analyzed for vitamin A by a method similar to that of Gallup and Hoefer (1946). Alfalfa hay used in Experiment II was analyzed for carotene by the A.O.A.C. (1955) procedure and carotenoids in the rumen and abomasum fluids were determined by the method of Kimble (1939). Nitrite in the samples was determined by a procedure adapted from Feigl (1958). The only statistical evaluation of experimental data was calculation of the treatment standard error.

TABLE 2. EXPERIMENTAL RATIONS USED IN SHEEP FEEDING EXPERIMENT

Ingredients	Treatment	
	Control	Control+KNO_2
	%	%
Chopped alfalfa hay [a]	50.00	50.00
Ground oats	48.50	46.50
Salt	0.25	0.25
Bonemeal	0.25	0.25
Vitamin A mix [b]	1.00	1.00
KNO_2	....	2.00

[a] Contained 16.2 mg. of B-carotene/lb.
[b] Contained 5,000,000 I.U. of vitamin A palmitate in gelatin/lb.

Results

The addition of KNO_2 to either abomasum or ventriculus fluid, in which the pH was below 4, resulted in destruction of most of the vitamin A by the end of the 60 minute incubation period (table 1). But in the rumen, crop and intestine fluids, in which pH was above 6, the presence of nitrite had little effect on vitamin A destruction. In the absence of nitrite there was little destruction of vitamin A in any of the fluids (table 1).

The destruction of vitamin A in ventriculus fluid was very rapid when KNO_2 was present (table 3). Approximately 54% of the added vitamin A was destroyed within 4 minutes after adding KNO_2 and 97.1% within 60 minutes. In ventriculus fluid without KNO_2 only 14.4% of the added vitamin A disappeared during the 60 minute incubation period.

In Experiment II, nitrite was not detected in either the rumen or abomasum fluid of sheep receiving the control or KNO_2 rations. The concentration of vitamin A and carotenoids in rumen fluid of control sheep was slightly higher than in sheep receiving KNO_2 (table 4). However, in abomasum fluid there was very little difference between the control and KNO_2-fed sheep in either vitamin A or carotenoid concentration. The pH of rumen fluid was above 6, and the pH of the abomasum fluid was slightly above 2.5.

In Experiment III nitrite was detected in ingesta from the three gut segments of the chick. The highest concentration of nitrite was found in the crop contents and the lowest concentration in intestine contents (table 5). The pH of ingesta from the crop and intes-

TABLE 3. EFFECTS OF KNO_2 UPON THE RATE OF VITAMIN A DESTRUCTION IN VENTRICULUS FLUID *IN VITRO*

Item	Vitamin A concentration (mcg./ml.)								
	0 min.	0.5 min.	1.0 min.	2.0 min.	4.0 min.	8.0 min.	10.0 min.	15.0 min.	60.0 min.
Ventriculus fluid	18.0	18.4	18.0	18.0	16.7	15.8	17.1	17.5	15.4
Ventriculus fluid +KNO_2	17.5	14.6	12.8	10.3	8.1	4.7	3.6	1.4	0.5
KNO_2 treatments as % of control	97.2	79.3	71.1	57.2	48.5	29.7	21.1	8.0	3.2

tine were above 6, compared to a pH of slightly above 4 for the ventriculus. Relatively high levels of vitamin A were found in the crop of birds receiving either the control or KNO_2 rations. The vitamin A concentration in ventriculus ingesta was 1.1 mcg./gm. in birds receiving KNO_2 and 9.7 mcg. in birds receiving the control ration. The concentration of vitamin A in ingesta from the intestine of birds receiving KNO_2 was higher than in ventriculus ingesta obtained from the same birds, but was lower than in the intestine ingesta collected from the control birds.

Discussion

Results from the *in vitro* experiment, in which attempts were made to simulate physiological conditions found in certain areas of the digestive tracts of sheep and chickens, indicate that the effect of nitrite on vitamin A destruction is dependent upon pH. In ventriculus and abomasum fluid, where pH was relatively low, there was almost complete destruction of the added vitamin A during the 60 minute incubation period when KNO_2 was present. In the rumen fluid, where pH was approximately 6, some vitamin A destruction occurred (18.5% was destroyed in fluid containing nitrite and 9% in the control fluid) and in intestine fluid, where pH was approximately 7, very little vitamin A was destroyed. These data suggest that if nitrite was present in either the abomasum of sheep or ventriculus of chickens rapid destruction of dietary vitamin A could occur. In addition, dietary vitamin A could be destroyed in the rumen of sheep either in the presence or absence of nitrite. King *et al.* (1962) observed approximately a 40% loss of carotene or vitamin A from the rumeno-reticulum of sheep and cattle, in 12 hours after feeding.

Data obtained from the *in vivo* experiment with sheep (table 4) indicate that the dietary nitrite disappeared from the rumen quite rapidly, and since no nitrite was detected in abomasum fluid, the nitrite was either metabolized or absorbed before reaching the abomasum.

The *in vivo* results suggested that dietary KNO_2 slightly accelerated the destruction of vitamin A and carotenoids in the rumen of sheep, but the concentrations of vitamin A and carotenoids found in the abomasum fluid did not substantiate this. In fact, slightly higher vitamin A and carotenoid levels were found in the abomasum fluid of sheep receiving KNO_2 than in sheep receiving the control ration; illustrating that there was no effect of 2% dietary nitrite on destruction of vitamin A and carotenoids.

It has been observed in cattle that dietary nitrate is rapidly converted to nitrite and am-

TABLE 4. EFFECTS OF FEEDING KNO_2 UPON VITAMIN A AND CAROTENOID CONCENTRATION AND pH OF RUMEN AND ABOMASUM FLUIDS OF SHEEP

Item	Treatment [a]			
	Control		Control+2% KNO_2	
	Rumen fluid	Abomasum fluid	Rumen fluid	Abomasum fluid
Vitamin A, mcg./10 ml.	23.0 ±0.4 [b]	6.8 ±1.8	21.5 ±1.6	7.5 ±0.9
Carotenoids, mcg./10 ml.	30.7 ±1.3	8.7 ±2.4	24.3 ±3.9	10.4 ±2.0
pH	6.18±0.1	2.59±0.1	6.67±0.02	2.66±0.19

[a] The rumen and abomasum fluids of sheep receiving both rations were analyzed for nitrite and none was detected.
[b] Standard error.

TABLE 5. EFFECTS OF FEEDING KNO_2 UPON VITAMIN A AND NITRITE CONCENTRATION AND pH OF INGESTA FROM THE CROP, VENTRICULUS AND INTESTINE OF BROILERS

	Treatment					
	Control			Control+KNO_2		
Item	Crop ingesta	Ventriculus ingesta	Intestine ingesta	Crop ingesta	Ventriculus ingesta	Intestine ingesta
Vitamin A, mcg./gm.	23.7 ±1.3[a]	9.7 ±2.1	8.5 ±1.1	34.5 ±1.5	1.1 ±0.2	4.5 ±0.9
KNO_2, mg./100 gm.	2.7 ±0.1	0	0	40.9 ±2.0	10.0 ±0.1	1.1 ±0.2
pH	6.33±0.10	4.06±0.12	7.58±0.04	6.42±0.04	4.26±0.04	6.91±0.12

[a] Standard error.

monia in the rumen (Wang *et al.*, 1961). Thus in the present sheep experiment if nitrate rather than nitrite had been fed similar results regarding vitamin A would be expected. Therefore, these data might offer a partial explanation as to why Smith *et al.* (1961) and Cline *et al.* (1962) did not observe any significant changes in the vitamin A status of sheep or cattle which had been fed nitrate.

In chickens a different picture was presented in regard to nitrite metabolism within the digestive tract. Data from the *in vivo* experiment with broilers showed that dietary nitrite was present in relatively high concentration in the crop. The vitamin A concentration of crop ingesta was higher for birds fed KNO_2 than for control birds; thus there was no apparent effect of nitrite on vitamin A in this area of the digestive tract. On the other hand, the vitamin A concentration was much lower in the ventriculus fluid of birds fed KNO_2 than in control birds. The simultaneous observation of nitrite in this fluid suggests that considerable vitamin A destruction occurred *in vivo*. However, there is the possibility that some of the observed destruction occurred between the time the ingesta was collected from the birds and when it was analyzed for vitamin A, which was approximately 45 minutes. The level of vitamin A in intestine ingesta from the KNO_2 fed birds was considerably lower than similar ingesta from control birds, and since the pH was approximately 7 very little vitamin A destruction would have occurred between the time of collection and analysis. These data support the suggestion that the nitrite effect was occurring in the ventriculus of the birds before they were killed.

In general these data corroborate the results obtained from the *in vitro* experiment, and offer an explanation for the exceptionally low liver vitamin A stores observed in chicks fed nitrite (Sell and Roberts, 1963). Furthermore, the digestive tract of the rat is somewhat similar to that of the chick, and it would be logical to assume that metabolism of dietary nitrite in the digestive tracts of these two species is similar. If so, the decrease in liver vitamin A stores in rats fed nitrite reported by O'Dell *et al.* (1960) Smith *et al.* (1961) and Emerick and Olson (1962) could be explained by destruction of the dietary vitamin A in the stomach of the rats (approximately pH 3) before it reached the small intestine where absorption takes place.

Summary

In vitro and *in vivo* studies were conducted to investigate some effects of nitrite on vitamin A destruction in sheep and chickens. The addition of KNO_2 to either abomasum or ventriculus fluid, where pH was below 4, resulted in rapid vitamin A destruction. But in rumen, crop or intestine fluid, where pH was above 6, relatively little vitamin A was destroyed in the presence of KNO_2 during a 60 minute incubation period. When 2.0% of KNO_2 was fed to fistulated sheep no nitrite was found in either rumen or abomasum fluid at approximately 90 minutes after feeding. Furthermore, there was relatively little difference in vitamin A concentration between abomasum fluids collected from sheep receiving the control and nitrite rations, suggesting that dietary nitrite did not enhance vitamin A destruction in the sheep. On the other hand, when broilers were fed 0.74% of KNO_2, nitrite was found in ingesta obtained from the crop, ventriculus and intestine at 2 hours after feeding. In addition, the concentration of vitamin A in both the ventriculus and intestine ingesta obtained from birds receiving KNO_2 was much less than in similar ingesta obtained from control birds. These data suggest that in the presence of nitrite vitamin A is destroyed in the ventriculus area of the digestive tract of the chick, where the pH is approximately 4.

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which on blanching, separating, and sorting furnished 3.7 pounds of testa, 2.0 pounds of germs, 2.5 pounds of objectionable or extraneous material and 86.7 pounds of sorted cotyledons for grinding into peanut butter. By removal of the 3.7 pounds of testa and the 2.0 pounds of germs the free fatty acids content of the finished product was reduced by 30%, while the oil content was increased approximately 2%. These byproducts are a rich source of oil since the 5.7 pounds of testa and germs contain 2 pounds of oil (37%).

SUMMARY

Data have been presented on the effects of roasting, blanching, and sorting on the oil contents of raw peanuts, intermediates, and finished products used in the preparation of stabilized and unstabilized peanut butters.

The data indicate that the roasted peanut kernels have a higher oil content than the corresponding raw kernels. This difference results presumably from loss of volatile matter in addition to moisture during roasting or from some effect of heat on the non-oil portion of the peanut.

The data also show that the sorted cotyledons resulting from the elimination of germs, testa and objectionable material during blanching, screening, and sorting contain a higher percentage of oil than the roasted kernels. Separation of components of lower oil content is responsible for this difference.

The testa of raw peanuts generally contains a very low percentage of oil. On the basis of the high percentage of oil found in the roasted testa it may be concluded that the testa has absorbed oil that was exuded from the peanut during roasting.

Examination of data reveals that the free fatty acids contents of the oils extracted from raw and roasted peanuts, sorted cotyledons, and peanut butters prepared with and without additives were uniformly low. It may also be concluded from these data that the roasting, blanching, and manual sorting operations in the manufacture of peanut butter are responsible for the slightly lower free fatty acids content of the oil in the sorted cotyledons than that found in the oil of the raw peanut kernels.

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Non-Bacterial Reduction of Nitrite in Pork[a]

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Soluble materials extracted from pork interfere in the determination of nitrite. The error thus introduced into analysis of cured pork products is relatively constant for any one method of extraction. Because of dilution, the error is negligible in the analysis of used tank pickles.

The reducing systems of fresh pork caused a progressive destruction of nitrite which was independent of bacterial contamination. The loss increased as the pH decreased, and as the temperature increased up to the temperature of protein denaturation.

The oxidation-reduction potential of suspensions of pork was approx. + 225 mV. at pH 5.9, but the factors controlling Eh did not control the destruction of nitrite. Some intermediary electron carriers were necessary for the reduction of nitrite by the reducing compounds of the meat. Hemoglobin, and 2:6-dichlorobenzenone indophenol were both capable of supplementing the electron carriers and increasing the loss of nitrite. Ascorbic acid, which lowered the Eh of the suspension, had less effect on the loss of nitrite.

Nitrite, whether added directly or formed by reduction of nitrate, is essential for good color development in the curing of pork products, but because of its undesirable properties the amount present must be controlled within narrow limits. The amount of a curing salt added to meat can be controlled by curing procedure, but the amount of nitrite found in meat by analysis is not entirely dependent upon the amount added.

Much of the variation in nitrite content is caused by bacterial action *(9)*, but work on the pH of muscles *(4)* indicates that other factors have a marked effect on the nitrite content of cured samples. These factors probably include destruction of nitrite by reaction with meat constituents, binding of nitrite by meat proteins so that it is not extracted by the procedure used, and interference of other extractable materials with the final analysis. A

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study was, therefore, made of the effect of these factors on the apparent nitrite content of pork.

MATERIALS AND METHODS

Two and one-half g. samples of finely ground psoas muscle[b] were macerated with a small amount of water and diluted to about 30 ml. in a wide-necked 50 ml. volumetric flask (sugar inversion type). Ten ml. of McIlvaine's citric acid-sodium phosphate buffer was added and the suspension held overnight at 40° F. (4.5° C.). The mixture was then warmed to room temperature, 125 μg. of sodium nitrite and/or other test solutions added, and the volume made to 50 ml. with water. Control solutions were prepared without pork. Separate flasks were prepared for initial (0-hour) analysis and for analysis after a 5 hr. reaction period on an end-over-end rotor in an air oven at 110° F. (43° C.).

For nitrite analysis, suspensions were centrifuged and a 1:5 dilution of the supernatant was filtered through a Whatman No. 42, 15 cm. paper. Color was developed in 10 ml. of filtrate by the sulfanilic acid-α naphthylamine method of Rider and Mellon *(6)*. Blank determinations without sulfanilic acid were prepared for each sample.

Nitrogen determinations were made by a micro-Kjeldahl technique, and the total solids determinations by air-drying for 18 hours, at 220° F. (105° C.). Bacterial counts were made on proteose peptone-tryptone agar incubated at 80.6° F. (27° C.) for 48 hours. Dyes used for estimating oxidation-reduction potentials were: 2,6-dichlorobenzenone indophenol (E. K.), 0-cresol-indo-2,6-dichlorophenol (B. D. H.), and 1-naphthol-2-sodium sulphonate-indophenol (B. D. H.). All dye solutions were prepared by dissolving 100 mg. of dye in 250 ml. of distilled water. Alpha-naphthylamine hydrochloride (E. K.) was recrystallized *(6)*, all other reagents were reagent grade.

INTERFERENCE OF SOLUBLE PROTEINS

Provided no rapid reaction occurs, the extent to which nitrite can be recovered immediately after addition to proteinaceous solutions should indicate the degree of interference of these materials in the determination. As shown by the data of Table 1, recovery in gelatin solutions was decidedly low. Use of gelatin that had been treated with excess nitrite and precipitated and washed with acetone did not improve the recovery, indicating that if binding had occurred it was reversed by acetone but not by the acid (pH 2.5) used in the determination.

TABLE 1

Interference of gelatin in the determination of nitrite

Gelatin concentration	Nitrite added	Nitrite recovered	Apparent loss
%	μg./ml.	μg./ml.	%
nil	0.43	0.43	0
1.4	0.43	0.30	30.0
2.1	0.43	0.24	44.0
2.1	0.64	0.41	36.1
3.2 treated[a]	0.45	0.14	69.3

[a] Gelatin that had been mixed with excess nitrite, then twice precipitated and washed with acetone.

More probably, the apparent loss was caused by interference of the gelatin with color development during analysis.

Table 2 shows that recoveries in aqueous extracts of fresh pork were also low. Lower recoveries occurred in a cold water extract than in a hot water extract. Partial precipitation of the solids from the cold water extract by heating the mixture after the addition of nitrite increased the recovery by about 20%. This suggests that nitrite was not bound to the heat coagulable protein, and that the extra 20% loss in the cold extract was caused by interference. The non-heat coagulable extractives may firmly bind some nitrite, but it is reasonable to assume that most of the apparent loss of nitrite from these solutions was caused by interference and not by destruction or binding of nitrite.

[b] Most muscles were obtained 24 hrs. after slaughter of the pigs. Samples stored between tests were frozen and held at —40° F. (—40° C.). No detectable change in the properties under study occurred in several weeks at this temperature.

TABLE 2

Interference of pork extract in the determination of nitrite (Total volume 50 ml.)

Pork extract added		Treatment of mixture	Nitrite added	Nitrite recovered	% loss
Type[a]	Amount				
	ml.		μg./ml.	μg./ml.	
	nil	nil	0.60	0.60	0
	nil	Heated, filtered[b]	0.60	0.60	0
Hot	10	nil	0.75	0.70	6.3
Hot	20	nil	0.75	0.67	10.9
Hot	20	nil	0.38	0.34	10.1
Cold	25	nil	0.60	0.40	32.7
Cold	25	Heated, filtered[b]	0.60	0.52	13.3

[a] Hot extract prepared by macerating lean pork tissue in hot water, heating for 20 minutes, diluting tenfold, centrifuging, and filtering the supernatant. Cold extract was prepared in the same manner but with cold water and no heating. The hot extract contained 0.03% nitrogen and 0.28% total solids, pH 6.55; the cold 0.080% nitrogen and 0.57% total solids, pH 5.98.

[b] Heated, after the addition of nitrite, for 20 min. in a boiling water bath, then filtered.

Attempts to reduce this interference by precipitation of the soluble protein were unsuccessful either because the precipitant itself prevented full color formation (trichloracetic acid), or because color developed in the blanks (ethanol, acetone).

NON-BACTERIAL DESTRUCTION OF NITRITE

Since it was not possible to obtain sterile muscle and to maintain aseptic conditions, the extent to which bacteria were responsible for reduction of nitrite was determined over a range of experimental conditions. The data of Figure 1, show that the effects of pH and of temperature on nitrite destruction did not parallel the effect on bacterial growth, and that bacterial numbers became negligible below pH 5.0 or above 113° F. (45° C.).

Loss of nitrite from pork suspensions increased with increasing temperature (Figure 1) until denaturation became marked, as indicated by a "cooked" appearance of the suspended meat particles, then decreased sharply.[f] Over a five-hour period, denaturation was obvious at 120° F. (49° C.), but not at 110° F. (43° C.).

At 110° F., recovery of nitrite decreased with decreasing pH (Figure 1). Within the range pH 7.0 to 5.0 (Table 3) losses from control samples without pork were within the error of the analysis, and the 0-hour recoveries from the pork suspensions were essentially constant. Below pH 5.0, nitrite loss increased still further, but there was also a marked decrease in the 0-hour recovery, and below pH 3.5 an extensive loss of nitrite occurred from control samples. Thus, within the pH range 7 to 5, differences between 0 and 5-hr. recoveries are a reliable measure of the extent of the reaction, but may not be so at lower pH levels.

Because the most probable mechanism of nitrite destruction appeared to be reduction, the oxidation-reduction potentials of the pork suspensions was estimated. Two and one-half g. of pork suspended in a volume of 50 ml. at pH 5.89 (buffer pH 5.5) reduced 2:6 dichlorobenzenone indophenol in 5 minutes, and 0-cresol-indo 2,6-dichlorophenol in 15 minutes, but did not reduce 1-naphthol-2 sodium sulphonate indophenol in 7 hrs. at 110° F. Since the E_o' of these dyes at pH 5.89 is 284, 248 and 190 mV. respectively, the Eh of the suspension was between 190 and 248 mV. The potential determined in this manner at 110° F. did not vary to a measurable degree with pH, but the total amount of 2,6-dichlorobenzenone indophenol that the meat could reduce in 5 hours at 110° F. (Table 4) decreased markedly with increasing pH.

[f] Heat denatured pork suspensions destroyed considerable quantities of nitrite at very high temperatures, e.g. at 250° F. (121° C.), with other conditions as outlined under "Methods." 44% of the nitrite was destroyed in 1 hr. [cf. *(8)* and *(5)*]. This destruction is probably caused by chemical reaction of nitrite with amino groups. A similar loss was observed in a 2.5% glycine solution.

TABLE 3

Effect of pH on the destruction of nitrite by suspensions of pork

Control samples				Pork suspensions			
pH	Nitrite recovery, %		Loss in 5 hr., %	pH	Nitrite recovery, %		Loss in 5 hr., %
	0 hr.	5 hr.			0 hr.	5 hr.	
3.17	96.6	80.0	16.6	3.17	33.8	9.4	24.4
3.52	100.6	91.4	9.2	3.39	42.2	8.0	34.2
4.02	99.1	96.6	2.5	4.10	73.5	26.6	46.9
				4.76	83.4	54.4	29.0
5.05	98.0	97.3	0.7	5.15	86.9	69.0	17.9
				5.68	87.2	76.6	10.6
6.09	98.7	98.0	0.7	6.20	86.0	75.8	10.2
				6.68	87.5	78.2	9.3
7.03	98.7	99.7	—1.0	7.13	86.9	81.0	5.9

TABLE 4

Amount of 2:6-dichlorobenzenone indophenol reduced by pork suspensions at various pH levels (2.5 g. pork in 50 ml., 0.1% dye solution)

pH	Ml. dye reduced during	
	2 hrs	5 hrs.
5.55[a]	15	17
5.83	10	12
6.38	7	7
6.62	2	4

[a] Lower pH levels were not studied because the dye is pH sensitive.

TABLE 5

Effect of curing salts on the reduction of dye by pork suspensions at 110° F. (43° C.) (2.5 g. pork plus salt and buffer equilibrated 18 hr. at 40° F. (4.5° C.), 2 ml. of 2:6-dichlorobenzenone indophenol added, and volume made to 50 ml.)

Salt added	pH of suspension	Time for complete reduction of dye
None	5.87	10 min.
15% NaCl	5.10	5 min.[b]
1% $NaNO_3$	5.71	10 min.
100 p.p.m. $NaNO_2$	5.88	15 min.
500 p.p.m. $NaNO_2$	5.90	More than 24 hr.

[b] The decrease in pH of this solution was sufficient to account for the rapid reduction.

Suspensions of meat that had been cured in a Wiltshire type pickle failed to reduce any of the dyes, and there was no loss of nitrite when the suspensions were held at 110° F. for 5 hr. The effect of curing salts on the potential was therefore investigated. Salts were added to the suspensions and the mixtures held at 40° F. (4.5° C.) overnight before addition of dye. As shown by Table 5, neither sodium chloride nor sodium nitrate significantly affected the rate of dye reduction, but 100 p.p.m. of sodium nitrite decreased the rate slightly and 500 p.p.m. raised the potential sufficiently to prevent reduction of the dye. Overnight exposure to nitrite apparently destroyed, or completely oxidized, the systems responsible for nitrite reduction.

No relation was found between the ability of fractions to reduce dye and their ability to destroy nitrite. Soluble and insoluble fractions of centrifuged pork suspensions each destroyed about half as much nitrite as the complete suspension. For example, when nitrite loss from entire suspension was 19.7%, losses from the soluble and insoluble portions were 8.7% and 9.4% respectively. The nitrite loss of the whole suspension was decreased by heating to only 3.7%. On the other hand, the potential of the fractions, and of the heated suspension, remained at approximately 225 mV., and the amount of dye destroyed in 5 hrs. was not altered.

Pork suspensions that had been equilibrated with 500 p.p.m. of nitrite for 18 hours, failed to reduce 2:6-dichlorobenzenone indophenol (Table 5). However, when nitrite and 2:6 dichlorobenzenone indophenol were added together the dye considerably increased the loss of nitrite (Table 6). Furthermore, the dye was capable of at least partially replacing those portions of the nitrite destroying system which were inactivated by heat. Destruction of nitrite by the suspensions must, therefore, have been limited by some compound(s) capable of transferring electrons across the gap in potential between the nitrite-nitric oxide system (+650 mV.) and that of the meat (+225 mV.).

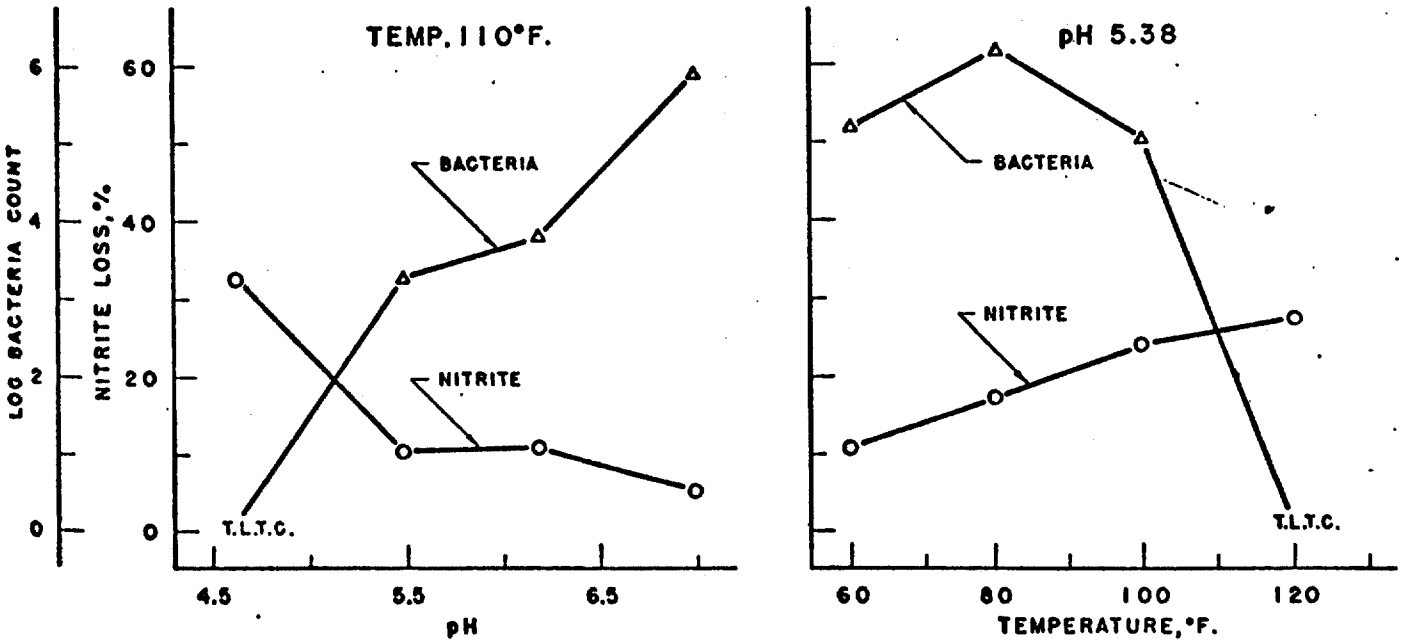

Figure 1. Effect of pH and of temperature on the destruction of nitrite and the growth of bacteria in pork suspensions.

TABLE 6

Effect of 2:6-dichlorobenzenone indophenol, oxyhemoglobin, potassium cyanide, and ascorbic acid on destruction of nitrite by pork suspensions (pH 5.8 to 5.9, 125 μg./50 ml. total volume)

Suspension	Reagent added	5 hr. loss of nitrite, %	
		Suspension only	Mixture
2.5 g. pork	13 ml. 2:6-dichlorobenzenone indophenol [a]	12.2	21.2
Pork solubles only	13 ml. 2:6-dichlorobenzenone indophenol [a]	7.8	17.0
Heated pork	13 ml. 2:6-dichlorobenzenone indophenol [a]	3.0	14.6
2.5 g. pork	13 p.p.m. KCN	10.2	5.2
2.5 g. pork	650 p.p.m. KCN	10.2	2.6 [b]
2.5 g. pork	1 ml. oxyhemoglobin [c]	9.0	38.4
2.5 g. pork	2 ml. oxyhemoglobin	9.0	56.0
2.5 g. pork	1 mg. ascorbic acid	10.6	18.6
2.5 g. pork	10 mg. ascorbic acid	12.4	19.6
2.5 g. pork	1 ml. oxyhemoglobin plus 1 mg. ascorbic acid	10.6	62.8
Water	1 mg. ascorbic acid	.....	—10.2 [d]

[a] 0.04% solution. The dye was not completely reduced by any of the suspensions.
[c] 1 to 1.5 mM. hemoglobin/litre.
[b] pH of mixture 6.43.
[d] Initial recovery 87.2%, 5 hr. recovery 97.4%.

The most probable carrier in this range would be a ferric-ferrous system, and the effects of cyanide and of added hemoglobin were therefore tested (Table 6). Potassium cyanide, at a concentration of 13 p.p.m., had a significant inhibitory effect, while 650 p.p.m. reduced the loss of nitrite to that found with heat-denatured suspensions. Added hemoglobin markedly increased the destruction of nitrite.

Combinations of hemoglobin and ascorbic acid, which lowers the Eh of the suspensions, were also studied (Table 6), but interpretation of the results was difficult because of lower recoveries in control mixtures of ascorbic acid and nitrite at 0 time than at the end of the 5-hour reaction period. However, it is evident that the destruction of nitrite was increased less by ascorbic acid than by the addition of either 2:6-dichlorobenzenone indophenol or oxyhemoglobin. Thus, in the destruction of nitrite, the electron transfer systems in the 650 to 190 mV. range were more limiting than the actual potential.

DISCUSSION AND CONCLUSIONS

Data presented in this paper suggest that the reported nitrite contents of bacons and hams analyzed by the sulfanilic acid—α naphthylamine method have been in error because some extractable materials interfere with the determination. Any one method of extraction should give solutions having relatively constant interference, and comparisons between samples analyzed by the same method should therefore be reliable. Data obtained by different extraction procedures may not be comparable. Used tank pickles also contain pork extractives, but most pickles require extensive dilution before analysis and the error is therefore minimized.

The destruction of nitrite by reducing systems in the meat probably affects the final nitrite level of the meat significantly. Under the conditions used, these systems were capable of destroying 10 μg. of nitrite per gram of meat in 5 hr. at 110° F. and up to 30 μg. in the presence of added hemoglobin. Even assuming that the experimental procedure did not decrease the activity of the systems involved, extrapolation of the temperature curve in Figure 1 to 40° F. indicates that a significant portion of the nitrite added to meat would be destroyed by the reducing systems during a normal 3 to 7 day cure. In accelerated cures less nitrite would be destroyed and the amount added should be reduced.

The potential of the pork suspensions used in these studies was between 190 and 250 mV. at pH 5.8. Hemoglobin and myoglobin present in the suspensions would be in the met- form [Eh of methemoglobin : 160 mV. at pH 7.0 *(3)*] and the chromogenic reactions involving hemoglobin and nitrite would differ from those occurring in meat. However, it is reasonable to assume that the effect of comminution and dilution has been to increase the availability of oxygen and thus raise the potential some 400 mV. [Eh of pork +200 mV. *(2)*] towards the positive side without changing the systems involved.

The measurements of oxidation-reduction potentials made in dilute suspensions were not sufficiently sensitive to indicate differences in the potential at different pH levels, but the total amount of dye reduced in 5 hr increased markedly with decreasing pH, thus paralleling the destruction of nitrite. However, the data on heated suspensions indicated that there was no direct relation between the reducing capacity towards dyes and towards nitrite. The ability of the suspensions to reduce dyes appeared to be controlled by various heat-stable components of the meat, while the reduction of nitrite was limited by some unknown, heat labile intermediaries.

From a practical point of view, these findings indicate some of the factors affecting the formation of nitric oxide hemoglobin. Although hemoglobin may react directly with nitrite to give a mixture of nitric oxide hemoglobin and methemoglobin *(1)* the relatively rapid reduction of nitrite suggests that hemoglobin will more probably react directly with nitric oxide. Since the amount of nitrite reduced increases with decreasing pH, the rate of color formation should be greater in muscles from rested or fed animals *(4, 7)*, and the lower residual nitrite in these muscles may be a factor in the greater color stability *(4)*. The limiting factor in the rate of nitrite reduction appears to be some intermediary electron carrier rather than the overall reducing potential. The artificial lowering of the potential by ascorbic acid, etc. can thus be of only limited value.

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食品中のニトロソアミンに関する研究（第1報）

In vitro および *in vivo* におけるジメチルニトロソアミンの生成

（昭和46年3月15日受理）

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Studies on Nitrosamines in Foods (I)

In vitro and *in vivo* Formation of Dimethylnitrosamine

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By the addition of nitrite as color fixative or preservative to marine fishes or fish roes which contain in high concentration of secondary amines, such as dimethylamine or diethylamine, the formation of nitrosamine in foods have to be presumed. The conditions in which dimethylnitrosamine was produced *in vitro* from sodium nitrite and dimethylamine hydrochloride were studied and it was found that the optimum pH for the production of dimethylnitrosamine in buffer solutions was near 3.6. Thirty minutes after oral dosage to rabbits, 5 mg of dimethylnitrosamine was detected in stomach contents when 1,000 mg of sodium nitrite and 500 mg of dimethylamine hydrochloride were administered. From the results of these experiments, the possibility of the formation of nitrosamines in foods was discussed which might occur in human stomach by the uptake of nitrite as food additive and when the foods which contain large quantities of nitrite and secondary amines were administered.

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I. まえがき

食品添加物として使用されている亜硝酸ナトリウムは，食肉製品，魚肉製品などの発色剤としてすぐれた効果を示すのみならず，保存料としての効果も期待できる．このためハム，ソーセージ，くん製魚類などの発色，防腐剤として各国で古くから利用されている．しかし近年ニトロソ化合物の発ガン性についての研究[1]が進み，とくにジメチルニトロソアミン (DMNA) は動物実験で飼料中 2~3 ppm の存在でも発ガン性を示すことから[2]，食品中のニトロソ化合物の存在が食品衛生上重大な問題として注目されてきた[3]．

ニトロソ化合物の発ガン性に関する報告は数多くなされており，その発ガン性の強力なこと，および臓器特異性を有することが知られているが，中でも DMNA の発ガン性はもっとも強いものに属する．この DMNA の前駆物質の一つであるジメチルアミン (DMA) あるいはトリメチルアミン (TMA) は魚類，とくに海産魚類に多く含まれていることは古くからいわれており，また最近の著者らの実験でも明らかにされている[4]．もう一つの前駆物質である亜硝酸塩は食品添加物として人為的に食品に添加されるほか，果実，野菜など自然界にも硝酸塩とともに広く分布存在している[5]．したがって多量の DMA を含む食品に亜硝酸塩を添加して製造加工する場合には食品中で，また多量の DMA を含む食品と，多量の亜硝酸塩を含む食品とをヒトが同時に大量に摂取した場合には，胃の中で DMNA が生成することは，理論的には考えられることである[6]．とくにヒトの胃液がニトロソ化には好適の強酸性であることから，ヒトの胃中での DMNA の生成の可能性は十分に考えられることである．Sander ら[6] はラットにジフェニルアミンと亜硝酸塩を，また Sen ら[6]はジエチルアミンと亜硝酸とを経口投与し，胃内容物よりそれぞれジフェニルニトロソアミンおよびジエチルニトロソアミンを検出しているが，さらにニトロソ化され難い DMA については実験をしていない．

著者らは食品中で，あるいはヒトの胃中でニトロソ化

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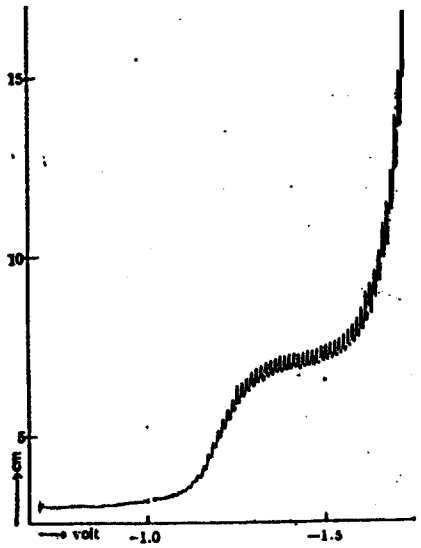
合物生成の可能性を考慮し，種々の条件下で DMNA 生成についての *in vitro* および *in vivo* の実験を行ない，2，3の知見を得た．すなわち，Sander ら[6]の推定しているとおり強塩基性の DMA と亜硝酸ナトリウムとからの DMNA 生成率は極めて低いが，pH 3 付近では確実に DMNA を生成している．またこの生成量は食肉製品の製造条件に近い60°～70°に加熱することにより，当然ながらその生成量は37°の約2.5倍と急増している．人工胃液中でも同じく生成率は低いが，亜硝酸塩の増加につれて，また pH が3に近づくにつれて DMNA の生成量は増加している．以上の結果に基づき，pH がヒトの胃液に近いウサギに大量の DMA と亜硝酸ナトリウムとを同時に経口投与し，30分後に胃内容物について分析した結果，最高 5 mg の DMNA の生成が確認された．

II. 実験方法および結果

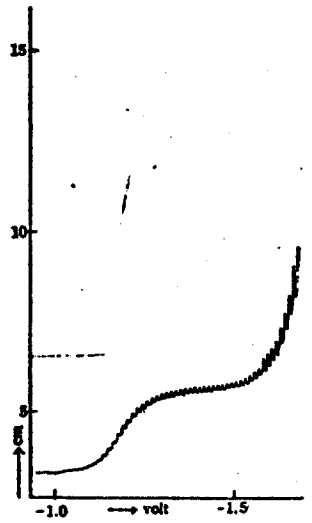
1. DMNA の分析

i） 緩衝液および人工胃液から DMNA の抽出法

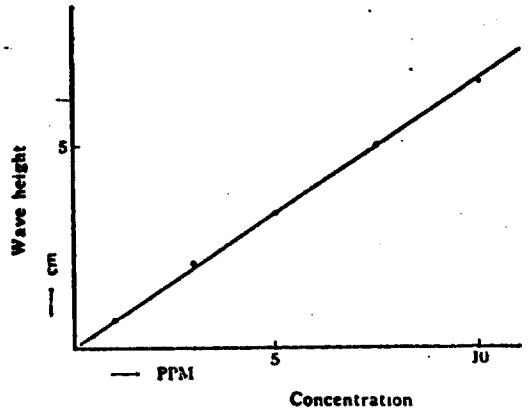
検体 20 ml を分液ロートにとり，炭酸カリウム 4 g を加えて DMNA 生成の反応を止めるとともに塩析効果を高める．ジクロロメタン 20 ml を加えて振とう機で3



1-1. Polarogram of dimethylnitrosamine (5 ppm)



1-2. Polarogram of diethylnitrosamine (5 ppm)



1-3. Calibration curve of dimethylnitrosamine

Fig. 1. Polarograms and calibration curve of nitrosamines in 0.01 M sulfosalicylic acid (vs.: Hg pool)

Sens.: 0.02 μA/mm,　Parallel cap.: 50 μF
Initial volt: −0.7 volt　Water temp: 25±0.5°C,
m: 0.7126 mg　*t*: 4.16 sec,
Hg press.: 1.00 kg/cm

分間抽出し，ジクロロメタン層を分取する．抽出を2回行ない，ジクロロメタン層を合し，0.01 M スルフォサリチル酸溶液 15 ml を加え，ロータリーエバポレーターで軽く吸引しながらジクロロメタンを留去する．留去後 20 ml のメスフラスコに移し，濃縮フラスコを少量の 0.01 M スルフォサリチル酸溶液で洗い，洗液はメスフラスコに合わせる．メスフラスコの内容液に 0.01 M スルフォサリチル酸溶液を加えて全量を正確に 20 ml とする．この液をポーラログラフ用検液とする．なお本抽出法による DMNA の回収率は96～102%であった．

ii) ウサギ胃内容物から DMNA の抽出法

胃内容物に胃洗浄液を合したものを遠心分離 (4000 r.p.m., 10分間) し，上澄をとり炭酸カリウム 40 g を加える．沈殿に蒸留水 30 ml を加えかく拌後，再び遠心分離して得られた上澄をさきの上澄と合わせる．必要ならばろ過する．ろ液（または上澄）の総量をはかり，その一定量をとり，同量のジクロロメタンと振とう機で10分間振とう抽出する．遠心分離 (1500 r.p.m., 10分間) してジクロロメタン層をとり，この一定量をはかり，ロータリーエバポレーター用濃縮フラスコに移す．0.01 M スルフォサリチル酸溶液 15 ml を加え，以下 i) と同様に操作してポーラログラフ用検液とする．本抽出法による DMNA の回収率は87～98%であった．

iii) DMNA のポーラログラフィー[7]

支持電解質として 0.01 M スルフォサリチル酸溶液を用い，水浴温度 25°，対極に水銀池を使用した．10分間窒素ガスを通じて除酸素したのち，直流ポーラログラフを用いて波高の測定を行ない，同じ支持電解質を使用して作製した検量線により定量した．この際の半波電位は −1.2 volts (vs. 水銀池) であった．なお装置は柳本交直両用ポーラログラフ PA 101 型を使用した (Fig. 1).

iv) DMNA の薄層クロマトグラフィー[8]

i) および ii) で得られたジクロロメタン層を無水硫酸ナトリウムで乾燥後，軽く吸引して濃縮する．プレートはシリカゲルスポットフィルム（東京化成製品），展開溶媒は n-ヘキサン・ジエチルエーテル・ジクロロメタン (4:3:2)，展開後 30% 酢酸をスプレーし，10分間紫外線照射（反応用 UV ランプ，10 W，照射距離約 5 cm）後，ニンヒドリン試液（ニンヒドリンスプレー，東京化成製）をスプレーし，熱風で加熱発色させる．なお確認のためグリース試薬による検出も同時に行なった．Rf 値は約0.3，DMNA 標準物質による確認限度はニンヒドリン試液で 0.2 μg，グリース試薬で 0.5 μg，またスポットフィルムのかわりにワコーゲル B-5 で自製したプレート (250 μ, 110°, 3時間) では確認限度は若干劣るがスポットのまとまりは良好である．なおプレートにスポットしたあとの操作はすみやかに行なう必要がある．スポットしたのち，長時間放置したり，スポットを熱風乾燥すると DMNA は蒸散しロスが大きいので注意を要する．

2. 緩衝液中における DMNA の生成

i) 37°, 3時間

各種 pH の Clark-Lubs 緩衝液中で，37°，3時間に生成する DMNA を抽出し，ポーラログラフで定量した．使用した DMA-HCl, 亜硝酸ナトリウムとも 5 mM である (Table 1).

ii) 37°, 12 週間

i) と同様の方法により 1～12 週間にわたる DMNA 生成量を測定した．使用した DMA (遊離型) は 2.5 mM，亜硝酸ナトリウムは 25 mM および 50 mM である．以上の実験により DMA は塩酸塩でも遊離型でもほぼ同程度の DMNA を生成すること，pH 3.3 付近が DMNA の生成に適していること，DMA 濃度が一定でも共存する亜硝酸ナトリウム濃度が増加するに伴い DMNA 生成量も増加していることがわかった (Table 2). なお DMNA 生成にもっとも適している pH の検討は60°で行なった．

iii) 60°, 3時間

i) と同様な方法により各種 pH の緩衝液中で60°，3時間加温した場合の DMNA 生成量を測定した．DMA-

Table 1. Formation of Dimethylnitrosamine from Sodium Nitrite and Dimethylamine Hydrochloride in Various pH Buffer Solution (37°C, 3 hours)

pH	1.2	2.2	3.3	4.2	5.2	6.0
DMNA* (ppm)	10.6	11.0	38.6	15.2	2.3	+
Yield** (%)	2.9	3.01	10.57	4.10	0.63	

* DMNA: Dimethylnitrosamine
** Theoretical yield calculated from dimethylamine
+: trace

Sodium nitrite and dimethylamine hydrochloride were dissolved in various pH buffer solution and incubated at 37°C for 3 hours. The reaction mixture was cleaned up and determined by polarography. The concentration of dimethylamine hydrochloride was 5 mM and of sodium nitrite was 50 mM. Clark-Lubs buffer solution was used and the amount of dimethylnitrosamine produced was expressed by ppm.

Table 2. Formation of Dimethylnitrosamine from Sodium Nitrite and Dimethylamine in Buffer Solution of Various pH (37°C, up to 12 weeks)

Conc. of $NaNO_2$ (mM)	25				50			
Weeks \ pH	3.4	4.4	5.4	6.2	3.4	4.4	5.4	6.2
	ppm							
1			0.9		19.8	13.2	2.9	0.6
2	15.9	10.6	1.3	1.0	24.5	22.4	5.4	0.8
4	17.5	12.0	3.4	1.1	29.3	23.5	9.6	2.3
8	15.2	12.6	5.4	2.0	32.2	23.8	15.6	4.1
12	15.5	13.5	7.7	3.4	31.9	23.7	19.5	5.6

Sodium nitrite and dimethylamine (free amine) were dissolved in buffer solutions of various pH and incubated at 37°C. An aliquot of the reaction mixture was cleaned up by extraction and determined by polarography. The concentration of dimethylamine was 2.5 mM. Clark-Lubs buffer solution was used and the amount of dimethylnitrosamine produced was expressed by ppm.

Table 3. Formation of Dimethylnitrosamine from Sodium Nitrite and Dimethylamine Hydrochloride in Various pH Buffer Solution (60°C)

Conc. of $NaNO_2$ (mM) \ pH	1.2	2.2	3.3	4.2	5.2	6.0	7.0
	ppm						
5	0.5	1.0	2.3	1.4			
25	5.3	8.9	39.2	21.1	2.6	0.8	+
50	13.7	22.0	91.6	73.8	9.4	1.6	+

+: trace

Sodium nitrite and dimethylamine hydrochloride were dissolved in buffer solutions of various pH and incubated at 60°C for 3 hours. The reaction mixture was cleaned up by extraction and determined by polarography. The concentration of dimethylamine hydrochloride was 5 mM and Clark-Lubs buffer solution was used. The amount of dimethylnitrosamine produced were expressed by ppm.

HCl は 5 mM, 亜硝酸ナトリウムは5, 25および 50 mM である. 対応する濃度の 37° と60° (ともに3時間加温) における生成量は 37° を 100% とするとき, pH 1.2 で 129%, pH 2.2 で 200%, pH 3.3 で 237%, pH 4.2 で 485%, pH 5.2 で 226% (いずれも60°加温時) である (Table 3). なお DMNA 生成には pH 3.3 付近が適当であることがわかったので, 細かく pH を区切り, pH と生成量との関係を検討した結果, 至適 pH は3.6 であることが明らかとなった.

3. 人工胃液中における DMNA の生成

人工胃液 (第七改正日本薬局方) に DMA-HCl および亜硝酸ナトリウムを各種濃度に溶解し, 37°, 3時間に生成する DMNA を測定した. なお亜硝酸ナトリウム濃度が増加すると pH は高くなるので, 最終的に pH 1.2 になるように塩酸で調整したものと, 調整しないものについて測定した. 対応する濃度の pH 1.2 の緩衝液中における生成率と, 人工胃液 (pH 1.2) 中における生成率を比較すると, 後者は前者の約84%であり, 人工胃液中の生成率は若干低い (Table 4).

また人工胃液中で TMA-HCl (50 mM) と亜硝酸ナトリウム (5~100 mM) とから生成する DMNA の量を測定したが, 37°, 3時間でも生成量はごく微量であり, 最高 7 ppm を認めたにすぎない. なおここで使用した TMA-HCl は, 不純物としての DMA をモル比で100分の1以上は含まないことを薄層クロマトグラフィーにより確認しいる (Table 5).

4. ウサギの胃における DMNA の生成

使用したウサギは体重 2.4~3.0 kg のメスで実験前日には水のみを投与, 実験日には何も投与しない. 一定量の DMA-HCl と亜硝酸ナトリウムを投与直前に約 70 ml の蒸留水に溶解し, 胃ゾンデを用いて経口投与する. 投与後30分で静脈に空気を注入して殺し, 直ちに開腹して全胃を摘出する. 胃内容物をとり出し, pH を測定後, 蒸留水約 100 ml で胃内壁を洗浄し, 洗浄液を胃

Table 4. Formation of Dimethylnitrosamine from Sodium Nitrite and Dimethylamine Hydrochloride in Synthesized Gastric Juice

Conc. of $NaNO_2$ (mM) \ Conc. of DMA-HCl* (mM)	5	50
	ppm	ppm
5	0.8	1.2
25	5.6	13.2
50	8.9	26.5
100	20.6	109.2

* DMA-HCl: Dimethylamine hydrochloride

Sodium nitrite and dimethylamine hydrochloride were dissolved in synthesized gastric juice (JP VII) and incubated at 37°C for 3 hours. The reaction mixture was cleaned up by extraction and determined by polarography.

Table 5. Formation of Dimethylnitrosamine from Sodium Nitrite and Trimethylamine Hydrochloride in Synthesized Gastric Juice

Conc. of $NaNO_2$ (mM)		25	50	100
DMNA produced (ppm)	−	+	1.6	7.0

−: Not detected by TLC method
+: Detected by TLC method

The mixture was incubated at 37°C for 3 hours, cleaned up and determined by polarography. The concentration of trimethylamine hydrochloride was 50 mM.

Table 6. Formation of Dimethylnitrosamine in Fasted Rabbit Stomach

No.	Body Weight (kg)	Dose			pH	DMNA* Produced (mg)	Yield*** (%)
		DMA-HCl (mg)	TMA-HCl+ (mg)	$NaNO_2$ (mg)			
1	2.4	100		100	2.2	0.09	0.09
2	2.4	100		100	3.0	0.12	0.13
3	3.3	250		500	2.5	0.57	0.25
4	3.0	250		500	4.5	1.25	0.55
5	3.0	500		1000	4.8	3.2	0.70
6	2.7	500		1000	4.5	5.0	1.10
7	2.8		1000	2000	4.1	>0.02**	
8	2.7		1000	2000	4.0	>0.02**	

* Amount of DMNA produced in whole stomach content
** Detected by TLC method
*** Theorectical yield calculated from dimethylamine
\+ TMA-HCl: Trimethylamine hydrochloride

30 minutes after dosage, rabbits were killed, stomach contents were taken out with the aid of water, cleaned up and determined by polarography.

内容物に合わせ，以後 1, ii) の方法により抽出して検液を調製し，生成した DMNA をポーラログラフィーにより定量する．対照実験としては24時間絶食させたウサギ(♀)に蒸留水 70 ml を胃ゾンデで経口投与し，上記の方法により胃内容物をとり出し検液を調製した．対照実験で得られた検液は，通常の測定電位 −0.7～−1.6 volt においてまったく妨害を認めなかった．またこの検液は薄層クロマトグラフィーによっても妨害するスポッ

トを示さない．また対照実験によって得られた胃内容物を遠心分離後，炭酸カリウムを加えてアルカリ性とした上澄液 100 ml に亜硝酸ナトリウム 400 mg，DMA-HCl 200 mg を加え，常法に従いポーラログラフィー用検液を調製した．この検液について得られたポーラログラムには，DMNA の定量を妨害するものは認められず，また DMNA も検出されていない．この対照実験の結果から，未反応の DMA-HCl と亜硝酸ナトリウムから抽出操作中に DMNA を生成することはないと考えられる (Table 6).

III. 考　　察

1. DMA あるいはジエチルアミンのような強塩基性アミンから，対応するニトロソアミンを生成する収率は一般に極めて低い．Sander ら[6] は pH 2 においてジプロピルアミンとジフェニルアミンのニトロソ化の速度を比較し，後者は前者の 1,000 倍も速やかにニトロソ化されることを報告している．したがって本実験で使用した DMA は第2級アミンの中でももっともニトロソ化されにくいアミンのうちの一つである．本実験の結果の示すとおり，DMNA 生成率は極めて低いが，pH 3.3 では比較的高い生成率を示した．

2. ハム，ソーセージなどの製造時には60°～80°に加熱処理されることが多いが，60°，3時間の加温により，37°，3時間の2～3倍の DMNA を生成している．また DMNA 生成の至適 pH を 60°，3 時間で測定した結果，pH 3.6 であることがわかった．

3. 第4報で報告するように畜肉中の DMA 量は極めて微量であるが，魚類，とくに海産魚類ではかなりの量の DMA が検出されている．したがって亜硝酸塩が畜肉に添加されても畜肉製品中に DMNA が生成される可能性はほとんどない．魚肉ハム，魚肉ソーセージの原料肉の一つである魚肉には，比較的多量の DMA を含むが，魚肉ハム，魚肉ソーセージに発色剤として亜硝酸塩が添加されたとしても，魚肉ハム，魚肉ソーセージの pH は 5.5～6.5であるので，生成率は極めて低い．第4報で報告するとおり，亜硝酸ナトリウムを添加した魚肉ハム，魚肉ソーセージから 100 ppb 以上の DMNA は検出されていない．今後さらに高感度の分析法で再検討されるとしても，魚肉ハム，魚肉ソーセージ中に検出される DMNA は 10～20 ppb 程度であろう．

4. 人工胃液での DMNA の生成実験では，pH を 1.2に調整したものよりも，調整しないものの方が，DMNA 生成の至適 pH に近く生成量も多い．DMA あるいは亜硝酸塩は食品とともに摂取されるので，ヒトの胃中の pH が1.2近辺にあるよりも，むしろ若干高い値を示すと考えるのが適当であろう．人工胃液中と緩衝液中とでの同一 pH での DMNA 生成量を比較すると，前者の方が約16%低い．この理由は明らかではないが，人工胃液中のタンパクによって亜硝酸塩がある程度消費されるので，DMNA 生成率が低下しているとも推定できる．

5. ウサギに DMA と亜硝酸ナトリウムを同時に経口投与した実験では，DMNA の生成率は低いが，投与量の増加とともに生成量は確実に増加している．Sander ら[6] のジフェニルアミンと亜硝酸ナトリウムをラットに投与し，胃内容物についてジフェニルニトロソアミンを検出した実験では，収率は理論値の17～31%に達している．これに対し著者らのウサギでの実験での DMNA の収率は，使用した DMA に対して 0.099～1.10% であり，はるかに低収率である．この理由は DMA の強塩基性によるものである．

6. TMA と亜硝酸ナトリウムから DMNA の生成率は極めて低い．ウサギに大量の TMA-HCl と亜硝酸ナトリウムを投与した場合でも，胃内容物から薄層クロマトグラフ法でわずかに検出し得る程度生成したのみで，定量はできなかった．したがって DMNA の前駆物質としては TMA を考慮する必要はない．

7. 以上の結果からヒトの胃中で，DMA と亜硝酸塩とから DMNA を生成する可能性は否定することはできない．しかしそのためには大量の DMA と，大量の亜硝酸塩を同時に摂取する必要がある．またその場合でも DMNA の生成率は低い．ヒトの1日の亜硝酸摂取量は約 1.5 mg といわれているので，この程度の摂取量で DMNA などのニトロソ化合物が生成するとは，ほとんど考えられない．本実験での DMA，亜硝酸塩の投与量は，ヒトの通常の摂取量と比較すると極端に大量である．*in vivo* の実験での最低投与量でも亜硝酸ナトリウムは 100 mg/2.4 kg であるので 42 mg/kg となり，体重 50 kg のヒトでは 2.1 g の投与量となる．これは前述の1日平均摂取量の1,400倍に相当し，しかもこの場合でも DMNA 生成はわずかに0.099%である．したがって本実験の結果から食品添加物として使用されている亜硝酸ナリウムを直ちに発ガン因子の一つとして考えるのは妥当性を欠くように思われる．

IV. ま と め

1. 緩衝液，人工胃液，ウサギ胃内容物中に生成する DMNA を抽出し，薄層クロマトグラフィーおよびポーラログラフィーにより DMNA の定性，定量を行なった．

2. 種々の pH の緩衝液中で，37°，3時間に生成する DMNA を定量し，pH 3.3 がもっとも高い生成率を示した．DMA からの生成率は理論値の10.57%，pH 2.2 および pH 4.2 にずれると，それぞれ 3.01，4.10 と減少する．さらに pH の間隔を細かくとり，60°，3時間で DMNA 生成の至適 pH を測定したところ，pH 3.6 に極大があることがわかった．

3. 比較的低濃度の DMA を亜硝酸ナトリウムを37°，4週間放置すると pH 6.2 でも 1.16～2.31 ppm（生成

率は理論値のそれぞれ0.63, 1.20%) の DMNA を生成する. この実験は食品の保存中における DMNA 生成の可能性を調べるために行なったもので, 12週間まで追跡した.

4. 食肉製品製造時に加えられる加熱条件として60°, 3時間における DMNA 生成量を測定した. また人工胃液中における生成量についても測定した.

5. ウサギに DMA と亜硝酸ナトリウムを同時に経口投与し, 30分後に胃内容物中の DMNA について定量した. DMNA の生成率は極めて低く, 理論値の0.09~1.10%であるが, 大量の DMA, 亜硝酸ナトリウムを投与するときには, 約 200g の胃内容物中に 5mg の DMNA を生成した. なお多量の TMA と亜硝酸ナトリウムを同様に経口投与した場合に生成する DMNA は痕跡程度であった.

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БЛАСТОМОГЕНЕЗ У КРЫС ПРИ КОМБИНИРОВАННОМ ДЕЙСТВИИ N-2-ФЛУОРЕНИЛАЦЕТАМИДА И N-НИТРОЗОДИМЕТИЛАМИНА

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Из лаборатории экспериментальных опухолей (зав.— канд. мед. наук Н. П. Напалков) НИИ онкологии им. проф. Н. Н. Петрова МЗ СССР (дир.— чл.-корр. АМН СССР проф. А. И. Раков)

При сочетанном действии различных канцерогенных соединений могут происходить как суммация, или усиление, так и ослабление их бластомогенного эффекта [1, 6], что, однако, невозможно предугадать заранее, без предварительной экспериментальной проверки. Наряду с этим суммация, или усиление бластомогенного эффекта, могут происходить и при последовательном применении канцерогенных веществ в малых дозах, которые сами по себе недостаточны для индукции опухолей [3, 4, 7]. Принимая во внимание, что в организм человека могут поступать различные количества нескольких канцерогенных соединений, необходимо дальнейшее накопление сведений об особенностях бластомогенеза при комплексном воздействии подобных веществ для выработки принципов рациональной профилактики злокачественных новообразований.

Было высказано предположение о том, что наибольшая вероятность суммации эффектов имеется при сочетанном действии агентов, обладающих одинаковой преимущественной органотропностью бластомогенного действия [8]. Это и побудило нас исследовать комбинированное действие N-2-флуоренилацетамида (2-ФАА) и N-нитрозодиметиламина (ДМНА), поскольку оба эти вещества вызывают появление опухолей печени у крыс. Ранее Weisburger и соавт. [9] установили, что сочетанное введение крысам 2-ФАА и некоторых симметричных нитрозаминов алифатического ряда сопровождалось увеличением частоты возникновения гепатом и сокращением латентного периода их развития по сравнению с опытами, в которых животные получали только 2-ФАА и в той же дозе, что и при комбинированном введении канцерогенов. Однако эти опыты не позволили сравнить эффект комбинированного действия на крыс 2-ФАА и нитрозаминов с действием одних только нитрозаминов, поскольку последние вводились в таких больших дозах, что вызывали появление гепатом у всех животных и в ранние сроки. Задача нашей работы заключалась в том, чтобы выяснить, как протекает бластомогенез у крыс при комбинированном введении им 2-ФАА и ДМНА по сравнению с опытами, в которых животные подвергались действию только одного их этих канцерогенных агентов, но в удвоенных дозах. Подобная организация эксперимента дает возможность для более четкого суждения о возможности суммации или потенцирования бластомогенных эффектов двух агентов, нежели их комбинированное применение в тех же дозах, что и при раздельном введении. В частности, для возможности сравнения результатов опытов необходимо выбрать такие дозы и такую продолжительность введения канцерогенных веществ, которые при их раздельном применении привели бы к возникновению опухолей лишь у части животных.

Материал и методика. В опытах были использованы 100 белых беспородных крыс-самцов весом 150—170 г. 2-ФАА и ДМНА вводили через зонд в желудок 6 раз в неделю в течение 55 дней. В зависимости от характера воздействия все животные были разделены на 4 группы. В первой группе каждой крысе вводили по

2 *мг* 2-ФАА, взвешенного в подсолнечном масле, во второй группе — по 0,2 *мг* ДМНА, растворенного в воде. Крысам третьей группы вводили одновременно 2-ФАА и ДМНА, но в половинных дозах. Животным четвертой группы вводили попеременно через день по 2 *мг* 2-ФАА и по 0,2 *мг* ДМНА, что в значительной мере ограничивало возможность прямого химического взаимодействия указанных соединений [5, 10]. Таким образом, для выявления различий в частоте появления опухолей у животных, получавших 2-ФАА и ДМНА порознь или совместно, были выбраны такие суммарные дозы и продолжительность введения этих веществ, которые значительно меньше применяющихся обычно для индукции опухолей [2, 10].

На 500—504-й день от начала введения 2-ФАА или ДМНА все оставшиеся в живых крысы были убиты. После вскрытия и макроскопического исследования животных их внутренние органы фиксировали в 10% формалине и подвергали обычной гистологической обработке. Срезы толщиной 5—6 μ окрашивали гематоксилином-эозином и по Ван-Гизону для последующего гистологического изучения.

Результаты опытов и их обсуждение. Опухоли развились у животных во всех группах (см. таблицу). Сведения о животных 3 и 4-й групп объединены, поскольку у крыс, получавших 2-ФАА и ДМНА одновременно или попеременно через день существенных различий в частоте и времени появления опухолей не было обнаружено.

Индукция опухолей у крыс при раздельном и сочетанном введении 2-ФАА и ДМНА

Характер воздействия	Суммарная доза введенных канцерогенов (в *мг* на крысу)	Кол-во крыс в начале опыта	Кол-во крыс, доживших до времени появления 1-й опухоли	Кол-во крыс с опухолями всех локализаций	Частота возникновения опухолей (%)	Кол-во крыс с опухолями печени	Частота возникновения опухолей печени (%)	Опухоли печени			Прочие опухоли	Всего опухолей
								цистохолангиомы	гепатомы	кавернозные гемангиомы		
2-ФАА	94,0	25	16	10	62,5±12,5	10	62,5±12,5	10	3	—	—	13
ДМНА	9,4	25	15	5	33,3±12,6	4	26,6±11,8	1	2	1	1	5
2-ФАА и ДМНА	47,0±4,7	50	27	11	40,7± 9,6	8	29,6± 9,6	3	5	1	4	13

У животных, получавших только 2-ФАА, опухоли возникли только в печени. Первое новообразование было обнаружено у крысы на 500-й день опыта. 10 из 13 опухолей, возникших у крыс этой группы, являлись цистохолангиомами (рис. 1, *а*). В их составе можно было выделить два основных типа структур. Один из них был представлен трубчатыми образованиями, выстланными призматическими эпителиальными клетками с округлыми светлыми пузырьковидными ядрами. Трубчатые структуры были различной величины и формы, а выстилающий их эпителий местами располагался в несколько слоев. Структуры второго типа представляли собой кисты, заполненные прозрачным, слегка зеленоватым содержимым. Чаще всего кисты были многокамерными и состояли из многих полостей, местами выстланных призматическими, а местами резко уплощенными эпителиальными клетками. В полости нередко вдавались сосочки, покрытые эпителием, образовывавшим многослойный пласт. Три опухоли оказались гепатомами, выбухавшими над поверхностью печени в виде ярко-розовых плотных узлов, четко отграниченных от окружающей ткани. Эти узлы состояли из крупных клеток, часто полиморфных и атипичных, во многих из которых отмечались признаки жировой или гидропической дистрофии (рис. 1, *б*). Эти клетки имели оксифильную цитоплазму и округлое или слегка овальное ядро с многочисленными, равномерно распределенными мел-

ками гранулами хроматина. В ядре имелось одно или два ядрышка, изредка встречались митозы. Подобные клетки образовывали атипичные печеночные балки.

