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NITRATES/NITRITES

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REFERENCE NUMBERS 602 - 1096

SET 2 OF 5

wird, ob Tauben künstliche Landmarken mit Haftschalen noch identifizieren können, sind im Gange.

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Activation of γ-Glutamyl Transpeptidase in Rat Liver by Toxic Doses of Dimethylnitrosamine

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The activation of γ-glutamyl transpeptidase (glutathionase) in rat liver is easily detectable within a few weeks of feeding with the carcinogens 3′-methyl-4-dimethyl aminoazobenzene [1] or 2-acetamidofluorene, but their obscure mode of action, dependence on the pituitary and relatively slow effects make it difficult to study how these hepatocarcinogens activate GT-ase.

Dimethylnitrosamine (DMN) offers greater promise. Its carcinogenicity A. appears to be connected with the methylation of the imidazole groups of guanine residues in nucleic acids (7-methylguanine) or of histidine residues in proteins by the intermediate diazoalkane [2, 3] and B. does not depend on the pituitary [4]. The question as to whether a rapid activation of GT-ase in rat liver could be elicited by DMN, perhaps by a single dose, was confirmed by two experiments (Fig. 1). In Experiment 1, male Sprague Dawley rats (av.w. 400 g) were injected (i.p.) six times at 8 and 15 h intervals with 5 mg DMN in 1 ml physiological saline. One third of the animals died after receiving the total dose, showing hemorrhagic lesions in the liver and bloody ascites in the abdominal cavity, but a progressive increase in the level of GT-ase occurred before any macroscopic pathological changes in liver or other organs became visible. In Experiment 2, a single dose of 30 mg DMN was given; 16—26 h after administration, an increase of GT-ase was recorded which at its maximum

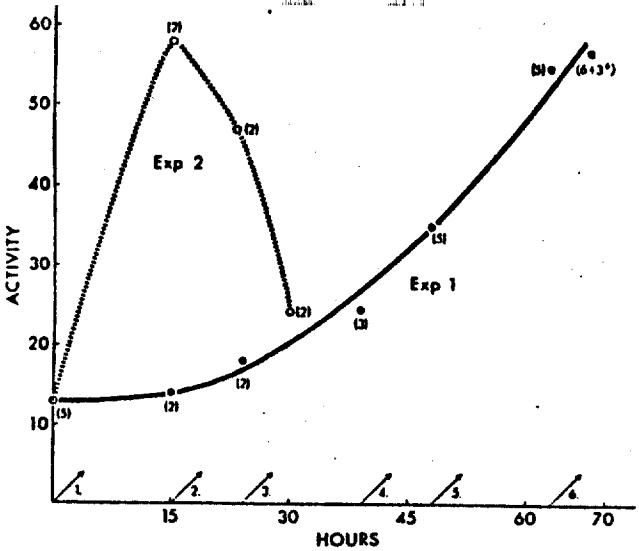

Fig. 1. Activity measured by the modified method of Orlowski and Meister [5]. — 10 mg acetone powdered liver incubated for 30 min at 37 °C in a mixture containing 22 μmoles glycylglycine, 5 μmoles L-γ-glutamyl-p-nitroanilide and tris buffer (0.1 M, pH 7.6) up to total volume of 4 ml. Reaction stopped by heating to 100 °C for 2 min. After centrifugation the amount of liberated p-nitroaniline measured as the difference in optical densities at 410 mμ between samples with and without substrate. Activity expressed in arbitrary units, corresponding to optical densities. (10 units equivalent to O.D. = 0.100). Number in brackets = number of animals.

corresponded to that elicited by a similar amount of DMN in Experiment 1. In animals showing an early maximum (16—20 h) of GT-ase activity no macroscopic pathological changes were noted. Thus, the activation of GT-ase by DMN may be one of the early manifestations of the alkylating effect of this carcinogen.

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Studium Generale. Zeitschrift für interdisziplinäre Studien/Journal for Interdisciplinary Studies. Vol. 24. Fasc. 2. 288 S. Berlin-Heidelberg-New York: Springer 1971. Einzelheft DM 10.—.

Die Zeitschrift legt im Heft 2 ihres 24. Jahrgangs 1971 vortreffliche Beiträge zu naturwissenschaftlichen Grundfragen vor: W. Lenz: Die genetische Grundlage der menschlichen Existenz; H. v. Bracken: Ein biologischer Aspekt der Intelligenz; G. Ziegelmayer: Rassengleichheit - Rassenmischung? Die anthropologischen Grundlagen; G. Osche: Mechanismen der Evolution und die Mannigfaltigkeit der Organismen; R. Bergius: „Begabung" als Ergebnis von Lernvorgängen?; W. Simonis: Zerstörung des biologischen Gleichgewichts; H. Querner: Ideologisch-weltanschauliche Konsequenzen der Lehre Darwins; W. Fuhrmann: Aktuelle Selektionsmechanismen; H. W. Jürgens: Die Großstadt als Lebensraum des Menschen. — Das Heft vermag vielfältige Anregungen zu geben.

F. L. Boschke (Heidelberg)

Principles of Holography. By Howard M. Smith. New York-London-Sydney-Toronto: John Wiley 1969. 239 S., 94 s.

Die seit 1962 sehr lebhaft anwachsende Original-Literatur über Holographie ist über einen sehr breiten Kreis verschiedenartiger und z.T. erst neu eingeführter Fachzeitschriften verteilt und daher für manchen Interessenten nur schwer zugänglich. Es kommt hinzu, daß, wie auf allen sich neu entwickelnden Arbeitsgebieten, auch in der Holographie die Originalarbeiten sehr häufig den Charakter von „Letters" tragen, also Mitteilungen von Beobachtungen und Gedanken sind, aber keine zusammenhängende Darstellung vermitteln. Gerade darin liegt das Bestreben und der Erfolg dieses Buches: Das Prinzip, die Möglichkeiten und die Grenzen der holographischen Bildspeicherung und -wiedergabe in geschlossener Form darzustellen. An Vorkenntnissen setzt der Autor nur ein Verständnis der optischen Abbildung und Schulkenntnisse der Mathematik voraus. Dennoch werden die einzelnen Gedankengänge nach qualitativer Erläuterung der

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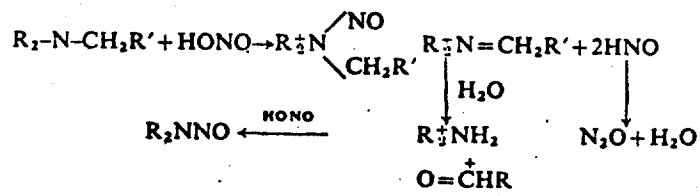
BIOLOGICAL SCIENCES

Formation of N-Nitrosodimethylamine from Naturally Occurring Quaternary Ammonium Compounds and Tertiary Amines

THE potential for the formation of N-nitrosamines in the human environment, either during the processing of foods or *in vivo* from nitrate and/or nitrite, and precursor amines has received considerable attention in recent years. Traditionally only secondary amines were thought to undergo N-nitrosation reactions. While secondary amines *per se* are not common in biological systems, tertiary amines and quaternary ammonium compounds do occur in plant and animal tissue. The possible formation of N-nitrosamines from these compounds must be considered because recent reports have appeared on the nitrosation of trimethylamine[1,2] and trimethylamine oxide[1] in connexion with the possible formation of N-nitrosamines in fish products cured with nitrate. The nitrosative cleavage of tertiary amines is not new and has been described before[3,4].

We are reporting the formation of dimethylnitrosamine (DMNA) from several quaternary ammonium compounds and some of their related tertiary amines in conditions simulating those found in comminuted meat products. The N-containing compounds (4.56 mmol) were reacted with $NaNO_2$ (22.8 mmol) in *p*H 5.6 buffer for 4 h at 78° C, then extracted with CH_2Cl_2; the extracts were dried with anhydrous Na_2SO_4 and concentrated in a Kuderna-Danish concentrator. DMNA was determined by a Varian aerograph model 1520 gas-liquid chromatograph using FID. The GLC conditions are as follows: column—2 feet × 1/8 inch absorbance 100–120 mesh Chromosorb 101'; flow rates (cm^3 min^{-1})—He 102, air 429, H_2 57; temperatures (°C)—detector 218; injector port 185°, column isothermal 150°. A gas-liquid chromatography–mass spectroscopy (GLC-MS) system with a DuPont model 492 mass spectrometer was used for all confirmation of identification. The results are shown in Table 1. DMNA is produced from tetramethylammonium chloride at almost the same level as from trimethylamine. The quaternary ammonium compound is demethylated, as shown by the accumulation of trimethylamine in the absence of nitrite. Smith[4] has proposed a pathway for the N-nitrosation of tertiary amines that appears to be applicable in this case:



The naturally occurring quaternary ammonium compounds, neurine, carnitine, betaine, choline and acetylcholine, also yield DMNA when reacted with $NaNO_2$ although at a considerably lower level than the tetramethylammonium compound. The results reflect the ability of the quaternary compound to dealkylate to trimethylamine or a mixed amine containing the Me_2N– group and another substituent. N,N-Dimethylglycine and its methyl ester are tertiary amines derived from the rearrangement of betaine in acidic conditions; 2-dimethylaminoethanol and its acetate derivative are similarly related to choline and acetylcholine respectively. The DMNA yield from the mixed alkyl tertiary amines is two to five times that obtained from trimethylamine and 4×10^3 to 2×10^4 times the DMNA formed from the parent quaternary ammonium compounds. In fact, 2-dimethylaminoethyl acetate forms DMNA almost one-half that formed by dimethylamine. It is interesting to note that larger quantities of DMNA are produced from the esters of the quaternary and tertiary compounds than from the parent compounds.

It is significant that DMNA can be formed from quaternary ammonium compounds, albeit at low levels, in the conditions described. We can therefore conclude that although quaternary ammonium compounds may represent a potential source of nitrosamine, the tertiary amines and free secondary amines present in food products are of greater concern.

Table 1 Formation of N-Nitrosodimethylamine from Naturally Occurring Quaternary Ammonium Compounds and Tertiary Amines

Structure	Name	mg DMNA mol amine*
$(CH_3)_2NH.HCl$	Dimethylamine . HCl	7,108
$(CH_3)_3N.HCl$	Trimethylamine . HCl	643
$(CH_3)_4\overset{+}{N}Cl^-$	Tetramethylammonium chloride	433
$(CH_3)_3\overset{+}{N}-CH=CH_2Cl$	Neurine chloride	133
$(CH_3)_3\overset{+}{N}-CH_2CH_2OAcCl^-$	Acetylcholine chloride	0.897
$(CH_3)_3\overset{+}{N}-CH_2CH_2OHCl^-$	Choline chloride	0.160
$(CH_3)_3\overset{+}{N}-CH_2CO_2^-$	Betaine	0.159
$(CH_3)_3\overset{+}{N}-CH_2CH(OH)CH_2CO_2HCl^-$	Carnitine chloride	0.044
$(CH_3)_2N-CH_2CH_2OAc$	2-Dimethylaminoethyl acetate	3,524
$(CH_3)_2N-CH_2CH_2OH$	2-Dimethylamino-ethanol	1,179
$(CH_3)_2N-CH_2CO_2Me$	Methyl ester of N,N-dimethylglycine	3,004
$(CH_3)_2N-CH_2CO_2H$	N,N-Dimethylglycine	1,405

* Quantification based on the use of cyclohexanone as an internal standard.

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N-Dimethylnitrosamine in Tobacco Smoke Condensate

THE possible presence of N-nitrosamines in cigarette smoke and/or condensate has been of interest for some years. Druckrey and Preussmann[1] postulated that N-nitrosamines could be produced in burning tobacco, but the difficulty of measuring these compounds prevented the testing of this postulate. We have developed[2] a gas chromatographic method for the selective detection of N-nitrosamines, and now report its application to the analysis of cigarette smoke condensate. We have identified N-dimethylnitrosamine (DMN) in cigarette smoke condensate, and shown that certain tobaccos grown in different conditions vary widely in their content of DMN.

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EFFECT OF SODIUM NITRITE CONCENTRATION ON N-NITROSODIMETHYLAMINE FORMATION IN FRANKFURTERS

INTRODUCTION

UNTIL 1926 nitrate salts were used in the United States in the fixing of color of meat products by the "corning" or curing process. Haldane (1901) demonstrated that this function takes place through the reduction of nitrate to nitrite. As a result of the work of Kerr et al. (1926), the Department of Agriculture permitted the use of sodium nitrite in meat curing. In addition to color fixation, nitrite serves to develop the characteristic flavor of the cured meat products (Brooks et al., 1940; Cho and Bratzler, 1970) and preserve them against bacterial spoilage (Silliker et al., 1958). In general cured meat products have enjoyed a long period of safe consumption. Concern over the use of food additives as potential public health hazards has increased in recent years. With it, the use of sodium nitrite has been questioned. One of the main reasons for this is that sodium nitrite, under acidic conditions, reacts with many amines, particularly secondary amines to form N-nitrosamines which may be tumorigenic or carcinogenic (Druckery et al., 1967).

The presence in meat products of free amines or amine precursors such as proteins, amino acids, phospholipids and other compounds poses a situation in which they may possibly react with nitrite to form N-nitrosamines. There have been reports of finding a nitrosamine in cured meat products (Möhler and Mayrhofer, 1968; Ender and Ceh, 1968; Freimuth and Gläser, 1970). The methods employed may have lacked the necessary sensitivity and selectivity to give positive identification. Other workers (Fazio et al., 1971; Fiddler et al., 1971; Telling et al., 1971) have not confirmed the presence of nitrosamines in various cured meat products at a level greater than 25 ppb. However, recently the presence of dimethylnitrosamine (DMNA) in three frankfurter samples, one of which contained 80 ppb, has been demonstrated (Wasserman et al., 1972). Studies to date indicate that DMNA occurrence is random and no adequate explanation is available. This could be due to a number of variables which are involved in the preparation of cured meat products e.g., the age and condition of the meat, the concentration of nitrite and the type and amounts of other ingredients used, the actual processing conditions, and subsequent time and temperature of storage. In view of the lack of information available on the effect of these processing factors on nitrosamine formation, a study was undertaken to determine the effect of nitrite concentration on DMNA formation in frankfurters containing sugar and salt, but without nitrate, ascorbate and delta-gluconolactone. The latter three ingredients are sometimes used commercially.

EXPERIMENTAL

Frankfurter preparation

Fresh beef and pork were trimmed to desired fat levels, 5 and 17% respectively, and ground through a 3/4-in. plate. The beef, pork and pork fat were stored under vacuum in Cryovac bags at 0°F. The day prior to processing, the frozen portions were removed from the freezer and allowed to thaw. The meat was ground through a 5/8-in. plate, then a 3/16-in. plate. A formulation based on the results of analyses of raw materials was used to produce frankfurters containing 9–10% added moisture and 30% fat. The weights (kg) of the components of the emulsion were: lean beef–3.934; lean pork–2.514; pork fat–3.552; ice–2.287; salt–0.251; and sugar–0.198. Nitrate, ascorbate and delta-gluconolactone were omitted from the formulation to reduce the number of variables that may effect nitrosamine formation. The ingredients were comminuted in a high-speed Stephan Universal Schnellkuter Type USF25 chopper equipped with a thermocouple and a sealable Plexiglass plate. Under 110 mm Hg vacuum, the initial temperature of the mixture was about 35°F. The chopping was continued up to a temperature of 60°F, taking approximately 5 min. The amount of sodium nitrite required was weighed, dissolved in 15 ml distilled water, and mixed thoroughly with 1.274 kg emulsion (equivalent to 1 kg of meat) using a Hobart Model N-50 mixer, to give the following levels of sodium nitrite added in ppm with respect to meat (lean plus fat): 150, 750, 1050, 1500 and 2500.

The emulsion was then stuffed into size 23 Nojax frankfurter casings using a screw-type stainless steel sausage stuffer having a capacity of 500g. The franks were linked using a Model MF Ty Linking Machine and cooked in a Dry-Sys Smokehouse using a 90-min commercial program of increasing heat and controlled humidity: 1/2 hr dry bulb (DB) 130°F, wet bulb (WB) 0; 1/2 hr DB 150°F, WB 130°F; 1/2 hr DB 170°F, WB 150°F. A light smoke was generated in a Mepaco apparatus and introduced into the smokehouse for the entire period. After the franks had reached an internal temperature of 160°F (about 2 hr), one batch containing different levels of added sodium nitrite was removed and the remainder was heated and smoked for an additional 2 hr (DB 170°F, WB 150°F). These frankfurters had an average internal temperature of 160°F. After removal from the smokehouse, the frankfurters were immersed in an ice-water bath for 3 min, dried, weighed and stored overnight at 36°F. A sample of the franks processed normally for approximately 2 hr at the 150 ppm level was analyzed according to AOAC procedures and found to contain 53.40% moisture, 29.42% fat and 14.42% protein. The remaining franks were then vacuum packaged, frozen and kept in a freezer at 5°F until analyses were carried out.

When needed, the frankfurters containing different levels of sodium nitrite were thawed, ground, mixed and an aliquot taken for analyses. The residual nitrite was determined by the AOAC method: the average loss in sodium nitrite in the 2-hr and 4-hr processed frankfurters was 48.6% and 55.4% respectively.

Table 1—The effect of sodium nitrite concentration on dimethylnitrosamine formation in frankfurters

	Processing time			
	2 Hr		4 Hr	
$NaNO_2$ added mg/kg meat	$NaNO_2$ residual mg/kg	DMNA μg/kg[a]	$NaNO_2$ residual mg/kg	DMNA μg/kg[a]
150	67	trace	53	trace
750	361	3	310	8
1050	574	8	473	12
1500	811	10	724	14
2500	1386	19 MS	1345	19 MS

[a]Corrected using recovery of sample with 20 μg/kg added; MS-confirmed by mass spectrometry.

Analytical procedures

The methodology employed for the determination of DMNA in frankfurters is a modification of the procedure described by Howard et al. (1970). The method is as follows: the ground frankfurter samples are subjected to extraction with methanolic potassium hydroxide followed by distillation from aqueous alkaline solution. The distillate is extracted with methylene chloride. The combined extracts are washed with base and dried over anhydrous sodium sulfate, concentrated and subjected to column chromatography using Florisil acidified with hydrochloric acid. After the column is washed with hexane, the sample is eluted with methylene chloride, concentrated and the DMNA present determined by GLC. The average recovery of DMNA in an aliquot of the same sample with 20 ppb added was 76%.

Gas liquid chromatography

Varian Aerograph Model 1740-1 gas chromatograph equipped with two 9 ft × 1/8 in. O.D. stainless steel columns packed with 15% Carbowax 20M-TPA on 60–80 Gas-Chrom P, was conditioned overnight at 180°C, and installed for on-column injection. The standard flame ionization detector was modified for use as an alkali flame ionization detector by the use of a potassium chloride coated coil as described by Howard et al. (1970). The flow conditions used were: helium 58, hydrogen 45 and air 188 ml/min. Helium flow was monitored continuously with Matheson Model LF 100 mass flowmeter. Hydrogen flow and, to a lesser extent, air flow were adjusted slightly from time to time in order to maintain desired detector sensitivity. Electrometer range used was 10^{-12} amp fsd. Injector port and detector temperatures were 190 and 250°C, respectively. Column temperature was 115° isothermal for routine analyses involving only DMNA.

GLC-mass spectrometric analysis

The analytical procedure described above, which used 25g of frankfurter, was repeated as needed to provide sufficient DMNA for confirmation of identity by mass spectrometry. An identical gas-liquid chromatograph equipped with a flame ionization detector and column was used as for the determination of DMNA. A column temperature of 115°C was used with injector port and detector temperatures of 200° and 230°, respectively. Helium, at a flow rate of 25 ml/min, was used as the carrier gas. The hydrogen and air flow rates were 40 and 335 ml/min, respectively.

The column effluent was split approximately 1:1 and passed into a DuPont Model 21-492 mass spectrometer equipped with a jet-type separator via an inlet line heated at 200°C. The mass spectra were obtained at an ionizing voltage of 70 ev and an ion source temperature of 200°C using a CEC model 5-124A recording oscillograph with a linear scan rate of 20 sec/decade.

RESULTS & DISCUSSION

A COOKING PROCESSING study was performed three times under the same 2- and 4-hr processing conditions covering a wide range of sodium nitrite concentrations added to frankfurter emulsion. Analyses of the frankfurters for DMNA yielded similar values for all of the studies. Representative data from one of these experiments is shown in Table 1.

Ideally the identification of DMNA in natural products should be unambiguous. Where very small amounts of DMNA are encountered, confirmation may be accomplished by mass spectrometry. In the present study, however, DMNA concentrations of less than 10 μg/kg in frankfurter samples prepared as described were insufficient for confirmation. Treating larger amounts of frankfurter and/or concentrating multiple extracts to obtain sufficient material resulted in the presence of artifacts which interfered with mass spectral interpretation. Therefore, a minimum level of DMNA 10 μg/kg was considered significant. When DMNA was not characterized by mass spectrometry, identity was based only on its GLC retention time. In the discussion of the results, the term "apparent" DMNA is used to indicate levels at which mass spectral confirmation could not be made.

The U.S. Code of Federal Regulations (1971) limits the quantity of sodium nitrite that may be added to comminuted meat products, including frankfurters, to 156 mg/kg (1/4 oz per 100 lb chopped meat). At levels of up to about five times this permissible concentration no significant amount of DMNA was observed when the normal 2-hr processing procedure was used; however, a DMNA concentration bordering on significance, i.e., 8 μg/kg meat, was obtained on heating an additional 2 hr. Concentrations of apparent or confirmed DMNA of 10 μg/kg and greater were found in frankfurters made with levels of sodium nitrite of 1500 mg/kg or higher, regardless of processing time. In addition to 2500 mg sodium nitrite per kg meat, levels of 3075 and 4250 mg/kg were also studied in another series of the same experiment, and concentrations of DMNA up to 60 μg/kg were found for the 4-hr processed frankfurters and confirmed readily by mass spectrometry. Sodium nitrite concentrations between 150 and 750 ppm sodium nitrite, for both the 2- and 4-hr processing times, gave negligible amounts of DMNA.

For most of the levels of added nitrite at which apparent or confirmed DMNA could be demonstrated, there was an increase in DMNA concentration when the frankfurters were treated an additional 2 hr. The extra heating period is not normally used commercially; however, its use here represents an extreme in experimental conditions.

Residual nitrite analyses showed that about one-half of the sodium nitrite added was still present after processing. Commercially prepared frankfurters made with 156 mg/kg sodium nitrite usually have residual nitrite levels ranging from 10–30% of the added material. In previous studies, we have observed that more nitrite is lost when processing in combination with ascorbate and nitrate, and the concentration of nitrite continues to decrease upon storage. Therefore the significance of the higher values found in this study cannot be evaluated at this time.

CONCLUSIONS

FRANKFURTERS conforming to U.S. Federal Regulations contain a concentration of sodium nitrite insufficient to produce, under the processing conditions described in this paper, amounts of DMNA measurable by our procedures. However, under laboratory conditions DMNA can be formed in a meat product. Concentrations of sodium nitrite up to about ten times the legal limit may lead to significant nitrosamine formation. This study does not explain why DMNA can be isolated from some commercial cured meat products containing the permissible level of sodium nitrite. Many variables have been suggested or can be conceived as contributing to DMNA formation in the products, e.g., localized high concentrations of nitrite in emulsions due to inadequate homogenization during processing, the effect of other cure ingredients, storage conditions, etc. Further work is needed to establish the contributions, if any, of these variables.

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Reference to brand names or firm name does not constitute endorsement by the U.S. Dept. of Agric. over others of a similar nature not mentioned.

DETERMINATION OF NITRITE AND NITRATE IN MEAT PRODUCTS

By M. J. FOLLETT and P. W. RATCLIFF

A rapid method, of improved accuracy, for the determination of nitrate and nitrite in meat products is described. Nitrate is determined by reduction to nitrite with cadmium, and subsequent development of the dye Orange I obtained with 1-naphthol/sulphanilic acid reagent. Nitrite present does not interfere and is separately determined with the same colorimetric reagent. In meat, a range of concentrations of nitrate and nitrite up to the equivalent of 2000 p.p.m. sodium nitrite may be determined.

Introduction

Several methods have been proposed for the determination of nitrite and nitrate in foodstuffs. Although nitrite may be readily and accurately determined in these materials, the methods for nitrate determination cannot be regarded as entirely satisfactory, especially when they are applied to the analysis of cured meat.

The determination of nitrate with phenoldisulphonic acid[1–3] is unreliable in the presence of carbohydrate and other organic matter, furthermore chloride and nitrite ions interfere. Colour stability is poor and sensitivity is limited to 5–60 μg. of N.[3] The brucine method[4–7] requires the use of high concentrations of strong acids, and the toxicity of the brucine reagent is another disadvantage. The nitration of brucine is not a completely reliable reaction, and the inconsistency of the standard curve makes it necessary to carry out a frequent re-calibration.[7] The 3,4-xylenol method[8, 9] requires elimination of chloride and nitrite, and organic matter interferes. The method is laborious and time-consuming, and the same criticism applies to the gasometric method[10] and to methods which depend upon reduction of nitrate to ammonia. Reduction to nitrite with zinc dust[11, 12] is not dependable as the results have been found to be materially affected by factors such as the particle size and the source of supply of the dust, and variations can also occur with the use of different production batches of the metal (unpublished work of the present authors).

Grau & Mirna[13] developed a method in which cadmium is used to reduce nitrate quantitatively to nitrite, the latter being estimated colorimetrically as the red azo dye obtained by reaction with sulphanilic acid and 1-naphthylamine, in acetic acid solution. This may perhaps be regarded as the most generally suitable method for the analysis of nitrate in cured meat. Experience with the analysis of cured meat in this laboratory has indicated, however, that this method may be troublesome under the conditions sometimes encountered.

Investigation has shown that the difficulties and disadvantages of the method of Grau & Mirna can be obviated by introducing appropriate modifications, which are detailed and discussed in this paper, and a modified and simplified form of the Grau & Mirna method is presented. Owing to its improved accuracy, greater speed and convenience in use, the method here described can be recommended for use in the routine determination of nitrite and nitrate in cured meats.

Experimental

Reagents

(A) Saturated solution of potassium aluminium sulphate.

(B) Buffer solution, pH 9·6–9·7. To 500 ml. of distilled water add 20 ml. of conc. HCl. Mix and then add 50 ml. of conc. aq. ammonia (sp. gr. 0·880) and make to 1 litre.

(C) Orange I reagent. Warm 360 ml. of distilled water and 50 ml. of glacial acetic acid to 50° and pour into a dark glass reagent bottle (600 ml.) containing 0·25 g. of powdered sulphanilic acid. Shake until dissolved, then add 0·20 g. of 1-naphthol and dissolve by shaking. Cool the solution to room temperature, and add 90 ml. of 10% aq. ammonia solution. The pH of this reagent should be 4·0 ± 0·05.

(D) Bromocresol purple. A solution containing 0·05% of the indicator in 20% ethanol.

Nitrite-free distilled water is used throughout.

Standard nitrite curve

Make a solution containing 0·25 g. of $NaNO_2$ in 500 ml. Take suitable aliquots (1–40 ml.), place in a series of 200-ml. flasks with 5 ml. of reagent (B), and make to the mark. Take 20 ml. of each solution and dilute to 100 ml. with distilled water. Transfer 5 ml. to a 20-ml. test tube and add 10 ml. of reagent (C) and 5 ml. of water. Place in a water bath 25–30° for 30 min. (in light) to develop the colour. Measure the extinction in a 1-cm. glass-cell at 474 mμ. (Hilger & Watts 'Uvispeck' spectrophotometer) against a blank consisting of 10 ml. of reagent (C) plus 10 ml. of water. Plot the values for extinction against the concentrations of nitrite. These concentrations, covering the range 2·5–100 μg. of $NaNO_2$ in the final solution obtained for colorimetric measurement, can be conveniently expressed in terms of the corresponding concentrations, 50–2000 p.p.m. of $NaNO_2$, in a 10-g. sample of meat.

Preparation of meat extract

Ten g. of meat are macerated with 70 ml. of hot distilled water (70°) + 5 ml. of reagent (B) in a M.S.E. Homogeniser for 15–30 sec., with addition of antifoam (0·1 ml. of nonanol). Filter through a small wire-wool plug into a 200-ml. graduated flask and wash the residue with hot water (70°). Place the flask on a water bath and raise the temperature to 80–90° to coagulate the protein, then add not more than 0·5 ml. indicator (D).* Add carefully the precipitating reagent (A) from a burette until the purple colour changes to a definite grey. The pH at this point should be between 5·5–6·5. Cool the solution carefully. When cool, the purple colour will reappear, and a further small addition of reagent (A) will be necessary to restore the grey colour of the required end-point. Make up to the mark with distilled water and filter through a No. 42 Whatman paper. The filtrate (solution E), is used for the determination of nitrite and nitrate. The final pH should be between 5·5 and 6·5.

Nitrite determination

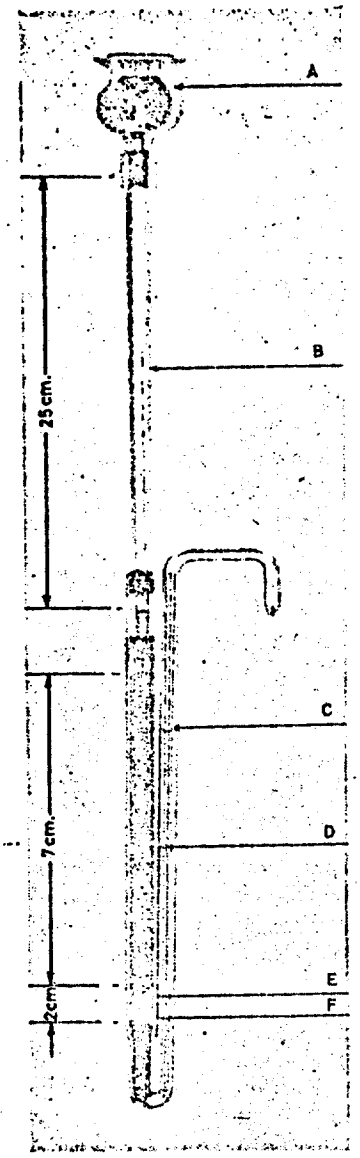
Take 20 ml. of the protein-free filtrate (E) in a 100-ml. flask and add 5 ml. of reagent (B). Make up to the mark with distilled water. Transfer 5 ml. to a 20-ml. test-tube and add 10 ml. of reagent (C) and 5 ml. of water. Proceed to develop the colour and determine the extinction as for the standard nitrite curve. By reference to the standard curve the concentration of nitrite can be read off directly as p.p.m. $NaNO_2$ in the meat.

Nitrate determination

Preparation of the cadmium column.—Place zinc rods in a 20% solution of cadmium sulphate. Remove the deposit of cadmium after 3–4 h. and place in a flask. Cover with dilute HCl and disintegrate for 1–2 sec. in the M.S.E. homogeniser. Wash with distilled water. A spongy material is obtained which can readily be washed into the column of the apparatus depicted in Fig. 1. The dimensions of the capillary inlet tube, which is attached to the top of the column, have been chosen to ensure that the flow of solution through the column is maintained at the optimum rate of ~5 ml./min. As it is essential to avoid drawing air into the column, the outlet capillary tube has been designed to provide a safeguard against the column running dry. Prior to a nitrate determination the column is washed with 25 ml. of dilute HCl (0·1N), followed by 50 ml. of distilled water, and finally with 25 ml. of diluted buffer reagent (B) (diluted 1 : 9 with distilled water). It is convenient to set up a battery of several columns for routine analyses as shown in Fig. 2.

Analysis of meat.—Take 20 ml. of the protein-free filtrate (E) and add 5 ml. of reagent (B). Pour the solution into the reservoir of the apparatus and pass through the column, rejecting the first 10 ml. of eluate. Wash the column with distilled water and collect 100 ml. of eluate in a graduated cylinder and shake to mix. Take a 5-ml. aliquot in a 20-ml. test tube, add 10 ml. of reagent (C) and 5 ml. of water, and proceed as in nitrate determination for development

* In dealing with materials such as brines, which contain smaller quantities of protein, the indicator added can be reduced to 0·1–0·2 ml.



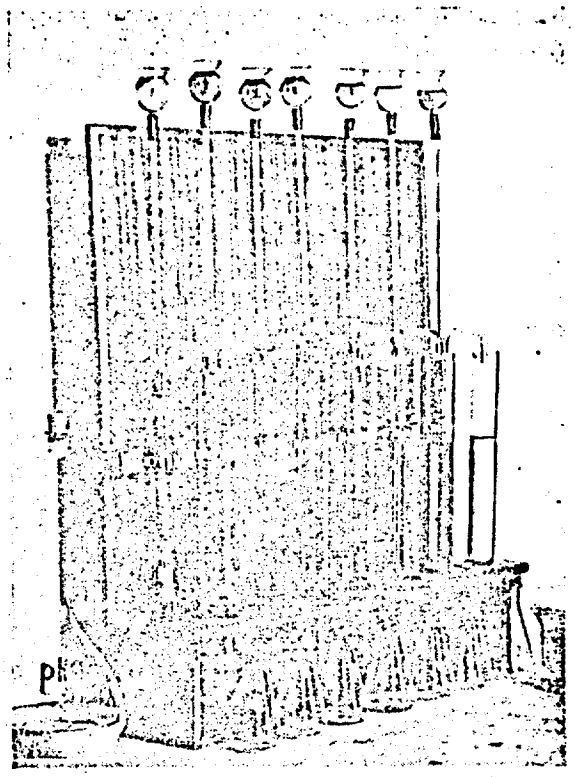


FIG. 2.—*Battery of cadmium columns as used in the laboratory*

FIG. 1 (left).—*Apparatus for nitrate reduction*

A 25-ml. reservoir
B Inlet capillary; 0·4 mm. i.d., 5–6 mm. o.d.
C Outlet capillary; 2 mm. i.d., 7–8 mm. o.d.
D Cadmium column; 1·2 cm. i.d., 1·4–1·5 cm. o.d.
E Silica granules; depth 1 in.
F Glasswool plug; depth 1 in.

of the Orange I colour. Read off total nitrite from the standard curve. After subtraction of the value for nitrite obtained in a separate nitrite analysis, the factor 1·465 is used to calculate p.p.m. KNO_3 in the meat.