У крыс второй группы под влиянием только ДМНА опухоли печени возникли у 4 животных (26,7±11,8%). Первое новообразование было найдено у крысы, убитой на 501-й день опыта. Помимо цистохолангиомы и гепатом описанного выше строения в одном случае была обнаружена кавернозная гемангиома печени. Она представляла собой крупный ярко-розовый узел мягкой консистенции, с поверхности разреза которого обильно стекала кровь. Опухоль состояла из крупных полостей, выстланных эндотелиальными клетками и отграниченных друг от друга

Рис. 1. Опухоли у крыс, получавших 2-ФАА.
а — цистохолангиома, *б* — гепатома. Гематоксилин-эозин. Ув. 112,5.

перегородками из грубых коллагеновых волокон. В некоторых перегородках располагались ряды печеночных клеток.

Наряду с опухолями печени у одной крысы из этой группы развилась диктиоцитарная ретикулосаркома. Это новообразование целиком занимало одну из долей легкого, которая имела плотную консистенцию. Поверхность разреза опухоли имела белесоватый цвет и была однородного характера. Основная масса опухолевой ткани состояла из крупных полиморфных клеток неправильной формы с большими резко гиперхромными ядрами и резко варьирующим ядерно-цитоплазменным соотношением. Опухолевые клетки не образовывали сомкнутого пласта, а были резко разобщены. Между отдельными клетками отчетливо определялись преколлагеновые и коллагеновые волокна, иногда образовывавшие пучки (рис. 2, *а*).

В объединенных третьей и четвертой группах у животных, получавших 2-ФАА совместно с ДМНА, опухоли печени (гепатомы, цистохолангиомы и кавернозная гемангиома) возникли у 8 крыс (29,6±±8,9%). Кроме того, у животных этой группы развилась одна аденома легкого, ретикулосаркома и две плазмоцитомы почки (рис. 2, *б*). В обоих случаях плазмоцитомы прорастали брюшную стенку с изъязвлением кожного покрова. Опухоли почек состояли из однородных округлых или овальных клеток с характерными эксцентрически расположенными

и небольшими ядрами округлой формы с грубыми глыбками хроматина, располагавшимися радиально. Между опухолевыми клетками обнаруживались русселевские тельца. Первая опухоль (плазмоцитома почки) в этой группе была обнаружена у крысы, убитой на 306-й день опыта.

Результаты описанных выше опытов позволяют заключить, что при введении крысам 4,7 *мг* (суммарная доза) ДМНА в сочетании с 47 *мг* 2-ФАА общая частота возникновения опухолей и скорость их индукции оказались такими же, как при введении одного ДМНА во вдвое большей дозе, равной 9,4 *мг*. Учитывая имеющиеся сведения о зависимости между дозой, продолжительностью введения канцерогенных нитрозаминов и интенсивностью бластомогенеза [3], можно полагать, что в этом

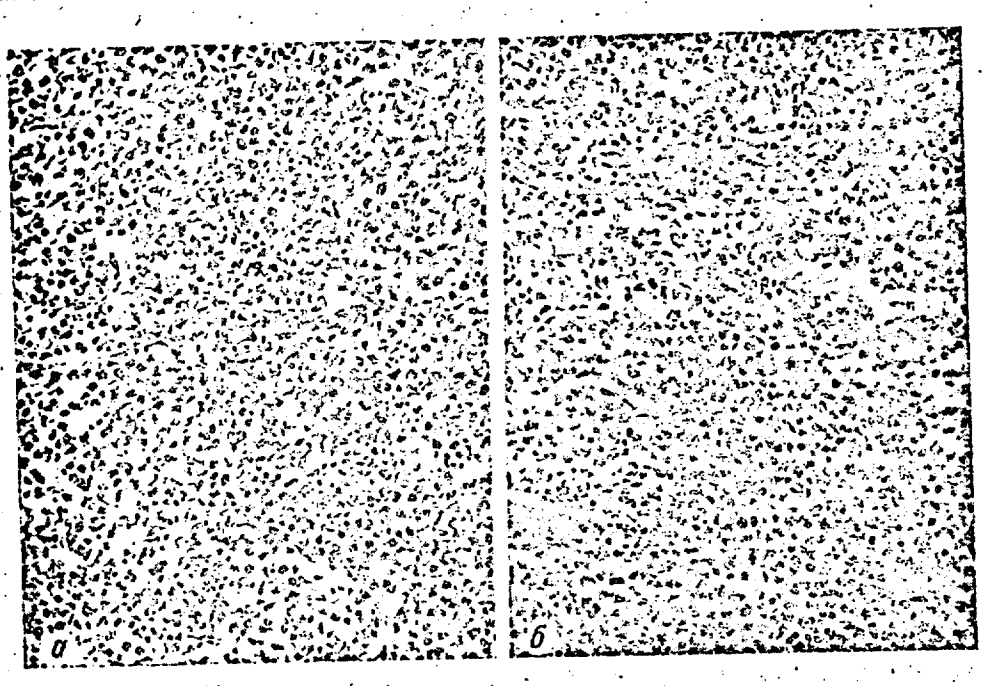

Рис. 2. Опухоли у крыс, получавших ДМНА (*а*) и 2-ФАА+ДМНА (*б*).
а — диктиоцитарная ретикулосаркома легкого, *б* — плазмоцитома почки с полным разрушением ее ткани. Гематоксилин-эозин. Ув. 200.

случае наблюдался аддитивный эффект двух веществ. Это заключение подтверждается как результатами сравнения частоты возникновения новообразований всех локализаций, так и опухолей только печени. Последние были отмечены у 26,7% крыс, получавших только ДМНА (9,4 *мг*) и у 29,6% крыс, которым ввели 4,7 *мг* ДМНА и 47 *мг* 2-ФАА.

Если сопоставить интенсивность бластомогенеза у крыс, которым вводили только 2-ФАА или только ДМНА, то видно, что примененные нами дозы этих веществ не совсем эквивалентны. Так, за одно и то же время у крыс, получивших 94,0 *мг* 2-ФАА, опухоли возникли значительно чаще, чем при введении 9,4 *мг* ДМНА. Это было особенно заметно при сравнении частоты развития опухолей печени (соответственно 62,5 и 26,7%; $p<0,05$). Нет оснований полагать, что половинные дозы указанных веществ также не будут различны по эффективности бластомогенного действия. Этим обстоятельством можно объяснить то, что в наших опытах замена половины количества 2-ФАА всего 4,7 *мг* ДМНА сопровождалось резким снижением частоты опухолей печени по сравнению с таковой у крыс, получивших полную дозу (94 *мг*) 2-ФАА (соответственно 29,6 и 62,5%; $p<0,05$). Если судить по частоте развития опухолей печени, то при комбинированном воздействии эффект оказался таким же, который можно было ожидать от половинной дозы

одного только 2-ФАА. Однако общая частота развития опухолей всех локализаций была несколько выше, так как у 3 крыс дополнительно развились новообразования, характерные для действия ДМНА (ретикулосаркома, плазмоцитомы почки). Таким образом, в данном случае аддитивный эффект при комбинированном применении двух агентов проявился в возникновении новообразований, вызываемых обычно лишь одним из этих веществ.

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BLASTOMOGENESIS IN RATS IN THE COMBINED EFFECT OF N-2-FLUORENILACETAMIDE AND N-NITROSODIMETHYLAMINE

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The experiments with the combined effect of two carcinogenic compounds N-2-fluorenylacetamide (2-FAA) and N-nitrosodimethylamine (DMNA) on rats have demonstrated that in DMNA injection in combination with 2-FAA the incidence of hepatic tumors was found to be the same (29.6±9.6%) as in injection of DMNA solely, but in a double dose (26.7±11.8%). An additive effect was manifested in the increased incidence of neoplasms of all localizations.

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КОКАНЦЕРОГЕННОЕ ДЕЙСТВИЕ ИШЕМИИ

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Вопрос о роли гипоксии и ишемии в канцерогенезе является одним из коренных и дискуссионных в онкологии [5, 12—14]. Уже было показано [3], что дополнительная ишемизация почек у крыс при имплантации пластмассовой капроновой пленки вокруг почек способствует увеличению относительной частоты папилломатоза лоханки и некоторому сокращению латентного периода развития сарком; в то же время сама по себе ишемизация почек не индуцирует развития опухолей, а вызывает только в отдельных случаях гиперплазию лоханочного эпителия. В настоящей работе исследовалось влияние дополнительной ишемизации почки на канцерогенное действие введенного в нее метилхолантрена.

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Kann Nitrit in der menschlichen Nahrung Ursache einer Krebsentstehung durch Nitrosaminbildung sein?

Von J. Sander

Mit 1 Abbildung

Seit im Jahre 1956 *Magee* und *Barnes* Dimethylnitrosamin zuerst als Lebergift und später (1959) als starkes Kanzerogen beschrieben haben, sind zahlreiche weitere N-Nitroso-Verbindungen untersucht worden. Einen besonders großen Anteil an der Aufklärung der biologischen Bedeutung dieser Stoffe verdanken wir dem Arbeitskreis von *Druckrey* (1961, 1962), der die Kanzerogenese durch Nitrosamine sowohl quantitativ wie qualitativ in Tierexperimenten untersuchte. *Es ist zu befürchten, daß Nitrosamine auch den Menschen durch Krebsbildung gefährden.* Bis jetzt ist aber noch nicht genügend geklärt, wo und in welchem Umfang Nitrosamine in der menschlichen Umgebung zu finden sind. Lediglich einige Anhaltspunkte sind bekannt. So wurde auf die Gefährdung von Chemikern und Arbeitern hingewiesen, welche mit den entsprechenden Stoffen umgehen (*Druckrey* 1964, *Brune* 1966). In letzter Zeit konnten Nitrosamine aus Weizenmehl isoliert werden (*Marquardt* 1966). Auch in einem eßbaren Pilz (Clitocybe suaveolens) wurde ein solches Kanzerogen gefunden (*Hermann* 1961). Vermutet wird die Beteiligung von Nor-Nikotin-Nitrosamin bei der Entstehung von Lungentumoren (*Boyland* 1964).

Dem Chemiker sind Nitrosamine seit langem bekannt. Sie haben die allgemeine Formel R-N(NO)-R. Um sekundäre Amine aus Amingemischen zu isolieren, benutzt man ihr Verhalten gegenüber salpetriger Säure, mit der sie Nitrosamine bilden. Die Reaktion findet nur in saurem Milieu statt. Unter dieser Voraussetzung können sich überall Nitrosamine bilden, wo sekundäre Amine und Nitrit gemeinsam vorkommen.

Nitrit kommt in lebenden Geweben nur in außerordentlich niedriger Konzentration vor (*Stieglitz* 1934). Hohe Werte werden in Nahrungsmitteln gefunden, wenn salpetrige Säure sich als Stoffwechselprodukt von Bakterien anhäufen kann, oder wenn sie künstlich hinzugefügt wird. Die Verwendung reinen Nitrits in der Nahrungsmittelindustrie ist in Deutschland verboten. Erlaubt ist ein Pökelsalz, welches auf 100 g Kochsalz 0,5 bis 0,6 g $NaNO_2$ enthält. Es wird Fleisch- und Wurstwaren zugesetzt, um ihnen eine ansprechende Farbe zu verleihen. Die gesetzliche Regelung besteht seit 1934. Trotzdem sind in den nachfolgenden Jahren zahlreiche Vergiftungen beschrieben worden, die auf den übermäßigen Gebrauch von Nitrit bei der Herstellung von Fleischwaren zurückzuführen waren (Literatur bei *Lachheim* 1960).

Pflanzen enthalten wechselnde Mengen Nitrat. Wird dieses durch Bakterien reduziert, so kann das dabei auftretende Nitrit Anlaß zu Vergiftungen geben. Beispiele für schwere Säuglingsintoxikationen nach Spinatmahlzeiten wurden beschrieben (*Sinois* 1965). Ebenso sind aus der Veterinärmedizin Nitritvergiftungen nach Verfütterung nitrathaltiger Pflanzen bekannt (*Liebenow* 1963). Die Pathogenese der sog. Brunnenwassermethaemaglobinaemie ist ähnlich. Sie tritt in Gegenden auf, deren Trinkwasser höhere Nitratkonzentrationen aufweist (*E. Gross* 1964).

Verabreicht man Laboratoriumstieren hohe Dosen $NaNO_2$, so tritt das Bild einer akuten Methaemoglobinaemie auf, die rasch zum Tod durch Sauerstoffmangel führt (LD_{50} bei der Maus = 215 mg/kg [*Riemann* 1950]). Bleibt bei geringerer Dosierung noch ein ausreichender Anteil normalen Haemoglobins übrig, so bildet sich die Veränderung des Blutfarbstoffes zurück. Weitere Schäden treten dann nicht auf.

Sekundäre Amine sind Stickstoffverbindungen, welche nach dem Schema R-NH-R aufgebaut sind. Man unterscheidet aromatische und aliphatische, wobei Systeme, die den Stickstoff im Ring selbst enthalten, wie aliphatische reagieren.

Mit der menschlichen Nahrung können viele sek. Amine in den Magen gelangen. Teils sind sie normalerweise in der Nahrung enthalten, auch als Stoffwechselprodukte von Bakterien und Pilzen, teils werden sie in Form von Medikamenten eingenommen. Auch Desinfektionsmittel, die in der Lebensmittelindustrie verwendet werden, können eine Rolle spielen. Die Tatsache, daß heute bereits etwa 70 verschiedene Nitrosamine auf ihre karzinogene Wirkung hin untersucht worden sind, weist darauf hin, wie groß die Zahl der N-nitrosierbaren Stoffe ist.

Wie eingangs erwähnt, können sich Nitrosamine bilden, wenn Nitrit und sekundäres Amin in saurem Milieu zusammentreffen. Es soll daher geprüft werden, ob eine Nitrosaminbildung auch im menschlichen Magensaft möglich ist. Außerdem ist zu überlegen, ob die hierfür notwendige Menge Nitrit mit der Nahrung aufgenommen wird.

Methodik

a) Nitritbestimmung in Lebensmitteln

Zur Herstellung eines wäßrigen Extraktes der Lebensmittel wurde 1 Gewichtsteil des zerkleinerten Materials mit 9 Teilen Wasser vereinigt. Einige Tropfen einer 25proz. Sodalösung machten die Reaktion leicht alkalisch. Nach kurzem Erhitzen auf 100° blieb der Ansatz unter gelegentlichem Umschütteln 3 Std. bei ZT stehen. Nach der Filtration

ergab sich in den meisten Fällen eine klare Lösung. Extrakte von frischem Obst und Gemüse sowie Mehl wurden im Rotationsverdampfer 1:20 eingeengt.

0,5 ml Testlösung, vermischt mit 4,5 ml Griess'schem Reagenz (Alpha-Naphthylamin und Sulfanilsäure in 30proz. Essigsäure), zeigte einen charakteristischen blauroten Farbton bei Anwesenheit von Nitrit. Die Ausbildung der Farbe war nach 30 min abgeschlossen. Ihre Lichtabsorption wurde im Zeiss Spektralphotometer bei 520 mμ gegen einen entsprechenden Leerwert gemessen, der anstelle des Griess'schen Reagenz 30proz. Essigsäure enthielt. Zur Konzentrationsbestimmung diente eine Eichkurve, deren Verlauf im angewendeten Bereich (unter 10 γ/ml) geradlinig war.

b) Synthese und Nachweis von Nitrosaminen im Magensaft

Zu je 5 ml menschlichem Magensaft (Nüchternsaft, pH 1,3) wurden 0,55 ml Natriumnitritlösung (50 γ NO_2^-/ml) und ein Kristall bzw. ein kleiner Tropfen sekundäres Amin hinzugefügt. *Die Amine waren Ephedrin, Diäthylamin, Dimethylamin und L-Prolin.* Der Ansatz wurde eine Stunde lang bei 37° C bebrütet. Zur Zerstörung der überschüssigen salpetrigen Säure diente Harnstoff, nach dessen Zugabe die Röhrchen nochmals eine Stunde bei 37° C bebrütet wurden. Danach war die Nitritprobe mit Griess'schem Reagenz negativ.

Der Nachweis der gebildeten Nitrosamine geschah nach einer neuen Methode, die an anderer Stelle ausführlich beschrieben werden soll. Ihr Prinzip besteht darin, Nitrit aus Nitrosaminen in alkalischer Lösung photochemisch abzuspalten. Zu diesem Zweck wurde der nitritfreie Magensaft mit 0,1 n KOH alkalisiert und 3 Std. mit langwelligem UV-Licht (λ max = 366 mμ) bestrahlt. Das gebildete Nitrit wurde mit Griess'schem Reagenz nachgewiesen. Als Kontrolle diente Magensaft, dem Nitrit, jedoch kein sek. Amin, zugesetzt worden war.

Ergebnisse

a) Nitritgehalt von Nahrungsmitteln

Die Ergebnisse der Untersuchung von insgesamt 157 Lebensmittelproben sind gruppenweise in Tab. 1 zusammengefaßt. Wie ersichtlich waren Milch und frisches Fleisch nitritfrei. Relativ wenig salpetrigsaure Salze fanden sich in frischem Gemüse und Obst und Gemüsekonserven. *Gefriergemüse dagegen wies einen erhöhten Nitritgehalt auf.* Obwohl die Mehlbleichung mit Stickoxyden in Deutschland nicht erlaubt ist, ergab die Nitritprobe bei Brot, Backwaren und Mehl einzelne positive Werte. Niedrig war die Konzentration der salpetrigen Säure in frischem Fisch und Fischkonserven, in Räucherfisch war sie erheblich höher.

Die Untersuchung von Wurstwaren ergab die höchsten Werte, allerdings bestand keine Relation zwischen der Intensität der Rotfärbung und der nachgewiesenen Menge Nitrit. Von der Herstellungsart der Wurst jedoch waren die Werte deutlich abhängig. Sogenannte Rohwurstarten machen bis zum Verkauf meist eine Reifung in Anwesenheit der als Pökelflora bezeichneten Mikroorganismen durch. Diese Sorten (Salami, Mettwurst u. ä.) waren meist nitritärmer als Koch- und Brühwürste (Leber-, Blut-, Gelbwurst bzw. Jagd-, Bier-, Bockwurst). Eine Ausnahme bildete nur die Blutwurst, in welcher die salpetrige Säure mit dem reichlich vorhandenen Haemoglobin reagieren kann. Hier ließ sich immer nur wenig Nitrit nachweisen.

Tabelle 1. Nitritgehalt von Nahrungsmitteln.

Nahrungsmittel	durchschnittl. Nitritgehalt der Nahrungsmittel (in mg% NO_2^-)	niedrigster	höchster	Zahl der Proben
Milch und Kondensmilch	0	0	0	5
Frischfleisch	0	0	0	3
Brot und Backwaren	0,01	0	0,05	8
Mehl	0,05	0	0,1	3
Gemüse, Obst, Konserven	0,02	0	0,07	15
Gefriergemüse	0,15	0,06	0,40	6
Fisch, Fischkonserven	0,09	0	0,19	12
Räucherfisch	0,97	0,23	1,45	3
Käse	0,21	0,03	0,81	22
Wurstwaren, einschl. Konserven und Schinken	1,85	0,05	6,15	80

Berechnet man aus den gemessenen Werten den durchschnittlichen Nitritgehalt der verschiedenen Wurstarten, so zeigt sich der Unterschied deutlich. Rohwürste enthielten im Mittel 1 mg% NO_2^-, während die beiden anderen Gruppen mit 2,3 mg% eine mehr als doppelt so hohe Konzentration aufwiesen.

In Konserven von Wurst und Fleisch fand sich weniger Nitrit. Der Durchschnitt von 7 Proben ergab 0,42 mg%.

b) Nitrosaminbildung im Magensaft

Die vermutete Synthese von Nitrosaminen im menschlichen Magensaft aus Nitrit und sek. Aminen konnte nachgewiesen werden, denn unter der Bestrahlung mit langwelligem UV-Licht trat Nitrit in allen Ansätzen auf, die zuvor salpetrige Säure und sek. Amin enthalten hatten. Dagegen blieb die Probe negativ, wenn sek. Amin im Reaktionsgemisch fehlte.

Diskussion

Will man die erzielten Resultate der Nitritbestimmung als repräsentativ ansehen, so läßt sich mit Hilfe des Statistischen Jahrbuches auf Grund der Angaben über den Lebensmittelverbrauch ungefähr berechnen, wieviel Nitrit im Durchschnitt von einer Person aufgenommen wird. Dieser Wert beträgt 22 μMol pro Tag. Abb. 1 gibt einen Überblick über den Verbrauch der wichtigsten Nahrungsmittel. In Säulen wurde der prozentuale Anteil der verschiedenen Lebensmittel am Gewicht der täglichen Nahrung dargestellt. Die jeweils zugehörige 2. Säule macht den Anteil des Nitrits deutlich, der mit den Speisen in den Magen gelangt. Obwohl Wurst und ähnliche Erzeugnisse nur 5 % des Nahrungsgewichtes ausmachen, wird mit ihnen die größte Menge Nitrit aufgenommen. Dies galt bisher als harmlos, denn Schäden durch langfristige Anwendung von Nitrit in niedriger Dosierung waren

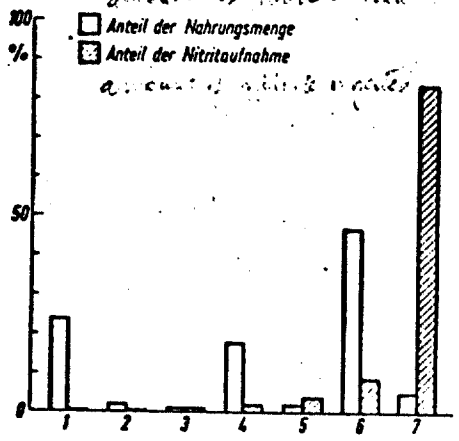

Abbildung 1. Nitritaufnahme mit verschiedenen Lebensmitteln. 1 = Milch, 2 = Frischfleisch, 3 = Fisch, 4 = Backwaren, 5 = Käse, 6 = Obst u. Gemüse, 7 = Wurstwaren (berechnet aus den Angaben des *Statistischen Jahrbuches* (1964).

nicht bekannt. Fütterungsversuche an Ratten mit relativ hohen Tagesdosen (100 mg/kg Ratte) ließen selbst bei Ausdehnung der Versuche über 3 Generationen keine Schädigung erkennen. Die gleichzeitige Gabe von Nitrit und sekundärem Amin (Diäthylamin) führte bei den Ratten nicht zur Krebsentstehung, da keine Nitrosamine gebildet wurden, was die Autoren auf eine unzureichende Säuerung des Magensaftes zurückführten (*Druckrey* 1963).

In unseren Versuchen konnte nun die Nitrosaminbildung aus Nitrit und sekundärem Amin im menschlichen Magensaft gezeigt werden. Wieviel Nitrosamin in vivo aus der zugeführten Nitritmenge entstehen kann, ist bis jetzt nicht bekannt. Sicher hängt dies von verschiedenen Faktoren ab. Zu den wichtigsten gehören die Konzentration der sekundären Amine, der Säuregrad des Speisebreies und die Verweildauer der Speisen im Magen. Nicht zu erwarten ist, daß häufig eine vollständige Umsetzung des aufgenommenen Nitrits mit sekundären Aminen stattfindet.

Für die Entstehung von Krebs genügt es nicht, daß überhaupt Nitrosamine gebildet werden, da Toxizität und Organspezifität bei einzelnen Nitrosaminen stark differieren. Andererseits ist seit langem bekannt, daß alle Carcinogengaben auch bei äußerst niedriger Dosierung voll summationsfähig sind (Summationsgifte, *Druckrey* 1948), und daß bei langdauernder Applikation die Zeit eine Verstärkung der carcinogenen Wirkung herbeiführt, womit die zur Tumorbildung erforderliche Gesamtdosis z. T. erheblich reduziert wird (*Druckrey* 1962). Dies kann besonders beim Menschen eine Rolle spielen, wo Jahrzehnte bis zum Auftreten von Tumoren vergehen können. Eine weitere Reduzierung der erforderlichen Carcinogendosis kann zumindest im Tierversuch durch sogenannte Cocarcinogene oder tumorrealisierende Stoffe bewirkt werden. Diese Substanzen sind selbst nicht cancerogen, können aber Tumoren zur Entwicklung bringen, die durch stark unterschwellige Carcinogendosen induziert worden sind.

Da selbst kleine Carcinogenmengen unter bestimmten Umständen zur Tumorbildung führen können, sollte dem Entstehen von Nitrosaminen im menschlichen Magensaft besondere Aufmerksamkeit geschenkt werden.

Zusammenfassung

Um zu prüfen, ob Nitrit in der menschlichen Nahrung Anlaß zu einer Nitrosaminbildung und damit eventuell Ursache einer Krebsentstehung sein könnte, wurde untersucht, welche Menge salpetriger Säure mit den Lebensmitteln aufgenommen wird und ob unter den Bedingungen des menschlichen Magens eine Nitrosaminsynthese aus Nitrit und sekundärem Amin stattfindet. Man kann annehmen, daß die hierzu notwendigen sekundären Amine in erforderlichem Maße in der Nahrung enthalten sind.

Auf Grund experimenteller Daten und statistischer Unterlagen wurde die durchschnittliche Nitritaufnahme einer Person mit 22 μMol pro Tag ermittelt. In In-vitro-Versuchen konnte gezeigt werden, daß Nitrosamine im Magensaft aus sekundären Aminen und Nitrit gebildet werden. Setzt man voraus, Nitrosamine verhielten sich hinsichtlich der Krebserzeugung beim Menschen ähnlich wie im Tierversuch, so wäre nur ein Teil des mit der Nahrung aufgenommenen Nitrits erforderlich, um durch Nitrosaminbildung Krebs zu verursachen.

Summary

In order to see whether nitrite in human nutrition is liable to cause the formation of nitrosamine and therefore possibly lead to cancerogenesis, it has been examined what amount of nitrous acid is taken up with the food and whether or not a nitrosamine synthesis is possible by nitrite and secondary amine under the conditions prevailing in the human stomach. One can assume that the quantities of secondary amines required for this process are contained in the food.

On the basis of experimental data and statistical information, a daily average nitrite uptake of 22 μMol has been calculated. In vitro, tests have shown that nitrosamine can be formed in the gastric juice from secondary amines and nitrite. Assuming that the behaviour of nitrosamine in cancerogenesis in man is similar to its behaviour in animal tests, only part of the nitrite taken up with human food would be required to cause cancer by nitrosamine formation.

Résumé

Afin de savoir si le nitrite contenu dans la nourriture de l'homme pouvait entraîner la formation de nitrosamines et par conséquent être la base de l'apparition du cancer, on a recherché quelle quantité d'acide azoteux était assimilée par voie alimentaire et si une synthèse des nitrosamines à partir du nitrite et d'amines secondaires pouvait avoir lieu dans des conditions habituelles pour l'estomac de l'homme. On peut supposer que les amines secondaires nécessaires sont en quantité suffisante dans les aliments.

A la suite de données expérimentales et statistiques, on a fixé l'assimilation moyenne de nitrite à 22 μMol par personne et par jour. Des expériences in vitro ont pu montrer que les nitrosamines dans les sucs gastriques étaient effectivement formées par liaison d'amines secondaires et du nitrite. En supposant que les nitrosamines jouent chez l'homme pour la formation du cancer le même rôle que dans les expériences qui ont été effectuées sur les animaux, une partie seulement du nitrite assimilé par voie alimentaire suffirait pour entraîner le cancer par formation de nitrosamines.

Resumen

Con el propósito de comprobar si el nitrito contenido en la nutrición del hombre da lugar a una formación de nitrosamina que, a su vez, podría ser la causa eventual de la formación de un cáncer, se investigó la cantidad de ácido nitroso que se consume con los alimentos y — tomando en consideración la constitución del estómago del hombre — si se produce una síntesis de nitrosamina del nitrito y de la amina secundaria. Se puede suponer que las aminas secundarias necesarias para este propósito se encuentran en la medida necesaria en los alimentos del hombre.

Sobre la base de datos experimentales y documentaciones estadísticas se comprobó un promedio de consumo de nitrito por persona, de 22 µMol como valor diario Mediante ensayos in vitro se logró comprobar que la nitrosamina en el jugo gástrico es formada por aminas secundarias y nitrito. Partiendo de la suposición de que las nitrosaminas se comportasen, en cuanto a la formación de cáncer en el hombre, en forma análoga que en los experimentos animales, sería suficiente solo una parte del nitrito que se consume con el alimento, para producir un cáncer debido a la formación de nitrosamina.

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Herrn Priv.-Doz. Dr. F. *Schneider*, Physiol.-chem. Institut Tübingen, danke ich für wertvolle Hinweise.

Aus dem Hygiene-Institut der Universität Kiel (Direktor: Prof. Dr. H. Gärtner) und aus dem Universitäts-Strahleninstitut der Universität des Saarlandes in Homburg (Direktor: Prof. Dr. F. Sommer)

Experimentelle Untersuchungen über den Einfluß des Staubes auf die Entstehung des Röntgenbildes bei den Pneumokoniosen*)

Von H. Gärtner und F. Sommer

Mit 5 Abbildungen

Das Röntgenbild (= Rö-Bild) der Lunge ist zwar in der Diagnostik der Staublungenerkrankungen von großer Bedeutung; es gibt aber keinen sicheren Anhalt für die Schwere der Krankheit selbst. Bekannt ist, daß röntgenologisch ausgedehnte Befunde klinisch kaum Erscheinungen zu machen brauchen und umgekehrt. Trotz eingehender Beschreibung des Rö-Bildes bei den verschiedenen Formen der Staublunge bestehen noch Unklarheiten.

Eine Frage, über die noch keine ganz einheitliche Meinung besteht, ist z. B. die, ob die für die Pneumokoniose typischen Veränderungen im Rö-Bild lediglich durch die Reaktion des Lungengewebes auf den eingeatmeten Staub bedingt sind, oder ob der letztere selbst in gewissem Maße an der Entstehung des Rö-Bildes beteiligt ist.

Boehme [1], *Haubrich* [4], *Lochtkemper* [6] und *Sommer* [9] haben in großen Reihenuntersuchungen differentialdiagn. Merkmale für die verschiedenen Staubberufe ausgearbeitet. Die Mehrzahl der gen. Autoren, aber auch *Ickert, Kaestle, Reichmann, Schulte* (Zit. nach [6]) u. a. sind dabei der Ansicht, daß sich Unterschiede bevorzugt durch die verschiedenartige fibrogene Reaktion des Lungengewebes ergäben, der Staub selbst aber im Rö-Bild nicht diagnostisch verwertbar in Erscheinung träte. Zur gleichen Frage betont aber *Haubrich* [4], daß bei der Entstehung der charakteristischen harten, feinfleckigen Rö-Bilder der *Siegerländer* Erzarbeiter die Mitwirkung des Staubes als wesentlich anzusehen sei. Auch *McLaughlin* [7] und Mit. stehen auf Grund ihrer Untersuchungen über Eisenoxydablagerungen in den Lungen auf dem Standpunkt, daß neben den fibrogenen Veränderungen auch die Dichte des eingeatmeten Staubes gegenüber den Rö-Strahlen zu beachten sei.

Ferner ist erörtert worden, ob den bei den Silikosen im Rö-Bild feststellbaren nodulären Veränderungen einzelne Knötchen im Lungengewebe entsprechen. Die hier zugrundeliegende Problematik ist auch in der allg. Röntgenologie bearbeitet worden; knötchenförmige Verschattungen im Rö-Bild wurden als Summationseffekte angesehen, und diese Auffassung muß auch für die Röntgenologie der Silikose gelten.

Zu beiden angeschnittenen Fragen steht die vollständige experimentelle Sicherung der herrschenden Ansichten noch aus. Die eigenen Untersuchungen bringen zwar im gegebenen Zusammenhang ebenfalls keine letzten Ant-

*) Die experimentellen Untersuchungen zu dieser Veröffentlichung sind 1956 auf Anregung des Erstautors, der auch das experimentelle Modell angab, und unter röntgenologischer Aufsicht des Mitautors von H. *Lenhart* [5] im Rahmen einer Doktorarbeit ausgeführt worden. Aus der Dissertation stammen die Abb. dieser Mitteilung. Dort finden sich weitere ausführliche photographische Vergleichsreihen.

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CAN NITRITE IN HUMAN NUTRITION INCOLVE CANCEROGENESIS THROUGH THE FORMATION OF NITROSAMINE? (Kann Nitrit in der menschlichen Nahrung Ursache einer Krebsentstehung durch Nitrosaminbildung sein?)

Author: J. Sander

Source: Archiv für Hygiene und Bakteriologie, 1967, 151:22-28

Since in 1956 Magee and Barnes described dimethyl nitrosamine first as hepatic poison and later on (1959) as highly carcinogenic, a large number of other N-nitroso-compounds have been investigated. We owe a considerable amount of clarification of the biological significance of these substances to the working circle of Druckrey (1961, 1962), who studied the cancerogenesis caused by nitrosamines quantitatively as well qualitatively in animal experiments. It is to be feared that nitrosamines imperil man also through cancer formation. So far, however, insufficient explanation has been provided as to where and to which extent nitrosamines are found in the human environment. A few indications are known only. Allusion was made to the endangerment of chemists and workers who handle such substances (Druckrey 1964, Brune 1966). In recent time, nitrosamines were isolated from wheat flour (Marquardt 1966). Such a carcinogenic substance was detected also in an n eatable mushroom (Clitocybe suaveolens) (Hermann 1961). The participation of Nor-nicotine-nitrosamine in the development of lung tumors is surmized (Boyland 1964).

Nitrosamines have been known for a long time by chemists. There is the general formula R-N(NO)-R. In order to isolate secondary amines from amine mixtures; one makes use of their behavior in relation to nitrous acid, with which they form nitrosamines. The reaction occurs only in acid medium. This prerequisite being fulfilled, nitrosamines can develop anywhere when secondary amines and nitrite occur together.

In living tissues, nitrite occurs only in extremely low concentration (Stieglitz 1934). High values are found in foodstuffs when nitrous acid can accumulate as

product of metabolism of bacteria, or when they are added artificially. The use of pure nitrite in the food industry is prohibited in Germany. A pickling salt containing 0.5 to 0.6 g $NaNO_2$ for 100 g of common salt is allowed. It is added to meat products and to sausages to impart color to them. The legal provision has existed since 1934. Nevertheless, in the following years, many poisonings have been described and attributed to the excessive use of nitrite in the manufacture of meat products (Literature with Lachheim 1960).

Plants contain varying amounts of nitrate. If nitrate becomes reduced by bacteria, the resulting nitrite can cause poisonings. Instances of severe infant poisonings after spinage ingestions were described (Sinois 1965). One knows also, through veterinary medicine, cases of nitrite poisonings when nitrate-containing plants were used in the fodder (Liebenow 1963). The pathogenesis of the so-called well-water methemoglobinemia is similar. It appears in regions the drinking water of which exhibit higher nitrate concentrations (E. Gross 1964).

If high doses of $NaNO_2$ are administered to laboratory animals, the picture of an acute methemoglobinemia develops. The lack of oxygen involved rapidly causes death(LD_{50} in mouse = 215 mg/kg (Riemann 1950). If, with a lesser dose, a sufficient amount of normal hemoglobin still remains, the change of the blood pigment forms again (? degenerates ?). Then other damages do not appear.

Secondary amines are N compounds which are synthesized as follows: R-NH-R. One distinguishes between aromatic and aliphatic compounds, the systems containing N in the ring itself, reacting like aliphatic compounds.

With the human food, many secondary amines can find their way into the stomach. Some are normally contained in the food, as products of metabolism from bacteria and fungi also; some are ingested in the form of medicines. A part is played also by disinfectants used in the food industry. Since today already about 70 different nitrosamines have been investigated as to their carcinogenic action, one can infer how high the number of substances that can be N-nitrosited.

As was mentioned at the beginning, nitrosamines can form if nitrite and secondary amine are found together in an acid medium. Hence the necessity to check whether nitrosamine formation is possible in man's gastric juice also, and whether the amount of nitrite, necessary for this, is ingested along with the food.

Procedure

a Nitrite Determination in Foodstuffs:

In order to produce an aqueous extract of the food stuffs, one part by weight of the comminuted material was combined with 9 parts of water. A few drops of a 25% soda solution rendered the reaction slightly alkaline. After briefly heating to 100 C, the batch was allowed to rest for three hours at room temperature, except for some occasional stirrings. After filtration, a clare solution resulted in the majority of the cases. Extracts of fresh fruitsand vegetables as well as of flour were 1:20 concentrated in a rotation evaporator.

An amount of 0.5 ml of test solution, mixed with 4.5 ml og Griess reagent (alpha-naphthylamine and sulphanilic acid in 30 % acetic acid), exhibited a characteristic blue-red shade when nitrite was present. The development of the color stopped after 30 minutes. The light absorption was measured with a Zeiss-spectrophotometer at 520 mμ against a corresponding blank value which contained 30% acetic acid instead of the Griess reagent. To determine the concentration, one used a calibrating curve whose shape was a straight line in the region utilized (below 10 γ/ml).

b Synthesis and Detection of Nitrosamines in Gastric Juice:

To 5 ml of human gastric juice (empty stomach juice, pH 1.3) 0.55 ml Na nitrite solution (50 γ NO_2^-/ml) and one crystal or a small drop of secondary amine were added. The amines were ephedrine, diethyl amine, dimethyl amine and L-proline. The mixture was allowed to brew for one hour at 37 C. Urea was used in breaking down the nitrous acid in excees. After urea had been added, the small tubes were allowed to rest one more hour at 37 C. Then the nitrite test with Griess reagent was negative.

The detection of the nitrosamines formed was performed by a new method which is supposed to be described in detail somewhere else. The principle involved consists in photochemically splitting nitrite from nitrosamines in alkaline solution. For this purpose, the nitrite free gastrict juice was alkalised with 0.1 n KOH, and irradiated for 3 hours with UV longwaves (λ max = 366 mμ). The nitrite formed was detected with Griess reagent. The control used in this case was gastric juice to which nitrite, but no secondary amine, had be added.

Results

a Nitrite Content of Food Stuffs:

Table 1: Nitrite content in Foodstuffs

a. Foodstuffs b. Average, lowest, highest nitrite content of the foodstuffs
c. Number of specimens d. Milk and evaporated milk e. Fresh meat
f. Bread and pastries g. Flour h. Vegetables, fruits, canned food
i. Fried vegetables j. Fish, canned fish k. Smoked fish l. Cheese
m. Sausages, canned food and ham

The results of the investigation of altogether 157 food specimens were grouped in Table 1. Obviously milk and fresh meat were free from nitrite. Relatively little nitric salts were present in fresh vegetables, fruits and canned vegetables. On the other hand, fried vegetables exhibited an elevated nitrite content. Although the bleaching of flour with nitric osides is not allowed in Germany, the nitrite tests gave positive values respectively with bread, pastry and flour. The concentration of the nitrous acid in fresh fish and canned fish was low, but was much higher in smoked fish.

The investigation of sausages yieled the highest values; it is true that no relation existed between the intensity of the red coloration and the amount of nitrite detected. Nevertheless, the value clearly depended on the type of preparation of the sausage. The so-called raw sausage types went in most cases through a ripening process in the presence of the microorganisms called pickling flora. This occurred before selling. These types (salami, Bologna sausage, among others)

were in the majority poorer in nitrite than sausages for cooking or boiling (liver sausages, black puddings, "yellow" sausages, beer-sausages, bock-sausages). Only black-pudding forms an exception; there nitrous acid can react with the hemoglobin present in abundance. Here always only little nitrite was detected.

If, on the basis of the values measured, one calculated the mean nitrite content in the different types of sausages, the difference appears clearly. Raw sausages contained an average of 1 mg% NO_2^-, whereas the other two groups, with 2.3 mg%, exhibited a concentration that was twice as high.

In canned sausage and meat, there was less nitrite. Seven specimens on the average resulted in 0.42 mg%.

b Nitrosamine Formation in the Gastric Juice:

It was possible to detect the surmized synthesis of nitrosamines in human gastric juice from nitrite and secondary amines, since under irradiation with longwave UV light, nitrite appeared in every batch which previously had contained nitrous acid or secondary amine. On the other hand, the test remained negative, when secondary amine was lacking in the reaction mixture.

Discussion:

If one wants to regard the results obtained about the nitrite determination as representative, then with the help of the Statistic Yearbook and on the basis of the data concerning food consumption one can calculate approximately how much nitrite is ingested on an average by one individual. This value amounts to 22 μmol per day.

Figure 1: Nitrite ingestion with different foodstuffs. 1 = milk, 2 = fresh meat, 3 = fish, 4 = pastries, 5 = cheese, 6 = fruits and vegetable, 7 = sausages (calculated from the data of the Statistic Yearbook (1964).

Figure 1 provides a synthetic view of the consumption of the main foodstuffs. The percentage of the different foodstuffs according to the weight of the daily intake was represented in columns. In each case the 2nd column shows the amount of nitrite which along with the food finds its way into the stomach. Although sausage and similar products constitutes only 5% of the food weight, it is with them that the highest amount of nitrite is absorbed. So far this was regarded as harmless, since injuries through prolonged utilisation of nitrite in low doses were unknown. Feeding tests involving the administration of relatively high daily doses to rats (100 mg/kg rat) revealed no injury, even when the experimentation was extended to three generations. The simultaneous intake of nitrite and secondary amine (diethyl amine) by the rats did not result in cancer, since no nitrosamines formed, which the Authors attributed to insufficient acidity of the gastric juice (Druckrey 1963).

Our tests revealed only the nitrosamine formation from nitrite and secondary amine in human gastric juice. How much nitrosamine in vivo candevelop from the nitrite amount introduced remains unknown so far. No doubt, this depends on different factors. To the most important of these belong the concentration of the secondary amines, the degree of acidity of the chyme and the time of sojourn of the food in the stomach. What is unexpected is that frequently a complete reaction of the nitrite ingested takes place with secondary amines.

For the development of cancer it does not suffice that nitrosamines be formed, since the toxicity and the organ specificity vary considerably in individual nitrosamines. On the other hand, it has been known for a long time that all carcinogen amounts administered, even in extremely dosage, are fully capable of summation (summation poisons, Druckrey 1948), and that in prologed application the time factor increases the carcinogenic action, so that the total dosis, necessary for the tumor formation, becomes considerably reduced (Druckrey 1962). This can play a part especially in man, where decades can elapse before the appearing of tumors- A further reduction of the carcinogenic dose required can be brought about at least in animal testing, through so-called cocarcinogens or tumor producing substances. These substances themselves are not cancerogenic, but can cause tumors, induced by very subliminal carcinogen doses, to develop. Since even small carcinogen amounts, under certain conditions, can lead to tumor formation, special attention should be given to the development of nitrosamines in human gastric juice.

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Virusforschung und Parasitologie

Originale

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Band 212

Mit 219 Abbildungen im Text und 6 zum Teil farbigen Tafeln

GUSTAV FISCHER VERLAG · STUTTGART
1969/70

8. Eine Probe von jedem Mittagessen (volle Portion) ist im Tiefkühlfach eines Kühlschrankes für mindestens 24 (48) Std. aufzubewahren.

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Die Bedeutung des Nitrit- und Nitratgehalts von Nahrungsmitteln für die Bildung kanzerogener Nitrosamine in menschlichen Magen

J. SANDER

Mit 2 Abbildungen im Text

Die toxische Wirkung von Nitrit wird vor allem in einer Methämoglobinbildung gesehen, die bei genügend hoher Dosierung rasch zum Tod durch Sauerstoffmangel in den Geweben führt. Bei niedriger Dosierung ist die Methämoglobinämie leicht rückbildungsfähig. Spätschäden treten nicht auf. Schädliche Wirkungen einer chronischen Nitritaufnahme in niedriger Dosis sind bisher beim Menschen nicht festgestellt worden. Chronische Applikation von Natriumnitrit an Ratten ließ in Untersuchungen von DRUCKREY et al. (Arzneimittel-Forsch. 13 (1963), 320) sowie in eigenen Untersuchungen eine wesentliche Beeinträchtigung der Entwicklung und der Lebenserwartung nicht erkennen. Die Auffassung, in Nitrit eine weitgehend ungefährliche Substanz vor sich zu haben, ist die Grundlage für die gesetzlich erlaubte Verwendung der Substanz bei der Herstellung bestimmter Nahrungsmittel.

Wenn Nitrit selbst in niedriger Dosis eine Schädigung des menschlichen Organismus nicht bewirkt, so ist jedoch in Erwägung zu ziehen, ob dieser reaktionsfreudige Stoff nicht Anlaß zur Bildung toxischer Umsetzungsprodukte geben kann. Hierbei ist besonders an die Möglichkeit einer Bildung kanzerogen wirkender Nitrosoverbindungen zu denken, deren Auswirkung sich nicht akut, sondern u.U. erst nach sehr langer Zeit zeigen würde.

Nitrosamine und Nitrosamide sind eine große Gruppe von Stoffen, von denen erst ein Teil auf eine kanzerogene Wirkung hin untersucht worden ist. Bis jetzt wurden etwa 70 verschiedene Nitrosoverbindungen geprüft. Die meisten davon erwiesen sich als krebserzeugend. Bei einigen davon genügt für die Induktion von Tumoren eine sehr geringe Menge.

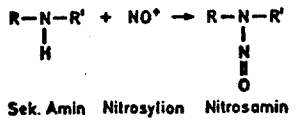

Abb. 1. Synthese von Nitrosaminen aus sekundären Aminen.

In Abbildung 1 wurde das allgemeine Reaktionsschema, nach dem Nitrosamine aus sekundären Aminen synthetisiert werden, dargestellt. Die Molekülreste R und R' können sehr verschiedenartig gebaut sein. Daraus ergibt sich eine große Zahl verschiedener Nitrosamine. Ist einer der Reste eine Carbonsäure, so spricht man von Nitrosamiden. Wie in der Mitte des Bildes angedeutet, erfolgt die Nitrosierung von Aminen durch das Nitrosyl Ion. Dieses wird aus Nitrit in Gegenwart von Säure freigesetzt. Unter diesem Blickwinkel muß man erwarten, daß eine saure Reaktion die Bildung von Nitrosaminen begünstigt. In Gegenwart von Säure bilden die Amine jedoch Salze, was wiederum eine Nitrosaminsynthese behindert. Die Synthese findet deshalb nicht bei maximaler Säuerung, sondern bei pH-Werten zwischen 1 und 3 mit optimaler Ausbeute statt. Diese Werte können auch im menschlichen Magen gemessen werden. Der Möglichkeit einer Nitrosaminbildung im Magen ist deshalb besondere Beachtung zu schenken. Will man sich ein Urteil über die Bedeutung einer Nitrosaminsynthese im Magen bilden, so ist zuerst zu fragen, ob die dafür notwendigen Ausgangsprodukte vorhanden sind.

Nitrit wird bestimmten Nahrungsmitteln zugesetzt. Es übt eine hemmende Wirkung auf Clostridien aus und hat die Eigenschaft, durch Reaktion mit Myoglobin eine ansprechende Rotfärbung bestimmter Wurst- und Fleischwaren zu bewirken. Es findet sich allerdings auch in solchen Erzeugnissen, in denen weder die eine noch die andere Wirkung erwartet werden kann, z. B. in Leberwurst, Weißwurst u.ä. Für die mit Wurst, Fisch und Käse oral aufgenommene Nitritmenge habe ich einmal den Wert von 22 μ Mol pro Tag und Person in der Bundesrepublik berechnet.

In den letzten Jahren sind wiederholt Nitritvergiftungen bei Mensch und Tier beschrieben worden, die auf die bakterielle Reduktion von in pflanzlichen Nahrungsmitteln enthaltenem Nitrat zurückzuführen waren. In solchen Nahrungsmitteln können u.U. Nitritkonzentrationen bis etwa 2000 μM in 100 g gefunden werden. Eine vergleichbare Situation ist bei der sogenannten Brunnenwassermethämoglobinämie gegeben.

Eine bakterielle Reduktion von Nitrat ist natürlich nicht nur in vitro möglich. Physiologischerweise finden sich in der Mundhöhle stets nitrat-reduzierende Bakterien, die ständig das im Speichel vorkommende Nitrat zu Nitrit reduzieren. Nach eigenen orientierenden Untersuchungen ist die mit dem Speichel verschluckte Menge Nitrit etwa in gleicher Größenordnung wie die Aufnahme mit Wurst- und Pökelwaren.

Die Möglichkeit einer Nitratreduktion im Magen-Darm-Trakt fand vor allem im Rahmen der Säuglingsmethämoglobinämie Beachtung. Eigene Untersuchungen, die in Zusammenarbeit mit der Medizinischen Poliklinik Tübingen durchgeführt wurden, dienten dem Zweck, zu prüfen, inwieweit eine bakterielle Nitritbildung auch im Magen des Erwachsenen möglich ist. Die Untersuchungen an etwa 80 Patienten führten zu folgenden Ergebnissen:

Nitrit wurde aus zugeführtem Nitrat nur gebildet, wenn das Nüchternsekret, wie das bei vielen Menschen der Fall ist, neutral oder nur schwach sauer reagierte. War der pH-Wert des Magennüchterninhaltes niedriger als drei, so wurde trotz der neutralisierenden Wirkung des Probetrunks kein Nitrit gebildet.

Man wird den Verhältnissen im Magen am ehesten gerecht, wenn man die vorhandene Bakterienflora als eine Art stationäre Kultur betrachtet, denn eine wesentliche Vermehrung der Bakterien findet in der zur Verfügung stehenden Zeit nicht statt. Den Bedingungen einer stationären Kultur entspricht es, daß neben Nitrat leicht metabolisierbare Stoffe, z.B. Glukose, angeboten werden müssen, um im Magen eine Nitritbildung stattfinden zu lassen. Dem entspricht weiterhin, daß die Steigerung des Nitratangebotes über eine gewisse Mindestmenge hinaus nicht zu einer Steigerung der Nitritbildung führte. Der Umfang der Nitratreduktion hing im wesentlichen von der dafür zur Verfügung stehenden Zeit ab, von derjenigen Zeitspanne also, die verging, bevor der pH-Wert unter 4 absank. Schon 10 Min. nach Verabreichung des Probetrunks war eine sehr deutliche Nitritbildung nachweisbar. Blieb der pH-Wert längere Zeit im Neutralbereich, so wurden Nitritkonzentrationen bis etwa ein Mikrogramm/ml gemessen. In einer etwas anderen Versuchsanordnung mit einer Versuchsdauer bis zu 3 Std. wurden bei Vorliegen einer Anazidität über 100 µg/ml gefunden. Es sei jedoch noch einmal daran erinnert, daß für die Synthese von Nitrosaminen eine saure Reaktion wichtig ist. Das heißt, eine maximale Nitrosaminsynthese, die auf eine bakterielle Nitritbildung im Magen zurückzuführen ist, ist nicht im Falle einer vollständigen Anazidität zu erwarten, sondern bei einer Subazidität, die zuerst eine bakterielle Nitratreduktion und zu einem späteren Zeitpunkt, nach Absinken des pH-Wertes, die Nitrosierung von Aminen besonders begünstigt. Diese Auffassung wurde durch Versuche an Patienten bestätigt, die eine sehr geringe Menge des untoxischen Diphenylamins zusammen mit dem Probetrunk erhielten. Das daraus entstehende Diphenylnitrosamin wirkt nicht kanzerogen.

Nitrit wird also regelmäßig in Mengen von 20–50 µMol pro Tag und Person aufgenommen. Diese Mengen können unter bestimmten Voraussetzungen, z.B. durch Verzehr einer Speise, in der nitratreduzierende Bakterien Nitrit gebildet haben, oder bei verminderter Säureproduktion der Magenschleimhaut um ein Vielfaches überschritten werden. Zur Orientierung seien die Dosen einiger Nitrosoverbindungen genannt, mit denen im Tierversuch Tumoren erzeugt wurden: DRUCKREY und Mitarb. erhielten Tumoren bei nahezu 100% der Versuchstiere nach Applikation von täglich 1–2 µMol Diäthylnitrosamin pro kg Körpergewicht, was einer Gesamtdosis von etwa 0,7–1,5 mMol pro kg entspricht. Bei Verwendung von N-Methyl-N-nitrosobenzylamin wurden Tumoren mit einer Gesamtdosis von weniger als 1 mMol pro kg erhalten. Verschiedene weitere Nitrosoverbindungen erwiesen sich als ähnlich stark wirkend. Die von DRUCKREY et al. angegebenen Dosen sind,

Im Magen können, wie umfangreiche eigene tierexperimentelle Untersuchungen ergaben, die stark basischen Dialkylamine nur sehr schwer, wenn überhaupt, nitrosiert werden. Verabreichung von 0,3% Diäthylamin und 0,3% Natriumnitrit zusammen mit dem Futter während eines halben Jahres führte bei Ratten dementsprechend nicht zur Induktion von Tumoren. Dagegen ließen sich durch Applikation der weniger stark basischen Amine Morpholin, N-Methylbenzylamin und N-Methylanilin zusammen mit Nitrit Tumoren bei allen Versuchstieren hervorrufen.

Eine Nitrosaminsynthese von praktisch interessierendem Ausmaß ist im Magen also nur möglich, wenn im Mageninhalt leicht nitrosierbare Stickstoffverbindungen anzutreffen sind, deren Nitrosoderivate darüber hinaus genügend kanzerogen wirken, was bei den leicht zu synthetisierenden aromatischen Nitrosaminen nicht immer zutrifft. Der für die Synthese verbrauchte Nitritanteil ist stets relativ klein. Er ist um so größer, je weniger stark basisch ein Amin ist und in je höherer Konzentration es vorkommt.

Die Bedeutung von Nitrit und Nitrat in Nahrungsmitteln ist also darin zu sehen, daß durch ihre Gegenwart die Möglichkeit zur Bildung kanzerogener Nitrosoverbindungen im Magen gegeben wird. Eine Induktion von Tumoren beim Menschen damit in Verbindung zu bringen, wäre wohl möglich, sofern sich der Nachweis einer oralen Aufnahme entsprechender nitrosierbarer Verbindungen erbringen ließe. Verschiedene Arzneimittel und Lebensmittelzusätze weisen eine solche Struktur auf. Über das natürliche Vorkommen derartiger Verbindungen liegen noch zu wenig Untersuchungen vor.

Dr. Johannes Sander, Hygiene-Institut, 74 Tübingen, Silcherstr. 7

Die Wiederverwendung von Abwasser

J. Borneff

Bei einer jährlichen Steigerung des Wasserverbrauches um 2–3% ist bis zum Jahre 2000 ein Mehrbedarf von ca. 60% zu erwarten (USA ca. 70%). Da wir zur Zeit etwa 15% des Grund- und Quellwassers nutzen, wird die Deckung des zusätzlichen Verbrauches in erster Linie mit Oberflächenwasser erfolgen müssen. Dieses enthält heute schon im Mittel 5% Abwasser, so daß ein Kreislauf „Wasser-Abwasser-Wasser" bereits in großem Stile existiert, allerdings noch ohne regelrechte Kurzschlüsse. Nach Meinung zahlreicher Fachleute sollte aber gerade dieser Schritt in der nächsten Zeit getan werden, d.h. neben der direkten Wiederverwendung des Abwassers auf dem industriellen Sektor und zur landwirtschaftlichen Bewässerung wäre eines Nutzung auch im öffentlichen Versorgungsbereich anzustreben.

Der Referent berichtet über die im Ausland bestehenden Anlagen und die in einem derartig hergestellten „Trinkwasser" zu beachtenden Schadstoffe, sowie über die modernen Methoden zu deren Eliminierung. Es dürfte danach außer Zweifel stehen, daß es mit diesem „advanced waste treatment" in Zukunft gelingen wird, ein – nach den Analysendaten – dem Grundwasser

DKD

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INDUCTION OF MALIGN TUMORS IN RATS BY ORAL ADMINISTRATION OF N,N'-DIMETHYL-UREA AND NITRITE (Induktion maligner Tumoren bei Ratten durch orale Gabe von N,N'-Dimethylharnstoff und Nitrit)

Author: J. Sander

Source: Arzneim.-Forsch. (Drug Res.) Vol 20, No 3, 1970, P. 418-419

Through food taken for nourishment or pleasure, and through medicines, nitrite and a large number of amines and alkyl amides reach the human stomach. Even in the stomach itself, when subacidity or anacidity is present, nitrite can develop through the bacterial reduction of nitrate contained in almost every food (meal) and can cause the formation of nitroso compounds (Ref. 1). Since many nitroso derivatives from amines and amides have proved to be highly cancerogenic (Ref. 2), it was purposeful to verify which secondary amines or alkyl amides, under the conditions prevailing in the stomach, can be converted there into cancerogenic nitrosocompounds. In previous publications it was expressed that only a few not highly basic secondary amines in the stomach of rats can be nitrosited to such extent as to cause the induction of cancer (Ref. 3, 4). In another investigation, the nitrositability under physiological conditions of alkylamides was verified in vivo and in vitro. Among the amides studied, only N-methyl urea and N-ethyl urea could nitrosite easily in the stomach of rats. The administration of these amides with the diet, when at the same time Na nitrite was given with the drinking water, exerted a very toxic action and was the cause of an early tumor formation in the animals tested (Ref. 5).

Procedure:

In this investigation now reported, N,N'-dimethyl urea was administered. Five groups, of 5 female rats respectively (SIV 50, Ivanovas, Kisslegg) were formed. When the experimentation was started, the animals weighed 120 g. The first group was given drinking water with an addition of 0.3% dimethyl urea, and a diet

(Altromin$^{(R)}$) without any addition. All the other groups were given food with an addition of 0.3% Na nitrite. Group 2 received in addition unchanged drinking water, whereas for group 3, 4 and 5, the following amounts of dimethyl urea were added to the water respectively: 0.1%, 0.3%, 0.9%. Food and water were given as desired by the animals. Administration was performed during 56 days.

Results:

Groups 1 and 2 exhibited no particular signs. The body weight of the animals increased with only very little delay as compared with what is normal. Food and water were consumed normally.

Groups 3 and 4 consumed food and water somewhat less. In group 5, both were consumed very reluctantly. The animals of this group died within the first four weeks of the test. No tumors were observed.

117 days after the administration of nitrite- and dimethyl urea was completed, the first female rat died in group 4. Before the 432nd day, all the animals of groups 3 and 4 died with malignant tumors of the central nervous system, of the heart, of the thymus, of the kidneys or of the thyroid gland, whereas in groups 1 and 2 no tumors developed. The pathological and anatomic findings will be reported somewhere else.

The investigations conducted so far show that some alkyl urea compounds can be nitrosited especially easily under physiological conditions. From the standpoint of preventive medicine and hygiene, it becomes necessary to investigate the nitroso compounds of alkyl ureas, which can reach the stomach, especially of those that are taken in regularly, such as many medicament for instance, with respect to a possible cancerogenic or related activity. One should also determine the possible significance of the development of nitrite in foodstuffs and in the stomach, in this connection, and which precautions should be taken. The admissibility of nitrite in the production of food should be precisely verified from the standpoint of the easy formation of cancerogenic nitroso compounds under physiological conditions.

English Summary at the end.

Die Analyse besagter Steroide erfolgte im wesentlichen wie an anderer Stelle [4, 5] beschrieben. Die Spaltung der in Plasma- oder Harnextrakten auftretenden Steroid-konjugate geschah durch Solvolyse in Äther/Perchlorsäure und Behandlung mit β-Glucuronidase (Ketodase, Gödecke & Co.). Die gesuchten freien oder freigesetzten Steroide wurden dann mittels der 2,4-Dinitrophenylhydrazin-Methode nach mehrfacher Dünnschichtchromatographie der freien Verbindungen und ihrer 2,4-Dinitrophenylhydrazone quantitativ erfaßt.

Ergebnisse und Diskussion

Die in Tab. 1 aufgeführten Plasmaspiegel von DHEA, Androsteron und Ätiocholanolon lassen nach 4—8wöchiger Gabe von täglich 10 mg DHEA-sulfat bei allen Probanden einen deutlichen Anstieg der genannten Steroide erkennen. Die Zunahme von DHEA und seinen Metaboliten bewegte sich zwischen 28 und 110% des jeweiligen Ausgangswertes. Erwartungsgemäß fand man auch eine merklich erhöhte Ausscheidung von DHEA, Androsteron und Ätiocholanolon im 24-h-Harn nach 2—8wöchiger Behandlung der Probanden (Tab. 2), die bei Männern und Frauen 29—64% über den Normalwerten lag. Die bei Versuchsende ermittelten Konzentrationen überstiegen die zugehörigen Kontrollwerte um insgesamt 1,92—3,47 mg, was 19,2—34,7% der verabreichten Dosis entspräche. Wenngleich derartige Ausscheidungsraten mit bisherigen Befunden weitgehend übereinstimmen [2, 6], können diese Ergebnisse nur als Annäherung an die tatsächlichen Werte betrachtet werden. Sind doch weder Tagesschwankungen in der adrenalen Sekretion von DHEA [7] und Ausscheidung von DHEA, Androsteron und Ätiocholanolon durch die Haut [8], noch der Einfluß des exogenen DHEA-sulfats bzw. des daraus gebildeten DHEA-sulfatids auf die Biosynthese von Steroiden in der Nebennierenrinde [9] berücksichtigt.

Immerhin dürfte auf Grund der vorliegenden Resultate die orale Zufuhr von 10 mg DHEA-sulfat pro Tag zumindest eine signifikante Vermehrung sulfokonjugierten DHEA's im Plasma hervorrufen, was für eine eventuelle Therapie des sogenannten DHEA-mangelsyndroms [10] von Bedeutung sein sollte.

Tab. 1: C_{19}-Steroide im Plasma vor und nach Gabe von Ro 6-6827/2 (µg/100 ml). D = Dehydroepiandrosteron; A = Androsteron; E = Ätiocholanolon.

Proband	Alter (J.)	Vor Behandlung				Dauer	Nach Behandlung			
		Cyclustag	D	A	E		Cyclustag	D	A	E
K. (w)	21	13	46,7	25,3	2,9	4 Wo.	12	82,6	61,8	5,2
M. (w)	23	12	56,4	28,6	1,7	4 Wo.	14	87,0	53,6	4,0
W. (w)	27	12	39,4	22,4	3,0	4 Wo.	15	72,1	59,4	4,7
W. (w)	28	14	62,6	28,0	2,8	4 Wo.	14	84,8	53,2	4,2
M. (m)	28		104,6	39,6	3,5	8 Wo.		132,9	71,8	5,1
W. (m)	28		112,4	40,2	4,1	8 Wo.		150,6	64,7	6,8
B. (m)	24		88,5	38,4	3,2	8 Wo.		126,0	62,1	4,0
D. (m)	23		124,8	29,6	2,8	8 Wo.		141,4	56,3	4,6

Tab. 2: C_{19}-Steroide im Harn vor und nach Gabe von Ro 6-6827/2 (mg/24 h). D = Dehydroepiandrosteron; A = Androsteron; E = Ätiocholanolon.

Proband	Alter (J.)	Vor Behandlung				Dauer	Nach Behandlung			
		Cyclustag	D	A	E		Cyclustag	D	A	E
K. (w)	21	13	1,26	2,12	2,80	4 Wo.	12	1,98	2,87	3,72
M. (w)	23	12	1,50	2,04	2,62	4 Wo.	14	2,26	3,82	4,05
W. (w)	28	14	1,06	2,20	2,46	4 Wo.	14	1,74	3,56	3,37
W. (w)	29	14	1,63	1,94	1,83	4 Wo.	12	1,86	2,72	2,74
M. (m)	28		1,82	2,64	2,90	8 Wo.		2,62	3,92	4,11
W. (m)	28		1,41	2,26	2,47	8 Wo.		2,44	4,06	3,54
B. (m)	24		2,04	3,46	3,14	8 Wo.		2,68	4,50	4,46
O. (m)	43		1,32	2,55	2,34	2 Wo.		2,11	3,82	3,44

Zusammenfassung

Nach oraler Gabe von 10 mg 3β-Hydroxy-androst-5-en-17-on (Dehydroepiandrosteron, DHEA)-sulfat über 2—8 Wochen stellte man bei normalen Personen einen beträchtlichen Anstieg der Plasmaspiegel von DHEA, Androsteron und Ätiocholanolon fest. Zugleich fand man eine vermehrte Ausscheidung besagter C_{19}-Steroide im 24-h-Harn.

Summary

Dehydroepiandrosterone, Androsterone, and Etiocholanolone in Plasma and Urine after Application of Dehydroepiandrosterone Sulfate

After oral administration of 10 mg of dehydroepiandrosterone (DHEA) sulfate over 2—8 weeks to normal subjects a distinct increase in plasma levels of DHEA, androsterone and etiocholanolone was observed. At the same time a higher excretion of these steroids in the 24 h urine was found.

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Aus dem Hygiene-Institut der Universität Tübingen: Direktor: Professor Dr. R.-E. Bader

Induktion maligner Tumoren bei Ratten durch orale Gabe von N,N'-Dimethylharnstoff und Nitrit*)

Von J. Sander

Nitrit und zahlreiche sekundäre Amine und Alkylamide gelangen mit Nahrungs-, Genuß- und Arzneimitteln in den Magen des Menschen. Nitrit kann bei Vorliegen einer Sub- oder Anacidität auch im Magen selbst durch bakterielle Reduktion von in fast allen Speisen enthaltenem Nitrat entstehen und Anlaß zur Bildung von Nitrosoverbindungen geben [1]. Nachdem sich viele Nitroso-Derivate von Aminen und Amiden als stark cancerogen erwiesen haben [2], war zu prüfen, welche sekundären Amine oder Alkylamide im Magen unter den dort vorkommenden Bedingungen in cancerogen wirkende Nitrosoverbindungen überführt werden können. In vorangehenden Publikationen wurde bereits dargestellt, daß nur einige nicht stark basische sekundäre Amine im Magen von Ratten in solchem Umfang nitrosiert werden können, daß dies die Induktion von Krebs bewirkt [3, 4]. In einer weiteren Untersuchung wurde die Nitrosierbarkeit unter physiologischen Bedingungen von Alkylamiden in vivo und in vitro geprüft. Von den untersuchten Amiden waren nur N-Methylharnstoff und N-Äthylharnstoff im Magen von Ratten leicht zu nitrosieren. Die Applikation dieser Amide mit dem Futter wirkte bei gleichzeitiger Gabe

*) Mit Unterstützung der Deutschen Forschungsgemeinschaft.

von Natriumnitrit mit dem Trinkwasser stark toxisch und war Ursache einer früh auftretenden Tumorbildung bei den Versuchstieren [5].

Methodik

In der jetzt mitgeteilten Untersuchung wurde N,N'-Dimethylharnstoff appliziert. Es wurden 5 Gruppen von je 5 weiblichen Ratten (SIV 50, Ivanovas, Kißlegg) gebildet. Die Tiere kamen mit einem Anfangsgewicht von 120 g in den Versuch. Die erste Gruppe erhielt Trinkwasser mit einem Zusatz von 0,5% Dimethylharnstoff und Futter (Altromin®) ohne Zusätze. Alle anderen Gruppen bekamen das Futter mit einem Zusatz von 0,5% Natriumnitrit. Gruppe 2 erhielt dazu unverändertes Trinkwasser, während dem Wasser in der Gruppe 3 0,1%, in der Gruppe 4 0,5% und in der Gruppe 5 0,9% Dimethylharnstoff zugesetzt war. Futter und Wasser wurden ad libitum gereicht. Die Applikationsdauer betrug 56 Tage.

Ergebnis

Gruppe 1 und 2 zeigten keine Besonderheiten. Das Körpergewicht der Tiere nahm mit gegenüber der Norm nur sehr geringer Verzögerung zu. Futter und Wasser wurden normal verbraucht.

Die Gruppen 3 und 4 verbrauchten Futter und Wasser in etwas reduziertem Umfang. In Gruppe 5 wurde beides sehr ungern genommen. Die Tiere dieser Gruppe starben innerhalb der ersten 4 Wochen des Versuchs. Tumoren wurden nicht beobachtet.

117 Tage nach Beendigung der Nitrit- und Dimethylharnstoff-Applikation starb die erste Ratte in Gruppe 4. Bis zum 452. Tag waren alle Tiere der Gruppen 3 und 4 mit malignen Tumoren des zentralen Nervensystems, des Herzens, des Thymus, der Nieren oder der Thyreoidea gestorben, während in den Gruppen 1 und 2 keine Tumoren auftraten. Über die pathologisch-anatomischen Befunde soll an anderer Stelle berichtet werden.

Die bisher angestellten Untersuchungen zeigen, daß einige Alkylharnstoffverbindungen unter physiologischen Bedingungen besonders leicht zu nitrosieren sind. Aus präventivmedizinisch-hygienischer Sicht ergibt sich hieraus die Notwendigkeit die Nitrosoverbindungen von Alkylharnstoffen, die in den Magen gelangen können, besonders solchen, die regelmäßig aufgenommen werden, wie z. B. manche Arzneimittel, auf eine mögliche cancerogene oder damit verwandte Wirkung hin zu untersuchen. Weiter ist zu prüfen, welche praktische Bedeutung eine Entstehung von Nitrit in Nahrungsmitteln und im Magen in diesem Zusammenhang haben kann und welche Maßnahmen dagegen getroffen werden können. Auch die Zulässigkeit von Nitrit bei der Herstellung von Nahrungsmitteln sollte angesichts der leichten Bildung von cancerogenen Nitrosoverbindungen unter physiologischen Bedingungen genau geprüft werden.

Zusammenfassung

Die Induktion von malignen Tumoren bei Ratten durch Gabe von N,N'-Dimethylharnstoff mit dem Trinkwasser und Natriumnitrit mit dem Futter wird beschrieben. Auf die besonders leicht ablaufende Nitrosierung der bisher geprüften Alkylharnstoffe im Magen so wie auf einige daraus zu ziehende Konsequenzen wird kurz hingewiesen.

Summary

Induction of Malign Tumours in Rats by Oral Administration of N,N'-Dimethylurea and Nitrite

The induction of malign tumours in rats by administering N,N'-dimethylurea with the drinking water and sodium-nitrite with the food is described. The easily effected nitrosation of the alkyl urea compounds tested hitherto in the stomach and some ensuing consequences are pointed out.

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GEDENKTAGE

In memoriam Professor Dr. B. Reichert

Am 10. Januar 1970 verstarb nach langem unheilbarem Leiden der apl. Professor für Pharmazeutische Chemie und Lebensmittelchemie der Universität München, Dr. phil. Benno Reichert.

Er wurde am 3. April 1906 in Rathenow/Havel geboren. Dort legte er im Jahre 1925 am Realgymnasium die Reifeprüfung ab. In der Altstädtischen Apotheke in Rathenow begann er seine Ausbildung als Apothekerpraktikant. Schon während dieser Praktikantenzeit zeichnete sich seine wissenschaftliche Begabung ab. 1925 erhielt er den 2. Preis der Meurer-Stiftung, 1926 den 1. Preis der gleichen Stiftung und 1929 die Silberne Medaille der Hagen-Buchholz-Stiftung. In Potsdam bestand er 1927 die Pharmazeutische Vorprüfung. Die anschließende Tätigkeit als Vorexaminierter absolvierte er ebenfalls in der Altstädtischen Apotheke.

1928 nahm er an der Universität Berlin seine pharmazeutischen und chemischen Studien auf. 1930 bestand er die Pharmazeutische Staatsprüfung und die Chemische Verbandshauptprüfung. 1932 promovierte er an der Universität Berlin zum Dr. phil. mit einer unter der Leitung von C. Mannich angefertigten Arbeit „Über eine Synthese von Chinolinderivaten mit basischer Seitenkette". Seine Approbation als Apotheker wurde ihm im gleichen Jahr zuerkannt. Die Hauptprüfung als Lebensmittelchemiker folgte 1933. Mit einer Arbeit „Betrachtungen über den Additionsverlauf von Wasserstoff und Brom an ω-Nitrostyrole und 7-Nitrostilbene" habilitierte er sich 1936 in Berlin; 1937 wurde er zum Dozenten für Pharmazeutische Chemie ernannt und 1943 zum apl. Professor an der Universität Berlin. Seinen Wehrdienst leistete er 1944 und 1945 als Oberapotheker und beratender Chemiker der Heeresgruppe Italien. Bis 1946 blieb er dort in Kriegsgefangenschaft. Die Nachkriegsjahre verbrachte er mit vorübergehender Tätigkeit in der Industrie und mit der Führung eines privaten Untersuchungs- und Forschungslaboratoriums. 1947 erreichte ihn ein Ruf an das Pharmazeutische Institut der Technischen Hochschule Karlsruhe, dem er jedoch aus persönlichen Gründen nicht folgen konnte.

Seit 1954 war er Lehrbeauftragter an der Universität München und habilitierte sich 1958 dorthin um.

Zweifellos erreichte er den Kulminationspunkt seiner wissenschaftlichen Laufbahn in Berlin am dortigen Pharmazeutischen Institut, das damals C. Mannich leitete und zu dessen Lieblingsschülern — die Witwe seines Lehrers versicherte es ihm häufig — er zählte. Aus dieser Zeit stammt eine Fülle von Veröffentlichungen auf organisch-chemischen und pharmazeutisch-chemischen Gebieten.

In Berlin wie in München legte er besonderen Wert auf seine Vorlesungen, die er mit großer Liebe und Sorgfalt vorbereitete; er war ständig bemüht, sie der raschen Entwicklung der pharmazeutischen Wissenschaft in den letzten Jahrzehnten anzupassen. Die vollen Hörsäle legten Zeugnis ab von der Gabe, sein Wissen mitzuteilen. Immer war er für seine Studenten da und man nannte ihn — sicher nicht zu unrecht — bisweilen „den Vater seiner Studenten", die ihn als einen bescheidenen und wissensreichen Menschen schätzten. In

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FURTHER EXPERIMENTS ON THE INDUCTION OF TUMORS BY ORAL ADMINISTRATION OF LOW DOSAGES OF N-METHYLBENZYLAMINE AND SODIUM NITRITE

(Weitere Versuche zur Tumor-Induktion durch orale Applikation niederer Dosen von N-Methylbenzylamin und Natriumnitrit)

Author: J. Sander

Source: Z. Krebsforsch. 76, 93 - 96 (1971) P. 93-96

Nitrite and different secondary amines find their way into the human stomach, so that a possible nitrosamine synthesis in this organ can be expected. In previous reports the induction of esophageal tumors in rats (Sander and Bürkle, 1969) and tumors of the antestomach and of the esophagus in mice (Sander, 1971) through the administration of standard fodder with an addition of methylbanzylamine and nitrite were described. In these first investigations, the rats had received during 56 days food with 0.5% methylbenzylamine and 0.5% Na nitrite. The lowest dosage with mice amounted to 0.1% amine and 0.15% Na nitrite, during 56 days, or 0.2% amine and 0.15% Na nitrite during 23 days. With this dosage, all the rats had developed tumors in the esophagus, and all the mice in the antestomach and in some cases in the esophagus also. Squamous celled carcinomas and papillomas were involved. Corresponding new growths were observed also after the administration of the N-nitroso-derivative of the amine used here (Druckrey and others, 1963; Thomas and So, 1969). The lower the doses of nitrite and amine necessary to induce cancer in animal experimentation, the sooner one could take into consideration nitrositing of naturally present methylbenzylamine or of other secondary amines, comparable from the standpoint of basicity, through nitrite, contained in food or bacterially developed in the stomach content (Sander and Seif, 1969), as the cause of cancer induction in man. Hence, further investigations on young female SIV-50-rats and NMRI-mice with the use of lower nitrite- and amine concentrations in the fodder, or with a shorter administration of the mixtures, were conducted in order to obtain a clue to the minimum doses of amine and nitrite, necessary to induce cancer. Nitrite was given with the fodder, and methylbenzylamine with the water, in order to induce tumors in rats. The results and dosages are shown in the Table.

Table: Presentation of the animal tests. R: female SIV-50-rats. Weight at the beginning of the tests 120 g. M: female NMRI-mice, weight at the beginning of the test 20 g. Total dose of methylbenzylamine: Groups 1 - 8 38 g/kg, Groups 10 and 11 5 g/kg

a. Group Number of animals b. Administration c. Duration in days d. Tumor induction e. No esophageal tumors f. All the rat had esophageal carcinoma g. No esophageal tumors in 28 months h. A carcinoma issuring from the nasal cavity i. All the mice had antestomach carcinomas within 12 months j. 3 mice had antestomach carcinomas within 12 month, the other animals are still under observation.

In these tests one found, with rats and mice, a dosage which, with the combined addition of nitrite and amine to the fodder, caused tumors with certainty in all the animals of an experimental group, and a lower dosage which no longer suffice to induce tumors with certainty. The "limit cancerogenic dose" of methylbenzylamine and nitrite, shown in these tests, is valid only for this special experimental setup. If other test animals or another diet were used, other results would probably be obtained..

During separate administration for 56 days in Group 9, methylbenzyl amine and nitrite have caused a tumor only in one animal. Two factors should be important for the low tumor production in separate administration, or for the higher occurrence in combined administration: If the amine and nitrite are given separately with the food or with the water, a less satisfactory mixing in the stomach occurs than when the two substances are added together to the fodder. In this way, because of the low stability of the nitrous acid, a lower part of the nitrite is available to react with the amine than in combined administration. Furthermore, in the case of a preliminary admixture of amine and nitrite already in the wet fodder itself, that is outside the stomach, there is a synthesis of small amounts of methyl benzyl nitrosamine detectable through thin layer chromatography and gas chromatography. The induction of cancer in the case of a joint addition of nitrite and amine to the fodder was thus to be attributed to the additive action of an extrastomachal and of an intrastomachal nitrosamine synthesis.

On the basis of the minimum doses determined in the test as to nitrite and methylbenzylamine, which were necessary to induce cancer, it does not follow at all that a concentration below 0.08% Na nitrite no longer permit such an induction in man. Methylbenzylamine, which in these tests was used in relative high dosage, no doubt is fairly more easily nitrosited than most of the secondary amines existing in food (Sander and others, 1968), and thus represents a special case among the natural amines. Other amines and amides, however - most of them synthetic, it is true, such as 2-imidazilidinone - are known (Sander, 1971) to be fairly more easily nitrosited under physiological conditions. In previous tests with 2-imidazolidinone, the addition of 0.05% of the amide to the fodder, with simultaneous administration of drinking water with 0.05% Na nitrite during 150 days was sufficient in order to induce already within 9 months malignant tumors in two out of 6 rats (Sander and Bürkle, 1971).

In every test, amine and nitrite were administered for a limited time only. During prolonged administration the probability of a cancer induction would be greater, since it is known that during continuous administration of nitrosamines, the longer the administration continues, the greater the decrease of the daily doses necessary for the cancer induction can be (Druckrey, 1967).

Nitrite concentrations of 80 or 50 mg $NaNO_2$ per 100 g exceed the limit value still definitely of 20 mg per 100 g (Souci and Mergenthaler, 1958) allowed in different countries in food treated with nitrite, or the values exceeding 6 mg nitrite per 100 mg (Sander, 1967) which we repeatedly found in our own investigations in salted meat. Although still too little is known about the presence of correspondingly slightly nitrositable amines and amides, whose nitroso-derivatives are cancerogenic, the margin of safety must already be regarded as to small, so that the utilization of nitrite in the distribution or in the production of food stuffs should not be accepted without further investigations. One bear in mind that in addition no insignificant amounts of nitrite are incorporated, which are formed through a bacterial reduction of nitrate (Sander, 1969).

English Summary at the beginning of the German text.

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Weitere Versuche zur Tumor-Induktion durch orale Applikation niederer Dosen von N-Methylbenzylamin und Natriumnitrit

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Further Experiments on the Induction of Tumors by Oral Administration of Low Dosages of N-Methylbenzylamine and Sodium nitrite

Summary. For 152 days-rats were fed a diet containing 0.25% of N-methylbenzylamine and sodium nitrite in concentrations from 0.32% down to 0.01%. Esophageal tumors were observed in all rats of a group when the concentration of sodium nitrite was 0.32%, 0.16% or 0.08%. No esophageal tumors were induced when the concentration was 0.06%, 0,4%, 0.02% or 0.01%. Out of 8 rats receiving a diet mixted with 0.3% of sodium nitrite and drinking water containing 0.6% N-methylbenzylamine one developed a carcinoma of the nose, none developed esophageal tumors. 10 out of 10 mice receiving for 16 days a diet containing 0.1% N-methylbenzylamine and 0.15% $NaNO_2$ developed carcinomas of the forestomach and partly papillomas of the esophagus but only 3 out of 10 mice died from carcinomas of the forestomach when the diet contained 0.1% of the amine and 0.1% $NaNO_2$ (fed for 16. days).

The problems involved in ingestion of low dosages of a secondary amine and nitrite are discussed shortly.

Zusammenfassung. Ratten erhielten 152 Tage lang Futter mit einem Zusatz von 0,25% N-Methylbenzylamin und Natriumnitrit in Konzentrationen von 0,32% bis 0,01%. Oesophagus-Tumoren wurden bei allen Tieren in den Gruppen beobachtet, die Futter mit 0,32%, 0,16% oder 0,08% Nitrit bekamen. Enthielt das Futter nur 0,6%, 0.04%, 0,02% oder 0,01% Nitrit, so traten keine Tumoren mehr auf. Bei Gabe von Futter mit einem Zusatz von 0.3% $NaNO_2$ und Trinkwasser mit 0,6% Methylbenzylamin trat nur bei einer von 8 Ratten ein von der Nasenhöhle ausgehendes Carcinom auf, keine wies Oesophagus-Tumoren auf.

Alle Mäuse einer Gruppe, die 16 Tage lang Futter mit Zusatz von 0,1% Methylbenzylamin und 0,15% $NaNO_2$ erhielten, entwickelten Carcinome des Vormagens und z. T. Papillome des Oesophagus. Bei Gabe von Futter mit 0,1% Amin und 0,1% $NaNO_2$ traten nur bei 3 von 10 Mäusen Vormagencarcinome auf.

Probleme, die mit der Inkorporation von niedrigen Dosen eines sekundären Amines und von Natriumnitrit zusammenhängen, werden kurz diskutiert.

Nitrit und verschiedene sekundäre Amine gelangen in den Magen des Menschen, so daß mit der Möglichkeit einer Nitrosaminsynthese im Magen gerechnet werden muß. In früheren Mitteilungen war die Induktion von Oesophagustumoren bei Ratten (Sander und Bürkle, 1969) und von Vormagen- und Oesophagustumoren bei Mäusen (Sander, 1971) durch Verabreichung von Standardfutter mit einem Zusatz von Methylbenzylamin und Nitrit beschrieben worden. Die Ratten hatten in diesen ersten Untersuchungen 56 Tage lang Futter mit 0,5% Methylbenzylamin und 0,5% Natriumnitrit erhalten. Die niedrigste Dosierung

* Frl. B. Roos danke ich für technische Mitarbeit. Der Deutschen Forschungsgemeinschaft danke ich für eine Sachbeihilfe.

betrug bei Mäusen 0,1% Amin und 0,15% Natriumnitrit, während 56 Tagen, bzw. 0,2% Amin und 0,15% Natriumnitrit während 23 Tagen. Bei dieser Dosierung hatten alle Ratten Tumoren im Oesophagus, alle Mäuse im Vormagen und z. T. ebenfalls im Oesophagus entwickelt. Es handelte sich um Plattenepithelcarcinome und Papillome. Entsprechende Neubildungen wurden auch nach Applikation des N-Nitrosoderivates des hier verwendeten Amins beobachtet (Druckrey et al., 1963; Thomas u. So, 1969). Eine Nitrosierung von natürlich vorkommendem Methylbenzylamin oder anderen, hinsichtlich der Basizität vergleichbaren sekundären Aminen, durch in Nahrungsmitteln enthaltenes oder im Mageninhalt bakteriell gebildetes Nitrit (Sander u. Seif, 1969) könnte als Ursache einer Krebsinduktion beim Menschen um so eher in Betracht gezogen werden, je niedriger die zur Induktion von Krebs im Tierversuch erforderlichen Dosen von Nitrit und Amin sind. Weitere Untersuchungen an jugendlichen, weiblichen SIV-50-Ratten und NMRI-Mäusen mit Anwendung niedrigerer Nitrit- und Aminkonzen-

Tabelle. *Zusammenstellung der Tierexperimente. R: weibliche SIV-50-Ratten, Gewicht zu Beginn der Versuche 120 g. M: weibliche NMRI-Mäuse, Gewicht zu Beginn der Versuche 20 g. Gesamtdosis Methylbenzylamin: Gruppen 1—8 38 g/kg, Gruppen 10 und 11 5 g/kg*

Gruppe	Zahl der Tiere	Applikationen	Dauer Tage	Tumorinduktion
1	4 R	0,25% N-Methylbenzylamin im Futter	152	keine Oesophagustumoren
2	4 R	wie 1, zusätzlich 0,32% $NaNO_2$ im Futter	152	alle Ratten hatten Oesophaguscarcinome
3	4 R	wie 1, zusätzlich 0,16% $NaNO_2$ im Futter	152	
4	4 R	wie 1, zusätzlich 0,08% $NaNO_2$ im Futter		
5	8 R	wie 1, zusätzlich 0,06% $NaNO_2$ im Futter	152	keine Oesophagustumoren in 28 Monaten
6	8 R	wie 1, zusätzlich 0,04% $NaNO_2$ im Futter	152	
7	8 R	wie 1, zusätzlich 0,02% $NaNO_2$ im Futter	152	
8	8 R	wie 1, zusätzlich 0,01% $NaNO_2$ im Futter	152	
9	8 R	0,3% $NaNO_2$ im Futter 0,6% N-Methylbenzylamin im Wasser	56	ein von der Nasenhöhle ausgehendes Carcinom
10	10 M	0,1% N-Methylbenzylamin 0,15% $NaNO_2$ im Futter	16	alle Mäuse hatten Vormagencarcinome innerhalb 12 Monat.
11	10 M	0,1% N-Methylbenzylamin 0,1% $NaNO_2$ im Futter	16	3 Mäuse hatten Vormagencarcinome innerhalb 12 Monaten, die übrigen Tiere werden weiterhin beobachtet

trationen im Futter, bzw. mit kürzerer Applikationsdauer der Mischungen, wurden deshalb durchgeführt, um einen Anhalt für die zur Krebsinduktion erforderlichen Mindestdosen von Amin und Nitrit zu erhalten. Außerdem wurde versucht, bei Ratten durch getrennte Gabe von Nitrit mit dem Futter und Methylbenzylamin mit dem Wasser Tumoren zu induzieren. Ergebnisse und Dosierungen wurden in der Tabelle zusammengestellt.

In diesen Versuchen wurde bei Ratten und Mäusen eine Dosierung gefunden, die bei gemeinsamem Zusatz von Nitrit und Amin zum Futter noch mit Sicherheit Tumoren bei allen Tieren einer Versuchsgruppe hervorrief, sowie eine niedrigere Dosierung, die für eine sichere Tumorinduktion nicht mehr ausreichte. Die in diesen Versuchen gezeigte „cancerogene Grenzdosis" von Methylbenzylamin und Nitrit gilt nur für diese spezielle Versuchsanordnung. Bei Verwendung anderer Versuchstiere oder anderer Diät würden wahrscheinlich andere Ergebnisse erhalten werden.

Bei getrennter Applikation während 56 Tagen in Gruppe 9, haben Methylbenzylamin und Nitrit nur bei einem Tier einen Tumor hervorgerufen. Zwei Faktoren dürften für die geringe Tumorausbeute bei getrennter Gabe, bzw. für die höhere Ausbeute bei gemeinsamer Gabe von Bedeutung sein: Werden das Amin und Nitrit mit dem Futter, bzw. dem Trinkwasser getrennt angeboten, so erfolgt im Magen eine weniger gute Durchmischung als beim gemeinsamen Zusatz zum Futtermittel. Auf diese Weise steht wegen der geringen Stabilität der salpetrigen Säure ein kleinerer Teil des Nitrits zur Reaktion mit dem Amin zur Verfügung als bei gemeinsamer Zugabe. Außerdem kommt es bei vorheriger Zumischung von Amin und Nitrit bereits im feuchten Futtermittel selbst, d. h. außerhalb des Magens, zu einer – durch Dünnschichtchromatographie und Gaschromatographie nachweisbaren – Synthese kleiner Mengen von Methylbenzylnitrosamin. Die Induktion von Krebs bei gemeinsamem Zusatz von Nitrit und Amin zum Futter, war deshalb auf die additive Wirkung einer extragastralen und einer intragastralen Nitrosaminsynthese zurückzuführen.

Auf Grund der in den Versuchen festgestellten Mindestdosen von Nitrit und Methylbenzylamin, die zur Krebsinduktion erforderlich waren, darf keinesfalls gefolgert werden, eine Konzentration unter 0,08% Natriumnitrit erlaube beim Menschen eine Krebsinduktion nicht mehr. Methylbenzylamin, das in diesen Versuchen in relativ hoher Dosierung verwendet wurde, ist zwar wesentlich leichter zu nitrosieren als die meisten in der Nahrung vorkommenden sekundären Amine (Sander et al,. 1968), stellt also gewissermaßen einen Sonderfall unter den natürlich vorkommenden Aminen dar, doch sind andere Amine und Amide – allerdings meist synthetische, wie z. B. 2-Imidazilidinon – bekannt (Sander, 1971), die unter physiologischen Bedingungen noch wesentlich leichter nitrosiert werden. In früheren Versuchen mit 2-Imidazolidinon genügte der Zusatz von 0,05% des Amids zum Futter, bei gleichzeitiger Verabreichung von Trinkwasser mit einem Gehalt von 0,05% Natriumnitrit während 150 Tagen, um schon innerhalb von 9 Monaten bösartige Tumoren bei zwei von sechs Ratten zu induzieren (Sander u. Bürkle, 1971).

In allen Versuchen wurden das Amin und Nitrit nur für begrenzte Zeit appliziert. Bei Verabreichung über längere Zeiträume wäre die Wahrscheinlichkeit einer Krebsinduktion größer, da bekannt ist, daß bei fortlaufender Applikation

von Nitrosaminen die zur Krebsinduktion erforderliche Tagesdosis um so stärker gesenkt werden kann, je länger die Anwendung fortdauert (Druckrey, 1967).

Nitritkonzentrationen von 80 bzw. 50 mg $NaNO_2$ pro 100 g überschreiten den in verschiedenen Ländern in mit Nitrit behandelten Nahrungsmitteln zugelassenen Grenzwert von 20 mg pro 100 g (Souci u. Mergenthaler, 1958), bzw. die in eigenen Untersuchungen in gepökelten Fleischwaren wiederholt gefundenen Werte bis über 6 mg Nitrit pro 100 mg (Sander, 1967) zwar noch deutlich; der Sicherheitsabstand muß aber – obwohl über das Vorkommen entsprechend leicht nitrosierbarer Amine und Amide, deren Nitrosoderivate cancerogen wirken, noch zu wenig bekannt ist – schon jetzt als zu gering angesehen werden, als daß man die Verwendung von Nitrit bei der Verarbeitung bzw. bei der Herstellung von Nahrungsmitteln ohne weitere Untersuchungen hinnehmen dürfte. Man muß dabei auch berücksichtigen, daß zusätzlich nicht unerhebliche Mengen Nitrit inkorporiert werden, die durch eine bakterielle Reduktion von Nitrat gebildet werden (Sander, 1969).

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INDUCTION OF MALIGNANT TUMORS IN RATS BY SIMULTANEOUS FEEDING OF NITRITE AND SECONDARY AMINES (Induktion maligner Tumoren bei Ratten durch gleichzeitige Verfütterung von Nitrit und sekundären Aminen)

Authors: Johannes Sander and Gernot Bürkle
Source: Z. Krebsforsch. 73, 54 - 66 (1969)

To different human foodstuffs, nitrite is added in order to obtain the coloration expected, or a preserving effect (SANDER, 1967). In vegetable food rich in nitrate, nitrite, partly in high concentration, can be formed through the bacterial reduction of nitrates. In spinage, unchanged as to its taste, which had been used as food for infants, concentrations of up to 73 mg NO_2^- per 100 g were measured, for instance (SINOIS and WODSAK, 1965). Our own investigations showed that in the human stomach also nitrate can be converted into nitrite, as long as a sufficient acid formation has not yet started. In cases of subacidity of anacidity, nitrite concentrations of up to 24 mg per 100 ml of stomach content were found (SANDER and SEIF, unpublished). Furthermore, often nitrite is detectable in the stomach content before any food ingestion, even when normal cidity is present.

A large number of secondary amines are present in natural substances, or are commercially produced. According to a still incomplete literature survey, at least 100 different secondary amines can gain access into the stomach of man.

It becomes obvious to think that the simultaneous presence of nitrite and secondary amines could cause the formation of cancerogenic nitrosamines. A nitrositing of secondary amines should be especially easy in the stomach where a low pH value facilitates the liberation of nitrate from nitrite. This concept was experimentally tested by DRUCKREY, STEINHOFF, BEUTHNER, SCHNEIDER and KLÄRNER (1963) as to its correctness. They gave rats diethylamine and nitrite in drinking water, or in the diet. Diethylnitrosamine is one of the most effective nitrosamines. It is stated that its development in the stomach should have resulted into the formation of tumors already with a slidht conversion of the amine applied. Yet no tumors developed.

With still higher dosage of diethylamine and nitrite (0.3% each) and the combined addition of the two components to the food - which ensured a better mixture of the stomach content with the two reaction partners - no tumors were obtained either in our own tests (SANDER, SCHWEINBENZ and MENZ, 1968). The investigations led to the result that secondary amines, which react basically so much like diethylamine, are not nitrosites to a measurable extent in the stomach, where a large number of other substances can react with nitrite. The less basic an amine was, the easier was its nitrositing under physiological conditions.

The results obtained through the chemical analysis of the stomach content of rats suggested the following question: With which secondary amines, nitrosamines - in the presence of nitrite in the stomach content - can be synthesized to such extent that tumors can thus be induced ? This question was tested by using morpholine, the basicity of which, with a pK_B-value of 5.6 was definitely lower than that of diethylamine, and with N.methylbenzylamine, which is still less basic (pK_B-value = 7.1). As previously, nitrite and the amines were added together to the food, although a slight reaction of these less basic amines with the nitrite was already possible in the nearly neutrally reacting food, that is outside the stomach.

Procedure: a Page 55

Table 1: Development of the body weight of the different experimental animal groups
a. Group b. Additions to the standard diet c. Body weight of the animals in g.
d. After 2 weeks, etc..

Six groups, each one consisting of 7 female Sprague-Dawley rats with an initial weight of 120 ± 3 g received a standard diet (altromine), to which for 8 weeks additions as in Table 1 were mixed. Afterwards the animals received the diet without additions. The body weight was currently checked. The food consumption, in the first 8 weeks, amounted to about 10 g per 100 g of body weight and per day. Out of this one calculates a total dose of 28 g of amine or Na nitrite per kg of body weight.

In the groups, which had received nitrite and a secondary amine, the animals were observed until their death. Then they were dissected and the organs were histologically examined. After a test duration of 270 days, the animals of Groups III and V were killed and dissected. Groups I and II are further observed.

Results:

The animals, which received only nitrite or only a secondary amines with their food, developed normally - except for a moderately marked decrease of the rate of growth. In the control groups, no animal died before the end of the test. Feeding with nitrite along with a secondary amine impaired growth considerably. Furthermore after a few weeks the animals looked unhealthy. Their skin was rough.

After the food additions had been interrupted, all the animals recovered very rapidly and their weight increased quickly. Especially the rats of Group IV, which had received morpholine and nitrite, did not look sick at ally until a short while before their death. In Group VI, the development of esophagus tumores prevented food ingestion, so that some of these animals, a short time before their death, lost much weight.

All the animals of Groups IV and VI died within a relatively narrowly limited period. Hepathic tumors were the cause of death in Group IV. Through the rupture of the livers in a hemorrhagic-necrotic tumor region, several test animals had bled to death in the peritoneal cavity. The first animal of this group died on the 150th, the last on the 234th day of experimentation. The cause of death for the animals of Group VI was in most cases a severe suppurative aspiration pneumonia, which no doubt had developed because of the highly constricting esophagus tumors. In this group, death occurred between the 202nd and 220th day of experimentation.

Histological Investigation

Test Group III: Macroscopically the findings were modest. In the liver only a very low up to a moderatly important fatty degeneration in the form of a fine dust, partly in the form of spots, partly acinocentral or peripheral, was detected.

Test Group V: Macroscopic or microscopic means revealed no pathological changes; especially the fine tissue structure of the esophagus of these animals was normal.

Test Group IV: (with 0.5% morpholine and 0.5% $NaNO_2$ addition to the altromine standard diet):

Macroscopically, it was possible to identify in most cases a large number of partly grey-white, partly black-red nodules reaching the size of a pea, on the surface or surface of section of the livers of the animals. Only in one animal there was an isolated formation of a tumor type and large as a hazelnut. Small cystic changes, partly filled with gelatinous masses, were present in several animals partly in the liver parenchyma, partly within the new growths.

In the lungs of two animals and in the kidney of one experimental animal, multiple small tumor nodules were found.

Microscopically, an organically typical structure of the liver parenchyma was found, except in one animal. The portal fields were not fibrously spread (? widened) or inflammatorily infiltrated. In all the experimental animals, the marked differences in cell and nucleus sizes of the hepatic epithelia were conspicuous. At times disseminated individual or group cell necroses were found, especially acinocentrally, partly with incipient inflammatory-cellular reaction. The Kupffer's cells as well as the perivascular macrophages contained Fe pigment in several animals. Only one animal exhibited a marked disorganization of the liver with formation of pseudo-lobuli of different sizes, which in most cases were surrounded by narrow binding tissue septa. Within these septa there were proliferating bile ducts, loose inflammatory-cellular infiltrates (mainly lymphocytes, plasma cells, and also some granulocytes) and some atrophic hepatic cells.

The multiple, differently large, grey-white, nodular new growth in the liver consisted of large polygonalcells resembling the liver epithelia; these polygonal cells were arranged in beams several cells in thickness. The cytoplasma of the cells was more or less highly colored and frequently contained (in the HE coloration) homogeneously eosin-red albumin globules. Their nuclei were relatively large and appeared to be poor in chromatin. In most cases, they contained several conspicuously large nucleoli. These surrounded regions, which were

partly sharply, partly unsharply delimited from the surrounding tissue, contained no well organized portal fields and were not arranged around a central vein. Within the nodules there were "puddles" with liquid which contain albumin; at times they contained pyknic nuclei or nuclear rests. In addition to the trabecularly structured nodules, there were also tubular or adenoid tissue formations, in which the cells seemed to be fairly differentiated, and exhibited no increased or atypic mitoses (Figure 1).

Figure 1: Adenoid growth form of an intrahepatic carcinoma from the experimental group IV - HE 140 x

Figure 2: Papillary tissue formation of a hepatocellular carcinoma from the experimental group IV - HE 140 x

Figure 3: Endothelial tumor cells (like in the malignant hemangioendethelium), which coat irregularly large cavities filled partly with erythrocytes (liver, experimental group IV) - HE 140 x

Figure 4: Cystadeno-carcinoma in the liver of an experimental animal from Group IV - HE 140 x

Frequently there were small centers of partly large blister-like, partly small blister-like cytoplasma alterations, which made the cells to appear like plant cells; more rarely larged nodules were made of the same cells.

There were apparently enclosed formations (grouped together, or also disseminated) varying in size, cystically widened, which were coated with flat to cubic epithelium and resembled irregularly proliferated bile duct formations. Those structures were partly surrounded by binding tissue, but partly bordered directly on liver tissue. At times these changes were tumorlike; they partly gave the impression of a malignant new growth.

In the regions, macroscopically described as black-red, but also in the individual grey-white nodules, there was tissue of definitely infiltrating and destroying growing epithelial new formations, which for the most part had developed multicentrically in the liver. Individual tumor nodules exhibited a trabecular structure.

The lines of tumor cells resembled normal liver trabeculae; the cells were polygonal and resembled hepatic cells; however, they had differences in the shape and color quality and exhibited a large amount of partly atypic mitoses. There were also papillary (Figure 2) and tubular as well as very anaplastic tumor nodules. In two test animals, the tumor cells had a spindle shape and formed many, mostly irregular, fissure-like cavities partly filled with erythrocytes (Figure 3). Here and there, the fissure-shaped cavities were coated with endethelial cells. Carvenoma formations existed also within these tumor nodules.

In another animal, the malignant new growth consisted of a multilocular cystic formation with papillomatous proliferations of cubic to highly cylindric cells, mostly arranged in single layers. These cells assumed the bluish-purple HE-coloration and definitely contrasted with the surrounding hepatic tissue (Figure 4). The nuclei of the epithelium lining the cysts were large and surprizing poor in chromatin. The cysts contained abundant albumin-containing liquid in which rather small groups of erythrocytes and macrophages rich in Fe pigment were.

Inside the tumor nodules and in their immediate vicinity there had been frequent extended bleedings and necroses. Several tumor tissue invasions into rather large branches of portal vein were observed.

The tumor nodules, observed in the lungs of two experimental animals corresponded by their cell structure to the malignant new growths detected in the liver of each. In the fine tissue investigation of the new growths developed in a kidney of an experimental animal, mainly papillary, but also some tubular renal cortex adenomas were found.

Test Group VI (with 0.5% N-methylbenzylamine and 0.5% $NaNO_2$ for the altromine standard diet):

The esophagus was the main place of manifestation of morbid alterations in this test group (whereas the liver exhibited nothing special). On the other hand, the cell and tissue changes and the development of tumors - in contrast with Group IV - were relatively uniform.

The macroscopic examination revealed exophytically growing tumors, from a very small size to the size of a lentil, with a surface that appeared to be partly smooth and partly projecting like a cowliflower. These tumors, detected after preparing the esophagi, existed in different numbers and mostly over the entire expanse of the organ.

The histological examination revealed that multicentrically developed epithelium hyperplasias and epithelial growths (Figure 5) were involved. In comparison with the controls, at first an induration of the cornifying pavement epithelium in connection with the entire esophagus was observed. This induration was partly due to an acanthosis with participation of all the cell layers, and partly due to hyperkeratosis. Very frequently there was a partly nodular partly flattened intraepithelial hyperplasia of basal cells, in which case the other epithelium layers had entirely or partly disappeared (Figure 6). The localized "basal cell-acanthosis" had here and there led to protuberances which stand out more or less above the mucous membrane level. Often, in partly very densely juxtaposed regions there was a definitely exophytic growth in most cases in papillomatous form. The multiple papilloma differed in size and in structure: In the center of the tumors there were mainly small, round, relatively uniform cells which looked like basal cells. In the direction of the lumen, these cells were covered by variously thick layers of the stratum spinosum and granulosum. In addition there was a very marked hyperkeratinization with formation of deep crypts filled with horny lamellas. Parakeratosis was recognizable over individual papilla tips. In the papillomatous formations the finger-shaped ramification of the papillas was variously developed and there were changes in the amount of the small basal cell-like and of the larger cornifying cells, and in the extent of the epithelial pearl formation.

Figure 5: Longitudinal section through the pharynx and larynx of an experimental animal of Group VI - HE 9.5x a. epiglottis b. trachea c. ventral side of the esophagus d. pavement epithelium carcinoma with starting destroying and infiltrating growth e. pavement epithelium papilloma in the treacheal mucosa f. pavement epithelium papilloma in the esophagus.

Figure 6: Localized intraepithelial basal cell-hyperplasia (esophagus from the test group VI) HE 140x

Figure 7: Disorganization of the layer arrangement of the cells, very different colorability and size of the cells; sporadic atypic mitotic forms (the so-called atypic epithelium) in an esophagus from test group VI - HE 1400x

Figure 8: Pavement epithelium-papilloma in the trachea (a). In basally remoted regions, malignant degenerated pavement epithelium papilloma in the esophagus (b). Animal group VI - HE 12.5x

In individual circumscribed regions of the esophagus epithelium, the formal cell arrangement was destroyed (Figure 7). There was a very different cell colorability and size. Partly, bi- and multinuclear cells and sporadically atypic mitotic forms were recognizable, although there was no growth tendency in depth. Occasionally, some individual cells exhibited vacuolar changes.

In most of the investigated experimental animals the cellular atypias were very marked. It developed there into invasive and destroying growth into deeper tissue layers, so that the precondition for the diagnosis of malignant new growths existed (Figure 8). To different amounts, the tumor masses consisted of differentiated prickle cells and horny cells and of no longer differentiated prickle cells. In addition to single cell cornification, nuclear hyperplasia, disturbed nucleus plasma relation and hyperchromasia, there was an increase in mitoses - also of the atypical type -. The normal stratification of the epithelium appeared to be fully disorganized. Epithelial pearl formation was observed in the cell lines and cell nests in depth. Granulocytes were loosely grouped between the epithelium proliferations. Rather often an inflammatory reaction around the tumors was found to be much more marked than with the previously described noninfliltrating and nondestroying new growths which in most cases were not accompanied by inflammatory cell reactions in the surrounding tissue.

Other alterations, special for individual experimental animals, are pavement epithelium metaplasia in the trachea and in the bronchial system, partly with formation of papillomatous proliferations, severe suppurative confluent broncho-pneumonia with signs of aspiration of plant food particles; development of a pavement epithelium carcinoma in the nasal cavities in one experimental animal.

Discussion:

The possibility of the formation of cancerogenic nitrosamine from secondary amines and nitrite in the stomach must be investigated as closely as possible because, as was mentioned at the beginning, amine and nitrite frequently gain access to the stomach or can develop there as bacterial products of metabolism. In our previous investigations involving chemical-analytic methods, the basic principles controlling the nitrosamine synthesis in the stomach were already presented. The possibly great practical importance of the formation of cancerogenic substances from inactive early stages in vivo demanded a further confirmation of the results. The evidence that a nitrosamine synthesis in the stomach can result in the induction of cancer was to be expected through animal tests after the application of amines which yield highly active nitroso derivatives and are less highly basic as diethylamines. N-methylbenzylamine and morpholine exhibit these properties. The basicity of the two amines is lower than that of diethylamine. What is involved, however, are definitely more alkalinely reacting compounds than the previously used aromatic amines (N-methylaniline (pK_B = 9.1) and diphenylamine (pK_B = 13.1)).

N-methylbenzylamine and morpholine were included in the tests because the former is found in plants, and the latter is a base widely used at the technical and industrial level.

Morpholine is utilised as solvent for a large amount of organic substances, such as resins, waxes, shellac, casein and dyes. It is an intermediate in the rubber industry and is applied as an anti-corrosive agent in steam-powered systems. With its help, volatile amines can be separated. Morpholine, with fatty acids, form soaps which have excellent emulsifying properties. These soaps are used in the manufacture of detergents of various type and are contained also in household cleaning products. The morpholine part in this case is loosely combined and is easily liberated. Morpholine derivatives are used in large amount in the detergent industry.

The extensive use of morpholine (BROWN, 1966; JEFFERSON, 1968) makes it probable that a large number of individuals comes into contact with this base at the professional or private level. The LD_{50} for rats is indicated as containing

1 g of morpholine/kg (SMYTH, CARPENTER, WEIL, POZZANI, 1954), or 1.5 mg of morpholine/kg or 5 g of morpholine-hydrochloride'kg (HÜLS, 1967). Undiluted morpholine irritates the skin and mucosa considerably. It is easily resorbed through the skin. The maximum permissible concentration in working premises has not yet been determined. Inhalation tests in rats, mice and guinea pigs, on the basis of a single inhalation of relatively high doses (100 mg/l air) during 4 hours, causes no enduring damages. A two-month diet with 250 mg of morpholine per kilogram of body weight and per day caused no detectable damage (HÜLS, 1967).

The conviction that morpholine is to a large extent harmless induced the discussion of its utilization in the domain of the food industry (SOUCI and MERGENTHALER, 1958). In 1962, the American Food and Drug Administration (FDA) approved several types of application; thus the addition of morpholine to vapor in 10 ppm concentrations was allowed for processes involving a contact between vapor and food. Morpholine salts from fatty acids can be used in coating vegetables and fresh fruits. The free base of its fatty acid salts are accepted as anticorrosive agents for metals coming into contact with food (Federal Register, 1962).

The use of morpholine in pharmaceutical production derives also some consideration. Not only the base itself is of interest in this case, but also the derivatives which exhibit a nitrositable N atom. The large number of derivates in which morpholine is combined through the N atom are of no interest in this connection.

The morpholine salt of salicylic acid can for instance be administered orally in doses of up to 6 grams daily for the treatment of different forms of rheumatism. A preparation, obtainable in Germany, is administered in the form of sugared pellets which dissolve not before they have reached the small intestine.

A morpholine derivative, which has found wide distribution, is the 3 methyl-2-phenyl morpholine

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It is recommended in the treatment of obesity. As far as we know, the literature contains no report regarding a test of the corresponding N-nitroso-derivatives as to a possible cancerogenic action.

N-methylbenzylamine was found in ephedra species (RAUEN, 1964). It is possible that the amine occurs in other plants and may find access to the human stomach along with food.

A compound akin to N-methylbenzylamine is dibenzethylene diamine

b P. 63

One can imagine it as being composed of two methylbenzylamine molecules. The substance is used in the fabrication of antibiotics. It can be taken orally by children in combination with pencillin G. As far as we know, the literature contains no publications of investigations of any possible cancerogenic effects of the corresponding nitroso derivatives.

The animal tests, described above, were conducted on purpose with a high daily dose of nitrite and amines, so that a safer statement was possible in case of a negative result. In conformity with the dosage, the tumors appeared very soon. From quantitative investigations by DRUCKREY, SCHILDBACH, SCHMÄHL, PREUSSMANN and IVANKOVIĆ (1963) it follows that with a reduction of the nitrosamine dose on the one hand the tumor induction period is prolonged, but on the other hand the required total dose of the cancerogenic product becomes much lower, in DRUCKREY's tests at least by the factor of 10. From this one must conclude than in our tests also tumors would have been induced if a definitely lower dosis of morpholine and N-methylbenzylamine had been applied. The dead of the experimental animals would then have occurred later.

In this connection it must be mentioned that the animals, alluded to in a previous publication (SANDER, SCHWEINSBERG, MENZ, 1968) and to which very low doses of N-methylaniline and nitrite had partly been administered, began to develop malignant tumors after a symptom free interval of about 8 months, during which theyreceived a normal diet without any additions. N-methylaniline is a constituent part of cigaret smoke and can along with saliva gain access to the stomach.

In all the animals of Group IV, multiple hepatocellular adenomatous formations and a large number of cysticly converted bile duct proliferates had developed.

Only one animal exhibited no malignant neoplasma; in this case multiple larger cysts and several nodules were observed, which appeared to be made of plant cell-like cells and to be sharply delimited. The other experimental animals exhibited multicentrically developed, mainly hepatocellular carcinoma with trabecular, papillary, tubular and adenoid tissue formations and extended, localized bleedings and necroses. Here and there, the new formations were very anaplastic. In two animals the morphological picture of the tumors was identical with that of a malignant hemangioendothelium. In one of the test animals, the tumor in the liver had to be considered as a multilocular cystadeno-carcinoma. In the other animals also, the bile duct proliferations were partly so irregular that a malignant excess of growth had to be assumed, especially since here and there invasive and destructive tissue formations were present. Only in one animal it was possible to detect at the same time a cirrhotic disorganization of the liver with partly subtotal, partly total necrosis, differing in age, of individual pseudolobuli. The other animals had a liver parenchyma, with a structure typical for the organ, with differently developed liver epithelium degenerations (individual and group cell necroses). Thus cirrhosis cannot be regarded as a necessary preliminary stage of the malignant new growths.

Lung metastases of the hepatocellular carcinomas were detected in two animals. In a test animal, multiple, mainly papillary, and also tubular renal cortex adenomas, of the size of a pin head , were found.

The fine tissue structure of the intrahepatic new growth corresponded essentially to the histological manifestations known through experiments with N-nitroso-morpholine and other cancerogenic substances (THOMAS, 1961; BANASCH and MÜLLER, 1964).

In Group VI, partly flattened, partly localized multicentrical basal cell proliferations, with in most case exophytic, more rarely endophytic, diffusion, developed. Everywhere one could observe a differently marked papillomatosis with heperkeratosis, mostly in the total extension of the esophagus, and partly also in the posterior wall of the pharynx. Intraepithelial cell atypias and cornifying pavement epithelium carcinomas have been induced, the multilocular development of which must be emphasized. Often epithelium metaplasias were observed in the upper air passages. There was no metastasis of the carcinoma into other organs.

A detailed histological investigation of the esophagus tumors induced by N-nitroso-N-methylbenzylamine, has not been performed as far as we know by the literature. DRUCKREY, PREUSSMANN, BLUM, INVANKOVIC, AFKHAM (1963), by using nitrosamines, were for the first time able to produce pavement epithelium carcinomas in rats, which had not succeeded previously with other known cancerogenic substances. GIBEL (1968), after administrating dinitrosopiperazine with the food, investigated the development stages of the esophagus carcinoma of rats and observed fine tissue alteragions of the esophagus, which correspond to a large extent to those obtained by us with N-nitroso-N-methylbenzylamine.

Consequences from the Standpoint of Preventive Medicine:

If high nitrite doses, mixed with the standard diet, are tolerated by rats without any essential damage, this means that the food used does not contain a high amount of secondary amines, which are easily nitrosited and have nitroso derivatives that are cancerogenic. Concluding from this, without any further investigation, that nitrite addition to human food is safe, is certainly unjustified. A very large number of different natural as well as synthetic secondary amines gains certainly access to the human stomach, as was mentioned at the beginning. Hence, it appears necessary to require a renewed very careful verification as to the admissibility of continuing to add nitrite to foodstuffs. At least severe measures should be applied to products the quality of which is not really improved by nitrite, such as liver puddings or fish.

In the toxicological investigation of medicines and food additions, a possible conversion to the corresponding nitroso-derivatives should be taken into account. On this question the literature contain no indications, even within the scope of the special question of how far medicines can exert carcinogenic effects (TRUHAUT, 1967). It should also be considered that nitroso compounds, any way at the level of animal testing, possess very teratogenic properties, and that in the case of unborn progeny, already relative low doses of cancerogenic substances can induce diaplacental cancer, which appears at a later age.

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Induktion maligner Tumoren bei Ratten durch gleichzeitige Verfütterung von Nitrit und sekundären Aminen

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Induction of Malignant Tumors in Rats by Simultaneous Feeding of Nitrite and Secondary Amines

Summary. Feeding of morpholine and nitrite or N-methylbenzylamine and nitrite induced malignant tumors in rats. It was thus proved, that cancerogenic nitroso-compounds may be formed in-vivo. Possible practical consequences of these results are suggested. The pathological-anatomical findings are shown.

Zusammenfassung. Verfütterung von Morpholin und Nitrit, bzw. von N-Methylbenzylamin und Nitrit an Ratten bewirkte die Induktion maligner Geschwülste. Damit wurde bewiesen, daß in vivo cancerogene Nitroso-Verbindungen gebildet werden können. Auf mögliche praktische Konsequenzen dieser Ergebnisse wurde hingewiesen. Die pathologisch-anatomischen Befunde wurden dargestellt.

Verschiedenen menschlichen Nahrungsmitteln wird *Nitrit* zugesetzt, um eine ansprechende Färbung oder um einen Konservierungseffekt zu erzielen (SANDER, 1967). In nitratreichen pflanzlichen Lebensmitteln kann Nitrit, z. T. in hoher Konzentration, durch bakterielle Reduktion von salpetersauren Salzen gebildet werden. In geschmacklich unverändertem Spinat, der als Nahrung für Säuglinge verwendet worden war, wurden beispielsweise Konzentrationen bis 73 mg NO_2^- pro 100 g gemessen (SINOIS u. WODSAK, 1965). Eigene Untersuchungen ergaben, daß auch im Magen des Menschen Nitrat in Nitrit überführt werden kann, solange eine ausreichende Säurebildung noch nicht eingesetzt hat. In Fällen von Sub- bzw. Anacidität wurden Nitritkonzentrationen bis 24 mg pro 100 ml Mageninhalt festgestellt (SANDER u. SEIF, unveröff.). Außerdem ist Nitrit oft im Nüchterninhalt des Magens, auch bei Vorliegen einer Normacidität, nachweisbar.

Zahlreiche *sekundäre Amine* kommen in Naturstoffen vor oder werden industriell erzeugt. Nach einer noch unvollständigen Durchsicht der Literatur können mindestens 100 verschiedene sekundäre Amine in den Magen des Menschen gelangen.

Der Gedanke, daß das Zusammentreffen von Nitrit und sekundären Aminen die Bildung cancerogener Nitrosamine bewirken könnte, liegt nahe. Eine Nitrosierung sekundärer Amine sollte besonders leicht im Magen möglich sein, wo durch einen niedrigen pH-Wert die Freisetzung salpetriger Säure aus Nitrit begünstigt wird. Diese Vorstellung wurde von DRUCKREY, STEINHOFF, BEUTHNER, SCHNEIDER

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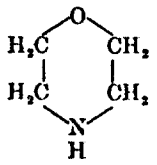
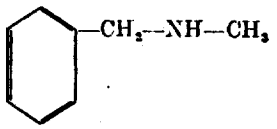
u. KLÄRNER (1963) experimentell auf ihre Richtigkeit geprüft. Sie gaben Ratten Diäthylamin und Nitrit im Trinkwasser, bzw. im Futter. Diäthylnitrosamin ist eines der wirksamsten Nitrosamine. Seine Entstehung im Magen hätte bereits bei nur geringem Umsatz des applizierten Amins zur Bildung von Tumoren führen müssen. Es traten jedoch keine Tumoren auf.

Bei noch höherer Dosierung von Diäthylamin und Nitrit (je 0,3%) und gemeinsamer Zugabe beider Komponenten zum Futter, was eine bessere Durchmischung des Mageninhaltes mit beiden Reaktionspartnern sicherte, wurden in eigenen Versuchen ebenfalls keine Tumoren erhalten (SANDER, SCHWEINBENZ u. MENZ, 1968). Die Untersuchungen führten zu dem Ergebnis, daß sich sekundäre Amine, die so stark basisch reagieren wie Diäthylamin, im Magen, wo zahlreiche weitere Stoffe mit Nitrit reagieren können, nicht in meßbarem Umfang nitrosieren lassen. Je weniger stark basisch ein Amin war, desto leichter ließ es sich unter physiologischen Bedingungen nitrosieren.

Die durch chemische Analyse des Mageninhaltes von Ratten gewonnenen Ergebnisse führten zu der Frage, mit welchen sekundären Aminen sich in Gegenwart von Nitrit im Mageninhalt Nitrosamine in solchem Umfang synthetisieren lassen, daß damit Tumoren induziert werden können. Diese Frage wurde unter Verwendung von Morpholin, dessen Basizität bei einem pK_B-Wert von 5,6 deutlich niedriger als diejenige des Diäthylamins war, und mit N-Methylbenzylamin, das noch weniger stark basisch ist (pK_B-Wert = 7,1), geprüft. Nitrit und die Amine wurden, wie früher, dem Futter gemeinsam zugesetzt, obwohl ein geringfügiger Umsatz dieser weniger stark basischen Amine mit dem Nitrit auch schon in dem nahezu neutral reagierenden Futter, d. h. außerhalb des Magens, stattfinden konnte.



Morpholin



N-Methylbenzylamin

Methodik

Tabelle 1. *Entwicklung des Körpergewichtes der verschiedenen Versuchstiergruppen*

Gruppe	Zusätze zur Standarddiät	Körpergewicht der Tiere in Gramm					
		nach 2 Wochen	nach 4 Wochen	nach 6 Wochen	nach 8 Wochen	nach 10 Wochen	nach 12 Wochen
I	—	160	205	220	230	240	250
II	0,5% $NaNO_2$	150	175	195	210	225	235
III	0,5% Morpholin	155	180	200	215	225	230
IV	0,5% Morpholin 0,5% $NaNO_2$	135	140	145	150	170	185
V	0,5% N-Methylbenzylamin	150	170	180	190	215	230
VI	0,5% N-Methylbenzylamin 0,5% $NaNO_2$	130	135	135	140	165	185

6 Gruppen von je 7 weiblichen Sprague-Dawley-Ratten mit einem Anfangsgewicht von 120 ± 3 g erhielten eine Standarddiät (Altromin), der 8 Wochen lang Zusätze, wie in Tab. 1 dargestellt, beigemischt wurden. Danach bekamen die Tiere die Diät ohne Zusätze. Das Körpergewicht wurde laufend kontrolliert. Der Futterverbrauch betrug in den ersten 8 Wochen etwa 10 g je 100 g Körpergewicht und Tag. Daraus berechnet sich eine Gesamtdosis von 28 g Amin bzw. Natriumnitrit je kg Körpergewicht.

In den Gruppen, die Nitrit und ein sekundäres Amin erhalten hatten, wurden die Tiere bis zum Tod beobachtet. Dann wurden sie seziert und die Organe histologisch untersucht. Die Tiere der Gruppen III und V wurden nach einer Versuchsdauer von 270 Tagen getötet und seziert. Die Gruppen I und II werden weiterhin beobachtet.

Ergebnisse

Tiere, die zusammen mit dem Futter nur Nitrit oder nur ein sekundäres Amin erhalten hatten, entwickelten sich – abgesehen von einer mäßig starken Verminderung der Wachstumsgeschwindigkeit – normal. In den Kontrollgruppen verstarb keines der Tiere vor Versuchsende. Verfütterung von Nitrit zusammen mit einem sekundären Amin bewirkte dagegen eine starke Beeinträchtigung des Wachstums. Die Tiere sahen außerdem nach einigen Wochen ungesund aus. Das Fell war struppig.

Nach Weglassen der Futterzusätze erholten sich alle Tiere sehr rasch und nahmen schnell an Gewicht zu. Besonders die Ratten der Gruppe IV, die Morpholin und Nitrit erhalten hatten, waren dann bis kurz vor dem Tod äußerlich nicht als krank zu erkennen. In der Gruppe VI führte das Auftreten von Oesophagustumoren zu einer Behinderung der Nahrungsaufnahme, so daß einige dieser Tiere kurz vor dem Tode stark an Gewicht verloren.

Alle Tiere der Gruppen IV und VI starben innerhalb eines relativ eng begrenzten Zeitraumes. Todesursache waren in der Gruppe IV Lebertumoren. Durch Ruptur der Leber in einem hämorrhagisch-nekrotischen Tumorbezirk hatten sich mehrere Versuchstiere in die Peritonealhöhle verblutet. Das erste Tier dieser Gruppe starb am 150., das letzte am 234. Versuchstag. Todesursache der Tiere in Gruppe VI war zumeist eine schwere eitrige Aspirations-Pneumonie, die sich wohl wegen der stark stenosierenden Oesophagus-Tumoren entwickelt hatte. Der Tod trat in dieser Gruppe zwischen dem 202. und 220. Versuchstag ein.

Histologische Untersuchung

Versuchstiergruppe III. Bei makroskopisch unauffälligem Befund ließ sich in der Leber lediglich eine sehr geringgradige bis mäßig starke feinstäubige Verfettung teils fleckförmig, teils acinozentral oder peripher nachweisen.

Versuchstiergruppe V. Weder makroskopisch noch mikroskopisch konnten pathologische Veränderungen festgestellt werden, insbesondere entsprach der feingewebliche Aufbau des Oesophagus dieser Tiere der Norm.

Versuchstiergruppe IV (mit 0,5% Morpholin- und 0,5% $NaNO_2$-Zusatz zur Altromin-Standard-Diät):

Makroskopisch waren auf der Ober- und Schnittfläche der *Leber* der Tiere meist zahlreiche teils grauweiße, teils schwarz-rote bis erbsgroße Knoten zu erkennen. Nur bei einem Tier fand sich ein solitäres mehr als haselnußgroßes tumorartiges Gebilde. Kleincystische, z. T. von gallertigen Massen angefüllte Veränderungen waren bei mehreren Tieren teils im Leberparenchym, teils innerhalb der Neubildungen vorhanden.

In den *Lungen* von zwei Tieren und in einer Niere eines Versuchstiers wurden multiple kleine Tumorknötchen festgestellt.

Mikroskopisch fand sich — ein Tier ausgenommen — ein organtypischer Aufbau des Leberparenchyms. Die Portalfelder waren nicht fibrös verbreitert oder entzündlich infiltriert. Auffällig waren bei allen Versuchstieren die ausgeprägten Zell- und Kerngrößenunterschiede der Leberepithelien. Nicht selten wurden disseminierte Einzel- und Gruppenzellnekrosen gefunden, insbesondere acinozentral, teilweise mit beginnender entzündlich-zelliger Reaktion. Die v. Kupfferschen Zellen ebenso wie perivasculäre Makrophagen enthielten bei mehreren Tieren Eisenpigment. Nur ein Tier zeigte einen ausgeprägten knotigen Umbau der Leber mit Ausbildung unterschiedlich großer Pseudolobuli, die zumeist von schmalen Bindegewebssepten umgeben waren. Innerhalb dieser Septen proliferierende Gallengänge, lockere entzündlich-zellige Infiltrate (überwiegend Lymphocyten, Plasmazellen, z. T. auch Granulocyten) und einzelne atrophische Leberzellen.

Die multiplen, unterschiedlich großen, grau-weißen, knotenförmigen Neubildungen in der Leber waren aus großen polygonalen den Leberepithelien ähnlichen Zellen zusammengesetzt, die in Balken von der Dicke mehrerer Zellen angeordnet waren. Das Cytoplasma der Zellen war verschieden stark angefärbt und enthielt häufig (in der HE-Färbung) homogen eosinrote Eiweißkugeln. Ihre Kerne waren relativ groß und erschienen chromatinarm. Sie enthielten meist mehrere auffallend große Nucleoli. Diese umschriebenen Bezirke, die teils scharf, teils unscharf von dem umgebenden Gewebe abzugrenzen waren, enthielten keine gut organisierten Portalfelder und waren nicht um eine Zentralvene angeordnet. Innerhalb der Knoten waren teilweise „Pfützen" mit eiweißhaltiger Flüssigkeit vorhanden, in die nicht selten pyknotische Kerne oder Kernreste eingeschlossen waren. Außer den *trabekulär* aufgebauten Knoten fanden sich aber auch *tubuläre* und *adenoide* Gewebsformationen, bei denen die Zellen relativ gut ausdifferenziert erschienen und keine vermehrten oder atypischen Mitosen aufwiesen (Abb. 1).

Häufig waren kleinherdige, teils fein-, teils grobblasige Cytoplasmaveränderungen, die den Zellen pflanzenzellähnliches Aussehen gaben, zu beobachten; seltener waren größere Knoten aus derartigen Zellen aufgebaut.

In herdförmiger Ansammlung, aber auch disseminiert, wurden verschieden große, cystisch erweiterte, gekammert erscheinende Gebilde beobachtet, die von flachem bis kubischem Epithel ausgekleidet waren und unregelmäßig proliferierten Gallengangsformationen ähnelten. Diese Gebilde waren teilweise von Bindegewebe umgeben, grenzten jedoch z. T. auch unmittelbar an Lebergewebe. Nicht selten waren diese Veränderungen tumorartig, teilweise machten sie sogar den Eindruck einer malignen Neubildung.

In den makroskopisch als schwarz-rot beschriebenen Bezirken, aber auch in einzelnen grau-weißen Knötchen fand sich Gewebe von eindeutig infiltrierend und destruierend wachsenden epithelialen Neubildungen, die sich meist multizentrisch in der Leber entwickelt hatten. Einzelne Tumorknoten zeigten dabei *trabekulären* Aufbau. Die Tumorzellstränge ahmten normale Leberbälkchen nach, die Zellen waren polygonal und ähnelten Leberzellen; sie hatten jedoch unterschiedliche Form- und Farbqualität und wiesen zahlreiche, z. T. atypische Mitosen auf. Außerdem kamen *papilläre* (Abb. 2) und *tubuläre* sowie *stark anaplastische* Tumorknoten vor. Bei zwei Versuchstieren hatten die Tumorzellen Spindelform

Abb. 1

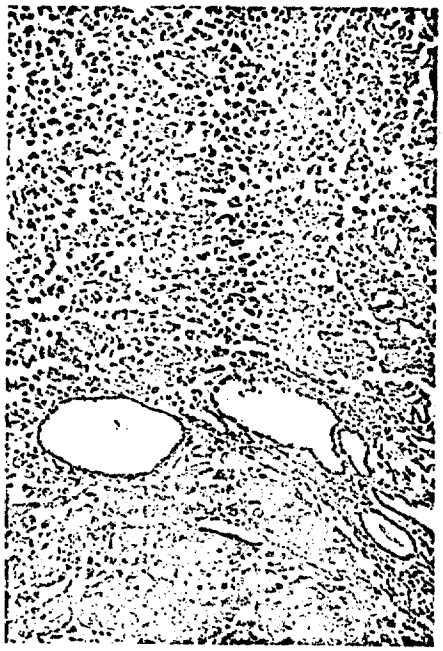


Abb. 2

Abb. 3

Abb. 4

Abb. 1. *Adenoide* Wuchsform eines intrahepatischen Carcinoms aus der Versuchstiergruppe IV — HE 140mal

Abb. 2. *Papilläre* Gewebsformation eines hepatocellulären Carcinoms aus der Versuchstiergruppe IV — HE 140mal

Abb. 3. Endothelartige Tumorzellen (wie beim malignen Hämangioendothelioma), die unregelmäßig große, z. T. von Erythrocyten ausgefüllte Hohlräume auskleiden (Leber, Versuchstiergruppe IV) — HE 140mal

Abb. 4. Cystadeno-Carcinom in der Leber eines Versuchstieres aus Gruppe IV — HE 140mal

und bildeten zahlreiche, meist unregelmäßige, spaltförmige Hohlräume, die z. T. von Erythrocyten angefüllt waren (Abb. 3). Stellenweise kleideten endothelartige Zellen die spaltförmigen Hohlräume aus. Auch kavernomartige Gebilde waren innerhalb dieser Tumorknoten zu erkennen.