Determination of the meat blank

The blank given by the meat sample can be found by removing nitrite and nitrate in a column of anion-exchange resin, Deacidite FF. The apparatus used is similar to that for the nitrate reduction, except that Deacidite resin replaces cadmium in the column, and the vertical capillary is of larger bore (2 mm.)

Before use, a saturated salt solution is passed through the column. This is then washed with distilled water until the eluate is free from chloride. To 30 ml. of the protein-free meat filtrate (E), add 2 drops of conc. HCl and pass through the column, collecting as soon as the eluate becomes acid (as shown by a wide-range indicator paper). When the solution has passed into the column, wash through with distilled water. Collect 30 ml. of eluate and to this add 2 drops of conc. aq. ammonia and shake well. Take 20 ml. of this solution and proceed as for nitrate determination, adding 5 ml. reagent (B) before passing through the cadmium column.

Reagent blank

The blank obtained on the reagents alone, after passing through the cadmium column, is equivalent to 5 p.p.m. of KNO_3. This should be added to the meat blank if the highest accuracy is required in calculating nitrate results.

Results and discussion

The protein precipitant

In the method of Grau & Mirna zinc hydroxide is used to precipitate protein in the aqueous extract obtained from the meat sample. The precipitation of the heated extract is effected by adding borax and zinc sulphate, the latter bringing the pH of the solution to 6·0–6·5. The pH is not, however, precisely controlled in this range, and a significant amount of zinc ions may remain in solution so that hydroxide tends to be precipitated at a later stage. Cloudy colloidal solutions may therefore be obtained before, during or after passage of the solution through the cadmium column. This is a distinct disadvantage of the Grau & Mirna procedure, which is avoided in the present method by use of potassium aluminium sulphate as the precipitant, under carefully controlled conditions of pH. The filtered extract obtained from the sample of meat is heated, and buffered so that addition of saturated potassium aluminium sulphate precipitates aluminium hydroxide, bringing the pH of the solution near the isoelectric point of the soluble protein, which is therefore effectively precipitated from solution. Care must be exercised in adding the correct amount of precipitant so that the pH is brought within the limits 5·5–6·5. Above this pH range the precipitation of protein is incomplete, whereas below it there will occur some loss of nitrite. Bromocresol purple is a suitable indicator for ensuring that the required pH is obtained. The pH of the hot solution increases on cooling, and it is necessary to add a further small quantity of the precipitant to the cooled solution, to obtain the required end-point. Under the conditions specified for this method, the pH of the cooled, protein-free filtrate should be between 5·5 and 6·5.

Nitrite and nitrate ions, which are readily washed from heat-coagulated protein, are not adsorbed to any appreciable extent by the residue of aluminium hydroxide.

Nonanol is the anti-foam agent recommended for use; silicones are to be avoided as they have a tendency to form stable colloidal suspensions.

Reduction of nitrate

The efficiency of the cadmium column in reducing nitrate is strongly dependent upon pH; the nitrate solution must be buffered at pH 9·5–9·7 to ensure complete reduction to nitrite. (See Fig. 3.)

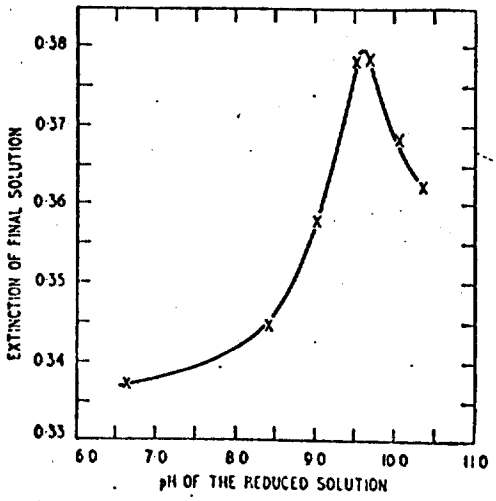


FIG. 3.—*Effect of pH on reduction of nitrate in the cadmium column*

Extinction values of the final solutions (containing the equivalent of 25 μg. of KNO_3) measured at 474 mμ

The Griess reagent

The original Griess reaction for nitrite[14] made use of a red azo-dye produced by coupling 1-naphthylamine with diazotised sulphanilic acid in sulphuric acid solution. In Ilosvay's modification,[15] which is adopted by Grau & Mirna,[13] the reagents were prepared in acetic acid solution, which greatly increased the rate of colour development. The coupling agent, however, contains a primary amine group which is capable of being diazotised, and may therefore couple with excess reagent.[16-19] The reaction is thus not completely reliable. The present authors have found that there is a tendency for the red azo-dye to change colour and to precipitate when the concentration of nitrite in the final solution, obtained by the method of Grau & Mirna, exceeds 75 μg. of $NaNO_2$. This corresponds to 750 p.p.m. of $NaNO_2$. Above this concentration, a further dilution would have to be made, after the preliminary analysis, or alternatively, the nitrite must be destroyed before proceeding to the analysis of nitrate. These authors[13] state that the latter procedure should be used when the nitrite content of the meat sample exceeds 50 p.p.m., and they suggest that Fischer esterification method[20] for this purpose.

This limitation, imposed by the unreliability and insolubility of the red azo-dye, is obviated in the present method by using 1-naphthol as the coupling agent. In the presence of diazotised sulphanilic acid, 1-naphthol couples to give the azo-dye Orange I.[21, 22] The greater reliability and solubility of Orange I is such that with the use of 1-naphthol a calibration curve can be prepared for concentrations of nitrite ranging from 0 to 2000 p.p.m. of $NaNO_2$ in meat—a range wide enough to cover all types of meat products likely to be met with in practice. It has been found that the dye will not precipitate even in the presence of as much as 4000 p.p.m. of $NaNO_2$.

The azo-dye, Orange I

The development of the colour, Orange I, is powerfully influenced by the pH of the solution. This effect of pH is shown graphically in Fig. 4, in which the extinction of the solution, measured at 474 mμ, is plotted against pH. At pH 4·0 the maximum development of colour intensity occurs, but this decreases quite steeply as the pH rises or falls from this critical value.

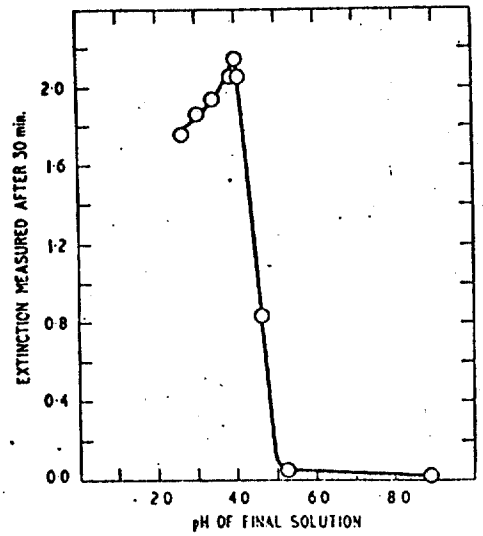


FIG. 4.—*Effect of pH on the development of Orange I*

Extinction read at 474 mμ after 30 min. at 25° (100 μg. of $NaNO_2$)

Temperature influences both the rate of development of colour, and the final value of the colour intensity obtained. It is recommended that temperature is standardised at 25–30° during development of the colour. Fig. 5 shows the relation between extinction at 474 mμ, and the time for maximum colour development at two different temperatures. At 25° the maximum intensity is practically reached after 20 min., with only a slight increase occurring in the next 10 min., and then remaining steady. At 10° the maximum, at a lower level, is barely reached in 30 min. The colour when fully developed remains stable for at least 48 h. when the solutions are kept in the dark. In the presence of light the excess reagent develops a colour which interferes.

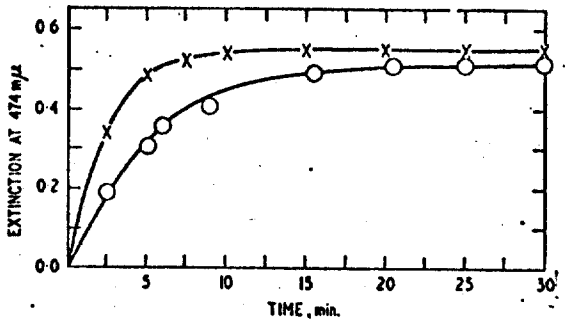


FIG. 5.—*Effect of temperature on the rate of colour development of Orange I at pH 4·0*
10° O 25° ×

The intensity of the Orange I colour, when developed under the conditions specified above, is measured in the spectrophotometer at a wavelength of 474 mμ. The extinction of the solution obeys Beer's Law over a wide range of concentrations at this wavelength and there is a linear relationship between concentration and extinction at 474 mμ for concentrations of nitrite within the range 0–2000 p.p.m. $NaNO_2$, calculated on the original sample taken. It is recommended that a separate curve with an enlarged scale is constructed for the range 0–250 p.p.m. sodium nitrite, in order to obtain higher accuracy when reading off these values for nitrite found. It is convenient also to construct curves for each of the concentration ranges 0–1000 and 1000–2000 p.p.m. sodium nitrite.

The value of the blank

In determining sodium nitrite in a meat sample, it is found that the value of the blank contributed by meat and reagents is insignificant. The analysis of nitrate, however, involves a reduction process which leads to the development of a reagent blank equivalent to approximately 5 p.p.m. of KNO_3, and to a meat blank which is also significant.

The *longissimus dorsi* muscle of fresh pork, for instance, contains substances which, after reduction of the protein-free extract in the cadmium column, react with Griess reagent. Under the conditions of the nitrate analysis a colour is developed which is equivalent to 13–30 p.p.m. of sodium nitrite. The nature of these colour-producing constituents in the meat is uncertain, although there is some indication that amino-acids such as glycine are involved.

Grau & Mirna[13] suggested that the nitrate values found, when their procedure is adopted for cured meats, should be corrected for a blank which they estimate as equivalent to 10 p.p.m. potassium nitrate (calculated on the meat). The values for the meat blank for different types of meat may, however, vary between 13 and 30 p.p.m. sodium nitrite (calculated on the same basis), and better accuracy will be obtained by correcting for the blank determined for each particular sample of meat. A direct determination of the meat blank can readily be made, using the protein-free meat filtrate from which nitrite and nitrate have been removed by passing the solution at pH 2·0 through a column of anion-exchange resin such as Deacidite FF.

Calibration of nitrite curve

In preparing the standard nitrite curve, the extinction was determined for solutions containing 2·5–100 μg. of $NaNO_2$ in the final solution for colorimetric determination. Twenty separately prepared solutions of nitrite were used for each of eight standard concentrations selected in this range, and the calibration curve was then constructed from the mean of the extinction values obtained for each concentration. These mean values are tabulated in Table I, which shows also the standard deviations and coefficients of variation about the mean of each set of 20 readings.

Accuracy of the method

Nitrite and nitrate were determined when present together in standard solutions containing the equivalent of either (1) 200 p.p.m. of $NaNO_2$ plus 500 p.p.m. of KNO_3, or (2) 200 p.p.m. of $NaNO_2$ plus 2000 p.p.m. of KNO_3, calculated on the basis of a 10-g. sample of meat. Ten solutions of each of these concentrations of mixed nitrite and nitrate were analysed, and the sodium nitrite found was 100% ± 2·5% of the known amounts present. Nitrate recovered

Table I

Calibration of the standard nitrite curve

Mean extinction values obtained, with standard deviations and coefficients of variation calculated for each value. Concentrations expressed as μg. of $NaNO_2$ in the final solution (20 ml.) for colorimetric measurement

Concentration of $NaNO_2$, μg.	Mean extinction $E_{1\,mc.}$ at 474 mμ	Standard deviation $\times 10^{-3}$	Coefficient of variation, %
2·5	0·058	±1·100	±1·910
5·0	0·115	±1·178	±1·490
12·5	0·275	±1·662	±0·604
25·0	0·549	±3·442	±0·627
37·5	0·823	±9·591	±1·165
50·0	1·101	±14·08	±1·278
75·0	1·645	±13·80	±0·839
100·0	2·165	±25·52	±1·179

(after subtracting the separately determined nitrite, and correcting for a reagent blank equivalent to 5 p.p.m. KNO_3) was 100% ± 2·8%.

Determinations were carried out on the *longissimus dorsi* of fresh pork to which was added sodium nitrite equivalent to 200 p.p.m. together with potassium nitrate equivalent to 500 p.p.m. In a total of 30 analyses, nitrite found was within 100 ± 2·0% of the known amount added (standard deviation ±1·586 and s.e. of the mean ±0·588 using 95% limits). Nitrate found, after correcting for both the meat blank and the reagent blank, was within 100 ± 1·3% (standard deviation ±4·199 and s.e. of the mean ±1·558 using 95% limits).

As little as 5 p.p.m. of sodium nitrite, calculated on the meat, can be determined. (This is equivalent to 0·25 μg. of $NaNO_2$ in the final 20 ml. of solution used for colorimetric measurement.)

Phosphate in amounts up to 5% (as PO_4^{3-}) in the meat does not interfere. Sugar and sodium chloride do not interfere when present in concentrations as high as 10% in the meat. Experience has shown that the columns will operate satisfactorily for at least 6 months with daily use. As there will be no visible signs of loss of efficiency apart from a slight darkening in colour of the cadmium, the columns should be periodically tested with a standard solution of potassium nitrate.

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Final revised pgs of 1972 Edition

(14)

POTASSIUM NITRATE

(621)

KNO_3 Mol. wt. 101.11

DESCRIPTION

Colorless, transparent prisms, or white granules or crystalline powder. It is odorless, has a salty taste, and produces a cooling sensation in the mouth. It is slightly hygroscopic in moist air. Its solutions are neutral to litmus. One gram dissolves in 3 ml. of water at 25°, in 0.5 ml. of boiling water, and in about 620 ml. of alcohol. A 1 in 10 solution gives positive tests for *Potassium*, page 928, and for *Nitrate*, page 927.

SPECIFICATIONS

Assay. Not less than 99.0 percent of KNO_3 after drying.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).
Chlorate. Passes test.
Heavy metals (as Pb). Not more than 20 parts per million (0.002 percent).
Lead. Not more than 10 parts per million (0.001 percent).
Loss on drying. Not more than 1 percent.

TESTS

Assay. Weigh accurately about 400 mg., previously dried at 105° for 4 hours, dissolve in 10 ml. of hydrochloric acid in a small beaker or porcelain dish, and evaporate to dryness on a steam bath. Dissolve the residue in 10 ml. of hydrochloric acid, and again evaporate to dryness, continuing the heat until the residue, when dissolved in water, is neutral to litmus. Transfer the residue with the aid of 25 ml. of water to a glass-stoppered flask, add exactly 50 ml. of 0.1 *N* silver nitrate, then add 3 ml. of nitric acid and 3 ml. of nitrobenzene, and shake vigorously. Add ferric ammonium sulfate T.S., and titrate the excess silver nitrate with 0.1 *N* ammonium thiocyanate. Each ml. of 0.1 *N* silver nitrate is equivalent to 10.11 mg. of KNO_3.

Arsenic. Dissolve 1 gram in 3 ml. of water, add 2 ml. of sulfuric acid, and evaporate to strong fumes of sulfur trioxide. Cool, wash down the sides of the container with water, and heat again to strong fuming. Repeat the washing and fuming three more times, then cool and dilute to 35 ml. with water. This solution meets the requirements of the *Arsenic Test*, page 865.

Chlorate. Sprinkle about 100 mg. of a dry sample upon 1 ml. of sulfuric acid. The mixture does not become yellow.

Heavy metals. A solution of 2 grams in 25 ml. of water meets the requirements of the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) and 1 gram of the sample in the control (*Solution A*).

2T3-53

Lead. A solution of 1 gram in 10 ml. of water meets the requirements of the *Lead Limit Test*, page 929, using 10 mcg. of lead ion (Pb) in the control.

Loss on drying, page 931. Dry at 105° for 4 hours.

Packaging and storage. Store in tight containers.

Functional use in foods. Preservative; color fixative in meat and meat products.

POTASSIUM NITRITE

KNO_2 **Mol. wt. 85.11**

DESCRIPTION

Small, white or yellowish, deliquescent granules or cylindrical sticks. It is very soluble in water, but is sparingly soluble in alcohol. A 1 in 10 solution is alkaline to litmus, and it gives positive tests for *Potassium*, page 928, and for *Nitrite*, page 928.

SPECIFICATIONS

Assay. Not less than 90.0 percent of KNO_2.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Heavy metals (as Pb). Not more than 20 parts per million (0.002 percent).

Lead. Not more than 10 parts per million (0.001 percent).

TESTS

Assay. Transfer about 1.2 grams, accurately weighed, into a 100-ml. volumetric flask, dissolve in water, dilute to volume, and mix. Pipet 10 ml. of this solution into a mixture of 50.0 ml. of 0.1 *N* potassium permanganate, 100 ml. of water, and 5 ml. of sulfuric acid, keeping the tip of the pipet well below the surface of the liquid. Warm the solution to 40°, allow it to stand for 5 minutes, and add 25.0 ml. of 0.1 *N* oxalic acid. Heat the mixture to about 80°, and titrate with 0.1 *N* potassium permanganate. Each ml. of 0.1 *N* potassium permanganate is equivalent to 4.255 mg. of KNO_2.

Arsenic. Dissolve 1 gram in 10 ml. of diluted sulfuric acid T.S., boil gently for 1 minute, cool, and dilute to 35 ml. with water. This solution meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. Dissolve 1 gram in 15 ml. of diluted hydrochloric acid T.S., and evaporate to dryness on a steam bath. To the residue add 2 ml. of hydrochloric acid, again evaporate to dryness, and dissolve the residue in 25 ml. of water. This solution meets the requirements of the

Heavy Metals Test, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Lead. A solution of 1 gram in 10 ml. of water meets the requirements of the *Lead Limit Test*, page 929, using 10 mcg. of lead ion (Pb) in the control.

Packaging and storage. Store in tight containers.

Functional use in foods. Color fixative in meat and meat products.

POTASSIUM PHOSPHATE, DIBASIC

Dipotassium Monophosphate; Dipotassium Phosphate

K_2HPO_4 Mol. wt. 174.18

DESCRIPTION

A colorless or white, granular salt which is deliquescent when exposed to moist air. One gram is soluble in about 3 ml. of water. It is insoluble in alcohol. The pH of a 1 percent solution is about 9. A 1 in 20 solution gives positive tests for *Potassium*, page 928, and for *Phosphate*, page 928.

SPECIFICATIONS

Assay. Not less than 98.0 percent of K_2HPO_4 after drying.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Fluoride. Not more than 10 parts per million (0.001 percent).

Heavy metals (as Pb). Not more than 20 parts per million (0.002 percent).

Insoluble substances. Not more than 0.2 percent.

Lead. Not more than 5 parts per million (0.0005 percent).

Loss on drying. Not more than 2 percent.

TESTS

Assay. Transfer about 6.5 grams of the sample, previously dried at 105° for 4 hours and accurately weighed, into a 250-ml. beaker, add 50.0 ml. of 1 *N* hydrochloric acid and 50 ml. of water, and stir until the sample is completely dissolved. Place the electrodes of a suitable pH meter in the solution, and titrate the excess acid with 1 *N* sodium hydroxide to the inflection point occurring at about pH 4. Record the buret reading, and calculate the volume (*A*) of 1 *N* hydrochloric acid consumed by the sample. Continue the titration with 1 *N* sodium hydroxide until the inflection point occurring at about pH 8.8 is reached,

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SODIUM NITRATE

$NaNO_3$ Mol. wt. 85.00

DESCRIPTION

Colorless, odorless crystals or crystalline granules. It is moderately deliquescent in moist air. It is freely soluble in water, and is sparingly soluble in alcohol. A 1 in 5 solution is neutral to litmus and gives positive tests for *Sodium*, page 928, and for *Nitrate*, page 927.

SPECIFICATIONS

Assay. Not less than 99.0 percent of $NaNO_3$ after drying.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Heavy metals (as Pb). Not more than 10 parts per million (0.001 percent).

Total chlorine. Passes test (approx. 0.2 percent).

TESTS

Assay. Weigh accurately about 350 mg., previously dried at 105° for 4 hours, dissolve in 10 ml. of hydrochloric acid in a small beaker or porcelain dish, and evaporate to dryness on a steam bath. Dissolve the residue in 10 ml. of hydrochloric acid, and again evaporate to dryness, continuing the heating until the residue, when dissolved in water, is neutral to litmus. Transfer the residue with the aid of 25 ml. of water to a glass-stoppered flask, add exactly 50 ml. of 0.1 *N* silver nitrate, then add 3 ml. of nitric acid and 3 ml. of nitrobenzene, and shake vigorously. Add ferric ammonium sulfate T.S., and titrate the excess silver nitrate with 0.1 *N* ammonium thiocyanate. Each ml. of 0.1 *N* silver nitrate is equivalent to 8.50 mg. of $NaNO_3$.

Arsenic. Dissolve 1 gram in 1 ml. of water, add 2 ml. of sulfuric acid, and evaporate to strong fumes of sulfur trioxide. Cool, wash down the sides of the container with water, and heat again to strong fuming. Repeat the washing and fuming three more times, then cool and dilute to 35 ml. with water. This solution meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. A solution of 3 grams in 25 ml. of water meets the requirements of the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) and 1 gram of the sample in the control (*Solution A*).

Total chlorine. Dissolve 1 gram in 100 ml. of water, add enough 6 percent sulfurous acid to give the solution a distinct odor of sulfur dioxide, boil gently until the odor of the sulfur dioxide is no longer apparent, and adjust the volume to 100 ml. by the addition of water. Add 1.0 ml. of 0.1 *N* silver nitrate followed by 3 ml. of nitric acid and 3 ml. of nitrobenzene, and shake vigorously. Add ferric ammonium sulfate T.S., and titrate the excess silver nitrate with 0.1 *N*

ammonium thiocyanate. No more than 0.6 ml. of the 0.1 *N* silver nitrate is consumed.

Packaging and storage. Store in tight containers.

Functional use in foods. Color fixative in meat and meat products.

SODIUM NITRITE

$NaNO_2$ **Mol. wt. 69.00**

DESCRIPTION

It occurs as a white to slightly yellow, granular powder, or as white or nearly white, opaque, fused masses or sticks. It has a mild, saline taste and is deliquescent in air. Its solution are alkaline to litmus. One gram of sodium nitrite dissolves in 1.5 ml. of water, but it is sparingly soluble in alcohol. Its solutions give positive tests for *Sodium*, page 928, and for *Nitrite*, page 928.

SPECIFICATIONS

Assay. Not less than 97.0 percent of $NaNO_2$ after drying.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Heavy metals (as Pb). Not more than 20 parts per million (0.002 percent).

Lead. Not more than 10 parts per million (0.001 percent).

Loss on drying. Not more than 0.25 percent.

TESTS

Assay. Dissolve about 3 grams, previously dried over silica gel for 4 hours and accurately weighed, in water to make 100.0 ml. Pipet 10 ml. of this solution into a mixture of 100.0 ml. of 0.1 *N* potassium permanganate, 50 ml. of water, and 5 ml. of sulfuric acid, keeping the tip of the pipet well below the surface of the liquid. Warm the solution to 40°, allow it to stand for 5 minutes, and add 25.0 ml. of 0.1 *N* oxalic acid. Heat the mixture to about 80°, and titrate with 0.1 *N* potassium permanganate. Each ml. of 0.1 *N* potassium permanganate is equivalent to 3.450 mg. of $NaNO_2$.

Arsenic. Dissolve 1 gram in 10 ml. of diluted sulfuric acid T.S., boil gently for 1 minute, cool, and dilute to 35 ml. with water. This solution meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. Dissolve 1 gram in a mixture of 10 ml. of water and 2 ml. of hydrochloric acid, and evaporate to dryness on a steam bath. Add another 2-ml. portion of hydrochloric acid, again evaporate to dryness, and dissolve the residue in 25 ml. of water. This solution

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meets the requirements of the *Heavy Metals Test*, page 920, using 20 mcg. of the lead ion (Pb) in the control (*Solution A*).

Lead. A solution of 1 gram in 10 ml. of water meets the requirements of the *Lead Limit Test*, page 929, using 10 mcg. of lead ion (Pb) in the control.

Loss on drying, page 931. Dry over silica gel for 4 hours.

Packaging and storage. Store in tight containers.

Functional use in foods. Color fixative in meat and meat products.

SODIUM PHOSPHATE, DIBASIC

Disodium Monohydrogen Phosphate; Disodium Phosphate

Na_2HPO_4 Mol. wt. 141.96

DESCRIPTION

Dibasic sodium phosphate is anhydrous or contains 2 molecules of water of hydration. It occurs as a white powder or crystalline solid. The anhydrous form is hygroscopic. Both forms are freely soluble in water and insoluble in alcohol. A 1 in 20 solution gives positive tests for *Phosphate*, page 928, and for *Sodium*, page 928.

SPECIFICATIONS

Assay. Not less than 98.0 percent of Na_2HPO_4 after drying.

Loss on drying. Na_2HPO_4 (anhydrous), not more than 5 percent; $Na_2HPO_4 \cdot 2H_2O$ (dihydrate), between 18 percent and 22 percent.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Fluoride. Not more than 50 parts per million (0.005 percent).

Heavy metals (as Pb). Not more than 10 parts per million (0.001 percent).

Insoluble substances. Not more than 0.2 percent.

TESTS

Assay. Transfer about 6.5 grams of the sample, previously dried at 105° for 4 hours and accurately weighed, into a 250-ml. beaker, add 50.0 ml. of 1 *N* hydrochloric acid and 50 ml. of water, and stir until the sample is completely dissolved. Place the electrodes of a suitable pH meter in the solution, and titrate the excess acid with 1 *N* sodium hydroxide to the inflection point occurring at about pH 4. Record the buret reading, and calculate the volume (*A*) of 1 *N* hydrochloric acid consumed by the sample. Continue the titration with 1 *N* sodium hydroxide until the inflection point occurring at about pH 8.8 is reached,

although this does not exclude the possibility of *in vivo* adsorption. Preliminary experiments with class-specific anti-M Ig suggest that the Ig on any one cell belongs to a single class. This result is most easily reconciled with the third hypothesis. The question of whether the surface Ig molecules function as antigen-specific receptors remains to be answered.

We thank Mr R. Lucken, Mr A. Savill and Miss J. Cressey for their technical assistance, and Dr B. A. Askonas for the anti-M Ig. This work was supported in part by the US National Multiple Sclerosis Society.

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Gas Chromatographic Determination of N-Alkyl Nitrosamines

THE carcinogenicity, at very low concentrations, of many N-nitrosamines is well established[1,2]. There is a need for improved methods of specific sensitive and quantitative determination of these compounds in samples of interest. Polarographic methods[3-6] are sensitive to rather less than one part per million but they lack specificity. Thin layer chromatography[7-9] offers a measure of discrimination between N-nitrosamines, but the methods so far developed are not entirely satisfactory. A specific and sensitive gas chromatographic method for N-alkylnitrosamines is described below.

The study has been limited to simple N-alkylnitrosamines which are volatile in steam; the separation procedure used has been that of Heath and Jarvis[3]. At the 1 p.p.m. level dimethyl and diethylnitrosamines are recovered to 95 per cent and 97 per cent, respectively, when added to corned beef. The gas chromatographic method is directly applicable to aqueous samples. Using microporous polymer beads as a column packing, water is rapidly eluted in a narrow band which does not interfere with the determination of N-alkylnitrosamines. A typical chromatogram is shown in Fig. 1. A lower limit of detection of 1 p.p.m. (corresponding to 10^{-2} μg in a 10 μl. injection on the column) was achieved using standard solutions, but the practical detection limit for sample extracts would depend upon the degree of purification achieved during the extraction stage. With suitable concentration techniques, such as repeated distillation or evaporation of a suitable solvent, amounts of nitrosamine of the order 0·01 p.p.m. should be detectable. The method is capable of direct extension to the analysis of more complex nitrosamines, but an alternative separation procedure, for example, solvent extraction, is necessary for non-volatile nitrosamines.

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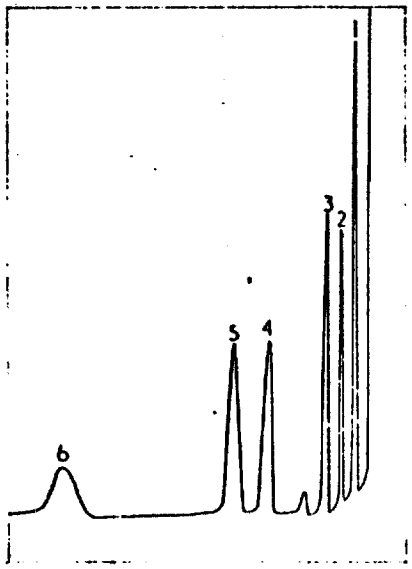

Fig. 1. Chromatogram of mixture of N-nitrosamines. 1, Dimethylnitrosamine; 2, methylethylnitrosamine; 3, diethylnitrosamine; 4, dipropylnitrosamine; 5, methylhydroxyethylnitrosamine; 6, dibutylnitrosamine. The gas chromatograph used was a Varian Aerograph 1200 with a 6 feet × ¼ inch (outside diameter) stainless steel column packed with Chromosorb 101, at 200° C, with a flame ionization detector. The flow rate was 25 cc/min, chart speed 4 inch/h; 5 μl. samples were

Adaptation to Hamsters of Neurotropic Measles Virus from Subacute Sclerosing Panencephalitis

SUBACUTE sclerosing panencephalitis (SSPE), a fatal neuropathy of children, has gained prominence recently because of its presumed aetiological association with measles virus[1]. Several successful isolations of the virus from affected brain tissues have been reported[2,3]. Preliminary characterization studies on these isolates have failed to show differences from classical measles virus strains.

This communication describes the adaptation of one of these SSPE measles isolates to brains of baby hamsters. Other strains of measles virus have been adapted to the brains of rodents in the past[4,5], but these strains were non-neural in origin. The virus used in our study was derived from the brain of a documented case of SSPE[6].

The Mantooth strain[2] of SSPE virus was obtained from Dr W. Zeman, Indiana University Medical Center. A pool of stock virus, which had been received as second passage fluids, was prepared in BSC-1 cells[7]; these cells were used throughout the study. To prepare hamster inocula, virus at a high multiplicity of infection was inoculated in bottles of cultured cells. After 24 h at 36° C, cells were scraped from bottles; cellular fluid-

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Formation of Nitric Oxide Myoglobin: Mechanisms of the Reaction with Various Reductants

SUMMARY—The concentration, temperature and pH dependences of the formation of nitric oxide myoglobin (NOMb) from metmyoglobin nitrite (MetMb · NO_2) were determined for nitrite and the reductants, ascorbic acid, cysteine, hydroquinone, nicotinamide adenine dinucleotide (NADH) and glyceraldehyde. The reaction for all reductants except glyceraldehyde involves the production of a nitroso-reductant intermediate which breaks down to release nitric oxide. The latter forms a nitric oxide metheme complex (Fe^{+++}) which is then reduced to the ferrous state (Fe^{++}). With cysteine and NADH there is a second pathway which probably involves the direct reduction of MetMb · NO_2. Ascorbate and hydroquinone form nitroso intermediates that are stabilized in alkali. The effects of oxygen, ethylenediaminetetraacetic acid and cytochrome *c* on the reaction were determined. Oxygen slows or inhibits the reaction, while the latter two have no effect on the reaction as studied.

INTRODUCTION

TO DATE, IMPROVEMENTS in cured meat color technology have been made on a largely empirical basis since the mechanisms of chromophore production in meat or meat products are unknown. It is known that the process is one of reduction of nitrous acid (from added nitrite) to nitric oxide and of metmyoglobin and methemoglobin from the ferric (met) to the ferrous state. But there are a number of reducing systems and/or reductants in meat which, in addition to or conjunction with added reductants, are capable of effecting the reduction.

Although isolated studies of the effects of various reductants and/or reducing systems have been made (Kelley *et al.*, 1957; Siedler *et al.*, 1959; Fox *et al.*, 1963; Walters *et al.*, 1965; Borys, 1965; Watts *et al.*, 1966), the accumulated data do not cover all conditions of pH, temperature and reactant concentrations encountered in cured meat production. The development of comprehensive data on the foregoing conditions for endogenous or added reductants can have a two-fold value, (1) by helping establish which reductants or systems are principally involved in meat curing, and (2) indicating the mechanism(s) of the reactions involved. Once these mechanisms are known it will be possible to determine the optimal conditions for obtaining maximal rates of color production conversions and stability.

We therefore undertook an *in vitro* study of the kinetics and thermodynamics of the formation of NOMb with the aim of producing coherent data on the pH, temperature and concentration dependences of the rate of nitric oxide heme pigment formation using reductants either normally added or endogenous to meat, or else potentially useful in the process. The data were used to develop equations that express the rate dependence of the reaction in terms of these various factors and reductants, and to determine the mechanisms of the reactions where possible.

EXPERIMENTAL

THE TECHNIQUES USED for the preparation of myoglobin have been described previously (Fox *et al.*, 1963). NADH was obtained from Nutritional Biochemicals Corporation; the rest of the chemicals were reagent grade. The nitric oxide came from the Matheson Company and the nitrogen was water-pumped prepurified grade.

The course of the reactions was followed by the change in absorption at 547.5nm as this was the wavelength of maximal absorption (β peak) in the visible portion of the spectrum as determined with a Cary Model 14 recording spectrophotometer calibrated to within $\pm$ 0.02nm. The Cary Model 14 or a Gilford attachment for a Beckman DU spectrophotometer was used to record the change in absorption; the Cary was used to record spectra. Temperature control was attained by means of a YSI thermoregulator with the thermistor probe either immersed in the cuvette or used as a surface probe fastened to the cuvette holder. The YSI unit controlled a Waco Refrigerated Visibility Bath. With this arrangement it was possible to control the cuvette to within plus or minus 0.05°C.

Reactions were run under three different conditions: (1) under nitrogen; (2) in open cuvettes without prior removal of oxygen; and (3) with air bubbling through the cuvette during the reaction. One centimeter cuvettes were fitted at the top with short sections of 6 mm tubing so that the cells might be sealed with size 000 serum bottle caps. Gases and solutions were introduced into the cuvettes through hypodermic needles inserted in the caps. Nitrogen gassing was conducted for a period of 10 min. Reagent solutions were made with nitrogen-gassed water.

NOMetMb was produced by bubbling nitric oxide through MetMb solutions after oxygen removal. A criterion of the conversion to NOMetMb was that the 575nm absorption maximum be higher than the 535nm absorption; lesser conversions gave poor results.

The reductants used in this study were ascorbate, cysteine, NADH, hydroquinone, and glyceraldehyde, chosen as being endogenous to meat or as containing functional groups corresponding to endogenous reductants. Table 1 summarizes the order of the dependences of the reaction rate on the concentration of the various reactants excepting glyceraldehyde. Table 2 lists a number of empirical equations for the various dependences on pH and temperature. With the exception of the reaction with NADH at pH

Table 1. Reaction rate on concentration.

Reductant	From metmyoglobin nitrite order of dependence, n				From nitric oxide myoglobin order of dependence, n			
			mM Nitrite					
	[R]	pH	<0.7	>0.7	[MMb]	[R]	pH	[MMb]
Ascorbate	0.50	−1.43[1]	1.0	0.41	0	1.0	0	1.0
Cysteine	0.48	−0.60	1.0	0.22	0	1.0	0	1.0
Hydroquinone	1.00	−1.2[1]	1.0	0.62	0	1.0	0	1.0
NADH	0.58	−1.19	~1.0		1.0[2] 0[3]	1.0	0.1[3]	1.0

[1] 20°C.
[2] pH 4.5.
[3] pH 5.5–6.5.

and below, the reactions were all zero order with respect to the concentration of pigment.

To determine the order of dependence of the reaction on the concentrations of reductant and nitrite, these concentrations were varied and the effect on the zero order rate constant (k_0) observed. The dependence was fractional order with respect to the concentration of cysteine and NADH and it was found necessary to make use of the logarithmic form (2) of the basic equation (1):

$$(1)\quad y = a\,x^n$$
$$(2)\quad \log y = \log a + n \log x$$

The plots are shown in Fig. 1. The value for n of 0.481 for cysteine is not significantly different from 0.500, and indicates that the reaction rate is dependent upon the square root of the cysteine concentration. The figure of n = 0.578 for the NADH dependence does differ significantly from 0.500, however. Fig. 2 shows the reaction sequences involved, and is as follows:

Nitrous acid, in the form of the anhydride reacts with the reductant to form a semi-stable intermediate, as proposed by Dahn *et al.* (1958, 1960), which may then react either to release free NO (reaction 4) or with another molecule of intermediate to reform the initial reactants (backward reaction, 3). Reaction 6, being the formation of the nitric oxide met-heme complex, is fast compared to the other reactions. Reaction 7 is a reduction step, and either it or reaction 4 is the rate-limiting step. Reaction 3 is the step which introduces the square root dependence on the reductant concentration into the rate expression, and if $k_3 > k_4$, the dependence will be exactly 0.5, i.e., cysteine and ascorbate. If $k_4 > k_3$ the reaction will be dependent directly upon the concentration of the reductant (n = 1.0), i.e., hydroquinone. It therefore follows that where $k_4 \cong k_3$ the dependence will be somewhere between 0.5 and 1.0, which is the case with NADH.

If $k_4 < k_7$ the overall rate expression will be zero order with respect to the pigment, but if $k_4 > k_7$ or if the reaction mechanism proceeds by some other pathway, for example, reaction 5, or reactions 8 and 9, the overall rate expression will be dependent upon the first power of the pigment concentration. The reaction sequence does not include the reduced form (ferrous) of the heme pigment, an omission based on the observation that at these pH values nitrite oxidizes the reduced heme pigments faster

Table 2. Coefficients of the equations describing the dependence of the rate of formation of nitric oxide myoglobin on pH and temperature.

Reductant	pH	t°C	Equation for log $10^2 k_0$[1,2]	ΔHa kcal/mole
Ascorbate	4.5	....	9.625 − 1804/T°K	8.26
	5.0	....	9.746 − 2050/T°K	9.38
	5.5	....	9.965 − 2323/T°K	10.63
	6.0	....	10.169 − 2593/T°K	11.87
	....	20°	9.907 − 1.43 pH	.....
	....	40°	9.805 − 1.32 pH	.....
Cysteine	4.5	....	16.212 − 4225/T°K	19.3
	....	20°	4.423 − .599 pH	.....
Hydroquinone	4.5	....	9.616 − 1902/T°K	8.71
	5.5	....	10.343 − 2549/T°K	11.67
	6.5	....	18.091 − 5092/T°K	23.30
NADH	....	20°	7.931 − 1.19 pH[3] log $10^2 k_{1st}$[4]	.....
NADH	4.5	....	12.467 − 2900/T°K	13.28

[1] $k_0 = \mu M/min$.
[2] The standard deviation ranged from 2 to 10%; the average and modal S. D. was 5%.
[3] pH 5.0–6.5.
[4] $k_{1st} = min^{-1}$.

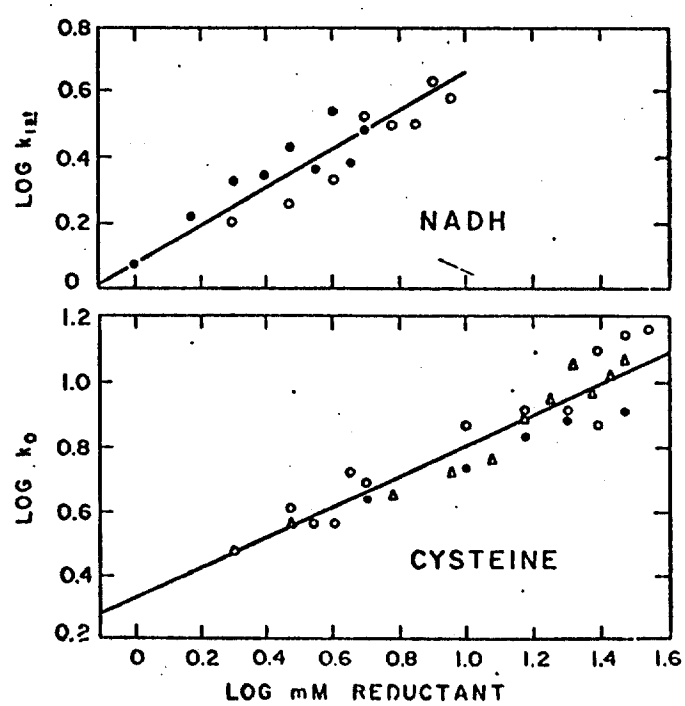


Fig. 1. Dependence of the rate constant for the formation of NOMb on the concentration of the reductants NADH and cysteine.

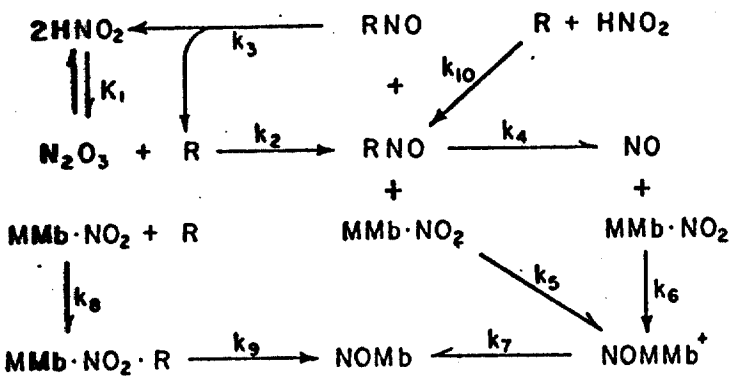


Fig. 2. Reaction sequences for the formation of NOMb from MetMb·NO₂.

than the nitric oxide heme pigments are formed, i.e., until the nitric oxide pigments are formed, the reduced heme pigments do not exist in solution in the presence of nitrite.

On the other hand nitric oxide combined with the oxidized pigment is probably stabilized against oxidation by oxygen and nitrite. The presence of the intermediate (NOMetMb) formed is observed spectrally during the course of the reaction from the position of the β peak (530–550 nm region), as follows. Since the maximum of NOMetMb appears at 532 nm, while the corresponding maximum for its reduction product NOMb lies at 547.5 nm, during the reaction a peak in this region appears first around 538–540 nm (indicating the presence of NOMetMb) and shifts towards a longer wavelength during the reaction until at the end of the reaction the final value of 547.5 nm is reached.

The variation with pH was fractional order with all four reductants as shown in Table 1. This fractional order may be the result of one or more of three causes: (1) the backward reaction 3 in Fig. 2 when the formation of the intermediate depends on pH; (2) two or more independent pathways to the same product with different pH dependences; and/or (3) two different forms of a semi-stable reaction intermediate, each form having a different heat of activation for breakdown. In the first case, the derived rate expression would be a precise mathematical derivation of the mechanism, but in the last two cases the expressions for the rate dependence are empirical. At the extremes of either pH or temperature the overall kinetics will be characteristic of one reaction mechanism or intermediate; at intermediate values the kinetics will reflect the behavior of all mechanisms and/or intermediates.

The reduction by ascorbate

The mechanism of this reaction has been discussed previously (Fox *et al.*, 1963) and was found to involve reactions 1, 2, 3, 4, 6 and 7. Reaction 4 was rate limiting, and, from the pH dependence, it was determined that the reaction was mainly dependent upon the unionized (acid) forms of the two acids, nitrous and ascorbic.

In the present study, the pH dependence was variable and somewhat less than 1.5 (1.43 at 20°C), in this case probably reflecting a variation in the stability of the nitroso-ascorbic acid intermediate. Since the heat of activation is the energy required to cleave the intermediate complex into an ascorbate radical and nitric oxide, its variation with pH is probably due to the ionization of the ascorbate carboxyl group, the ionized (alkaline) form being the more stable. It is important to note that the equations in Table 2 hold true only for the pH range 4.5 to 6.0. At 40° and pH 4.5 some cleavage of the protein-heme bond occurred producing some nitric oxide hemochrome; above pH 6.0 the reaction rate begins to increase with pH.

Reduction by cysteine

The basic assumption in solving the reaction sequence from the observed dependences is that the intermediates occur at constant concentration, "steady state," (Fox *et al.*, 1963). The desired rate expression which describes the principal reaction at pH 4.5 is the same for cysteine as for ascorbate, with different rate constants. The solution is obtained by assuming the reaction sequence includes reaction steps 1, 2, 3, 4, 6 and 7, with steady state concentrations of N_2O_3, AHNO, NO and NOMetMb. Reaction 4 is assumed to be rate limiting. The pH dependence $k_0 \sim [H]^{0.6}$, is very much different from what would be expected from a reaction involving only the unionized forms of the reactants.

Since the sulfhydryl group does not ionize appreciably in this pH range, it would be expected that the pH dependence would be due to the nitrous acid ionization and that k_0 would be proportional to $[H]^{1.0}$. From the low value of the order of dependence and the lack of variation with pH of the heat of activation, it is apparent that there is a second reaction which is an important pathway, particularly at the higher pH values. Since there were at least two mechanisms operative, we attempted to further elucidate the reaction mechanism by varying the concentration of the reactants at pH 6.5 where the effect of the reaction of the unionized forms would be minimal.

The rate of conversion to NOMb tended to be intermediate between zero and first order with respect to the pigment at higher pH values when the concentrations of cysteine and nitrite were 5m*M*. At nitrite concentrations above 5m*M* at pH 6.5, the reactions were all exactly first order with respect to pigment, the nitrite dependence disappeared completely, and the dependence of the first order rate constant on the cysteine concentration was very low ($n = 0.15$ in equation [2]).

The results indicate saturation of the system with both reductant and nitrite at the concentrations studied, and are consistent with a reaction sequence represented by reactions 8 and 9 in Fig. 2, showing the direct reduction by cysteine of $MetMb \cdot NO_2$. The heat of activation did not vary with pH, indicating that the scission of the S-N bond was not affected by ionizations in this pH range, regardless of the mechanism of the reaction. The value obtained is normal for the reduction reactions of cysteine, ca. 20 kcal/mole (Tarbell, 1961).

Reduction by hydroquinone

Hydroquinone was chosen as representative of the quinoid-type reductants that occur in animal tissue such as the tocopherols (E-Vitamins), K-vitamins, coenzyme Q and ubiquinone. Using the usual method of steady state analysis, it was determined that the reduction involved in sequence pathways 10, 4, 6 and 7, with reaction 4 again

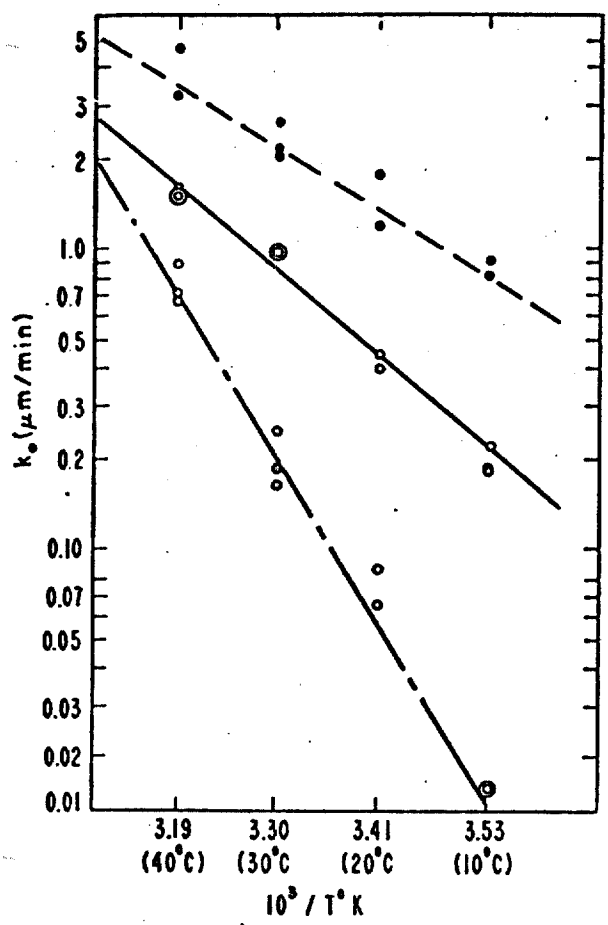
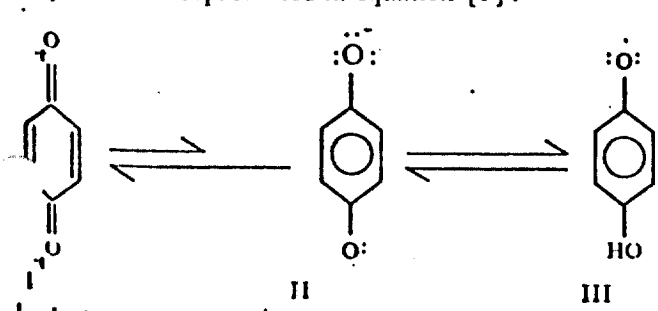


Fig. 3. Arrhenius plots for the formation of NOMb with hydroquinone as reductant. pH 4.5, - - -; pH 5.5, ——; pH 6.5, — —.

the limiting step. The release of NO and the formation of NOMetMb was attested to by the appearance of the β peak at 540 nm. The variation with temperature of the rate of the reaction increased sharply with decreasing hydrogen ion concentration, as shown in Fig. 3.

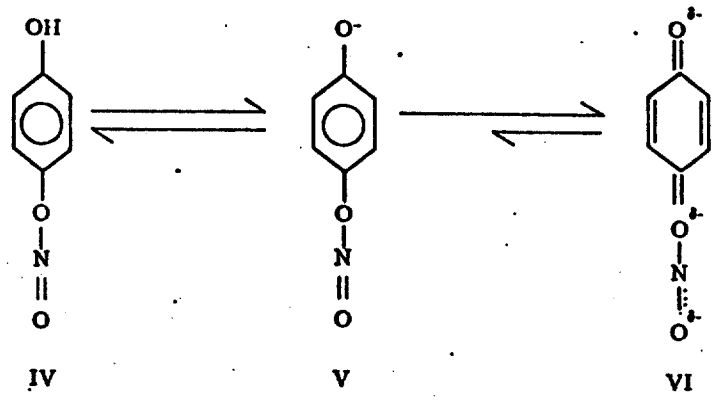
The heats of activation calculated from the Arrhenius plots are, in kcal/mole: pH 4.5, 8.7; pH 5.5, 11.7, and pH 6.5, 23.3. This increase is the result of the increased stability of the quinone intermediate at higher pH values as was also noted for the ascorbate intermediate. This increased stability in alkali is typical of quinoid structures containing oxygen and is the result of the delocalization of the charge of the ionized species in the π-bond structure of the semi-quinone (Gould, 1959).

The theory of the resonance stability of the alkaline semi-quinones is represented in equation [3]:



In I the undissociated proton interferes with the participation of the p-orbital electrons of the oxygen in the resonant structure. In III, the structure is stabilized by the further inclusion of the two oxygen atoms in the π-bond system and the delocalization of the charge.

We therefore postulate the nitroso-semiquinone intermediate to be a structure containing both hydro-quinone and nitric oxide which may be formulated as follows:



Structure VI is the classical resonance representation of the stabilized structure and indicates the extensive delocalization of the π-bond electrons over the entire moelcule. It was not possible to assign specific heats of activation to either form of the intermediate because in the narrow range of pH 4.5 to 6 the reaction involved mixtures of both forms in unknown quantities.

The cleavage which releases nitric oxide is a one electron transfer presumably resulting in the formation of a semi-quinone. We postulate that it is the ease of formation and stability of the semi-quinone as compared with the relative difficulty of formation of the radical products of the other reductants which accounts for the linear dependence of the rate constants on the concentration of hydroquinone and the fractional order dependences of ascorbate, cysteine, and NADH.

In a bimolecular reaction between two intermediate reductant-nitric oxide complexes, the bimolecular intermediate may either react to produce nitric oxide and the radical form of the reductant, or revert to the original electronic states of the reduced reductant and nitrous acid. The first reaction is in the forward direction. When the steady state analysis is made, the order of this reaction disappears and does not affect the order of the total reaction. The second reaction is in the backward direction and, if favored, introduces a fractional order dependence on the reductant. The quinone bimolecular intermediate will readily form the relatively stable radical product (first reaction) with the release of nitric oxide. In the case of the acid quinone, the formation of the meriquinone dimer may further stabilize the semi-quinoid product resulting from the release of nitric oxide, and contribute toward the ease of electron transfer.

In contrast, the other reductants, whose radical forms are not as fully stabilized as is the semiquinone structure, would tend to revert to their original state of reduction from the dimer rather than form the radical product, hence introducing a fractional order dependence.

Reduction by NADH

The total reaction sequence, as determined from the dependences of Table 1, involves reaction steps 1, 2, 3, 4,

6 and 7. At pH 4.5 the reaction is first order with respect to the pigment, but at pH 5.5 and higher values the conversion of pigment is zero order. The rate of the reaction at pH 4.5 must therefore be governed by a slow reaction after the involvement of the pigment. In the sequence of Fig. 2 it would be reaction 7, the reduction of NOMetMb. If the reduction of the last named pigment is the rate limiting step, then its rate should be equal to the rate of the reduction of MetMb·NO_2.

Table 3 summarizes the rate constants for the two reactions, and, as can be seen, at pH 4.5 the rates of the two reactions are the same. Comparing the turnover rates at higher pH values for the two reactions (0 order rate versus $k_{1st} \times 50\ \mu M$ pigment), it is seen that the rate of NOMetMb reduction is several magnitudes greater than the rate of MetMb·NO_2 reduction, hence the reduction of the latter is zero order. Such a situation did not prevail with the other reductants; at all pH values studied the rate of NOMetMb reduction was greater than the rate of nitric oxide production.

As in the case of cysteine the fractional order dependence of the NADH reduction is probably due to two or more different reaction mechanisms since the heat of activation did not vary with pH. However, we could not obtain data to support any further conclusions as to possible sequences, in part due to the formation of a reaction product between nitrite, NADH and heme pigment, which was not NOMb. The first reaction observed was the formation of NOMb followed by a reaction resulting in the formation of a stable heme pigment with a distinctive absorption spectrum, Fig. 4. The product is water-soluble and therefore presumably contains native protein, but we have not yet been able to convert it to any other recognizable heme pigment form.

Reduced nicotinamide adenine dinucleotide phosphate (NADPH, TPNH) was tried as a reductant. Although a reddening of the solutions was observed, the NADPH denatured the protein with concomitant increase in turbidity.

Reduction by glyceraldehyde

The solutions of pigment, glyceraldehyde and nitrite did not show any spectral changes for periods up to 3 days at any pH between 4.5 to 6.5 and 20°C. It was necessary to raise the temperature to 40°C before any appreciable reactions were observed. At pH 4.5, the observed reaction was the production of nitrimetmyoglobin, the green heme pigment observed by Fox *et al.* (1964), while at pH 6.0 conversion to NOMb occurred overnight. The reaction

Table 3. Rates of reduction of metmyoglobin nitrite and nitric acid myoglobin by NADH.

pH	Pigment	Order	Rate constant	Turnover number [MMb]=50 μM
4.5	MMb·NO_2	1	0.187 min^{-1}	9.35
	NOMMb	1	0.211 min^{-1}	10.55
5.5	MMb·NO_2	0	0.71 μM/min	0.71
	NOMMb	1	0.224 min^{-1}	11.2
6.5	MMb·NO_2	0	0.083 μM/min	0.083
	NOMMb	1	0.182 min^{-1}	9.10

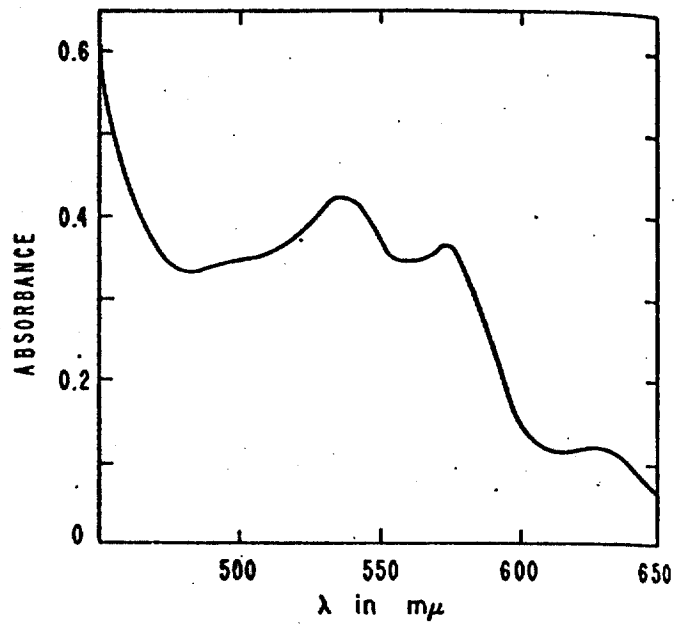


Fig. 4. Spectra of final product of reaction between NADH, nitrite and MetMb.

rate increased with increasing pH values above pH 6.0 and is probably a reaction similar to that observed for the other reductants above pH 6.5. At intermediate pH values, no reactions were observed up to 40°C.

Effect of nitrite

The concentration of nitrite was varied over a range of 0.05 to 50.0 m*M* at pH 4.5, 20°C in nitrogen, with each of the four reductants. The results were plotted on a log-log scale and the calculated values of n (Equation 2) are shown in Table 1. Two effects are noted: (1) the fractional dependence was observed only above 0.7 m*M* nitrite and (2) the fractional order varied with the reductant. The 0.7 m*M* nitrite corresponds to ca. 0.05 m*M* undissociated nitrous acid at pH 4.5, the same concentration as the pigment, and suggests an activation process involving the pigment. The fractional order dependences suggest multiple reactions between the reductant and the nitrite, with nitric oxide being but one of a number of products. In fact Evans *et al.* (1956) have found this to be true with the reaction between nitrite and NADH or ascorbate producing N_2O and N_2 as well as NO.

Reduction of NOMetMb

The reduction of NOMetMb was first order with respect to the pigment and with respect to the various reductants tested (ascorbate, cysteine, hydroquinone and NADH). The calculated rate constants were consistent with the observed rates of NOMb formation from MetMb·NO_2 and, except with NADH, did not vary with pH. The reduction of NOMetMb by NADH showed a low order dependence on pH (Table 1) which may be an effect of steric hindrance. NADH is a large molecule, and may not fit as well or as easily into the cleft in which the heme lies (Kendrew *et al.*, 1960; Perutz *et al.*, 1960). The pH dependence thus reflects changes in the protein conformation making the heme more accessible as the pH decreases

Table 4. Effect of air on the reaction, MMb·NO_2 → NOMb.

Reductant	Gas	pH	Temp	k_a μM/min	k_{1st} min^{-1}	Initial turnover rate μM/min
Asc	Air	4.5	20	....	0.315	13.3
	N_2	4.5	20	22.7 ± 2.5	....	22.7
	Air	4.5	30	....	0.649	23.1
	N_2	4.5	30	40.3 ± 3.6	....	40.3
	Air	4.5	40	....	1.23	33.0
	N_2	4.5	40	61.4 ± 0.3	....	61.4

Effect of oxygen

The reaction studied by Fox *et al.* (1963) took place in open cuvettes with no precautions to remove dissolved oxygen. All reactions discussed in this paper were run in closed absorption cells that had prepurified nitrogen bubbled through the solutions to remove oxygen. The rates of the latter reactions showed less variation and the rate constants were higher than in the previous study. In studying the effect of oxygen on the reaction, we bubbled air through the reacting solutions to maintain a constant level of oxygen in the solutions. The results are summarized in Table 4.

Two principal effects were noted; the reactions in air were first order with respect to pigment and the reaction rates were reduced, as indicated by turnover numbers in Table 4. This reduction in rate is probably a result of the relatively rapid oxidation of nitric oxide by oxygen. When the proposed nitroso-ascorbic acid intermediate breaks down to yield nitric oxide, in the absence of oxygen the only reactant available is the heme pigment.

With oxygen present, a competition between heme pigment and oxygen exists, and the relative amounts of nitric oxide consumed by the two pathways are dependent on the relative concentrations of pigment and oxygen, and the respective rate constants of the two reactions. With nitric oxide produced at a constant rate and the oxygen concentration constant, the observed fate of NOMb formation will depend on the concentration of MetMb available for complexing nitric oxide. Since this latter concentration decreases as the pigment is converted, the reaction becomes first order.

Effect of cytochrome c

Walters *et al.* (1965), studying the reduction of nitrite by mitochondria, found that ferrocytochrome *c* was oxidized by the system with the formation of nitric oxide. Since experience had shown us that our myoglobin preparations were sometimes contaminated with small amounts of cytochromes, we considered it advisable to determine what effect such contamination might have on the reaction. Our studies of the chromatography of myoglobin on carboxymethyl-cellulose showed that cytochrome *c* adhered firmly to the top of the column.

We placed myoglobin preparations on carboxymethyl cellulose columns under conditions that cause cytochrome *c* to adhere (0.05 *M* phosphate, pH 7.0), but found none, probably because it was completely dialyzed out of the solution in the two dialysis steps applied in preparing myoglobin. We therefore tested the effect of reduced cytochrome *c* on the reaction rate at 0.05 m*M*, 0.0005 m*M*, and zero concentration levels, but found no differences in the reaction rates. We therefore conclude that cytochrome *c* would have had no effect on the formation of NOMb in our reaction mixtures.

Effect of high pH

All the reductants exhibited a decreasing rate of MetMb·NO_2 reduction with increasing pH up to pH 6.0 to 6.5. Above this pH the rate versus pH curve rose sharply. Even the reduction by glyceraldehyde showed an appreciable reaction rate above pH 7.0 at 20.0°C. From preliminary studies it appeared that the reaction mechanisms above pH 6.5 are different from those below pH 6.5 and that further study will be required to characterize them.

Effect of ethylenediamine tetraacetic acid (EDTA)

Siedler *et al.* (1959) and Weiss *et al.* (1953) reported that the addition of iron increased the formation of nitric oxide hemochrome; Pascal *et al.* (1957) reported that the oxidation of cysteine by peroxide was probably metal-catalyzed. We wished to know whether or not contamination was affecting our results, so we examined the effect of the addition of EDTA on our systems. The results were negative; the addition of EDTA neither changed the rate of the reaction, nor improved the precision of the measurements. This result is consistent with the observed negative pH dependence of the reactions, since metal catalyses are generally associated with positive rate-pH dependences (Pascal *et al.*, 1957).

DISCUSSION

OUR RESULTS EMPHASIZE some of the important aspects of the role performed by reductants in cured meat color production. Some of these aspects are (1) the effectiveness of any given reductant over the range of pH encountered in meat products; (2) its effectiveness in the elimination of oxygen and/or forming the nitric oxide pigment in the presence of oxygen; (3) the temperatures required to produce the nitric oxide heme pigments within reasonable time periods, and (4) the number of side reactions which take place that either use up the available reductants or produce nitrogen oxides no longer available for the production of cured meat pigments.

With regard to (1) and (3) it has been observed (Fox *et al.*, 1967) that cysteine is fully as effective as ascorbate in color production in frankfurter emulsions, after the oxygen has been eliminated and the emulsions are cooked. This is a happenstance of the pH and temperature dependences of cysteine and ascorbate. Thus, from the data of Table 2, it may be calculated that at pH 6.0 and 100°F (40°C) cysteine reduces MetMb·NO_2 as fast as does ascorbate.

Sufficient oxidizable sulfhydryl groups do exist in meat to account for the observed rates; Hamm *et al.* (1965, 1966) found 17.5 m*M* (1965) and 25 m*M*-SH (1966) by amperometric titration. Assuming a 1:1 dilution in frankfurter emulsions, the sulfhydryl content of the added meat alone could account for the observed rates of nitric oxide hemochrome formation in frankfurter emulsions (Fox *et al.*, 1967). Sulfhydryl groups alone are only part of the available reductants in muscle tissue; Thomson *et al.*

1962) and Regier *et al.* (1956) reported total reducible substance concentrations ranging from 50 to 100 mM.

Chromophore production itself is only one of a number of requirements. As Watts *et al.* (1966) and Fox *et al.* (1967) have shown, removal of oxygen, when present, is a vital part of cured meat color production. Ascorbate is more effective than cysteine in this respect, since the former reacts faster with oxygen and is capable of driving the reaction of $MetMb \cdot NO_2 \rightarrow NOMb$ to completion in the presence of oxygen. The latter reaction was accomplished only at the highest temperature studied (40°C) by cysteine.

Side reactions have been given little if any consideration to date, but may be of great importance. ten Cate (1962) obtained evidence of N_2O in the gases above curing pickles, showing that such side reactions do occur in practice. Our studies show that these reactions are particularly important at nitrite concentrations common to cured meat products (30-200 ppm, 0.7–5.0 m*M*) and that the relative rates vary with the reductant.

Since these side reactions produce nitrogen in oxidation states no longer available for the formation of nitric oxide, part of the overall effectiveness of any given reductant may depend upon the extent to which the reductant produces these compounds. As a general conclusion then, these studies suggest that no one reductant may be optimally effective in all cured meat products, but that consideration should be given to using different reductants or combinations thereof, depending on what kind of products are made and how they are made.