Bei einem weiteren Tier bestand die maligne Neubildung aus einem mehrkammerigen cystischen Gebilde mit papillomatösen Wucherungen aus kubischen bis hochcylindrischen Zellen, meist einschichtig angeordnet, die sich in der HE-Färbung bläulich-violett anfärbten und gegenüber dem umgebenden Lebergewebe deutlich abhoben (Abb. 4). Die Kerne des die Cysten auskleidenden Epithels waren groß und auffallend chromatinarm. In den Cysten fand sich reichlich eiweißhaltige Flüssigkeit, in der kleinere Erythrocytenansammlungen und reichlich eisenpigmenthaltige Makrophagen enthalten waren.

Innerhalb der Tumorknoten und in deren unmittelbarer Umgebung war es häufig zu ausgedehnten Blutungen und Nekrosen gekommen. Mehrfach war ein Tumorgewebseinbruch in größere Pfortaderäste zu beobachten.

Die in den *Lungen* bei zwei Versuchstieren festgestellten Tumorknötchen entsprachen in ihrem Zellaufbau den jeweils in der Leber nachgewiesenen malignen Neubildungen. Bei der feingeweblichen Untersuchung der in einer *Niere* eines Versuchstieres entwickelten Neubildungen ergab sich die Diagnose überwiegend papillärer, teilweise auch tubulär strukturierter Nierenrinden-Adenome.

Versuchstiergruppe VI (mit 0,5% N-Methylbenzylamin und 0,5% $NaNO_2$ zur Altromin-Standard-Diät):

Der *Oesophagus* war der hauptsächliche Manifestationsort krankhafter Veränderungen in dieser Versuchstiergruppe (während die Leber keine Besonderheiten aufwies). Dabei waren die Zell- und Gewebsveränderungen und die Entwicklung von Geschwülsten — im Gegensatz zur Tiergruppe IV — relativ einheitlich.

Makroskopisch fand sich nach Präparation der Speiseröhren in unterschiedlicher Zahl und meist in der gesamten Ausdehnung des Oesophagus multiple kleinste bis über linsengroße exophytisch wachsende Tumoren, deren Oberfläche teils glatt, teils blumenkohlartig gehöckert erschien.

Histologisch handelte es sich um multizentrisch entwickelte Epithelhyperplasien und epitheliale Gewächse (Abb. 5). Im Vergleich zu den Kontrolltieren fiel zunächst eine nahezu die gesamte Speiseröhre betreffende Verdickung des verhornenden Plattenepithels auf. Diese war teilweise bedingt durch eine Acanthose unter Beteiligung aller Zellschichten, teilweise durch Hyperkeratose. Sehr häufig fand sich eine teils knötchenförmige, teils flächenhafte intraepitheliale Hyperplasie von Basalzellen, wobei die übrigen Epithelschichten ganz oder teilweise verschwunden waren (Abb. 6). Die herdförmige „Basalzellen-Acanthose" hatte stellenweise zu Protuberanzen geführt, die das Schleimhautniveau mehr oder weniger überragten. Häufig war es in z. T. sehr dicht aneinanderliegenden Bezirken zu einem eindeutig exophytischen Wachstum meist in papillomatöser Wuchsform gekommen. Die multiplen unterschiedlich großen Papillome waren verschiedenartig aufgebaut: Im Zentrum der Geschwülste überwogen kleine, runde, relativ gleichförmige Zellen, die wie Basalzellen aussahen. Lumenwärts waren diese Zellen von unterschiedlich dicken Schichten des Stratum spinosum und granulosum bedeckt. Dazu fand sich eine stark ausgeprägte Hyperkeratinisation mit Aus-

bildung tiefer Krypten, die von Hornlamellen gefüllt waren. Über einzelnen Papillenspitzen war Parakeratose erkennbar. In den papillomatösen Gebilden war die fingerförmige Verzweigung der Papillen verschieden stark, auch wechselte die Quantität der kleinen basalzellähnlichen und der größeren verhornenden Zellen, ebenso das Ausmaß der Hornperlenbildung.

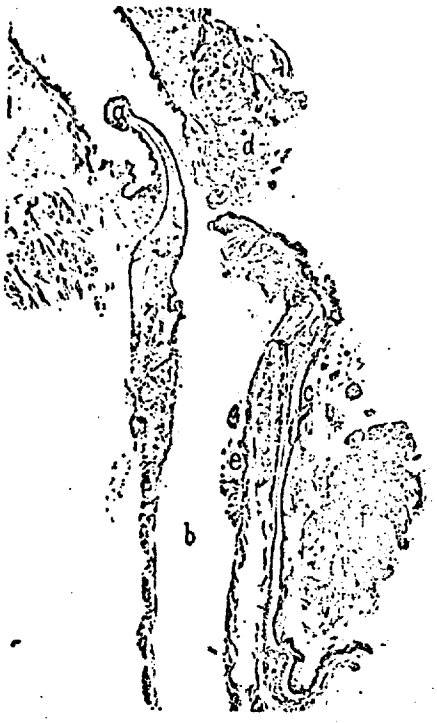


Abb. 5

Abb. 6

Abb. 5. Längsschnitt durch Pharynx und Larynx eines Versuchstieres aus Gruppe VI — HE 9,5mal. a) Epiglottis; b) Trachea; c) Ventralseite des Oesophagus; d) Plattenepithel-Carcinom mit beginnend destruierendem und infiltrierendem Wachstum; e) Plattenepithel-Papillom in der Tracheal-Schleimhaut; f) Plattenepithel-Papillom im Oesophagus

Abb. 6. Herdförmige intracepitheliale Basalzell-Hyperplasie (Oesophagus aus der Versuchstiergruppe VI) HE 140mal

In einzelnen umschriebenen Bezirken des Oesophagusepithels war die regelrechte Zellschichtung aufgehoben (Abb. 7). Dort kam eine stark unterschiedliche Zellfärbbarkeit und Zellgröße zur Darstellung. Teilweise waren zwei- und mehrkernige Zellen und vereinzelt atypische Mitoseformen zu erkennen, noch ohne daß eine in die Tiefe gerichtete Wachstums-Tendenz bestand. Gelegentlich waren einzelne Zellen vacuolär verändert.

Bei dem überwiegenden Teil der untersuchten Versuchstiere waren die cellulären Atypien sehr stark ausgeprägt. Es war dort außerdem zu invasivem und destruierendem Wachstum in tiefere Gewebsschichten gekommen, so daß die

Voraussetzung für die Diagnose maligner Neubildungen gegeben waren (Abb. 8). Die Tumormassen waren zu unterschiedlichen Anteilen aus differenzierten Stachel- und Hornzellen und aus entdifferenzierten Stachelzellen zusammengesetzt. Neben Einzelzellverhornung, nucleärer Hyperplasie, gestörter Kern-Plasma-Relation und Hyperchromasie war eine Zunahme von Mitosen — auch atypischer Art —

Abb. 7 Abb. 8

Abb. 7. Desorganisation der Zellschichtung, stark unterschiedliche Zellfärbbarkeit und Zellgröße; vereinzelt atypische Mitoseformen (sog. atypisches Epithel) in einem Oesophagus aus der Versuchstiergruppe VI — HE 1400mal

Abb. 8. Plattenepithel-Papillom in der Trachea (a). In basisfernen Bezirken maligne entartetes Plattenepithel-Papillom im Oesophagus (b) — Tiergruppe VI — HE 12,5mal

festzustellen. Die normale Schichtung des Epithels erschien völlig desorganisiert. Hornperlenbildung war auch in den in die Tiefe dringenden Zellsträngen und -nestern zu beobachten. Herdförmig waren Granulocyten in lockerer Ansammlung zwischen den Epithelproliferationen vorhanden. Eine entzündliche Reaktion in der Tumorumgebung war hier öfters zu erkennen und erheblich stärker als bei den oben beschriebenen nicht infiltrierend und destruierend wachsenden Neubildungen, die entzündlich-zellige Reaktionen im umgebenden Gewebe meist vermissen ließen.

Als weitere und für einzelne Versuchstiere spezielle Veränderungen sind zu erwähnen: Plattenepithelmetaplasie in der Trachea und im Bronchialsystem,

teilweise mit Ausbildung papillomartiger Wucherungen, schwere eitrige confluierende Bronchopneumonie mit Zeichen der Aspiration pflanzlicher Nahrungsteilchen; Entwicklung eines Plattenepithelcarcinoms in den Nasenhöhlen bei einem der Versuchstiere.

Diskussion

Die Möglichkeit einer Bildung cancerogener Nitrosamine aus sekundären Aminen und Nitrit im Magen muß deshalb möglichst exakt untersucht werden, weil, wie eingangs erwähnt, sekundäre Amine und Nitrit häufig in den Magen gelangen oder dort als bakterielle Stoffwechselprodukte entstehen können. In unseren früher durchgeführten Untersuchungen mit chemisch-analytischen Methoden waren die grundlegenden Prinzipien, denen eine Nitrosaminsynthese im Magen gehorcht, bereits dargestellt worden. Die möglicherweise große praktische Bedeutung, die einer Bildung cancerogener Stoffe aus inaktiven Vorstufen in vivo zukommt, erforderte eine weitere Bestätigung der Ergebnisse. Der Beweis, daß eine Nitrosaminsynthese im Magen zur Induktion von Krebs führen kann, war durch Tierversuche nach Applikation solcher Amine zu erwarten, die stark wirkende Nitrosoderivate liefern und die wenigr stark basisch sind als Diäthylamin. N-Methylbenzylamin und Morpholin weisen diese Eigenschaften auf. Die Basizität beider Amine ist geringer als die des Diäthylamins. Es handelt sich jedoch um noch wesentlich stärker alkalisch reagierende Verbindungen als die früher verwendeten aromatischen Amine [N-Methylanilin ($pK_B = 9{,}1$) und Diphenylamin ($pK_B = 13{,}1$)].

N-Methylbenzylamin und Morpholin wurden auch deshalb in die Versuche einbezogen, weil ersteres in Pflanzen vorkommt und letzteres eine in großem Umfang technisch und industriell verwendete Base ist.

Morpholin dient als Lösungsmittel für eine große Zahl organischer Substanzen, z. B. von Harzen, Wachsen, Schellack, Kasein und Farbstoffen. Es ist Zwischenprodukt in der Gummiindustrie und findet als Mittel zur Korrosionsverhütung in dampfbetriebenen Systemen Anwendung. Mit seiner Hilfe können flüchtige Amine getrennt werden. Morpholin bildet mit Fettsäuren Seifen, die ausgezeichnete emulgierende Eigenschaften haben. Diese werden bei der Herstellung von Putzmitteln verschiedener Art verwendet und sind auch in Haushaltspflegemitteln enthalten. Der Morpholinanteil ist dabei nur lose gebunden und wird leicht in Freiheit gesetzt. Derivate des Morpholins werden in großer Menge in der Waschmittelindustrie gebraucht.

Die ausgedehnte Anwendung von Morpholin (BROWN, 1966; JEFFERSON, 1968) macht es wahrscheinlich, daß ein großer Personenkreis beruflich oder im Privatbereich mit dieser Base in Berührung kommt. Morpolin ist eine mäßig stark toxisch wirkende Substanz. Die LD_{50} für Ratten wird mit 1 g Morpholin/kg (SMYTH, CARPENTER, WEIL, POZZANI, 1954), bzw. mit 1,5 g Morpholin/kg oder 5 g Morpholin-Hydrochlorid/kg (HÜLS, 1967) angegeben. Unverdünntes Morpholin reizt Haut und Schleimhäute stark. Es wird leicht durch die Haut resorbiert. Die Festlegung einer maximal zulässigen Arbeitsplatzkonzentration ist bisher nicht erfolgt. Inhalationsversuche an Ratten, Mäusen und Meerschweinchen führten bei einmaliger Einatmung verhältnismäßig hoher Dosen (100 mg/l Luft) während 4 Std zu keinen nachhaltigen Schäden. Eine zweimonatige Verfütterung von 250 mg

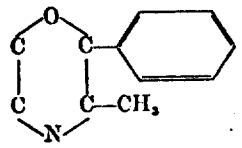
Morpholin pro kg Körpergewicht und Tag blieb ohne nachweisbare Schädigung (Hüls, 1967).

Die Überzeugung, in Morpholin eine weitgehend ungefährliche Substanz vor sich zu haben, war Anlaß, ihre Verwendung im Bereich der Lebensmittelindustrie zu diskutieren (Souci u. Mergenthaler, 1958). Die amerikanische Food and Drug Administration (FDA) genehmigte 1962 mehrere Anwendungsarten: So wurde der Zusatz von Morpholin zu Dampf in Konzentrationen von 10 ppm für Prozesse gestattet, in denen der Dampf mit Nahrungsmitteln in Berührung kommt. Morpholinsalze von Fettsäuren dürfen zum Überziehen von Gemüse und frischen Früchten verwendet werden. Die freie Base oder ihre fettsauren Salze sind als Korrosionsschutzmittel für Metalle, die mit Nahrungsmitteln in Berührung kommen, zugelassen (Federal Register, 1962).

Beachtung verdient auch die Verwendung von Morpholin bei der Arzneimittelherstellung. Nicht nur die Base selbst ist dabei von Interesse, sondern auch Derivate, die ein nitrosierbares Stickstoffatom aufweisen. Die große Zahl der Derivate, in denen Morpholin über das Stickstoffatom gebunden ist, sind dagegen in diesem Zusammenhang ohne Belang.

Das Morpholinsalz der Salicylsäure kann z. B. oral in Dosen bis zu 6 g täglich zur Behandlung verschiedener Formen des Rheumatismus gegeben werden. Ein in Deutschland erhältliches Präparat wird allerdings in Form von Dragees appliziert, die sich erst im Dünndarm auflösen.

Ein Morpholinderivat, das große Verbreitung gefunden hat, ist das 3-Methyl-2-Phenylmorpholin:

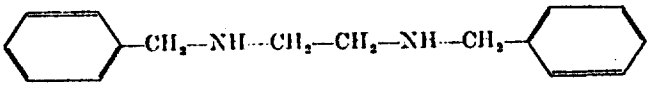


a

Es wird zur Behandlung der Fettleibigkeit empfohlen. Über eine Prüfung des entsprechenden N-Nitroso-Derivates auf eine etwaige cancerogene Wirkung liegt u. W. in der Literatur kein Bericht vor.

N-Methylbenzylamin wurde in Ephedraarten gefunden (Rauen, 1964). Es ist nicht ausgeschlossen, daß sich das Amin auch in anderen Pflanzen findet und evtl. mit Nahrungs- oder Genußmitteln in den menschlichen Magen gelangt.

Eine mit N-Methylbenzylamin verwandte Verbindung ist das Dibenzäthylendiamin:



b

Man kann es sich aus zwei Methylbenzylaminmolekülen zusammengesetzt vorstellen. Die Substanz wird in der Antibioticaherstellung benutzt. Sie kann in Verbindung mit Penicillin G von Kindern oral aufgenommen werden. In der Literatur liegen u. W. keine Veröffentlichungen vor, in denen beschrieben würde, daß die entsprechenden Nitrosoderivate auf ihre cancerogene Wirkung untersucht worden wären.

Die vorstehend geschilderten Tierversuche wurden absichtlich mit einer hohen Tagesdosis von Nitrit und Aminen durchgeführt, um bei einem evtl. negativen Ausgang eine sichere Aussage machen zu können. Die Tumoren traten der Dosierung entsprechend sehr früh auf. Aus quantitativen Untersuchungen von Druckrey, Schildbach, Schmähl, Preussmann u. Ivankovic (1963) geht hervor, daß bei einer Reduzierung der Nitrosamindosis einerseits die Tumorinduktionszeit verlängert wird, andererseits aber die erforderliche Gesamtdosis des Cancerogens wesentlich geringer wird, in Druckreys Versuchen mindestens um den Faktor 10. Man darf daraus folgern, daß in unseren Versuchen auch dann Tumoren induziert worden wären, wenn eine wesentlich niedrigere Dosis von Morpholin und N-Methylbenzylamin appliziert worden wäre. Der Tod der Versuchstiere wäre dann entsprechend später aufgetreten.

Es soll in diesem Zusammenhang erwähnt werden, daß die in einer vorausgehenden Veröffentlichung (Sander, Schweinbenz, Menz, 1968) erwähnten Tiere, die N-Methylanilin und Nitrit in z. T. sehr niedriger Dosierung erhalten hatten, nach einem symptomfreien Intervall von etwa 8 Monaten, während dessen sie normales Futter ohne Zusätze bekamen, maligne Tumoren zu entwickeln begannen. N-Methylanilin ist Bestandteil des Zigarettenrauches und kann zusammen mit dem Speichel in den menschlichen Magen gelangen.

Bei allen Tieren der Gruppe IV hatten sich multiple hepatocelluläre adenomartige Gebilde und zahlreiche teils cystisch umgewandelte Gallengangsproliferate entwickelt. Nur bei einem Tier konnte ein malignes Neoplasma nicht festgestellt werden; hier waren multiple größere Cysten und mehrere Knoten zu beobachten, die aus pflanzenzellähnlichen Zellen aufgebaut und scharf begrenzt erschienen. Die übrigen Versuchstiere zeigten multizentrisch entstandene, überwiegend hepatocelluläre Carcinome mit trabekulären, papillären, tubulären und adenoiden Gewebsformationen und ausgedehnten, herdförmigen Blutungen und Nekrosen. Stellenweise waren die Neubildungen stark anaplastisch. Bei zwei Tieren war das morphologische Bild der Geschwülste mit dem eines malignen Hämangioendothelioms identisch. Bei einem der Versuchstiere war der Tumor in der Leber als multilokuläres Cystadeno-Carcinom anzusprechen. Die Gallengangswucherungen waren auch bei den übrigen Tieren teilweise so unregelmäßig, daß man einen malignen Wachstumsexceß annehmen mußte, zumal stellenweise invasive und destruierende Gewebsformationen vorhanden waren. Nur in einem Tier war gleichzeitig ein cirrhotischer Umbau der Leber mit teils subtotaler, teils totaler, unterschiedlich alter Nekrose einzelner Pseudolobuli nachzuweisen; die übrigen Tiere hatten ein organtypisch aufgebautes Leberparenchym mit verschieden stark in Erscheinung tretenden Leberepitheldegenerationen (Einzel- und Gruppenzellnekrosen). Die Cirrhose kann also nicht als notweniges Vorstadium der malignen Neubildungen angesehen werden.

Lungenmetastasen der hepatocellulären Carcinome konnten bei zwei Tieren nachgewiesen werden. Bei einem Versuchstier wurden multiple, bis stecknadelkopfgroße, überwiegend papillär, aber auch tubulär aufgebaute Nierenrinden-Adenome festgestellt.

Der feingewebliche Aufbau der intrahepatischen Neubildungen entsprach im wesentlichen den histologischen Erscheinungsformen, die aus Experimenten mit

N-Nitrosomorpholin und anderen Cancerogenen bekannt sind (THOMAS, 1961; BANNASCH u. MÜLLER, 1964).

Bei der Versuchstiergruppe VI ist es zu teils flächenhaften, teils herdförmigen multizentrischen Basalzellproliferationen mit meist exophytischer, seltener endophytischer Ausbreitung gekommen. Auch war überall eine unterschiedlich ausgeprägte Papillomatose bei Hyperkeratose festzustellen, meist in der gesamten Ausdehnung der Speiseröhre, teilweise auch im Bereich der Rachenhinterwand. Es sind intraepitheliale Zellatypien und verhornende Plattenepithelcarcinome induziert worden, wobei deren multilokuläre Entwicklung hervorzuheben ist. Mehrfach konnten auch Epithelmetaplasien in den oberen Luftwegen beobachtet werden. Eine Metastasierung der Carcinome in andere Organe ist nicht aufgetreten.

Eine ausführliche histologische Untersuchung der durch N-Nitroso-N-Methylbenzylamin induzierten Oesophagustumoren ist uns aus dem Schrifttum nicht bekannt. DRUCKREY, PREUSSMANN, BLUM, IVANKOVIC, AFKHAM (1963) haben durch die Verwendung von Nitrosaminen erstmals Plattenepithel-Carcinome bei Ratten im Oesophagus erzeugen können, was mit anderen bekannten Cancerogenen zuvor nicht gelungen war. GIBEL (1968) hat nach alimentärer Zufuhr von Dinitrosopiperazin die Entwicklungsstadien des Oesophagus-Carcinoms der Ratte untersucht und dabei feingewebliche Veränderungen des Oesophagus beobachtet, die den von uns durch N-Nitroso-N-Methylbenzylamin erzielten weitgehend entsprechen.

Präventivmedizinische Konsequenzen

Wenn hohe Nitritdosen, vermischt mit Standard-Diät, von Ratten ohne wesentliche Schädigung toleriert werden, so deutet dies darauf hin, daß in dem verwendeten Futter sekundäre Amine, die leicht zu nitrosieren sind und deren Nitroso-Derivate cancerogen wirken, nicht in erheblichem Umfang vorkommen. Es ist sicherlich nicht berechtigt, daraus ohne weitere Untersuchung zu folgern, daß eine Nitritzugabe zu menschlichen Nahrungsmitteln unbedenklich sei. In den menschlichen Magen gelangen, wie eingangs erwähnt, mit Sicherheit sehr viele verschiedene sekundäre Amine, natürlich vorkommende ebenso wie synthetische. Deshalb scheint es notwendig zu fordern, es solle noch einmal sehr sorgfältig überprüft werden, ob Nitrit weiterhin Lebensmitteln zugesetzt werden darf. Zumindest sollten strenge Maßstäbe an solche Erzeugnisse angelegt werden, deren Qualität durch Nitrit nicht wesentlich verbessert wird, wie z. B. Leberwurst oder Fisch.

Bei der toxikologischen Untersuchung von Arzneimitteln und Lebensmittelzusätzen sollte eine mögliche Umsetzung zu den entsprechenden Nitroso-Derivaten berücksichtigt werden. Darüber finden sich in der Literatur keine Hinweise, auch nicht im Rahmen der speziellen Frage, inwieweit Arzneimittel carcinogene Effekte auszuüben vermögen (TRUHAUT, 1967). Es sollte auch bedacht werden, daß Nitrosoverbindungen, jedenfalls tierexperimentell, starke teratogene Eigenschaften haben, und daß darüber hinaus bei ungeborenen Nachkommen schon durch relativ niedrige Dosen von Cancerogenen diaplacentar Krebs, der in späterem Lebensalter auftritt, induziert werden kann.

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Induktion maligner Tumoren bei Ratten durch orale Gabe von 2-Imidazolidinon und Nitrit

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Induction of Malignant Tumors in Rats by Oral Administration of 2-Imidazolidinone and Nitrite

Summary. Two groups of 6 female rats each received for 150 days standard diet, which contained 2-Imidazolidinone (0.1%). One group got, in addition, drinking water with 0.12% sodium nitrite. In this group nephroblastomas developed in 5 rats and a squamous cell carcinoma in 1 rat. In the group that did not get nitrite no tumors were observed.

Zusammenfassung. 2 Gruppen von je 6 weiblichen Ratten bekamen Standardfutter, dem 150 Tage lang 0,1% 2-Imidazolindion zugesetzt wurde. Eine Gruppe erhielt außerdem Trinkwasser mit einem Zusatz von 0,12% Natriumnitrit. In dieser Gruppe entwickelten sich Nephroblastome in 5 Ratten und ein Plattenepithelcarcinom in einer Ratte. In der Gruppe, die kein Nitrit erhielt, wurden Tumoren nicht beobachtet.

Im Rahmen der Krebsursachenforschung wird der Gruppe der N-Nitrosoverbindungen zunehmendes Interesse entgegengebracht. Dies ist vor allem auf die weite Verbreitung von solchen Aminen und Amiden in der Umwelt des Menschen, aus denen cancerogene Nitrosoverbindungen synthetisiert werden können, zurückzuführen. In eigenen Untersuchungen wurde geprüft, ob in vivo aus sekundären Aminen oder Alkylamiden die entsprechenden Nitrosoderivate entstehen können, wenn gleichzeitig Nitrit angeboten wird. Die Frage ist von besonderer Bedeutung, da außer Alkylamiden und sekundären Aminen auch Nitrit inkorporiert wird oder bei Vorliegen einer Sub- oder Anazidität im Mageninhalt gebildet werden kann (Sander u. Seif, 1969). Während sich stark basische sekundäre Amine im Magen nur sehr schwer nitrosieren ließen, wurden die weniger stark basisch reagierenden Amine Morpholin und N-Methylbenzylamin leicht genug nitrosiert, um bei simultaner Gabe von Nitrit Leber- bzw. Oelsophaguscarcinome hervorzurufen (Sander u. Bürkle, 1969). Auch durch Vermischen von Standardfutter mit N-Methylanilin (0,03%) und Natriumnitrit (1%) wurden Oelsophaguscarcinome induziert (Sander, 1970).

Die leichte Überführung der wenig basischen sekundären Amine in entsprechende Nitrosoverbindungen war Anlaß, Alkylamide, die noch weniger stark basisch reagieren, in die Untersuchungen einzubeziehen. In vitro erwiesen sich

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Alkylharnstoffverbindungen als unter physiologischen Bedingungen leicht nitrosierbar (Sander et al., 1970). Daraufhin erhielten Ratten in mehreren Versuchsgruppen Methyl-, Äthyl- oder N,N'-Dimethylharnstoff und Nitrit jeweils getrennt im Trinkwasser oder im Futter in unterschiedlicher Dosierung (0,1% bis 1%). Bisher traten bei 24 von 40 Ratten, die einige Tage lang Methylharnstoff und Nitrit erhalten hatten, Tumoren des zentralen Nervensystems oder der Nieren sowie einige anders lokalisierte Tumoren auf. 3 von 4 Ratten, die Äthylharnstoff und Nitrit bekommen hatten, entwickelten maligne Tumoren (Sander, 1970). Auch alle 10 Ratten, die N,N'-Dimethylharnstoff und Nitrit bekamen, starben an malignen Tumoren (Sander, 1970).

Die weiteren Untersuchungen wurden mit 2-Imidazolidinon, einem Amid, das industrielle Anwendung findet, durchgeführt. Das Nitrosoderivat ist relativ instabil. Es induzierte bei subcutaner Injektion Tumoren am Injektionsort (Druckrey et al., 1967). Das Amid wurde auch im Hinblick auf die Möglichkeit gewählt, hiermit die Bedeutung einer Synthese einer chemisch instabilen, lokal wirksamen Nitrosoverbindung als Ursache von Tumoren am Entstehungsort, d. h. im Magen zu prüfen. Der Nachweis einer Krebsinduktion im Magen durch orale Gabe eines Alkylamids und Nitrit wäre für die Krebsursacheforschung von Interesse, da die eine Nitrosaminbildung im Magen begünstigende Sub- und Anazidität statistisch mit einer erhöhten Magenkrebsmorbidität korreliert ist.

Methodik

Es wurden 2 Gruppen von je 6 weiblichen SIV 50-Ratten (Züchter: Ivanovas, Kisslegg/Allgäu) gebildet. Die Tiere wogen zu Beginn der Versuche 120 g. Sie bekamen als Alleinfutter Altromin® in Pulverform, dem zur Herstellung eines knetbaren Teiges je kg 450 ml Wasser zugesetzt wurden. Das Futter wurde mit 0,1% 2-Imidazolnidion zubereitet. Während Gruppe I unverändertes Trinkwasser erhielt, war dem Wasser in Gruppe II 0,12% Natriumnitrit zugesetzt. Die Applikation dauerte 150 Tage. Futter und Wasser wurden ad libitum gereicht. In den ersten 3 Wochen des Versuchs wurden die Tiere regelmäßig gewogen.

Ergebnisse

2-Imidazolidinon wurde in der applizierten Dosis in Gruppe I gut vertragen. Eine Beeinträchtigung der Entwicklung war nicht festzustellen. In Gruppe II, die gleichzeitig mit dem Trinkwasser Nitrit erhielt, wurde das Futter ungern aufgenommen. Dementsprechend nahmen diese Tiere verlangsamt zu, so daß das durchschnittliche Körpergewicht nach 3 Wochen in Gruppe II nur 123 g gegenüber 175 g in Gruppe I betrug.

Bis zum 311. Versuchstag waren alle 6 Ratten der Gruppe II gestorben oder wegen äußerlich erkennbarer Tumoren getötet worden. Pathologisch-anatomisch wurden bei 5 der Ratten stecknadelkopf- bis hühnereigroße Nierentumoren nachgewiesen. In 2 Tieren hatten sich die Nierengeschwülste doppelseitig entwickelt. Sie zeigten eine höckerige Oberfläche, waren von weißlicher bis rotgrauer Farbe und wiesen eine relativ weiche Konsistenz auf.

Histologisch handelte es sich um Neubildungen, deren Aufbau am ehesten den menschlichen Nephroblastomen entsprach. Die Tumoren zeigten infiltrierendes und destruierendes Wachstum, unterschiedlichen Zellreichtum, herdförmige Polymorphie der im übrigen überwiegend spindelförmigen Zellkerne. Innerhalb der Tumoren fanden sich teilweise atypische Tubuli und glomerulumähnliche

Strukturen, myxomatöse Veränderungen, ausgedehnte Nekrosen und Blutungen, Züge und Bündel glatter Muskulatur und streifige Fibrosen. Eines der Tiere hatte eine ausgedehnte Metastasierung in die Lungen.

Ein Tier wies außer dem Nierentumor noch ein gut haselnußgroßes z. T. nekrotisches, überwiegend papilomatöses Mamma-Adenom auf. Das Tier, bei dem sich keine Nierengeschwulst entwickelt hatte, zeigte im Bereich der Parotis ein haselnußgroßes, verhornendes Plattenepithelcarcinom. In dem Vormagen eines der Tiere fand sich eine umschriebene Basalzell-Hyperplasie. Bei der orientierenden histologischen Untersuchung der parenchymatösen Organe der Tiere konnten sonstige pathologische Befunde nicht erhoben werden.

In Gruppe I überlebten alle Tiere bis zum Versuchsende. Sie wurden getötet, nachdem in Gruppe II das letzte Tier gestorben war. Tumoren oder sonstige pathologisch-anatomischen Veränderungen wurden nicht nachgewiesen.

Diskussion

Die Induktion maligner Tumoren durch orale Gabe von 2-Imidazolidinon und Nitrit zeigt, daß das Amid in vivo leicht in das entsprechende cancerogen wirkende Nitrosoderivat überführt werden kann. Die Induktion von Tumoren erfolgte mit relativ niedrigen Dosen, die nur für eine begrenzte Zeit appliziert worden sind. In weiteren, noch nicht abgeschlossenen Versuchen mit noch niedrigeren Tagesdosen wurden in einer Gruppe, die 150 Tage lang nur 0,05% Nitrit und 0,05% 2-Imidazolidinon mit dem Futter bzw. dem Trinkwasser bekam, nach einer Versuchszeit von 9 Monaten bereits bei 2 von 6 Ratten Tumoren induziert (1 Nephroblastom, 1 Plattenepithelcarcinom). Die Versuchsergebnisse machen es wahrscheinlich, daß bei chronischer Applikation noch wesentlich niedrigere Dosen der beiden Substanzen Krebs hervorrufen können.

Eine Induktion von malignen Tumoren durch In-vivo-Synthese von Nitrosoverbindungen ist somit mit verschiedenen Alkylharnstoffen, zu denen auch 2-Imidazolidinon gerechnet werden kann, sehr leicht möglich, wesentlich leichter als mit den meisten sekundären Aminen. In eigenen Untersuchungen genügte dazu eine nur wenige Tage dauernde Anwendung von Nitrit und Alkylharnstoffen. Seit den Untersuchungen von Druckrey und seinen Mitarbeitern ist bekannt, daß nitrosierte Alkylharnstoffe, diaplacentar oder bei sehr jungen Tieren appliziert, eine noch wesentlich stärkere cancerogene Wirkung entfalten als beim erwachsenen Tier (Druckrey et al., 1970). Bei Verfütterung von Alkylharnstoffverbindungen und Nitrit an schwangere Versuchtstiere dürfte deshalb eine Senkung der für eine Tumorinduktion ausreichenden Dosen möglich sein.

Carcinome des Drüsenmagens sind bei Verfütterung von Alkylamiden und Nitrit bisher nicht nachgewiesen worden. Das bedeutet jedoch nicht, daß Tumoren dieser Lokalisation durch orale Applikation eines Amids und Nitrit nicht induziert werden könnten. Eine Induktion von Magenkrebs bei Meerschweinchen durch N-Methylnitrosoharnstoff und Methylnitrosourethan wurde von Druckrey et al. (1968) beschrieben. Mit zwei weiteren Nitrosoverbindungen konnten bei Ratten Tumoren des Drüsenmagens induziert werden. Sugimura und Fujimura induzierten Magenkrebs durch Anwendung von N-Methyl-N-nitroso-N'-nitroguanidin (1967).

Druckrey et al. beobachteten Drüsenmagencarcinome nach oraler Gabe von N-Methyl-N-nitroso-N'-acetylharnstoff (1970). Möglicherweise spielt auch bei diesen instabilen Verbindungen neben einer lokalen Wirkung auf die Schleimhaut des Drüsenmagens eine h-organotrope Wirkung, wie sie von Druckrey et al. bei vielen anderen Nitrosoverbindungen nachgewiesen wurde, eine Rolle.

2-Imidazolidinon wurde stellvertretend für viele andere Alkylamide, die mit Nahrungs-, Genuß- oder Arzneimitteln oral aufgenommen werden können, untersucht. Die cancerogene Wirksamkeit des Nitrosamids war bekannt. Von den meisten von Menschen inkorporierten Alkylamiden ist jedoch über die Nitrosierbarkeit in vivo oder über die cancerogene Wirkung der Nitrosoderivate nichts oder nur ungefähres bekannt. Angesichts des nicht seltenen Vorkommens von Nitrit im Mageninhalt sollte die Möglichkeit einer Nitrosierung von oral aufgenommenen Alkylamiden berücksichtigt werden. Dies gilt besonders für Nahrungsmittelfremdstoffe und Arzneimittel.

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BACTERIAL REDUCTION OF NITRATE IN THE HUMAN STOMACH, AS A CAUSE FOR NITROSAMINE FORMATION (Bakterielle Reduktion von Nitrat im Magen des Menschen als Ursache einer Nitrosamin-Bildung)

Authors: Johannes Sander and Fritz Seif

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Investigations concerning the formation of cancerogenic nitroso compounds under physiological conditions have revealed that a nitrosamine synthesis in the human stomach, because of the H ion concentration to be found there, can easily be started as long as suitable secondary amines and nitrite are present in the stomach content. Many different secondary amines can enters the stomach, as constituents of food or medicines. Nitrite, added to food or developing in food stuffs through the bacterial reduction of nitrate, is orally ingested in a certain amount (Reference 1). As is shown by further investigations, a fraction of the nitrite present is always available for the nitrositing of secondary amines in the stomach, since a very large amount of other food constituents reacts with the nitrous acid (Reference 2). In most cases, a lack of nitrite will limit the formation of nitrosamine in the stomach; hence this formation should be favored by an increase availabitily of nitrite in the same organ.

In its empty state, the human stomach offen contains a neutrally, or slightly acid reacting secretion. Here, a large number of bacteria can develop and increase in number. Nitrate reducing types exist among them also. Since nitrate is now present in high concentration in drinking water and in many food stuffs, the possibility of its bacterial reduction to nitrite in the stomach had to be tested. Then the question arose whether, when and under which prerequisites a bacterial nitrite formation in the stomach can cause or increase a nitrosamine synthesis. A certain significance as cause or concomitant cause of cancer in man should be ascribed probably to a measurable amount of nitrosamine formation in the human stomach.

Procedure:

After a few ml of empty stomach content had been obtained, 31 patients were administered a test dring containing 100 mg Na hydrogen carbonate, 300 mg Na nitrate and 1000 mg glucose in 40 ml water. To this solution, 10 mg diphenyl amine in 3 ml ethyl alcohol were added. When added to the aqueous solution, diphenyl amine precipitated in the form of small crystals.

During 50 minutes, every 10 minutes, a part of the stomach content was obtained. The nitrite content was measured with the help of the reagent according to Griess-Ilosvay, and the pH was determined. All the gastric juice portions of a patient were nutralized, combined, and were extracted with dichloromethane. In the extract, diphenylnitrosamine was quantitative determined through photolytic splitting, and qualitatively in two-dimensional film chromatography as previously described (References 3, 4).

Results

a Nitrite in Empty Stomach Content:

In 8 of the patients investigated, the empty stomach juice contained nitrite. The concentrations measured ranged between a still detectable trace of nitrite in the case of one patient, and a little above 1 μg NO_2^- per ml with another. Concentration exceeding 0.5 μg/ml were measured only when the pH of the empty stomach content was higher than 5. On the other hand, in several patients whose stomach content reacted almost neutrally, no nitrite was found.

b Nitrite Formation after Administration of the Drink Specimen:

The reaction of the empty stomach content was of great significance for the nitrite formation following the administration of the drink specimen. If, namely, the pH value of the empty stomach secretion was below 3, then, despite a neutralizing effect of the drink specimen, it was impossible to detect a measurable nitrite

formation during the time available, except in the case of one patient. If the pH of the empty stomach content exceeded 4, nitrite, after the administration of the drink specimen, was always formed, as long as the pH value did not already decrease below 4 within the first 10 minutes (1 case).

Figure 1: Nitrite concentrations in stomach juice specimens the pH of which exceeded 5. (Two extremely high concentrations which were measured in one patient with anacidity (25 and 26 µg/ml after 40 or 50 minutes were not entered in the diagram.

Figure 1 exhibits the nitrite concentrations measured in specimens the pH of which was higher than 5 at the time, indicated on the abscissa, following the administration of the drink specimen. The concentrations, measured at lower pH values, were not represented, since as a rule they were definitely lower. Nevertheless, in several specimens, pH values up to 1.5 nitrite amounts were found; they were only slightly under those found in the previous specimen with a still high pH. In these cases, however, nitrite was not present in the mobile (watery) freshly extracted stomach juice. It was found exclusively in mucous parts, which obviously protected the nitrate-reducing bacteria and their immediate environment for a time from the action of the acid.

Through the multiplication of the nitrite concentrations measured by the corresponding amount of ml of stomach juice obtained, it was possible to calculate the minimum amount of nitrite formed in the stomach of one patient. Amounts between about 1 µmole and 4 µmole of nitrite were found, when the pH at least for 30 minutes did not drop below 5. If the acid formation was higher, then less nitrite was formed. With one patient, who was clinically suspected of a stomach carcinoma, there was complete anacidity. In this case, 12 µmole of nitrite were formed within 50 minutes.

c Nitrosamine Formation:

In 11 of the patient examined, a considerable nitrite formation as well as a subsequent pH decrease from the neutral range to values below 3 within the time available were found. The nitrosamine amounts, detectable in these cases, are exhibited in Figure 2. Despite the considerable dispersion of the values,

it can be seen that a nitrosamine formation can be intensified when enough time is available for this.

Figure 2: Dependence of the amount of diphenylnitrosamine, detected in the stomach content, from the time that elapsed before the pH of the stomach content dropped below 3.

In 5 patients, the acid secretion did not suffice in order to exhaust the buffer capacity of the drink specimen. In these cases, nitrosamine amounts below 1 µg were detected, despite higher nitrite concentrations.

In 15 patients no nitrite formation was detectable. Most of the quantitative nitrosamine determination gave negative results. In individual cases, nitrosamine amounts between 0.2 and 0.5 µg were found. This is due to nitrosing through nitrite contained in the saliva.

In the case of nitrosamine contents exceeding 1 µg, the quantitative determination was completed by a film chromatographic detection. R_f values and staining in each case agreed with the control substance.

Discussion:

Conclusions concerning possible connections between a cancer induction in man and the formation of cancerogenic nitroso compounds in the stomach can be drawn only is a sufficient knowledge of the possibilities of nitrositing suitable N compounds in the stomach exists. Investigations in man are necessary in order to take into account all the factors which can induce a nitrosamine formation in the human stomach. Animal tests and tests in vitro are not sufficient for this. Nevertheless, they can be used as a complement, since many problems cannot be investigated in man because of the risk involved for his health.

In our investigations, we tried to exclude in advance any health risk for the people under examination. Hence, no water soluble amine, which would have been easily nitrosited, was usable, since the corresponding nitroso-derivates are cancerogenic

Diphenylamine, a practically nontoxic substance, yield a nitrosamine which has no cancerogenic eff ct (Reference 5). It was a disadvantage that the amine in the drink specimen was crystalline, hence only to a small extent accessible to the attack exe ted by the nitrous acid. This explains the certainty with which we found, in the stomach content, nitrosamine concentrations quite below the values that would have been obtained through the additi n of a higher amount of a solubilizer or through the use of a corresponding soluble amine.

In evaluating the results, one must also bear in mind that every 10 minutes a large part of the stomach content was drawn with a sound. While the test was progressing, a considerable dilution of the drink specimen was occurring through secretions of the gastric mucosa. With the use of a solid test meal with a longer period of dwell, correspondingly higher amounts of nitrite and nitrosamine might be expected. It was not possible such a test meal, since the investigation served at the same time to the performance of a clinical functional diagnosis of the grastric mucosa.

Despite these limitations, the following facts can be derived from the test results:

1. A nitrosamine formation from nitrite and from a secondary amine can take place in the human stomach. The detection of the nitrosamine formation confirms what was to be expected on the basis of previous investigations regarding the synthesis of cancerogenic nitrosamines under physiological conditions.

2. It is not necessary for the nitrite, required for a nitroamine synthesis, to enter the stomach in food stuffs. The nitrate, contained in many food stuffs, can be reduced to nitrite in the stomach, under suitable conditions.

3. Nitrosamine formation in the stomach is especially facilitated if, through a slow decrease of the pH, optimum conditions are successively obtained for bacterial nitrite formation and nitrosamine synthesis. A considerable buffer capacity of the food can be responsible for a slowed-down decrease of the pH value, as a restricted function of the gastric mucosa. If there is no decrease of the pH, only a slight nitrosamine formation takes place, despite high nitrite concentrations.

Nitrosamine formation, measurable to some extent by chemical methods, is possible, as was previously described (Reference 2), if the stomach contains, in addition to nitrite, such secondary amines that do not have too basic a reaction. No doubt, however, strongly basic amines are nitrosited in very little amount. Since these strongly basic amines enter the stomach with greater frequency and in higher amount than less basic ones, the corresponding nitrosamines might perhaps acquire a practical significance through summation over long years. Certain acylalkylamides and other N compounds, by reacting in the stomach with nitrous acid, can probably be converted in cancerogenic derivatives also. Investigations along this line have been started.

In the toxicological testing of medicines and of food or food additives, one should make allowance for the possibility of nitrositation in the stomach. This is the case especially for substances that are ingested in larger amount or for a longer time, and for such as easily react with nitrous acid. The urgency of pertinent investigations is based on the recent successful production of cancer in rats through the fatty degeneration of a diet containing morpholine and nitrite or N-methylbenzylamine and nitrite. (Reference 6).

The statistically acertained higher frequency with which persons suffering from a chronic atrophic gastritis are attacked by stomach cancer suggests the investigation of a connection with a possible cancerogenic formation in this organ. As is well known, an atrophic gastritis is connected with a functional loss of the gastric mucosa, this loss progressing from a subacidity to an anacidity. Now a subacidity - according to the investigations presented - facilitates the synthesis of cancerogenic nitroso compounds.

What significance should be ascribed to the formation of cancerogenic nitroso compounds in the stomach, is an especially topical question today, since it has been possible to induce tumors in animal tests with a large number of different nitrosamines and nitrosamides (Reference 7). All the findings obtained so far indicate that these cancerogenic substances produce cancer in man also, if they penetrate the human organism.-

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tion of the carbonyl-C-atom or the α-C-atom by a phenyl rest or the presence of such rests at both C-atoms were discovered to be the prerequisite for a good antimicrobial activity.

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Aus dem Hygiene-Institut (Direktor: Professor Dr. R.-E. Bader) und der Medizinischen Poliklinik (Direktor: Professor Dr. F. Heni) der Universität Tübingen

Bakterielle Reduktion von Nitrat im Magen des Menschen als Ursache einer Nitrosamin-Bildung

Herrn Professor Dr. H. Druckrey zum 65. Geburtstag gewidmet

Von Johannes Sander und Fritz Seif

Untersuchungen über die Bildung cancerogener Nitroso-Verbindungen unter physiologischen Bedingungen haben ergeben, daß eine Nitrosamin-Synthese im menschlichen Magen auf Grund der dort anzutreffenden H-Ionen-Konzentration leicht vonstatten gehen könnte, sofern geeignete sekundäre Amine und Nitrit im Mageninhalt vorhanden sind. Viele verschiedene sekundäre Amine können als Bestandteile von Nahrungs-, Genuß- oder Arzneimitteln in den Magen gelangen. Auch Nitrit, das Lebensmitteln zugesetzt wird oder in ihnen durch bakterielle Reduktion von Nitrat entstehen kann, wird oral in gewissem Umfang aufgenommen [1]. Wie aus weiteren Untersuchungen hervorgeht, steht für eine Nitrosierung sekundärer Amine im Magen immer nur ein Bruchteil des vorhandenen Nitrits zur Verfügung, da sehr viele andere Bestandteile der Nahrung mit der salpetrigen Säure reagieren [2]. In den meisten Fällen wird ein Mangel an Nitrit eine Nitrosamin-Bildung im Magen begrenzen; ein vermehrtes Angebot von Nitrit im Magen müßte sie deshalb begünstigen.

Der Magen des Menschen enthält im nüchternen Zustand häufig ein neutral oder nur schwach sauer reagierendes Sekret. Hierin können sich zahlreiche Bakterien erhalten oder vermehren. Unter ihnen befinden sich auch Nitrat-reduzierende Arten. Da Nitrat im Trinkwasser und in vielen Nahrungsmitteln in z. T. hoher Konzentration vorkommt, war zu prüfen, ob es im Magen bakteriell zu Nitrit reduziert werden kann. Das führte uns weiter zu der Frage, ob, und wenn ja, unter welcher Voraussetzung eine bakterielle Nitrit-Bildung im Magen eine Nitrosamin-Synthese verursachen oder verstärken kann. Einer Nitrosamin-Synthese meßbaren Umfanges im menschlichen Magen müßte man vermutlich eine Bedeutung als Ursache oder Mitursache von Krebs beim Menschen zusprechen.

Methodik

31 Patienten erhielten nach Gewinnung von einigen ml Magennüchterninhalt einen Probetrunk, der in 40 ml Wasser 100 mg Natriumhydrogencarbonat, 500 mg Natriumnitrat und 1000 mg Glukose enthielt. Außerdem wurde der Lösung 10 mg Diphenylamin in 5 ml Äthylalkohol zugesetzt. Diphenylamin fiel bei Zugabe zu der wäßrigen Lösung in Form kleiner Kristalle aus.

Während 50 min wurde alle 10 min ein Teil des Mageninhaltes gewonnen. Es wurden der Nitritgehalt mit Hilfe des Reagenz nach Griess-Ilosvay und der pH-Wert gemessen. Alle Magensaftportionen eines Patienten wurden neutralisiert, vereinigt und mit Dichlormethan extrahiert. Im Extrakt wurde Diphenylnitrosamin quantitativ durch photolytische Spaltung und qualitativ in zweidimensionaler Dünnschichtchromatographie wie früher beschrieben [3, 4], bestimmt.

Ergebnisse

a) Nitrit im Magennüchterninhalt

Bei 8 der untersuchten Patienten enthielt der Nüchternsaft Nitrit. Die gemessenen Konzentrationen lagen zwischen einer gerade noch feststellbaren Spur Nitrit bei einem Patienten und etwas über 1 μg NO_2^- pro ml bei einem anderen. Konzentrationen über 0,5 μg/ml wurden nur gemessen, wenn der pH-Wert des Magennüchterninhaltes höher als 5 war. Andererseits wurde bei mehreren Patienten, deren Mageninhalt nahezu neutral reagierte, kein Nitrit gefunden.

b) Nitrit-Bildung nach Verabfolgung des Probetrunks

Für eine Nitrit-Bildung nach der Verabreichung des Probetrunks war die Reaktion des Nüchterninhaltes von großer Bedeutung. Lag nämlich der pH-Wert des Nüchternsekretes unter 5, so konnte trotz einer neutralisierenden Wirkung des Probetrunks eine meßbare Nitrit-Bildung in der zur Verfügung stehenden Zeit mit Ausnahme eines Patienten nicht festgestellt werden. Lag der pH-Wert des Nüchterninhaltes über 4, so wurde nach Verabreichung des Probetrunks stets Nitrit gebildet, sofern der pH-Wert nicht schon innerhalb der ersten 10 min unter 4 absank (1 Fall).

In Abb. 1 sind die Nitrit-Konzentrationen eingetragen, die in Proben gemessen wurden, deren pH-Wert zu der auf der Abszisse angegebenen Zeit nach Verabfolgung des Probetrunks noch höher als 5 war. Die bei niedrigeren pH-Werten gemessenen Konzentrationen wurden nicht dargestellt, da sie in der Regel wesentlich niedriger waren. Jedoch wurden in mehreren Proben mit pH-Werten bis zu 1,5 Nitrit-Mengen festgestellt, die nur wenig unter denen lagen, die in der vorangehenden Probe mit noch hohem pH-Wert gefunden worden waren. In diesen Fällen war aber Nitrit nicht in dem dünnflüssigen frisch sezernierten Magensaft enthalten. Es fand sich ausschließlich in Schleimpartien, die offenbar die Nitrat-reduzierenden Bakterien und ihre unmittelbare Umgebung eine Zeit lang vor der Einwirkung der Säure schützten.

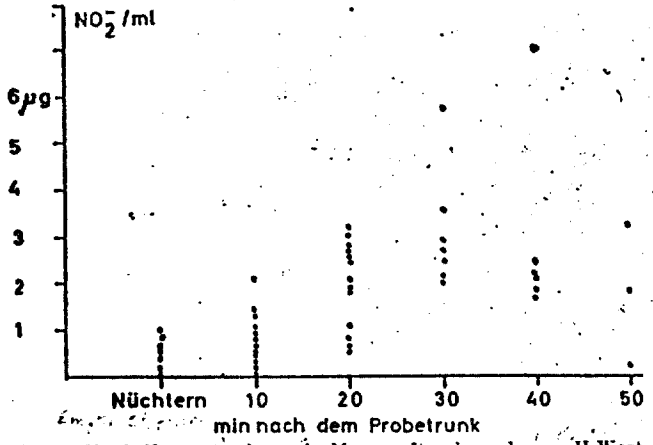

Abb. 1: Nitrit-Konzentrationen in Magensaftproben, deren pH-Wert über 5 lag. (Zwei extrem hohe Konzentrationen, die bei einem Patienten mit Anacidität gemessen wurden (25 und 26 µg/ml nach 40 bzw. 50 min wurden nicht dargestellt.)

Durch Multiplikation der gemessenen Nitrit-Konzentrationen mit der entsprechenden Anzahl der gewonnenen ml Magensaft ließ sich die Mindestnitrit-Menge, die im Magen eines Patienten gebildet wurde, errechnen. Mengen zwischen etwa 1 µMol und 4 µMol Nitrit fanden sich, wenn der pH-Wert mindestens 30 min lang nicht unter 5 absank. War die Säurebildung stärker, so wurde entsprechend weniger Nitrit gebildet. Bei einem Patienten, bei dem der klinische Verdacht auf ein Magencarcinom bestand, fand sich eine vollständige Anacidität. Hier wurden innerhalb von 50 min 12 µMol Nitrit gebildet.

c) Nitrosamin-Synthese

Bei 11 der untersuchten Patienten wurden sowohl eine starke Nitrit-Bildung als auch eine daran anschließende pH-Wert-Senkung vom Neutralbereich auf Werte unter 3 innerhalb der zur Verfügung stehenden Zeit gefunden. Die Nitrosamin-Mengen, die in diesen Fällen nachgewiesen werden konnten, sind in Abb. 2 dargestellt. Man erkennt, trotz starker Streuung der Werte, daß eine Nitrosamin-Bildung verstärkt werden kann, wenn eine entsprechend lange Zeit dafür zur Verfügung steht.

Bei 5 Patienten reichte die Säuresekretion nicht aus, um die Pufferkapazität des Probetrunks zu erschöpfen. In diesen Fällen wurden trotz hoher Nitrit-Konzentrationen Nitrosamin-Mengen unter 1 µg nachgewiesen.

Bei 15 Patienten war eine Nitrit-Bildung nicht nachweisbar. Die quantitative Nitrosamin-Bestimmung ergab meist negative Werte. In Einzelfällen wurden Nitrosamin-Mengen zwischen 0,2 und 0,5 µg gefunden. Dies wird auf eine Nitrosierung durch im Speichel enthaltenes Nitrit zurückgeführt.

Bei Nitrosamin-Gehalten über 1 µg wurde die quantitative Bestimmung durch einen dünnschichtchromatographischen Nachweis ergänzt. R_f-Werte und Anfärbung stimmten in allen Fällen mit der Kontrollsubstanz überein.

Diskussion

Rückschlüsse auf eventuelle Zusammenhänge zwischen einer Induktion von Krebs beim Menschen und

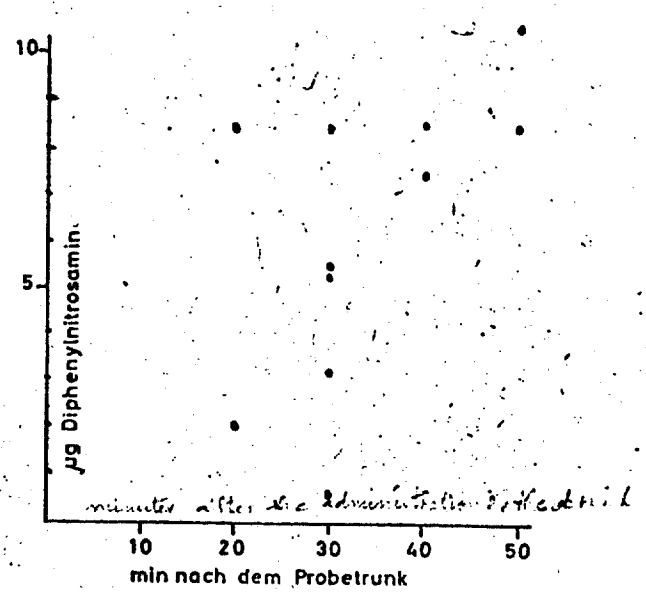

Abb. 2: Abhängigkeit der im Mageninhalt nachweisbaren Menge Diphenylnitrosamin von der Zeit, die verstrich, bevor der pH-Wert des Mageninhaltes unter 3 absank.

der Bildung cancerogener Nitroso-Verbindungen im Magen können nur gezogen werden, wenn ausreichende Kenntnisse über die Möglichkeiten einer Nitrosierung geeigneter Stickstoff-Verbindungen im Magen vorliegen. Untersuchungen am Menschen sind notwendig, um alle Faktoren berücksichtigen zu können, die auf eine Nitrosamin-Synthese im menschlichen Magen Einfluß nehmen. Tierversuche und Tests in vitro reichen dafür nicht aus. Sie stellen jedoch eine Ergänzung dar, denn manche Fragestellungen können wegen eines damit verbundenen gesundheitlichen Risikos am Menschen nicht untersucht werden.

In den eigenen Untersuchungen wurde die Untersuchungsmethodik darauf ausgerichtet, eine gesundheitliche Gefährdung der Untersuchten von vornherein auszuschließen. Es konnte deshalb kein wasserlösliches Amin, das relativ leicht zu nitrosieren gewesen wäre, verwendet werden, da die entsprechenden Nitroso-Derivate cancerogen wirken. Diphenylamin, eine praktisch untoxische Substanz, liefert ein Nitrosamin, das nicht cancerogen wirkt [5]. Von Nachteil war, daß das Amin im Probetrunk kristallin vorlag und somit dem Angriff der salpetrigen Säure nur zu einem kleinen Teil zugänglich war. Mit Sicherheit wurden deshalb im Mageninhalt Nitrosamin-Konzentrationen nachgewiesen, die ganz wesentlich unter den Werten lagen, die durch Zugabe einer größeren Menge eines Lösungsvermittlers oder durch Verwendung eines entsprechenden löslichen Amins erhalten worden wären.

Bei der Beurteilung der Ergebnisse ist weiterhin zu berücksichtigen, daß alle 10 min ein großer Teil des Mageninhaltes durch eine Sonde abgezogen wurde. Eine starke Verdünnung des Probetrunks durch Sekrete der Magenschleimhaut war bei fortschreitender Versuchsdauer die Folge. Bei Verwendung einer festen Probemahlzeit mit längerer Verweildauer wären entsprechend größere Nitrit- und Nitrosamin-Mengen zu erwarten. Eine solche Probemahlzeit konnte nicht verwendet werden, da die Untersuchung gleichzeitig einer klinischen Funktionsdiagnose der Magenschleimhaut diente.

Trotz dieser Einschränkungen lassen sich folgende Tatsachen aus den Versuchsergebnissen ableiten:

1. Eine Nitrosamin-Synthese aus Nitrit und einem sekundären Amin kann im menschlichen Magen stattfinden. Der Nachweis der Nitrosamin-Bildung bestätigt, was auf Grund früherer Untersuchungen über die Synthese cancerogener Nitrosamine unter physiologischen Bedingungen zu erwarten war.

2. Es ist nicht notwendig, daß das für eine Nitrosamin-Synthese erforderliche Nitrit mit Nahrungsmitteln in den Magen gelangt. Das in vielen Nahrungsmitteln enthaltene Nitrat kann im Magen unter geeigneten Bedingungen zu Nitrit reduziert werden.

3. Eine Nitrosamin-Bildung im Magen wird besonders begünstigt, wenn durch langsames Absinken des pH-Wertes für eine bakterielle Nitrit-Bildung und eine Nitrosamin-Synthese nacheinander optimale Bedingungen geschaffen werden. Eine starke Pufferkapazität der Nahrung kann ebenso für eine verlangsamte pH-Wert-Senkung verantwortlich sein wie eine eingeschränkte Funktion der Magenschleimhaut. Das Ausbleiben einer pH-Wert-Senkung erlaubt, trotz hoher Nitrit-Konzentrationen, nur eine geringgradige Nitrosamin-Synthese.

Eine Nitrosamin-Synthese in mit chemischen Methoden meßbarem Umfang ist, wie früher beschrieben [2], möglich, wenn im Magen neben Nitrit solche sekundären Amine vorhanden sind, die nicht zu stark basisch reagieren. In sehr geringem Umfang werden aber zweifellos auch stark basische Amine nitrosiert. Da diese wesentlich häufiger und in größerer Menge in den Magen gelangen als wenig basische, könnten die entsprechenden Nitrosamine durch langjährige Summation vielleicht doch eine praktische Bedeutung erhalten. Wahrscheinlich können auch bestimmte Acylalkylamide und andere Stickstoff-Verbindungen im Magen durch Reaktion mit salpetriger Säure in cancerogen wirkende Derivate überführt werden. Entsprechende Untersuchungen sind begonnen worden.

Bei der toxikologischen Prüfung von Arznei- und Genußmitteln oder Nahrungsmittelzusätzen sollte man die Möglichkeit einer Nitrosierung im Magen berücksichtigen. Dies gilt besonders für Stoffe, die in größerer Menge oder langfristig aufgenommen werden, und solche, die leicht mit salpetriger Säure reagieren. Die Dringlichkeit entsprechender Untersuchungen geht auch aus der Tatsache hervor, daß es kürzlich gelang, durch Verfütterung einer Diät, die Morpholin und Nitrit bzw. N-Methylbenzylamin und Nitrit enthielt, an Ratten Krebs zu erzeugen [6].

Die statistisch gesicherte größere Häufigkeit, mit der Personen, die an einer chronisch-atrophischen Gastritis leiden, von Magenkrebs befallen werden, legt es nahe, eine Verknüpfung mit einer möglichen Cancerogen-Bildung im Magen zu suchen. Eine atrophische Gastritis ist bekanntlich mit einer von Sub- zu Anacidität fortschreitenden Funktionseinbüße der Magenschleimhaut verbunden, und gerade eine Subacidität begünstigt, entsprechend den vorgelegten Untersuchungen, die Synthese cancerogener Nitroso-Verbindungen.

Die Frage, welche Bedeutung einer Bildung cancerogener Nitroso-Verbindungen im Magen zuzusprechen ist, ist von besonderer Aktualität, weil mit vielen verschiedenen Nitrosaminen und Nitrosamiden im Tierversuch Tumoren induziert werden konnten [7]. Alle bisherigen Befunde deuten darauf hin, daß diese Cancerogene auch beim Menschen Krebs hervorrufen können, sofern sie in den menschlichen Organismus gelangen.

Zusammenfassung

Untersuchungen an 31 Patienten ergaben, daß im menschlichen Magen Nitrat bakteriell zu Nitrit reduziert werden kann. Im Magen gebildetes Nitrit ermöglichte die Synthese von Diphenylnitrosamin aus zugeführtem Diphenylamin. Eine Nitrosamin-Synthese im Mageninhalt wurde besonders begünstigt, wenn, ähnlich wie bei einer Subacidität, durch ein langsames Absinken des pH-Wertes zunächst für die bakterielle Nitrat-Reduktion und anschließend für die Nitrosierungs-Reaktion optimale Voraussetzungen gegeben waren. Praktische Konsequenzen einer Nitrosamin-Synthese im Magen wurden kurz diskutiert.

Summary

Bacterial Reduction of Nitrate in the Human Stomach as a Cause for Nitrosamine Formation

Studies on 31 patients yielded that in the human stomach nitrate can be reduced to nitrite by bacteria. Nitrite formed in the stomach made the synthesis of diphenylnitrosamine from administered diphenylamine possible. The synthesis of nitrosamine in the stomach was specially favoured when, similar to subacid conditions, bacterial nitrate reduction and subsequent nitrosation reaction received optimal conditions by gradual decrease of the pH value. The practical consequences of a synthesis of nitrosamine in the stomach were discussed in short.

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Aus der Wissenschaftlichen Abteilung der H. F. & Ph. F. Reemtsma, Hamburg

Stickstoffverbindungen des Tabakrauches

Herrn Professor Dr. H. Druckrey zum 65. Geburtstag gewidmet

Von Georg Neurath

Den Stickstoffverbindungen im Tabak und Rauch galt seit dem Nachweis des Nicotins, als dem anregenden Prinzip der Tabakpflanze, durch Posselt u. Reimann 1828 [96] die besondere Aufmerksamkeit der Untersucher. 1843 gelang es Melsens [60], das Nicotin aus dem Rauch von Pfeifentabak zu isolieren und die Bruttoformel aufzustellen. Dies geschah immerhin 50 Jahre vor der endgültigen Aufklärung der Konstitution des Nicotins, die nach einer Vermutung von Pinner [93], durch die Synthese von Pictet [92] vollzogen wurde. Als weitere Stickstoffverbindung wies Vogel [127] im Jahre 1858 Cyanwasserstoff im Tabakrauch nach. Die ersten heterocyclischen Stickstoffverbindungen, Pyridin, Picolin und Collidin, fanden Vohl u. Eulenberg 1871 [128]. Bogen wies 1929 Ammoniak [7] und Gabelya [27] im gleichen Jahre Methylamin, Dimethylamin und Trimethylamin im Tabakrauch nach.