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SEVEN CASES OF POISONING BY SODIUM NITRITE*

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With the ready availability of sodium nitrite, it is surprising that cases of poisoning caused by the ingestion of this salt in food are uncommon occurrences. Sodium nitrite is manufactured chiefly for its use in the dye industry. It is also employed as an anti-corrosive in anti-freeze mixtures and used as a counter against rust when surgical instruments are sterilized. It is used either alone or mixed with saltpetre (sodium nitrate) in the curing of meat, to bring out the red colour and as a preservative. In South Africa, the Food, Drugs and Disinfectants Act No. 13 of 1929, as amended, permits the use of nitrites in the curing of meat provided the end product contains not more than 200 parts per million (ppm) calculated as sodium nitrite. The same 'maximum permissible amount' is laid down in the USA by the Bureau of Animal Industry of the Department of Agriculture. Heat used in cooking destroys nitrites, but this cannot be depended on since the amount destroyed varies with the degree of heat and the length of cooking. Sodium nitrate is more commonly used in curing than is sodium nitrite. While saltpetre (*US Pharmacopeia*) is 99·5% pure, commercial saltpetre frequently has a large admixture of sodium nitrite.

Meat curing was a process originally aimed at the preservation of meat without refrigeration. Its primary feature was the addition of salt to the meat, but through the centuries variations have been introduced and combinations have been made with other processes so that today a cured-meat product may not only be salted, but also smoked and/or dried; sugar-cured; treated with sodium nitrite and/or sodium nitrate in order to 'fix' the colour, i.e. render the colour heat-stable; or spiced and aged to improve flavour. The ready availability of refrigeration today has reduced the need for meat preservation with salt.

Basically there are two types of curing processes. The first involves the addition of the curing agents in the dry state and the second in the form of a brine. The curing agents customarily used are: salt, sugar, sodium nitrate, sodium nitrite, and vinegar. Salt is primarily a flavouring agent, but is also a preservative. Sugar is largely added for its flavour, though not exclusively. As already stated, the nitrite and nitrate of sodium are colour-fixation agents, though the nitrate is also bacteriostatic. This colour-fixation enables cured meats to retain their redness after cooking.

Sodium and potassium nitrate were the first colour-fixation agents used. Later, when it was realized that the nitrates were merely sources of nitrites, the direct use of nitrites was permitted. Many contend that the best results in curing meat are achieved by a combination of the two curing agents.

CASE REPORTS

At 1.30 p.m. on Sunday 21 May 1961, a White family, consisting of the father aged 31, the mother aged 29, a male child aged 14 months and a female child aged 5 years, together with 2 adult females and 1 adult male who were visiting their household, partook of a meal consisting of cocktail sausages, cheese pancakes, potatoes in their jackets, bread and butter, and tea with milk. The only item which was consumed by all 7 was the sausages.

Case 1 — G.F., female child aged 5 years. Within 30 minutes of her meal, which consisted of 4 sausages and potato, the child complained of severe headache and palpitations. Examination revealed an alert child who was well orientated and lucid. She was, however, rather unsteady on her feet and wanted to lie down. There was no vomiting or diarrhoea, but she had some nausea. As the examination proceeded, however, she became drowsy and tended to fall asleep. A striking feature was the marked tachycardia, the heart rate being 170 a minute, while the pulse was of good volume and the blood pressure was 100/60 mm. Hg. While she was being transferred to a nursing home, cyanosis of the lips and mucous membranes became evident, but there was no respiratory distress. No adventitious lung sounds were detected. Drowsiness increased, but a good response to supra-orbital pressure remained. Gastric lavage yielded large quantities of undigested and partially digested sausage. Treatment was continued in an oxygen tent. Thereafter the child's condition improved considerably—drowsiness disappeared fairly soon and the cyanosis vanished after about 2 hours' stay in the oxygen tent. The heart rate returned to normal more slowly; 12 hours later it was 100 a minute and the blood pressure was 110/65 mm. Hg. Thereafter she remained well and suffered no after-effects.

Case 2 — G.F., male child aged 14 months. This child had eaten only 1 or 2 sausages. He was irritable, but had no vomiting or diarrhoea. Examination showed that the child's condition was good. There was no cyanosis or respiratory distress. The temperature was 99°F., but the heart rate was 180 a minute. Some cyanosis of the lips and tongue became evident about 1 hour later, that is about 1½ hours after the meal. No other abnormal physical signs were detected. Gastric lavage yielded a good quantity of sausage. The child was placed in an oxygen tent where the cyanosis soon disappeared. The heart rate of 180 a minute persisted for 2 hours and then gradually slowed down, being 110 a minute the following day. There was no recurrence of the cyanosis and the child remained well.

Case 3 — Mr. R.M.H.F., male aged 31 years.
Case 4 — Mrs. J.R.F., female aged 29 years.
Case 5 — Mr. B.H.U., male aged 64 years.
Case 6 — Mrs. M.U., female aged 63 years.
Case 7 — Mrs. L.T., female aged 50 years.

Within 30 minutes of their meal, all the adults experienced palpitations and a sensation of constriction in the throat, followed by headache, dizziness and numbness of the fingers. There was epigastric discomfort and some nausea, but no diarrhoea, vomiting or colic. They all became somewhat drowsy. The headache was disturbing and was described as throbbing and bounding. Examination of all the adults revealed a tachycardia of 120 - 140 a minute; blood pressures

*Clinical description by Dr. H. Altman, B.Sc., M.B., B.Ch., M.R.C.P. (Lond. and Edin.), D.C.H. and Dr. D. Meskin, M.B., B.Ch., both of Johannesburg.

varied from 100/60 to 135/95 mm. Hg; all were apyrexial. Cyanosis of the lips and buccal mucosa became evident 1 hour after the meal, but there was no respiratory distress. There were no other abnormal physical signs.

All the adults were encouraged to vomit and, with the assistance of an emetic, 3 succeeded. Mr. R.F. and Mrs. L.T. had gastric lavages which yielded undigested sausage and partially digested remnants of the recent meal. None of the adults was detained in hospital. They complained of some headache for 2-3 days. A mild diarrhoea developed the following day and, with general lassitude, remained for 2-3 days, by which time all had recovered completely.

ACTION BY JOHANNESBURG CITY HEALTH DEPARTMENT

The sources of the sale and the manufacture of the sausages were immediately traced. Samples of uncooked as well as the original cooked sausages, together with samples of the stomach contents of the 2 children, were submitted to the South African Institute for Medical Research for bacteriological analysis, and subsequently to the City Health Department's chemical division. Careful inspection at the home of the victims revealed no insecticide, detergent or other poison which might have been added inadvertently in cooking the sausages, nor was any clue apparent at the delicatessen store which sold the product.

At one (A) of the 2 butcheries which manufactured the sausages there were grounds for suspecting that saltpetre and borax might well have been added to the sausage meat in excessive quantities. Samples of the saltpetre, borax and the cornflour used in the processing were submitted for chemical analysis, together with samples of the sausages.

The borax and the cornflour were chemically pure, but the saltpetre, which should be practically 100% sodium nitrate, was found to be 100% sodium nitrite. Meanwhile, bacteriological reports from the South African Institute for Medical Research on the sausages and the gastric contents showed no pathogenic organisms. The following further chemical analyses then became available:

Description	*Sodium nitrite (parts per million)*
Uncooked sausage from butchery A	4,700
Cooked sausage from butchery A	7,400
Stomach contents of each affected child	48
Cornflour from butchery A	nil
Borax from butchery A	nil
Uncooked sausage from second butchery (B)	178

From these analyses it was clear that the poisoning was due to excess sodium nitrite in the sausages caused by faulty supplies of 'saltpetre' to butchery A.

The supplier of the contaminated saltpetre was immediately traced and a stop-order placed on all his stocks of this product. The names and addresses of all butchers and suppliers of meat and meat products throughout the country who had bought saltpetre from this firm during the previous 2 months were obtained, and each one was contacted by telephone. An appeal to return suspected stocks was also made through the press. No fewer than 43 firms had to be contacted, many from as far afield as Bethal, Zeerust, Swaziland, Sabie, Pietersburg, Letaba Tzaneen, Ermelo, etc. Health departments at these places were also informed.

The supplier's stocks of saltpetre consisted of: 101 × 50 kg. hessian bags, 48 × 100 lb. paper packets, 47 × 8 lb. bags (dispensed), and 27 × 20 lb. boxes (dispensed).

Each container was tested and found to be in order, the average sodium nitrite content being no more than 10 ppm.

A bin containing 280 lb. of 'saltpetre', from which cash orders for small quantities were dispensed, was found to contain a mixture of saltpetre and sodium nitrite.

Stocks of saltpetre from the 43 firms mentioned above were returned to the supplier and all were tested by the City Health Department. Six of the 43 consisted of a mixture of saltpetre and sodium nitrite and were confiscated. Of these, 4 were from the Johannesburg area, 1 from the Orange Free State, and 1 from the East Rand. Stocks of the remaining 37 were satisfactory.

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Standard pack: Single jute or hessian bags.

Also available: Jute bags with polyethylene inner bag; iron and fibre drums.

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DISCUSSION

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The *US Pharmacopeia* gives the average pharmacological dose of sodium nitrite as 0·060 G. or 1 grain, the *British Pharmacopoeia* as 0·03 - 0·12 G., or ½ - 2 grains. It has been established that a dose of 3 grains in an adult causes low blood pressure, pallor, sweating, nausea, dizziness and finally loss of consciousness.

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Possible Contamination of Underground Water Supplies

In January 1959 all 3 infants of a set of triplets died at the Krugersdorp Hospital when 39 days old, following the administration of sodium nitrite instead of sodium chloride. This was the second recorded instance in South Africa of death from sodium nitrite poisoning, the first being at Krugersdorp as well. In 1953, 3 African children aged 11 months, 2 years and 5 years, died on that occasion.

At the 1959 inquest proceedings, Prof. D. G. Steyn of the University of Pretoria expressed the opinion[17] that, though only these 3 fatal cases had been recorded in this country up to then, 'we must have had hundreds of cases of cyanosis from it (sodium nitrite). It appears that many of the deaths of so-called "blue babies" have been incorrectly diagnosed because the necessary tests are not made'. Professor Steyn ascribed this to the fact that the semi-arid regions of South Africa have 'underground water supplies (which) are frequently contaminated by nitrates and nitrites, and we are faced with the terrific danger of cyanosis to babies because their feeds are prepared from such water. Hundreds, perhaps thousands, of wells contain nitrite in amounts dangerous to infants'. Many authorities will not agree with this view.

There are two sources of nitrogen, as nitrites and nitrates, in South African soils and waters:

1. Oxidation of organic matter.
2. A small amount by fixation of atmospheric nitrogen, either through the aid of symbiotic bacteria in the soil, or through electrical discharges during thunderstorms, when nitric acid will be formed and washed down by rain.

Dr. G. W. Bond[18] stated that the majority of wells or borehole waters show small amounts of nitrates as the result mainly of pollution. He found very few instances

of nitrite pollution, and then only in small amounts. This is understandable because, during the oxidation of organic matter, nitrites are an intermediate stage between ammoniacal nitrogen and nitrates and they are readily oxidized in water and soil to nitrates. This makes the risk of nitrite poisoning from wells and boreholes a small one.

SUMMARY

1. Seven cases of acute poisoning by sodium nitrite are described. They resulted from the ingestion of sausages, in the processing of which sodium nitrite instead of saltpetre (sodium nitrate) had accidentally been used.

2. All 7 patients recovered completely without ill-effects.

3. The public health investigations which followed the outbreak are described and an appeal is made for far more stringent identification measures when dealing with this highly toxic substance.

4. The literature is reviewed and the treatment described.

5. Finally, comments are made on the differing views regarding the degree of contamination of underground water supplies in South Africa with nitrites.

Thanks are due to my colleagues in the Johannesburg City Health Department, particularly in the Food Section, for their assistance and the meticulous investigations which followed the outbreak; to the South African Institute for Medical Research for the bacteriological investigations; to our Department's Chief Chemist, Dr. E. G. White, Ph.D., for all the chemical investigations and for information on underground water supplies; and finally to the Medical Officer of Health, Johannesburg, for his interest and permission to publish.

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A LONG-ACTING DIURETIC SUITABLE FOR OUTPATIENT TREATMENT OF FLUID RETENTION IN OEDEMA OF PREGNANCY AND THE PREMENSTRUAL TENSION SYNDROME

HERBERT KRAMER, M.B., B.CH., F.R.C.S. (EDIN.), F.I.C.S., M.R.C.O.G., *Obstetrician and Gynaecologist, Johannesburg*

Before the discovery of salidiuretics the treatment of oedema of pregnancy presented a difficult problem. Owing to the risk of renal damage the physician had to be extremely cautious in prescribing diuretic drugs, especially since the investigations of Fahr[1] and Volhard[2] suggested that organic lesions of the kidney were responsible for the symptoms of toxaemia of pregnancy. Clearance tests and renal biopsies have in the meanwhile showed that toxaemia of pregnancy is chiefly caused by functional disorders which are reversible after parturition. The earlier passive attitude (usually only rest in bed and dietetic measures in the form of a diet rich in proteins and vitamins and low in salt and sodium, were prescribed) has therefore been largely superceded by active diuretic therapy. The development of oedema is caused by retention of sodium. Sodium is also retained during normal pregnancy. Careful balance tests have shown that the values in the last 2 months of pregnancy attain 70 - 170 mEq. weekly; this corresponds to a total of 10 - 15 G. of sodium. In toxaemia of pregnancy the sodium retained amounts to a multiple of this value.

Hormonal factors (heightened secretion of follicular and adrenocortical hormones) play a significant part in producing this increase in sodium retention during normal pregnancy. The excessive retention of sodium in toxaemia, however, is most probably the result of a reduction in the glomerular filtration rate and the simultaneous increase in tubular resorption of sodium and water. This mechanism is also of significance in the development of cardiac oedema. Whereas in normal pregnancy sodium retention causes an increase in extracellular fluid of 4 litres at the most (not taking into account foetal fluid-containing cavities), which results merely in a physiological increase in tissue fluid and possibly in a latent tendency to oedema, an excessive retention of sodium leads to manifest oedema and, under certain conditions, to the well-known clinical signs of toxaemia of pregnancy. It is the duty of the practising obstetrician to recognize its occurrence and to treat it at an early stage.

Only if one bears in mind that even under optimum treatment the foetal mortality in late toxaemia may be as high as 25%, does one realize the magnitude of this task and of the responsibility which the physician has to bear.

THIS STUDY

The following study deals with outpatient, antenatal welfare and treatment of excessive fluid retention in private practice. It is concerned chiefly with the practical problems that determine the therapeutic procedure. Contrary to the old opinion that pregnant women do not require any particular attention until the last trimester of pregnancy, the present belief is that controls should be carried out already in the early months of pregnancy so as to recognize and avoid impending toxaemia. In the first place, the weight gain of the women should be recorded. In addition, the blood pressure is measured and the urine examined. Determination of the protein content of the urine alone is insufficient, since albuminuria only occurs in advanced stages of the disease and is a severe sign of pre-eclampsia.

Theoretically, in normal pregnancy the gain in weight is estimated at 10 - 12 kg. (20 - 25 lb.). Half of this pertains to the product of pregnancy and the amniotic fluid; the remaining 50% arises from the increase in size of the uterus, the breasts, and above all, from the extra-cellular fluid. Weight gains exceeding 0·5 kg./week or 1·5 - 2 kg./month are no longer physiological and suggest the beginning of toxaemia. In these cases it is necessary to search for oedema; which is found not only in the lower limbs, but also in the hands and face and over the sacrum. For the differential diagnosis it is important to know that isolated oedema of the legs is mostly due to static disorders, and disappears during the night. Excessive weight gains without oedema are almost invariably the result of overeating. Here a reduction of calorie intake is indicated, possibly in conjunction with appetite-inhibiting drugs.

Choice of Diuretic

Carrying out this form of antenatal welfare in our private practice, we observed, in the course of 3 months, 30 patients with manifest oedema which did not respond to dietetic therapy alone, and therefore required active diuretic treatment. In our search for a suitable diuretic we excluded from the beginning mercurial preparations, since their parenteral administration produces abrupt shifts in the water and electrolyte balance and the oral administration leads to gastro-intestinal intolerance in a relatively large number of cases. For several reasons the

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SEVEN CASES OF POISONING BY SODIUM NITRITE*

M. L. FREEDMAN, *O.B.E.*, M.B., CH.B., D.P.H., *Assistant Medical Officer of Health, Johannesburg*

With the ready availability of sodium nitrite, it is surprising that cases of poisoning caused by the ingestion of this salt in food are uncommon occurrences. Sodium nitrite is manufactured chiefly for its use in the dye industry. It is also employed as an anti-corrosive in anti-freeze mixtures and used as a counter against rust when surgical instruments are sterilized. It is used either alone or mixed with saltpetre (sodium nitrate) in the curing of meat, to bring out the red colour and as a preservative. In South Africa, the Food, Drugs and Disinfectants Act No. 13 of 1929, as amended, permits the use of nitrites in the curing of meat provided the end product contains not more than 200 parts per million (ppm) calculated as sodium nitrite. The same 'maximum permissible amount' is laid down in the USA by the Bureau of Animal Industry of the Department of Agriculture. Heat used in cooking destroys nitrites, but this cannot be depended on since the amount destroyed varies with the degree of heat and the length of cooking. Sodium nitrate is more commonly used in curing than is sodium nitrite. While saltpetre (*US Pharmacopeia*) is 99·5% pure, commercial saltpetre frequently has a large admixture of sodium nitrite.

Meat curing was a process originally aimed at the preservation of meat without refrigeration. Its primary feature was the addition of salt to the meat, but through the centuries variations have been introduced and combinations have been made with other processes so that today a cured-meat product may not only be salted, but also smoked and/or dried; sugar-cured; treated with sodium nitrite and/or sodium nitrate in order to 'fix' the colour, i.e. render the colour heat-stable; or spiced and aged to improve flavour. The ready availability of refrigeration today has reduced the need for meat preservation with salt.

Basically there are two types of curing processes. The first involves the addition of the curing agents in the dry state and the second in the form of a brine. The curing agents customarily used are: salt, sugar, sodium nitrate, sodium nitrite, and vinegar. Salt is primarily a flavouring agent, but is also a preservative. Sugar is largely added for its flavour, though not exclusively. As already stated, the nitrite and nitrate of sodium are colour-fixation agents, though the nitrate is also bacteriostatic. This colour-fixation enables cured meats to retain their redness after cooking.

Sodium and potassium nitrate were the first colour-fixation agents used. Later, when it was realized that the nitrates were merely sources of nitrites, the direct use of nitrites was permitted. Many contend that the best results in curing meat are achieved by a combination of the two curing agents.

*Clinical description by Dr. H. Altman, B.Sc., M.B., B.Ch., M.R.C.P. (Lond. and Edin.), D.C.H. and Dr. D. Meskin, M.B., B.Ch., both of Johannesburg.

CASE REPORTS

At 1.30 p.m. on Sunday 21 May 1961, a White family, consisting of the father aged 31, the mother aged 29, a male child aged 14 months and a female child aged 5 years, together with 2 adult females and 1 adult male who were visiting their household, partook of a meal consisting of cocktail sausages, cheese pancakes, potatoes in their jackets, bread and butter, and tea with milk. The only item which was consumed by all 7 was the sausages.

Case 1 — G.F., female child aged 5 years. Within 30 minutes of her meal, which consisted of 4 sausages and potato, the child complained of severe headache and palpitations. Examination revealed an alert child who was well orientated and lucid. She was, however, rather unsteady on her feet and wanted to lie down. There was no vomiting or diarrhoea, but she had some nausea. As the examination proceeded, however, she became drowsy and tended to fall asleep. A striking feature was the marked tachycardia, the heart rate being 170 a minute, while the pulse was of good volume and the blood pressure was 100/60 mm. Hg. While she was being transferred to a nursing home, cyanosis of the lips and mucous membranes became evident, but there was no respiratory distress. No adventitious lung sounds were detected. Drowsiness increased, but a good response to supra-orbital pressure remained. Gastric lavage yielded large quantities of undigested and partially digested sausage. Treatment was continued in an oxygen tent. Thereafter the child's condition improved considerably—drowsiness disappeared fairly soon and the cyanosis vanished after about 2 hours' stay in the oxygen tent. The heart rate returned to normal more slowly; 12 hours later it was 100 a minute and the blood pressure was 110/65 mm. Hg. Thereafter she remained well and suffered no after-effects.

Case 2 — G.F., male child aged 14 months. This child had eaten only 1 or 2 sausages. He was irritable, but had no vomiting or diarrhoea. Examination showed that the child's condition was good. There was no cyanosis or respiratory distress. The temperature was 99°F., but the heart rate was 180 a minute. Some cyanosis of the lips and tongue became evident about 1 hour later, that is about 1½ hours after the meal. No other abnormal physical signs were detected. Gastric lavage yielded a good quantity of sausage. The child was placed in an oxygen tent where the cyanosis soon disappeared. The heart rate of 180 a minute persisted for 2 hours and then gradually slowed down, being 110 a minute the following day. There was no recurrence of the cyanosis and the child remained well.

Case 3 — Mr. R.M.H.F., male aged 31 years.
Case 4 — Mrs. J.R.F., female aged 29 years.
Case 5 — Mr. B.H.U., male aged 64 years.
Case 6 — Mrs. M.U., female aged 63 years.
Case 7 — Mrs. L.T., female aged 50 years.

Within 30 minutes of their meal, all the adults experienced palpitations and a sensation of constriction in the throat, followed by headache, dizziness and numbness of the fingers. There was epigastric discomfort and some nausea, but no diarrhoea, vomiting or colic. They all became somewhat drowsy. The headache was disturbing and was described as throbbing and bounding. Examination of all the adults revealed a tachycardia of 120 - 140 a minute; blood pressures

varied from 100/60 to 135/95 mm. Hg; all were apyrexial. Cyanosis of the lips and buccal mucosa became evident 1 hour after the meal, but there was no respiratory distress. There were no other abnormal physical signs.

All the adults were encouraged to vomit and, with the assistance of an emetic, 3 succeeded. Mr. R.F. and Mrs. L.T. had gastric lavages which yielded undigested sausage and partially digested remnants of the recent meal. None of the adults was detained in hospital. They complained of some headache for 2 - 3 days. A mild diarrhoea developed the following day and, with general lassitude, remained for 2 - 3 days, by which time all had recovered completely.

ACTION BY JOHANNESBURG CITY HEALTH DEPARTMENT

The sources of the sale and the manufacture of the sausages were immediately traced. Samples of uncooked as well as the original cooked sausages, together with samples of the stomach contents of the 2 children, were submitted to the South African Institute for Medical Research for bacteriological analysis, and subsequently to the City Health Department's chemical division. Careful inspection at the home of the victims revealed no insecticide, detergent or other poison which might have been added inadvertently in cooking the sausages, nor was any clue apparent at the delicatessen store which sold the product.

At one (A) of the 2 butcheries which manufactured the sausages there were grounds for suspecting that saltpetre and borax might well have been added to the sausage meat in excessive quantities. Samples of the saltpetre, borax and the cornflour used in the processing were submitted for chemical analysis, together with samples of the sausages.

The borax and the cornflour were chemically pure, but the saltpetre, which should be practically 100% sodium nitrate, was found to be 100% sodium nitrite. Meanwhile, bacteriological reports from the South African Institute for Medical Research on the sausages and the gastric contents showed no pathogenic organisms. The following further chemical analyses then became available:

Description	*Sodium nitrite (parts per million)*
Uncooked sausage from butchery A	4,700
Cooked sausage from butchery A	7,400
Stomach contents of each affected child	48
Cornflour from butchery A	nil
Borax from butchery A	nil
Uncooked sausage from second butchery (B)	178

From these analyses it was clear that the poisoning was due to excess sodium nitrite in the sausages caused by faulty supplies of 'saltpetre' to butchery A.

The supplier of the contaminated saltpetre was immediately traced and a stop-order placed on all his stocks of this product. The names and addresses of all butchers and suppliers of meat and meat products throughout the country who had bought saltpetre from this firm during the previous 2 months were obtained, and each one was contacted by telephone. An appeal to return suspected stocks was also made through the press. No fewer than 43 firms had to be contacted, many from as far afield as Bethal, Zeerust, Swaziland, Sabie, Pietersburg, Letaba, Tzaneen, Ermelo, etc. Health departments at these places were also informed.

The supplier's stocks of saltpetre consisted of: 101 × 50 kg. hessian bags, 48 × 100 lb. paper packets, 47 × 8 lb. bags (dispensed), and 27 × 20 lb. boxes (dispensed).

Each container was tested and found to be in order, the average sodium nitrite content being no more than 10 ppm.

A bin containing 280 lb. of 'saltpetre', from which cash orders for small quantities were dispensed, was found to contain a mixture of saltpetre and sodium nitrite.

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Possible Contamination of Underground Water Supplies

In January 1959 all 3 infants of a set of triplets died at the Krugersdorp Hospital when 39 days old, following the administration of sodium nitrite instead of sodium chloride. This was the second recorded instance in South Africa of death from sodium nitrite poisoning, the first being at Krugersdorp as well. In 1953, 3 African children aged 11 months, 2 years and 5 years, died on that occasion.

At the 1959 inquest proceedings, Prof. D. G. Steyn of the University of Pretoria expressed the opinion[17] that, though only these 3 fatal cases had been recorded in this country up to then, 'we must have had hundreds of cases of cyanosis from it (sodium nitrite). It appears that many of the deaths of so-called "blue babies" have been incorrectly diagnosed because the necessary tests are not made'. Professor Steyn ascribed this to the fact that the semi-arid regions of South Africa have 'underground water supplies (which) are frequently contaminated by nitrates and nitrites, and we are faced with the terrific danger of cyanosis to babies because their feeds are prepared from such water. Hundreds, perhaps thousands, of wells contain nitrite in amounts dangerous to infants'. Many authorities will not agree with this view.

There are two sources of nitrogen, as nitrites and nitrates, in South African soils and waters:

1. Oxidation of organic matter.
2. A small amount by fixation of atmospheric nitrogen, either through the aid of symbiotic bacteria in the soil, or through electrical discharges during thunderstorms, when nitric acid will be formed and washed down by rain.

Dr. G. W. Bond[18] stated that the majority of wells or borehole waters show small amounts of nitrates as the result mainly of pollution. He found very few instances

of nitrite pollution, and then only in small amounts. This is understandable because, during the oxidation of organic matter, nitrites are an intermediate stage between ammoniacal nitrogen and nitrates and they are readily oxidized in water and soil to nitrates. This makes the risk of nitrite poisoning from wells and boreholes a small one.

SUMMARY

1. Seven cases of acute poisoning by sodium nitrite are described. They resulted from the ingestion of sausages, in the processing of which sodium nitrite instead of saltpetre (sodium nitrate) had accidentally been used.

2. All 7 patients recovered completely without ill-effects.

3. The public health investigations which followed the outbreak are described and an appeal is made for far more stringent identification measures when dealing with this highly toxic substance.

4. The literature is reviewed and the treatment described.

5. Finally, comments are made on the differing views regarding the degree of contamination of underground water supplies in South Africa with nitrites.

Thanks are due to my colleagues in the Johannesburg City Health Department, particularly in the Food Section, for their assistance and the meticulous investigations which followed the outbreak; to the South African Institute for Medical Research for the bacteriological investigations; to our Department's Chief Chemist, Dr. E. G. White, Ph.D., for all the chemical investigations and for information on underground water supplies; and finally to the Medical Officer of Health, Johannesburg, for his interest and permission to publish.

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A LONG-ACTING DIURETIC SUITABLE FOR OUTPATIENT TREATMENT OF FLUID RETENTION IN OEDEMA OF PREGNANCY AND THE PREMENSTRUAL TENSION SYNDROME

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Before the discovery of salidiuretics the treatment of oedema of pregnancy presented a difficult problem. Owing to the risk of renal damage the physician had to be extremely cautious in prescribing diuretic drugs, especially since the investigations of Fahr[1] and Volhard[2] suggested that organic lesions of the kidney were responsible for the symptoms of toxaemia of pregnancy. Clearance tests and renal biopsies have in the meanwhile showed that toxaemia of pregnancy is chiefly caused by functional disorders which are reversible after parturition. The earlier passive attitude (usually only rest in bed and dietetic measures in the form of a diet rich in proteins and vitamins and low in salt and sodium, were prescribed) has therefore been largely supereeded by active diuretic therapy. The development of oedema is caused by retention of sodium. Sodium is also retained during normal pregnancy. Careful balance tests have shown that the values in the last 2 months of pregnancy attain 70 - 170 mEq. weekly; this corresponds to a total of 10 - 15 G. of sodium. In toxaemia of pregnancy the sodium retained amounts to a multiple of this value.

Hormonal factors (heightened secretion of follicular and adrenocortical hormones) play a significant part in producing this increase in sodium retention during normal pregnancy. The excessive retention of sodium in toxaemia, however, is most probably the result of a reduction in the glomerular filtration rate and the simultaneous increase in tubular resorption of sodium and water. This mechanism is also of significance in the development of cardiac oedema. Whereas in normal pregnancy sodium retention causes an increase in extracellular fluid of 4 litres at the most (not taking into account foetal fluid-containing cavities), which results merely in a physiological increase in tissue fluid and possibly in a latent tendency to oedema, an excessive retention of sodium leads to manifest oedema and, under certain conditions, to the well-known clinical signs of toxaemia of pregnancy. It is the duty of the practising obstetrician to recognize its occurrence and to treat it at an early stage.

Only if one bears in mind that even under optimum treatment the foetal mortality in late toxaemia may be as high as 25%, does one realize the magnitude of this task and of the responsibility which the physician has to bear.

THIS STUDY

The following study deals with outpatient, antenatal welfare and treatment of excessive fluid retention in private practice. It is concerned chiefly with the practical problems that determine the therapeutic procedure. Contrary to the old opinion that pregnant women do not require any particular attention until the last trimester of pregnancy, the present belief is that controls should be carried out already in the early months of pregnancy so as to recognize and avoid impending toxaemia. In the first place, the weight gain of the women should be recorded. In addition, the blood pressure is measured and the urine examined. Determination of the protein content of the urine alone is insufficient, since albuminuria only occurs in advanced stages of the disease and is a severe sign of pre-eclampsia.

Theoretically, in normal pregnancy the gain in weight is estimated at 10 - 12 kg. (20 - 25 lb.). Half of this pertains to the product of pregnancy and the amniotic fluid; the remaining 50% arises from the increase in size of the uterus, the breasts, and above all, from the extra-cellular fluid. Weight gains exceeding 0·5 kg./week or 1·5 - 2 kg./month are no longer physiological and suggest the beginning of toxaemia. In these cases it is necessary to search for oedema, which is found not only in the lower limbs, but also in the hands and face and over the sacrum. For the differential diagnosis it is important to know that isolated oedema of the legs is mostly due to static disorders, and disappears during the night. Excessive weight gains without oedema are almost invariably the result of overeating. Here a reduction of caloric intake is indicated, possibly in conjunction with appetite-inhibiting drugs.

Choice of Diuretic

Carrying out this form of antenatal welfare in our private practice, we observed, in the course of 3 months, 30 patients with manifest oedema which did not respond to dietetic therapy alone, and therefore required active diuretic treatment. In our search for a suitable diuretic we excluded from the beginning mercurial preparations, since their parenteral administration produces abrupt shifts in the water and electrolyte balance and the oral administration leads to gastro-intestinal intolerance in a relatively large number of cases. For several reasons the

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Effect of Potassium Nitrate Solutions on Blood Pressure of Rats*

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HYPERTENSION CAN be induced in rats by giving them diets rich in sodium chloride[1] or by giving them either isotonic[2] or hypertonic sodium chloride solutions[3,4] as their sole drinking fluid. Vascular disease[1,2] and cardiac hypertrophy[3] accompany the elevation of blood pressure. The rate of development of sodium chloride-induced hypertension appears to be increased by increasing the concentration of sodium chloride in the drinking water. Although these facts are well known, it cannot be stated with certainty that the sodium ion *per se* is responsible for the development of this type of hypertension. Therefore, it seemed worthwhile to test whether hypertension could be induced in rats by a salt other than NaCl, thereby testing the specificity of sodium chloride in production of hypertension. To this end, solutions of potassium nitrate have been used.

METHODS AND RESULTS

Two separate experiments were performed, both using male rats of the Holtzman strain. The similarities of experimental procedure in the two experiments are as follows: All rats were kept three to a cage in a room illuminated from 8 A.M. to 6 P.M. and maintained at a temperature of 26 ± 1°C. The food given was Purina laboratory checkers. Drinking fluids were given in bottles of the type described by Lazarow.[5] These consisted of nursing bottles with cast aluminum caps. Blood pressures were measured weekly using the microphonic manometer technic of Friedman and Freed, but without anesthesia.[6] The rats were accustomed to the procedure of blood pressure measurement for 1 week before the experiment began. During this period blood pressure was measured daily.

EXPERIMENT 1

Two groups of rats with five rats per group were used. At the end of a control period lasting 1 week, *group 1* was given 0.15 M. KNO_3 as the sole drinking fluid (Fig. 1). At the end of the third experimental week, 0.25 M. KNO_3 replaced 0.15 M. KNO_3. Treatment continued for 16 weeks. At the end of the nineteenth week, 0.30 M. KNO_3 was substituted for 0.25 M. This group was maintained on 0.30 M. KNO_3 until the experiment ended 16 weeks later. *Group 2* received tap water to drink during the first 10 experimental weeks. At the end of the tenth week, 0.15 M. KNO_3 solution replaced the water. At the end of the thirteenth week, 0.25 M. KNO_3 solution was given in place of 0.15 M. These rats received 0.25 M. KNO_3 solution until the end of the experiment. Concentrations of KNO_3 solution were increased gradually because previous experience with NaCl solutions had taught that rats given hypertonic concentrations often refuse to drink. This occurred less often when the concentration was increased gradually. Blood pressures were measured weekly for each group of rats.