Im letzten Jahrzehnt wirkten die modernen physikalisch-chemischen Analysenmethoden, wie auf anderen Gebieten, auch in der Untersuchung der Stickstoffverbindungen des Tabakrauches stark beschleunigend. So sind in der 1959 erschienenen, zusammenfassenden Arbeit über die Inhaltsstoffe von Tabak und Tabakrauch von Johnstone u. Plimmer [49] ca. 50 Stickstoffverbindungen aufgeführt, während heute 181 Stickstoffverbindungen im Tabakrauch sicher bekannt sind.

Die Kenntnis über die Zusammensetzung des Tabakrauches ist zur Zeit wegen der Bedeutung, die man seinen biologischen Wirkungen beimißt, in starker Ausdehnung begriffen. Zu den pharmakologisch und toxikologisch interessantesten Stoffen zählen seine stickstoffhaltigen Verbindungen. Es scheint daher nützlich, die bekannten Stickstoffverbindungen zusammenfassend darzustellen. Andererseits ist die Literatur über die biologischen Wirkungen der Stickstoffverbindungen außerordentlich umfangreich, so daß Angaben über Vorhandensein und Konzentration eines Stoffes bereits begrenzte Hinweise auf die biologische Bedeutung geben.

Trotz dieser zunehmenden Ausweitung unseres Wissens weisen Elmenhorst u. Harke [23] in einer 1968 erschienenen Veröffentlichung darauf hin, daß zur Zeit nur etwa 70% des Stickstoff-Gehaltes von Tabakrauchkondensaten bestimmten Verbindungen zugeordnet werden können. Die Autoren vermuten, daß mindestens 30% des Stickstoffes Verbindungen entstam-

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Notes

The Editor wishes to point out that the pages of the Journal are open for the inclusion of short notes dealing with analytical practice and kindred matters. Such notes are submitted to the Publication Committee in the usual manner.

POISONING BY SODIUM NITRITE

RECORDED cases of poisoning by sodium nitrite are so rare that it is perhaps as well to record the following fatal cases which recently occurred at Middlesbrough, as the result of mistaking sodium nitrite for common salt.

On Sunday, May 31st, 1936, a man, aged 44 years, his wife, aged 42 years, and a daughter of the wife by a former husband, aged 5 years, collapsed and died shortly after eating their dinner.

So far as could be ascertained, the man and woman died approximately within an hour after partaking of the meal. The child was removed to the local infirmary and its stomach washed out, but it died after an illness of about 3 hours. The symptoms observed previous to death were characteristic of this type of poisoning, namely, difficulty in breathing, marked cyanosis, vomiting, and finally stupor and collapse.

The dinner consisted of meat, potatoes, cabbage, Yorkshire pudding, rhubarb tart and custard. A salt-cellar on the table contained an upper layer of a salt of a faint yellow colour and a lower layer of white salt similar in appearance to prepared table salt.

A basin was subsequently found containing about 4 ounces of salt similar in appearance to that of the upper layer in the salt-cellar. The contents of the basin and the upper layer in the salt-cellar were found to be sodium nitrite of commercial purity containing 98 per cent. of the pure salt.

The composition of the lower layer in the salt-cellar corresponded with that of prepared table salt containing 98·2 per cent. of sodium chloride.

The following amounts of nitrites (as sodium nitrite) were found in the organs, etc., received for examination:

	Sodium nitrite g.
Man's stomach	4·275
Woman's stomach	1·284
Child's stomach (washed out previous to death) ..	0·005
Child's vomit	1·047

The unconsumed portions of the foodstuffs forming the meal contained the following percentages of nitrites (as sodium nitrite).

	Sodium nitrite Per Cent.
Yorkshire pudding	4·50
Potatoes	0·75
Cabbage	6·50
Joint of meat (outer layer)	0·15
Joint of meat (interior)	0·015
Rhubarb tart	Nil
Custard	0·015

It was impossible to form a definite idea of the quantity of sodium nitrite taken by any of the three persons, as, unfortunately, neither the vomit of the man nor of the woman, nor the contents of the child's stomach previous to washing

out were retained for examination, but the quantity must have been appreciably in excess of the amounts subsequently recovered from the organs, etc.

No definite evidence could be obtained as to the origin of the sodium nitrite, but the man was employed at the Billingham Factory of the Imperial Chemical Industries, Ltd., and had access to the sodium nitrite plant at those works.

A sample of the sodium nitrite found in the house was examined by Mr. W. C. Hughes—chief analyst at the factory referred to—who stated that its composition was similar to that of the product made by his firm, and that was as far as it was possible to go in the matter.

The method used for the determination of the nitrite-content of the specimens examined was extraction with water, precipitation of the proteins with basic lead acetate, addition of potassium iodide, and titration of the liberated iodine with sodium thiosulphate in an atmosphere of carbon dioxide; the Griess alpha-naphthylamine and sulphanilic acid colorimetric method was also used. A. SCHOLES

Note.—A case of poisoning by nitrite is recorded in THE ANALYST, 1936, 614.—EDITOR.

THE DETECTION OF NITRITES

A REAGENT consisting of dimethylaniline and sulphanilic acid in equi-molecular proportions has been found useful for the detection of nitrites. It is prepared by dissolving 1 g. of dimethylaniline and 1·5 g. of sulphanilic acid in 100 ml. of $N/2$ hydrochloric acid.

In applying the test 2 drops of the reagent are added to about 10 ml. of the solution under examination; in the presence of a nitrite a red colour is produced. Alternatively, the test can be carried out with one drop of the solution on a filter-paper.

Very dilute solutions should be left to stand for ten minutes to allow the colour to develop, and it is advisable to use rather more of the reagent (1 ml.). A solution containing 0·05 mg. of NO_2 in 50 ml. (1 in 1,000,000) will develop a colour in 10 minutes, which is perceptible if the tube is compared with a similar tube containing no nitrite.

The colour is due to the formation of methyl orange, which is turned red by the acid present. The sulphanilic acid is diazotised by the nitrite, and the product immediately couples with the dimethylaniline.

With more concentrated solutions (exceeding 1 in 100,000) a yellow colour is first produced, and this, on standing, changes to orange and ultimately to red. This appears to be due to the interaction of nitrous acid and dimethylaniline, with the formation of a yellow nitroso-compound, *p*-nitroso-dimethylaniline. It should be noted, however, that the mixture of dimethylaniline with sulphanilic acid is much more sensitive than dimethylaniline alone, the limits being approximately 1 in 1,000,000 and 1 in 100,000, respectively.

The formation of a yellow colour in more concentrated solutions can be avoided by adding the two reagents separately. If sulphanilic acid alone is first added, followed after a short interval by a solution of dimethylaniline in $N/2$ hydrochloric acid, a pink colour is produced.

The test can be applied colorimetrically in Nessler glasses for the determination of the approximate concentration of very dilute solutions of nitrites—ranging from 1 in 1,000,000 to 1 in 100,000. As, however, the intensity of colour depends upon time as well as upon concentration, it is essential that the standard colours should be produced as nearly as possible simultaneously with that in the solution under examination.

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COMMENTS ON NITRITE POISONING

(Über Nitritvergiftungen)

Author: G. Schrader

Source: Deutsche Zeitschrift für die Gesamte Gerichtliche Medizin, Vol. 32, 1940, P. 391 - 397.

The nitrous acid and its salts (nitrites) produce severe blood poisons involving the conversion of the blood pigment into methemoglobin or hematin (heme). Poisonings in this case are relatively rare. Occasionally they occur when such medical processes are applied like radioscopy. Abnormal fermentations developing in the intestinal canal, nitrite is able to form from the otherwise harmless opaque meal bismuth subnitrate through reduction processes, and to give rise to ~~intoxications~~ cases of poisoning (Starkenstein, Rost, Pohl). Another quite unusual medical poisoning was induced through Vasoklin tablets. Ten of these had been swallowed by a 2 1/2 year-old child. Each one contains, beside theobromine, 0.053 g of Ca nitrite, so that the child had taken about 0.53 g of nitrite. Death occurred within 2 to 3 hours. Before this event, nothing of a harmful or even lethal action of Valoklin had been known (Lochte and Putschar).

Beside these extremely rare cases of medical poisoning, industrial poisonings by nitrites are reported every now and then in the literature. These are nitrites used in dye works, in metal and rubber industries, etc., for technical purposes. Apart from general data (Flury-Zangger, F. Reuter), more precise descriptions concerning such cases, especially lethal poisonings of this sort, cannot be found in the literature, so that they seem to be of slight importance from the standpoint of industrial pathology.

The food trade, especially the salting of meats, plays the main role in the development of nitrite poisonings. Previously, the salt used in this process was added with a dangerous amount of nitrite in many cases, since through reduction of hemoglobin, nitrite imparts to the meat a pleasant bright red color. This hazardous use of nitrites was recently restricted in most countries.

In Germany, a 1934 legal provision allows only 0.5 to 0.6 kg of Na nitrite for 100 kg of salt to be used (this salt consisting mainly of halite with addition of K nitrate and sugar. This low nitrite content involves as a rule no harful concentration in the salted material. Nevertheless, injuries have become known on repeated occasions. F. Reuter explains this as follows: In many cases, according to his experience, inadmissible nitrite concentrations are still taking place in salt mixtures, despite the interdiction to use nitrite, or in spite of the legal restriction imposed. On the other hand it is maintained (Behre), except for an epidemic following the ingestion of chopped meat (which had been treated with a considerable amount of K nitrate), other poisonings with nitrite salted meat cannot be ascertained. Where really more serious ~~intoxications~~ cases of poisoning were observed now and then, it must have been cases of unfortunate confusions, such as Na nitrite being used instead of the harmless nitrate (K nitrate) or of kitchen salt. Such mistakes normally comes to one's mind, since the nitrites (KNO_2 and $NaNO_2$) often carry the designation of "salt" and lood fairly like common salt. Behre, however, points still to another possible explanation: the bacterial reduction of nitrate residues (from K nitrate), contained in the salted meat, to nitrite, when the meat was not quite faultless when salting was started. The chemical processes taking place in the salting brine are still unexplained. There is certainly a reduction of K nitrate to nitrite, especially since the brine is used for an average period of two months which may occasionally extend up to two years, without any testing of the nitrite content. Under the bacterial effects previously mentioned, the usually harmless low nitrite content is apt to increase suddenly from 2 - 5 mg to 80 mg per 100 g of meat. Since amounts of 200 -300 mg of nitrite can already involve toxic consequences in man, one can see in such seldom occurring reactions a cause for many explainable poisoning occurrences (Behre).

Unprofessional handling in the manufacture of pickling salt can at times induce injury through the chronic action of nitrite. This is brought out by an observation made by Schulz relatively to a 52 year old supervisor of pickling in a meat packing plant (more exactly: a large butchery business). For several years this man had to manufacture a nitrite salt. Each time, 900 g of nitrite were thoroughly mixed in the proper machine with 3 hundredweight of rock salt. Although a considerable amount of dust was developed, no protective mask was used.

Increasing cardiac troubles, starting with hypotony, finally resulted in disablement. The causal connection with the long years of nitrite dust inhalation was recognized.

Several of our own observations of nitrite poisonings, which were difficult to explain, induced us to conduct a closer study of the clinical and anatomic processes, and to undertake some animal tests. As to the clinical aspect, the nitrite action is characterized for once by a decrease of the muscular tone in the arterial wall and by corresponding vasodilatation. With severe poisoning there are also a conversation of the blood pigment into methemoglobin and a blood decomposition. From this follow, in case of oral ingestion, in addition to gastroenteritic processes, quite a noticeable cyanosis especially of the mucous membranes and a dark to grey-blue coloration of the skin (methemoglobin). According to the extent of the blood transformation, respiratory troubles, morbid sleepness up to deep unconsciousness follow. Death is usually marked by suffocation or respiratory paralysis.

The autopsy revealed no characteristic pathological and anatomic changes. The detection of methemoglobin cannot be performed successfully on the corpse blood, since post mortem reduction processes may cause the methemoglobin to disappear again. It remains detectable for the longest time in the skin vessels (F. Reuter).

The present observations concern a group poisoning (8 persons) and two individual lethal poisonings. We will now describe these cases in details, since the poisoning pattern and also the genesis of such rate intoxications are not well known, so that it is often difficult to explain them.

1. Mass Poisoning in a Butchery Business: For the noon meal a milk soup was cooked in the sausage shop, since the kitchen range was out of order. A flour remnant mixed with baking powder, was used for this purpose. Immediately after the ingestion of this soup, 8 persons in age ranging from 1 1/2 to 80 years suffered from violent poisoning symptoms. Only a young butcher was spared; he had refused the soup because of its unpleasant taste. The poisoning symptoms consisted in nausea, retching and actual vomiting. Several exhibited an unusual dark blue coloration of the lips and a cyanosis of the face. The poisoning symptoms in some developed in collapse and in unconsciousness. The youngest, a 1 1/2 year old girl, died at the hospital about 2 1/2 hours later; the others recovered after 1 to 2 days.

The initial suspicion of a bacterial food poisoning had to be abandoned because of the negative results of the bacteriological investigation. The chemical investigation (conducted here by Dr. Klauer in the chemical section of the Institute) revealed Na nitrite in remnants of the milk soup as well as in the pumped out stomach content of one of the diseased; thus nitrite poisoning was ascertained. As tests were netative. The obduction of the child, performed somewhere else, revealed no characteristic changes. Histologically, there were moderate pulmonary oedema and considerably capillary congestion of the lungs, as well as a moderate fatty degeneration of the liver cells. On the other hand, the myocardium was free fon any changes. Chemically a nitrite detection in the corpse parts, especially in the stomach content, was unsuccessful. It must be borne in mind, however, that repeated vomiting and intense gastric lavages had probably eliminated the poison to a large extent, and that small poison remnants had decomposed before being caught. The spectroscopic examination of the corpse blood for methemoglobin was negative. Nevertheless, a lethal nitrite poisoning was undeniable, in view of the other results of examination. How the poison reached the soup, could no longer be explained. The subsequent chemical investigation of the components used in preparing the soup were negative also. The explanation nearest to mind had to be that nitrite had been taken for salt in the sausage shop where nitrite was quite probably kept for pickling purposes.

2. Poisoning after Sausage Ingestion: Shortly after sausage ingestion, a 9 year old boy fell ill with repeated and violent vomiting and diarrhoea. Because of the extremely severe poisoning symptoms, immediate hospital admission was necessary, the predominent sign being a grey blue coloration of the face. Considerable restlessness was present. The members felt cold. At times the boy was unconscious. After two days, he died with symptoms of increasing circulatory insufficiency and respiratory paralysis. Bacteriologically, no indication of bacterial food poisoning was found. The obduction, performed in other quarters, revealed no worth noticing changes. Our assumption of nitrite poisoning was confirmed, through the chemical detection of nitrite in the stomach content (Dr. Klauer). On the other hand, the spectroscopic investigation for methomoglobin was negative. Histologically, there was an extreme capillary congestion in the parenchymatous organs. Other symptoms were localized emphysema and slight edema of the pulmonary tissue,

extensive adiposis in the form of fine droplets in the myocardium yet without any inflammatory inclusions, sparse fatty degenerations of the kidneys and of the liver, and also of the germinating centers of the spleen. For lack of any other possibility of explanation, nitrite poisoning had to be admitted in this case. No further specimens of the problematic sausage was available, unfortunately. The entire genesis of this case, however, pointed to the sausage as the source of the poison. It was not possible to determine with certainty how nitrite arrived there.

3. Fatal Poisoning by Drinking Pickling Brine: One afternoon, the 2 1/2 year old daughter of a professional butcher, unattended for a moment, drank an undertermined amount of salt brine consisting of a solution of K nitrate, cane sugar and a so-called pickling fix. It was described by the father as nitrite solution whose chemical examination revealed an amount of 0.86 mg Na nitrite per 1 ccm. About half an hour after this, threatening symptoms developed in the form of violent vomiting and a blue coloration of the lips. Immediately, the child was transported to the hospital. She was already unconscious on her arrival. Her face and members were bluish. One and a half hour later (about 2 to 2 1/2 hour after the ingestion of the brine) death appeared in the form of respiratory paralysis. The autopsy revealed no anatomically ascertainable cause of the death. The mucous membrane of the stomach and (as we later ascertained in parts of the corpse transmitted to us) also the mucous membrane of the upper small intestine exhibited a peculiarly bright red color. Methemoglobin was detected in the blood. On the other hand, no nitrite or nitrate were detectable in the intestine content. One must bear in mind that the low nitrite amounts, as far as they had not been already resorbed into the circulation at the time of the hospitalization, were expelled no doubt by the violent vomiting and by the gastric lavages. Histologically, a maximum vasodilatation with capillary stasis occurred in the heart, lung, liver, kidneys, paranephros and brain. Except for a slight haemorrhageous pulmonary edema, a slight fine-droplet fatty degeneration of the Kupffer's cells in the spleen and a localized fatty degeneration of the liver, no important findings were performed. The myocardium fibers were free from fatty degenerations. The microscopic investigation of the renal tissue for possible methemoglobin was negative.

On the basis of the course of the disease and of the results of investigation as a whole (As tests negative) the child's death had to be ascribed to nitrite poisoning. It was afterwards no longer possible to determine absolutely whether this poisoning was due exclusively to the pickling brine, relatively low in nitrite content, that the child drank, or whether the child had not ingested perhaps a brine of a higher nitrite content which had been diluted before it was confiscated by the Police and was sent to us.

A survey of these different observations with altogether 10 poisonings, among which 3 fatal cases, results in the following important details. Two of these events involving 9 poisonings occurred in meat shops where the dangerousness of nitrite-containing pickling salt or pickling brine was not considered with the necessary care. This is the only to understand how nitrite salt found its way into the food during the preparation of the noon meal in the sausage shop, or how it was possible for a child to ingest a pickling brine left unguarded and containing a risky amount of nitrite. In contrast with these two cases, the third poisoning occurred through sausage ingestion. Like in the observations made by Behre, the genesis must very probably be traced back to a bacterial reduction of nitrate to nitrite as a result of the not quite faultless condition of the sausage ingredients. It is remarkable also that three deaths involved young persons, that is children of 1 1/2, 2 1/2 and 9 years of age. We must leave undecided whether this involved a particular sensitivity of this age range for nitrite poisoning.

All the poisoning cases involved had not been clearly identified when the victims were still living. Despite the prominent and conspicuous cyanosis, due to the formation of methemoglobin, no spectroscopic determination through an examination of the blood had been performed, which to be regretted. It is this important diagnostic determination that must come to mind in similar unclear cases of poisoning, since, as was learnt from our experiences, the chemical nitrite detection in dead body parts is difficult or barely possible, and the spectroscopic determination of methemoglobin in the blood of the corpse - especially if this arrives from outside in a state of decomposition - cannot not always be performed with certainty. During the post mortem reduction processes the methemoglobin progressively disappears from the blood of the dead body (F. Reuter). We were able to confirm this fact

in our experimental animal studies in connection with afore-mentioned poisoning observations. The heart blood of a rabbit, which died from an oral nitrite ingestion, was examined immediately once the animal had been dissected. It contained methemoglobin. After a four day storage at room temperature and with the exclusion of air - in order to prevent later O diffusion - it was no longer possible to detect methemoglobin. On the other hand, with a second rabbit, under similar external conditions, the detection of methemoglobin in the stored blood was possible over a much longer period of time. It was not possible to find out with certainty the external factors to which such differerence of behavior was due.

The post mortem findings and the histological changes after nitrite poisoning are quite uncharacteristic and, diagnostically, can scarcely be evaluated with the required certainty. In the foreground of our observations consistently stood the capillary stasis, which can no doubt be connected with the suffocation processes as a result of the methemoglobin formation. The fatty degeneration of the myocardium, detectable in one case only and with a remarkably protracted course (in contrast with the other two deaths, the end here occurred not before two days had elapsed), must certainly attributed also to the severe and continuous anoxemic state as a result of methemoglobin formation. In animal tests with protracted nitrite poisoning (esophageal sound feeding of rabbits), these changes were not reproducible. Thus, in the expertise of such death, the anatomic investigation will be tantamount actually to the exclusion of other causes of death. The diagnosis "nitrite poisoning" will have to be based on the sum of all external circumstances, the clinical picture, the result of chemical ans spectroscopic examination and finally on the autopsy, the primary problem of As poisoning having to be solved as a differential diagnosis.

Summary:

Ten nitrite poisoning cases, among which 3 deaths, were observed. The majority of them occurred in a butchery business in the form of a mass poisoning involving 8 people. Unprofessional storage of nitrite-containing picling salt or pickling brine was the cause. In a case of poisoning after sausage ingestion, a bacterial reduction of nitrate to nitrite must be assumed as the cause. Death struck only young people ranging from 1 1/2, 2 1/2 and 9 years of age. The clinical

picture was characterized by gastroenteritic processes and complicated cases (to be ascribed to methemoglobin formation). On the basis of the uncharacteristic postmortem result and of the difficult nitrite detection in the dead body parts, the spectroscopic detection of methemoglobin while the victims are still living or this detection in the dead body blood a short time after death is especially important.-

Legt man diese Werte zugrunde, so muß das 3,4 kg schwere Kind bei Annahme der gleichen Wirkung wie bei älteren Kindern erheblich weniger als 6,2 mg Tl/kg, das sind im ganzen 21 mg Tl = 26 mg Thalliumsulfat, aufgenommen haben, denn es hatten sich wie oben bereits erwähnt, außer leichtem vorübergehendem Durchfall keine Krankheitserscheinungen gezeigt, vielmehr war es stets munter geblieben.

Zusammenfassung.

Es wird über einen Mordversuch mit Zeliopaste an einem 4 Wochen alten Säugling berichtet. Der Nachweis der Giftaufnahme wurde geführt durch spektroskopischen Nachweis des Thalliums im Kot und die Feststellung, daß der Kot den gleichen Farbstoff enthielt wie die Zeliopaste. Abgesehen von einem vorübergehenden Durchfall traten bei dem Säugling keine Krankheitserscheinungen auf. Die aufgenommene Thalliummenge wird auf erheblich weniger als 26 mg (Thalliumsulfat) geschätzt.

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(Aus dem Institut für gerichtliche und soziale Medizin der Universität Halle. Direktor: Prof. Dr. *G. Schrader*.)

Über Nitritvergiftungen.

Von

G. Schrader.

Die salpetrige Säure und ihre Salze (Nitrite) stellen heftige Blutgifte dar, unter deren Einwirkung der Blutfarbstoff zu Methämoglobin bzw. Hämatin umgewandelt wird. Vergiftungen damit sind verhältnismäßig seltene Ereignisse. Sie kommen gelegentlich bei *medizinaler Anwendung* (Röntgendurchleuchtung) vor, indem bei abnormen Gärungsvorgängen im Darmkanal aus dem an sich ungiftigen Kontrastmittel Bismut. subnitric. durch Reduktionsvorgänge Nitrit entstehen und zu Intoxikationen Anlaß geben kann (*Starkenstein, Rost, Pohl*). Eine andere ganz ungewöhnliche medizinale Vergiftung ereignete sich durch *Vasoklintabletten*, von denen ein $2^1/_2$jähriges Kind 10 Stück verschluckt hatte. Diese Tabletten enthalten neben Theobromin jeweils 0,053 g Calciumnitrit, so daß das Kind etwa 0,53 g Nitrit eingenommen hatte. Der Tod trat innerhalb von 2—3 Stunden ein. Bis zu diesem Vorfall war von einer schädlichen oder gar tödlichen Wirkung des Vasoklin nichts bekannt (*Lochte* und *Putschar*).

Neben diesen außerordentlich seltenen medizinalen Vergiftungen sind im Schrifttum gelegentlich über *gewerbliche Vergiftungen* mit Nitriten berichtet, die in Färbereien, Metall- und Kautschuk-Industrien u. a. zu technischen Zwecken verwandt werden. Abgesehen von allgemeinen Angaben (*Flury-Zangger, F. Reuter*) sind genauere Schilderungen über entsprechende Vorfälle, insbesondere tödliche Vergiftungen dieser Art, im Schrifttum nicht aufzufinden, so daß die gewerbepathologische Bedeutung gering zu sein scheint.

Die wichtigste Rolle für das Zustandekommen von Nitritvergiftungen spielt das *Nahrungsmittelgewerbe*, und zwar das Pökeln von Fleischwaren. Früher wurde dem Pökelsalz Nitrit in oft bedenklicher Menge beigefügt, da dieses durch Reduktion des Hämoglobins dem Fleisch die schöne hellrote Farbe verleiht. Durch Verbote wurde in den meisten Ländern in neuerer Zeit diese bedenkliche Verwendung von Nitriten eingeschränkt. Bei uns ist gemäß gesetzlicher Verfügung vom Jahre 1934 nur eine Verwendung von 0,5—0,6 kg Natriumnitrit auf 100 kg Pökelsalz (das in der Hauptsache aus Steinsalz mit Zusatz von Salpeter und Zucker besteht) gestattet. Bei diesem geringen Nitritgehalt ist im allgemeinen eine schädliche Anreicherung in der Pökelware nicht zu erwarten. Trotzdem sind mehrfach Schädigungen

bekannt geworden. *F. Reuter* erklärt dies damit, daß seiner Erfahrung nach trotz Verbots der Nitritverwendung bzw. trotz der gesetzlich verfügten Einschränkung doch vielfach unzulässige Nitritkonzentrationen in Pökelsalzgemischen vorkommen. Von anderer Seite (*Behre*) wird dagegen geltend gemacht, daß, abgesehen von einer Massenerkrankung nach Genuß von Hackfleisch (das mit erheblicher Salpetermenge behandelt war), sonstige Vergiftungen an nitritgepökeltem Fleisch nicht sichergestellt seien. Wo wirklich ernstere Intoxikationen gelegentlich beobachtet wurden, dürfte es sich um unglückselige Verwechslungen gehandelt haben, wie von Natriumnitrit mit dem ungiftigen Nitrat (Salpeter) oder mit Kochsalz. Gerade letztgenannte Verwechslungsmöglichkeit liegt insofern nahe, als auch die Nitrite (KNO_2 und $NaNO_2$) vielfach die Bezeichnung „Salz" tragen und dem Kochsalz weitgehend ähnlich sehen. *Behre* weist jedoch noch auf eine andere Erklärungsmöglichkeit hin, nämlich die bakterielle Reduktion von im Pökelfleisch enthaltenen Nitratresten (aus dem Salpeter) zu Nitrit, wenn das Fleisch bei Beginn der Pökelung nicht ganz einwandfrei war. Die chemischen Vorgänge in der Pökellake sind noch ungeklärt. Es findet sicherlich eine Reduktion von Salpeter zu Nitrit statt, zumal die Lake durchschnittlich 2 Monate lang, gelegentlich bis zu 2 Jahren benutzt wird, ohne daß der Nitritgehalt nachgeprüft wird. Der für gewöhnlich ungefährliche geringe Nitritgehalt des Fleisches kann unter den vorgenannten bakteriellen Einflüssen plötzlich von 2—5 mg auf 80 mg pro 100 g Fleisch ansteigen. Da Mengen von 200—300 mg Nitrit schon beim Menschen sich toxisch auswirken können, wird in solchen selten vorkommenden Umsetzungen eine Ursache für manche unerklärbare Vergiftungserscheinung gesehen (*Behre*).

Durch unsachgemäßes Verhalten bei der Herstellung von Pökelsalz kann gelegentlich durch chronische Nitriteinwirkung eine Schädigung zustande kommen. Das geht aus einer Beobachtung von *Schulz* hervor, die einen 52jährigen Pökelmeister in einem großen Fleischereibetrieb betraf. Dieser hatte über mehrere Jahre hin ein Nitritpökelsalz herzustellen. Und zwar wurden jeweils 900 g Nitrit mit 3 Ztr. Steinsalz in einer Mischmaschine durchmischt. Trotz erheblicher Staubentwicklung war ein Maskenschutz nicht angewandt worden. Zunehmende Herzbeschwerden, beginnend mit Hypotonie, führten schließlich zur Berufsunfähigkeit. Der Kausalzusammenhang mit der jahrelangen Nitritstaubeinatmung wurde anerkannt.

Mehrere eigene Beobachtungen von Nitritvergiftungen, deren Aufklärung auf Schwierigkeiten stieß, veranlaßten uns zu näherem Studium der klinischen und anatomischen Erscheinungen sowie zur Vornahme einiger Tierversuche. Was zunächst das klinische Bild anlangt, so ist die Nitritwirkung einmal in einer Senkung des Muskeltonus in der

Arterienwand und entsprechender Gefäßerweiterung gekennzeichnet. Bei schweren Vergiftungen tritt weiter die Umwandlung des Blutfarbstoffes in Methämoglobin sowie ein Blutzerfall hinzu. Daraus folgt bei oraler Aufnahme neben gastroenteritischen Erscheinungen eine besonders auffallende Cyanose speziell der Schleimhäute und dunkle bis graublaue Verfärbung der Haut (Methämoglobin!). Je nach dem Ausmaß der Blutumwandlung folgen Atemstörungen, Schlafsucht bis zu tiefer Bewußtlosigkeit. Der Tod tritt gewöhnlich unter den Zeichen der Erstickung bzw. Atemlähmung ein.

Charakteristische pathologisch-anatomische Veränderungen werden bei der Leichenöffnung nicht gefunden. Auch der Methämoglobinnachweis gelingt am Leichenblut nicht regelmäßig, da infolge von postmortalen Reduktionsvorgängen das Methämoglobin wieder verschwinden kann. Am längsten soll es noch in den Hautgefäßen nachweisbar sein (*F. Reuter*).

Die *hiesigen Beobachtungen* betreffen eine Gruppenvergiftung (mit 8 Personen) und zwei tödliche Einzelvergiftungen. Diese Beobachtungen seien hier näher geschildert, da das Vergiftungsbild und auch die Genese solcher seltenen Intoxikationen wenig bekannt ist und deshalb deren Aufklärung häufig auf Schwierigkeiten stößt.

1. *Massenvergiftung in einem Fleischerhaushalt.* Zum Mittagessen war eine Milchsuppe in der Wurstküche der Fleischerei gekocht worden, da der Küchenherd nicht in Ordnung war. Zur Verwendung kam dabei ein Mehlrest, der mit Backpulver vermengt war. Unmittelbar nach dem Genuß dieser Suppe erkrankten 8 Personen im Alter von $1\frac{1}{2}$—80 Jahren unter heftigen Intoxikationserscheinungen. Nur ein Fleischergeselle blieb verschont, der wegen des schlechten Geschmacks die Suppe zurückgewiesen hatte. Die Vergiftungserscheinungen bestanden in Übelkeit, Brechreiz und Erbrechen. Bei mehreren fiel eine ungewöhnliche dunkelblaue Verfärbung der Lippen und eine Cyanose des Gesichtes auf. Die Vergiftungserscheinungen steigerten sich bei einigen der Erkrankten bis zu Kollaps und Bewußtlosigkeit. Die jüngste Person, ein $1\frac{1}{2}$jähriges Mädchen, verstarb im Krankenhaus etwa $2\frac{1}{2}$ Stunden später, während sich die übrigen Erkrankten nach 1—2 Tagen wieder erholten. Der zunächst aufgetauchte Verdacht einer bakteriellen Nahrungsmittelvergiftung mußte auf Grund des negativen bakteriologischen Untersuchungsergebnisses fallen gelassen werden. Die hiesige chemische Untersuchung (durch Dr. *Klauer* in der chemischen Abteilung des Instituts) ließ in Resten der Milchsuppe sowie in dem ausgeheberten Mageninhalt von einer der erkrankten Personen Natriumnitrit nachweisen, so daß damit eine Nitritvergiftung sichergestellt war. Die Arsenproben fielen negativ aus. Die von anderer Seite durchgeführte Obduktion des Kindes ergab keine charakteristischen Veränd-

rungen. Histologisch fand sich mäßiges Lungenödem und erhebliche capillare Stauung in den Lungen sowie eine mäßige Leberzellverfettung. Der Herzmuskel war dagegen frei von jeglichen Veränderungen. Chemisch gelang an den Leichenteilen, insbesondere am Mageninhalt, ein Nitritnachweis nicht. Dabei muß allerdings berücksichtigt werden, daß durch das wiederholte Erbrechen und intensive Magenspülungen wahrscheinlich das Gift zum größten Teil entfernt war und geringe Giftreste durch Zersetzungsprozesse der Erfassung entzogen wurden. Die spektroskopische Untersuchung des Leichenblutes auf Methämoglobin fiel negativ aus. Trotzdem war an einer tödlichen Nitritvergiftung im Hinblick auf die übrigen Untersuchungsergebnisse nicht zu zweifeln. Auf welche Weise das Gift in die Suppe kam, konnte nicht mehr geklärt werden. Die nachträgliche chemische Untersuchung der bei der Suppenbereitung verwendeten Bestandteile verlief ebenfalls negativ. Die nächstliegende Erklärung dürfte eine Verwechslung von Salz mit Nitrit in der Wurstküche sein, wo höchstwahrscheinlich Nitrit für Pökelzwecke aufbewahrt war.

2. *Vergiftung nach Wurstgenuß.* Kurz nach einem Wurstgenuß erkrankte ein 9jähriger Knabe mit mehrfachem heftigen Erbrechen und Durchfällen. Wegen der außerordentlich schweren Intoxikationserscheinungen wurde alsbald Krankenhausaufnahme erforderlich, wobei eine graublaue Färbung des Gesichtes das hervorstechendste Merkmal darstellte. Es bestand erhebliche Unruhe. Die Gliedmaßen fühlten sich kalt an. Zeitweilig war der Knabe bewußtlos. Nach zweitägigem Verlauf verstarb er unter den Erscheinungen zunehmender Kreislaufschwäche und Atemlähmung. Bakteriologisch fand sich kein Anhaltspunkt für eine bakterielle Lebensmittelvergiftung. Die von anderer Seite durchgeführte Obduktion förderte keine bemerkenswerten Veränderungen zutage. Unsere Vermutung auf Nitritvergiftung wurde durch den chemischen Nitritnachweis im Mageninhalt (Dr. *Klauer*) bestätigt. Die spektroskopische Untersuchung auf Methämoglobin fiel dagegen negativ aus. Histologisch fand sich an den parenchymatösen Organen eine stärkste capilläre Stauung. Ferner am Lungengewebe herdförmiges Emphysem und geringes Ödem; am Herzen umfangreiche feintropfige Muskelfaserverfettungen, jedoch ohne jede entzündliche Einlagerungen; an Niere und Leber spärliche Verfettungen, desgleichen auch in den Keimzentren der Milz. Mangels jeder anderen Erklärungsmöglichkeit mußte auch hier eine Nitritvergiftung angenommen werden. Von der in Frage stehenden Wurst standen leider keine weiteren Proben zur chemischen Überprüfung zur Verfügung. Doch deutete die ganze Genese der Intoxikation auf die Wurst als Giftquelle hin. Wie das Nitrit dort hineingeriet, war nicht sicher zu ergründen.

3. *Tödliche Vergiftung durch Trinken von Pökellauge.* Es handelte sich um die $2^1/_2$jährige Tochter eines Fleischermeisters. Das Kind hatte eines Nachmittags in einem unbeaufsichtigten Augenblick von einer sog. Pökelsole eine nicht näher bestimmte Menge getrunken. Die Pökelsole bestand aus einer Lösung von Salpeter, Rohrzucker und sog. Pökelfix. Sie wurde von dem Vater des Kindes als Nitritlösung bezeichnet. Bei ihrer chemischen Untersuchung fand sich ein Gehalt von 0,86 mg Natriumnitrit pro 1 ccm. Etwa eine halbe Stunde nach diesem Vorfall traten bedrohliche Krankheitserscheinungen in Gestalt von heftigem Erbrechen und Blaufärbung der Lippen auf. Das Kind wurde sofort ins Krankenhaus überführt. Bei seiner Ankunft war es bereits bewußtlos. Gesicht und Gliedmaßen waren bläulich verfärbt. Unter den Zeichen einer Atemlähmung trat $1^1/_2$ Stunden später (etwa 2 bis $2^1/_2$ Stunden nach dem Trinken der Pökellauge) der Tod ein. Bei der von anderer Seite durchgeführten Leichenöffnung war eine anatomisch faßbare Todesursache nicht festzustellen. Die Schleimhaut des Magens und (wie später von uns an den übersandten Leichenteilen festgestellt werden konnte) auch die Schleimhaut des oberen Dünndarms zeigten eine auffallend hellrote Farbe. Im Leichenblut fiel die Untersuchung auf Methämoglobin positiv aus. Dagegen war im Darminhalt weder Nitrit noch Nitrat nachweisbar. Es muß jedoch dabei berücksichtigt werden, daß die geringen Nitritmengen, soweit sie nicht bei der Krankenhausaufnahme bereits in den Kreislauf resorbiert waren, sicherlich durch das heftige Erbrechen und die Magenspülungen herausbefördert wurden. Histologisch fand sich in Herz, Lunge, Leber, Niere, Nebenniere und Gehirn eine maximale Gefäßerweiterung mit capillärer Stase. Abgesehen von geringem hämorrhagischem Lungenödem, einer geringen feintropfigen Verfettung der *Kupffer*schen Sternzellen in der Milz und einer herdförmigen Leberverfettung, wären keine bemerkenswerten Befunde zu erheben. Die Herzmuskelfasern waren frei von Verfettungen. Die mikroskopische Untersuchung des Nierengewebes auf Methämoglobin fiel negativ aus.

Auf Grund des Krankheitsverlaufes und des gesamten Untersuchungsergebnisses (Arsenproben negativ) war der Tod des Kindes auf eine Nitritvergiftung zurückzuführen. Es ließ sich jedoch hinterher nicht mehr einwandfrei klarstellen, ob diese Vergiftung einzig auf die von dem Kinde getrunkene Pökelsole mit ihrem verhältnismäßig schwachen Nitritgehalt zurückzuführen war, oder ob das Kind nicht etwa eine stärker nitrithaltige Pökellauge getrunken hatte, die vor der polizeilichen Beschlagnahmung und Einsendung an uns verdünnt worden war.

Überblickt man diese verschiedenen Beobachtungen mit insgesamt 10 Vergiftungen, darunter 3 tödlichen, so ergeben sich daraus folgende

bemerkenswerte Einzelheiten. 2 dieser Vorfälle mit 9 Vergiftungen ereigneten sich in Fleischereibetrieben, wo die Gefährlichkeit von nitrithaltigem Pökelsalz bzw. Pökelsole nicht mit der nötigen Sorgfalt beachtet war. Nur dadurch ist es verständlich, daß Nitritsalz bei Herstellung der Mittagsmahlzeit in der Wurstküche in die Speise geriet bzw. eine unbeachtet stehen gelassene Pökelsole mit einem nicht unbedenklichen Nitritgehalt von einem Kinde getrunken werden konnte. Abweichend von diesen beiden Vorkommnissen entstand die dritte Vergiftung durch Wurstgenuß. Deren Genese ist, ähnlich wie in den Beobachtungen *Behres*, mit größter Wahrscheinlichkeit auf eine bakterielle Reduktion von Nitrat zu Nitrit infolge nicht ganz einwandfreier Beschaffenheit der Wurstbestandteile zurückzuführen. Weiterhin ist bemerkenswert, daß sämtliche 3 Todesfälle Personen im jugendlichen Alter betrafen, nämlich Kinder von $1^1/_2$, $2^1/_2$ und 9 Jahren. Ob es sich dabei um eine besondere Empfindlichkeit dieser Altersstufen gegenüber Nitritintoxikationen handelt, muß offen gelassen werden.

Sämtliche Vergiftungsfälle waren zu Lebzeiten nicht klar erkannt worden. Trotz der im Vordergrund stehenden auffallenden Cyanose, die auf Methämoglobinbildung zurückzuführen ist, war leider eine *spektroskopische Sicherstellung durch entsprechende Blutuntersuchung noch zu Lebzeiten* der Vergifteten unterblieben. An diese wichtige diagnostische Sicherstellung wird aber bei ähnlich liegenden unklaren Vergiftungsfällen gedacht werden müssen, da, wie unsere Erfahrungen lehrten, der *chemische Nitritnachweis an den Leichenteilen nur schwer oder kaum zu erbringen ist* und auch die spektroskopische Methämoglobinfeststellung am Leichenblut — zumal wenn es bei Einsendung von auswärts zersetzt eintrifft — nicht immer sicher durchgeführt werden kann. Denn unter den postmortalen Reduktionsvorgängen verschwindet das Methämoglobin allmählich aus dem Leichenblut (*F. Reuter*). Diese Erfahrung konnten wir bei unseren *tierexperimentellen Studien* im Anschluß an oben genannte Vergiftungsbeobachtungen bestätigen. Es wurde Herzblut eines Kaninchens, das einer peroralen Nitriteinbringung erlegen war, unmittelbar nach der Sektion des Tieres untersucht und erwies sich als methämoglobinhaltig. Nach 4tägiger Aufbewahrung bei Zimmertemperatur unter Luftabschluß (zur Vermeidung nachträglicher Sauerstoffdiffusion) war Methämoglobin *nicht mehr nachzuweisen*. Bei einem zweiten Kaninchen gelang dagegen unter äußerlich ähnlichen Umständen der Methämoglobinnachweis in dem aufbewahrten Blut noch über weit längere Zeit. Welche äußere Faktoren ein solches unterschiedliches Verhalten bedingen, ließ sich nicht sicher ergründen.

Der Leichenbefund und die histologischen Veränderungen nach Nitritvergiftung sind sehr uncharakteristisch und lassen sich diagnostisch kaum mit der nötigen Sicherheit auswerten. Im Vordergrund unserer Beobachtungen stand durchweg die *capilläre Stase*, die wohl mit den Erstickungsvorgängen infolge der Methämoglobinbildung in Zusammenhang gebracht werden kann. Die *Herzmuskelverfettung*, die nur in einem Fall festzustellen war, und zwar bei auffallend protrahiertem Verlauf (Todeseintritt im Gegensatz zu den beiden anderen Todesfällen erst nach 2 Tagen), ist sicherlich ebenfalls auf den schweren und langanhaltenden anoxämischen Zustand infolge der Methämoglobinbildung zurückzuführen. In *Tierversuchen* mit protrahierter Nitritvergiftung (Schlundsondenfütterung an Kaninchen) ließen sich diese Veränderungen nicht reproduzieren. Somit wird bei der Begutachtung derartiger Todesfälle die anatomische Untersuchung im wesentlichen nur auf den Ausschluß anderweitiger Todesursachen hinauslaufen. Die Diagnose „Nitritvergiftung" wird sich auf einer Zusammenfassung sämtlicher äußerer Umstände, des klinischen Bildes, des chemisch-spektroskopischen Untersuchungsergebisses und schließlich der Leichenöffnung aufbauen müssen, wobei differentialdiagnostisch in erster Linie die Frage einer Arsenvergiftung zu ventilieren ist.

Zusammenfassung. Es wurden 10 Fälle von Nitritvergiftung, darunter 3 Todesfälle, beobachtet. Die Mehrzahl davon ereignete sich in einem Fleischereibetrieb in Form einer Massenvergiftung von 8 Personen. Unsachgemäße Aufbewahrung von nitrithaltigem Pökelsalz bzw. Pökelsole war die Ursache. Bei einer Vergiftung im Anschluß an Wurstgenuß muß eine bakterielle Reduktion von Nitrat zu Nitrit als Ursache angenommen werden. Die Todesfälle betrafen nur jugendliche Personen im Alter von $1^1/_2$, $2^1/_2$ und 9 Jahren. Das klinische Bild war durch gastroenteritische Erscheinungen und auffallende Cyanose (auf Methämoglobinbildung zurückzuführen) gekennzeichnet. Bei dem uncharakteristischen Sektionsbefund und dem schwierigen Nitritnachweis in den Leichenteilen kommt dem spektroskopischen Methämoglobinnachweis zu Lebzeiten oder im Leichenblut kurz nach dem Tode besondere Bedeutung zu.

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Part III: Nitrate and Nitrite

Several quantitative methods are available for determining nitrate and nitrite in food, especially in meat, yet no satisfactory procedure has been devised. Nitrite may be readily and accurately determined; nitrate determinations have shown great variation in results, particularly in cured meat samples.

Quantitative Determination of Nitrate

Analytical methods for the determination of nitrate can be grouped into three general categories: nitration of a phenolic-type component; oxidation of an organic compound by the nitrate ion; and reduction of the nitrate ion to nitrite or ammonium ion.

Nitration of phenolic group.—The phenoldisulfonic acid (1–3) and the 2,4-xylen-1-ol (4) methods that are most commonly used belong to this group. Nitrite, chloride, and carbohydrates interfere in both methods. Furthermore, these methods are cumbersome and tedious. In the 2,4-xylen-1-ol method, the resultant *o*-nitrophenolic chromogene has a low molar absorptivity. Direct analysis by these methods is impossible with mixtures containing low nitrate concentrations. In 1964, Landmann and Worland (5) stated that the 2,4-xylen-1-ol method was subject to systematic errors and large random errors resulting in only fair precision. Recently, Davidek *et al.* (6) used the volatile property of 6-nitro-2,4-xylen-1-ol to eliminate the interfering substances in the determination of nitrate, but this added step is time consuming.

The 3,4-xylen-1-ol method (7–9) also requires the elimination of nitrite and chloride and it too is time consuming. Moreover, the silver sulfate used as a masking agent often contains nitrate. The same criticism applies to the distillation method (10).

Other methods of this type include the chromotropic acid method (11); its modification (12), which uses a silver oxide column to eliminate the interfering formaldehyde; the 4-methylumbelliferone method (13); and the 2,6-xylenol method (14). This last method permits a direct determination since a *p*-nitrophenolic chromogene has a higher molar absorptivity than the *o*-nitrophenolic type. Andrews (15) has improved the 2,6-xylen-1-ol method, and West and Ramachandran (16) proposed further improvements by using masking agents to eliminate potential critical interferences such as chloride, chlorine, iron, and antioxidants. To quantitatively determine nitrate at low levels (down to 10 ppm), Hatcher and Schall (17) isolated the nitrate by thin layer chromatography to eliminate interferences and measured the nitrate by a modified 3,4-xylenol procedure.

Two indirect polarographic methods have recently been proposed; both methods require the absence of nitrite and halogens. Dhont (18) used nitrophenoldisulfonic acid and obtained a well-defined reduction wave. Davidek and Davidková (19) used 2,4-xylenol as a reagent and obtained a reaction product that can be isolated by steam distillation. These analysts (19) stated that the polarographic method was more sensitive than colorimetric methods and that results were linear over a wide range of concentrations.

Oxidation by nitrate.—The determination of nitrates by redox reactions is not selective and is generally inferior to nitration methods (20).

The method based upon the oxidation of

brucine (21, 22) requires a high concentration of sulfuric acid, does not completely oxidize brucine, and requires frequent recalibration because of the inconsistent standard curve; three nitrate standards should be included with each group as a check on the standard curve. Landmann *et al.* (21) studied the method collaboratively and found it very variable and unsatisfactory.

Diphenylamine (23), strychnine and strychnidine (24), salicylic acid (introduced especially for mineral water by Müller and Widemann (20)), and 1-aminopyrene (25) are also used as reagents. The 1-aminopyrene method appears to have greater sensitivity and is simpler than the 2,4- or 2,6-xylenol, brucine, phenoldisulfonic acid, and chromotropic acid methods. In the 1-aminopyrene method, nitrite is eliminated by amidosulfuric acid, and the oxidation products are measured colorimetrically.

Although it may not belong to this group, the method based upon the selective interference of nitrate in the determination of rhenium by α-furyldioxime (26) is highly sensitive and is easily adapted to high speed multi-analysis. Nitrite may interfere to a small extent at high concentrations, probably caused by a partial disproportionation of the nitrous acid to nitrate ion, and it is advantageous to remove large amounts of nitrite ion with excess sulfamic acid, which does not interfere in the reaction. The method using 2-phenylpyridine ketoxime (27) as a lignand is less sensitive than the above method.

Reduction to nitrite or ammonium.—Several methods have been proposed to reduce nitrate to nitrite and to determine nitrite by conventional diazotization and coupling reactions. Microorganisms (28, 29), zinc (30–33), and cadmium (34–36) have been suggested as reductants. Nitrite can be determined colorimetrically as the red azo dye obtained by reaction of sulfanilic acid with 1-naphthylamine (34) or with 1-naphthol (32). The color (Orange I) formed with 1-naphthol is less prone to precipitate than the corresponding 1-naphthylamine derivative and is more stable; 1-naphthol is also less carcinogenic. Benassi (37) used phenol instead of α-naphthylamine to produce a more stable color. Peach (38) proposed resorcinol as a coupling agent. Braunsdorf (39) used Rieglers's reagent, a mixture of 1,4-naphthionic acid and 2-naphthol. Kamm *et al.* (40) proposed a new colorimetric method for nitrite in which 1-naphthylamine is diazotized by nitrite and coupled with excess of the amine; the color is extracted into chloroform, the extract is acidified, and the absorbance is measured at 555 mμ. Another colorimetric procedure for nitrite in meat employs phenothiazine as the chromogenic material (41). Lichtenberg (42) proposed a method in which the nitrite is diazotized with procaine and coupled with thymol to produce a red dye with an absorption maximum at 450 mμ. Lambert and Zitomer (30) used 4-aminophenyltrimethylammonium ion as the diazotizable amine, and *N,N*-dimethyl-1-naphthylamine as the coupling agent. Bogs and Göthe (43) proposed a very sensitive test in which the nitrite is coupled with antipyrene in the presence of potassium dichromate.

A new means to eliminate the nitrite ion for the nitrate determination is the exchange of the diazonium cation onto a nuclear sulfonic ion exchange resin before reduction of the nitrate ion. The blood-red azo dye produced by coupling of diazotized benzidine with an excess of benzidine in glacial acetic acid can also be used to determine nitrite content (44).

Nitrate is reduced to ammonia by ferrous sulfate (45), titanous chloride (46), or chromous sulfate (47). The ammonium ion can be determined titrimetrically or colorimetrically (45).

Quantitative Determination of Nitrite

The use of nitrite in meat preservation has stimulated interest in determining the nitrite content of meat. The ability of nitrite to diazotize is commonly used as the basis for its determination. (These methods were mentioned for the determination of nitrate.)

Kureš and Lát (48) applied polarography directly to aqueous extracts of brine after removal of albumin. Dhont (49) suggested an indirect polarographic method in which the nitrite is first converted to 1-nitroso-2-naphthol. Davídek and Záčková (50) de-

scribed an interesting polarographic method for determining nitrate and nitrite in succession: First, nitrite is eliminated as methyl nitrite, and nitrate is isolated by steam distillation as 6-nitro-2,4-xylenol and determined polarographically; second, nitrite is oxidized with potassium permanganate, and nitrate is determined as before. Nitrite is calculated by difference. In contrast, Hartley and Bly (51) directly determined nitrite by polarography; nitrate interferes, but the appropriate potentials for diffusion current measurement of the nitroso- and nitro-xylenols are sufficiently separated so that a hundred-fold excess of nitro-xylenols can be tolerated without interference.

Colorimetric techniques can also be used (6).

Legal Regulations

Belgium.—Nitrate is detected with diphenylamine; the method is described in *Laboratoriumbuch für den Lebensmittelchemiker*, by W. Diemair, 8th Ed., page 8 (1963). If nitrites are present, they are destroyed with urea in acid solution. For the quantitative determination of nitrate, the method proposed by Henrioul (52) and based on the reaction of nitrite with *m*-diaminobenzene is used. For the qualitative determination, the nitrite ion is detected with starch and zinc iodide.

Germany.—No official methods of analysis exist in food laws for the determination of nitrate and nitrite. The method proposed by Möhler (36) is often used; in principle, this method is the same as that proposed by the International Organization for Standardization (ISO).

Italy.—Nitrites are determined by a colorimetric method based upon the reaction of nitrites with sulfanilic acid and 1-naphthylamine; mercuric chloride is used to clarify a turbid solution. Nitrates are quantitatively determined in absence of nitrites by the brucine method described by Landmann *et al.* (21). Any nitrites present are destroyed by adding acidified methanol and volatilizing the reaction product by a stream of nitrogen (37). The third method used in the presence and/or absence of nitrites is the colorimetric method of Szekely and Riviere (53), which is based upon the reaction of nitrate with diphenylamine and 4,4'-diaminodiphenylsulfone in the presence of a mixture of sulfuric and phosphoric acids.

Luxembourg.—The diphenylamine method is used to determine nitrate. The Griess reagent is used for nitrite determination.

The Netherlands.—Analytical methods for the determination of nitrate and nitrite are specified in food laws. For their qualitative determination, a slightly alkaline solution of the sample is filtered and clarified with mercuric chloride and hydrochloric acid, sodium chloride is added, and the solution undergoes reaction with diphenylamine for nitrate and nitrite, with Griess-Romein reagent for nitrite, and with brucine-sulfuric acid for nitrate.

For the quantitative determination of nitrate, the sample is macerated with water and filtered; the filtrate is treated with silver sulfate and potassium ferrocyanide and again filtered. This filtrate is evaporated, and the residue is treated with phenolsulfuric acid. The color intensity of the ammoniacal solution is measured visually against solutions of known nitrate content. For the quantitative determination of nitrite, a slightly alkaline solution of the sample is filtered, clarified with mercuric chloride-hydrochloric acid, and again filtered. The filtrate is treated with Griess-Romein reagent or a solution of this reagent, and color intensity is measured visually against solutions of known nitrite content.

Scandinavian area.—The Nordisk Metodik Komite for Levnedsmidler recommended a method of analysis for nitrite: Nitrite ion diazotize sulfanilamide in hydrochloric acid medium, and the diazonium salt produces an intensely reddish azo compound with *N*-(1-naphthyl)-ethylenediamine dihydrochloride. The color intensity is measured either visually against a solution of known nitrite content or spectrophotometrically against water at 540 mμ.

United Kingdom.—No official methods are found in food laws. For the nitrate and nitrite determination the Government Laboratory uses the AOAC methods, 10th Ed., page 347 (1965).

United States.—The Association of Official Analytical Chemists (AOAC) proposed a collaborative study of the method of Follett for both nitrate and nitrite and a further study of the proposed modification of the official, first action xylenol method for nitrates. For the determination of nitrite in dry cure mix and pickle mix, an iodometric procedure by Landmann was adopted as official, first action by the AOAC. The General Referee (54) recommended that the method be adopted as official, final action, but Subcommittee C did not recommend final action status. In 1965–1966 the method was further studied on meat. Landmann (55) has abandoned this method.

Summary

The use of nitrate and nitrite as a preservative is restricted to meat and meat products. The nitrate-nitrite content of baby food, especially spinach, received recent attention in connection with the appearance of methemoglobinemia with cyanosis in babies.

A literature review clearly indicates that the direct determination of nitrate (gravimetrically, spectrophotometrically, or colorimetrically) is not very reliable. The colorimetric method of Davidek *et al.* (6) is based on the formation of 6-nitro-2,4-xylenol and its subsequent isolation by steam distillation. In this method, nitrate is determined in a solution that contains only a minor amount of interfering material, but the method is time consuming. More attention should be given to the method based upon the selective interference of nitrate in the determination of rhenium by α-furyldioxime.

Nitrite may be readily and accurately determined by using its diazotization property. In recent literature there is a tendency to prefer the method by which the nitrate is reduced to nitrite with cadmium. In samples containing both nitrate and nitrite, nitrite is directly determined as well as total nitrate-nitrite after reduction; nitrate is calculated by difference. The method proposed by the ISO, based upon the colorimetric determination of nitrite, seems to be a synthesis of many other methods.

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Part IV: Benzoic Acid

Since benzoic acid is a commonly used preservative in food, numerous methods have been published for its analysis. The following is a selected review of these methods.

Isolation

Methods for the isolation of sorbic acid from food (*see* Part II), i.e., steam distillation after acidification, incidental extraction of the steam distillate with an organic solvent, or direct extraction of the food with an organic solvent after acidification of this food are also used to isolate benzoic acid. When both sorbic and benzoic acids are present, they are isolated together. Part IV deals with benzoic acid in the absence of sorbic acid; Part V describes the analysis of benzoic and sorbic acids together.

Steam distillation is used to isolate benzoic acid from wines (1, 2), bread and baked products (3), and mayonnaise, mustard, and margarine (4); however, interfering substances often distill with the acid. Benzoic acid can be isolated by diethyl ether extraction of the steam distillate (4, 5) and possibly subsequent extraction of the ether layer with sodium bicarbonate solution (2).

A method that employs ether extraction of the food after acidification is often used. Chloroform (6–8) and benzene (9) are sometimes used instead of ether. Further purification can be made by extracting the organic layer with an alkaline solution of sodium bicarbonate or ammonium hydroxide. The alkaline solution can be acidified and re-extracted with ether, or it can be evaporated to dryness and the residue acidified, transferred to a volumetric flask, and diluted to a known volume. This procedure is used for jams, jellies, soft drinks, fruit juices, and beverages (10); wines (11, 12); jams (13); meat after digestion with perchloric acid (14, 15); foods in general (6); and milk and yoghurts (16).

Roos and Versnel (17) extracted benzoic acid from margarine and butter with acidified methanol.

Ford (18) isolated benzoic acid from beverages, soft drinks, and fruit bases by ion exchange chromatography on De-Acidite FF anion exchange resin.

1953

Effects of Dietary Nitrite on the Chick: Growth, Liver Vitamin A Stores and Thyroid Weight

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Experimental evidence has been presented showing that dietary nitrate accelerates the depletion of vitamin A from body stores of ruminants (Hatfield et al., '61)[1] and that dietary nitrite or nitrate exerts similar effects on the rat (Smith et al., '61).[2] Welsch et al.[3] observed enlarged thyroid glands in rats consuming a ration containing 2.5% KNO_3. The data reported herein describe the effects of dietary nitrite on growth, vitamin A status and thyroid gland weight of the chick when vitamin A and carotene were fed or when vitamin A was administered by intramuscular injection.

METHODS AND MATERIAL

Experiment 1. One-day-old, White Leghorn cockerels obtained from a commercial hatchery were fed a practical, vitamin A-free ration for one week. After the depletion period, 25 chicks were randomly allotted to each of 12 pens in electrically heated chick batteries, and 6 treatment groups were randomly assigned pens in a replicated block design.

As shown in table 1, treatments were divided into groups A and B. The treatments in group A were applied to 20 chicks per experimental pen. Group B was established by randomly selecting 5 chicks per experimental pen and applying the treatments listed under group B, table 1, for the entire experimental period. The only difference between groups A and B was that chicks used in group B received a high level of vitamin A by intramuscular injection in addition to vitamin A supplied in ration.

The basal ration contained 510 IU of vitamin A/kg and consisted of the following: (expressed as percentage of the total ration), ground wheat, 67.0; soybean meal (45% protein), 22.0; meat scrap (50% protein), 5.5; rapeseed oil, 3.0; defluorinated rock phosphate, 1.0; vitamin premix, 1.0; salt premix, 0.5.

The vitamin premix contributed the following per kilogram of ration: vitamin A, 510 IU; vitamin D_3, 818 ICU; vitamin E, 22 IU; and the following expressed as milligrams per kilogram of ration: riboflavin, 5.5; niacin, 16.5; pantothenic acid, 11; choline, 275; menadione, 1.1; vitamin B_{12}, 0.01; the salt premix supplied the following (expressed as milligrams per kilogram of ration), sodium chloride (iodized), 4,600; manganese, 120; iron, 17; zinc, 11; copper, 6.6.

The vitamin A used in the rations was an accurately assayed gelatin encapsuled material.[4] Nitrite, as potassium nitrite, was premixed with finely ground wheat and a sufficient quantity of this premix was substituted for an equal amount of wheat contained in the basal ration to give the desired level of nitrite.

Feed and water were supplied ad libitum, with feed consumption and body weights being recorded weekly.

A commercial preparation of vitamin A in oil[5] was diluted with corn oil immediately prior to injection. Chicks were injected intramuscularly every second day with 0.1 ml of the corn oil solution. The quantity of vitamin A injected per chick

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[4] Kindly supplied by Chas. Pfizer of Canada Ltd.

[5] Injectable vitamin A kindly supplied by Hoffmann La Roche, Limited.

TABLE 1

Outline of treatments used in experiment 1

Treatment no.	Dietary nitrite, 0.4%	Source of vitamin A[1]		Total vitamin A[1] (oral or oral plus injected)
		Ration	Injected	
		Group A		
1	—	510	0	510
1+N	+	510	0	510
2	—	4080	0	4080
2+N	+	4080	0	4080
3	—	510	3570	4080
3+N	+	510	3570	4080
		Group B		
1	—	510	10,720	11,230
1+N	+	510	10,720	11,230
2	—	4080	10,720	14,800
2+N	+	4080	10,720	14,800
3	—	510	10,720	11,230
3+N	+	510	10,720	11,230

[1] Vitamin A values expressed as international units per kilogram of ration or the equivalent thereof.

TABLE 2

Outline of treatments used in experiment 2

Treatment no.	Dietary nitrite, 0.4%	Source of vitamin A[1]			Total vitamin A[1] (oral or oral plus injected)
		Ration		Injected	
		Vitamin A	Carotene		
1	—	0	0	0	0
1+N	+	0	0	0	0
2_o	—	4080	0	0	4080
2_o+N	+	4080	0	0	4080
3_o	—	16,320	0	0	16,320
3_o+N	+	16,320	0	0	16,320
2_i	—	0	0	4080	4080
2_i+N	+	0	0	4080	4080
3_i	—	0	0	16,320	16,320
3_i+N	+	0	0	16,320	16,320
2_c	—	0	4080	0	4080
2_c+N	+	0	4080	0	4080
3_c	—	0	16,320	0	16,320
3_c+N	+	0	16,320	0	16,320

[1] Vitamin A values expressed as international units per kilogram of ration or the equivalent thereof.

in group A was computed on the basis of the difference between the vitamin A intake of chicks consuming rations containing 4,080 IU of vitamin A/kg (treatments 2 and 2+N) and those consuming the same ration with respect to nitrite but containing only 510 IU of vitamin A/kg (treatments 3 and 3+N). These computations were made at two-day intervals between injections. Sufficient vitamin A was injected into chicks consuming the low vitamin A ration (group A) to equalize vitamin A intake (oral vs. oral plus injected) between each respective treatment pair (2 vs. 3 and 2+N vs. 3+N). The quantity of vitamin A injected into chicks of group B was similarly calculated, except that all chicks were injected with vitamin A equivalent to 10,720 IU/kg of ration consumed.

All chicks not receiving vitamin A by injection were injected with 0.1 ml of corn oil carrier every second day.

Experiment 2. In this trial, chicks of the same type and from the same source as those in experiment 1 were used. Fifteen chicks were randomly allotted to each of 42 experimental pens at one day of age and 14 treatments, as outlined in table 2

were randomly assigned to the pens according to a triplicated block design. The basal ration listed under experiment 1 was used and was identical in composition except vitamin A was not included in the vitamin premix. Feed was supplied ad libitum for the first week of the experiment. A depression in feed intake of nitrite-fed chicks was observed; consequently feed consumption was restricted for the remaining three weeks of experiment 2. The chicks in all treatments were restricted to the quantity of feed consumed per chick in the treatment consuming the least feed. Chicks consuming the nitrite, vitamin A-free ration (1+N) ate the least feed. The quantity of feed allowed the remaining chicks was calculated daily on a per chick basis. Feed restriction was initiated to: (1) study the influence of nitrite on chick performance when feed intake was equalized, and (2) more precisely control the vitamin A intake among treatments to facilitate more valid comparison of the influence of nitrite on vitamin A status of the chick. Body weights were recorded weekly.

The quantities of vitamin A to be injected were computed as described for experiment 1. Carotene levels used in the rations were computed on the basis of a 2:1 conversion ratio by weight of β-carotene to vitamin A. From these computations, levels of dietary β-carotene were derived which theoretically would be approximately equivalent to the two levels of dietary vitamin A. Stabilized synthetic β-carotene[6] was used in this study.

Liver samples were taken from three chicks per experimental pen after 4 weeks of the experiment for analysis as described under experiment 1. At the same time, thyroid glands were removed from the sacrificed birds and weighed.

RESULTS

The data from experiment 1 (table 3) show that dietary nitrite significantly decreased weight gain ($P \leq 0.05$) and generally reduced efficiency of feed utilization by the chicks regardless of vitamin A level or method of administration. Accompanying this reduction in weight gain was a marked drop in feed consumption. An attempt to relate these observations more precisely by covariance analysis was precluded because the regression coefficients of weight gain on feed consumption for the nitrite and non-nitrite treatments were significantly different.

[6] β-Carotene kindly supplied by Hoffmann La Roche, Limited.

TABLE 3

Influence of nitrite on weight gain, feed conversion, liver vitamin A and thyroid gland weight of chicks, experiment 1

Treatment no.	Weight[1] gain	Feed[2] conversion	Liver vitamin A	Thyroid gland wt	Mortality
	gm		*μg/gm fresh wt*	*mg/100 gm body wt*	%
			Group A		
1	194±13[3]	2.10±0.04	2.96±0.08	8.80±1.16	10
1+N	116±2	2.76±0.18	1.14±0.49	15.70±0.30	44
2	223±4	1.86±0.03	31.58±1.55	6.86±1.25	2
2+N	125±2	2.32±0.06	2.00±0.27	13.88±1.40	8
3	184±22	1.89±0.02	13.68±0.17	7.90±0.22	8
3+N	126±6	2.17±0.07	5.90±2.18	9.62±0.47	10
			Group B		
1	207±17	—	36.32±6.28	8.66±1.53	0
1+N	123±13	—	34.32±4.13	10.91±2.16	0
2	228±11	—	49.52±4.86	7.30±0.97	0
2+N	132±8	—	42.30±8.21	8.12±0.86	0
3	197±5	—	46.85±4.55	8.33±0.56	0
3+N	121±14	—	39.76±2.14	9.47±0.97	0

[1] Weight gain per cockerel from zero to 4 weeks of age.
[2] Grams of feed required per gram of gain. Weight gain of cockerels from groups A and B were pooled to derive at total weight gain per experimental pen. Total gain was used to compute feed conversion.
[3] Mean ± SE.

A marked influence of dietary nitrite on liver level of vitamin A of chicks in group A occurred. Whether the higher level of vitamin A was fed (treatment 2+N) or intramuscularly injected (treatment 3+N) nitrite significantly reduced liver vitamin A level ($P \leq 0.05$) as compared with that of chicks not receiving nitrite (treatments 2 and 3).

The liver vitamin A data of treatments 2 and 3, group A, also indicate that chicks receiving vitamin A by injection did not utilize the vitamin as well, in terms of liver storage, as chicks receiving vitamin A as part of the ration. However, this had little effect on interpretation of these data since nitrite reduced liver vitamin A levels of chicks receiving the vitamin by either method to essentially the same level (treatments 2+N and 3+N).

In contrast with the liver vitamin A data of group A, the liver vitamin A level of chicks in group B, which received injections of massive doses of vitamin A, was not markedly influenced by dietary nitrite. Evidently this level of injected vitamin A greatly surpassed the quantity of vitamin A that was being lost for liver storage under the influence of nitrite, and as a result, vitamin A levels of the liver were increased.

The continued growth depression caused by nitrite in group B, even though liver vitamin A levels were high, suggests that the low liver vitamin A levels induced by nitrite in group A were a secondary effect of nitrite and not necessarily the cause of or correlated with reduced growth. It was obvious that the depression in weight gain induced by nitrite in groups A and B was of similar magnitude, and this depression in growth persisted even though liver vitamin A levels were high (group B). Exact comparison of liver vitamin A levels between chicks consuming nitrite and those not receiving nitrite may be questioned because of differences in feed intake, which led to differences in oral vitamin A intake. However, these differences were small compared with the differences observed in liver vitamin A levels of chicks receiving appreciable quantities of vitamin A either orally or *per os* plus injection.

The feeding of nitrite significantly increased thyroid gland weight of chicks in group A ($P \leq 0.05$) except when vitamin A was injected. Nitrite-fed chicks receiving vitamin A by injection (treatment 3+N) exhibited enlarged thyroid glands compared with non-nitrite fed chicks (treatments 1, 2 and 3). However, these were significantly smaller than the thyroid glands of nitrite fed chicks receiving vitamin A orally (treatment 1+N and 2+N) ($P \leq 0.05$). This indicates that vitamin A administered by injection partially overcame the effect of nitrite on the thyroid gland whereby hypertrophy was induced. On the other hand, an equivalent level of oral vitamin A failed to counteract this effect. This phenomenon was further substantiated by the weight of the thyroid glands of chicks in group B. These chicks received massive doses of vitamin A by injection and exhibited thyroid glands which had undergone little, if any, hypertrophy.

The percentage of mortality (table 3) was high when nitrite accompanied the low vitamin A level (treatment 1+N) but was reduced when 4,080 IU of vitamin A/kg of ration or the equivalent thereof accompanied dietary nitrite. Mortality was most severe during the first 10 days of the experiment with relatively few losses encountered thereafter. All chicks receiving nitrite, and especially those simultaneously receiving the low level of vitamin A exhibited anorexia, mild ataxia and dyspnea. These chicks were pale and quickly became exhausted when excited. At no time during the experiment were any specific symptoms of vitamin A deficiency observed during antemortem or postmortem examination. Supplemental vitamin A partially overcame the acute toxicity of dietary nitrite in group A and massive doses of vitamin A completely prevented mortality in group B.

In experiment 2, when daily feed consumption by all groups was equalized, differences between weight gain (1 to 4 weeks of age) of chicks fed nitrite and those receiving no nitrite were not observed except where vitamin A-free rations were fed (table 4). Chicks consuming the nitrite, vitamin A-free ration (treatment

TABLE 4

Influence of nitrite on weight gain, liver vitamin A and carotene, and thyroid gland weight of chicks, experiment 2

Treatment[1] no.	Weight[2] gain	Liver vitamin A	Liver carotene	Thyroid gland wt	Mortality
	gm	*μg/gm fresh wt*	*μg/gm fresh wt*	*mg/100 gm body wt*	%
1	72±3[3]	0.88±0.27	1.05±0.19	7.67±0.65	7
1+N	51±3	0.19±0.14	1.14±0.07	16.97±2.62	33
2_o	88±2	24.13±7.20	1.03±0.07	6.66±0.89	0
2_o+N	86±2	1.26±0.34	1.14±0.15	13.59±2.83	7
3_o	94±6	87.25±4.67	1.16±0.21	5.67±0.88	0
3_o+N	80±3	15.87±5.98	1.05±0.50	10.21±0.78	16
2_i	91±1	35.39±7.70	1.47±0.10	8.13±0.59	9
2_i+N	85±2	8.20±1.78	1.27±0.13	12.98±1.20	2
3_i	85±4	69.80±4.27	1.54±0.14	6.74±0.91	11
3_i+N	86±1	55.58±5.78	1.16±0.10	10.92±0.92	2
2_c	92±3	4.55±2.03	1.41±0.17	5.93±1.03	4
2_c+N	79±6	1.35±1.27	1.05±0.27	12.31±2.55	13
3_c	92±6	36.66±4.31	3.05±0.25	6.32±1.08	4
3_c+N	85±6	2.55±0.49	1.04±0.15	10.01±2.55	2

[1] The subscript used after each treatment number denotes the following: (o) vitamin A-fed, (i) vitamin A injected and (c) carotene fed and was the vitamin A source.
[2] Weight gain per cockerel from 1 to 4 weeks of age.
[3] Mean ± SE.

1+N) gained significantly less ($P \leq 0.05$) than all other treatment groups although average feed consumed per chick was the same. The consistent improvement in weight gain when a source of vitamin A was administered to chicks fed nitrite as compared with chicks fed the nitrite, vitamin A-free ration, indicates that vitamin A or β-carotene largely overcame the growth depressing effect of nitrite when feed intake was equalized. Although nitrite generally depressed weight gain regardless of level or source of vitamin A, the magnitude of depression was not large in most instances and within treatment pairs (3_i+N vs. 3_i, etc.) was not consistent. The data show that vitamin A, given as part of the ration or injected, and ration carotene were essentially equal in overcoming the growth depressing effect of nitrite under the conditions of this experiment.

Liver vitamin A data (table 4) demonstrate the influence of dietary nitrite, whereby storage of vitamin A was markedly reduced, with the exception of where the high level of vitamin A was injected (1+N). These data support those of experiment 1. It was also demonstrated that nitrite exerted nearly the same effect on liver vitamin A level when β-carotene was the source of vitamin A for the chick as when vitamin A was administered. The results of experiment 2 also show that a high level of injected vitamin A would markedly increase liver vitamin A in the presence of dietary nitrite, and that this occurred only with injected vitamin A. On the other hand, an equivalent quantity of vitamin A administered in the ration failed to increase liver vitamin A to the same extent. This would suggest an increase in destruction of ration vitamin A prior to absorption due to nitrite or to interference by nitrite in absorption of vitamin A from the digestive tract, or both.

Although only obvious when high levels of β-carotene were fed (3_c and 3_c+N), the effect of dietary nitrite on storage of carotene in the liver was parallel to its effect on vitamin A storage. Nitrite significantly decreased ($P \leq 0.05$) liver carotene level of chicks in treatment 3_c+N as compared with 3_c.

Dietary nitrite caused a consistent enlargement of the thyroid gland (table 4). As in the case with weight gain, this effect was most severe with the vitamin A-free ration (treatment 1+N). With each successively higher level of vitamin A or β-carotene consumed, thyroid gland enlargement became less. Again vitamin A, fed or injected, and ration β-carotene were essentially equal in this respect. Degree of thyroid enlargement, although not completely consistent, was generally inversely related

to weight gain during the three-week experimental period.

Early mortality due to feeding nitrite was observed in experiment 2. Nitrite induced the most severe losses when chicks consumed the vitamin A-free ration (1+N). As in experiment 1, vitamin A or β-carotene partially alleviated acute nitrite toxicity although this influence was not completely consistent in all treatments. Specific vitamin A deficiency symptoms were not observed in any treatment.

DISCUSSION

Inhibition of growth by dietary nitrite or nitrate has been reported with cattle,[7] swine[8] and rats (Smith et al., '61).[9] The data reported herein describe similar effects of dietary nitrite on the chick under ad libitum feeding conditions. However, when feed intake was equalized among nitrite-fed and chicks not fed nitrite, growth was not greatly influenced by nitrite, except in chicks that did not receive vitamin A or vitamin A precursor. This suggests that nitrite depressed growth primarily by reducing feed consumption of chicks receiving supplemental vitamin A. Weinchethal et al.[10] reported reduced feed consumption by cattle receiving 1% of $NaNO_3$ in the ration and Hale et al. ('62) observed similar results in cattle when 1% KNO_3 was fed.

Muhrer et al. ('55) reported symptoms of vitamin A deficiency in cattle consuming nitrate and O'Dell et al.[11] reported that a diet containing 0.3% of KNO_3 caused xerophthalmia and reduced liver vitamin A level in rats. Smith et al. ('61) presented data showing that a ration containing 0.8% of KNO_3 reduced liver vitamin A levels and growth of rats, and this "chronic nitrite toxicity" could be largely overcome by supplementing the ration with vitamin A or carotene. In the present study the chicks did not exhibit any specific vitamin A deficiency symptoms when fed 0.4% of nitrite for 4 weeks. However, giving supplemental vitamin A to nitrite-fed chicks largely overcame the mortality caused by nitrite when low levels of vitamin A were fed, but failed to alleviate the growth depressing effect of nitrite under ad libitum feeding conditions. In contrast, when feed intake was equalized, supplemental vitamin A or β-carotene almost completely overcame the growth depressing effect of nitrite. These results strongly suggest that nitrite inhibits growth of the chick primarily by decreasing feed consumption.

Nitrite also accelerated the loss of injected vitamin A from the chicks as determined by the quantity stored in liver. However, chick growth and liver vitamin A data of both experiments failed to show that reduction in liver vitamin A levels caused by nitrite, was the major reason for growth depression. In the case when nitrite accompanied the vitamin A-free ration, growth was most severely depressed. On the other hand, marked increases in liver stores induced by massive doses of vitamin A injected intramuscularly failed to overcome the growth depressing effect of nitrite in experiment 1.

The liver vitamin A levels in experiment 2 are of interest from the standpoint of the effect of dietary nitrite on utilization of ration vitamin A. In both instances where equivalent levels of vitamin A were administered either as part of the ration or by injection, liver vitamin A levels of nitrite-fed chicks receiving ration vitamin A were appreciably lower than in chicks receiving vitamin A by injection. This was particularly true of chicks receiving high levels of vitamin A. These results suggest that nitrite either accelerated the destruction of vitamin A in the ration or decreased absorption of vitamin A from the digestive tract, or both, thereby, decreasing utilization of ration vitamin A as compared with that given by injection. Under practical circumstances this would be an important mechanism by which dietary nitrite could adversely affect the vitamin A status of animals. Nitrite also decreased liver vitamin A level by either interfering with the storage mechanism or accelerating loss of vitamin A from the

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[9] See footnote 2.

[10] See footnote 7.

[11] See footnote 2.

liver, or both. This was shown by the relative liver vitamin A levels of chicks receiving vitamin A by injection with or without dietary nitrite. Nitrite appreciably decreased liver vitamin A level of the injected chicks as compared with chicks not receiving nitrite. However, in the case of high levels of injected vitamin A, nitrite did not decrease liver levels as much as in chicks fed a comparable level of vitamin A.

Nitrite consistently caused enlargement of the thyroid gland when low levels of vitamin A (510 IU/kg of ration or less) were given. Bloomfield et al. ('61) reported that dietary nitrate adversely influenced iodine metabolism by the rat and sheep, thereby, suggesting interference with thyroid gland function. Welsch et al.[12] observed enlarged thyroid glands in rats consuming a ration containing 2.5% of KNO_3. The results of the present investigation indicate that nitrite exerted a goitrogen-like influence on the thyroid gland of the chick. The present data on chicks also illustrate that the highest percentage of mortality and marked growth depression caused by nitrite was accompanied by the most severe thyroid gland hypertrophy. However, under ad libitum feeding conditions, the same degree of growth depression was apparent even though massive doses of vitamin A partially overcame the thyroid gland hypertrophy induced by nitrite. Under conditions of equalized feed intake, degree of thyroid gland enlargement was inversely related to the level of vitamin A or carotene supplementation. This illustrates that thyroid gland hypertrophy due to dietary nitrite was alleviated by vitamin A supplementation, but that degree of thyroid enlargement was not necessarily related to the growth of nitrite-fed chicks.

The data presented suggest that dietary nitrite depresses growth of chicks primarily by reducing feed consumption. The effect of nitrite on liver vitamin A depletion and thyroid gland hypertrophy can be partially alleviated by supplementing the ration with high levels of vitamin A or carotene, or by injecting vitamin A intramuscularly. However, overcoming these effects of dietary nitrite will not alleviate growth inhibition when chicks are supplied with feed ad libitum. This suggests that the underlying mechanism of the influence of nitrite on feed intake and consequent growth of the chick is located in some other physiological processes than those in which vitamin A or the thyroid gland or both, are critically involved.

SUMMARY

Experiments were conducted to determine the effect of 0.4% dietary nitrite (as potassium nitrite) on growth, vitamin A status and thyroid gland weight of chicks. Nitrite depressed growth under ad libitum feeding conditions regardless of whether vitamin A was administered orally or by intramuscular injection. However, when feed intake between nitrite and non-nitrite fed chicks was equalized a depression in growth was observed only in chicks receiving the vitamin A-free ration containing nitrite. Liver vitamin A levels were low in chicks fed the various levels of vitamin A or carotene along with nitrite. However, when high levels of vitamin A were injected, which were equivalent to levels fed, liver vitamin A concentration was relatively high in nitrite-fed chicks. Thyroid gland hypertrophy was observed in chicks receiving nitrite and was largely overcome by intramuscular injection of massive does of vitamin A. Nitrite increased chick mortality rate but specific vitamin A deficiency symptoms were not observed. The data suggest that nitrite depresses growth of chicks primarily by reducing feed consumption. In addition, these data indicate that the influence of nitrite on feed intake and consequent growth of the chick resides in some other physiological processes than those in which either vitamin A or the thyroid gland, or both, are critically involved.

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CHROM. 4831

Gas–liquid chromatographic determination of dimethylnitrosamine as dimethylnitramine at picogram levels

In view of the extreme carcinogenicity of some of the nitrosamines in different animal species[1,2] and their reported occurrence in foodstuffs[3–6] considerable interest has developed, during the past few years, in chromatographic and other procedures for the detection of these compounds[7–13]. Polarographic[14], thin-layer (TLC) and gas–liquid chromatographic (GLC) methods have been used for their detection in foods with varying degree of success[7–13]. The polarographic method is only sensitive to 1 p.p.m. levels and it lacks specificity[12,13]. The TLC and GLC methods are more sensitive and specific than the polarographic technique but they require a large sample size (0.2–1 kg), and the clean-up steps are quite laborious and lengthy. These methods are unsuitable for analyzing small amounts of biological samples that are encountered during the metabolic studies of nitrosamines in laboratory animals. There is a need, therefore, for a more sensitive method of detecting these compounds.

An attempt to increase the sensitivity of the method by reducing the nitrosamines to hydrazines, prior to GLC, has proven unsuccessful[8]. The detection limit of the hydrazines was no better than that of the nitrosamines. During the course of our investigation we have observed that dimethylnitramine (DMNA), the oxidation product of dimethylnitrosamine (DMN), is extremely sensitive to electron capture detection, and this technique can be used to detect DMN at minute quantities. This communication reports the results of our findings.

Experimental

Reagents and solvents. All reagents were of analytical grade. Ethyl acetate, methylene chloride, *n*-pentane and *n*-hexane were distilled in all-glass apparatus.

Preparation of DMNA. Crystalline DMNA was prepared by nitrolysis of N,N-dimethylformamide according to the method of ROBSON[15]. The product was recrystallized from diethyl ether and *n*-pentane, and the final material had a m.p. of 52° (literature value, 54–56°). The IR spectrum (KBr pellet) showed peaks at 1470, 1437, 1385 and 1310 cm^{-1} indicating the presence of a nitro group. The compound was dissolved in ethyl acetate to give a concentration of 1 mg/ml and subsequent dilutions were carried out in *n*-pentane.

Conversion of DMN to DMNA. The method of EMMONS AND FERRIS[16] was used with minor modifications. About 1–5 μg of DMN in 2–10 μl of methylene chloride was added to 9 ml of trifluoroacetic acid and 50% hydrogen peroxide mixture (5:4) and the solution was allowed to stand at room temperature for 12–24 h. The mixture was poured on 10–15 g ice, made alkaline (pH 10–11) by careful addition of 30–40 ml 20% potassium carbonate, and extracted with two 50 ml portions of methylene chloride. The methylene chloride extract was dried over anhydrous sodium sulfate, filtered, and concentrated to *ca.* 5 ml by evaporating on a steam bath. The concentrated extract was quantitatively transferred into a glass-stoppered graduated test tube and 1 ml *n*-hexane was added. The solution was then concentrated to 0.2 ml on a hot water bath in a stream of nitrogen (care should be taken to avoid complete drying).

The solution was made up to 5–10 ml with *n*-pentane and a 1–4 μl aliquot was used for GLC analysis.

Isolation of DMN from nitrite-treated fish. The sample was prepared and extracted as described by us earlier[17]. The only difference was that all the concentration steps were carried out by evaporation through Snyder columns (macro and micro) instead of in a stream of nitrogen. The spot corresponding to DMN on the TLC plate was eluted with methylene chloride using a micro-Soxhlet apparatus. About 1 ml water was added to the extract and the mixture heated on a water bath (Snyder column) until all the methylene chloride was driven off. The aqueous solution was cooled to room temperature and the DMN present in the solution was converted to DMNA by the method described above. The preparation was passed through an alumina column (Woelm basic, cationotropic, activity grade 1; 2.5 cm × 1 cm diameter) and the interfering materials were removed by washing with 50 ml *n*-pentane. Finally, the adsorbed DMNA was eluted with 100 ml diethyl ether, the eluate concentrated to 0.5 ml, and a 0.2μl aliquot used for GLC analysis.

GLC analysis. A Varian Aerograph gas chromatograph, Model 1200, equipped with an electron capture detector (^{3}H) and a 1 mV recorder was used. Conditions:

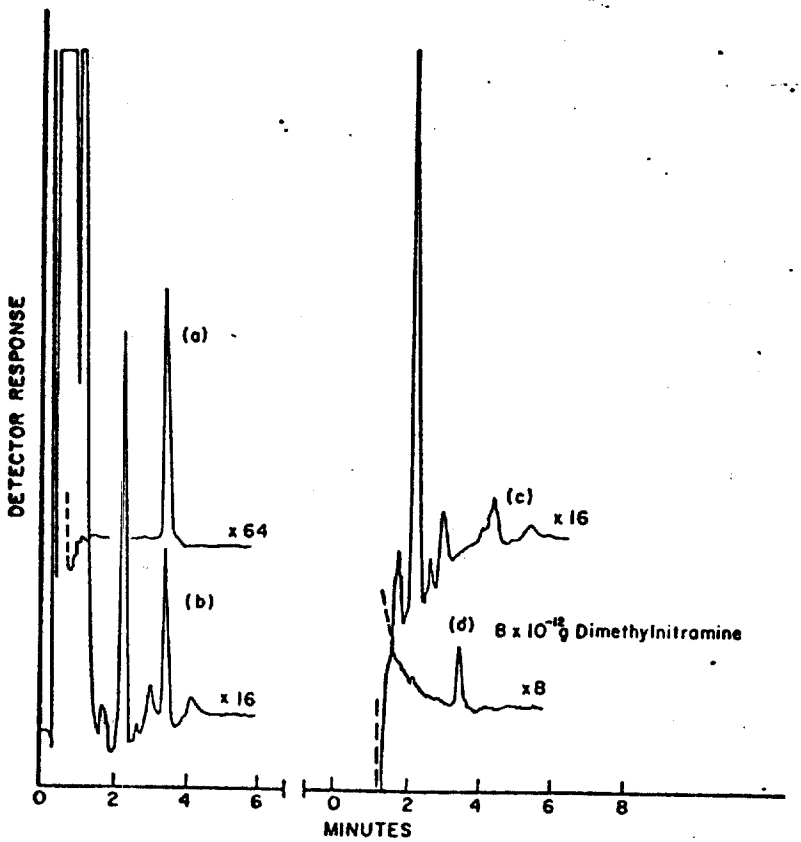

Fig. 1. GLC diagrams: (a) DMNA from nitrite-treated smoked hake (after clean-up through the alumina column), (b) DMNA prepared from DMN by pertrifluoroacetic acid oxidation, (c) pertrifluoroacetic acid oxidation blank and (d) DMNA prepared by nitrolysis of dimethylformamide. Range setting, 1; attenuator setting as shown in the diagrams. For other conditions see text.

10% Carbowax 20
stainless-steel colu
ml/min.

Results and discuss

The use of an
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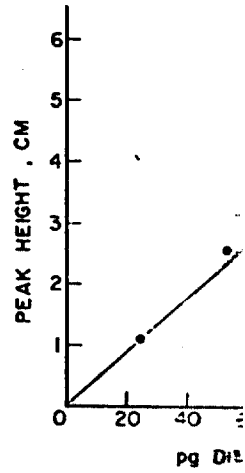

Fig. 2. Standard curve

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at room temperatu
5 μg DMN was pre
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Thus far, we h
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10% Carbowax 20 M on 60–80 mesh chromosorb W (HMDS treated), 6 ft. × 1/8 in. stainless-steel column; column 152°, injector 225°, detector 210°, nitrogen flow 24 ml/min.

Results and discussion

The use of an electron capture detector made the GLC method very sensitive; about 8 pg of DMNA (or 16 pg DMN) could be detected under the conditions used (Fig. 1). This is about a thousand times more sensitive than the detection limit of nitrosamines by the hydrogen flame detector[12,17]. The peak heights were proportional to the amount of DMNA at least up to 100 pg (Fig. 2). A blank carried out through all the steps did not show the corresponding peak for DMNA (Fig. 1). The DMNA prepared by nitrolysis of dimethylformamide was undistinguishable from that prepared by pertrifluoroacetic acid oxidation of DMN.

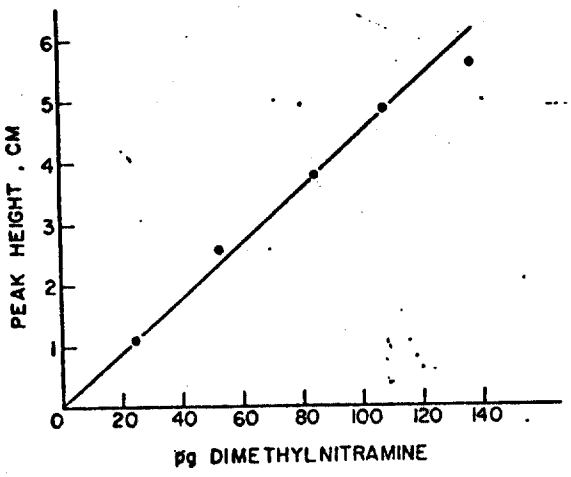

Fig. 2. Standard curve for DMNA.

The percentage conversion of DMN to DMNA varied with the conditions used but the results were reproducible. About 60% of the theoretical yield was obtained when the reaction mixture was allowed to stand for 24 h. An overnight reaction period at room temperature gave about 50% yield. The amount of DMNA formed from 5 μg DMN was proportional to that obtained from a 1 μg sample. The use of 90% hydrogen peroxide would be expected to produce a higher yield[16]. Due to relative unavailability of 90% hydrogen peroxide and the hazard involved in its use no attempt was made to use the concentrated reagent.

Thus far, we have shown that DMN can be oxidized to DMNA on a micro scale and detected by GLC at picogram levels. A great deal of work remains to be carried out before it can be directly applied to food extracts. Proper clean-up procedures need to be developed and applied to different foods. However, the technique, as it stands now, can be used to confirm the identity of nitrosamines isolated from foods by the existing procedures. To demonstrate this, we isolated DMN from a sample of nitrite-

treated smoked hake which was found to contain DMN in our previous studies[17]. A strong peak corresponding to DMNA (Fig. 1) thus confirms the identity of DMN.

Although we have only used the technique for the determination of DMN, it is anticipated that other volatile nitrosamines could be similarly converted to the nitramines and detected by GLC. It is hoped that the extreme sensitivity of the technique will be useful for studying the metabolism of various nitrosamines as well as for detecting their presence in the environment.

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530

CHROM. 4846

Separation of carcinogenic nitrosamines on Sephadex LH-20

Chromatographic separations are well-established methods for the analysis of N-nitroso compounds. Thin-layer chromatography on silica gel or alumina[1] and a variety of gas chromatographic methods have been successfully utilised for the separation and identification of these substances[2]. During our investigations of suitable methods for trace analysis of N-nitroso compounds[3-5] we examined whether gel chromatography could be employed as a separation technique for nitrosamines and possibly also for the removal of interfering contaminants.

Materials and m

All nitrosa
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A wheat flo
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(rotatory) and the
phase was evapo
adsorbed residue
50 ml were collec

Sephadex L
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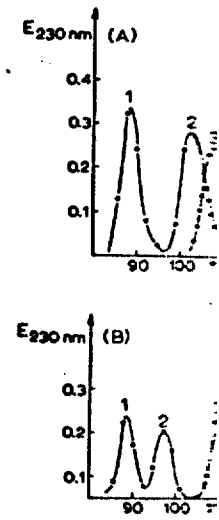

Fig. 1. Gel chromat
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nitrosamine; 2 = 2
1 = 10.5 μg dimet
nitrosamine; 4 = 5
di-n-hexylnitrosam

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was performed at
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using a Zeiss DM

Results and disc

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an efficient sepa

Materials and methods

All nitrosamines investigated were synthesised in our laboratory and purified by known methods; their purity was established by a gas chromatographic examination. Fifty percent aqueous methanol, analytical grade, was the solvent system of choice even for highly lipophilic N-nitroso compounds such as di-*n*-hexylnitrosamine.

A wheat flour concentrate was prepared by extraction of 1000 g of commercial wheat flour with dichloromethane in a Soxhlet for 12 h. The solvent was evaporated (rotatory) and the residue was partitioned in *n*-heptane–acetonitrile[3]. The acetonitrile phase was evaporated together with 100 g of alumina (neutral). The alumina with the adsorbed residue was filled in a column and eluted with 50% aqueous methanol; 50 ml were collected.

Sephadex LH-20 was allowed to swell for 12 h in the solvent and was then packed into a 1 × 100 cm column. The gel was washed with the solvent until the eluate showed no absorption in UV light at 230 nm. To prevent photolytic cleavage

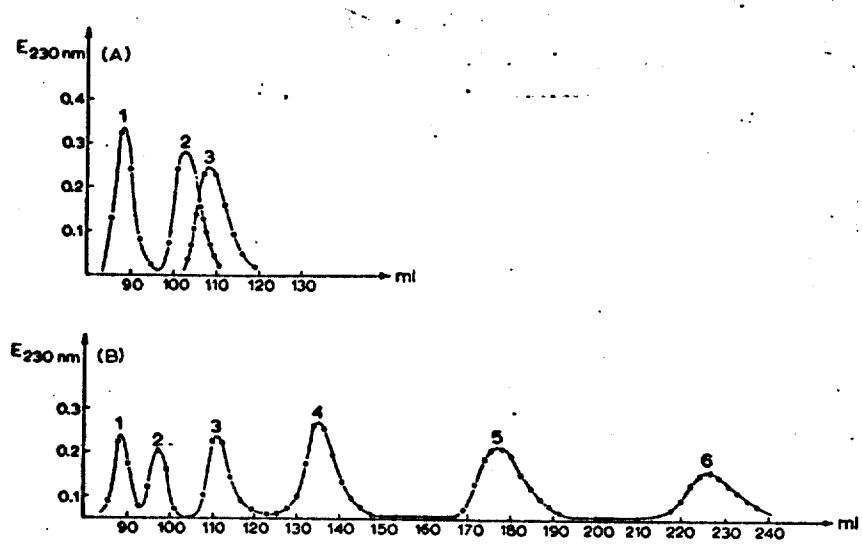

Fig. 1. Gel chromatography on Sephadex LH-20. Column, Sephadex LH-20, 100 × 100 mm; eluent, methanol–water (1 : 1), 4.3 ml/h; sample volume, 0.5 ml. (A) 1 = 19.4 μg methylethylnitrosamine; 2 = 26.4 μg methylbutylnitrosamine; 3 = 25.3 μg methylpentylnitrosamine. (B) 1 = 10.5 μg dimethylnitrosamine; 2 = 14 μg diethylnitrosamine; 3 = 21.5 μg di-*n*-propylnitrosamine; 4 = 50 μg di-*n*-butylnitrosamine; 5 = 74 μg di-*n*-pentylnitrosamine; 6 = 52 μg di-*n*-hexylnitrosamine.

of nitrosamines during chromatography, the column was screened from direct light with black paper. The compounds were applied in a sample volume of 0.5 ml, elution was performed at a rate of 4.3 ml/h and fractions of 2 or 3 ml were collected. Nitrosamine concentrations in the fractions were determined by UV spectroscopy at 230–235 nm, using a Zeiss DMR 21 recording spectrophotometer.

Results and discussion

As can be seen from Fig. 1, gel chromatography on Sephadex LH-20 resulted in an efficient separation of all six symmetrical di-*n*-alkylnitrosamines under the con-

ditions described above. Dimethylnitrosamine was eluted first, followed by diethylnitrosamine, di-*n*-propylnitrosamine and the higher homologues.

The elution sequence of the methylalkylnitrosamine homologues follows the same principle; thus methylethylnitrosamine is eluted before methylbutylnitrosamine which is followed by methylpentylnitrosamine. A similar separation as that shown in Fig. 1 was obtained with the application of 140–270 μg of symmetrical dialkylnitrosamines using a 2 × 100 cm column.

Whilst dialkylnitrosamine homologues which differ in two methylene groups were invariably well separated, the separation of nitrosamines with a difference of only one methylene group was incomplete (methylbutyl- from methylpentylnitrosamine).

The above results indicate that the separation is not based upon a gel filtration process. Apparently the elution behaviour of symmetrical and unsymmetrical nitrosamines under these conditions is determined primarily by absorption effects. The affinity of a given nitrosamine to the Sephadex LH-20 matrix increases with its lipophilic character and can be correlated with the distribution coefficient of the compounds in the system *n*-hexane–aqueous buffer (ref. 6, p. 115).

The usefulness of gel chromatography for the separation of micro-amounts of nitrosamines from biological materials was also investigated. A similar mixture of nitrosamines was dissolved in a 2-ml aliquot of the wheat flour concentrate and applied on the 2 × 100 cm column. Over the whole elution range in which nitrosamines were to be expected, UV-absorbing contaminants from the extract prevented the determination of the substances in the eluate by UV spectroscopy.

Although mixtures of pure nitrosamines can be successfully separated by gel chromatography on Sephadex LH-20, the method as described cannot be used for the separation of nitrosamines from interfering biological contaminants.

This investigation was carried out under contract with the International Agency for Research on Cancer.

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FOOD ADDITIVES

Diethylnitrosamine and Other N-Nitrosamines in Foods

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Dimethyl-, diethyl-, di-*n*-propyl, and other nitrosamines in foods are determined semiquantitatively as follows: (*a*) initial extraction of the nitrosamines from foods into suitable solvents, (*b*) isolation of the nitrosamines from other interfering materials by steam distillation and ion exchange cleanup, (*c*) detection and semiquantitative estimation by TLC, and (*d*) final identification by GLC analysis of the separated compounds. The detection limit for dimethylnitrosamine in foods was 0.15 ppm and for diethyl- and di-*n*-propylnitrosamines, 50 ppb. The method has been successfully applied to wheat flour, fish, cheese, and spinach samples.

The discovery by Magee and Barnes (1) in 1956 of the carcinogenicity of dimethylnitrosamine (DMN) excited much interest and many reports have appeared concerning the carcinogenic, mutagenic, and toxic characters of various nitrosamines (2, 3). Nitrosamines have been shown to be carcinogenic in a wide range of species (4) and to be capable of inducing tumors at different sites of the body. In spite of their rapid decomposition in the living systems, some of them can cause cancer after only one dose (5).

Nitrosamines are easily formed from secondary amines and nitrous acid or nitrous gases (N_2O_3, NO_2, etc.). Under certain conditions even tertiary amines react with nitrous acid and give rise to the formation of N-nitrosamines through a sequence of complex reactions (6, 7). Since many foods contain large amounts of amines and small amounts of nitrite it is possible that nitrosamines occur in foods. The presence of 4-methylnitrosaminobenzaldehyde in an edible mushroom (8), DMN in herring meal (9, 10), N-nitrosopiperidine (NP) and other N-nitrosamines in tobacco (11, 12), and diethylnitrosamine (DEN) in wheat flour (12, 13), has been reported. In view of the highly carcinogenic properties of many of these compounds, sensitive and specific methods for their analysis in foods are highly desirable. In this paper a procedure is reported whereby the presence of nitrosamines in various foods can be detected at very low levels and with more certainty than previous methods (12, 13).

METHOD

Apparatus and Reagents

(a) *Gas chromatograph.*—Varian Aerograph Model 1200, with hydrogen flame detector and 1 mv recorder. GLC conditions: (*1*) 6% Reoplex 400 on 60–80 mesh Chromosorb W, 1/8" × 5' stainless steel column, nitrogen flow 29 ml/min, column temperature 115°C, range setting 10, and attenuation 8; (*2*) 5% SE-30 on acid-washed, dimethylchlorosilane-treated 60–80 mesh Chromosorb W, 1/8" × 5' stainless steel column, nitrogen flow 27 ml/min, column temperature 48°C, range setting 10, and attenuation 1.

(b) *Explosion-proof blender,*

This paper was presented at the 82nd Annual Meeting of the Association of Official Analytical Chemists, Oct. 14–17, 1968, at Washington, D.C.

(c) *Ultraviolet lamp.*—Short wavelength (λ_{max}. 253 mμ), without filter (General Electric Co.).

(d) *Silica gel-MN.*—Kieselgel G-HR (Macherey, Nagel and Co., Germany).

(e) *Solvents.*—Glass-distilled. Peroxide-free diethyl ether was prepared by the conventional method (14).

(f) *Ion exchange resin.*—Amberlite CG-120, strongly acidic cation exchange resin, sodium form (or Rexyn 101). Soak in water 24 hr and wash thoroughly with water.

(g) *Phosphate buffer mixture.*—Mix 5.0 ml KH_2PO_4 (1*M*) with 20.0 ml Na_2HPO_4 (0.5*M*); pH of mixture when diluted to ca 225 ml should be 7–7.5.

(h) *Spray reagents.*—(*1*) 1.5% diphenylamine in ethanol and 0.1% palladium (II) chloride in 0.2% NaCl solution (4:1); store solutions at 4°C and mix just before use. (*2*) *Griess reagent:* 1% sulfanilic acid and 0.1% α-naphthylamine; prepare both solutions in 30% acetic acid. Store at 4°C and mix just before use (1:1). (*3*) 0.3% ninhydrin in ethanol containing 2% pyridine. For details of spray reagent *see* Preussman *et al.* (15) and Kröller (12).

(i) *Nitrosamine standards.*—DMN, DEN, di-*n*-propylnitrosamine (DPN), di-*n*-butylnitrosamine (DBN), nitrosopiperidine (NP), and phenylbenzylnitrosamine (PBN) were purchased commercially. All other nitrosamines (Table 1) were prepared by heating aqueous solution (pH 2–2.5) of corresponding secondary amines (500 mg) with 1 g $NaNO_2$ 20 min at 37°C. The unreacted amines were removed by passing reaction mixtures (buffered at pH 4.5 with sodium acetate) through Amberlite CG-120 column (1 × 4 cm). The nitrosamines were isolated by extracting the Na_2CO_3-saturated column eluate with ether. For some compounds (e.g., dinitrosopiperazine, methylbenzylnitrosamine, and dibenzylnitrosamine), the nitrosamines separated from the reaction mixture either as a crystalline solid or an oily liquid. Several dilutions (10 and 1 mg/ml and 50 μg/ml) of working standards were prepared in peroxide-free dry ether and were kept in glass-stoppered tubes. To prevent concentration of the solutions by evaporation and decomposition of the compounds, the tubes were placed inside a closed jar containing some ether and stored at −10°C.

Extraction

(a) *Wheat flour.*—Extract 100 g flour with 500 ml peroxide-free ether 3–4 hr in 6 × 18 cm Soxhlet thimble. Add ca 100 ml water to ether extract and distill mixture in current of steam. Continue distillation until ca 200 ml aqueous distillate is collected. Add 20 g K_2CO_3 to aqueous distillate, dissolve by stirring, and transfer solution to separatory funnel. Extract solution twice with equal volumes (ca 210 ml) of ether by shaking vigorously 8–10 min. Collect ether extracts in 1 L glass-stoppered Erlenmeyer flask and add ca 20 g anhydrous Na_2SO_4. Let extract stand over Na_2SO_4 30–45 min, swirling mixture occasionally. Filter extract through Whatman No. 1 paper, wash Na_2SO_4 on filter paper with 20–30 ml anhydrous ether, and concentrate combined filtrate and washing to ca 20 ml on hot water bath in stream of nitrogen. Quantitatively transfer concentrated extract (ca 10 ml at a time) into 15 ml graduated glass-stoppered test tube and continue evaporation until final volume is 0.5 ml.

(b) *Cheese or fish.*—Cut 100 g sample into small pieces and homogenize in blender 5 min with 400 ml CH_2Cl_2 and 5 g K_2CO_3. Filter homogenate through glass wool and collect filtrate in 1 L distilling flask. Wash residue on glass wool with ca 50 ml CH_2Cl_2 and add washing to main filtrate. Add 25.0 ml phosphate buffer mixture and ca 200 ml water to filtrate and remove CH_2Cl_2 by heating flask in boiling water bath. After all CH_2Cl_2 is distilled, continue heating for 10–15 min. Discard CH_2Cl_2 distillate.

Connect distilling flask to steam generator and distill mixture in current of steam until ca 200 ml distillate is collected (gently heat distilling flask to prevent condensation of steam). Add 1 ml 5% sodium acetate (buffer) to distillate, adjust pH to 4.5, and pass solution (flow rate, 1 ml/min) through Amberlite resin column (2.5 × 2.5 cm). Wash column with ca 10 ml water and add washing to column effluent. Make mixture alkaline by adding 20 g K_2CO_3 and extract it twice with 210 ml of CH_2Cl_2. Dry CH_2Cl_2 extract over anhydrous Na_2SO_4 and then concentrate to 0.5 ml, as described in (a).

(c) *Spinach.*—Proceed as outlined in (b), except omit preliminary distillation of CH_2Cl_2. Distill food extract in current of steam and extract aqueous distillate with CH_2Cl_2 (use CH_2Cl_2 from the steam distillate) at alkaline pH. (As amines were found to be absent in steam distillate, the ion exchange cleanup procedure was also omitted.)

Thin Layer Chromatography

Prepare TLC plates in conventional manner with 250 μ layer of silica gel G-HR. Spot 1–5 μg* for standard nitrosamines and 100–200 μl aliquots for food extracts. Spot standards and food extracts in duplicate on both halves of plate (divided by imaginary vertical line). After de-

Table 1. R_f Values of different nitrosamines in various solvent systems[a]

Nitrosamines[b]	*n*-Hexane: Diethyl Ether: CH_2Cl_2 4:3:2	5:7:10	10:3:2
DMN	0.32	0.35	0.10
DEN	0.57	0.55	0.24
DPN	0.77	0.63	0.38
DBN	0.87	0.72	0.46
PBN	0.93	0.74	0.53
NP	0.56	0.52	0.23
N-Methyl-N'-nitrosopiperazine	0.09	0.28	0.64
Dinitrosopiperazine	0.09	0.31	0
Methyl-*n*-propylnitrosamine	0.48	0.76	0.27
Diisopropylnitrosamine	0.66	0.89	0.41
Ethyl-*n*-propylnitrosamine	0.61	0.87	0.37
Methyl-*n*-butylnitrosamine	0.57	0.83	0.33
Dibenzylnitrosamine	0.83	0.92	0.63
Methylbenzylnitrosamine	0.58	0.85	0.32

[a] *See* Preussmann *et al.* (15).
[b] *See* text for abbreviations.

veloping plate in appropriate solvents (Table 1) spray left side of plate with Griess reagent and right side with 30% acetic acid. Irradiate plate under UV light (without filter) 5–10 min. (Red-violet spots should appear on left side within this period.) Heat plate 5–10 min in jet of warm air to evaporate acetic acid. Spray right side of plate with ninhydrin reagent and heat plate at 80°C. (Red-purple spots should start to appear within 10–15 min.) Estimate amount of nitrosamines (if present) in food extracts from relative intensities of colored spots compared to those of standards. The R_f values of various nitrosamines are given in Table 1.

Gas Chromatography

If food extract gives positive test for a nitrosamine by TLC with both the reagents (Griess and ninhydrin), spot remaining extract as streak on TLC plate and develop chromatogram in suitable solvent. (Prepare fresh extract if necessary.) Scrape off band with same R_f as unknown spot and elute with water-saturated ether or CH_2Cl_2. Dry eluate over anhydrous Na_2SO_4 and concentrate to 0.3–0.5 ml. Use 2–4 μl aliquot of concentrated eluate for GLC analysis. Diagrams of typical chromatograms are presented in Fig. 1.

Results and Discussion

By a combination of the TLC and GLC techniques described, the presence of DMN, DEN, DPN, and possibly other nitrosamines in foods can be detected and identified at very low levels (0.05–0.15 ppm). The method of Marquardt and Hedler (13) for determining DEN in wheat flour uses a crude ether extract for GLC and TLC analyses; we have experienced many difficulties with their technique because of the presence of interfering substances in the extract. Although Thewlis (16) used the same method and reported that DEN (at the 1 ppm level) added to the concentrated flour extract could be detected by GLC analysis, no data on the per cent recovery of the added DEN were presented. Moreover, by using the Marquardt-Hedler method (13) we were unable to get a satisfactory recovery (<30%) of DEN when we added it to the flour (before extraction) instead of to the extract.

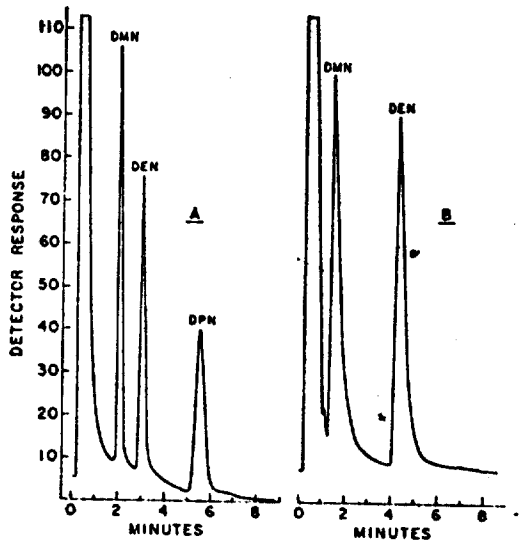

FIG. 1—GLC separation of DMN, DEN, and DPN standards: A, 2 μg each of DMN, DEN, and DPN in 2 μl ether, using GLC conditions 1; B, 1 μg each of DMN and DEN, using GLC conditions 2.

In a recent communication, Kröller (12) reported a method similar to the one described in this paper. However, we found it unsuitable for the analysis of nitrosamines in cheeses and fish (herring). Large amounts of amines, present in both fish and cheese, and some yellow pigments in cheese interfered with the identification and location of the nitrosamines on TLC plates. The addition of K_2CO_3 during the extraction of food samples, in our method, gave a more effective extraction of nitrosamines into CH_2Cl_2, and the treatment of the steam-distillate with the Amberlite resin helped to remove the interfering amines. The nitrosamines passed through the resin column unabsorbed under our conditions. Also, preliminary heating of the food extracts for 10–15 min before steam distillation coagulated proteinaceous and other materials that caused excessive foaming during steam distillation. If foaming still persisted, it could be easily controlled by adding 5 g USP beeswax to the final food extract during the steam distillation process.

Yellow spots that occasionally appeared on thin layer plates of cheese and fish extracts were separated from most of the nitrosamines by developing the plates in either of the following solvents: (*a*) dry diethyl ether, (*b*) dry CH_2Cl_2, and (*c*) benzene:glacial acetic acid: water (10:10:5; the top layer was used). The last solvent was found to be most suitable for this purpose. As the nitrosamines are unstable in acidic pH, especially when exposed to UV light, TLC plates should, preferably, be developed in the dark when using solvent (*c*). Trace amounts of acetic acid present in the thin layer band eluate should also be removed by extracting with 10 ml 10% K_2CO_3 solution before the eluate is subjected to GLC analysis.

The minimum detection limit for different nitrosamines in food depended on the volatility of the compounds. For example, DEN and DPN could be detected when added to different foods at 50 ppb levels whereas the more volatile DMN could consistently be detected only at or above 0.15 ppm levels. The approximate per cent recoveries of the nitrosamines added to different foods are presented in Table 2. These data are based on the TLC analysis of food extracts, using the Griess reagent and the ninhydrin solution as the location reagents. We could isolate DMN, DEN, and DPN from various foods spiked with suitable levels of different nitrosamines (0.3 ppm for DMN and 0.1 ppm for DEN and DPN) and identify them by GLC.

Thin layer chromatograms of food extracts, when sprayed with the diphenylamine reagent, gave many false-positive spots for nitrosamines. The compounds responsible for these spots failed to give color reactions with either of the other two reagents. Therefore, it seems that the ninhydrin spray technique of Kröller (12) and the Griess reagent are more specific for nitrosamines than the diphenylamine spray. The replacement of 10% HCl with 30% acetic acid, with our modification, gave better and faster results than were obtained with the use of 10% HCl. The minimum detection limits for various nitrosamine standards were about 0.3–0.6 μg with Griess reagent and slightly better (about 30% more sensitive) with the ninhydrin reagent. The

Table 2. Recovery of DMN, DEN, and DPN added to various food samples (100 g)

Food	Compd Added	Amount Added, μg	Approx. % Rec.[a]
Cheese (Havarti)	DMN	15	65
	DEN	5	65
	DPN	5	65
Cheese (Cheddar)	DEN	10	65
	DPN	20	50
Fish (herring)	DMN	20	70
	DEN	6	70
	DPN	5	70
	DEN	10	80
	DPN	20	80
	DEN	25	80
	DEN	10	80
Corned beef[b]	DMN	5	40
	DEN	5	50
	DPN	5	50
Canned luncheon meat[b]	DMN	5	30
	DEN	5	70
	DPN	5	70
Wheat flour (white)	DEN	10	65
	DEN	400	90
	DEN	30	90
	DEN	25	65
Whole-wheat flour	DEN	10	40
Dark rye flour	DEN	10	60

[a] Results based on visual estimation of spots on TLC plates. See *Method* for details.

[b] Method same as for cheese. The GLC technique has not yet been applied to the meat samples.

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mpd ed	Amount Added, µg	Approx. % Rec.[a]
MN	15	65
EN	5	65
N	5	65
N	10	65
N	20	50
MN	20	70
N	6	70
N	5	70
EN	10	80
PN	20	80
N	25	80
N	10	80
MN	5	40
EN	5	50
N	5	50
MN	5	30
EN	5	70
PN	5	70
EN	10	65
N	400	90
EN	30	90
EN	25	65
EN	10	40
EN	10	60

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spots were usually stable for 30 min and then either gradually disappeared or were overshadowed by the background color.

Griess and ninhydrin reagents are generally used for detecting nitrous acid and compounds containing primary or secondary amino groups, respectively (12). Since N-nitrosamines produce secondary amines and nitrous acid upon irradiation with UV light at an acidic pH, a positive reaction to both the reagents can be considered specific for N-nitrosamines. Other compounds such as organic nitrites and nitroso compounds (17–19) and free amines, although rarely present in the final food extracts, give positive tests with these reagents usually without irradiation with UV light. However, these interfering compounds can be easily differentiated from the N-nitrosamines by the fact that the latter group of compounds gives positive tests with both the reagents only after irradiation with UV lights. It must be emphasized that a positive reaction with one spray reagent should not be used as a proof of the presence of a nitrosamine in food extracts.

We have examined ten samples of flour (both whole wheat and white), six of Cheddar cheese, eight of frozen or pickled herring, and four of spinach. Most of the samples did not contain DMN, DEN, and DPN. In a few cases, the extracts from Cheddar cheese and herring gave positive spots for nitrosamines (with the Griess reagent) but the amounts of the available unknown compounds and samples of food were too small to try other spray reagents and therefore could not be definitely identified. Heating the flour samples 6 hr at 150–160°C did not produce any new nitrosamine.

It is possible that amines and nitrite in food might produce nitrosamines in the stomach under suitable conditions. To test this hypothesis we have incubated aqueous extracts of stomach contents from rabbits, dogs, and rats with diethylamine and $NaNO_2$ and isolated DEN from the reaction mixture in each case. The amount of DEN produced increased with decrease in pH of the stomach extracts. For example, gastric juices from rabbits (pH 1–2.5) produced more DEN than the gastric juices from rats (pH 4–5). Our observations confirm the finding of Sander (20) who has recently shown that nitrosamines can be formed *in vitro* from secondary amines and nitrite when mixed together in human gastric juice. We have also fed $NaNO_2$ and diethylamine (enclosed in separate capsules) to rabbits and cats; after 20 min the animals were killed and the gastric juices were examined. In each case we could demonstrate the presence of DEN in the gastric juice, thus establishing the fact that the nitrosation reaction also takes place *in vivo*. The full details of this work will be published later.

Similarly, nitrite and secondary amines in foods might react with each other during storage or processing and produce the nitrosamines. As the occurrence of secondary amines (21–25) and nitrite (26–29) in foods is a well established fact and in some countries nitrites are permitted as a food additive (28), any possible risk to human health arising from the formation of trace amounts of nitrosamines *in vivo* deserves special attention and requires further investigation. A constant check on the possible occurrences of nitrosamines in various foods should also be exercised to minimize the health hazards from their presence in foods.

Acknowledgments

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PESTICIDE FORMULATIONS

Gas Chromatographic Determination of Ethyl O,O-Dimethyldithiophosphoryl-l-Phenyl Acetate in Technical Grade Cidial and Its 50% Liquid Formulation

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A gas chromatographic method has been developed for analysis of ethyl O,O-dimethyldithiophosphoryl-l-phenyl acetate. The insecticide or a convenient amount of an insecticidal preparation is dissolved in toluene, together with a suitable internal standard, and analyzed by gas chromatography, using a thermal conductivity detector. The precision (P = 0.05) of the method is ±1.5% of the concentration found.

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The activity of Cidial® as an insecticide and acaricide and its physiochemical properties have been discussed in previous work (1, 2). A thin layer chromatographic method for the determination of the active ingredient in the technical product and its liquid formulation (Cidial 50 L) has been reported by Bazzi *et al.* (3). In this paper a gas chromatographic

Cidial® is the registered trademark of the Montecatini Edison Co. The proposed common name is phenthoate; the chemical name, in accordance with the International Union of Pure and Applied Chemistry regulations, is S-α-ethoxycarbonylbenzyl dimethyl phosphorothiolothionate.

1961

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FOOD ADDITIVES

Diethylnitrosamine and Other N-Nitrosamines in Foods

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Dimethyl-, diethyl-, di-*n*-propyl, and other nitrosamines in foods are determined semiquantitatively as follows: (*a*) initial extraction of the nitrosamines from foods into suitable solvents, (*b*) isolation of the nitrosamines from other interfering materials by steam distillation and ion exchange cleanup, (*c*) detection and semiquantitative estimation by TLC, and (*d*) final identification by GLC analysis of the separated compounds. The detection limit for dimethylnitrosamine in foods was 0.15 ppm and for diethyl- and di-*n*-propylnitrosamines, 50 ppb. The method has been successfully applied to wheat flour, fish, cheese, and spinach samples.

The discovery by Magee and Barnes (1) in 1956 of the carcinogenicity of dimethylnitrosamine (DMN) excited much interest and many reports have appeared concerning the carcinogenic, mutagenic, and toxic characters of various nitrosamines (2, 3). Nitrosamines have been shown to be carcinogenic in a wide range of species (4) and to be capable of inducing tumors at different sites of the body. In spite of their rapid decomposition in the living systems, some of them can cause cancer after only one dose (5).

Nitrosamines are easily formed from secondary amines and nitrous acid or nitrous gases (N_2O_3, NO_2, etc.). Under certain conditions even tertiary amines react with nitrous acid and give rise to the formation of N-nitrosamines through a sequence of complex reactions (6, 7). Since many foods contain large amounts of amines and small amounts of nitrite it is possible that nitrosamines occur in foods. The presence of 4-methylnitrosaminobenzaldehyde in an edible mushroom (8), DMN in herring meal (9, 10), N-nitrosopiperidine (NP) and other N-nitrosamines in tobacco (11, 12), and diethylnitrosamine (DEN) in wheat flour (12, 13), has been reported. In view of the highly carcinogenic properties of many of these compounds, sensitive and specific methods for their analysis in foods are highly desirable. In this paper a procedure is reported whereby the presence of nitrosamines in various foods can be detected at very low levels and with more certainty than previous methods (12, 13).

METHOD

Apparatus and Reagents

(a) *Gas chromatograph.*—Varian Aerograph Model 1200, with hydrogen flame detector and 1 mv recorder. GLC conditions: (*1*) 6% Reoplex 400 on 60–80 mesh Chromosorb W, 1/8" × 5' stainless steel column, nitrogen flow 29 ml/min, column temperature 115°C, range setting 10, and attenuation 8; (*2*) 5% SE-30 on acid-washed, dimethylchlorosilane-treated 60–80 mesh Chromosorb W, 1/8" × 5' stainless steel column, nitrogen flow 27 ml/min, column temperature 48°C, range setting 10, and attenuation 1.

(b) *Explosion-proof blender.*

This paper was presented at the 82nd Annual Meeting of the Association of Official Analytical Chemists, Oct. 14–17, 1968, at Washington, D.C.

(c) *Ultraviolet lamp.*—Short wavelength (λ_{max}, 253 mμ), without filter (General Electric Co.).

(d) *Silica gel-MN.*—Kieselgel G-HR (Macherey, Nagel and Co., Germany).

(e) *Solvents.*—Glass-distilled. Peroxide-free diethyl ether was prepared by the conventional method (14).

(f) *Ion exchange resin.*—Amberlite CG-120, strongly acidic cation exchange resin, sodium form (or Rexyn 101). Soak in water 24 hr and wash thoroughly with water.

(g) *Phosphate buffer mixture.*—Mix 5.0 ml KH_2PO_4 (1*M*) with 20.0 ml Na_2HPO_4 (0.5*M*); pH of mixture when diluted to ca 225 ml should be 7–7.5.

(h) *Spray reagents.*—(*1*) 1.5% diphenylamine in ethanol and 0.1% palladium (II) chloride in 0.2% NaCl solution (4:1); store solutions at 4°C and mix just before use. (*2*) *Griess reagent:* 1% sulfanilic acid and 0.1% α-naphthylamine; prepare both solutions in 30% acetic acid. Store at 4°C and mix just before use (1:1). (*3*) 0.3% ninhydrin in ethanol containing 2% pyridine. For details of spray reagent *see* Preussman *et al.* (15) and Kröller (12).

(i) *Nitrosamine standards.*—DMN, DEN, di-*n*-propylnitrosamine (DPN), di-*n*-butylnitrosamine (DBN), nitrosopiperidine (NP), and phenylbenzylnitrosamine (PBN) were purchased commercially. All other nitrosamines (Table 1) were prepared by heating aqueous solution (pH 2–2.5) of corresponding secondary amines (500 mg) with 1 g $NaNO_2$ 20 min at 37°C. The unreacted amines were removed by passing reaction mixtures (buffered at pH 4.5 with sodium acetate) through Amberlite CG-120 column (1 × 4 cm). The nitrosamines were isolated by extracting the Na_2CO_3-saturated column eluate with ether. For some compounds (e.g., dinitrosopiperazine, methylbenzylnitrosamine, and dibenzylnitrosamine), the nitrosamines separated from the reaction mixture either as a crystalline solid or an oily liquid. Several dilutions (10 and 1 mg/ml and 50 μg/ml) of working standards were prepared in peroxide-free dry ether and were kept in glass-stoppered tubes. To prevent concentration of the solutions by evaporation and decomposition of the compounds, the tubes were placed inside a closed jar containing some ether and stored at −10°C.

Extraction

(a) *Wheat flour.*—Extract 100 g flour with 500 ml peroxide-free ether 3–4 hr in 6 × 18 cm Soxhlet thimble. Add ca 100 ml water to ether extract and distill mixture in current of steam. Continue distillation until ca 200 ml aqueous distillate is collected. Add 20 g K_2CO_3 to aqueous distillate, dissolve by stirring, and transfer solution to separatory funnel. Extract solution twice with equal volumes (ca 210 ml) of ether by shaking vigorously 8–10 min. Collect ether extracts in 1 L glass-stoppered Erlenmeyer flask and add ca 20 g anhydrous Na_2SO_4. Let extract stand over Na_2SO_4 30–45 min, swirling mixture occasionally. Filter extract through Whatman No. 1 paper, wash Na_2SO_4 on filter paper with 20–30 ml anhydrous ether, and concentrate combined filtrate and washing to ca 20 ml on hot water bath in stream of nitrogen. Quantitatively transfer concentrated extract (ca 10 ml at a time) into 15 ml graduated glass-stoppered test tube and continue evaporation until final volume is 0.5 ml.

(b) *Cheese or fish.*—Cut 100 g sample into small pieces and homogenize in blender 5 min with 400 ml CH_2Cl_2 and 5 g K_2CO_3. Filter homogenate through glass wool and collect filtrate in 1 L distilling flask. Wash residue on glass wool with ca 50 ml CH_2Cl_2 and add washing to main filtrate. Add 25.0 ml phosphate buffer mixture and ca 200 ml water to filtrate and remove CH_2Cl_2 by heating flask in boiling water bath. After all CH_2Cl_2 is distilled, continue heating for 10–15 min. Discard CH_2Cl_2 distillate.

Connect distilling flask to steam generator and distill mixture in current of steam until ca 200 ml distillate is collected (gently heat distilling flask to prevent condensation of steam). Add 1 ml 5% sodium acetate (buffer) to distillate, adjust pH to 4.5, and pass solution (flow rate, 1 ml/min) through Amberlite resin column (2.5 × 2.5 cm). Wash column with ca 10 ml water and add washing to column effluent. Make mixture alkaline by adding 20 g K_2CO_3 and extract it twice with 210 ml of CH_2Cl_2. Dry CH_2Cl_2 extract over anhydrous Na_2SO_4 and then concentrate to 0.5 ml, as described in (a).

(c) *Spinach.*—Proceed as outlined in (b), except omit preliminary distillation of CH_2Cl_2. Distill food extract in current of steam and extract aqueous distillate with CH_2Cl_2 (use CH_2Cl_2 from the steam distillate) at alkaline pH. (As amines were found to be absent in steam distillate, the ion exchange cleanup procedure was also omitted.)

Thin Layer Chromatography

Prepare TLC plates in conventional manner with 250 μ layer of silica gel G-HR. Spot 1–5 μg for standard nitrosamines and 100–200 μl aliquots for food extracts. Spot standards and food extracts in duplicate on both halves of plate (divided by imaginary vertical line). After de-

Table 1. R_f Values of different nitrosamines in various solvent systems[a]

Nitrosamines[b]	*n*-Hexane: Diethyl Ether: CH_2Cl_2 4:3:2	5:7:10	10:3:2
DMN	0.32	0.35	0.10
DEN	0.57	0.55	0.24
DPN	0.77	0.63	0.38
DBN	0.87	0.72	0.46
PBN	0.93	0.74	0.53
NP	0.56	0.52	0.23
N-Methyl-N'-nitrosopiperazine	0.09	0.28	0.64
Dinitrosopiperazine	0.09	0.31	0
Methyl-*n*-propylnitrosamine	0.48	0.76	0.27
Diisopropylnitrosamine	0.66	0.89	0.41
Ethyl-*n*-propylnitrosamine	0.61	0.87	0.37
Methyl-*n*-butylnitrosamine	0.57	0.83	0.33
Dibenzylnitrosamine	0.83	0.92	0.63
Methylbenzylnitrosamine	0.58	0.85	0.32

[a] See Preussmann *et al.* (15).
[b] See text for abbreviations.

veloping plate in appropriate solvents (Table 1) spray left side of plate with Griess reagent and right side with 30% acetic acid. Irradiate plate under UV light (without filter) 5–10 min. (Red-violet spots should appear on left side within this period.) Heat plate 5–10 min in jet of warm air to evaporate acetic acid. Spray right side of plate with ninhydrin reagent and heat plate at 80°C. (Red-purple spots should start to appear within 10–15 min.) Estimate amount of nitrosamines (if present) in food extracts from relative intensities of colored spots compared to those of standards. The R_f values of various nitrosamines are given in Table 1.

Gas Chromatography

If food extract gives positive test for a nitrosamine by TLC with both the reagents (Griess and ninhydrin), spot remaining extract as streak on TLC plate and develop chromatogram in suitable solvent. (Prepare fresh extract if necessary.) Scrape off band with same R_f as unknown spot and elute with water-saturated ether or CH_2Cl_2. Dry eluate over anhydrous Na_2SO_4 and concentrate to 0.3–0.5 ml. Use 2–4 μl aliquot of concentrated eluate for GLC analysis. Diagrams of typical chromatograms are presented in Fig. 1.

Results and Discussion

By a combination of the TLC and GLC techniques described, the presence of DMN, DEN, DPN, and possibly other nitrosamines in foods can be detected and identified at very low levels (0.05-0.15 ppm). The method of Marquardt and Hedler (13) for determining DEN in wheat flour uses a crude ether extract for GLC and TLC analyses; we have experienced many difficulties with their technique because of the presence of interfering substances in the extract. Although Thewlis (16) used the same method and reported that DEN (at the 1 ppm level) added to the concentrated flour extract could be detected by GLC analysis, no data on the per cent recovery of the added DEN were presented. Moreover, by using the Marquardt-Hedler method (13) we were unable to get a satisfactory recovery (<30%) of DEN when we added it to the flour (before extraction) instead of to the extract.