Results: Mean systolic blood pressure of group 1 rose within a week after initiation of 0.15 M. KNO_3 as the sole drinking fluid. Blood pressure increased from a mean level of 145 to 155 mm. Hg. Blood pressure was maintained at this higher level for 19 weeks in spite of the fact that the concentration of KNO_3 was increased to 0.25 M. at the end of the third experimental week. Blood pressure rose to levels in the vicinity of 160 mm. Hg within 3 weeks after substituting 0.30 M. KNO_3 for 0.25 M. KNO_3. This latter blood pressure was maintained for the remainder of the experiment.

Mean systolic blood pressures of group 2 varied from 141 to 149 mm. Hg during the first 10 experimental weeks while water was the sole drinking fluid. After substituting 0.15 M. KNO_3 for water at the end of the tenth week, blood pressure rose to approximately 155 mm. Hg. Substitution of 0.25 M. KNO_3 for 0.15

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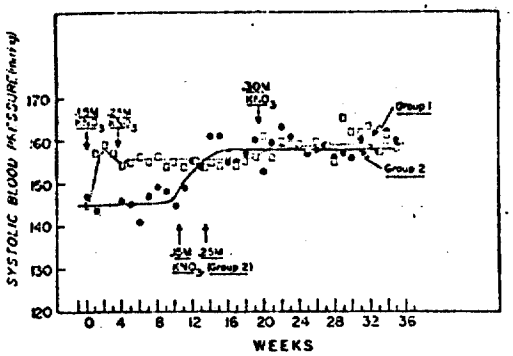

FIG. 1. The effect of KNO_3 solutions on systolic blood pressure of rats. *Group 1* (□) received 0.15 M. KNO_3 as the sole drinking fluid at time indicated as 0 on the abscissa. After 3 weeks, 0.25 M. KNO_3 solution was substituted for 0.15 M. At the end of the nineteenth week 0.3 M. KNO_3 was substituted for 0.25 M. This group continued to receive 0.3 M. KNO_3 until the end of the experiment. *Group 2* (●) received tap water to drink during the first 10 experimental weeks. At the end of the tenth week, 0.15 M. KNO_3 was substituted for tap water. Three weeks later 0.25 M. KNO_3 was substituted for 0.15 M. This group continued to receive 0.25 M. KNO_3 until the end of the experiment.

M. KNO_3 at the end of the thirteenth experimental week was accompanied by a further slight rise to a mean systolic level of approximately 157 mm. Hg. Blood pressure was maintained at this level for the remainder of the experiment.

EXPERIMENT 2

This experiment was performed to repeat and extend the observations made in experiment 1. A total of sixteen rats were used. At the end of a 2 week control period, eight of the rats were given only 0.15 M. KNO_3 solution to drink. The concentration of the KNO_3 solution was increased to 0.25 M. at the end of the fifth experimental week and to 0.30 M. at the end of the tenth experimental week. These rats were maintained on 0.30 M. KNO_3 for the duration of the 28 week experimental period. The remaining eight rats received tap water to drink throughout the experiment. All rats were given the drinking fluids and Purina laboratory checkers *ad libitum*. Blood pressures and body weights were measured weekly.

At the end of the twentieth week of the experiment the rats were placed in individual cages and given finely ground Purina laboratory checkers as food and their usual drinking fluid (either water or 0.30 M. KNO_3 solution). Fluid containers were the same as those used in experiment 1. Food containers have been described in detail in a publication dealing specifically with them.[7] After allowing the rats 4 days to accustom themselves to their new environment, individual food and fluid intakes were measured daily for 10 days. A sample of food was wet-ashed

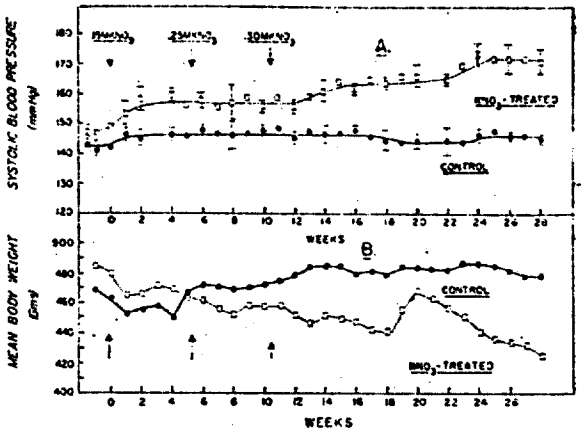

FIG. 2. A, the effect of KNO_3 solutions on systolic blood pressure of rats. *Treated rats* (□) were given 0.15 M. KNO_3 solution as sole drinking fluid at time indicated as 0 on the abscissa. After 5 weeks 0.25 M. KNO_3 solution was substituted for 0.15 M. Five weeks later 0.3 M. KNO_3 solution was substituted for 0.25 M. This group continued to receive 0.3 M. KNO_3 solution for the remainder of the experiment. *Control rats* (●) were given tap water to drink at all times during the experiment. ±1 standard error is set off at the means. B, *mean body weight* of KNO_3-treated (□) and control (●) rats throughout the experiment. Arrows indicate times when concentration of KNO_3 solution was changed.

with concentrated nitric acid and analyzed in duplicate for potassium by flame photometry. The potassium content of the food was found to be 206 mEq. per kg. The objective was to calculate the total potassium intake of the treated group.

Results: Some results of this experiment are shown in Figure 2. Systolic blood pressure of the KNO_3-treated group increased within 2 weeks after initiation of 0.15 M. KNO_3 as the sole drinking fluid (Fig. 2A). No further elevation of pressure was observed after treatment with 0.25 M. KNO_3. However, within 5 weeks after initiation of 0.30 M. KNO_3, blood pressures rose to levels of 160 mm. Hg and by the end of the experiment were 170 mm. Hg. Blood pressures of treated rats were significantly higher ($p < 0.01$) than control levels after the first experimental week. A single exception to this statement occurred during the eighth experimental week when the difference between means was not significant. We have no explanation for the greater variability of blood pressure which occurred at this time. Control rats maintained a relatively constant blood pressure throughout the experiment. Mean systolic blood pressure levels varied from 142 to 147 mm. Hg.

Mean body weights of the two groups of rats is

TABLE I

Ratio of Organ Weight to Body Weight (mg./100 gm. Body Weight) of Some Organs of the Rats in Experiment

Group	No. Rats	Mean Body Weight (gm.)	Mg./100 gm. Body Weight							
			Heart	Kidneys	Testis	Adrenal	Thyroid	Thymus	Seminal Vesicles	Prostate
Control	8	476	314.9 ± 6.0*	756.3 ± 22.4	740.7 ± 30.1	13.4 ± 1.2	8.7 ± 0.7	42.3 ± 3.1	141.8 ± 6.1	136.1 ± 9.5
			(0.34†)	(<0.01)	(<0.01)	(0.02)	(0.14)	(0.27)	(0.95)	(0.59)
KNO_3-treated	8	423	330.0 ± 13.9	966.2 ± 37.2	908.0 ± 41.8	20.0 ± 2.1	10.0 ± 0.5	50.6 ± 6.4	142.5 ± 9.0	126.7 ± 13.9

* ±1 standard error of mean.
† Probability value.

shown in Figure 2B. Body weight of control rats increased slightly after an initial fall. Mean body weight at the end of the experiment was about 10 gm. greater than initial body weight. In contrast, body weight of the KNO_3-treated rats fell during the course of the experiment. Body weight increased during the nineteenth and twentieth week but decreased thereafter to reach a level at the end of the experiment approximately 60 gm. below initial body weight.

The food intakes of the two groups of rats measured during the twenty-first week of the experiment were: 4.1 ± 0.3 (S.E.) gm. per 100 gm. body weight per day for control rats and 4.2 ± 0.2 gm. per 100 gm. body weight per day for KNO_3-treated rats. The difference between means is not significant ($P = 0.87$). The fluid intakes were: 10.4 ± 0.4 (S.E.) ml. per 100 gm. body weight per day for the control rats drinking water and 8.4 ± 0.3 ml. per 100 gm. body weight per day for the rats drinking 0.3 M. KNO_3. The difference between these means is significant ($p < 0.01$). The latter rats were ingesting 3.4 mEq. K per 100 gm. body weight per day; control rats ingested 0.84 mEq. per 100 gm. body weight per day. The treated rats ingested approximately four times as much potassium as did the control rats.

Organ Weights: At the end of the experiment all rats were killed by ether inhalation and organs, including heart, kidneys, testis, adrenals, thyroid, thymus, seminal vesicle and prostate, were removed, blotted on filter paper and weighed on a torsion balance.

Table I shows the weights of various organs of control and KNO_3-treated rats removed at autopsy. Ratios of kidney weight to body weight, testis weight to body weight and adrenal weight to body weight were increased significantly ($p < 0.01$) by treatment with KNO_3. The other organs of treated rats, including heart, thyroid, thymus, seminal vesicles and prostate, were not significantly changed from control values.

Statistical analyses of the data were performed using the Student "t" test for the 95 per cent confidence limit.[8]

COMMENTS

These studies represent preliminary observations on the physiologic effects of KNO_3 solutions administered to rats in their drinking water. Systolic blood pressures of rats drinking this salt solution became elevated within 2 weeks after treatment began. The maximal elevation of blood pressure occurred when 0.3 M. KNO_3 solution was given, although administration of isotonic KNO_3 solution also elevated blood pressure to a modest degree in both experiments (Fig. 1 and 2A).

Role of Potassium: The total potassium intake during administration of 0.3 M. KNO_3 as the sole drinking fluid was fourfold greater than that of controls given tap water to drink. Total fluid intake of treated rats was 81 per cent of that of control subjects. The virtual intake concentration of potassium (total potassium/total fluid times 1,000) for treated rats was 405 mEq. per L. while that for control rats was 81 mEq. per L. Treated rats ingested potassium in a five-fold greater concentration than control rats. While it is only possible to speculate concerning the mechanism of blood pressure elevation by KNO_3, apparently the osmolar concentration ingested is a factor. Presumably this is a factor in the range of concentrations from 0.23 to 0.3 M. since this

represents the upper limit of concentrating ability of the kidneys for potassium, sodium and chloride ions in both rats and man.[9,10] It is noteworthy that maximal elevation of blood pressure also occurred when 0.3 M. NaCl was given as the sole drinking fluid in earlier studies.[11] A greater yield of hypertension may then be induced in rats when the virtual intake concentration of either sodium or potassium is near the limit of the kidney to concentrate these ions in the urine. Perhaps continued maximal work of the kidneys plays a role in the development of salt hypertension.

A complete explanation of "salt" hypertension must also take into account the possibility that specific ions contribute to the production of hypertension. It would be difficult otherwise to explain why isotonic solutions of NaCl and KNO_3 are able to elevate blood pressure and to produce vascular lesions.[2]

Role of Nitrate Ion: It is also possible to speculate that the nitrate ion contributes indirectly to the elevation of blood pressure. Hiatt[12] observed that intravenous administration of large doses of isotonic $NaNO_3$ over a period of 1 to 13 weeks was accompanied by lowered plasma chloride concentration, elevated plasma bicarbonate concentration, elevated blood pH and decreased plasma osmolarity in dogs on a low chloride diet. The conditions of the present experiment were not the same as those of Hiatt; therefore, it can only be supposed that similar effects occurred. If so, the contribution of this altered acid-base balance to the elevation of blood pressure will need to be evaluated.

Other Contributing Factors: Clues to other factors that may contribute to elevation of blood pressure in KNO_3-treated rats are found in Table I. An increase in the ratio of organ to body weight of kidney, testis and adrenal were observed in KNO_3-treated rats (Table I). The increased kidney weight apparently reflects the *increased osmotic work to which the kidney was subjected* and is similar in degree (30 per cent increase) to that observed when the same concentration (0.3 M.) of NaCl is given to rats.[11]

The ratio of testis weight to body weight of treated rats increased 29 per cent above that of control rats. The same percentage increase in testis weight ratio was observed in rats given the same concentration of NaCl solution as their sole drinking fluid.[11] It seems unlikely that this increase in weight resulted from an increase in tissue water content since all tissues of the treated rats did not increase in weight (Table I). The increased testicular weight is not accompanied by increased testosterone secretion as assessed by increase of weight in the seminal vesicle and prostate gland. Weights of these organs of treated rats were not different from control rats (Table I). The increased testicular weight may reflect *increased secretion of gonadotrophic hormones* (*FSH*) by the anterior pituitary gland. Whatever the mechanism involved in its increase in size, we are left with the interesting question: Does the increase in testicular size play a role in the development of salt hypertension?

The increased ratio of adrenal weight to body weight of treated rats reflects *increased secretion of adrenocorticotrophic hormone* (*ACTH*) by the pituitary gland. Presumably, this arose as a result of undefined "stress" associated with the requirement to ingest a hypertonic KNO_3 solution. The thymus glands of treated rats did not show the decrease which characteristically accompanies an increase in glucocorticoid output. This lack of effect could be attributed to the fact that such a response is more difficult to observe in older rats in whom the thymus is already small. Under the conditions of this experiment one might expect maximal sodium-retaining activity to be manifest by the adrenal gland with consequent *increase in aldosterone secretion* accompanying the increased urinary sodium loss brought about by the nitrate ion.[13] We cannot state at present whether the adrenal cortex plays a role in the elevation of blood pressure under these conditions. The answer to this must await further study.

The ratio of heart weight to body weight of treated rats was larger than that of control rats but the difference between means was not significant ($p = 0.34$). Thus, the elevation of systolic blood pressure of the treated rats was not accompanied by a significant cardiac hypertrophy. In this single respect, treatment with hypertonic KNO_3 differs from treatment with hypertonic NaCl. Further studies are in progress to confirm this finding and to probe its significance.

Toxic Side Effects: Administration of KNO_3 appeared to be accompanied by minimal toxic side effects. A gradual weight loss occurred but the rats continued to eat and to keep themselves clean. Greene and Hiatt[11] also reported that there was no evidence of a severe toxicity in dogs given $NaNO_3$. Their dogs became ano-

rexic only after blood chloride levels had dropped to 65 per cent of normal.

Perhaps the concentration, time of administration, specific ion, renal concentrating ability and changes in acid-base balance all contribute to the elevation of blood pressure of salt-treated rats. The possibility of endocrine involvement further complicates the problem. Elucidation of the physiologic mechanisms by which KNO_3 elevates blood pressure must await further detailed study.

Summary

Administration of KNO_3 solutions to rats as their sole drinking fluid was accompanied by an elevation of systolic blood pressure. The maximal elevation of blood pressure occurred when the rats were ingesting 0.3 M. KNO_3 solution, although even an isotonic solution elevated blood pressure to a modest degree. Treatment was accompanied by a gradual loss of body weight. Ratios of organ weight to body weight of kidneys, testis and adrenals were increased by treatment with KNO_3 while organ weight ratios of heart, seminal vesicles, prostate, thyroid and thymus were not affected. A similarity exists between the physiologic responses of rats treated with the same concentrations of NaCl and KNO_3.

Acknowledgment

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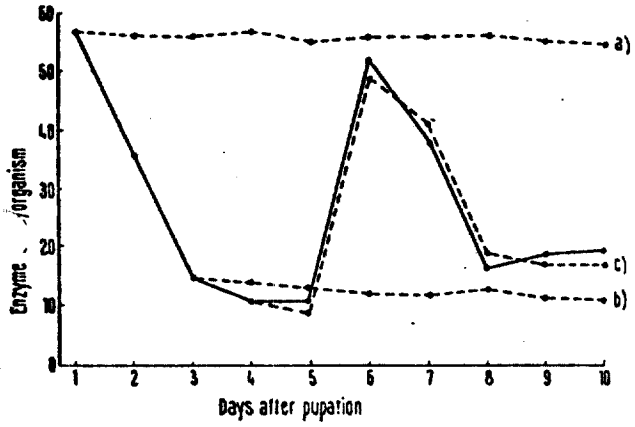
essitated by implantation of 5FU crystals, but without any material implanted. The assay for ADH activity has been described earlier[5].

Treatment with 5FU during the first 3 days following pupation inhibits the pattern of change in ADH activity, the level of activity remaining constant to at least the 10th day of pupal life. As seen in the Figure, a high level is maintained with treatment on the 1st day after pupation, while a constant low level results from treatment on the 3rd day after pupation. When 5FU is administered on the 4th day after pupation or later, the ensuing pattern of ADH activity is not appreciably different from that of normal development. For the sake of clarity only 3 experimental series and 1 control series are included on the Figure.

Apparently the entire program of developmental change in ADH activity is dependent on a 5FU-sensitive process which is completed by the 4th day following pupation. Treatment with 5FU during the first 3 dasy is inhibitory to the realization of this program, the level of ADH activity being locked at the time of treatment. MUTH[2] has shown that the sensitivity of pupal development to 5FU treatment changes drastically between the 3rd and 4th days after pupation. Pupae treated during the first 3 days show severe inhibition of the development of most adult structures, and they die after the 13th pupal day. However, treatment from the 4th day permits development of a viable adult emerging on the 13th day after pupation (on schedule with normal development), although a few adult structures may still be abnormal[3,4]. Therefore, the establishment of the pattern of change in ADH activity may be dependent on the same process(es) necessary for the establishment of the major portion of the program of metamorphic changes in the *Ephestia* pupa. This process is quite likely the synthesis of specific nucleic acids during early pupa.



Effect of 5FU on developmental changes in ADH activity. Solid lines indicate changes in control animals wounded 1 day after pupation. The pattern is essentially the same as described for normal development[5]. Dashed lines a) b) and c) represent animals treated 1, 3 and 4 days after pupation, respectively. The first points for a), b) and c) are control values, since the earliest assays for ADH activity were at 2, 4 and 5 days, respectively. Each point represents a determination on 5–10 individuals.

The effect of 5FU on nucleic acid metabolism has been extensively studied[6]. As an analog of uracil, 5FU can be incorporated into all classes of RNA molecule, and such 5FU-RNA can result in miscoding, or in inhibition of translation. 5FU can also inhibit DNA synthesis, presumably by being converted to 5-fluorodeoxyuridylic acid, an inhibitor of thymidylate synthetase[7]. Thus, these effects could be inhibitory to any developmental program which requires the synthesis of specific nucleic acid(s) during a specific time period.

Zusammenfassung. Behandlung von Puppen der Mehlmotte *Ephestia kühniella* mit 5-Fluorourazil während der ersten 3 Puppentage unterdrückt die charakteristischen Schwankungen der ADH-Aktivität während der weiteren Entwicklung. Behandlung am 4. Tag oder später hat keinen Einfluss auf das Enzym. Die Wirkungen von 5-Fluorourazil auf ADH decken sich mit früher beobachteten Wirkungen auf morphogenetische Prozesse.

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Inhibition of Mouse Liver Protein and Nuclear RNA Synthesis Following Combined Oral Treatment with Sodium Nitrite and Dimethylamine or Methylbenzylamine

Nitrosamines appear to be a major class of carcinogens that are likely to be causally related to human cancer in industrialized society[1]. High concentrations of nitrosamines have been found in nitrite-preserved fish meal[2] and in fermented liquors[3]; trace concentrations of nitrosamines have also been reported in tobacco smoke, cheese, meat, grains and alcoholic beverages[4-7]. Even more widespread and of possibly greater significance are precursors of nitrosamines-nitrites and secondary amines[1]. Secondary amines can be nitrosated in vitro[8-11] and in vivo[12] to form corresponding nitrosamines. Chronic feeding of rats with nitrite combined with secondary amines produced synergistic carcinogenicity[13]. More recently, combined oral administration of nitrite and secondary amines produced synergistic acute toxicity and liver necrosis in mice[14]. We report here acute inhibition of liver protein and nuclear RNA synthesis following combined oral administration of nitrite and secondary amines to mice.

Male Swiss albino mice (ICR/Ha) weighing between 23 and 28 g were housed in porcelainized tubs and given food and water ad libitum. Single doses of dimethylamine-hydrochloride (DMA) and methylbenzylamine-hydrochloride (MBA) were administered to mice by gavage at concentrations of 2000 and 1200 mg/kg either alone or in combination with sodium nitrite ($NaNO_2$) at concentrations of 150 or 100 mg/kg, respectively (Tables I–III).

For the ^{3}H-leucine uptake studies, mice were injected with 1.0 µc of ^{3}H-leucine (55 mC/µmoles; New England

Table I. Synergistic effects of dimethylamine-hydrochloride and sodium nitrite on incorporation of ^{3}H-cytidine into mouse liver nuclear RNA

Groups	^{3}H-Cytidine incorporation[a]					
	At 2 h		At 6 h		At 18 h	
	No. of replicates	Mean ± S.E. (% of control values)	No. of replicates	Mean ± S.E. (% of control values)	No. of replicates	Mean ± S.E. (% of control values)
Control	4	3.06 ± 0.32	4	10.0 ± 0.8	4	13.3 ± 13
Sodium nitrite (150 mg/kg)	4	2.88 ± 0.48 (94%)	4	7.9 ± 0.8 (79%)	4	10.3 ± 0.8 (77%)
Dimethylamine (2000 mg/kg)	4	2.98 ± 0.10 (97%)	4	10.5 ± 0.7 (105%)	2	14.4 ± 0.5 (108%)
Sodium nitrite and Dimethylamine	4	2.06 ± 0.20 (67%)	4	3.8 ± 1.4[b] (38%)	4	8.1 ± 1.4 (60%)

[a] Expressed as cpm/μg DNA; [b] Statistically significant interaction, $p < 0.01$.

Table II. Synergistic effects of dimethylamine-hydrochloride and sodium nitrite on incorporation ^{3}H-leucine into mouse liver protein

Group	Mean ^{3}H-leucine incorporation[a]			
	At 2 h		At 18 h	
	No. of replicates	Mean ± S.E. (% of control values)	No. of replicates	Mean ± S.E. (% of control values)
Control	9	39.7 ± 4.1	5	41.7 ± 2.9
Sodium nitrite (150 mg/kg)	9	39.7 ± 4.1 (100%)	9	39.5 ± 3.7 (95%)
Dimethylamine-hydrochloride (2000 mg/kg)	10	54.4 ± 10.2 (137%)	10	41.6 ± 2.2 (100%)
Sodium nitrite and dimethylamine-hydrochloride	10	15.0 ± 3.5[b] (38%)	10	27.9 ± 5.2 (67%)

[a] Expressed as cpm/mgP; [b] Statistically significant interaction, $p < 0.01$.

Nuclear) 30 min prior to sacrifice; mice were sacrificed 6 or 18 h after administration of DMA alone or with nitrite and 6 h after administration of MBA alone or with nitrite. Uptake of ^{3}H-leucine into whole liver protein was determined with aliquots of liver homogenates from individual mice. Following precipitation with 10% TCA, protein pellets were washed consecutively twice with 10% TCA, with 10% TCA at 90°C for 15 min, with 95% ethanol containing 10% potassium acetate, with absolute ethanol, twice with ethanol-ether (3:1), and twice with ether[15]. Duplicate aliquots of dried protein were dissolved in Protosol and counted in a Beckman liquid scintillation counter.

For nuclear RNA studies, mice were injected with 2.5C of ^{3}H-cytidine (5.9 μC/mMole, New England Nuclear) 45 min prior to sacrifice; mice were sacrificed 2, 6 or 18 h after administration of DMA alone or with nitrite. Highly purified liver nuclei were isolated from 2 pooled livers by sedimentation through 2.1 M sucrose, containing 1 m*M* $MgCl_2$ and 3.5 m*M* potassium phosphate at pH 6.8[9]. Nuclei were lysed with 0.1% sodium lauryl sulfate and aliquots were washed consecutively with 4% PCA, twice with 2% PCA, with absolute ethanol, and twice chloroform-methanol (1:1). After drying, the nucleic acids were hydrolyzed with 5% PCA at 90°C for 30 min; aliquots were counted for radioactivity and analyzed for DNA, by the diphenylamine procedure[16].

The effects of combined administration of DMA and $NaNO_2$ on nuclear RNA synthesis, as measured by incorporation of ^{3}H-cytidine into nuclear RNA, are shown in Table I. Incorporation of ^{3}H-cytidine into nuclei of control mice and mice treated with either DMA or $NaNO_2$ were similar. However, combined administration of DMA and $NaNO_2$ inhibited cytidine incorporation by 33, 62 and 40% at 2, 6 and 18 h following treatment, respectively (Table I).

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Table III. Synergistic effects of methylbenzylamine-hydrochloride and sodium nitrite on mouse liver protein synthesis

Treatment	No. of replicates	Mean ^{3}H-leucine incorporation ± S.E.[a] (% of control values)
Control	8	53.4 ± 4.8
Sodium nitrite (100 mg/kg)	8	52.6 ± 2.3 (99%)
Methylbenzylamine-hydrochloride (1200 mg/kg)	8	56.3 ± 4.8 (105%)
Sodium nitrite and methylbenzylamine-hydrochloride	9	40.4 ± 4.2[b] (76%)

[a] Expressed as cpm/mgP; [b] Statistically significant interaction, $p < 0.01$.

The effects of DMA and $NaNO_2$ on uptake of ^{3}H-leucine into mouse liver protein are shown in Table II. Neither DMA nor $NaNO_2$ alone induced any statistically significant inhibition. However, DMA and $NaNO_2$ together produced 63 and 33% inhibition of leucine uptake at 6 and 18 h after treatment, respectively (Table II). Comparable inhibition of leucine incorporation was produced by combined administration of MBA and $NaNO_2$ (Table III).

In the present experiments, DMA or MBA when administered in combination with $NaNO_2$ produced synergistic acute inhibition of liver protein and nuclear RNA synthesis; similar inhibitory effects are also induced by dimethylnitrosamine[17] and methylbenzylnitrosamine[18]. These data confirm and extend findings of previous studies on induction of synergistic acute toxicity and hepatic nerosis in mice following oral administration of DMA or MBA together with $NaNO_2$[14] and afford strong presumptive evidence of in vivo nitrosamine synthesis from nitrite and amine precursors. The relevance of these findings to potential human hazards from continued use of nitrate and nitrite as food additives and from drinking of water with elevated nitrate merits further consideration.

Zusammenfassung. Orale Gabe von Dimethylamin oder Methylbenzylamin zusammen mit Natriumnitrit bewirkte eine synergistische Hemmung der Protein-Synthese in der Leber von Mäusen. Ausserdem hemmte die kombinierte Gabe von Dimethylamin und Nitrit synergistisch die Synthese nuklearer RNS in der Leber.

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Encephalitogenic Protein: a β-Pleated Sheet Conformation (102–120) Yields a Possible Molecular Form of a Serotonin Receptor

Carnegie[1] has recently suggested that a specified segment of the basic A1 protein composes one of the serotonin receptors in the central nervous system. The conformation suggested by Carnegie is based on a previous model of ours[2]. We wish to suggest an alternative model that accounts for the mode of action of the unusual aminoacid methyl-arginine. The model suggested by Carnegie is based on a coil conformation for the polypeptide and the role of methyl arginine is not accounted for. An α-helical conformation would be unlikely owing to the two glycine moieties in the sequence and the protein itself contains no α-helix[3]: but a β-conformation is possible. The conformation is illustrated in Figure 1 for this segment of the protein itself with 3 turns and 11 cross C=O... HN hydrogen bonds. This locates the 3 key aminoacids —Trp, Arg (Lys) and Gln—in a conformation capable of binding 5HT (in its preferred conformation)[3] in the manner shown in Figure 2. The β-structure gives a rigid molecule and the binding groups for 5HT are relatively fixed. As may be seen in Figure 2, the methyl-Arg and Phe moieties and the Gln (120), Arg (121) and Lysine (104) hydrocarbons form a lipophilic 'bed' at the bottom of which lies the indole ring of the Trp moiety, fixed by lipophilic interactions and steric hindrance. If the 5HT molecule in its preferred conformation is bound by π-π stacking to the Trp molecule, it attains the following additional contacts: 1. lipophilic: β-hydrocarbon to Trp π cloud and β-hydrocarbon and Arg γ-hydrocarbon: α-hydrocarbon to Arg

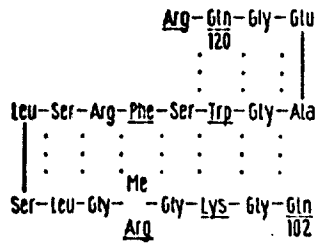

Fig. 1. The suggested conformation of the encephalitogenic polypeptide. The dots indicate CO..HN hydrogen bonds.

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Table 1. TRITIUM AND CARBON-14 ACTIVITY OF 3-METHOXY-4-HYDROXYMANDELIC ACID AND THE NEUTRAL METABOLITE ISOLATED FROM LIVER AND KIDNEY OF A CAT INFUSED WITH NOREPINEPHRINE-7-^{3}H AND EPINEPHRINE-1-^{14}C

Compound	Liver			Kidney		
	c.p.m. per 1 gm. tissue ^{3}H	c.p.m. per 1 gm. tissue ^{14}C	^{3}H/^{14}C	c.p.m. per 1 gm. tissue ^{3}H	c.p.m. per 1 gm. tissue ^{14}C	^{3}H/^{14}C
3-Methoxy-4-hydroxymandelic acid	810	270	3/1	1,200	360	3·3/1
Neutral metabolite	350	100	3·5/1	520	165	3·1/1

In cats untreated with iproniazid the final chromatogram developed from liver and kidney extracts showed in addition to the presence of 3-methoxy-4-hydroxymandelic acid another radioactive peak. The mobility of this peak was found to be greater than the mobility of 3-methoxy-4-hydroxymandelic acid. In cats treated with iproniazid, however, these peaks were not found.

The fast-moving peak was demonstrated to behave as a neutral compound since it was extractable into ethyl acetate from the aqueous phase in the *p*H range of 1–8. This compound was readily acetylated and from its mobility in the Bush 'C' solvent system[4] it appeared to be a polar substance. The ^{3}H : ^{14}C ratio for 3-methoxy-4-hydroxymandelic acid was found to be the same as for the neutral compound (Table 1). This shows that the neutral compound is formed from norepinephrine and epinephrine in the same proportion as 3-methoxy-4-hydroxymandelic acid.

The chromatographic behaviour of the neutral compound that we have isolated was found to be similar to that of 3-methoxy-4-hydroxyphenyl glycol. In the butanol–alcohol–water system, the R_F value of this compound was identical with the R_F value of 3-methoxy-4-hydroxyphenyl glycol (Axelrod, J., private communication). 3-Methoxy-4-hydroxyphenylacetaldehyde was postulated by Axelrod[1] to be a common precursor for 3-methoxy-4-hydroxymandelic acid and 3-methoxy-4-hydroxyphenyl glycol. The fact that the same ratio of ^{3}H : ^{14}C was found for 3-methoxy-4-hydroxymandelic acid and the neutral compound indicates that both have a common precursor.

The finding that in cats treated with iproniazid 3-methoxy-4-hydroxyphenyl glycol as well as 3-methoxy-4-hydroxymandelic acid were not detectable demonstrates that both compounds arise from the action of monoamine oxidase on norepinephrine and epinephrine. On the basis of the evidence given here, we conclude that the neutral compound isolated from liver and kidney is identical with 3-methoxy-4-hydroxyphenyl glycol.

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Levels of Pyridine Nucleotides in Liver Poisoning

Gallagher and Simmonds[1] have shown that rats are protected against the lethal effects of carbon tetrachloride by prophylactic treatment with precursors of pyridine nucleotides. Such treatment was found also to protect against the loss of pyridine nucleotides from liver cells and to prevent necrosis of liver cells after dosing with carbon tetrachloride[2,3]. Rees, Spector and Sinha[4] found that the administration of 'Phenergan' (10-(2-dimethylamino-*iso*propyl) phenothiazine hydrochloride) to animals poisoned with carbon tetrachloride protects the liver cells against necrosis. In the present investigation we examined the effect of treatment with 'Phenergan' on the pyridine nucleotides of liver cells in carbon tetrachloride poisoning. The work was also extended to other liver poisons such as thioacetamide and dimethyl nitrosamine.

Table 1. EFFECT OF 'PHENERGAN' ON THE LEVELS OF PYRIDINE NUCLEOTIDES IN THE LIVER IN RATS POISONED WITH CARBON TETRACHLORIDE

Treatment	E_{260} absorption
Nil	1·26
'Phenergan'	1·40
Carbon tetrachloride	0·65
Carbon tetrachloride + 'Phenergan'	1·13

Rats were killed 24 hr. after poisoning with carbon tetrachloride (1·25 ml./kgm. body-weight). 'Phenergan' dosing was at 0 and 6 hr. following poisoning (25 mgm. and 12·5 mgm./kgm. body-weight respectively). The E_{260} reading is corrected to 10 mgm. fat-free dry weight of liver extracted with 5 ml. 5 per cent w/v trichloracetic acid solution.

Table 1 shows that the administration of 'Phenergan' to rats poisoned with carbon tetrachloride prevented largely the fall in the level of pyridine nucleotide which followed the action of carbon tetrachloride alone. As was shown by Rees and Sinha (ref. 5, and unpublished work) the hepatotoxic agents carbon tetrachloride, dimethyl nitrosamine and thioacetamide lead to a different chronological pattern of leakage of liver enzymes into the blood stream. Consequently we determined the level of pyridine nucleotides in the liver and the serum at different times during the course of poisoning to determine the time relationships of the change.

Table 2 shows that as early as 5½ hr. after the administration of carbon tetrachloride there is a considerable fall in the E_{260} of the acid extracts of liver homogenates, but at this time there is no fall in the mitochondrial E_{260}. However, 10½ hr. after administration of carbon tetrachloride the mito-

Table 2. LEVELS OF PYRIDINE NUCLEOTIDES IN LIVER AND SERUM FOLLOWING POISONING OF RATS WITH CARBON TETRACHLORIDE, THIOACETAMIDE AND DIMETHYL NITROSAMINE

Treatment	Time (hr. after poisoning)	E_{260} Liver homogenate	E_{260} Mitochondria	E_{260} Serum
Nil	—	1·26	0·55	0·54
Carbon tetrachloride	5·5	0·97	0·55	—
	9	—	—	0·90
	10·5	0·82	0·34	—
	20	0·78	0·33	—
Thioacetamide	5·5	1·30	0·54	—
	9	—	—	0·52
	10·5	1·17	0·50	—
	20	1·18	0·59	—
Dimethyl nitrosamine	5·5	1·10	0·54	—
	9	—	—	0·66
	10·5	0·86	0·39	—
	20	0·68	0·40	—

Rats were dosed with carbon tetrachloride, 1·25 ml./kgm. body-weight; thioacetamide, 200 mgm./kgm. body-weight, and dimethyl nitrosamine, 100 mgm./kgm. body-weight. The E_{260} reading is corrected to 10 mgm./fat-free liver-weight for the liver homogenate, to 1 mgm. nitrogen for the mitochondria and to 1 ml. of serum. In all cases extraction of the tissues was made with 5 ml. 5 per cent w/v trichloracetic acid solution.

chondrial-levels had also fallen. This is in good agreement with the data on the leakage of enzymes from the liver in carbon tetrachloride poisoning. Rees and Sinha[4] have shown that at 6 hr. there is loss from the liver of cytoplasmic *iso*citric dehydrogenase and malic dehydrogenase and that the loss of mitochondrial enzymes occurs later, at about 12 hr. Consequently it appears that the initial effect of carbon tetrachloride is on the cell membrane, leading to a leakage of cytoplasmic constituents, and that mitochondrial damage occurs later in the course of the poisoning. In the case of thioacetamide poisoning, no fall in the E_{260} of either liver homogenates or mitochondria is observed during the 20-hr. course of the poisoning (Table 2). This is in contrast to the enzyme leakage from the liver cells and mitochondria, and suggests that the effect of carbon tetrachloride and thioacetamide on semi-permeable membranes may be different. Finally, dimethyl nitrosamine does not affect the E_{260} of either the liver homogenate or mitochondria at 5½ hr., but the E_{260} of both is depressed by 10½ hr. (Table 2). This finding agrees with the observed time relationships of the leakage of cytoplasmic constituents (Rees and Sinha, unpublished work) but indicates that pyridine nucleotides are lost from the mitochondria at a time when glutamic dehydrogenase is not leaking. This again suggests a type of damage to the semi-permeable membrane different from that occurring with the other two poisons.

Measurement of the serum E_{260} values at 9 hr. (Table 2) after poisoning with each of the above agents showed that there is a rise in the E_{260} following carbon tetrachloride poisoning, no change in the case of thioacetamide and a smaller rise in the case of dimethyl nitrosamine. These results are in good agreement with the levels of pyridine nucleotides in the livers of animals poisoned with each agent.

This work has shown that treatment with 'Phenergan', like pretreatment with precursors of pyridine nucleotides, maintains normal levels of pyridine nucleotides following carbon tetrachloride poisoning. However, it is probable that whereas the pyridine nucleotide precursors maintain this level by stimulating replacement of the nucleotides lost as a result of carbon tetrachloride poisoning, 'Phenergan' appears to act by preventing the leakage of pyridine nucleotides and of enzymes across the mitochondrial and cell membranes.

This investigation also shows that the loss of pyridine nucleotides from liver cells subsequent to carbon tetrachloride poisoning is an early event in the course of the poisoning and is not simply a result of necrosis of liver cells. The results with dimethyl nitrosamine and thioacetamide indicate that the three hepatotoxins have different effects on the semi-permeable membranes. In the case of carbon tetrachloride there is a loss of cytoplasmic and mitochondrial enzymes as well as cytoplasmic and mitochondrial pyridine nucleotides. Dimethyl nitrosamine leads to a loss of cytoplasmic enzymes but not of mitochondrial enzymes, and to a loss of pyridine nucleotides from both. Finally, thioacetamide, although leading to a loss of both cytoplasmic and mitochondrial enzymes, does not lead to the loss of cytoplasmic or mitochondrial pyridine nucleotides. Thus it appears that at least two types of membrane damage can occur, one leading to a loss of enzymes only (for example, thioacetamide), and the other to the loss of pyridine nucleotides only from the mitochondria, as in dimethyl nitrosamine, while a combination of these effects or a more general third group leads to the loss of both as in carbon tetrachloride poisoning.

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Isolation of a δ-Lactone Compound from the Leaves of *Clitoria marina* Linn.

THE plant *Clitoria marina* Linn. belongs to the Papilionaceae sub-group of Leguminosae, and is distinguished from *Clitoria ternatea* Linn. by its light-blue to almost white flowers. The leaves of the plant are used in medicine in India. A systematic chemical examination of the leaves has therefore been undertaken, and isolation of a δ-lactone compound from the alcoholic extract of the leaves has been achieved.

Sun-dried and powdered leaves were exhaustively extracted with 80 per cent ethyl alcohol in a Soxhlet for 36 hr. The extract was filtered hot, the filtrate reduced to half its original volume under reduced pressure and kept at 0° for 10 hr. when a solid substance settled down. The solid was separated, boiled with a large excess of ethyl alcohol, filtered and the filtrate again kept at 0° for 3 hr. The separated solid was refluxed with ethyl alcohol and a little activated animal charcoal and filtered hot. The filtrate on cooling deposited a white amorphous substance, m.p. 92° (yield 0·1375 per cent on dry weight basis). Further purification did not alter the melting point, and the purity of the product was established by chromatographic examination.

The substance was insoluble in water, cold ethyl alcohol, chloroform, petroleum ether and ether; but sparingly soluble in hot ethyl alcohol, benzene, pyridine and ethyl acetate. It did not decolorize bromine water or alkaline potassium permanganate and gave no acetyl derivative. It also did not reduce Fehling's solution and did not give tests for the presence of a keto group. It was found to be lævorotatory. Found: C, 78·95; H, 12·82 per cent, mol. wt. (Rast) 386; $C_{26}H_{50}O_2$ requires C, 79·18; H, 12·69 per cent, mol. wt. 394.

The bromo-derivative of the substance prepared by the usual bromination procedure was obtained as a pale yellow powder, m.p. 80°. It was soluble in benzene, chloroform and ether. Found: Br, 16·65 per cent. $C_{26}H_{51}O_2Br$ requires Br, 16·84 per cent. On treatment with freshly distilled hydrogen iodide (b.p. 126°), an ash-coloured product, m.p. 68°, was obtained. Found: I, 24·00 per cent. $C_{26}H_{51}O_2I$ requires I, 24·33 per cent.

The methyl ester of the substance prepared by usual esterification procedure gave a white amorphous powder, m.p. 74°, soluble in ether and chloroform.

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EFFECT OF SODIUM NITRATE ON THE VITAMIN A NUTRITION OF SHEEP[1]

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FORAGES with a high nitrate content have been reported to affect the vitamin A nutrition of animals (Garner, 1958; Garner *et al.*, 1958; Muhrer et al., 1955; Smith *et al.*, 1961). O'Dell *et al.* (1960) observed an increased depletion rate of liver vitamin A stores in rats fed a diet containing nitrite. Emerick and Olson (1962) showed that nitrite, but not nitrate, reduced liver vitamin A storage in rats when preformed vitamin A was administered orally but not when it was injected. Liver storage of vitamin A from dietary carotene, however, was lowered by both nitrite and nitrate with the greatest effect resulting from nitrite. Under *in vitro* conditions, Olson *et al.* (1963) found no effect of nitrate on carotene destruction in rumen fluid even though a relatively high level of nitrite had accumulated. However, instances have been reported in which low liver vitamin A levels in sheep have been thought to be due to feeding of rations with a high content of nitrate or nitrite (Hatfield *et al.*, 1961; Holst et al., 1961).

The experiments described herein were conducted to determine the effects of sodium nitrate on carotene and vitamin A levels in liver and plasma of sheep.

Experimental Procedure

Trial 1. Eighty Texas lambs that had been previously fed a ration of alfalfa hay for about 1 month were given 3-mg. diethylstilbestrol implants and allotted to eight lots of 10 lambs each on the basis of weight and sex (nine ewes and one wether per lot). Four replicated treatments were as follows: (1) control, (2) 3000 I.U. of vitamin A per lamb daily, (3) 2.5% of sodium nitrate and (4) 3000 I.U. of vitamin A plus 2.5% of sodium nitrate.

The daily ration was gradually increased to 2 lb. of ground alfalfa hay and 1 lb. of a concentrate mixture per head during a 2-week preliminary period and then held constant for the remainder of the 84-day trial. This provided a uniform daily intake of vitamin A and sodium nitrate. Vitamin A palmitate was included in the concentrate mixture from the start of the experiment while the sodium nitrate was added immediately after the preliminary period.

The control concentrate mix was composed of 94.9% ground corn and 5.1% trace mineralized salt. Sodium nitrate, when used, replaced the trace mineralized salt and a portion of the corn and comprised 7.5% of the concentrate mix to give 2.5% in the total ration. The amounts of salt and sodium nitrate resulted in the rations, with and without nitrate, being about equal in sodium content. All lots of lambs were offered trace mineral salt and dicalcium phosphate free-choice. Analyses of the concentrate mixes and alfalfa hay by the A.O.A.C. (1960) method showed that they contained 0.17 and 3.26 mg. of β-carotene per pound respectively.

Blood methemoglobin values were determined periodically by the method of Evelyn and Malloy (1938). Plasma carotene and vitamin A values were determined at the start, approximately the middle and at the termination of the experiment by the method of Koehn and Sherman (1940). Differences between treatment means were evaluated according to the procedure outlined by Duncan (1955).

Trial 2. Two hundred and sixty-eight native ewes and wethers weighing about 94 lb. and previously fed alfalfa hay for about 5 months were utilized in this trial. A control-hay-concentrate mixture was fed to all sheep for a 2-week preliminary period to get them on full feed. They were then allotted to four treatment groups of 67 each on the basis of weight and sex. Treatments were as follows: (1) control, (2) 4100 I.U. of vitamin A per lamb daily, (3) 3% of sodium nitrate and (4) 4100 I.U. of vitamin A per lamb daily plus 3% of sodium nitrate.

The control ration consisted of a hay-corn

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mixture containing 25% ground alfalfa hay and 75% ground corn, and a protein-mineral supplement containing 50% ground corn, 38% soybean meal, 6% trace mineralized salt, 3% dicalcium phosphate and 3% ground limestone. The hay-corn mixture was fed *ad libitum* and the protein-mineral supplement was fed at the rate of 0.25 lb. per head daily. Analysis showed the protein content of the control hay-corn mix and control supplement to be 11.6 and 21.0% respectively. The 3% of sodium nitrate replaced an equal amount of corn in the hay-corn mixture and soybean meal in the protein-mineral supplement. Vitamin A palmitate was added to the appropriate protein supplements, and the concentration by analysis was 16,400 I.U. per pound of supplement. Analysis by A.O.A.C. (1960) procedure showed a β-carotene content of 3.7 mg. per lb. of hay-corn mix and 0.51 mg. per lb. of supplement.

Twelve sheep, the heaviest five ewes and seven wethers, were slaughtered from each group initially and at 2-week intervals during the 8-week experiment. This procedure was used to obtain data on liver vitamin A at periodic intervals. In this manner, 60 sheep were used from each treatment. Extra animals in each lot provided adequate numbers to allow for death losses.

Blood and liver data were determined for each animal slaughtered. Methemoglobin, carotene and vitamin A in blood and statistical analyses were determined as described for trial 1. Liver vitamin A was determined by the method of Johnson and Baumann (1947).

Results and Discussion

Trial 1. Results of the first trial are presented in table 1. The rates of gain were rather low for all lots. These were mostly ewe lambs, feed was limited and they were handled and bled frequently. Feeding rations containing 2.5% of sodium nitrate (about 34 gm. per head daily) and/or 3000 I.U. of vitamin A per head daily did not significantly affect rate of gain, feed efficiency or carcass grade. The amount of feed offered was completely consumed in almost all instances, although less readily at first by the sheep fed sodium nitrate. While 1% of sodium nitrate has been reported to significantly reduce weight gains of fattening cattle (Weichenthal *et al.*, 1963), 4% of sodium nitrate was reported to have no effect on weight gains of sheep (Smith *et al.*, 1962).

There were no visible signs of nitrate toxicity during trial 1, and methemoglobin values were low in all instances. The low plasma carotene values obtained in this experiment are characteristic of sheep (Maynard and Loosli, 1962) and did not appear to be influenced by the treatments. Initial plasma vitamin A values averaged approximately 25 mcg. per 100 ml. Values for sheep on the control and vitamin A treatments declined to about 20 mcg. of vitamin A after 1 month, and remained near this level for the remainder of the trial. After 1 month on trial, there was an increase in the average plasma vitamin A level of the sheep fed sodium nitrate with and without vitamin A. At this time the values were significantly ($P<.01$) higher than those for sheep fed rations without nitrate. However, the values for these groups declined by the end of the experiment to about the same levels as those not fed nitrate.

Trial 2. A critical measure of weight gain was not obtained in trial 2 because the slaughter of animals at 2-week intervals resulted in some being on the experiment only a short time. Sheep fed the control and vitamin A rations consumed an average of about 3.5 lb. of feed per head daily. Feed consump-

TABLE 1. TRIAL 1—PERFORMANCE AND BLOOD DATA OF SHEEP FED RATIONS WITH AND WITHOUT SODIUM NITRATE AND/OR VITAMIN A

	Treatment			
Item	Control	3000 I.U. vitamin A	2.5% of sodium nitrate	2.5% of sodium nitrate plus 3000 I.U. of vitamin A
No. of lambs	20	20	20	20
Av. initial wt., lb.	75.9	75.8	75.8	75.6
Av. final wt., lb.	99.3	95.0	98.2	96.7
Av. daily gain, lb.	0.28	0.23	0.27	0.25
Av. daily feed, lb.	3.00	3.00	3.00	3.00
Feed/100 lb. gain, lb.	1051	1288	1096	1166
Methemoglobin, gm./100 ml.				
Sept. 13 (initial)	..	..	0.14	0.10
Sept. 20	..	..	0.43	0.54
Sept. 28	..	..	0.28	0.54
Oct. 12	..	..	1.66	1.06
Oct. 27	..	..	0.62	0.68
Nov. 10	..	..	0.28	1.00
Nov. 17 (final)	..	..	0.94	0.94
Plasma carotene, mcg./100 ml.[a]				
Initial	1.48	1.78	2.19	2.18
Oct. 12	5.69	5.80	4.92	5.08
Final	3.89	3.20	3.66	2.07
Plasma vitamin A, mcg./100 ml.				
Initial	24.78	25.82	24.84	25.78
Oct. 12	19.82[b]	18.26[b]	29.61[c]	31.12[c]
Final	19.45	19.36	20.81	20.09

[a] Error due to substances absorbing light at 440 mμ is probably high.

[b, c] Means on the same line having different superscript letters differ significantly ($P<.01$).

tion was 5.6 and 2.8% less when 3% of sodium nitrate and sodium nitrate plus vitamin A were included in the rations. This reduction in feed consumption resulting from the use of rations with added sodium nitrate is in agreement with work using cattle (Weichenthal *et al.*, 1963; Hale *et al.*, 1962).

Several deaths occurred during this trial. Four death losses occurred in the lot of sheep fed sodium nitrate, one in the lot fed vitamin A and five in the lot fed sodium nitrate plus vitamin A. One death loss in each of these three lots of sheep occurred late in the experiment and was due to urinary calculi. The other losses occurred during the first 3 weeks of the experiment. These animals died suddenly, and it was not possible to obtain blood samples for methemoglobin analyses. However, there were no appreciable amounts of methemoglobin present at the regular 2-week bleedings. Average methemoglobin values never exceeded 0.4 gm. per 100 ml. of blood for any of the groups. Apparently 3% of sodium nitrate (about 42 gm. per head daily) was toxic in this experiment, and vitamin A did not appear to lessen the toxic effect: Vitamin A has also been reported to be ineffective in overcoming the lowered performance attributed to the feeding of sodium nitrate to cattle (Weichenthal *et al.*, 1963).

Plasma and liver carotene values were low for sheep in all groups as was true for trial 1. Therefore, the values are not reported. Initial plasma and liver vitamin A values (table 2) averaged from 23–25 mcg. per 100 ml. and 22–28 mcg. per gm. respectively. There was an increase in plasma vitamin A values during the experiment with the values being slightly higher in most instances for the sheep receiving vitamin A. Sodium nitrate did not appear to influence plasma vitamin A values as in trial 1. However, plasma vitamin A values remained at considerably higher levels for sheep fed rations without nitrate in trial 2. The extent to which this may have influenced the difference in results between the two trials is not known.

Average liver vitamin A values for sheep fed the control ration decreased to about 11 mcg. per gm. in 6 weeks. The value remained at this level until the termination of the 8-week experimental period. When vitamin A was supplemented at 4100 I.U. per head daily, liver vitamin A values remained at approximately the initial level. Guilbert *et al.* (1940) reported that 1.4 mg. of carotene per 100 lb. of body weight was adequate to meet the requirement of sheep and that three times this amount was needed to promote liver storage. The decrease in liver vitamin A from the initial level of approximately 22 mcg./gm. in the control animals occurred while the sheep were consuming an average of 12.3 mg. of carotene per head daily. There was some indication, however, that the average liver vitamin A level of this group was becoming stabilized at approximately 11 mcg. per gm. of liver.

A depletion of liver vitamin A stores was observed in the sheep receiving sodium nitrate. This is in contrast to data reported for cattle fed 1% of sodium or potassium nitrate (Weichenthal *et al.*, 1963; Hale *et al.*, 1962) and sheep fed 4% of sodium nitrate (Smith

TABLE 2. TRIAL 2—PLASMA AND LIVER VITAMIN A VALUES OF SHEEP FED RATIONS WITH AND WITHOUT SODIUM NITRATE AND/OR VITAMIN A

Days on trial	No. of sheep per treatment	Treatment: Control (1)	4100 I.U. vitamin A(2)	3% of sodium nitrate (3)	3% of sodium nitrate plus 4100 I.U. vitamin A(4)
Plasma vitamin A, mcg./100 ml.					
Initial	12	24.24 ±1.07[a]	22.78 ±1.12	24.66 ±1.11	24.92 ±1.20
14	12	32.39[b] ±1.36	33.23[b] ±1.59	24.68[c] ±1.82	33.28[b] ±2.25
28	12	39.11[b] ±1.50	44.62[c] ±2.28	36.92[b] ±2.17	45.83[c] ±2.64
42	12	32.86[b] ±1.66	41.29[c] ±3.03	31.59[b] ±2.01	44.90[c] ±1.44
56	12	37.88 ±2.60	37.93 ±4.00	33.67 ±1.67	41.85 ±1.52
Liver vitamin A, mcg./gm.					
Initial	12	22.02 ±3.54	22.60 ±3.56	28.15 ±4.64	23.27 ±6.46
14	12	18.38 ±2.94	21.74 ±2.87	17.26 ±2.17	24.43 ±3.57
28	12	14.83[b] ±2.30	22.51[c] ±2.48	11.84[b] ±2.43	23.66[c] ±2.58
42	12	11.10[b] ±1.51	20.32[c] ±1.82	12.16[b] ±2.54	14.08[b, c] ±2.84
56	12	11.87[b] ±1.50	25.50[c] ±5.70	6.41[b] ±0.82	14.00[b] ±1.66

[a] Mean±standard error. Values are averages of 12 analyses per period for each treatment with the exception that treatment 4 at 14 days and treatment 2 at 28 days contained 11 values, and treatment 3 at 14 days and treatment 1 at 28 days contained 10 values.

[b, c] Means on the same line having different superscript letters differ significantly ($P<.05$).

et al., 1962). However, in the former study with cattle, liver vitamin A levels were low in all instances and the latter study with sheep was of a much shorter duration than that reported here.

Vitamin A supplementation, while apparently limiting the extent of depletion in sheep fed sodium nitrate, failed to prevent it. In each lot of sheep receiving sodium nitrate, final average liver vitamin A values were about one-half as high as those obtained in corresponding lots of sheep not receiving the sodium nitrate. When vitamin A was fed, the difference between final liver vitamin A values for groups with and without nitrate was significant ($P<.01$). While nitrate appeared to exert a similar effect in the groups not receiving vitamin A, this difference was not statistically significant. Statistical analysis showed that ewes and wethers did not differ significantly in plasma or liver vitamin A levels, and there were no sex x treatment interactions for any of the sampling periods.

Variable results have been reported concerning the correlation between plasma and liver vitamin A levels. Ralston and Dyer (1959) and Diven *et al.* (1960) found no apparent relationship between plasma and liver vitamin A. However, Braun (1945) and Hibbs and Krauss (1947) reported that plasma and liver vitamin A concentrations were related when liver vitamin A storage was low. The data from this experiment were grouped according to the concentration of vitamin A in the liver, and correlation coefficients were calculated. Correlation coefficients of —.03, —.05, 0.11, —.02, 0.19 and —.15 were obtained between liver and plasma vitamin A concentrations for liver vitamin A values in the ranges of 0–4.99, 5.0–9.99, 10.0–14.99, 15.0–19.99, 20.0–24.99 and over 25.0 mcg. per gm. None of these correlations were statistically significant, indicating that plasma vitamin A concentrations are of little value in predicting the extent of liver vitamin A stores in sheep.

Data obtained from these experiments indicate that levels of nitrate approaching acutely toxic levels can have an effect on tissue vitamin A levels of sheep. The importance of the long-term effect of these high levels of nitrate on the vitamin A status of livestock, however, appears to be of secondary importance in view of the immediate hazard, i.e., the possibility of death due to primary toxic effects.

Summary

Rations containing 2.5 and 3.0% of added sodium nitrate were used in separate experiments to determine their effect on tissue vitamin A levels of sheep. Only small amounts of methemoglobin were found at regular bleeding periods in sheep receiving either level of nitrate, although seven sheep were lost from suspected acute nitrate poisoning while being fed 3% of sodium nitrate. Vitamin A offered no protection from nitrate poisoning.

A transient increase in plasma vitamin A levels occurred about the middle of the first trial in sheep fed 2.5% of sodium nitrate and 2.5% of sodium nitrate plus 3000 I.U. of vitamin A. By the end of the trial, however, the values for these lots of sheep approximated the values obtained in sheep receiving corresponding non-nitrate treatments. No effect of nitrate on plasma vitamin A levels was observed in the second trial. However, lower liver vitamin A stores were found in the nitrate-fed sheep. After 56 days on experiment, sheep fed 3% of sodium nitrate had liver vitamin A concentrations that were 46.0% lower than those fed the control ration. Similarly, sheep fed 3% of sodium nitrate plus 4100 I.U. of vitamin A daily had liver vitamin A values 45.1% lower than those fed 4100 I.U. of vitamin A without nitrate.

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Effect of Curing Agents on the Growth and Survival of Food-Poisoning Strains of Clostridium perfringens

SUMMARY

The effects of different combinations of curing salts (NaCl, $NaNO_3$, $NaNO_2$) and temperature on the growth, survival, and heat resistance of several strains of *Clostridium perfringens* were investigated. These strains were shown to survive and grow in concentrations of curing salts considerably higher than those used in normal curing operations. When used in conjunction with heat, the concentrations of curing salts required to affect cell survival were still well above those usually employed. Hams were pumped with curing brines inoculated with low concentrations of spores of a food-poisoning strain of *C. perfringens*, then cured and smoked according to routine procedures. *C. perfringens* could still be isolated from the hams after cooling.

A simple procedure for preparing reproducible inocula of aged spores is described. These aged spores showed much greater resistance to heat after storage in meat-brine mixtures than did spores from young sporulating cultures.

INTRODUCTION

The role of *Clostridium perfringens* as a causative agent of food poisoning has received increasing attention in the last few years, particularly in the United States and England (Hobbs, 1962; Angelotti *et al.*, 1962). Hall *et al.* (1963), in a study of 83 strains of *C. perfringens* associated with cases of food poisoning in both countries, reported that the spores from the English strains were considerably more heat-resistant. It was suggested that cases of *C. perfringens* food poisoning in England came primarily from foods contaminated prior to cooking, and in the United States from food contaminated after cooking. Barnes *et al.* (1963) reported that there is a greater possibility of *C. perfringens* food poisoning if meat is contaminated prior to cooking.

Studies on the resistance of anaerobic bacteria to curing agents have indicated that *C. botulinum* and putrefactive anaerobes will survive and sometimes grow in the presence of appreciable concentrations of curing agents (Silliker, 1959). An outbreak of food poisoning from salted beef contaminated with *C. perfringens* was reported by Hobbs (1962). She also reported that unpublished data from her laboratory had indicated that vegetative cells of *C. perfringens* would survive six days on raw meat immersed in 22% NaCl brine and that spores would germinate in 5% NaCl. No details of this work were presented, however, and no heat-resistance determinations were made. Studies by Strong *et al.* (1963) have shown a rather high incidence of contamination of fresh meats with *C. perfringens*. Hall and Angelotti (1965) have also shown a high incidence of fresh meat contamination with *C. perfringens*, but found only a small percentage of these to produce heat-resistant spores. These studies, however, do not give any data on the survival of *C. perfringens* in hams.

With increased interest in *C. perfringens* as a cause of food poisoning, the present investigation was undertaken to obtain more definitive data regarding the effects of curing salts on the growth and heat resistance of this microorganism as well as on its survival in a normal curing process.

EXPERIMENTAL PROCEDURES

Preparation of vegetable cell and spore suspensions. When only vegetative cells of *C. perfringens* were required, a culture in the log phase of growth (3–4 hr) from fluid thioglycollate medium (Difco) was used. When a sporulating culture was needed, a 24-hr SEC culture, as described by Angelotti *et al.* (1962), was used. Although the freshly sporulated SEC cultures were adequate for the studies on heat resistance and survival in curing salts, a more stable preparation, primarily containing spores, was desired for inoculation into hams. Spore inocula prepared by heating a culture at 80°C for 5 min were unsatisfactory because this treatment decreased their heat resistance, probably through activation of the spores (Barnes *et al.*, 1963). Attempts failed to separate spores from vegetative cells by the procedures of Sacks

and Alderton (1961), Stewart and Halvorson (1953), and Long and Williams (1958). Strong and Canada (1964) used cultures stored on sterile soil for their spore inocula, and the procedure employed in our study was similar except that filter-paper discs were substituted for soil. Onto each sterile filter-paper disc (Schleicher and Schuell Co., No. 740-E) was placed 0.05 ml of a sporulating culture grown in SEC medium; these were dried over $CaSO_4$ in a desiccator at room temperature. As indicated in Fig. 1, the unheated count (i.e., vegetative cells and any spores not requiring heat shock for germination) of the inoculated discs decreased rapidly during the first day after the culture was added to the disc, then slowly declined. The spore count (heated 80°C, 5 min), however, increased rapidly during the first day, decreased somewhat the second day, and declined very slowly thereafter. If all cells appearing in the unheated count are considered vegetative cells, this means that over 99% of the vegetative cells have lost their viability by the end of two days. Even if they are considered spores not requiring heat shock, they represent less than 5% of the total spore count. Therefore, use of a count on the spore discs after heat shock is a valid indication of total spore population. The discs afforded an easy way of obtaining spore inocula of approximately known concentrations without having to weigh the sample, as is necessary in using soil stocks. To use the discs, one or more were placed in a tube of sterile distilled water and agitated vigorously. The discs disintegrated completely, and the filter-paper pulp could be removed by filtration through sterile cheesecloth with no decrease in spore count.

Plate counts and incubation. Plate counts were made on SPS agar, on which strains of *C. perfringens* associated with food poisoning produce black colonies (Angelotti *et al.*, 1962). All plates and tubes (including thioglycollate tubes) were incubated at 37°C in an anaerobic incubator (National Appliance Co.) flushed four times with a mixture of 90% N_2 and 10% CO_2. All plates were incubated for 48 hr.

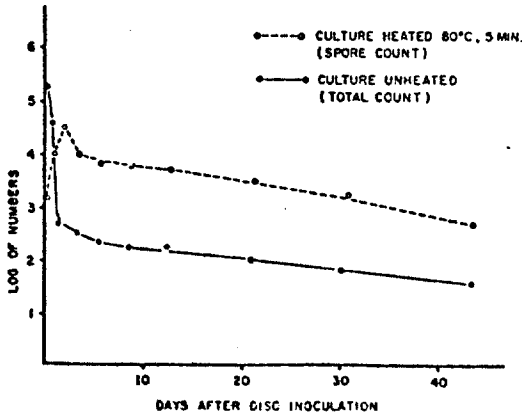

Fig. 1. Changes in total count and spore count of *C. perfringens* No. 15 on filter-paper discs.

Effect of curing salts on growth of C. perfringens in an artificial medium. Fluid thioglycollate medium was prepared containing various concentrations of curing salts. Castellani and Niven (1955) have shown that sterilization of glucose and nitrite together considerably reduced the apparent concentration of nitrite required to inhibit anaerobic growth of *Staphylococcus aureus*. Results were similar in our study with *C. perfringens*. Therefore, the nitrite solutions were sterilized by filtration through membrane filters and added aseptically to the fluid thioglycollate medium. Duplicate thioglycollate tubes containing each of the salt concentrations were inoculated with 0.1 ml of a vegetative cell culture. After 24 hr of incubation, the tubes were examined visually for extent of growth. Table 1 shows that 6% (w/v) NaCl, 10,000 ppm $NaNO_3$, or 400 ppm $NaNO_2$ are required to inhibit the growth of *C. perfringens* significantly.

Survival of C. perfringens in solutions of curing salts. Four solutions were prepared containing the following range of concentrations of curing salts: from 7.5% (w/v) NaCl, 3,700 ppm $NaNO_3$, and 370 ppm $NaNO_2$ to 17% NaCl, 23,000 ppm $NaNO_3$, and 2,300 ppm $NaNO_2$. They were sterilized by filtration, and 15 ml were placed in sterile screw-cap tubes (15 × 150 mm). One-half milliliter of a 24-hr SEC spore culture of each of five strains of *C. perfringens* was inoculated in triplicate into these solutions, and the inoculated solutions were stored at 3°C. (This approximates the temperature at which meats are held while being cured.) One-half-milliliter samples were withdrawn periodically from these tubes and inoculated into fluid thioglycollate medium. Four of the five strains tested survived at least 48 days in all of the stored brine solutions. In a subsequent trial, a spore disc culture of one of the most heat-resistant strains was used. Fifty percent of the spores (original inoculum 30/ml) remained viable for at least 35 days in a brine containing 21.5% NaCl, 1,800 ppm $NaNO_3$, and 1,200 ppm $NaNO_2$. This brine is similar in concentration to that pumped into hams in routine curing operations (Fields and Dunker, 1952).

Additional tests were carried out to determine the effect of curing salts on the heat resistance. In these tests the cultures survived 100°C for at least 30 min in the presence of 6% NaCl, 30,000 ppm $NaNO_3$, or 2,000 ppm $NaNO_2$, used individually. The cells also survived 80°C for at least

Table 1. Growth of 18 strains of *C. perfringens* in various concentrations of NaCl, $NaNO_3$, and $NaNO_2$ contained in thioglycollate medium.

Extent of growth	NaCl (w/v%)			$NaNO_3$ (ppm)			$NaNO_2$ (ppm)		
	4	6	8	8,000	10,000	12,000	300	400	500
Good	14[a]	1	0	18	0	0	18	0	0
Slight	4	8	1	0	11	0	0	13	0
None	0	9	17	0	7	18	0	5	18

[a] Number of strains.

6 hr in a combination of 10% NaCl, 3,000 ppm $NaNO_3$, and 1,000 ppm $NaNO_2$, a combination of heat and curing salts well above that normally encountered in cured meats.

Survival of C. perfringens under simulated curing conditions in the laboratory. Fresh meat was aseptically removed from hams and ground in a sterile grinder, and sterile concentrated solutions of the curing salts were added to give a final concentration of 3% NaCl, 500 ppm $NaNO_3$, and 200 ppm $NaNO_2$. These represent the maximum allowable concentration of $NaNO_2$ as well as the usual maximums of NaCl and $NaNO_3$ found in cured meats. A brine solution without meat was also prepared and placed in several tubes. Spore suspensions, treated as described in Table 2, were inoculated into these meat-brine and brine samples. The inoculated tubes were held at 3°C for 3 days and then heated at 80°C for 60 min. Plate counts were made, and the number of cells of *C. perfringens* type was determined after 48 hr of incubation. The uninoculated meat had an aerobic count of < 50 per gram; no anaerobic bacteria were encountered.