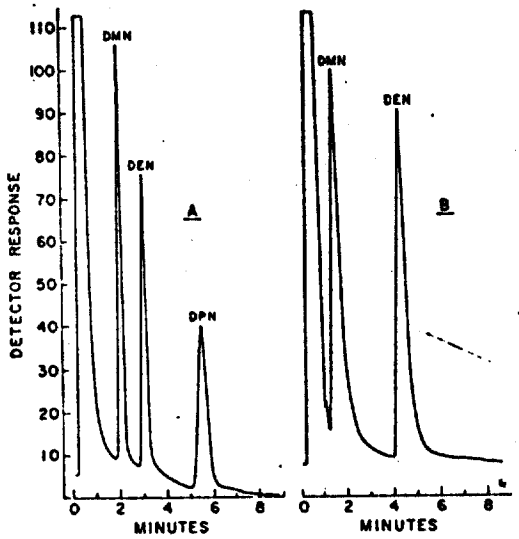

FIG. 1—GLC separation of DMN, DEN, and DPN standards: A, 2 μg each of DMN, DEN, and DPN in 2 μl ether, using GLC conditions 1; B, 1 μg each of DMN and DEN, using GLC conditions 2.

In a recent communication, Kröller (12) reported a method similar to the one described in this paper. However, we found it unsuitable for the analysis of nitrosamines in cheeses and fish (herring). Large amounts of amines, present in both fish and cheese, and some yellow pigments in cheese interfered with the identification and location of the nitrosamines on TLC plates. The addition of K_2CO_3 during the extraction of food samples, in our method, gave a more effective extraction of nitrosamines into CH_2Cl_2, and the treatment of the steam-distillate with the Amberlite resin helped to remove the interfering amines. The nitrosamines passed through the resin column unabsorbed under our conditions. Also, preliminary heating of the food extracts for 10–15 min before steam distillation coagulated proteinaceous and other materials that caused excessive foaming during steam distillation. If foaming still persisted, it could be easily controlled by adding 5 g USP beeswax to the final food extract during the steam distillation process.

Yellow spots that occasionally appeared on thin layer plates of cheese and fish extracts were separated from most of the nitrosamines by developing the plates in either of the following solvents: (*a*) dry diethyl ether, (*b*) dry CH_2Cl_2, and (*c*) benzene:glacial acetic acid: water (10:10:5; the top layer was used). The last solvent was found to be most suitable for this purpose. As the nitrosamines are unstable in acidic pH, especially when exposed to UV light, TLC plates should, preferably, be developed in the dark when using solvent (*c*). Trace amounts of acetic acid present in the thin layer band eluate should also be removed by extracting with 10 ml 10% K_2CO_3 solution before the eluate is subjected to GLC analysis.

The minimum detection limit for different nitrosamines in food depended on the volatility of the compounds. For example, DEN and DPN could be detected when added to different foods at 50 ppb levels whereas the more volatile DMN could consistently be detected only at or above 0.15 ppm levels. The approximate per cent recoveries of the nitrosamines added to different foods are presented in Table 2. These data are based on the TLC analysis of food extracts, using the Griess reagent and the ninhydrin solution as the location reagents. We could isolate DMN, DEN, and DPN from various foods spiked with suitable levels of different nitrosamines (0.3 ppm for DMN and 0.1 ppm for DEN and DPN) and identify them by GLC.

Thin layer chromatograms of food extracts, when sprayed with the diphenylamine reagent, gave many false-positive spots for nitrosamines. The compounds responsible for these spots failed to give color reactions with either of the other two reagents. Therefore, it seems that the ninhydrin spray technique of Kröller (12) and the Griess reagent are more specific for nitrosamines than the diphenylamine spray. The replacement of 10% HCl with 30% acetic acid, with our modification, gave better and faster results than were obtained with the use of 10% HCl. The minimum detection limits for various nitrosamine standards were about 0.3–0.6 μg with Griess reagent and slightly better (about 30% more sensitive) with the ninhydrin reagent. The

Table 2. Recovery of DMN, DEN, and DPN added to various food samples (100 g)

Food	Compd Added	Amount Added, μg	Approx. % Rec.[a]
Cheese (Havarti)	DMN	15	65
	DEN	5	65
	DPN	5	65
Cheese (Cheddar)	DEN	10	65
	DPN	20	50
Fish (herring)	DMN	20	70
	DEN	6	70
	DPN	5	70
	DEN	10	80
	DPN	20	80
	DEN	25	80
	DEN	10	80
Corned beef[b]	DMN	5	40
	DEN	5	50
	DPN	5	50
Canned luncheon meat[b]	DMN	5	30
	DEN	5	70
	DPN	5	70
Wheat flour (white)	DEN	10	65
	DEN	400	90
	DEN	30	90
	DEN	25	65
Whole wheat flour	DEN	10	40
Dark rye flour	DEN	10	60

[a] Results based on visual estimation of spots on TLC plates. See *Method* for details.
[b] Method same as for cheese. The GLC technique has not yet been applied to the meat samples.

on isolate DMN, various foods spiked ifferent nitrosamines 0.1 ppm for DEN them by GLC.

ams of food extracts, diphenylamine re- lse-positive spots for ounds responsible for color reactions with reagents. Therefore, it in spray technique of ess reagent are more es than the diphenyl- lacement of 10% HCl ith our modification, esults than were ob- 10% HCl. The mini- r various nitrosamine 3–0.6 μg with Griess etter (about 30% more hydrin reagent. The

N, DEN, and DPN added to samples (100 g)

Compd Add	Amount Added, μg	Approx. % Rec.[a]
DMN	15	65
DEN	5	65
DPN	5	65
DEN	10	65
DPN	20	50
DMN	20	70
DEN	6	70
DPN	5	70
DEN	10	80
DPN	20	80
DEN	25	80
DEN	10	80
DMN	5	40
DEN	5	50
DPN	5	50
DMN	5	30
DEN	5	70
DPN	5	70
DEN	10	65
DEN	400	90
DEN	30	90
DEN	25	65
DEN	10	40
DEN	10	60

isual estimation of spots on for details.
heese. The GLC technique to the meat samples.

spots were usually stable for 30 min and then either gradually disappeared or were overshadowed by the background color.

Griess and ninhydrin reagents are generally used for detecting nitrous acid and compounds containing primary or secondary amino groups, respectively (12). Since N-nitrosamines produce secondary amines and nitrous acid upon irradiation with UV light at an acidic pH, a positive reaction to both the reagents can be considered specific for N-nitrosamines. Other compounds such as organic nitrites and nitroso compounds (17–19) and free amines, although rarely present in the final food extracts, give positive tests with these reagents usually without irradiation with UV light. However, these interfering compounds can be easily differentiated from the N-nitrosamines by the fact that the latter group of compounds gives positive tests with both the reagents only after irradiation with UV lights. It must be emphasized that a positive reaction with one spray reagent should not be used as a proof of the presence of a nitrosamine in food extracts.

We have examined ten samples of flour (both whole wheat and white), six of Cheddar cheese, eight of frozen or pickled herring, and four of spinach. Most of the samples did not contain DMN, DEN, and DPN. In a few cases, the extracts from Cheddar cheese and herring gave positive spots for nitrosamines (with the Griess reagent) but the amounts of the available unknown compounds and samples of food were too small to try other spray reagents and therefore could not be definitely identified. Heating the flour samples 6 hr at 150–160°C did not produce any new nitrosamine.

It is possible that amines and nitrite in food might produce nitrosamines in the stomach under suitable conditions. To test this hypothesis we have incubated aqueous extracts of stomach contents from rabbits, dogs, and rats with diethylamine and $NaNO_2$ and isolated DEN from the reaction mixture in each case. The amount of DEN produced increased with decrease in pH of the stomach extracts. For example, gastric juices from rabbits (pH 1–2.5) produced more DEN than the gastric juices from rats (pH 4–5). Our observations confirm the finding of Sander (20) who has recently shown that nitrosamines can be formed *in vitro* from secondary amines and nitrite when mixed together in human gastric juice. We have also fed $NaNO_2$ and diethylamine (enclosed in separate capsules) to rabbits and cats; after 20 min the animals were killed and the gastric juices were examined. In each case we could demonstrate the presence of DEN in the gastric juice, thus establishing the fact that the nitrosation reaction also takes place *in vivo*. The full details of this work will be published later.

Similarly, nitrite and secondary amines in foods might react with each other during storage or processing and produce the nitrosamines. As the occurrence of secondary amines (21–25) and nitrite (26–29) in foods is a well established fact and in some countries nitrites are permitted as a food additive (28), any possible risk to human health arising from the formation of trace amounts of nitrosamines *in vivo* deserves special attention and requires further investigation. A constant check on the possible occurrences of nitrosamines in various foods should also be exercised to minimize the health hazards from their presence in foods.

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PESTICIDE FORMULATIONS

Gas Chromatographic Determination of Ethyl O,O-Dimethyldithiophosphoryl-l-Phenyl Acetate in Technical Grade Cidial and Its 50% Liquid Formulation

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A gas chromatographic method has been developed for analysis of ethyl O,O-dimethyldithiophosphoryl-l-phenyl acetate. The insecticide or a convenient amount of an insecticidal preparation is dissolved in toluene, together with a suitable internal standard, and analyzed by gas chromatography, using a thermal conductivity detector. The precision (P = 0.05) of the method is ±1.5% of the concentration found.

Received November 30, 1967.

The activity of Cidial® as an insecticide and acaricide and its physiochemical properties have been discussed in previous work (1, 2). A thin layer chromatographic method for the determination of the active ingredient in the technical product and its liquid formulation (Cidial 50 L) has been reported by Bazzi *et al.* (3). In this paper a gas chromatographic

Cidial® is the registered trademark of the Montecatini Edison Co. The proposed common name is phenthoate; the chemical name, in accordance with the International Union of Pure and Applied Chemistry regulations, is S-α-ethoxycarbonylbenzyl dimethyl phosphorothiolothionate.

Fd Cosmet. Toxicol. Vol. 7, pp. 301–307. Pergamon Press 1969. Printed in Great Britain

1962

Formation of *N*-Nitrosamines from Secondary Amines and Nitrite in Human and Animal Gastric Juice

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Abstract—A study was made of the possible formation of diethylnitrosamine (DEN) from nitrite and diethylamine in the acidic environment of the stomach of humans and various laboratory animals. The *in vitro* formation of DEN was demonstrated when diethylamine and sodium nitrite were incubated with gastric juices from rats, rabbits, cats, dogs and man. Human and rabbit juices (pH 1–2) produced more DEN than did rat gastric juice (pH 4–5). The nitrosation reaction has been shown to occur *in vivo* in cats and rabbits. The yields of DEN were low but the identity of the DEN formed was confirmed by both thin-layer and gas chromatography. The possible formation of traces of nitrosamines over a prolonged period under the acid conditions prevailing in the stomach could constitute a hazard to human health. It is suggested that, for the further tests necessary to establish whether a significant hazard does exist, the rabbit, cat or ferret, with gastric juices similar in pH to that of man, may be more suitable test species than the rat.

INTRODUCTION

N-Nitrosodialkylamines are among the most potent chemical carcinogens known (Magee & Schoental, 1964; Schmähl & Osswald, 1967; Terracini, Magee & Barnes, 1967). It has been suggested that nitrite and secondary amines present in food might react with each other in the acidic environment of the human stomach to form these carcinogens (Druckrey, Steinhoff, Beuthner, Schneider & Klärner, 1963). By using a colorimetric test, Sander (1967) showed that nitrosamines are formed when sodium nitrite and various secondary amines are incubated with human gastric juice at pH 1·3. However, in this work the identity of the nitrosamines was based on a colorimetric assay with whole incubation mixtures. The amounts of nitrosamines found in the various reaction mixtures were not given.

In view of the highly carcinogenic properties of the nitrosamines, it is desirable to know the amount of nitrosamines that could be formed from such reactions. Knowledge of the ratios of amine and nitrite required to form a minimum detectable amount of a nitrosamine will aid the assessment of the implications to human health.

The present communication provides quantitative data obtained from the gastric juices of man and a range of laboratory animals. A brief outline of this work has already been published (Sen, Smith, Schwinghamer & Marleau, 1969).

EXPERIMENTAL

Animals and diets. Male Wistar rats (mean body weight 200 g) and New Zealand White Albino rabbits (mean body weight 4·4 kg) were used. Dogs (mean body weight 20 kg) and

cats (mean body weight 3 kg) were obtained from local sources and were of unidentified strains. Before use all the animals were allowed laboratory food and water *ad lib.* and caged for at least 2 days.

Human gastric juice. Samples were obtained through the courtesy of Mrs. J. Draper of Ottawa Civic Hospital. Only highly acidic samples were selected for this experiment. The patients involved had received no drugs for 48 hr prior to the withdrawal of gastric juice.

In vitro *studies*

Sodium nitrite and diethylamine were incubated at 37°C with gastric juices from the rat, rabbit, dog, cat and man and the rate of formation of diethylnitrosamine (DEN) was studied. Two different methods were used to isolate and determine the amount of DEN formed, method A being suitable when the amount of DEN produced was over 10 μg while method B was used for detecting DEN at lower levels, down to less than 1 μg. Experience indicated the amount of DEN to be expected and the method was chosen accordingly.

Method A. The animals were killed by a blow on the head or were anaesthetized with ether, and the stomachs were removed. In most cases, the entire stomach contents (including undigested food) and 100–200 ml water were mixed manually using a glass rod and the mixture was filtered through glass wool and then filter paper. Suitable amounts (Table 1) of diethylamine hydrochloride and sodium nitrite were added to the final filtrate and the mixture was incubated for 20–60 min at 37°C. The reaction mixture was then filtered through

Table 1. *Amount of DEN detected by method A in gastric juices of various species incubated* with diethylamine and sodium nitrite*

Species	Volume of gastric juice or stomach extract (ml)	pH	Chemicals added: Diethylamine hydrochloride (mg)	Chemicals added: Sodium nitrite (mg)	DEN (μg) detected in reaction mixtures by: TLC	DEN (μg) detected in reaction mixtures by: GLC
Rabbit	100	2	450	300	720	630
	100	2	450	300	700	1700
	100	2	—	—	0†	
Rat‡	100	4·6	450	300	400	500
	125	4·9	450	300	80	
	100	4·4	—	—	0	
Dog	700 (100 ml used in each test)	5·4	450	300	10	
			450	300	10	
			500	500	30	
			—	—	0	
	120 (60 ml used in each test)	7·4	450	300	0	
			450	300	0	
Cat	110 (45 ml used in each test)	2·9	450	300	450	1000
			—	—	0	
Man	22	1·7	450	300	1400	1520
	22	1·3	—	—	0	0
	24	1·3	450	300	3000	2140
	24	1·3	—	—		0
	15	1·9	1	0·1–0·2	0	0

* Incubation for 20 min at 37°C.
† Zero values indicate levels undetectable by the method used.
‡ Stomachs from 25 rats pooled in each case.

Whatman No. 1 filter paper and the filtrate adjusted to pH 4·5. The solution was passed through a cation-exchange Amberlite-resin (CG-120, sodium form) column (2·5 cm × 2·5 cm) at a flow rate of 0·5–1·0 ml/min. The column was washed with 10 ml water and the washings were added to the column effluent.

The combined effluent and washing was adjusted to pH 10·9 and passed through an anion-exchange column (Amberlite IRA-400, chloride form; 5 cm × 2·5 cm). After the column had been washed with 10 ml water, the combined washing and effluent was made alkaline with 20 g potassium carbonate and the solution was extracted twice with 200 ml portions of peroxide-free ether or methylene chloride. The organic extract was dried over anhydrous sodium sulphate, concentrated to 0·5–1·0 ml and analysed by thin-layer (TLC) and gas–liquid (GLC) chromatography (Sen *et al.* 1969). When the pH of the stomach extract exceeded 4·5, the order of passage through the two ion-exchange columns was reversed.

Method B. Samples of human gastric-juice (5 ml) were incubated in the dark with 1 ml diethylamine hydrochloride and 1 ml sodium nitrite in solutions of appropriate concentration (Table 2) for 20–60 min at 37°C, after which 5 ml potassium dihydrogen phosphate (1 M), 20 ml disodium hydrogen phosphate (0·5 M) and about 100 ml water were added to the reaction mixture and the pH of the solution was adjusted to 7–7·2. Any DEN present in the mixture was isolated by steam distillation, cation-exchange treatment of the steam distillate and extraction of the potassium carbonate-saturated column eluate with ether or methylene chloride according to the method previously described (Sen *et al.* 1969). As the amount of DEN present in these samples was very small, all the extract obtained from one experiment was concentrated to 0·2 ml and spotted as a single spot on TLC plates. *n*-Hexane–diethylether–methylene chloride (4:3:2) or water-saturated diethyl ether was used for developing the TLC plates. Ninhydrin and Griess reagents were used to locate DEN on the developed chromatoplates (Sen *et al.* 1969).

Table 2. *Amount of DEN detected by method B in 5-ml samples of human gastric juice incubated at 37°C with diethylamine and sodium nitrite*

Gastric-juice sample no.	pH	Chemicals added: Diethylamine hydrochloride (mg)	Chemicals added: Sodium nitrite (mg)	Incubation period (min)	DEN in reaction mixture* (μg)
1	1·9	100	2	20	0·5
2	1·7	100	1	20	0·3
1	1·9	100	0·5	20	0·2
3	1·5	100	0·5	20	0·5
4	1·2	100	0·5	60	0·4
1	1·9	100	0·2	20	0†
1	1·9	50	1	20	0
4	1·2	50	0·2	60	0·2
5	1·7	10	1	20	0
1	1·9	1	10	20	0

* Values are means of two or three experiments.
† Zero values indicate levels undetectable by the method used.

In vivo *experiments*

Rabbits and cats were fasted for 12 hr and then given 20–25 g of food. After a further 20–30 min, suitable amounts (Table 3) of diethylamine hydrochloride and sodium nitrite crystals were administered orally, either enclosed in separate gelatine capsules or, in the

case of the rabbits, dissolved separately in water and fed through a stomach tube. After 20–30 min, the animals were killed and the entire stomach was taken out. The stomach contents were extracted with 100 ml water and the extracts analysed for DEN by method A. No attempt was made to determine the amount of DEN lost by absorption through the intestinal walls into the circulating blood during the *in vivo* incubation period.

Table 3. *Amount of DEN detected in the stomach extracts of animals given an oral dose of diethylamine and sodium nitrite*

Species	Oral dose: Diethylamine hydrochloride (mg)	Oral dose: Sodium nitrite (mg)	pH of stomach extract	DEN (μg) detected in stomach extract by: TLC	DEN (μg) detected in stomach extract by: GLC
Rabbit	1000	1000	4·5	2000	2000
	450	300	4·8	200	180
	450	300	3·8	100	
Cat	450	300	5·9	68	63

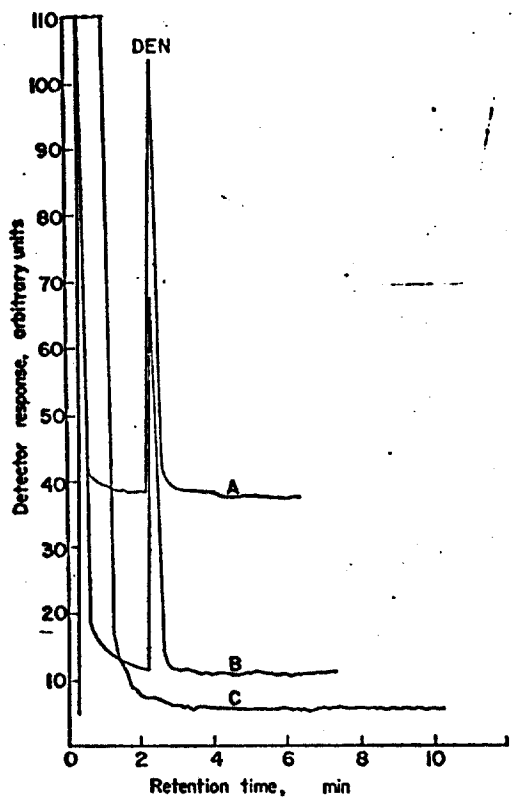

FIG. 1. Gas-chromatographic identification of DEN. (A) 1 μl of final extract of incubation mixture containing 22 ml human gastric juice and suitable amounts of diethylamine and sodium nitrite (Table 1, sample no. 1); (B) 2 μg DEN; (C) 2 μl of extract from the gastric juice used for (A) but incubated without diethylamine and sodium nitrite. Conditions: 6% coating of Reoplex 400 on 60–80 mesh chromosorb W in stainless steel column 6 ft × ⅛ in. ID; nitrogen flow rate 29 ml/min; column temperature 125°C; range setting at 10 and attenuator at 4 or at 1 (sample C); Varian Aerograph gas chromatograph, Model 1200, equipped with hydrogen flame detector and 1-mv recorder.

RESULTS

Tables 1–3 show the amounts of DEN formed from diethylamine and sodium nitrite after incubation with gastric juice taken from different animals or man, or after feeding of the two compounds to the rabbit or cat. In the *in vivo* experiments the chemicals administered, particularly at the higher dose level, caused some cyanosis and shallow, rapid breathing. As expected, the rate of formation of DEN depended on the pH of the gastric juice or stomach extract and on the initial concentrations of the reactants. Although the human gastric juices were highly acidic, about 0·2–0·5 mg sodium nitrite and nearly a 200-fold excess of diethylamine were needed to demonstrate the formation of a detectable amount (0·2 μg) of DEN.

We have added 1, 5 and 100 μg aliquots of DEN to 5 ml portions of human gastric juices and, after incubating these for 20–60 min at 37°C in the dark, have been able to detect DEN in the final extracts by method B. The average percentage recoveries of the added DEN ranged between 30 and 40%. Therefore, the amount of DEN formed in the experiments giving negative tests for DEN (Table 2) must have been well below 1 μg. None of the results (Tables 1–3) has been corrected for percentage recoveries and the actual amounts of DEN produced, especially those in Table 2, must have been at least 50% higher.

The identity of DEN formed was confirmed by both TLC and GLC. The DEN isolated from the reaction mixtures had the same R_F values as authentic DEN in the two solvent systems used, and also gave similar colour reactions with ninhydrin and Griess reagents. The GLC retention time of the isolated DEN corresponded exactly to that of standard DEN (Fig. 1). When the amounts of DEN formed were very small, the GLC technique was used only for qualitative identification, as the peaks obtained were too small for quantitative estimation. Corresponding blanks in all cases gave negative tests for DEN (Table 1; Fig. 1).

DISCUSSION

Our results confirm Sander's (1967) observations that secondary amines and nitrite, if present in ingested foods, can produce traces of nitrosamine in the human stomach. Sander was able to detect the formation of DEN on incubation of a 5 ml aliquot of human gastric juice (pH 1·3) with only 40 μg of sodium nitrite and a few crystals of diethylamine hydrochloride. We have failed to detect any DEN at this level, but as Sander did not report any data on the detection limit of his method or on the amounts of DEN formed, it is very difficult to compare our results with his and to come to any definite conclusion as to the cause of the discrepancies between the two sets of results.

We have tried the Sander (1967) technique for determining DEN in human gastric juice but found it unsuitable for our samples. When analysed by his method, the human gastric juice samples gave false-positive tests for nitrosamines even though they did not contain any added nitrite or secondary amines. The same samples gave negative tests for DEN when analysed by method B, as mentioned above.

Small amounts of nitrite and fairly large amounts of secondary amines have been reported to occur in human foods (Kamm, McKeown & Smith, 1965; Phillips, 1968; Wick, Underriner & Panceras, 1967). For example, fish (Wick *et al.* 1967; Beatty, 1938; Shewan, 1937), vegetables (Preusser, 1966) and fruit juices (Stewart, Newhall & Edwards, 1964) contain large amounts of secondary amines, and in certain cases a single meal may contain up to 100 mg of these amines. Moreover, nitrite is used as a food additive (up to 200 ppm

in meats and smoked fish) in many countries (Canadian Food and Drugs Act and Regulations, Table XI; The Preservatives in Food Regulations 1962; Statutory Instrument 1962, no. 1532, HMSO, London; US Code of Federal Regulations, Title 21, Chapter I, Sec. 121.1064). The added nitrite in meat is mostly present in bound forms as nitrosomyoglobin and haemochromogen (a denatured form of nitrosomyoglobin), but a small amount of free nitrite is always available for reaction with secondary amines (Watts, 1954). Therefore in certain cases the amounts of secondary amines and nitrite could reach the danger level and trace amounts of nitrosamines could be formed in the stomach under acidic conditions.

It has been shown that on reducing the dietary intake of DEN the total dose required to induce tumors also decreases, indicating an "acceleration process" (Schmähl & Thomas, 1965). Therefore, the formation of trace amounts of nitrosamines in the stomach over a long period of time could be hazardous to human health. However, the ultimate answer to these questions must be found in actual *in vivo* studies with different animals. Druckrey *et al.* (1963) have already carried out such studies with rats but failed to produce any carcinogenic or teratogenic effects in the animals after feeding diethylamine and sodium nitrite for the entire life span of the rats over two generations. These authors attributed the negative results to the low acidity (pH > 4) of the stomach fluid in the rats used in their study. The results of our investigations suggest that other animals such as rabbits, cats and possibly ferrets, with gastric juices similar in pH to that of man, will be more suitable than rats for such experiments.

Addendum

After this manuscript was sent for publication the paper by Sander, Schweinsberg & Menz (1968) came to our attention. These authors concluded that the ease of formation of the nitrosamines depends greatly on the basicity of the amines. The synthesis of nitrosamines from nitrite and secondary amines in the stomach of rats could be demonstrated only after feeding weakly basic amines, such as diphenylamine, *N*-methylaniline and *N*-methylbenzylamine, but not with the strongly basic amine, diethylamine. The failure of these authors to detect any DEN in the stomach of the experimental animals, even after the feeding of large amounts of nitrite and diethylamine (0·3% of each in the diet), could have been due either to the use of an insensitive method or to the different conditions used in their experiment. Although the weakly basic amines readily undergo nitrosation, the resulting nitrosamines are either not carcinogenic at all (e.g. diphenylnitrosamine) or are less carcinogenic than dimethylnitrosamine and DEN (Sander *et al.* 1968). Moreover, *N*-methylaniline and *N*-methylbenzylamine are not commonly encountered in foods.

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Formation de *N*-nitrosamines à partir d'amines secondaires et de nitrite dans le suc gastrique de l'homme et des animaux

Résumé—Les auteurs ont examiné si le nitrite et la diéthylamine peuvent se transformer en diéthylnitrosamine (DEN) dans le milieu acide de l'estomac de l'homme et de différents animaux de laboratoire. La formation de DEN a été démontrée *in vitro* par incubation de diéthylamine et de nitrite de sodium avec des sucs gastriques de rats, de lapins, de chats, de chiens et d'humains. Le suc gastrique des lapins et des humains (pH 1–2) produit plus de DEN que le suc gastrique des rats (pH 4–5). On a démontré que la réaction de nitrosation se produisait *in vivo* chez le chat et chez le lapin. La quantité de DEN ainsi formée est faible, mais l'identité de cette DEN a été confirmée par la chromatographie, tant en couche mince qu'en phase gazeuse. La formation de traces de nitrosamines dans les conditions acides dominant dans l'estomac pourrait constituer à la longue un danger pour la santé humaine. Les auteurs se demandent si le lapin, le chat et le furet, dont le suc gastrique présente un pH similaire à celui du suc gastrique humain, ne conviendraient pas mieux que le rat pour les essais ultérieurs indispensables pour déterminer l'existence d'un risque significatif.

Bildung von *N*-Nitrosaminen aus sekundären Aminen und Nitrit im Magensaft von Mensch und Tier

Zusammenfassung—Die mögliche Bildung von Diäthylnitrosamin (DEN) aus Nitrit und Diäthylamin in der sauren Umgebung des Magens des Menschen und verschiedener Laboratoriumstiere wurde untersucht. Die Entstehung von DEN *in vitro* wurde nachgewiesen, wenn Diäthylamin und Natriumnitrit mit dem Magensaft von Ratten, Kaninchen, Katzen, Hunden vom Menschen inkubiert wurden. Der Magensaft von Mensch und Kaninchen (pH 1–2) erzeugte mehr DEN als der Magensaft der Ratte (pH 4–5). Die Nitrosierungsreaktion *in vivo* wurde für Katzen und Kaninchen nachgewiesen. Die DEN-Ausbeuten waren gering, aber die Identität des entstandenen DEN wurde durch Dünnschicht- und Gaschromatographie bestätigt. Die mögliche Entstehung von Spuren von Nitrosaminen in längeren Zeiträumen unter den im Magen vorherrschenden sauren Bedingungen könnte eine gesundheitliche Gefährdung des Menschen bedeuten. Bei weiteren Versuchen, die notwendig sind, um festzustellen, ob eine nennenswerte Gefahr besteht, müssten statt der wohl weniger geeigneten Ratten als Versuchstiere Kaninchen oder Frettchen verwendet werden, die einen im pH-Wert dem des Menschen ähnlichen Magensaft besitzen.

1963

Formation of Nitrosamines in Nitrite-Treated Fish

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Abstract

An investigation of the formation of nitrosamines from the naturally occurring amines in fish and added nitrite (sometimes used as a preservative) was carried out. Eighteen samples of smoked, and 5 of canned fish were analysed after cooking with or without sodium nitrite (up to 200 ppm). The results indicate that certain kinds of fish, especially those rich in amines, can form dimethylnitrosamine (DMN) during cooking with nitrite. The identity of the DMN isolated from the fish samples was verified by both thin-layer and gas chromatography.

Résumé

La formation de nitrosamines par l'action des amines naturelles du poisson avec le nitrite de sodium (ajouté quelquefois comme préservatif au poisson) a été étudiée. Dix-huit échantillons de poisson fumé et cinq de poisson en conserves ont été examinés pour leur contenu de nitrosamines après cuisson avec ou sans nitrite de sodium (jusqu'à 200 p.p.m.). La présence du diméthylnitrosamine (DMN) est évidente dans le poisson riche en amines naturelles. L'identité DMN isolé du poisson a été vérifiée par chromatographie en couche mince et par chromatographie en phase gazeuse.

Introduction

Sodium nitrite is widely used as a preservative various foods, especially in fish and meat products, ... many countries (Canadian Food and Drugs Act and Regulations, Table XI; The Preservatives in Food Regulations, H. M. Stationery Office, U.K., 1962; United States FDA Food Additive Regulations, 121.1064). Either alone or in combination with sodium chloride, it is believed to inhibit the growth of harmful bacteria in foods (Pivnick, Rubin, Barnett, Nordin, Ferguson and Perrin, 1967; Tarr 1961) and it also imparts an attractive red color to meat products (Watts, 1954). However, recent studies (Kroller, 1967; Ender and Ceh, 1967; Sen, Smith and Schwinghamer, 1969a) point out that the indiscriminate use of nitrite in foods may be hazardous due to the formation of carcinogenic nitrosamines from nitrite and amines, which occur naturally in foods or are produced during storage. This was demonstrated by the outbreak of a hepatotoxic disorder in domestic animals fed herring meal that had been preserved with sodium nitrite (Ender, Harve, Helgebostad, Koppang, Madsen and Ceh, 1964; Ender, Harve, Madsen, Ceh and Helgebostad, 1967). The hepatotoxic factor was later isolated and confirmed to be dimethylnitrosamine (DMN) which is one of the most potent and versatile carcinogens known to exist (Magee and Barnes, 1956; Schmahl and Osswald, 1967).

In a series of studies, Ender and his associates (Ender *et al.* 1964, 1967; Ender, 1966) demonstrated that various methylamines, especially dimethylamine trimethylamine, present in herring meal are responsible for the formation of DMN. However, their studies were limited only to herring meal for animal feed and in most cases, the amount of nitrite used was very large (up to 1.2%). Since sodium nitrite is used as a preservative in certain kinds of smoked fish (United States FDA Food Additive Regulations, 121.1064), which are used for human consumption, it is possible that these products may contain trace amounts of nitrosamines. Evidence that such formation of nitrosamines from the amines in smoked fish and added nitrite could take place under normal cooking conditions is presented in this report. The recent improvements in the method of analysis of nitrosamines in fish are also described.

Experimental

Analysis of uncooked samples

The method of Sen, Smith, Schwinghamer and Marleau (1969b) and Sen and Smith (1969) was used with a few modifications below. The sample size was 200 g instead of 100 g, and during extraction, 20 ml of 20% potassium carbonate was used to facilitate extraction of the nitrosamines into the methylene chloride phase. Also, three 250 ml portions of methylene chloride were used for extraction instead of one 400 ml portion. The ion-exchange resin column was replaced with a combined ion-exchange resin and polyamide (Alupharm Chemicals) column (bottom layer - 2.5 cm polyamide; top layer - 2.5 cm ion-exchange resin; 2.5 cm diam.). Five ml 1 N sodium hydroxide solution and 10 g USP beeswax (or Dow Corning 'Antifoam A') were added to the mixture just before steam distillation.

The steam distillate (250 - 300 ml) was adjusted to pH 4.5 and passed through the combined ion exchange-polyamide column and the combined effluent and washing was extracted with methylene chloride as described before (Sen *et al.* 1969b). The methylene chloride extract was dried over anhydrous sodium sulfate, filtered, and concentrated to approx. 5 ml and then mixed with 45 ml anhydrous n-pentane. The mixture was passed through an alumina column (Woehm basic, cationotropic, activity grade 1; bottom layer-alumina, 2.5 cm x 1 cm diam., top layer - anhydrous sodium sulfate, 2.5 cm) and the column was washed with 20-50 ml anhydrous ether (elution of the main yellow pigment should be avoided). The combined effluent and washing was concentrated to 0.3 ml, spotted as a single spot, and the amount of nitrosamines present in the extract was semiquantitatively determined by thin-layer chromatography (Griess reagent) using the method of Sen *et al.* (1969b). When the test was positive, a duplicate extract was prepared and examined by the ninhydrin reagent. Two solvent systems - n-hexane: diethyl ether:methylene chloride in the ratio of (a)

4:3:2 and (b) 10:3:2 - were used for TLC analysis.

For gas liquid chromatographic (GLC) identification the nitrosamine spots from TLC plates were scraped off and eluted with diethyl ether using a micro soxhlet extraction apparatus. The ether extract (5-10 ml) was concentrated to ca 0.3 ml and a 2-4 μl aliquot was injected for GLC analysis.

Preparation and analysis of cooked samples:

a) Preparation and cooking. 200 g of fish were cut into small pieces and homogenized in a blender for 3-4 min. The homogenized fish was transferred into a beaker and mixed with 40 ml 0.1% sodium nitrite (200 ppm), The beaker was covered with aluminum foil and placed in an oven (110°C) for 60-70 min.

b) Water extraction. 200 ml water was added to the cooked fish, mixed well, and the mixture allowed to settle. The supernatant was carefully decanted and filtered through a glass wool mat in a Buchner funnel (no suction). The sample in the beaker was successively washed with 200 ml phosphate buffer mixture (Sen et al. 1969b) and 100 ml water, and the washings filtered as above. The filtrate and washings were combined in a 1-liter flask, heated in a boiling water bath for 15-20 min, and then steam distilled as described above. Extraction then proceeded as with the uncooked samples. This water extraction method was used, because the original methylene chloride extraction (as used for uncooked samples) formed an emulsion during homogenization in the blender.

c) Modified methylene chloride extraction. This was carried out in the same way as the original method except that 200 g anhydrous sodium carbonate, instead of potassium carbonate solution, and 100 g anhydrous sodium sulfate were added to the samples, and mixed well in a blender prior to extraction. At this stage, 40 mg sodium nitrite was added to the control samples (after cooling) and extracted in the usual manner.

Results and Discussion

Altogether 18 samples of smoked fish - 8 cod, 2 herring, 2 haddock, 2 halibut, 2 mackerel, 1 hake and 1 salmon - were each cooked with 200 ppm sodium nitrite and analyzed for nitrosamines. In addition, 3 samples of canned mackerel and 2 of canned salmon were similarly cooked with sodium nitrite and analyzed. Some of the samples tested gave positive tests for nitrosamines after cooking with sodium nitrite (Table 1); others gave negative results. In each case, the identity of the nitrosamines was confirmed by the double-spray technique (Sen *et al.* 1969b). The isolated nitrosamines from the fish samples had the same R_f as that of the respective standards (Sen *et al.* 1969b) when developed in solvent system (a). The DMN isolated from each of samples 3-7 was further confirmed by chromatography using solvent system (b). In addition, the identity of DMN isolated from samples 5 and 7 was verified by GLC on two different columns (Fig. 1-2). The GLC and TLC results for samples 5 and 7 compared well with each other. For example, the GLC estimations of DMN for the two samples were 8.2 μg and 1 μg respectively. The respective TLC values were 7 μg and 4 μg. However, we want to emphasize that both sets of data are very approximate figures and should be considered semiquantitative only.

Table 1 Amount of nitrosamines formed after cooking various fish samples with or without sodium nitrite

Code No.	Kind	Nitrite levels ppm	Approx. quantity of nitrosamines detected, μg/200 g (uncorrected for percentage recoveries)
1	Smoked Haddock	0 (control)	N.D.
		200	(DEN) 1
2	Smoked Cod	0	N.D.
		50	(DPN) 1
		200	(DPN) 2.5; (DMN) 0.7
		200[a]	(DPN) 0.7; (DMN) 2.5
3	Smoked Cod	0	N.D.
		200	(DPN) 0.5; (DMN) 2
4	Canned Mackerel, Brand A	0	N.D.
		200	(DMN) 8
5	"	0	N.D.
		200	(DMN) 7
6	Smoked Mackerel	0	N.D.
		200	(DMN) 1
7	Smoked Hake	0	N.D.
		200	(DMN) 4
8	Canned Mackerel, Brand B	0	N.D.
		200	(DMN) 4
9	Canned Salmon	0	N.D.
		200	(DMN) <0.5
10	Canned Mackerel[c], Brand A	200 (control)	N.D.
		200	(DMN) 5
11	Smoked Mackerel[c]	200 (control)	N.D.
		200	(DMN) 0.5
12	Smoked Hake[b,c]	200 (control)	N.D.
		200	(DMN) 9

[a]Cooked at 210°C for 25 min,. [b]cooked for 20 min. under 20 psi. All other samples were cooked for 60-70 min. at 110°C

[c]Analyzed by the methylene chloride extraction procedure. Sodium nitrite was added to the control samples after cooking the fish and cooling.

Abbreviations: N.D., Not detectable; DMN, dimethylnitrosamine; DEN, diethylnitrosamine; and DPN, di-n-propylnitrosamine.

As proof of the identity of DMN isolated from the nitrite-treated fish samples, we have presented TLC evidence on two solvent systems using two spray reagents, and GLC evidence using two different columns. Ender *et al.* (1964), working with much larger levels of nitrite and nitrosamines, have demonstrated that DMN isolated from nitrite-treated fish and authentic DMN have identical ultraviolet and infrared spectra. Therefore, it is highly unlikely that the isolated nitrosamine in this study (at least for samples 5 and 7) was incorrectly identified. Because of extremely low levels of DMN and the presence of other impurities in the final extracts, it was not possible to carry out such spectrophotometric comparisons in this study.

All the samples gave negative tests for nitrosamines when analyzed as received or after cooking in the absence of nitrite (Table 1). In certain cases, the intensity of the nitrosamine spots on TLC plates increased with increase in nitrite concentration. Due to insufficient sample size or very low levels of nitrosamines in some samples, the identity of the nitrosamines in all cases could not be verified by GLC, or by TLC in the second solvent system.

The use of polyamide in conjunction with the ion-exchange resin, and subsequent fractionation on

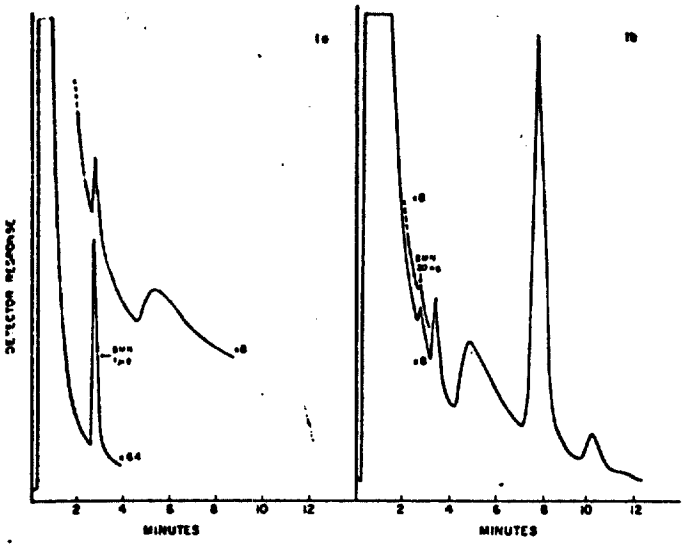

Fig. 1. GLC identification of DMN. (a) isolated from Mackerel (No. 5), and (b) from Hake (No. 7). GLC conditions: 6% Reoplex 400 on 60-80 mesh chromosorb W, ⅛" x 5' stainless steel column, nitrogen flow 27 ml/min, column temperature 105°C. Gas chromatograph — same as in Sen et al. (1969a).

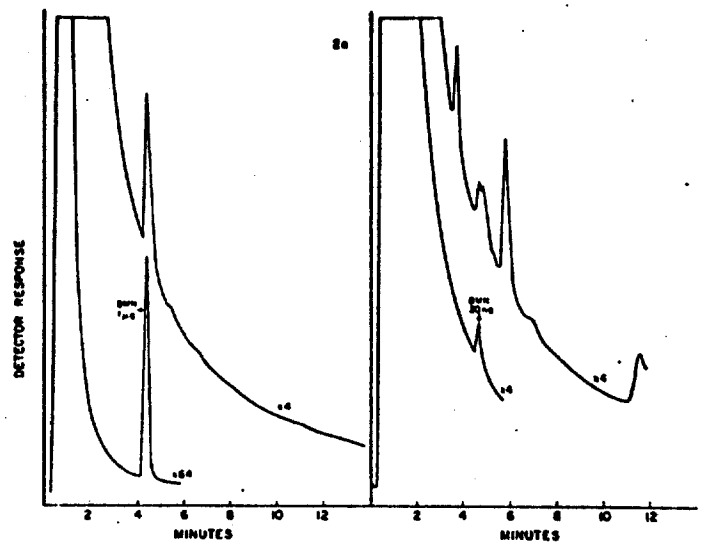

Fig. 2. GLC identification of DMN. (a) from Mackerel (No. 5), and (b) from Hake (No. 7). GLC conditions: 10% Carbowax 20M on 60-80 mesh chromosorb W (HMDS treated), ⅛" x 6' stainless steel column, nitrogen flow 26 ml/min, column temperature 103-105°C.

the alumina column resulted in a much better clean-up and produced a cleaner background on TLC plate than that was obtained in the earlier method (Sen *et al.* 1969b). Based on the analysis of spiked samples, the detection limit was found to be about 20 ppb. Since the percentage recovery of nitrosamines added to fish at this level is very low (40%) the actual amounts of nitrosamines formed in the cooked samples must have been much more than that detected.

Toward the end of this study we found that nitrosamines could be formed from nitrite and secondary amines (if present in large amounts) during steam distillation, even at a strongly alkaline pH. To eliminate this source of error, the aqueous extraction method for the cooked fish was abandoned in favor of the methylene chloride method. Three fish samples (No. 5-7), which had been shown to contain fairly large amounts of DMN when analysed by the aqueous extraction method, were reanalyzed by the methylene chloride method (reported as No. 10-12). Each test sample (cooked with nitrite) contained some DMN; although two of them at reduced concentration.

Methylene chloride seems not to take up excess nitrite from the sample, thereby preventing the formation of DMN during steam distillation. Thus, control samples, which contained same amount of sodium nitrite as the test samples, did not contain any detectable amount of DMN. Insufficient quantity prevented analysis of the other samples by this method. The smoked Hake (No. 12), when cooked with nitrite under pressure, produced the largest amount of DMN.

It is interesting to note that Ender and Ceh (1967) have found traces of DMN (1-15 μg/Kg) in a few smoked fish samples. However, their report does not state whether these samples were preserved with nitrite. The hydrazine method, which was used by the above-mentioned workers, for detecting nitrosamines in various foods appears to be more sensitive than our method but it has certain disadvantages. In their method, DMN is first converted to dimethylhydrazine which in turn is reacted with p-dimethylaminobenzaldehyde to produce a yellowish derivative. The color intensity of the derivative is finally measured with a spectrophotometer. The method indirectly measures DMN and, therefore, can be subject to interference by foreign materials giving yellowish solution, or it can give false-positive results if samples are contaminated with microgram amounts of dimethylhydrazine. Whereas, in our method, the nitrosamines are measured directly and they can be confirmed by TLC using different solvent systems and the double-spray technique or by GLC on different columns.

Ender and Ceh (1967) use a hot (90°C) acidic solution for extraction of the samples, and nitrosamines could be formed as artefacts during extraction especially in samples containing large amounts of nitrite and secondary amines. In addition, the overall procedure employed by Ender and Ceh is both very lengthy involving 3 or 4 distillation steps and uses a large sample (1 Kg).

The secondary and tertiary amine contents of various fish can vary widely (Shewan, 1937; Rea and Shewan, 1949) which may explain why nitrosamines were not detected in all the samples analyzed. The concentration of amines in fish depends on various factors such as species, age, environment, bacterial flora and storage conditions (Shewan, 1937; Rea and Shewan, 1949). Amines are usually present in larger quantities in marine fish than in fresh-water fish (Rea and Shewan, 1949; Varela and Wojciech, 1956). It is, therefore, difficult to predict whether a particular sample of fish, especially a marine fish, will be free of these amines and be safe to treat with nitrite. Our preliminary findings, as reported in this paper, indicate that the use of nitrite in fish in certain cases can lead to the

formation of nitrosamines in the finished cooked product. Nitrite is not permitted in fish in Canada and therefore, we were unable to obtain and analyze any ample that had been preserved with nitrite.

Acknowledgement

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Formation of Nitrosamines in Nitrite-Treated Fish

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Abstract

An investigation of the formation of nitrosamines from the naturally occurring amines in fish and added nitrite (sometimes used as a preservative) was carried out. Eighteen samples of smoked, and 5 of canned fish were analysed after cooking with or without sodium nitrite (up to 200 ppm). The results indicate that certain kinds of fish, especially those rich in amines, can form dimethylnitrosamine (DMN) during cooking with nitrite. The identity of the DMN isolated from the fish samples was verified by both thin-layer and gas chromatography.

Résumé

La formation de nitrosamines par l'action des amines naturelles du poisson avec le nitrite de sodium (ajouté quelquefois comme préservatif au poisson) a été étudiée. Dix-huit échantillons de poisson fumé et cinq de poisson en conserves ont été examinés pour leur contenu de nitrosamines après cuisson avec ou sans nitrite de sodium (jusqu'à 200 p.p.m.). La présence du diméthylnitrosamine (DMN) est évidente dans le poisson riche en amines naturelles. L'identité DMN isolé du poisson a été vérifiée par chromatographie en couche mince et par chromatographie en phase gazeuse.

Introduction

Sodium nitrite is widely used as a preservative in various foods, especially in fish and meat products, in many countries (Canadian Food and Drugs Act and Regulations, Table XI; The Preservatives in Food Regulations, H. M. Stationery Office, U.K., 1962; United States FDA Food Additive Regulations, 121.1064). Either alone or in combination with sodium chloride, it is believed to inhibit the growth of harmful bacteria in foods (Pivnick, Rubin, Barnett, Nordin, Ferguson and Perrin, 1967; Tarr 1961) and it also imparts an attractive red color to meat products (Watts, 1954). However, recent studies (Kroller, 1967; Ender and Ceh, 1967; Sen, Smith and Schwinghamer, 1969a) point out that the indiscriminate use of nitrite in foods may be hazardous due to the formation of carcinogenic nitrosamines from nitrite and amines, which occur naturally in foods or are produced during storage. This was demonstrated by the outbreak of a hepatotoxic disorder in domestic animals fed herring meal that had been preserved with sodium nitrite (Ender, Harve, Helgebostad, Koppang, Madsen and Ceh, 1961; Ender, Harve, Madsen, Ceh and Helgebostad, 1967). The hepatotoxic factor was later isolated and confirmed to be dimethylnitrosamine (DMN) which is one of the most potent and versatile carcinogens known to exist (Magee and Barnes, 1956; Schmahl and Osswald, 1967).

In a series of studies, Ender and his associates (Ender *et al.* 1961, 1967; Ender, 1966) demonstrated that various methylamines, especially dimethylamine and trimethylamine, present in herring meal are responsible for the formation of DMN. However, their studies were limited only to herring meal for animal feed and in most cases, the amount of nitrite used was very large (up to 1.2%). Since sodium nitrite is used as a preservative in certain kinds of smoked fish (United States FDA Food Additive Regulations, 121.1064), which are used for human consumption, it is possible that these products may contain trace amounts of nitrosamines. Evidence that such formation of nitrosamines from the amines in smoked fish and added nitrite could take place under normal cooking conditions is presented in this report. The recent improvements in the method of analysis of nitrosamines in fish are also described.

Experimental

Analysis of uncooked samples

The method of Sen, Smith, Schwinghamer and Marleau (1969b) and Sen and Smith (1969) was used with a few modifications below. The sample size was 200 g instead of 100 g, and during extraction, 20 ml of 20% potassium carbonate was used to facilitate extraction of the nitrosamines into the methylene chloride phase. Also, three 250 ml portions of methylene chloride were used for extraction instead of one 400 ml portion. The ion-exchange resin column was replaced with a combined ion-exchange resin and polyamide (Alupharm Chemicals) column (bottom layer - 2.5 cm polyamide; top layer - 2.5 cm ion-exchange resin; 2.5 cm diam.). Five ml 1 N sodium hydroxide solution and 10 g USP beeswax (or Dow Corning 'Antifoam A') were added to the mixture just before steam distillation.

The steam distillate (250 - 300 ml) was adjusted to pH 4.5 and passed through the combined ion exchange-polyamide column and the combined effluent and washing was extracted with methylene chloride as described before (Sen *et al.* 1969b). The methylene chloride extract was dried over anhydrous sodium sulfate, filtered, and concentrated to approx. 5 ml and then mixed with 45 ml anhydrous n-pentane. The mixture was passed through an alumina column (Woelm basic, cationotropic, activity grade 1; bottom layer-alumina, 2.5 cm x 1 cm diam., top layer - anhydrous sodium sulfate, 2.5 cm) and the column was washed with 20-50 ml anhydrous ether (elution of the main yellow pigment should be avoided). The combined effluent and washing was concentrated to 0.3 ml, spotted as a single spot, and the amount of nitrosamines present in the extract was semiquantitatively determined by thin-layer chromatography (Griess reagent) using the method of Sen *et al.* (1969b). When the test was positive, a duplicate extract was prepared and examined by the ninhydrin reagent. Two solvent systems - n-hexane: diethyl ether:methylene chloride in the ratio of (a)

4:3:2 and (b) 10:3:2 - were used for TLC analysis.

For gas liquid chromatographic (GLC) identification the nitrosamine spots from TLC plates were scraped off and eluted with diethyl ether using a micro soxhlet extraction apparatus. The ether extract (5-10 ml) was concentrated to ca 0.3 ml and a 2-4 μl aliquot was injected for GLC analysis.

Preparation and analysis of cooked samples:

a) *Preparation and cooking.* 200 g of fish were cut into small pieces and homogenized in a blender for 3-4 min. The homogenized fish was transferred into a beaker and mixed with 40 ml 0.1% sodium nitrite (200 ppm). The beaker was covered with aluminum foil and placed in an oven (110°C) for 60-70 min.

b) *Water extraction.* 200 ml water was added to the cooked fish, mixed well, and the mixture allowed to settle. The supernatant was carefully decanted and filtered through a glass wool mat in a Buchner funnel (no suction). The sample in the beaker was successively washed with 200 ml phosphate buffer mixture (Sen et al. 1969b) and 100 ml water, and the washings filtered as above. The filtrate and washings were combined in a 1-liter flask, heated in a boiling water bath for 15-20 min, and then steam distilled as described above. Extraction then proceeded as with the uncooked samples. This water extraction method was used, because the original methylene chloride extraction (as used for uncooked samples) formed an emulsion during homogenization in the blender.

c) *Modified methylene chloride extraction.* This was carried out in the same way as the original method except that 200 g anhydrous sodium carbonate, instead of potassium carbonate solution, and 100 g anhydrous sodium sulfate were added to the samples, and mixed well in a blender prior to extraction. At this stage, 40 mg sodium nitrite was added to the control samples (after cooling) and extracted in the usual manner.

Results and Discussion

Altogether 18 samples of smoked fish - 8 cod, 2 herring, 2 haddock, 2 halibut, 2 mackerel, 1 hake and 1 salmon - were each cooked with 200 ppm sodium nitrite and analyzed for nitrosamines. In addition, 3 samples of canned mackerel and 2 of canned salmon were similarly cooked with sodium nitrite and analyzed. Some of the samples tested gave positive tests for nitrosamines after cooking with sodium nitrite (Table 1); others gave negative results. In each case, the identity of the nitrosamines was confirmed by the double-spray technique (Sen *et al.* 1969b). The isolated nitrosamines from the fish samples had the same R_f as that of the respective standards (Sen *et al.* 1969b) when developed in solvent system (a). The DMN isolated from each of samples 3-7 was further confirmed by chromatography using solvent system (b). In addition, the identity of DMN isolated from samples 5 and 7 was verified by GLC on two different columns (Fig. 1-2). The GLC and TLC results for samples 5 and 7 compared well with each other. For example, the GLC estimations of DMN for the two samples were 8.2 μg and 1 μg respectively. The respective TLC values were 7 μg and 1 μg. However, we want to emphasize that both sets of data are very approximate figures and should be considered semiquantitative only.

Table 1 Amount of nitrosamines formed after cooking various fish samples with or without sodium nitrite

Code No.	Kind	Nitrite levels ppm	Approx. quantity of nitrosamines detected, μg/200 g (uncorrected for percentage recoveries)
1	Smoked Haddock	0 (control)	N.D.
		200	(DEN) 1
2	Smoked Cod	0	N.D.
		50	(DPN) 1
		200	(DPN) 2.5; (DMN) 0.7
		200[a]	(DPN) 0.7; (DMN) 2.5
3	Smoked Cod	0	N.D.
		200	(DPN) 0.5; (DMN) 2
4	Canned Mackerel, Brand A	0	N.D.
		200	(DMN) 8
5	"	0	N.D.
		200	(DMN) 7
6	Smoked Mackerel	0	N.D.
		200	(DMN) 1
7	Smoked Hake	0	N.D.
		200	(DMN) 4
8	Canned Mackerel, Brand B	0	N.D.
		200	(DMN) 4
9	Canned Salmon	0	N.D.
		200	(DMN) <0.5
10	Canned Mackerel[c], Brand A	200 (control)	N.D.
		200	(DMN) 5
11	Smoked Mackerel[c]	200 (control)	N.D.
		200	(DMN) 0.5
12	Smoked Hake[b,c]	200 (control)	N.D.
		200	(DMN) 9

[a]Cooked at 210°C for 25 min,. [b]cooked for 20 min. under 20 psi. All other samples were cooked for 60-70 min. at 110°C

[c]Analyzed by the methylene chloride extraction procedure. Sodium nitrite was added to the control samples after cooking the fish and cooling.

Abbreviations: N.D., Not detectable; DMN, dimethylnitrosamine; DEN, diethylnitrosamine; and DPN, di-n-propylnitrosamine.

As proof of the identity of DMN isolated from the nitrite-treated fish samples, we have presented TLC evidence on two solvent systems using two spray reagents, and GLC evidence using two different columns. Ender *et al.* (1964), working with much larger levels of nitrite and nitrosamines, have demonstrated that DMN isolated from nitrite-treated fish and authentic DMN have identical ultraviolet and infrared spectra. Therefore, it is highly unlikely that the isolated nitrosamine in this study (at least for samples 5 and 7) was incorrectly identified. Because of extremely low levels of DMN and the presence of other impurities in the final extracts, it was not possible to carry out such spectrophotometric comparisons in this study.

All the samples gave negative tests for nitrosamines when analyzed as received or after cooking in the absence of nitrite (Table 1). In certain cases, the intensity of the nitrosamine spots on TLC plates increased with increase in nitrite concentration. Due to insufficient sample size or very low levels of nitrosamines in some samples, the identity of the nitrosamines in all cases could not be verified by GLC, or by TLC in the second solvent system.

The use of polyamide in conjunction with the ion-exchange resin, and subsequent fractionation on

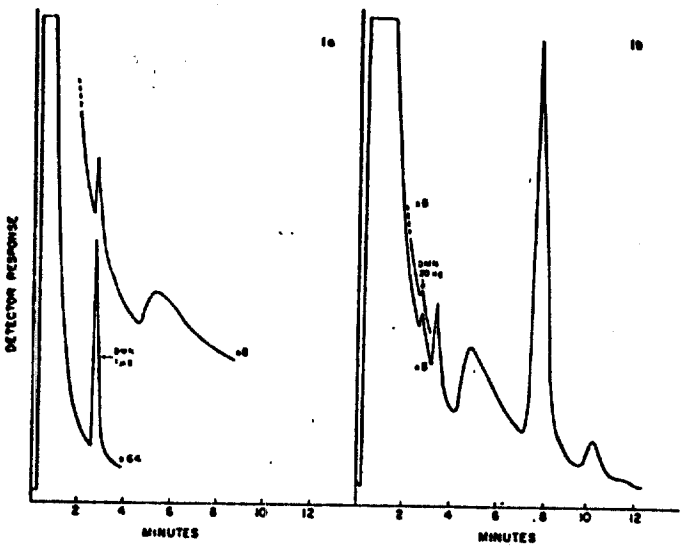

Fig. 1. GLC identification of DMN. (a) isolated from Mackerel (No. 5), and (b) from Hake (No. 7). GLC conditions: 6% Reoplex 400 on 60-80 mesh chromosorb W, ⅛" x 5' stainless steel column, nitrogen flow 27 ml/min, column temperature 105°C. Gas chromatograph — same as in Sen et al. (1969a).

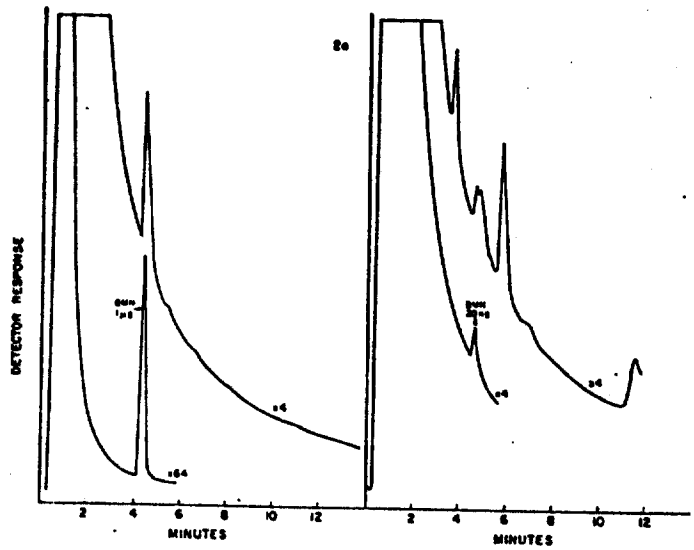

Fig. 2. GLC identification of DMN. (a) from Mackerel (No. 5), and (b) from Hake (No. 7). GLC conditions: 10% Carbowax 20M on 60-80 mesh chromosorb W (HMDS treated), ⅛" x 6' stainless steel column, nitrogen flow 26 ml/min, column temperature 103-105°C.

the alumina column resulted in a much better clean-up and produced a cleaner background on TLC plate than that was obtained in the earlier method (Sen *et al.* 1969b). Based on the analysis of spiked samples, the detection limit was found to be about 20 ppb. Since the percentage recovery of nitrosamines added to fish at this level is very low (40%) the actual amounts of nitrosamines formed in the cooked samples must have been much more than that detected.

Toward the end of this study we found that nitrosamines could be formed from nitrite and secondary amines (if present in large amounts) during steam distillation, even at a strongly alkaline pH. To eliminate this source of error, the aqueous extraction method for the cooked fish was abandoned in favor of the methylene chloride method. Three fish samples (No. 5-7), which had been shown to contain fairly large amounts of DMN when analysed by the aqueous extraction method, were reanalyzed by the methylene chloride method (reported as No. 10-12). Each test sample (cooked with nitrite) contained some DMN; although two of them at reduced concentration.

Methylene chloride seems not to take up excess nitrite from the sample, thereby preventing the formation of DMN during steam distillation. Thus, control samples, which contained same amount of sodium nitrite as the test samples, did not contain any detectable amount of DMN. Insufficient quantity prevented analysis of the other samples by this method. The smoked Hake (No. 12), when cooked with nitrite under pressure, produced the largest amount of DMN.

It is interesting to note that Ender and Ceh (1967) have found traces of DMN (1-15 μg/Kg) in a few smoked fish samples. However, their report does not state whether these samples were preserved with nitrite. The hydrazine method, which was used by the above mentioned workers, for detecting nitrosamines in various foods appears to be more sensitive than our method but it has certain disadvantages. In their method, DMN is first converted to dimethylhydrazine which in turn is reacted with p-dimethylaminobenzaldehyde to produce a yellowish derivative. The color intensity of the derivative is finally measured with a spectrophotometer. The method indirectly measures DMN and, therefore, can be subject to interference by foreign materials giving yellowish solution, or it can give false-positive results if samples are contaminated with microgram amounts of dimethylhydrazine. Whereas, in our method, the nitrosamines are measured directly and they can be confirmed by TLC using different solvent systems and the double-spray technique or by GLC on different columns.

Ender and Ceh (1967) use a hot (90°C) acidic solution for extraction of the samples, and nitrosamines could be formed as artefacts during extraction especially in samples containing large amounts of nitrite and secondary amines. In addition, the overall procedure employed by Ender and Ceh is both very lengthy involving 3 or 4 distillation steps and uses a large sample (1 Kg).

The secondary and tertiary amine contents of various fish can vary widely (Shewan, 1937; Rea and Shewan, 1949) which may explain why nitrosamines were not detected in all the samples analyzed. The concentration of amines in fish depends on various factors such as species, age, environment, bacterial flora and storage conditions (Shewan, 1937; Rea and Shewan, 1949). Amines are usually present in larger quantities in marine fish than in fresh-water fish (Rea and Shewan, 1949; Varela and Wojciech, 1956). It is, therefore, difficult to predict whether a particular sample of fish, especially a marine fish, will be free of these amines and be safe to treat with nitrite. Our preliminary findings, as reported in this paper, indicate that the use of nitrite in fish in certain cases can lead to the

formation of nitrosamines in the finished cooked product. Nitrite is not permitted in fish in Canada and therefore, we were unable to obtain and analyze any sample that had been preserved with nitrite.

Acknowledgement

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N-Nitrosodimethylamine in Fish Meal

Trace amounts of *N*-nitrosodimethylamine (0.12–0.45 ppm) were detected in fish meal which had been implicated in the liver disease of mink in Canada. The identity of the isolated compound was confirmed by both thin-layer and gas-liquid chromatographic (glc) analysis, as well as by combined glc–mass spectrometry. No nitrite could be detected in these samples.

N-Nitrosodimethylamine (DMN) is a potent carcinogen (Magee and Barnes, 1956), and the reported presence of this compound in the environment has recently aroused a great deal of concern among scientists (Lijinsky and Epstein, 1070; Magee, 1971). An outbreak of hepatotoxic disorders in farm animals in Norway was shown to be due to the presence of DMN in the nitrite-treated fish meals that were fed to the animals (Bohler, 1960; Ender *et al.*, 1964; Koppang and Helgebostad, 1966). It was established that various methylamines, mainly dimethylamine and trimethylamine, present in the fish reacted with the added nitrite and formed toxic levels of DMN (Ender *et al.*, 1964, 1967; Sakshaug *et al.*, 1965). The concentration of DMN in six of the toxic feeds ranged from 30 to 100 ppm. No DMN was detected (detection limit, 10–15 ppm) in the nontoxic batches of herring meal. In recent years, trace amounts of DMN and other nitrosamines have been reported to be present in foods for human consumption; a brief outline of these findings has been published elsewhere (Magee, 1971; Sen, 1972).

Recent reports (Kirk, 1971) of the death of a large number of mink in Canadian farms prompted us to examine the mink feeds for nitrosamines. Some of these feeds contained a large proportion (55–60%) of herring meal and, according to the manufacturer, none were preserved with nitrate or nitrite. In addition to the analysis for nitrosamines, a few samples were also analyzed for nitrate and nitrite. The results of the findings are presented in this communication.

EXPERIMENTAL

Determination of DMN. The method used is similar to that reported by us previously (Sen, 1971; Sen *et al.*, 1969, 1970). It can be briefly described as follows. The sample (25–50 g) was moistened with 3 *N* potassium hydroxide, extracted in a blender with methylene chloride, and the extract was distilled over 3 *N* potassium hydroxide (200 ml) until all the methylene chloride was removed. The residual mixture was distilled and about 150 ml of distillate was collected. The distillate was made alkaline and reextracted with methylene chloride. Interfering amines were removed by washing the extract with glycine–hydrochloric acid buffer (pH 2.1). Further purification was achieved by chromatography on a basic alumina column. Finally, DMN was detected and semiquantitatively estimated by thin-layer chromatography (tlc) and gas–liquid chromatography (glc) using a Coulson electrolytic conductivity detector (Rhoades and Johnson, 1970). Final confirmation was carried out by glc–mass spectrometry. The details of the method have been reported earlier (Sen, 1971, 1972).

Glc Conditions. 10% Carbowax 20M on 60–80 mesh Chromosorb W (HMDS treated), 6 ft × 1/8 in. stainless steel column, helium flow 30 ml/min, column temperature 105° for first 3 min, then programmed to 175° at 10°/min. The effluent from the column was allowed to vent in the air for the first 3 min. A Varian gas chromatograph, Model 2700, connected to a Coulson electrolytic conductivity detector (furnace 500°, pyrolytic mode) was used. Detector voltage 30, and attenuator at 16.

Determination of Nitrate and Nitrite. Determined by the method of Kamm *et al.* (1965).

RESULTS AND DISCUSSION

Seven samples of fish meals or products containing fish meal were analyzed and the results are presented in Table I. All but one sample contained considerable amounts of DMN. Although small amounts of nitrate were detectable in all of the four samples analyzed, none contained any detectable amount of nitrite. Our failure to detect any nitrite (detection limit 0.1 ppm) in these feeds corroborates the manufacturer's claim that no nitrite was used in preparation of these products. It is, however, not clear how the formation of DMN took place in the absence of nitrite. One possibility is that small amounts of nitrite naturally present in the raw fish may have contributed to the formation of DMN. Such a small level of nitrite would be completely destroyed by reaction with various amino compounds in the fish meal, and the residual amount of nitrite would be too small to be detected in the final product. Hot fire gases, obtained from the combustion of fuel oils, may be another source of the nitrite. Ender and Ceh (1967) have pointed out that stack gases from fuel oil contain up to 1000 ppm of nitrogen oxides and the industrial use of such gases for drying fish meal may lead to the formation of DMN. However, further research is needed to establish this point.

Various methods have been described for the determination of DMN in food for human and animal consumptions. These include ultraviolet (Ender *et al.*, 1964) and infrared (Moehler and Mayrhofer, 1968), spectroscopic, polarographic (Heath and Jarvis, 1955; Walters *et al.*, 1970), colorimetric (Eisenbrand and Preussmann, 1970), tlc (Preussmann *et al.*, 1964; Sen *et al.*, 1969), glc (Howard *et al.*, 1970; Sen *et al;* 1969, 1970) methods. Many of these methods are unsuitable for trace analysis of nitrosamines in biological mixtures mainly because of the lack of sensitivity or specificity. The tlc method (Sen *et al.*, 1969) with the double-spray technique is very specific for *N*-nitrosamines, but additional confirmation is necessary for unequivocal proof of the identity. Recent studies by Rhoades and Johnson (1970) indicated that amines and certain dilakylnitrosamines can be selectively detected by glc analysis using a Coulson electrolytic conductivity detector operating in the pyrolytic mode. If free amines and other basic compounds are removed by an acid wash of the extract (as was done in this study) the technique then becomes more selective and detects only nitrosamines and other neutral compounds which produce amines upon pyrolysis. In addition to the glc evidence (Figure 1), the tlc and glc–mass spectroscopic (Figure 2) confirmation greatly strengthens the validity of our findings.

DMN is extremely toxic to fur-bearing animals, as well as to cattle and sheep (Koppang, 1966, 1970). Mink appears to be the most susceptible species. A dietary level of 2.5 or 5 ppm of DMN has been shown to produce toxic symptoms within 7–11 days and 100% mortality within 34 days (Carter *et al.*, 1969). Multiple liver tumors have been produced in

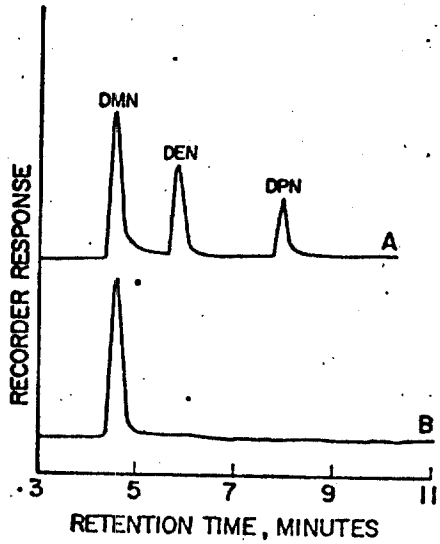

Figure 1. Glc analysis of nitrosamines. (A) 90 ng each of DMN, diethylnitrosamine (DEN), and di-*n*-propylnitrosamine (DPN). (B) 10.5-μl aliquot of the final methylene chloride extract (1.9 ml) from mink feed no. 2 (Table I)

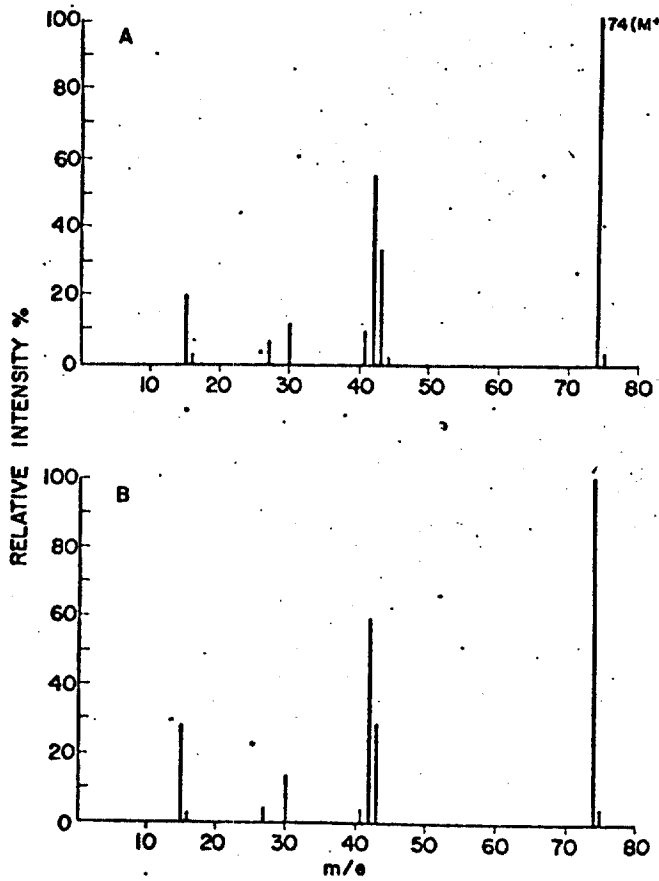

Figure 2. Mass spectrum of DMN. (A) DMN standard, (B) DMN isolated from sample no. 5. The background signals due to methylene chloride solvent and column bleed (scanned just prior to the emergence of the DMN peak) were subtracted in each case. A Hitachi Perkin-Elmer RMS-4 mass spectrometer coupled with a Perkin-Elmer, Model 990, gas chromatograph was used

weight/day (Koppang, 1970). Therefore, it is highly likely that some of the feeds analyzed in this study would be toxic to mink. It is of interest that the first two samples were reported to be toxic to mink, and many deaths occurred due to liver damage.

In view of the above findings it can be concluded that significant amounts of DMN can be present in some fish meals used as animal feeds. Therefore, it would be highly desirable to carry out routine analysis of fish meals for DMN. Such a practice may help prevent the occurrence of liver disease in animals and reduce their mortality rate due to poisoning by DMN.

Table I. DMN, Nitrate, and Nitrite Levels in Various Fish Meals

Sample	DMN, ppm	Nitrate,[a] ppm	Nitrite,[a] ppm
Mink feed	0.40[b]		
	0.18	22	N.D.[c]
Whole herring meal	0.30[b]		
	0.42	22	N.D.
	0.45[b]	17	N.D.
Fish meal	N.D.	13	N.D.
	0.12		

[a] Results expressed as sodium salt. [b] Confirmed by glc–mass spectrometry. [c] N.D. = not detectable. Detection limit for DMN is 0.01 ppm and for nitrite is 0.1 ppm.

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The Effect of Nitric Oxide on Bacteria

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Abstract

Shank, J. L. (Swift and Company, Chicago), J. H. Silliker, and R. H. Harper. The effect of nitric oxide on bacteria. Appl. Microbiol. **10**:185–189. 1962.—Nitric oxide, as well as several other oxides of nitrogen, were assayed for their antibacterial action. It is shown that nitric oxide has virtually no effect on bacteria, whereas both $NaNO_3$ and $NaNO_2$ appear to have either neutral or stimulatory effects. It is suggested that the formation of nitrous acid is mainly responsible for the quantitative as well as the qualitative changes that occur in the bacterial flora of cured meat. A pH-dependent "nitrite cycle" is presented to account for the production of nitrous acid in cured meat systems.

The addition of nitrate or nitrite to fresh meat ultimately results not only in pigment changes, but also, along with sodium chloride, brings about quantitative changes as well as qualitative changes in the bacterial population. Members of the genera *Pseudomonas* and *Achromobacter* are mainly responsible for the spoilage of fresh meat (Stewart, 1932; Haines, 1933; Ayres, Ogilvy, and Stewart, 1951). In cured meats, on the other hand, this predominately gram-negative flora of aerobic saprophytes is replaced by gram-positive facultative bacteria of the lactic acid group (*unpublished data*). Since the chemical and bacteriological aspects of curing occur as a function of nitrate or nitrite addition to meat, it is not unreasonable to postulate that the same general mechanism may be involved in both cases.

In meats, nitrate (NO_3^-) is converted to nitrite (NO_2^-) through bacterial reduction. Nitrite, in the slightly acid environment of meat (pH 5.5 to 6.5), exists in equilibrium with nitrous acid (HNO_2). Nitrous acid, under the prevailing reducing conditions of the meat, is reduced to nitric oxide (NO) which reacts with myoglobin to yield the precursor of myochromogen, the stable red pigment of cured meat (Haldane, 1901; Hoaglund, 1914; Urbain and Jensen, 1940; Jensen, 1951).

That nitric oxide may also play an important role in the bacteriology of curing thus becomes an inviting hypothesis. Since nitric oxide reacts with the heme pigments of meat, it could react with the heme-containing enzymes of the gram-negative bacteria, thereby poisoning them. This hypothesis could account, a priori, for the overgrowth of gram-positive, catalase-negative bacteria on cured meat. Although Hatton (1881) states that bacteria connected with the spoilage of meat extracts are able to develop in an atmosphere of nitric oxide, Warburg (1927) concluded that nitric oxide is capable of two distinct reactions with the respiratory enzyme of yeast cells. One reaction is "reversible" and does not permanently affect the cell, whereas the other is "irreversible" and kills the cell. Ingram (1939) reported that the oxygen uptake by *Bacillus cereus* was inhibited by nitrite (at pH 6), thus implying that the cytochrome system of this organism was involved. This hypothesis was denied by Tarr (1941*a*) whose data show that it is unlikely that nitrite inhibits growth by affecting the aerobic respiratory enzymes of bacteria. Tarr's conclusion is substantiated by the work of Castellani and Niven (1955) who were able to inhibit streptococci with nitrite, even though these bacteria have no heme pigments.

Since nitrite is most bacteriostatic under acid conditions (Tarr, 1941*a*, *b*) and since nitric oxide would certainly be involved in the pathway of nitrite reduction at reduced pH ranges (Corbet, 1934), a series of experiments was conducted to study the effect of the various oxides of nitrogen, including pure nitric oxide, on bacteria.

Materials and Methods

Analytical. Nitric oxide[1] was washed through 40% sodium hydroxide before use to remove any residual nitrogen dioxide. High purity nitrogen,[2] passed through hot copper threads, was used for flushing the system. Where reported, nitrate was determined quantitatively using the ferrous chloride method (AOAC, 1960). Nitrite was determined colorimetrically with modified Griess reagent (AOAC, 1960). Nitric oxide was qualitatively estimated by entrapment of the gas in agar. That this was nitric oxide could be inferred by the immediate formation of a brown gas upon exposure to oxygen (NO_2).

Bacteriological. Staphylococcus aureus, Streptococcus durans, and *Proteus vulgaris* were obtained from the American Type Culture Collection, Washington, D. C. *Clostridium* 3679, species PA-29, was originally obtained from C. F. Schmidt, Continental Can Company, Chicago, Ill. *Lactobacillus* K7B was obtained from R. H. Deibel of the American Meat Institute Foundation, Chicago, Ill. *Pseudomonas fluorescens* was isolated from meat in our laboratory and identified according to *Bergey's Manual of*

[1] The Matheson Company, Inc., East Rutherford, N. J.
[2] National Cylinder Gas Company, Chicago, Ill.

Determinative Bacteriology (Breed, Murray, and Smith, 1957).

Lactobacillus K₇B was maintained by daily transfers in Trypticase soy broth,[3] whereas the other bacteria were subcultured in brain heart infusion broth.[4] *Clostridium* 3679 was enumerated by making serial dilutions in Peptone colloid[1] followed by 7 days of incubation at 37 C; development of turbidity constituted a positive reaction. *S. durans* was enumerated using Trypticase soy broth, with 24 hr of incubation at 37 C. The other bacteria were estimated by plate count procedures on Tryptone glucose yeast extract agar (TGY).[4] For analysis of mixed cultures, *P. fluorescens* was determined on King's medium (Silliker, Shank and Andrews, 1958); *S. aureus* on tellurite glycine (Zebovitz, Evans, and Niven, 1955), and *S. durans* by serial dilution in Azide dextrose broth.[4]

RESULTS

Effect of Nitric Oxide and Nitrogen Dioxide on Various Bacteria

This preliminary experiment was designed to ascertain the gross effects of $\overline{NO}$ and $\overline{NO_2}$ on bacterial cells. Eighteen-hour broth cultures of the test bacteria were centrifuged, washed in distilled water, and resuspended in 0.001 M phosphate buffer, pH 7.2. The organisms were individually absorbed onto separate 12.7-mm penicillin assay filter discs and the discs placed in a vacuum desiccator. The desiccator was evacuated to 29 in. Hg and backfilled with oxygen-free nitrogen gas. This operation was repeated three times. After the fourth vacuumization, nitric oxide was introduced to atmospheric pressure. The bacteria on the discs were kept in this atmosphere for 30 min. The desiccator was then evacuated and refilled with nitrogen. This operation was repeated three times. The discs were then removed from the desiccator under flowing nitrogen and transferred to individual tubes of sterile Trypticase soy broth. After breaking up the filter discs with sterile broken glass, an aliquot was removed from each tube for bacteriological analysis. This procedure was repeated with 0.5% (by volume) and 1.0% (by volume) air being injected into the nitric oxide chamber, resulting in the formation of approximately 0.18% and 0.36% $\overline{NO_2}$, respectively.

The results of this experiment are given in Table 1 where it is clearly shown that none of the test bacteria were completely inactivated by exposure to the relatively pure nitric oxide. Except for *P. fluorescens*, the nitric oxide counts compared quite favorably with the control (vacuum) counts. That this system was contaminated by trace amounts of oxygen is suggested by the slight drop in pH (6.1 to 5.8). This could account for the inactivation of *P. fluorescens* in this experiment. As the amount of air increased, however, a significant reduction in all bacteria occurred. It is noted that the *Lactobacillus* was the least sensitive of the test organisms.

[3] Baltimore Biological Laboratory, Inc., Baltimore, Md.
[4] Difco Laboratories, Inc., Detroit, Mich.

Effect of Nitrous and Nitric Acids on Bacteria

That pH alone was not responsible for the results shown in Table 1 is indicated by experiments in which 1% aqueous solutions of sodium nitrite and sodium nitrate were reacted with an equivalent amount of HCl to form nitric and nitrous acids plus sodium chloride. The acid solutions were prepared individually and used immediately. For this test, 1.0 ml from 18-hr broth cultures was added to 10 ml of the acid solutions. Three trials were run for each test organism. Contact time between the cells and the acid during these trials averaged 86 sec, after which the cells were absorbed onto sterile Millipore discs and washed with 100 ml of 0.001 M phosphate buffer. The Millipore filter discs were subsequently broken up and counts made on TGY agar. It will be noted from Table 2 that the pH of the nitric acid suspensions was lower than the nitrous acid suspensions, but that the greatest antibacterial effect was associated with the nitrous acid.

Exposure of bacteria to >200 ppm $\overline{NO}$. Since nitric oxide has a limited solubility in water (at 25 C, 760 mm, only 56.3 ppm nitric oxide will be dissolved), it was decided to determine the effect of materially increasing the amount of nitric oxide available to react with the bacteria. Accordingly, two stainless steel bombs were set up, each contain-

TABLE 1. *Effect of $\overline{NO}$ and $\overline{NO_2}$ on various bacteria*

Organism	Total count per ml			
	Vacuum only	$\overline{NO}$	$\overline{NO}$ + 0.18% $\overline{NO_2}$	$\overline{NO}$ + 0.36% $\overline{NO_2}$
Pseudomonas fluorescens	800,000	100	<100	<100
Staphylococcus aureus	160,000,000	9,000,000	500	100
Streptococcus durans	1,000,000	1,000,000	100,000	1,000
Lactobacillus K₇B	41,000,000	20,000,000	1,000,000	195,000
Clostridium 3679	10,000	1,000	100	<10
pH of filter pads....	6.1	5.8	4.6	4.3

TABLE 2. *Effect of HNO_2 and HNO_3 on bacteria*

Bacteria	Acid	pH of suspension	Average total count (three trials)
Staphylococcus aureus	—	—	1,000,000,000
	HNO_3	1.4	10,000,000
	HNO_2	2.8	<1,000
Proteus vulgaris	—	—	1,000,000,000
	HNO_3	1.4	14,000,000
	HNO_2	2.8	<1,000
Pseudomonas fluorescens	—	—	350,000,000
	HNO_3	1.4	11,000
	HNO_2	2.8	<1,000

ing 1 liter of 0.85% NaCl, 0.05% of K_2HPO_4 and 0.01% of KH_2PO_4 at pH 7.4. Both bombs were autoclaved for 15 min at 121 C. One was maintained anaerobic by bubbling nitrogen gas through the buffer. The other was cooled under filtered air. When both reached 25 C (room temperature), they were inoculated with the test bacteria. Both bombs were then evacuated for 30 min (29 in. Hg). At this time, nitric oxide was introduced into both bombs until a gauge pressure of 40 psi was reached. With this pressure, at 25 C, 203.8 ppm nitric oxide are forced into solution. Samples for bacteriological examination, under a nitrogen blanket, were taken initially (just after inoculation), immediately after the pressure reached 40 psi, and after 15 min at 40 psi. The results are given in Table 3. It is evident that the nitric oxide in the "anaerobic" bomb failed to show any significant antibacterial activity. In contrast, the "aerobic" bomb showed complete inactivation of the bacteria after 15 min.

Effect of $\overline{NO}$ and $\overline{NO_2}$ on Clostridium spores. In attempting to achieve maximal nitric oxide purity under experimental conditions, it was found that white mineral oil is chemically inert (within the limits of the experiment) to the presence of the various oxides of nitrogen. Accordingly, a glass cylinder was completely filled with mineral oil and inverted over a mineral oil reservoir. The cylinder mineral oil was then displaced with washed nitric oxide. Millipore discs, previously inoculated with *Clostridium* 3679 (a mixture of vegetative cells and spores), were introduced on the end of a curved stainless steel fork under the mineral oil lock and up into the nitric oxide atmosphere for 30 min. The discs were then removed under flowing nitrogen and broken up in sterile water. Serial dilutions in Peptone colloid were made. This test was repeated in the presence of 0.45% (by volume) and 0.91% (by volume) nitrogen dioxide formed from the injection of air through a rubber seal into the nitric oxide. Millipore filters inocu-

TABLE 3. *Exposure of bacteria to 203 ppm $\overline{NO}$*

	Total count per ml		
	Clostridium 3679	*Streptococcus durans*	*Pseudomonas fluorescens*
"Anaerobic" System:			
Initial	1,000,000	1,000,000	1,000,000
NO, 40 psi, 0 min	1,000,000	1,000,000	1,000,000
NO, 40 psi, 15 min	1,000,000	1,000,000	100,000
"Aerobic" System:			
Initial	1,000,000	100,000	1,000,000
NO, 40 psi, 0 min	100,000	100	<10
NO, 40 psi, 15 min	<10	<10	<10

TABLE 4. *Effect of $\overline{NO}$ and $\overline{NO_2}$ on Clostridum 3679*

Condition	pH Millipore	Count per ml Millipore filter	
		Before pasteurization*	After pasteurization*
Control (*a*)	7.1	10,000,000	100,000
Control (*b*)	7.4	10,000,000	100,000
$\overline{NO}$ (*a*)	6.9	1,000,000	1,000
$\overline{NO}$ (*b*)	6.9	1,000,000	10,000
$\overline{NO}$ + 0.45% $\overline{NO_2}$ (*a*)	6.4	1,000	<100
$\overline{NO}$ + 0.45% $\overline{NO_2}$ (*b*)	6.1	<100	<100
$\overline{NO}$ + 0.91% $\overline{NO_2}$ (*a*)	6.1	<100	<100
$\overline{NO}$ + 0.91% $\overline{NO_2}$ (*b*)	6.5	<100	<100

* At 70 C, 15 min.

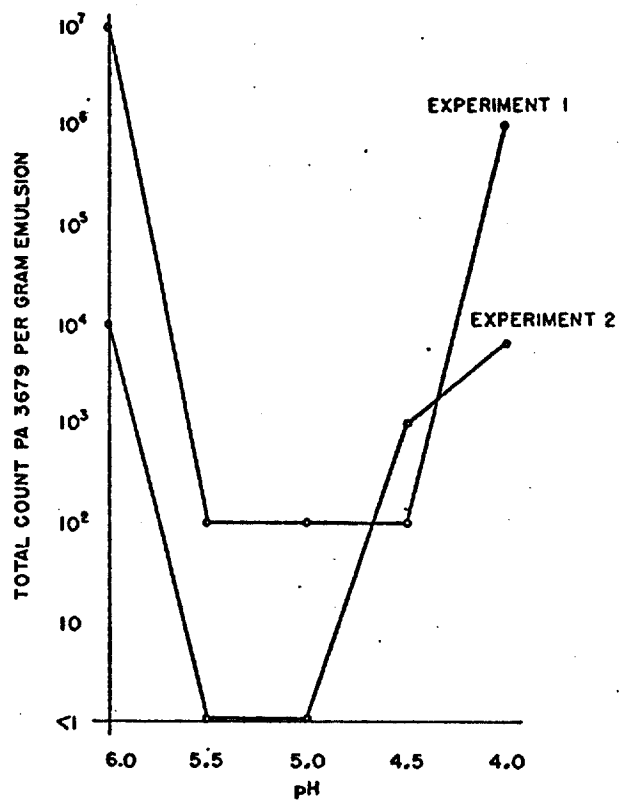


FIG. 1. *Effect of pH on the activity of nitrite. Experiment 1: Clostridial counts after 4 days of incubation, initial pH 5 count, after pasteurization = 100,000 PA3679 per g. Experiment 2: Clostridial counts after 2 days of incubation, initial pH 5 count, after pasteurization = 100,000 PA3679 per g.*

TABLE 5. *Oxidation of nitrite with decreasing pH**

pH	NO_2^-	NO_3^-	Qualitative $\overline{NO}$ production
7.0	600	10	−
6.5	530	10	−
6.0	376	10	+
5.5	45	70	2+
5.0	12	150	4+
4.5	10	140	4+
4.0	8	140	3+

* In brain heart infusion broth containing 0.15% agar and 0.06% $NaNO_2$. pH adjustments made with lactic acid. Determinations were made after 24 hr at 25 C.

lated with *Clostridium* 3679 were also exposed to these systems for 30 min. Duplicate tests were made for each condition. The results, summarized in Table 4, establish that the vegetative bacterial cell, as well as the spore, are both inactivated in the presence of nitrogen dioxide, but not in the presence of nitric oxide.

Effect of pH on activity of nitrite in meat against spores of Clostridium 3679. Sodium nitrite, 160 ppm, was added to 50 lb of ground meat emulsion, previously inoculated with spores of *Clostridium* 3679 to give 3×10^6 spores per g. The emulsion was then split into five lots and the pH of each lot adjusted with lactic acid from pH 4.0 to pH 6.0 at 0.5 pH increments. Control emulsion, similarly adjusted but lacking nitrite, was included. After appropriate mixing, each emulsion was packaged under nitrogen in thermal death time cans and pasteurized at 64 C for 35 min. The cans were then placed in a 37 C incubator. Figure 1 shows the results of two separate experiments. The initial counts, immediately after pasteurization, for both the control and the nitrite emulsions, were comparable. In experiment 1 the clostridial counts of the nitrite-treated meat, after the cans had been incubated for 4 days, are given. In experiment 2, the results after a 2-day incubation are given. It is seen that the bactericidal effect is greatest in the pH range 4.5 to 5.5. On either side of this range, that is, at pH 6 and at pH 4, the antibacterial effect of the added nitrite was far less significant. It had been reported that, at pH 7.5 or above, nitrite favors the growth of bacteria; at pH 5.6 to 5.8, nitrite exerts a very distinct bacteriostatic action; at pH 5.3 and below, nitrite disappears and again no bacteriostatic action is found (Henry, Joubert, and Goret, 1954). As nitrite disappears in an acid environment through the mediation of nitrous acid, nitrate and nitric oxide are formed (Table 5). These materials represent the more stable end products of nitrite destruction at a low pH.

Discussion

According to Taylor, Wignal, and Cowley (1927), nitrous acid in an aqueous solution decomposes in two stages:

$$2HNO_2 \rightleftharpoons N_2O_3 + H_2O \quad (1)$$

$$N_2O_3 \rightleftharpoons NO + \overline{NO}_2 \quad (2)$$

In this scheme, the hydration of nitrogen dioxide would account, ultimately, for the formation of nitrate, whereas the nitrite, also resulting, would again be available for subsequent formation of the anhydride, N_2O_3:

$$2NO_2 \xrightarrow{H_2O} HNO_2 + HNO_3 \quad (3)$$

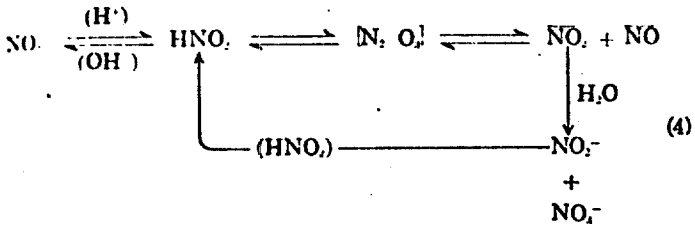
The net result of these reactions is the accumulation of nitrate and nitric oxide.

The fate of sodium nitrate or sodium nitrite added to a meat system is determined not only by the chemistry of these compounds but is profoundly affected by the biological condition of the muscle itself, as well as the numbers and kinds of bacteria that may be present. The development of the cured meat color, as indeed the accompanying antibacterial effect, is not a clear-cut phenomenon for each may be attributed to many particular components. Cured meat color may be developed by $\overline{NO}$, $\overline{NO}_2$, as well as HNO_2 (*unpublished data*), and we have shown in this paper that $\overline{NO}_2$ and HNO_2 are both powerful bactericidal agents. In a system as complex as meat, therefore, one is forced to speak in terms of generalities. If one says that NO cures meat it is meant that a reserve reducing capacity and anaerobiocity has increased the tendency for this system to produce and accumulate nitric oxide resulting in a *more nearly nitric oxide type* of cure. If one says that nitrous acid is bactericidal it is meant that HNO_2 has become the *main component* of an equilibrium mixture that involves $\overline{NO}_2$, NO_2^-, NO_3^-, as well as $\overline{NO}$. In other words, maximal color development probably occurs when meat reacts with $\overline{NO}$, whereas maximal bacteriostasis occurs in the presence of HNO_2.

In reviewing the literature it was found that the U-curves in Fig. 1 have previously been observed by Tarr (1942). He found that *Clostridium botulinum* spores grew in an anaerobic fish digest broth (pH 4.6) to which 200 ppm sodium nitrite had been added. At pH 5.1 no growth occurred. When these systems were shaken in the presence of air, no growth took place. An explanation of this may lie in the fact that on the slightly acid to alkaline side of the U-curve, sodium nitrite occurs principally as a salt. The early work of Lewis and Moran (1930) showed that sodium nitrite, at levels as high as 900 ppm (pH 7.0), actually stimulated the growth of *Clostridium sporogenes*. The inability of sodium nitrite, per se, to inactivate bacteria (at the levels commonly used in meat curing) has also been reported by Bulman and Ayres (1952) and Yesair, Bohrer, and Cameron (1944). In the pH range from 4.5 to 5.5, nitrite becomes very bactericidal, because nitrite exists principally as nitrous acid. Below pH 4.5 on the more acid side of the U-curve, nitrous acid is rapidly converted to nitrate and nitric oxide. In the presence of oxygen (e.g., Tarr's aerobic broth), however, nitric oxide is oxidized to nitrogen dioxide which, in an aqueous system, forms nitric and nitrous acids. We have presented data to show that of these two acids, nitrous acid exhibits by far the greatest antibacterial activity toward both vegetative cells and spores. In the absence of available oxygen, on the other hand, only nitric oxide and nitrate accumulate and neither of these possess antibacterial characteristics. In fact, it has been shown that nitrate stimulates aerobic sporeformers (Silliker, Greenberg, and Schack, 1958).

The data presented in this paper suggest that nitric oxide is itself relatively inert. With respect to this conclusion it is of interest to note the review on this subject by Gray (1959) who states that in a mixture of NO and NO_2 gases, the toxicity of the mixture is about proportional to the amount of NO_2 present. If NO and NO_2

are excluded as important toxiological agents, it seems reasonable to postulate that the antibacterial activity of nitrite, except for the possible formation of hydroxylamine (Lindsey and Rhines, 1932) is due principally to the formation of nitrous acid. The dynamics of nitrous acid production may now be visualized in a cyclic reaction where nitrite undergoes a concomitant oxidation-reduction reaction resulting in the formation of nitrate, nitric oxide, and nitrogen dioxide. Nitrogen dioxide, reacting with water, would generate more nitrate and nitrite with the nitrite re-entering the cycle again (equation 4).



(4)

At low pH levels (pH 3 to 4), the cycle rapidly forms NO_3^- and $\overline{NO}$, virtually exhausting its bactericidal capacity. At intermediate pH levels (pH 4.5 to 5.5) the cycle rotates more slowly. The presence of HNO_2 is prolonged, thereby increasing its reaction potential. This is the level of maximal bactericidal activity. At higher pH levels (pH 6 to 7), the equilibrium shifts toward $NaNO_2$, the cycle is prevented from functioning, and no bactericidal effects are noted.

Nitrous acid, formed as indicated above or by the oxidation of $\overline{NO}$, would have two fundamental areas of reaction. One would be with the bacterial cell itself (Quastel and Woolridge, 1927; Philpot and Small, 1938) and the other with various constituents of the medium making them unavailable for subsequent metabolism (Castellani and Niven, 1955). Either or both of these reactions could result in bactriostasis. The reason for the selection of gram-positive bacteria over gram-negative bacteria in cured meat systems, apart from the effects of hypertonicity, may eventually be explained in these terms.

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Studies reported here are part of a program to reevaluate current nitrate and nitrite standards for water and food. Included are field studies among infants in areas with varying concentrations of nitrates in water supplies as well as experimental studies on laboratory animals. Findings are reported and discussed. The detection of irreversible electrical brain activity changes in rats receiving low doses of nitrites in drinking water is of particular concern.

Epidemiological and Toxicological Aspects of Nitrates and Nitrites in the Environment

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Introduction

Nitrates and nitrites are frequently ingested by large segments of the population in drinking water and food. The ever-increasing concentrations of nitrogen salts in water are mainly a result of pollution by organic solid and liquid wastes and inorganic chemical fertilizers used extensively in agriculture. Nitrates and nitrites are found as natural components of many vegetables such as spinach and rhubarb but also are used widely as food additives in sausages and other forms of processed meat.

Infant methemoglobinemia resulting from the consumption of water with high nitrate concentrations was first recognized clinically by Comly in 1945.[1] Since then about 2,000 cases, including many fatal poisonings in infants resulting from ingestion of water containing nitrates have been recorded in various countries throughout the world.[2] The Public Health Service Drinking Water Standards[3] recommend that water consumed by infants not contain over 45 mg/l of nitrates as NO_3. The World Health Organization has made a similar recommendation. These standards were originally based on limited epidemiological evidence gathered by Walton in 1951,[4] indicating that no cases of methemoglobinemia had been reported in the United States when water containing less than 45 mg/l of NO_3 was consumed. More recent studies in Europe have, however, reported on clinical and subclinical cases of the disease in infants consuming water having less than 45 mg/l of NO_3.[5]

Recently there has been pressure to relax the nitrate standard in drinking water in areas exposed to increasing nitrate pollution of ground water in which overt cases of infant methemoglobinemia are rarely reported. The Department of Health in California has issued administrative orders which require surveillance of infants' health in communities supplying water containing up to 90 mg/l NO_3 while recommending the discontinuation of the source only at higher concentrations. The W.H.O. Recommended Drinking Water Standard for Europe - 1970 more or less have followed suit. The 1971 International Drinking Water Standards of the W.H.O. retained, however, the recommended maximum limit of 45 mg/l.

Allowable concentrations of nitrates and nitrites have been set in the U.S. by the Food and Drug Administration at 200 ppm for most "corned" meat products. This standard is supported by toxicological work carried out prior to the establishment of the rigorous testing procedures currently practiced.[6] Methemoglobinemia, in some cases fatal, has been reported from the consumption of meat products containing nitrates in excess of allowable concentrations as well as from the consumption of unadulterated spinach naturally containing high concentrations of nitrates.[7]

The series of studies reported upon here are part of a broad spectrum program to re-evaluate the current nitrate and nitrite standards for water and food, and include epidemiological field studies among infants in areas with medium-high and low concentrations of nitrates in the water supplies as well as various experimental studies in laboratory animals. Of particular interest was the possible presence of chronic sub-clinical methemoglobinemia in infants in the areas of Israel having medium-high concentrations of nitrates in the water supply but few overt clinical cases, as reported on by Knotek and Schmidt in Czechoslavakia.[5]

Field Studies

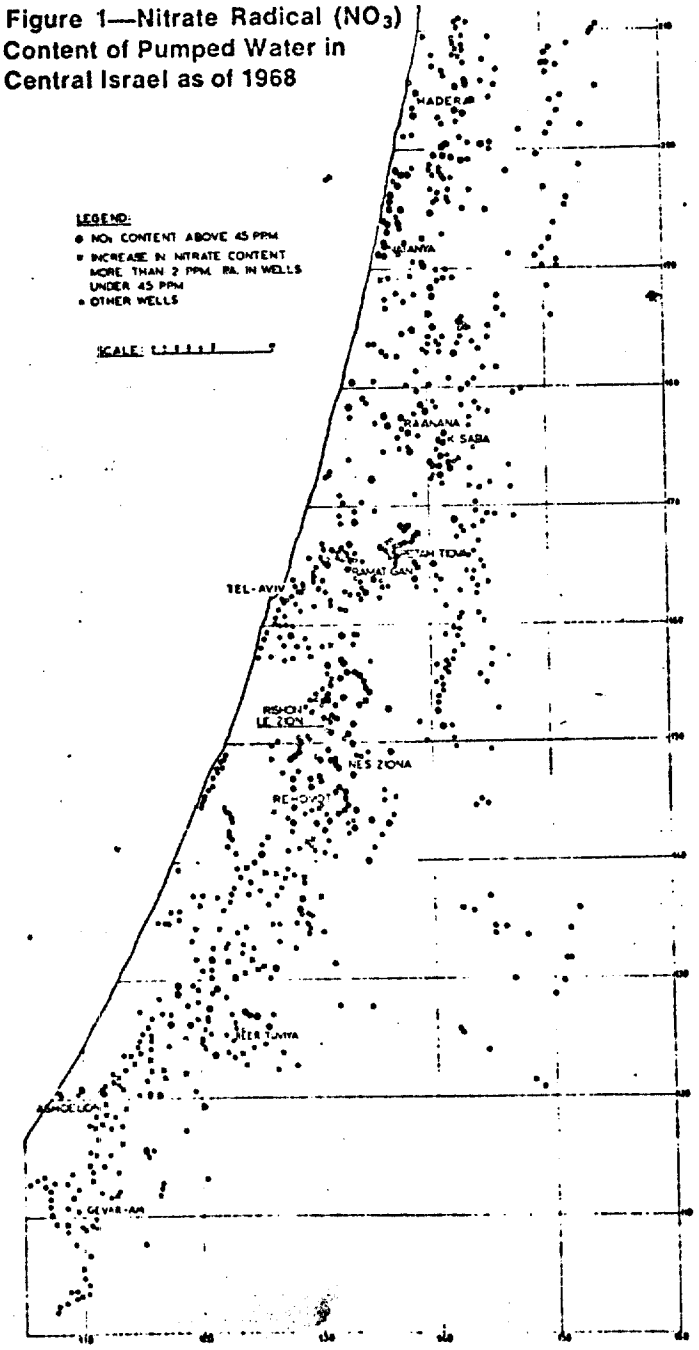
2,473 infants were studied in communities with medium-high and low concentrations of nitrates in the drinking water. The study area is on the coastal plain of Israel and includes the towns of Rehovot, Rishon le Zion, and Nes Ziona as well as a number of small agricultural settlements near Natanya (see Figure 1). The nitrate concentration is most of the water supply wells in these communities varied from 50-90 mg/l. However, since the integrated water supply systems in each community are served by a number of wells of different nitrate content, the actual NO_3 concentration at the tap in a given home may vary from hour to hour depending on the pumping rates and water demand. Infants were examined at well-baby clinics run on a routine basis by the Ministry of Health, or by the Kupat Holim-Medical Insurance scheme, so that it was not practical to determine the exact concentration of nitrates at the home tap of the infant examined at the time of examination.

For the purposes of this analysis all 1,702 infants from the study area are considered as one group with a mean of about 70 mg/l. Jerusalem, with 5 mg/l of nitrates in the water supply was chosen as a control area, and 758 infants were examined at Municipal Health Department well-baby clinics.

A 0.2 ml fingertip blood sample was taken from each infant studied by project staff nurses and examined for hemoglobin and methemoglobin (MetHb) in the laboratory in Jerusalem. An accurate and precise micromethod for testing MetHb was developed to meet the needs of this study and similar field studies. The method is a modification of the classical Evelyn and Malloy[8] method and has been reported upon previously.[9] Blood samples are stabilized so that they can be taken in distant communities and transported to the laboratory for assay within 24 hou rather than within less than an hour as required by the co ventional procedure. This method can show differences MetHb as small as .1% which is essential in a study aim at detecting slightly raised levels of MetHb in a healt population where normal MetHb levels are expected to about 1% of total hemoglobin.

A detailed questionnaire was filled in by the proje nurse for each infant, based on information supplied by t mother and the files of the well-baby clinic. Details on ag sex, weight, ethnic background, health status and nut tional regime were recorded and together with the labora ry results were punched on cards for computer analys Particular attention was given to intake of tap water w milk formula or from other sources.

Figure 1—Nitrate Radical (NO_3) Content of Pumped Water in Central Israel as of 1968



Results of Field Study

There are essentially no differences between t mean MetHb levels in the study group and the cont group as shown by Table 1. The mean MetHb level for entire population studied was 1.04% with a standard dev tion of .72%. For the most susceptible age group of 1- days the mean MetHb for the study group is 1.38% wh being 1.30% for the controls. The mean for both group 1.33%. The differences are not significant. For all ages, mean MetHb per cent in the control population is actua higher than that of the study population, but this is not s tistically significant. However, on further analysis it w shown that 31.5% of the infants up to 60 days of age in study population have MetHb levels higher than 1.7% against 21.9% for the controls.

Only 6% of the infants studied received powde milk formula made up with tap water. The remaining 9- of the infants were either breast-fed and/or received wh cow's milk. Those infants fed with powdered milk form in the study area showed somewhat higher MetHb per c than those fed exclusively on other types of milk (see Ta 2). There are only 36 infants in this category and the ferences are not significant.

49% of the infants in the 1-60 day age group c sumed either citrus or tomato juice or both. For the 61 day group 80% and over 91 days 91% consumed such tamin C rich foods. The mean consumption for the t population studied was 87%. In Table 3 the MetHb le in infants with or without vitamin C rich foods in their are presented. The 1-90 day age group of the study pop tion consuming citrus or tomato juice show somewhat lo MetHb levels as compared to the non-consumers. In control group and in those over 91 days in age there w no differences.

In the 1-90 day age group infants reported to be fering from diarrhea on the day of examination or wi the last month showed higher MetHb in both the study control area (see Table 4). In the study area, 1-90 day fants with diarrhea had a mean of 1.78% MetHb as c pared to 1.16% in infants not suffering. Infants of days with diarrhea from the study area showed hi MetHb than those from the control area. The number this category are small and the differences are not sig cant.

There were essentially no differences among inf above 91 days in age.

Table 1—Methemoglobin in Infants in Areas With High and Low Nitrate Concentrations in Drinking Water

	Mean NO_3 Mg/1	1-60 days		61-90 days		91+ days		All ages		
		n	MetHb%	n	MetHb%	n	MetHb%	n	MetHb%	S.D.
Control area	5	96	1.30	75	1.24	556	.97	758*	1.11	.72
Study area	50-90	71	1.38	188	1.14	1426	.99	1702*	1.01	.72
Total		167	1.33	263	1.17	1995	.98	2473*	1.04	.72

* Total includes infants with age unknown.

Table 2—Methemoglobin in Infants Drinking Powdered Milk Formula and Only Other Forms of Milk

	Powdered Milk		Only other Forms of Milk	
	n	MetHb%	n	MetHb%
Control area	111	.98	664	1.14
Study area	36	1.17	1666	1.00
Total	147	1.01	2310	1.04

MetHb is significantly higher in the first 60 days of life in both study and control populations (Table 1). Table 5 shows that the mean for both groups is 1.33% for the 1-60 day group, and 1.17% for the 61-90 day group. Infants over 90 days show MetHb levels of about 1%, with the mean for the total population studied of 1.04%. The same patterns are found with present weight as shown in Table 6.

Results of Laboratory Studies

Many questions concerning the toxicological effect of exposure to nitrates and nitrites in drinking water or food can be elucidated only by experimental studies carried out under laboratory conditions. Some of the major findings of our toxicological investigations on nitrites will be reported upon here.

Chronic Exposure of Rats to Sodium Nitrite in Drinking Water

Previous chronic studies by others have not shown significant MetHb levels or other pathological findings in rats consuming water containing nitrites as high as 2000 mg/l.[10] Our studies of the kinetics of MetHb disappearance after the administration of nitrites showed that the MetHb level is reduced by 50% every 90 minutes. We have also shown that rats being nocturnal animals consume 80% of their water at night and that by 10 A.M. they may no longer show significant MetHb levels, even when consuming water high in nitrites. Our measurements showed a peak in water consumption and MetHb level in the middle of the night. In designing our chronic studies we adjusted the "day" and "night" hours in the animal rooms so that blood examinations would be taken at about the rats "midnight," so as to detect the expected maximum daily MetHb concentration.

Five groups of male 3-month-old rats were used

Table 3—Methemoglobin in Infants With and Without Citrus or Tomato Juice in Diet

	Age 1-90 days				Age 91+ days			
	with		without		with		without	
	n	MetHb%	n	MetHb%	n	MetHb%	n	MetHb%
Control area	65	1.28	106	1.27	452	1.03	104	1.09
Study area	226	1.19	33	1.30	1366	.97	73	.98
Total	291	1.21	139	1.28	1818	.98	.77	1.04

Table 4—Methemoglobin in Infants With and Without Diarrhea

	Age 1-90 days				Age 91+ days			
	with		without		with		without	
	n	MetHb%	n	MetHb%	n	MetHb%	n	MetHb%
Control area	33	1.43	137	1.23	186	.99	369	1.06
Study area	20	1.78	239	1.16	165	1.01	1254	.96
Total	53	1.56	376	1.18	352	1.00	1635	.93

Table 5—Methemoglobin as a Function of Age

Age in Days	n	MetHb%
1-60	167	1.33
61-90	263	1.17
91-120	235	1.07
121-180	393	.95
181-270	461	1.00
271-360	411	.94
361-450	277	.99
451-540	161	.97
541+	57	.86
Unknown	48	-
All ages	2473	1.04

Table 6—Methemoglobin by Present Weight

Wgt in Kgs.	n	MetHb
0- 4.0	38	1.37
4.1- 5.0	204	1.30
5.1- 6.0	303	1.13
6.1- 7.0	341	1.08
7.1- 8.0	408	.99
8.1- 9.0	431	.96
9.1-10.0	317	.93
10.1-12.0	323	.99
12 -	38	.97
Unknown	70	-
Total	2437	1.04

with 8 per group. The first group "A" received normal tap water was the control. The four other groups received drinking water containing sodium nitrite at the following concentrations: "B" - 100, "C" - 1000, "D" - 2000 and "E" - 3000 mg/l.

Rats were weighed monthly and samples of tail blood were taken for determination of MetH and Hb. After 24 months, the animals were sacrificed and examined for gross pathology as well as for histological differences.

In summary, the findings of this study which can be considered only as a pretest for the full scale chronic toxicity experiments in progress are as follows: a) There were no significant differences in growth and development or mortality between the controls and the experimental group. b) There were no significant differences in Hb levels between the controls and experimental group. c) The MetHb levels of experimental groups C, D and E were significantly raised throughout the study and averaged respectively 5%, 12% and 22%. The MetHb level of "B"—receiving 100 mg/l of sodium nitrite was slightly raised above the controls for the first 60 days of the study only. Afterwards the level of "B" and "A", the controls, were identical. d) Examinations for blood glucose, pyruvate and lactate did not show any differences between the controls and experimental group. e) Pathology: At the end of 24 months' exposure to nitrites, the five groups were sacrificed. The animals were anesthetized with ether, bled, and their internal organs were inspected, weighed, and fixed in 10% neutral formaline.

Histological examinations were done on tissues from the heart, lungs, kidneys, liver, spleen, pancreas, adrenals and some brains.

The last three organs showed no pathological features in any of the rats. The liver and spleen were frequently congested, while the kidneys sometimes showed focal inflammatory and degenerative changes. The main pathological changes in the experimental group were noted in the lungs and heart.

In the lungs, the bronchi were frequently dilated, their walls infiltrated with lymphocytes, while the mucosa and muscle were often atrophied. Frankly purulent bronchial exudate also occurred. Interstitial round cells and fibrosis were sometimes encountered, emphysema was the rule. These changes, while present in one or two rats of the control group and in group "B" (100 mg/l NO_2), were found with increasing frequency and severity in the last three groups treated with higher NO_2 doses.

In the heart, there were small foci of cells and fibrosis in some animals, while a mode diffuse interstitial cellularity with pronounced degenerative foci was frequent in the highest NO_2 groups only. The oil red O stain showed no increase lipid deposits in the hearts of the experimental animals.

The intramural coronary arteries provided the surprising feature of this study. In most of the control animals the blood vessels showed some degree of thickening and often even a marked hypertrophy and narrowing. In the experimental groups, and especially in group E, who received about 250-350 mg/kg of $NaNO_2$ for 2 years, the coronaries were thin and dilated, their appearance not what is usually seen in animals of advanced age.

The findings in the lungs and heart, although per se non-specific, may be directly related to the experimental procedures, but intercurrent etiological factors must not be excluded. The lack of the usual aging changes in the coronary arteries is noteworthy and will be further investigated.

In each group tissue was taken from the heart for electronmicroscopic examinations. Full details of the pathological findings are to be reported upon elsewhere.

The Effects of the Administration of Sodium Nitrite in Drinking Water on Pregnant Rats and their Newborn

Two experimental groups were used, each containing twelve pregnant rats. Group I was given 2000 mg/l sodium nitrite and Group II, 300 mg/l in their drinking water. The control groups, of seven pregnant rats, received water without added nitrite.

The pregnant rats exhibited higher susceptibility to nitrites than non-pregnant rats. They showed higher MetHb levels than non-pregnant rats receiving the same doses.

Pregnant rats receiving 2000 mg/l in their water (Group I) developed enemia with Hb levels of 10.3 ± 1.5 gm % as compared to 14.2 ± 0.9 gm % Hb in non-pregnant controls.

There was a pronounced effect on mortality, among newborn rats of dams receiving 2000 mg/l (Group I) and 3000 mg/l (Group II) in their water, particularly in the three-week period up till weaning.

The litters in the control group contained 10 fetuses

9.5 in Group I and 8.5 fetuses in Group II. The general mortality was 6% in the control, as opposed to 30% in Group I and 53% in Group II. Birthweights were similar with 5.5 gm. for each group. However, after birth, pups in Group I and II lagged behind the controls in their growth rates. For example, after 1 week the mean weights were 16.5 gm. in the control group, 12.0 gm. in Group I and 9.5 gm. in Group II. After 21 days (at the end of the period of giving nitrites to the dams), the mean weight in the control group was 51.5 gm. as compared to 29.5 mg. in Group I and 18.5 gm. in Group II.

Apart from the weights, a characteristic difference observed in the pups of the experimental groups was that the fur thinned and lost its luster. After separation from their dams and being put on water, there was an improvement in growth in the experimental groups. At 62 days the control group attained a mean weight of 213 gm., Group I, 181 gm. and Group II, 172 gm.

During the whole period from birth to weaning, the pups showed no abnormally high MHb. The mean hemoglobin of the pups from the experimental groups was low—about 20% less than that of the current group.

Induced Methemoglobinemia by Transplacental Transport of Nitrites in Rats

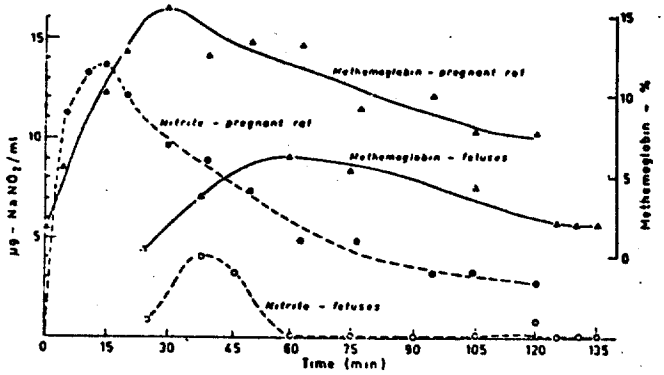
Since clinical methemoglobinemia from nitrates in water apparently appears only in babies, one possible solution proposed for areas with high nitrate water is to supply the infants with bottles of low nitrate content water from alternative sources. This measure will not exclude the risk of the exposure to nitrates in the prenatal stage, i.e., the transfer of nitrates or nitrites through the placenta to the fetus, where methemoglobinemia may be induced. Pregnant women can also consume nitrites in "corned" meat products.

The possibility of this occurring was tested on pregnant rats. Nitrites were given in drinking water or by injection to the pregnant rats and subsequently nitrites and methemoglobinemia were tested in the fetal blood.[11]

Suckling rats whose dams got nitrites in the drinking water only after giving birth showed no rise in MetHb levels. By contrast, their dams showed high MetHb levels. This demonstrates that nitrites are apparently not transferred in appreciable amounts to the pups via the milk.

The transfer of nitrites to the fetus in situ and the production of MetHb was tested in the following experiment. Pregnant white albino rats were used in this experiment. Each pregnant rat was weighed and anesthetized with ether. Suitable quantities of blood were collected from the tail at regular intervals. After opening the abdomen, the fetuses were removed serially over a two-hour period, the umbilical cords being cauterized and the fetuses washed in saline solution at 37° C, and then decapitated. Blood was collected and the initial MetHb and nitrite levels were determined. From 2.5 - 50 mg/kg of sodium nitrite were given orally or by injection to the pregnant rat and the kinetics of nitrites and MetHb in the dam as well as in the fetuses were measured. The micro-methods developed by our group for determining MetHb and nitrites in blood enabled us to carry out these experiments with the small amount of blood available from each fetus.[9,12]

Figure 2—Kinetics of Nitrite and Methemoglobin in Blood of a Pregnant Rat and the Fetuses (30 mg /kg $NaNO_2$ per os)



The characteristic picture obtained is shown in Figure 2. Nitrites rose in the fetal blood though with a lag of about 20 minutes behind the mother. This rise in nitrite was followed by a rise in MetHb. The kinetic picture was similar between the dam and the fetuses. Different concentration of nitrites caused similar effects differing only in their timing and MetHb levels. The possibility that the placenta was damaged during the experiment and that this led to increased permeability was excluded when sodium nitrite was given to the pregnant rat after the birth had started. The first fetus showed a normal MetHb level of 1.6% but those who were born after the chemical had been given showed levels of 10.1% MetHb and 0.3 ug/ml of sodium nitrite in their blood. The births were unassisted.

The threshold of the transplacental transfer effect was at a sodium nitrite does of 2.5 mg/kg. The increase in effect was steep with increased sodium nitrite dosage. Pregnant rats exhibited a higher susceptibility to develop methemoglobinemia from nitrite doses than non-pregnant rats in acute or chronic experiments.

These experiments demonstrate the transfer of nitrites through the placenta and the production of MetHb in the fetus. The low levels of methemoglobin found in pups 12 hours after birth from dams receiving nitrites in their water can be explained by the rapid recovery of the methemoglobinemia even in the newborn rats. In mature rats, the time needed for the reduction of half of the MetHb level is about 90 hours. The results underline the possible risk of intrauterine methemoglobinemia when water or food containing nitrates or nitrates is given during pregnancy. It is, however, difficult to extrapolate from these acute animal experiments to the situation that may exist with humans consuming low levels of MetHb inducing chemicals. A study of MetHb levels in infants cord blood among mothers from high and low nitrate areas is planned to help clarify this question.

Behavioral Studies in Mice Chronically Exposed to Sodium Nitrites in Water

In an effort to develop sensitive tests to detect possible effects of sub-clinical methemoglobinemia, behavioral

studies with mice were undertaken.[13] Groups of 57 black 6J male mice were given nitrites in their drinking water at doses aimed at producing MetHb levels varying from slightly above normal to 15% which can be considered in the sub-clinical range. $NaNO_2$ doses in water were 100, 1000, 1500, 2000 mg/l. Controls received tap water. The level of motor activity was determined in a special activity box designed for psychological studies with mice.[14]

Analysis of the results shows a decided and significant reduction of overall motor activity in the groups receiving the highest levels of nitrites. There is a significant inverse relation between MetHb level and activity with a coefficient of correlation of .65. An effort to counteract the methemoglobinemia was made by giving the group receiving the highest level of nitrites (2000 mg/l) vitamin C. The effect was to reduce the MetHb levels close to normal but the activity level of the group so treated remained low and about equal to the equivalent group receiving no antidote.

These experiments seem to indicate that nitrites have some form of sedative effect on the mice treated, not necessarily associated with the development of methemoglobinemia. The E.E.G. studies of rats treated with nitrites were expected to throw some light on these findings.

Brain Electrical Activity Changes in Rats Resulting from Chronic Exposure to Sodium Nitrite in Water

Studies were initiated to determine whether there were any detectable brain electrical activity changes in rats exposed to varying levels of sodium nitrite in their drinking water.[15] It was the specific objective of these studies to develop sensitive neurophysiological parameters to measure the effects of nitrite consumption leading to sub-clinical methemoglobinemia.

Electrodes were implanted on the cortex of 4 groups of 3-month-old male rats. Group A, the controls, received tap water; Group B, 100 mg/l; Group C, 300 mg/l; and D, 2,000 mg/l of $NaNO_2$. For auto-controls E.E.G. recordings were made on each rat several times before starting the regime of nitrites in their drinking water. After exposure to nitrites regular E.E.G. recordings were made. After 2 months, nitrites were removed from the drinking water. E.E.G. recordings were taken at intervals for another 4½ month period. Analysis of the results shows that there is an increase in the frequency of the E.E.G. waves in the experimental group D over the controls, while groups B and C showed lower frequencies than the controls. Rats in all the experimental groups showed paroxysmal outbursts not appearing in the control group or in their own pre-nitrite E.E.G. recordings. On ending of nitrite intake the electrical outbursts disappeared only in Group B which received 100 mg/l in their water, which nevertheless showed diffused spikes and sharp waves. All other groups showed continuation of the same type of E.E.G. outbursts over the recovery period as appeared during the exposure to nitrites. This appears to indicate some form of irreversible brain electrical changes resulting from chronic exposure to nitrites even at the 100 mg/l level.

This study will serve as a pretest for a longer, more detailed study of these phenomena, during which it is hoped to further define the characteristics of the E.E.G. changes, and their cause.

Discussion

Field Studies

The fact that there are no significant differences b tween the mean MetHb levels in the 1,720 infants in t study area with a mean nitrate concentration of 70 mg/l the drinking water as compared to the 758 infants in t control area does not support the findings of subclinic methemoglobinemia reported by Knotek and Schmidt[5] Czechoslovakia. A possible explanation for this lies in t differences in infant feeding practices found in Israel whe only 6% of the infants included in the study receiv appreciable amounts of tap water together with formu prepared from powdered milk. Preliminary measuremen of fluid intake in infants 2-3 months of age indicate th even during hot dry periods their intake of tap water giv as a supplement is about 20% of their total daily liquid i take, which is mainly made up of mothers' milk or cow milk. Apparently all of the infants studied by Knotek ar Schmidt received appreciable amount of tap water rich nitrates since infant feeding was almost universally based c milk powder formula. Another factor may be the wide practiced feeding of vitamin C rich foods such as citrus ar tomato juice to infants. Both foods are inexpensive ar available during most of the year in Israel. 87% of the i fant population studied consumed such vitamin C ric foods which are known to be an effective antidote t methemoglobinemia. In Central Europe it can be assume that such vitamin C rich food supplements are expensiv and not always in supply so that they are much le frequently used.

The finding that infants in the study area receivin powdered milk formula or no vitamin C rich food suppl ments had slightly higher MetHb than their controls is sup portive of this hypothesis, although the number of infants i each category was small and the differences are not stati tically significant.

The higher MetHb levels in infants (1-90 day reported to be suffering from diarrhea in both the study an control area supports clinical findings in Israel of an assoc ation between infant diarrhea and methemoglobinemia.[1] The fact that in the study area the level of MetHb in infan with diarrhea was higher than similar infants in the contr area may be suggestive of an additive effect resulting fro the higher exposure to nitrates in water. But there again th number of infants in those categories is small and the dif ferences not statistically significant.

The finding of higher MetHb levels in the first 6 days of life in both study and control groups supports simi lar findings by Shearer et al.[17]

One conclusion from this study is that no apparen public health problem associated with infant methemoglo binemia was detected in the study area, despite the fact th most of the wells were supplying water with nitrate concen trations above that generally recommended by public healt authorities. However, it would be premature to extrapolat from these findings as to the situation that may exist i other areas where infants consume appreciable amounts o tap water together with milk formula and where vitamin C rich foods are not widely given as diet supplements to suc young infants.[18]

Laboratory Studies

Previous work on the toxicology of nitrates and nitrites has not given rise to any serious concern[6,10] and both compounds are widely used as legally approved food additives. 200 ppm of $NaNO_2$ are allowed in sausages and other "corned" meat products, despite the fact that $NaNO_2$ is many fold more toxic than $NaNO_3$.

Nitrates are also widely found as a natural component of many foods.[7] In our own studies[19] we detected as much as 4,850 ppm of NO_3 and 233 ppm of NO_2 in a local variety of spinach. Samples of beets, cauliflower, cabbage, rhubarb and radishes were all found to contain over 1,000 ppm of NO_3. Under certain conditions of preparation and storage a portion of the nitrates can be reduced to nitrites by bacterial flora:[7,19] The nitrite form is more toxic by far. Numerous cases of methemoglobinemia, some fatal, have been reported on in connection with infants who ate spinach.

Phillips[7] calculated that in a typical Canadian meal, adults may consume some 313 mg of nitrate from various fresh foods or about 4.5 mg/kg. Under normal circumstances this is not considered detrimental. If all of that, or a major portion, was converted to nitrites prior to eating, or by stomach flora after eating, the effect might be toxic.

Our findings of distinct pathology in the heart and lungs of rats chronically exposed to 2,000 and 3,000 mg/l of $NaNO_2$ in their drinking water for a two-year period, providing a dose of about 250-350 mg/kg is striking although difficult to extrapolate to humans, both because the pathology was detected only at massive dose levels and since there is always the possibility of species specific toxic effects.

The fact that nitrites can be passed through the placenta of rats and cause raised levels of MetHb in the fetus, as well as other irreversible effects in newborn rats, may give rise to concern particularly since this effect was still detectable at $NaNO_2$ doses of 2.5 mg/kg. Such doses of nitrites can be consumed by humans under not particularly extreme conditions. The most disquieting finding is the fact that we have detected irreversible brain electrical activity changes in the E.E.G. recordings of rats exposed to 100 and 300 mg/l $NaNO_2$ in their drinking water for a period of 2 months. The level of exposure to nitrites in these experiments comes uncomfortably close to commonly found levels of nitrates in food and water. Here, too, we must suggest caution in extrapolating the results of such small scale preliminary experimental studies with $NaNO_2$ in rats to the possible effects on humans. But these latter electrophysiological findings, together with the other experimental studies reported upon here, suggest that if these results are confirmed by more extensive retests now in progress there may well be a need to re-examine the present standards for nitrates and nitrites used as food additives or for that matter allowed in drinking water.

Summary and Conclusions

2,473 infants in areas with medium-high (50-90 mg/l as NO_3) and low nitrate (5 mg/l as NO_3) concentrations in drinking water were studied in an effort to determine whether there is any association between methemoglobin (MetHb) levels and nitrates in drinking water.

No differences were found between the mean MetHb level in the study and control areas.

A possible explanation for this finding lies in the fact that only 6% of the infants consumed appreciable amounts of tap water together with powdered milk formula. The remainder were breast-fed or consumed whole cow's milk. 87% of the infants were fed vitamin C rich foods such as citrus or tomato juice which are known for their anti-MetHb effects. It cannot be assumed that there is no danger from nitrates in drinking water in areas where infants consume larger amounts of tap water with powdered milk formula and/or little vitamin C rich foods.

MetHb levels are highest in the first 60 days of life with a mean of 1.33%. The mean MetHb level for the entire infant population studied was 1.04 ± .72%.

Rats chronically exposed to 2,000 and 3,000 mg/l $NaNO_2$ (250-350 mg/kg) in their drinking water for two years showed distinct pathology in the heart and lung tissues as compared to controls.

Pregnant rats given acute doses of $NaNO_2$ varying from 2.5 - 50 mg/kg show transplacental passage of the chemical with the production of MetHb in the fetuses.

Rats born of dams chronically exposed to nitrites in drinking water during the gestation period showed high mortality rates and poor growth and development as compared to controls.

Mice chronically exposed to 1,000-2,000 mg/l $NaNO_2$ in their water showed lowered motor activity as compared to controls.

Rats with electrodes implanted on the cortex were chronically exposed to nitrites in their drinking water in concentrations ranging from 100-2,000 mg/l of $NaNO_2$. All experimental groups showed major brain electrical activity changes as shown by E.E.G. recordings after two weeks as compared to their own controls and the control group. On cessation of exposure to nitrites after 2 months, brain electrical activity changes in E.E.G. recordings remained, suggesting some form of irreversible damage.

The findings of these small scale preliminary toxicological studies are being rechecked in more extensive tests, but, if found to be correct, would suggest that standards for allowable concentrations of nitrite and nitrate in food and water should be re-examined in light of possible toxic effects on man.

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