As seen in Table 2, both heated and unheated spores from SEC cultures survive in stored brine solutions better than in meat-brine mixtures, where nutrients are available. It is also evident that spores maintained on filter-paper discs have a much higher percentage of survival than unaged spores from regular SEC cultures. Results were similar with four other strains.

Table 2. Survival of spores of *C. perfringens* in simulated curing procedures.

Inoculum[a]	Substrate[b]	Spore count[c]	% survival[d]
Unheated SEC	Meat + brine	4,500	<0.1
	Brine	2,280	30.0
Unheated disc	Meat + brine	70	39.0
	Brine	850	64.0
Heated SEC	Meat + brine	4,500	<0.1
	Brine	1,800	4.0
Heated disc	Meat + brine	850	0.4
	Brine	850	3.0

[a] Spore culture unheated or heated 80°C, 5 min.
[b] Final concentrations in the ground ham and brine without meat were 3% NaCl, 0.05% $NaNO_3$, and 0.02% $NaNO_2$.
[c] Number of spores added per gram of meat or ml of brine.
[d] After inoculation, held 3 days at 3°C, heated 60 min at 80°C.

Survival of C. perfringens in cured hams. Whole hams were cured and smoked by a procedure similar to Type I of Fields and Dunker (1952). Hams were spray-pumped with a brine containing 17.9% (w/v) NaCl, 1,500 ppm $NaNO_3$, and 980 ppm $NaNO_2$. They were then cured for seven days at 3°C in a cover pickle containing 15.8% NaCl and 1,300 ppm each of $NaNO_3$ and $NaNO_2$. The hams were smoked for 19–20 hr, during which time the internal temperature reached 61°C and remained there for about 1 hr. Twelve hams were pumped to 10% net weight increase with brine inoculated with a low concentration (20/ml) of *C. perfringens* spores prepared from paper discs. At various stages of the curing and smoking process, two hams were removed and a count was made of typical *C.-perfringens*-type colonies. Samples were cut from three locations in the center and bone areas of the hams, and plate counts were made from the meat taken from these areas and from the fluid that drained into the cavity remaining after removal of the meat sample.

As indicated by Table 3, *C. perfringens* consistently survived both curing and smoking processes. The fluid probably contained exudate from the tissue as well as brine remaining in the intermuscular spaces. This fluid consistently had a higher count than the meat samples. In hams in which a heavier inoculum was used (40/ml brine), it was possible to isolate *C. perfringens* from the meat as well as from the fluid after smoking and cooling.

DISCUSSION

Barnes *et al.* (1963) showed that heat-shocking 48-hr spore cultures caused them to lose their heat resistance when subsequently cooled to 10°C within 3 hr in a meat medium. Curran and Pallansch (1963) have also demonstrated incipient germination of *Bacillus* spores at sub-minimal growth temperatures. Our investigation showed a similar loss of resistance on storage at 3°C for 3 days for the heat-shocked cells from both

Table 3. Survival of *C. perfringens* during routine curing procedures.

	Treatment of sample			
	Unheated		Heated[b]	
Phase of process[a]	Fluid[c]	Meat[c]	Fluid[c]	Meat[c]
After pumping	+	+	+	+
After curing	+	+	+	+
Smoked to 30°C[d]	+	−	+	−
Smoked to 45°C[e]	+	±	+	±
After smoking (not cooled)	+	+	+	+
After smoking and cooling	+	+	+	±
Uninoculated controls				
Fresh ham	−	−	−	−
After smoking and curing	−	−	−	−

[a] Two hams were removed for examination at each phase of the process.
[b] Samples heated 5 min at 80°C.
[c] Source of sample.
[d] Required 6 hr in smokehouse to reach 30°C.
[e] Required 13 hr in smokehouse to reach 45°C.

SEC cultures and spore disc cultures inoculated into a meat-brine substrate. However, for the spores that were stored 3 days at 3°C without prior heat shock < 0.1% of the spores from the SEC cultures retained their heat resistance whereas 39% of the spores from the discs were resistant. These data suggest that, while the young spores in the SEC culture had developed heat resistance, their immaturity permitted much more germination at sub-minimal growth temperatures than occurred in the mature spores. Since a mature spore is the type more likely to be encountered as a contaminant from the environment, it would appear that aged spores should be used in studies relating to the survival of *C. perfringens* in foods.

Although there are definite differences in the heat tolerance of different strains of *C. perfringens*, there was little difference in the effect of curing salts on the various strains even at levels well above that found in commercial curing operations. Not only will they survive, but they may actually grow if the suspending medium and temperature are favorable for growth. This ability to survive in concentrated brines indicates the need for proper sanitary precautions in storing commercial brines to be used for pumping hams and cover pickle.

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THE USE OF A MOLECULAR SEPARATOR IN THE DETERMINATION OF TRACE CONSTITUENTS BY COMBINED GAS CHROMATOGRAPHY AND MASS SPECTROMETRY

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SUMMARY

A method combining gas chromatography and mass spectrometry for the detection and estimation of traces of volatile nitrosamines in extracts is described. A membrane separator is used and details of its construction and performance are given. By peak matching against a reference compound a detection limit of 2 mg/l of the nitrosamines is achieved. By incorporating a peak cutting system into the gas chromatograph the detection limit is lowered to 0.2 mg/l, which represents a limit of 0.2 μg/kg on the original material.

INTRODUCTION

Combined gas chromatography and mass spectrometry (GC–MS) is used extensively for the analysis of multi-component mixtures. A recent survey of the literature revealed that the most frequently used interface is the Watson–Biemann separator. This may be attributed partly to the fact that the device is satisfactory, although not necessarily ideal, for the vast majority of analyses, and that it is commercially available from several mass spectrometer manufacturers. The separator does, however, have a number of potential disadvantages, which become significant, for example, when the analysis of trace constituents is attempted. The separator has a large surface area and volume, within which losses by adsorption and band spreading may occur. The separator functions on the basis of Graham's law, and hence the efficiency is relatively low for compounds of low molecular weight.

The separator introduced by LLEWELLYN AND LITTLEJOHN[1] offers advantages in that the dead volume and surface area are small, and sample enrichment is achieved predominantly as a result of partition within a silicone rubber membrane, and not by diffusion. In common with the Watson–Biemann separator, the original membrane separator required intermediate pumping with a rotary pump. A single-stage membrane separator has more recently been described by HAWES *et al.*[2], which has the added advantage that the membrane is pumped directly by the mass spectrometer pump system, so that no sample is lost through an intermediate stage. The high pres-

sure side of the membrane is vented to atmosphere (and is often connected to a flame ionisation detector), so that connecting the gas chromatograph to the mass spectrometer does not result in any change in carrier gas flow rate within the column. Changes do occur when a Watson–Biemann separator is used. Potential disadvantages are that significant residence times within the membrane may occur, particularly if too thick a membrane is employed. A compromise is necessary since a workable ion source pressure may not be obtainable with a thin membrane in a single-stage separator. Irreversible adsorption may occur on the membrane, resulting in substantial or complete loss of sample. The maximum operating temperature of the system is governed by the stability of the GC stationary phase as with all interface systems, and also by the thermal stability of the membrane.

A membrane separator has been constructed in the laboratory, and its use for the transfer of nanogram quantities of materials eluted from a GC column to a mass spectrometer has been evaluated. Bearing in mind the limitations mentioned above, it has been found to be significantly more useful for this work than a Watson–Biemann separator. A description of the apparatus and the results of the study are described below.

APPARATUS

The mass spectrometer is an AEI model MS902 double focusing instrument having a maximum scan speed of 10 sec per decade mass range, and fitted with peak matching facilities. The gas chromatograph is a Philips Research instrument, fitted with a flame ionisation detector. The membrane separator is housed in the GC oven, and is connected to the mass spectrometer with 0.5 mm I.D. stainless steel tubing, contained within 12 mm flexible metal tubing to ensure uniform heating. The tubing

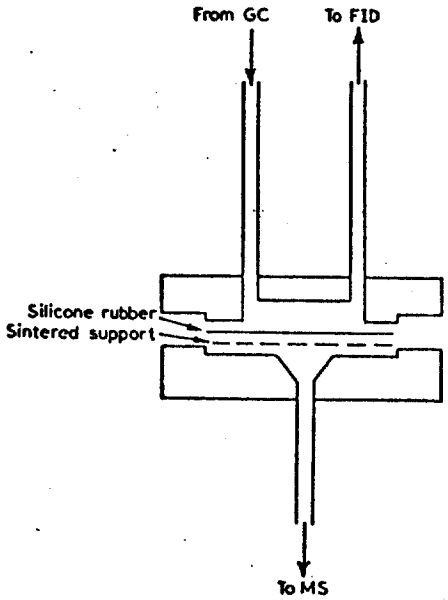


Fig. 1. Diagram of membrane separator.

is wrapped in heating tape and the line temperature controlled with a variable voltage transformer.

The transfer line contains a stainless steel bakeable bellows on/off valve (Nupro type H), so that the mass spectrometer can if necessary be isolated from the GC, and both instruments used independently. The temperature of the complete interface system is monitored at several points with chromel–alumel thermocouples.

The separator consists of a sheet of 0.05 mm × 21 mm diameter silicone rubber sheet (available from Sandev Ltd., Harlow, Essex) supported on a metal sintered disc (Perkin-Elmer filter disc) clamped between stainless steel flanges (see Fig. 1). About 8 sq.mm of membrane come into direct contact with GC effluent. The separator is assembled by placing the sinter and rubber on the lower flange, the perimeter of the rubber is coated with a silicone rubber moulding paste (Esco Rubber type SR600), and the upper flange, which is fitted with a gold 'O' ring, is placed on the membrane, and the assembly clamped together. The paste is allowed to polymerise at room temperature, after which the separator is heated for several days at 200° in a stream of nitrogen. It is then tested for leaks simply by confirming that no gas flows at the exit tube to the mass spectrometer, and for free passage across the membrane surface to the venting line. The separator is then connected to the mass spectrometer with the GC inlet and venting lines blanked off, to check that there is no leakage between the flanges to atmosphere. The GC column and detector are connected, and a final leakage test is made by noting the ion source pressure. The whole system is baked at 220° until an acceptable background spectrum is obtained. A pressure about one order of magnitude higher than is normally obtained in the ion source is usual with a new separator. It has been found that after prolonged use (several weeks) the instrument pressure will slowly decrease to a minimum. The separator used in the present work has been in continuous operation between 130° and 200° for ten months, and has shown no signs of deterioration.

DISCUSSION

Much of the work carried out on the GC–MS system has involved the detection of traces of volatile nitrosamines, and as an example of the value of the system, it is this application which will be considered in detail.

For the satisfactory recording of the spectra of GC effluent, at least partial resolution of the constituents is necessary. However, the separation of trace constituents, from extraneous material present in substantially larger amounts in extracts, is difficult to achieve. Capillary columns may be of some value[3], but they have not been used in the present work, firstly because the sample loading required to obtain reasonable amounts of the constituents under study in the ion source would result in gross overloading of the column, and secondly because a quantitative estimate of the amounts of nitrosamines was required. Additional criteria were that the initial qualitative analysis of an extract for the presence of nitrosamines must be complete in a reasonable time (less than 1 h per sample), and that all the nitrosamines must be resolved from each other. For a standard mixture of volatile dialkyl and heterocyclic nitrosamines these conditions can be satisfied and a chromatogram of such a mixture, in water, is shown in Fig. 2a. Operating conditions are given in Table II. No difficulty was encountered in obtaining a complete spectrum for each nitrosamine. A chromato-

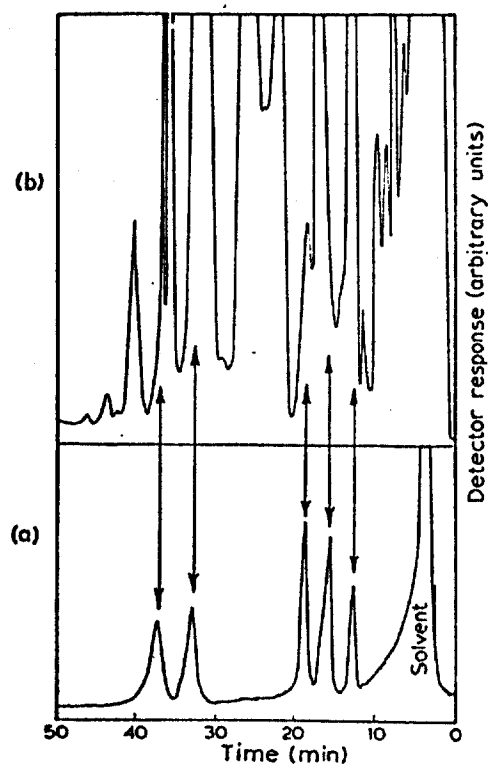


Fig. 2. Comparison of chromatograms of nitrosamine standard solution, and an extract. (a) 5-μl standard (50 ng per nitrosamine). Attenuation $\times$ 10. (b) 5-μl extract. Attenuation $\times 10^3$.

gram of an extract run under identical conditions is shown in Fig. 2b, from which it can be seen that nitrosamines are completely masked. A spectrum run at the point at which diethylnitrosamine is expected is clearly useless, particularly when it is realised that the detection of 50 ng of each nitrosamine requires a sensitivity increase of 2×10^3 over that used to obtain the spectrum illustrated (Fig. 3). The detection of traces of known materials can, however, be achieved even under such conditions of gross interference by monitoring, under high resolution, a characteristic and abundant ion in the spectrum, rather than by attempting to run a complete low resolution

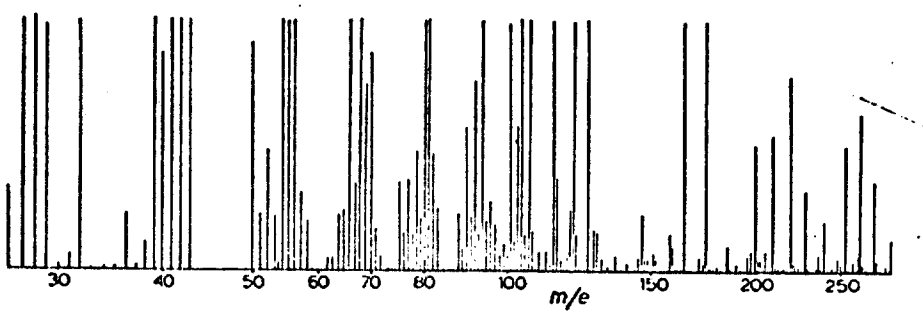


Fig. 3. Spectrum of GC effluent.

spectrum for characterisation. For the detection of a series of nitrosamines the appropriate parent ion was chosen. Since the retention time of each nitrosamine can be determined using standard mixtures, the analysis of an extract for several nitrosamines can be carried out in a single run, provided that the interval between the elution of successive nitrosamines is sufficient to allow the mass spectrometer to be set for the appropriate parent ion.

The resolution necessary for such a procedure will depend on the extent of interference expected from other compounds of the same nominal mass, eluted at the same time. Account must also be taken of fragment ions in assessing the resolution. Resolution requirements will be more stringent the higher the molecular weight of the nitrosamine, but against this the probability of interference will decrease with retention time.

The coincident elution of volatile nitrosamines with other compounds present in foodstuffs is obviously widespread, so that GC analysis alone[4], even using nitrogen-specific detectors[5], will not give unequivocal results. Even GC–MS analysis must be used with caution. Bearing in mind that for trace analysis the maximum possible sensitivity is required, a resolving power was used which would unequivocally detect the presence of nitrosamines in mg/l amounts.

The GC detection limit, using a standard solution of dimethylnitrosamine in water, with a flame ionisation detector is 1 mg/l (= 5 ng). With a rubidium sulphate thermionic detector a similar value is obtained, and with a Coulson coulometric detector the limit is 0.5 mg/l (ref. 6). Some workers[7,8] have improved the detection limit by converting nitrosamines to the corresponding nitramines and detecting by electron capture. A limit of 30 μg/l (= 16 pg) has been quoted[7]. Even with a specific detector, the limits are substantially higher when extracts are analysed, and the probability of erroneous results due to interference is still high. A sample which gives a positive result for nitrosamines by GC should be subjected to GC–MS analysis for confirmation.

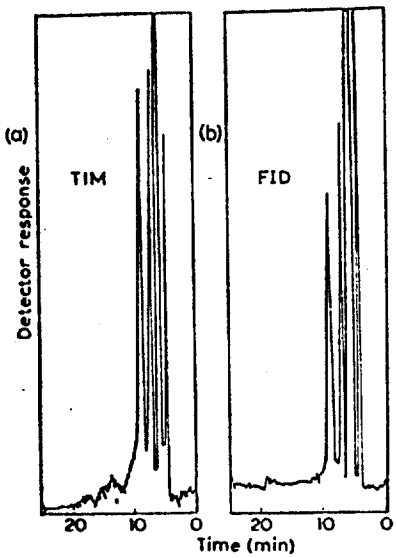


Fig. 4. Comparison of (a) total ion monitor and (b) flame ionisation detector responses (0.05 μl ketone mixture).

TABLE I

LIST OF NITROSAMINES STUDIED

Nitrosamine	*Abbreviation*	*Molecular weight*	*Base peak*	*Relative intensity*	*Reference mass*	*Ratio*
Dimethyl	DMN	74.0480	74	100.00	73.9968	1.000692
Methylethyl	MEN	88.0637	88	100.00	92.9952	1/1.05600
Diethyl	DEN	102.0793	102	100.00	100.9984	1.010702
Dipropyl	DPN	130.1106	70	24.73	130.9920	1/1.006774
Ethylbutyl	EBN	130.1106	58	34.68	130.9920	1/1.006774
Dibutyl	DBN	158.1419	84	14.74	156.9969	1.007293
Piperidyl	PIPN	114.0793	42	91.26	113.9967	1.000725
Pyrrolidyl	PYRN	100.0366	41	92.86	99.9936	1.000701

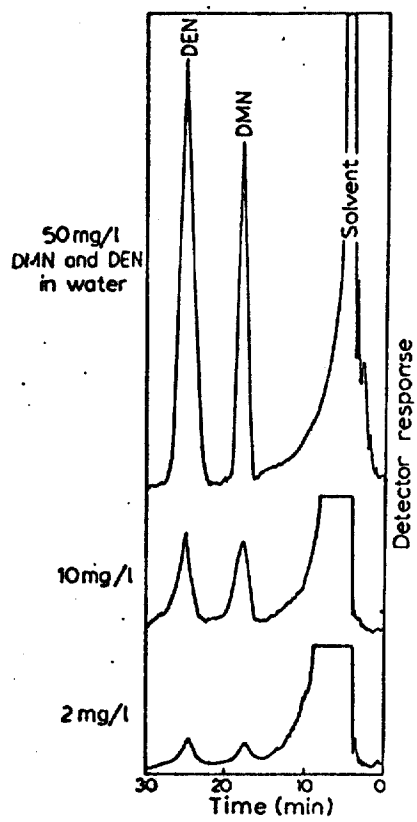
RESULTS

Preliminary experiments were carried out to show that the membrane separator did not suffer any memory effects, at least for the components under study, and that resolution losses between the GC column and the mass spectrometer were negligible. Fig. 4 shows a comparison between the flame ionisation detector response and total ion monitor response, for a trial four-component ketone mixture. Satisfactory spectra were obtained by scanning at 10 sec per decade mass in the region of the effluent peak maxima. Similar experiments were carried out using a series of nitrosamines (molecular weight 74 to 158), and satisfactory results were obtained. The detection of trace amounts of nitrosamines to the 50 mg/l level was satisfactory, but below this difficulty was encountered. However, treatment of the separator and transfer line to the mass spectrometer with bis(trimethylsilyl)ether resulted in a significant improvement and by peak matching 2 mg/l amounts of nitrosamines could be detected under the conditions quoted in Table II. A list of nitrosamines studied, with their accurate masses and the mass of the appropriate fragment ion of the reference compound

TABLE II

OPERATING CONDITIONS FOR GC–MS ANALYSIS OF NITROSAMINES

Gas chromatograph	Philips Research Model
Carrier gas	Helium
Flow rates	10 ml min^{-1} and 15 ml min^{-1}
Temperatures	
injection port	160°
column	130° and 160°
detector	175°
transfer line	160°
Column	5.1 m × 2 mm I.D. stainless steel, 15% FFAP on 80–100 BS mesh a.w. Chromosorb W
Sample size	5 μl
Mass spectrometer	MS 902
Accelerating voltage	8 kV
Trap current	100 μA
Electron beam voltage	60 eV (tuned for maximum monitor response)
Multiplier voltage	2 kV
Ion source pressure (helium)	3×10^{-6} mm Hg
Resolution (10% valley)	7000 or 12,000



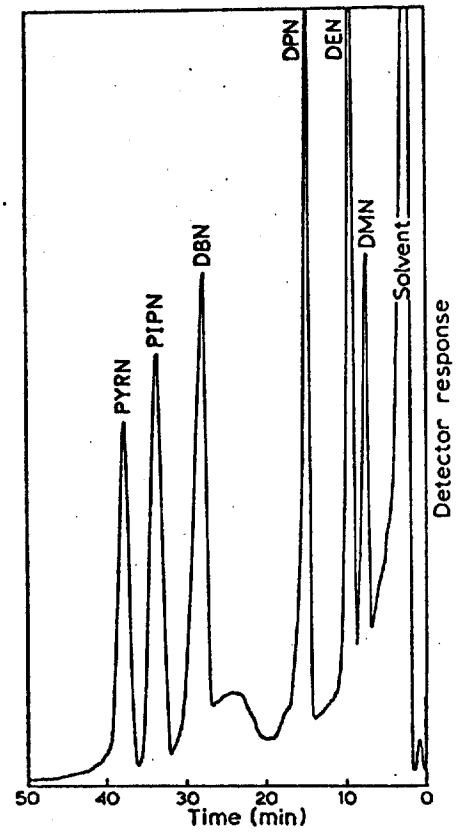


Fig. 5. Chromatograms of standard dimethyl- (DMN) and diethylnitrosamine (DEN) solutions 130°).)

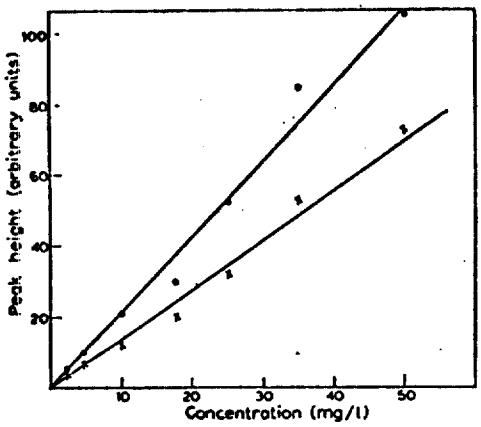
Fig. 6. Chromatogram of a standard nitrosamine solution (160°). For abbreviations, see Table I. Concentration: 33 mg/l of each nitrosamine in water.

(heptacosafluorotributylamine) is given in Table I. Relative intensities of the parent ions are also given[9].

Parent ion monitoring for the nitrosamines requires that all the nitrosamines are well resolved from each other in the gas chromatograph (except ethylbutyl from dipropyl), since a finite time is involved in setting the mass spectrometer for each ion in turn. Further, although nitrosamines can be detected even under a solvent peak, sensitivity is suppressed. It was found necessary to carry out the analysis of a mixture for the first three nitrosamines (Table I) under different GC conditions to that for the remainder of the nitrosamines. Where a large number of samples were to be analysed this was found to effect more economy of time than the temperature programming of each mixture for the analysis of all the nitrosamines in a single run.

Experimental conditions are given in Table II.

The two sets of conditions given for carrier gas flow rate and column temperature refer to the conditions used for the analysis of early and late members of the series, respectively. Chromatograms of standard mixtures of nitrosamines of various concentrations are shown in Figs. 5 and 6. Calibration of the mass spectrometer was carried out using standard mixtures in water, and calibration curves covering the



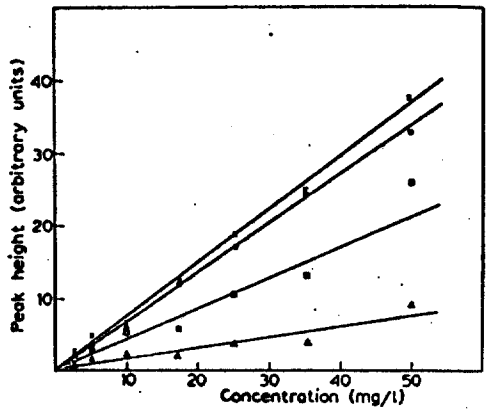


Fig. 7. Calibration of mass spectrometer for dimethyl- (○) and diethylnitrosamine (×) (GC at 130°).

Fig. 8. Calibration of mass spectrometer for higher nitrosamines (GC at 160°). □, Dipropyl-; △, dibutyl-; ×, piperidyl-; and ○, pyrrolidylnitrosamine.

range 2 to 50 mg/l are shown in Figs. 7 and 8. It has been found, however, that there are significant variations in mass spectrometer response with time even under standard operating conditions, and calibration must be carried out regularly. Fig. 9 shows the variation of response to a 5-μl sample of 10 mg/l dimethylnitrosamine in water over a period of four months.

Under normal operating conditions a detection limit of 2 mg/l for dimethylnitrosamine in an extract is regularly attainable, using a resolution of 7,000. The limits are similar for the other nitrosamines. For a 5-μl injection this is equivalent to 10 ng. Extracts of solid samples are concentrated by three orders of magnitude prior to analysis, and hence the detection limit on the original material is 2 μg/kg. A resolution of 12,000 is used in cases of doubt arising from masking of the parent ion due to excessive amounts of extraneous material in the same region. The detection limit is increased to about 5 μg/kg in such cases.

A substantial improvement in the absolute detection limit could be achieved by introducing a greater volume of extract into the chromatograph, but this has several drawbacks, *viz.* overloading of the column, rapid deterioration of the column and ion

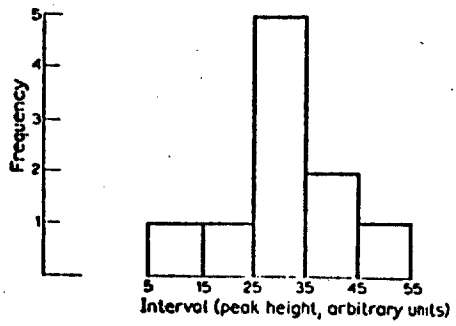


Fig. 9. Variation of mass spectrometer response to dimethylnitrosamine.

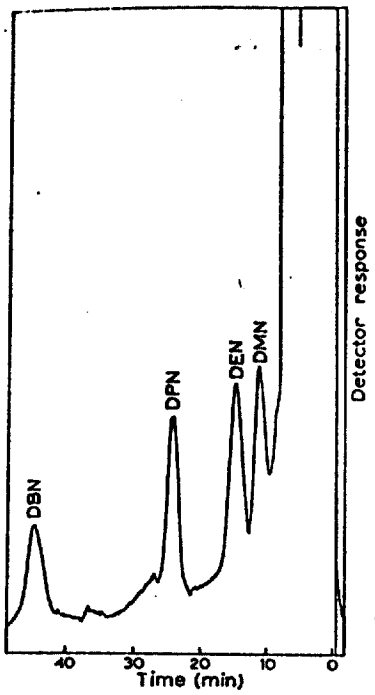


Fig. 10. Chromatogram of a 50-μl standard nitrosamine mixture containing 10 mg/l of each nitrosamine using a peak cutting system. For abbreviations, see Table I.

source, and an unacceptable increase in source pressure. To overcome this, a peak cutting system[10] has been built and incorporated into the chromatograph, in which the first column is a preparative scale column, and the second an analytical scale column. Detection at the exit of the first column is by means of a micro-volume katharometer. It is thus possible to inject say 50 μl of extract on to the preparative column and to resolve the nitrosamines from the solvent which is vented to atmosphere, at the end of the preparative column. The remainder of the extract is passed on to the second column for further GC resolution, and is detected by the mass spectrometer in the manner described above. Materials of excessively long retention

TABLE III

OPERATING CONDITIONS FOR PEAK CUTTING

Carrier gas flow rate	23 ml min^{-1}
Temperatures	
injection	175°
columns	175°
detectors	175°
transfer line	160°
Columns	
first	1.8 m × 4 mm I.D. stainless steel, 15% Carbowax 20 M on 60–80 BS mesh a.w. Chromosorb W
second	5.4 m × 2 mm I.D. stainless steel, 5% Carbowax 20 M on 80–100 BS mesh a.w. Chromosorb W

time may be backflushed. A chromatogram obtained using this system is shown in Fig. 10 under the conditions given in Table III. The limit of detection is lowered by about one order of magnitude for all the nitrosamines studied.

CONCLUSIONS

The membrane separator is a satisfactory device for the transfer of trace quantities of nitrosamines from a GC column to a mass spectrometer. Volatile nitrosamines can be unequivocally detected by peak matching with a reference compound under high resolution. By conventional GC separation a detection limit in solution of 2 mg/l can be routinely obtained, and by using a peak cutting device amounts as low as 0.2 mg/l can be detected. For sample extracts concentrated by three orders of magnitude this is equivalent to 0.2 μg/kg in the original material. The detection limits are of the same order both for standard samples and extracts. Quantitative estimates of the amounts of nitrosamines present can be obtained, but frequent calibration of the system is necessary to obtain even moderate precision.

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THE REACTION OF HEMOGLOBIN WITH NITRITE

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Although nitrite has been extensively used as an agent for the formation of methemoglobin, the quantitative relations of the reaction appear to be uncertain; three widely different values have been reported for the amount of methemoglobin formed to nitrite utilized. The first statement of a quantitative relationship was made by Barcroft and Müller (1) in 1911. In a preliminary report containing no experimental data or equations, and for which none was supplied in subsequent publications, they indicated the molar ratio of nitrite utilized to methemoglobin formed as 2; that is, 2 molecules of nitrite react with 1 molecule of hemoglobin to produce 1 molecule of methemoglobin. This relation was accepted by Stadie (2) in 1921 in his investigations on methemoglobin formation. From experimental determinations, Van Slyke and Vollmund (3) in 1925, Meier (4) in 1925, and von Issekutz (5) in 1939 reported that 1 molecule of nitrite was used in forming 1 molecule of methemoglobin; *i.e.*, a molar ratio of 1. Austin and Drabkin (6) in 1935 reported a ratio of 0.5 to 0.7 and one of approximately 0.5 can be calculated from data published in 1942 by Darling and Roughton (7) dealing with the effect of methemoglobin on the oxygen dissociation curve.

The reasons for some of these discrepancies will be dealt with in detail in subsequent discussion. An obvious source of error in several of the investigations was in the assumption that all nitrite added to blood or solutions of hemoglobin was quickly and completely utilized in methemoglobin formation. Analyses were not made for residual nitrite. In the investigation reported here, this source of error was avoided. The amount of nitrite reacting with hemoglobin was determined and the influence of temperature, concentration of nitrite, and of hydrogen ion concentration on the reaction was studied.

EXPERIMENTAL

Adult white rats were used for experiments *in vivo* and freshly shed heparinized[1] rat blood for experiments *in vitro*. Hemoglobin and methemoglobin were determined by the method of Evelyn and Malloy (8). The concentration of nitrite in the blood was determined by a modification of

[1] Crystalline liquaemin was kindly supplied by the Roche-Organon Corporation.

the method of Stieglitz and Palmer (9) with sulfanilic acid instead of 2-naphthylamine-6,8-disulfonic acid.

To three 2 cc. portions of whole blood containing 8.20 mM of hemoglobin per liter there were added 0.02, 0.04, and 0.06 cc. of a 1.0 per cent solution of sodium nitrite yielding 1.45, 2.90, and 4.35 mM of nitrite per liter in the respective portions. The blood was maintained at 20°. At the end of 30 minutes, 1 hour, and each hour thereafter, determinations were made for

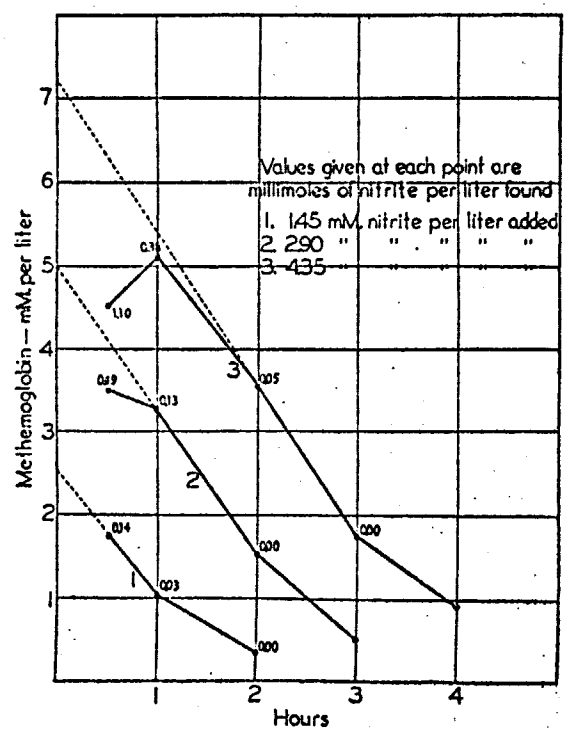

FIG. 1. Methemoglobin and residual nitrite in whole blood after addition of sodium nitrite.

methemoglobin and nitrite. The findings are recorded in Fig. 1. The residual nitrite, as mM per liter, is shown by the small figure at each point plotted.

The curves express the relation between the rate of formation of methemoglobin from the gradually disappearing nitrite and the rate of the continuous reduction of methemoglobin. At the time intervals used, the maximum formation of methemoglobin was found for the two smaller amounts of nitrite at 30 minutes and for the largest at 1 hour. At these times, 9 to 17 per cent of the nitrite was still present; it did not disappear

until the 2nd or 3rd hour. The rate of disappearance of methemoglobin increased as the nitrite concentration approached zero. At the low concentrations of methemoglobin reached by the time all, or nearly all, of the nitrite had disappeared, the rate of reduction slowed, as has been indicated by Gelinsky (10) and Cox and Wendel (11). On the assumption that, except at these low levels, the rate of reduction is at a uniform rate (11), an approximation can be made of the total methemoglobin formation from the nitrite by extending the curve for the maximum rate of reduction back to zero time. These extensions are shown by the dotted lines in Fig. 1; the amounts of methemoglobin indicated by these extrapolations are 2.5, 5.0, and 7.2 mM per liter for 1.45, 2.9, and 4.35 mM of nitrite respectively. The corresponding ratios of nitrite utilized to methemoglobin formed are 0.58, 0.55, and 0.59. In another series of experiments, ratios of 0.55, 0.52, and 0.53 were obtained. The possible sources of error in this extrapolation would tend toward a high rather than a low ratio: (*a*) during the period of maximum rate of reduction of methemoglobin a small amount of nitrite was still present; (*b*) the rate of reduction from which the extrapolation was made occurred when the reduction was more than half complete and if, as reported by Gelinsky (10), but contrary to Cox and Wendel (11), the rate of reduction varies with the concentration, the slope of the curve expresses a rate less than the average.

In the second series of experiments, laked blood was employed in order to eliminate the reduction of methemoglobin. To each of two portions of red cells laked with distilled water and containing 6.65 mM of hemoglobin per liter, 2.84 mM per liter of sodium nitrite were added. One portion was kept at 20° and the other at 37°. At 30 minutes, 1 hour, and each hour thereafter, methemoglobin was determined, and at 5 hours, the residual nitrite. The findings are given in Fig. 2. The rate of methemoglobin formation was more rapid at 37° than at 20°; at 5 hours the respective concentrations were 4.5 and 2.75 mM. The residual nitrites were 0.57 and 1.50 mM per liter. The molar ratios of methemoglobin formed to nitrite utilized were 0.49 and 0.51. Thus, although the rate of methemoglobin formation in laked blood is markedly influenced by temperature, the molar ratio of the reaction of hemoglobin and nitrite is not affected.

In a third series of experiments, sodium nitrite was added to each of six portions of red cells laked by water or blood laked by saponin in amounts sufficient to give molar ratios of 0.5 to 1.05 with the hemoglobin present. The temperature was 20°. At 1 and 3 hours, determinations were made of methemoglobin and residual nitrite. The molar ratios of methemoglobin formed to nitrite used, Table I, at both times were between 0.48 and 0.57; they were not influenced by the concentration of nitrite present.

In a fourth series of experiments, nitrite was added to blood laked by

saponin in quantities to give molar ratios of 0.15 to 1.16 with the hemoglobin present; potassium dihydrogen phosphate was then added to make the blood acid and the concentrations of methemoglobin and nitrite were determined 3 and 5 minutes later. These short intervals were employed to avoid appreciable autoxidation of hemoglobin in the acid medium (12) and because the reaction between nitrite and hemoglobin proceeds rapidly in such a medium. As seen from the data of Table II, even within these short periods all the nitrite had disappeared when the hemoglobin was in excess and all the hemoglobin had reacted when the nitrite was in excess.

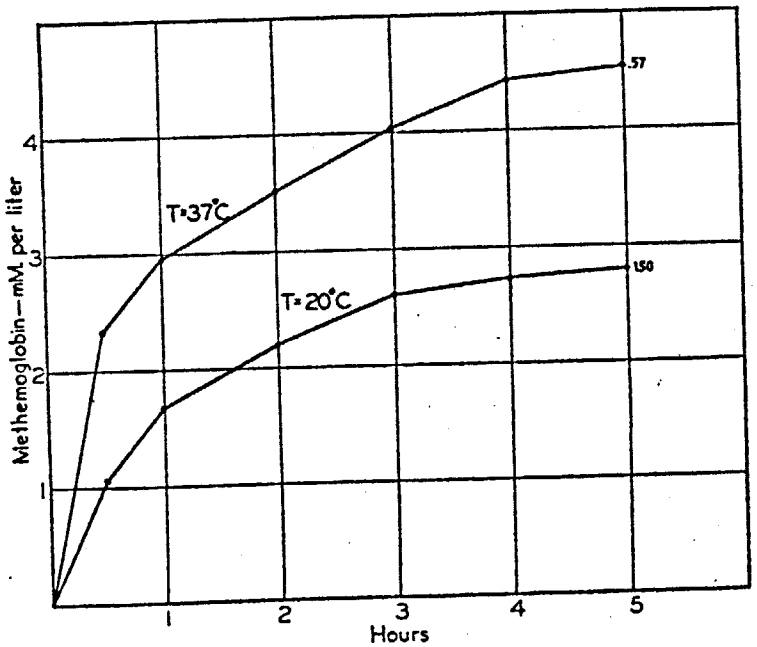

FIG. 2. Methemoglobin and residual nitrite in laked blood after addition of 2.84 mM of sodium nitrite per liter.

Acidification had no influence upon the molar relation of methemoglobin formed to nitrite utilized; the ratios as found ranged from 0.49 to 0.54.

In a final series of experiments, the methemoglobin formed from nitrite was determined in the living animal to test the validity of the findings of von Issekutz (5). He injected sodium nitrite in doses of 8 to 30 mg. per kilo (0.116 to 0.435 mM per kilo) and from the maximum concentration of methemoglobin in the blood, which developed in 30 minutes to 1 hour, he calculated an average nitrite-methemoglobin molar ratio of nearly 1. In the experiments here, rats were given, by intraperitoneal injection, 0.218 and 0.435 mM per kilo of sodium nitrite. At 30 minutes, 1 hour, and each

hour thereafter, to a total of 4 hours, the methemoglobin was determined and, in contrast to the work of von Issekutz (5), the concentration of nitrite. With a dose of 0.218 mM per kilo the methemoglobin concentration reached a maximum of 1.84 mM per liter at 30 minutes; the corresponding nitrite value was 0.218 mM per liter. With a dose of 0.435 mM per kilo the concentration of methemoglobin reached a maximum of 3.85 mM per liter in 1 hour; that of nitrite was then 0.333 mM. In both experiments there were

TABLE I

Formation of Methemoglobin in Laked Blood As Determined from Nitrite Added and Nitrite Utilized

Red cells laked with	Moles nitrite added / Moles Hb	1 hr., moles nitrite / moles Hb calculated from		3 hrs., moles nitrite / moles Hb calculated from	
		Nitrite added	Nitrite utilized	Nitrite added	Nitrite utilized
Water	0.5			1.42	0.48
"	1.00			1.62	0.50
"	1.03	2.65	0.50		
Saponin	0.52	2.03	0.48	1.46	0.51
"	1.03	2.65	0.53	1.75	0.54
"	1.05	2.70	0.57	1.78	0.51

TABLE II

Formation of Methemoglobin in Acidified Blood

Moles nitrite added / Moles Hb	MHb formed	Nitrite utilized	Nitrite remaining	Moles nitrite utilized / Moles MHb
	mM per l.	*mM per l.*	*mM per l.*	
0.15	2.11	1.03	0.00	0.49
0.24	2.82	1.46	0.00	0.52
0.30	3.91	2.05	0.00	0.52
0.50	5.46	2.75	0.00	0.50
0.59	6.10	3.23	0.87	0.53
1.16	7.10	3.85	0.35	0.54

still appreciable amounts of nitrite in the blood at the time the maximum amount of methemoglobin was present. In these experiments, and also in those of von Issekutz (5), no allowance was made for the elimination of nitrite in the urine. Thus for two reasons it cannot be assumed that the maximum amount of methemoglobin reached in the blood expresses the reaction of hemoglobin with the total amount of nitrite given. In addition, the maximum amount of methemoglobin observed is the algebraic sum of the amount produced and the amount reduced; this point has already been discussed. The only conclusion justified from such experiments is

that the molar ratio of nitrite utilized to methemoglobin formed is less than 1.

DISCUSSION

Barcroft and Müller (1) define the relation of methemoglobin formation to nitrite utilization in the statement: "Methemoglobin is formed quantitatively when potassium nitrite is added to blood, the amount of hemoglobin converted containing an amount of dissociable oxygen equivalent to that necessary to convert the nitrite to nitrate." Since each molecule of hemoglobin contains 1 molecule of dissociable oxygen for each atom of iron, it follows from this statement that 2 molecules of nitrite react with 1 molecule of hemoglobin to form 1 molecule of methemoglobin. From present knowledge of methemoglobin as a ferric compound (13) expressed by the formula Hb_2O(or HbOH), it is impossible to write an equation fulfilling the conditions in the statement of Barcroft and Müller (1). Thus the equation

$$4NO_2^- + 2HbO_2 \rightarrow Hb_2O + 4NO_3^-$$

cannot be balanced. In any possible reaction between nitrite and hemoglobin to form methemoglobin and nitrate the amount of hemoglobin converted must always contain an amount of dissociable oxygen greater than that necessary to convert the nitrite to nitrate.[2] One may thus write the equation

$$3NO_2^- + 2HbO_2 \rightarrow Hb_2O + 3NO_3^-$$

The nitrite-methemoglobin molar ratio in this reaction is 1.5. Other equations may be written in which free oxygen is liberated as a product of the reaction,

$$NO_2^- + 2HbO_2 \rightarrow NO_3^- + Hb_2O + O_2$$

or

$$4NO_2^- + 4HbO_2 \rightarrow 4NO_3^- + 2Hb_2O + O_2$$

In the first of these equations, the nitrite-methemoglobin molar ratio is 0.5; in the second, it is 1.0.

In the experimental work of Van Slyke and Vollmund (3) from which

[2] Since 2 molecules of methemoglobin are formed by each molecule of nitrite, 0.5 molecule of oxygen should be liberated per molecule of hemoglobin reacting, if the nitrite goes only to nitrate. We have found in four determinations, using no excess of nitrite, a liberation of 0.80, 0.77, 0.79, and 0.80 mole of oxygen per mole of hemoglobin. The determinations were made by the micro gasometric method of Roughton and Scholander (14). These data would indicate that products other than nitrate are produced in the reaction.

they reported a nitrite-methemoglobin molar ratio of 1, laked blood was employed. To 5 cc. portions containing 7.30 mM of hemoglobin per liter there were added 0.05, 0.1, 0.2, and 0.4 cc. of a 1.0 per cent solution of sodium nitrite. These investigators calculated these amounts as representing 0.1, 0.2, 0.4, and 0.8 mole of nitrite per mole of hemoglobin. An arithmetical error appears in this calculation; the amounts of nitrite used are correctly 0.2, 0.4, 0.8, and 1.6 moles per mole of hemoglobin—twice the amounts they state. This recalculation results in a corresponding change in the nitrite-methemoglobin molar ratio from 1, which they report, to 2.

The explanation for this high ratio obtained after correction of the calculations of Van Slyke and Vollmund may be in the facts that the determinations of methemoglobin were made at the end of 30 minutes at room temperature and that none was made for residual nitrite. From a preliminary experiment in which they found virtually all of the hemoglobin converted to methemoglobin in 20 minutes at room temperature, they concluded that 30 minutes were adequate for the reaction. This conclusion would be justified were it known that all of the nitrite added had been utilized. If, as shown here, 1 mole of nitrite reacts with 2 moles of hemoglobin, nitrite was present in considerable excess in their experiment. The rate of reaction of nitrite and hemoglobin depends upon their relative concentrations (15). Thus with an excess of nitrite its relative concentration would become greater as the hemoglobin disappeared and the reaction would proceed at an undiminishing rate. If, however, the hemoglobin were in excess, the relative concentration of nitrite would become progressively smaller with a correspondingly diminishing rate of reaction. The results of the present investigation have, in fact, shown that except in acidified hemoglobin solutions, or when the nitrite is in excess, the reaction is far from complete after 30 minutes, particularly at room temperature. This is in agreement with the observations of Austin and Drabkin (6) who found that upon addition of small amounts of nitrite to hemoglobin solutions the maximum formation of methemoglobin did not occur for many hours.

Meier (4), in estimating a nitrite-methemoglobin ratio of 1, made no determinations of methemoglobin but measured the amount of free oxygen liberated in the reaction. When he added large excesses of nitrite to acid blood, oxygen was taken up. This he explained by the oxidation of nitrite to nitrate which he showed would occur in the presence of acid, in air, without hemoglobin.

$$2HNO_2 + O_2 \rightarrow 2HNO_3 \qquad (1)$$

At a concentration of nitrite, however, which was just sufficient to convert all of the hemoglobin to methemoglobin Meier found that an amount of

oxygen was liberated equal to one-fourth of the amount in oxyhemoglobin. For this reaction, he therefore wrote the equation

$$4HNO_2 + 4HbO_2 \rightarrow 4HNO_3 + 2Hb_2O + O_2 \quad (2)$$

in which 1 mole of nitrite forms 1 mole of methemoglobin. The error made by Meier was that he arbitrarily chose 1 mole of nitrite per mole of hemoglobin as the amount just sufficient to convert all of the hemoglobin to methemoglobin. Had he used 0.5 mole of nitrite per mole of hemoglobin, he would have found that this too was enough to convert all of the hemoglobin. The equation for this reaction is

$$HNO_2 + 2HbO_2 \rightarrow HNO_3 + Hb_2O + O_2 \quad (3)$$

in which one-half of the oxygen in the oxyhemoglobin is liberated instead of one-fourth of it, as found by Meier. Since he added twice as much nitrite as is necessary, only half of it reacted with the hemoglobin according to Equation 3; the other half reacted with oxygen according to Equation 1, utilizing half of the oxygen liberated in the formation of methemoglobin and resulting in a sum of reactions which is expressed by Equation 2. Meier gave no evidence that all of the nitrite he added reacted exclusively with hemoglobin.

The determination of the nitrite-methemoglobin ratio for the living animal by the method used by von Issekutz (5) is, as pointed out, impossible, since (*a*) the maximum concentration of methemoglobin occurs at a time when there is considerable residual nitrite, (*b*) before the maximum is reached, there is reduction of methemoglobin, (*c*) part of the nitrite is excreted in the urine (16).

Since the nitrite-methemoglobin ratio *in vitro* does not appear to be dependent upon temperature, concentration, or pH and appears to be constant for the reaction between nitrite and hemoglobin, it is probably the same in the living animal as *in vitro*.

SUMMARY

1. In the reaction between nitrite and hemoglobin, *in vitro*, 1 molecule of nitrite reacts with 2 molecules of hemoglobin to form 2 molecules of methemoglobin.

2. Temperature, concentration of nitrite, and pH are without influence upon the ratio of the nitrite utilized to methemoglobin formed.

3. In an acid medium the reaction of nitrite with hemoglobin is complete in a short time.

4. In a neutral or slightly alkaline medium, the reaction may take many hours for completion, depending upon the concentration of nitrite.

5. The amount of methemoglobin formed per mole of nitrite utilized cannot be determined directly in the living animal but is probably the same as it is *in vitro*.

Acknowledgment is made of the technical assistance of Evelyn Shukovsky.

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Outbreak of Sodium Nitrite Poisoning

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SODIUM nitrite is manufactured chiefly for its use in the dye industry. It is also employed as an anti-corrosive in anti-freeze mixtures. For many years it has been used either alone or mixed with saltpeter in the curing of meat in order to bring out the red color, and as a preservative. The Bureau of Animal Industry of the Department of Agriculture allows nitrites to be used in curing meat, provided the finished product contains not more than 200 p.p.m. The salt is sold freely on the market. Heat used in cooking destroys nitrites, but it cannot be depended upon, since the amounts destroyed vary with the degree of heat and the length of cooking. Saltpeter, or sodium nitrate, is the salt more commonly used in curing meat. U.S.P. saltpeter is 99.5 per cent pure, but commercial saltpeter frequently has a large admixture of sodium nitrite. It is surprising that in spite of the ready availability of sodium nitrite, cases of food poisoning caused by the ingestion of this salt in food are unusual occurrences. No mention of such food poisoning is made in the standard texts of Jordan, Tanner, or Dack. The report of an outbreak of nitrite poisoning is therefore of interest.

On September 25, 1944, a report was received by the Department of Health from a hospital in lower New York City that 11 old men had been admitted to the hospital with cyanosis and shock. The investigation indicated that all of them had become ill that morning between 7:15 and 10 A.M. In each instance illness occurred within 5 to 30 minutes after eating breakfast in a cafeteria on Chatham Square. All were between 60 and 80 years of age and all were of the derelict type, living in cheap rooming houses on the Bowery. Seven of those ill lived in the same rooming house; the other four lived in different rooming houses of the same type, and were unacquainted with each other.

The clinical picture was similar in all. Within 5 to 30 minutes after breakfast, the involved individuals became dizzy, felt weak, and complained of abdominal cramps. Eight of them vomited and all had diarrhea. Five of them became unconscious shortly after onset of symptoms. All were admitted to the hospital by ambulance.

What impressed the admitting physician was the picture of shock that the patients presented. They had subnormal temperatures, cold extremities, and low blood pressures. In addition, all had cyanosis. In one patient, the cyanosis was over the entire body; in the rest it was chiefly of the lips, nose, ears, fingers, and toes. Blood counts and urine examinations were normal for the most part. They were treated for shock with bed rest, heat, and stimulant drugs. They also received gastric lavage and the more cyanotic patients got oxygen therapy. All but one patient recovered. This man, age 82, died the following morning. Autopsy revealed bronchopneumonia. The only other abnormal finding was a diffuse

mahogany brown discoloration of the organs.

In view of the cyanosis, the possibility of poisoning with one of the coal tar derivatives was thought of, and specimens of blood were obtained from each of the patients. These were examined spectroscopically.* Absorption bands for methemoglobin were found in all. The men were closely questioned about the use of drugs prior to their illness. All denied such use.

The restaurant in which the men ate is located in a poor section of the city and caters to men of very restricted means. It does not rate high in sanitation, lighting, or adequate kitchen equipment.

The men had become ill shortly after eating breakfast. All but one had eaten oatmeal, rolls, and coffee. One ate oatmeal only. The investigation was therefore directed toward this food. It was prepared by the cook at 5 A.M. and allowed to boil for 2½ to 3 hours. The dry oatmeal was obtained on the market in 5 lb. paper cartons. Four gallons of water were added, and a handful of salt. A similar handful of this when actually weighed equalled 101 gm. The mixture was stirred well before and during cooking and was kept simmering on the stove until all servings had been made. There were 6 gallons in the finished product and about 125 people were served, which made a serving equal to about 6 oz. There seemed no reason to suspect the dry oatmeal which was purchased in the usual commercial carton. The water used was from the city supply. The salt used for cooking was in a can near the stove. It had a faint yellow color. The cook stated that he filled the can from a supply on a shelf. There were two containers on this shelf, one filled with what appeared to be ordinary white-colored salt and the other filled with a yellowish looking salt. The owner stated that the latter was saltpeter, which he used in curing meat. Both containers and the can were embargoed and examined in the chemical laboratory of the Department of Health. The substance which looked like salt did actually turn out to be sodium chloride. The material in the other container and in the can was curing salt of which 92 per cent was sodium nitrite. The can had been filled from this container by mistake. None of the oatmeal was left, so that no determination was possible of the amount of nitrite ingested in the oatmeal by each individual. Since about 125 portions of oatmeal had been served, inquiry was made at the various hospitals serving the area and at rooming houses in the vicinity to determine if any other men had become ill. No evidence was obtained of any more cases. It appeared improbable that the oatmeal was the sole source of poisoning since it had been eaten by 125 men and only 11 had become ill. It occurred to us that the same error might have been made in filling saltshakers as in filling the can. Accordingly, we obtained 17 saltshakers used in the restaurant. The salt from 10 of them was pooled and chemical examination revealed 0.074 per cent of sodium nitrite. We requested examination of the contents of each of the remaining 7 saltshakers separately. One contained 0.137 per cent of sodium nitrite, 2 had only faint traces, and 4 had only sodium chloride.

As soon as we became aware of the possibility of nitrite poisoning, a chemical examination of the blood of the men in the hospital was requested. A pooled sample of the blood from the ten survivors was dialyzed through a collodion membrane. The dialysate was evaporated to a concentrated form with

* We are indebted to Dr. Alexander O. Gettler of the Chief Medical Examiner's Office for the spectroscopic determinations and for the chemical examination of blood.

low heat, and tested for the presence of nitrates and nitrites. The results indicated the presence of nitrites.

Since none of the cooked oatmeal was available, a batch was cooked for 2½ hours, using 1¼ lb. of oatmeal, 25 gm. of the "salt" from the can and 1 gallon of water. The ingredients used were in the same proportion and the cooking for the same length of time as in the original batch. A sample of the mixture contained 0.6 per cent of sodium nitrite before cooking and 0.1 per cent of sodium nitrite after cooking.* In the usual portion of 6 oz., there would be 0.18 gm. (about 2½ grains) of sodium nitrite.

COMMENT

The U. S. Pharmacopeia gives the average pharmacologic dose of sodium nitrite as 0.060 gm. or 1 grain. The British Pharmacopeia gives the dose as 0.03 to 0.12 gm. (½ to 2 grains). Soma Weiss and his coworkers conducted several studies on the effect of sodium nitrite on normal subjects. In one of these studies they established that a dose of 3 grains caused low blood pressure, pallor, sweating, nausea, dizziness, and finally loss of consciousness, when the subjects were tilted from a lying position to an angle of 75°.[1] The addition of a small amount of sodium nitrite to the portion of cooked oatmeal eaten would bring the total dose up to the toxic level.

As we reconstruct the picture, a number of individuals in the restaurant ate the oatmeal either without the addition of salt or with the addition of sodium chloride from the saltshakers that contained no nitrites. The 11 men who became ill must have used one of the few saltshakers that contained considerable admixture of sodium nitrite, bringing the intake up to toxic levels.

A search of the literature yields only a few reports of cases of nitrite poisoning. Most of these are in individuals, a few in family groups of two or three. The clinical picture in all of them is so much alike that one seems to be reading the same case history repeatedly. Onset of symptoms is dramatically sudden, very soon after eating the contaminated food. Nausea and vomiting are common, but the characteristic picture is one of cyanosis, particularly of the fingertips, nose, and ears, and the rapid loss of consciousness, frequently leading to death. The spectroscopic examination of the blood gives an absorption band characteristic of methemoglobin and at autopsy the only positive finding is a chocolate colored discoloration of the organs.

In most of the reported cases the nitrite was used in food, as in our report, under the mistaken notion that it was table salt. Padberg and Martin[2] report poisoning which caused death in three men who prepared a stew with salt obtained from a manufacturing plant. Examination of the stomach contents of the dead men as well as of the beef stew showed the presence of nitrites.

McQuiston[3] also reports on the death of three persons, a man, woman, and child, who used salt in cooking and in a saltshaker, which the man obtained from the plant where he worked. Analysis of the remaining food and of the salt in the saltshaker as well as of the stomach washings, showed large quantities of sodium nitrite.

A similar occurrence in three members of a family who used sodium nitrite in cooking is reported by Ruegg.[4] Here, too, the salt was brought home from the factory under the impression that it was sodium chloride. All three recovered after treatment.

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* Andrew J. Pensa, chemist, Department of Health, performed the chemical determinations of the oatmeal as well as of the salts in the containers and saltshakers.

H. Huziter-Kramer[5] reported sodium nitrite poisoning in two small groups. In one of these, a father, mother, and daughter became ill soon after a meal in which sodium nitrite, brought home from the factory by the father, was mistakenly used for salt. Typical symptoms of vomiting, cyanosis, and loss of consciousness occurred and the patients were removed to a hospital. Nitrites were found in the stomach, and methemoglobin in the blood. All made recoveries. In the other group, two men became ill after eating pickled cabbage brought to the factory by a fellow worker, which was liberally sprinkled with salt from the factory. Both men died, and sodium nitrite was recovered from the stomach as well as in the remains of the lunch.

Four cases described by Musso[6] were given sodium nitrite in a drugstore in Algeria in a purgative lemonade. The native druggist took the nitrite from a bottle labeled "sodium tartrate." All four died. A similar error in which a druggist gave two infants aged 3 months and 2 months sodium nitrite instead of sodium citrate as a cathartic caused illness in both and death in one of them.[7]

Single cases of sodium nitrite poisoning have been reported by Palmer,[8] Arbuckle and Thies,[9] Naidu and Rao,[10] and Molitaris.[11] All were fatal cases. A fatal case of poisoning with potassium nitrite is reported by Farinand and Woltz.[12]

The uniformity of clinical symptomatology and the high fatality rate is striking in the reports from the literature. The chances of death depend largely, in all probability, on the amount of nitrites ingested. In the outbreak reported here only one death occurred among 11 cases. According to our calculations, most of the men took a little more than the pharmacologic dose, so that although they became seriously ill, they made an eventual recovery.

SUMMARY

1. An outbreak is reported in which 11 men were poisoned by eating oatmeal in which sodium nitrite was used instead of table salt, and on which, in all probability, additional sodium nitrite was added from saltshakers.

2. Symptoms began immediately after the meal and consisted of nausea, vomiting, cyanosis, shock, and loss of consciousness. One death occurred.

3. Methemoglobin was found in the serum of each case and nitrites were recovered from a pooled specimen of the bloods.

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NITRITE IN THE CONTROL OF CLOSTRIDIUM BOTULINUM

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The main thrust of my comments today will be the presentation of the results and conclusions of a study on the effect of nitrite and nitrate on botulinal toxin formation in canned ham. This work was funded by the American Meat Institute. The protocol design was a joint effort of the American Meat Institute Foundation Scientific Advisory Committee, and USDA and FDA scientists. While I am here in the limelight discussing the results of the work, the researchers who actually did the work were Bruce Tompkin, Lee Christiansen, and Ted Kueper of the Swift R&D Center. The study was carried out at Swift and Company for a very basic reason. Our Research and Development Center at Oak Brook, Illinois is equipped with a botulism pilot plant and a staff of immunized scientists. However, the project should be viewed by everyone as a joint meat industry-government attempt to factually assess the role of nitrate and nitrite in preventing botulism in canned ham. Indeed, we are indebted to Ralph Johnston of USDA and Don Kautter of FDA for their hard physical labor in the actual preparation and inoculation of the test product. This is the first of a series of such studies dealing with various categories of cured meat products. A bacon study is underway, and a frankfurter test protocol is in the final stages of design.

There is a certain mystique, an aura of fascination concerning botulism. Botulinal toxin is the most poisonous poison. You have all been exposed to the conundrum that the amount of toxin in an aspirin-sized tablet could kill the total population of New York City. The practicality of such a proposition breaks down when one attempts to assess the likelihood of distributing precisely the lethal dose to each and every inhabitant of that troubled metropolis, but the point is well made. Additionally, man is pretty well conditioned to the thought that he may get bitten by a venomous snake, should he wander off the beaten track in certain geographical areas, and accepts with equanimity the knowledge

that 50,000 Americans will perish in traffic accidents this year, but the thought of getting zapped at the kitchen table is simply not acceptable to Western civilization. When a homemaker gives botulism to her family with something she improperly canned in her own kitchen, it makes the newspapers. When a commercial processor is the source, it makes headlines.

The commercial botulism outbreaks which have occurred in North America over the last 25 years are in Table 1. Nine persons perished (compared to about 700 botulism deaths from home canned foods over the same time). The economic disasters suffered by the affected companies should serve to alert all critics of the food industry that no altruistic spirit is needed to drive food processors to assuring beyond any reasonable doubt that their products will not be botulism hazards.

Table 1. Commercial Botulism Outbreaks

Item	Year	Fatalities	Cause
Tuna, canned	1963	2	Faulty cans
Smoked fish, vacuum packaged	1963	5	No refrigeration
Liver paste, canned	1963	1	Underprocessing
Vichyssoyse, canned	1971	1	Underprocessing
Chicken vegetable soup, canned	1971	0	Underprocessing

There is much conflicting information in the literature concerning the effect of nitrite on botulinum toxin production in cured meat products. Some very knowledgeable researchers have concluded that nitrite, at levels permitted under USDA regulation, does not prevent botulinal toxin development (Tanner and Evans, 1934; Stumbo, 1945). Other workers state that nitrite is essential for preventing botulism in these products (Pivnick et al., 1967; Johnston et al., 1969). As you sift through these publications, the reasons for this dichotomy emerge.

First, each class of cured meat product is a story unto itself. Ham as a biologically reactive system is totally different from bacon, which in turn is different from dried sausage, etc., etc., etc. One cannot make general assessments about the importance of nitrite (and nitrate) upon all cured meat systems without running appropriate studies on each and every one.

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Secondly, the question of absolute safety is one which food microbiologists have been wrangling about for many years and will continue to do so for many more. Everybody agrees that the number of botulinal spores with which we challenge a product system has a profound effect upon the results of the test. It is also generally agreed that the botulinal spore exists in low numbers in meat products. There is much argument as to what level of inoculation constitutes a sufficient challenging inoculum to permit conclusions as to total botulinal safety. Our survey of the incidence of clostridia in meats and poultry in packing plants throughout North America demonstrated that the expected level of botulinal spores occurring in nature is quite low (Greenberg, 1966). This can be balanced against published data in which the presence of Clostridium botulinum has been demonstrated at the retail store level in luncheon meat (Taclindo et al., 1967), frankfurters (Insalata et al, 1969), and cooked ham (Abrahamsson and Riemann, 1971). Abrahamsson and Riemann noted that "the compositions of the samples (of ham) containing C. botulinum would permit multiplication at the proper temperature". In terms of the amount of NaCl organoleptically tolerable in these products, they are certainly correct. But, does the nitrite (and/or nitrate) in such products play a protective role?

Canned cured meat products have enjoyed an unblemished botulism safety record in the United States. It is generally agreed that the public health record of shelf-stable canned cured meat is due to an interdependent combination of brine, nitrite, mild thermal processing and a low incidence of botulinal spores in the raw material. Perishable canned ham does not receive sufficient thermal processing to prevent botulinal toxin production in the product and thus is labeled "keep under refrigeration". It is also recognized that this class of product is often temperature abused at the retail and consumer level.

The objective of this test was to determine the role which nitrite and nitrate have in retarding or preventing toxin production in this product. The test indicated nitrite to be an effective agent in retarding or preventing toxin production by C. botulinum; nitrate, however, had little effect.

Eight levels of nitrite (0-500 ppm) and four levels of nitrate (0-2000 ppm) were tested. This provided data above and below the current levels permitted by Federal Regulations.

Two levels of spores (100 and 10,000/gm) were tested, along with uninoculated controls. The inocula consisted of a mixture of five strains of type A and 5 strains of type B C. botulinum. The inocula were mixed into the meat immediately after the nitrate and nitrite were added. The product was then placed into cans and given a water cook, to an internal temperature of 155°F, which is a standard industry practice.

The bulk of the canned product was abused by holding at 80°F. and observed over 6 months. Product was examined as to spoilage and tested for the presence of botulinal toxin. Uninoculated product was followed during storage to determine the rate of decrease of nitrite and nitrate.

The product consisted of fresh, whole, boneless pork hams, ground and formulated to contain 2.5% salt, 0.5% dextrose, and 0.02% sodium isoascorbate. Analysis of the formulated product after manufacture showed salt 2.2%, moisture 55.9% (brine 3.7%), fat 27.4%, and pH 6.24.

Most probable number analysis of C. botulinum in finished product resulted in an average count for the low inoculum level to be 90 spores per gram, and the average for the high inoculum level 5,000 per gram.

Table 2 depicts nitrite levels as formulated originally into the product and as analyzed in the raw and cooked product. There was a reduction of nitrite as a result of contact with the raw meat. A comparison of formulated NO_2 and Before Cook NO_2 for each nitrite level demonstrated approximately 1/4 of the added nitrite was lost after addition to raw meat. Nitrite analysis immediately after cook was very similar to that in the raw product.

Table 2. Nitrite Levels (ppm) as Formulated and as Analyzed

Time of analysis	Nitrite level as formulated							
	0	50	100	150	200	300	400	500
Raw, after formulation	0	29	70	110	144	158	280	378
After cook	0	37	65	96	139	155	268	352

Nitrite levels were reduced during storage of the cooked product. At given initial nitrite levels, the rate of depletion was dependent on time and temperature of storage. Several linear and non-linear models were fitted for prediction of residual nitrite.

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144	158	280	378
39	155	268	352

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The best fitting model showed a geometric reduction of nitrite with time.

The residual nitrite values at 500 ppm and 100 ppm initial input during 80°F storage are in Figure 1. You will note that even at 500 ppm initial input, residual nitrite was below 100 ppm within 2 weeks.

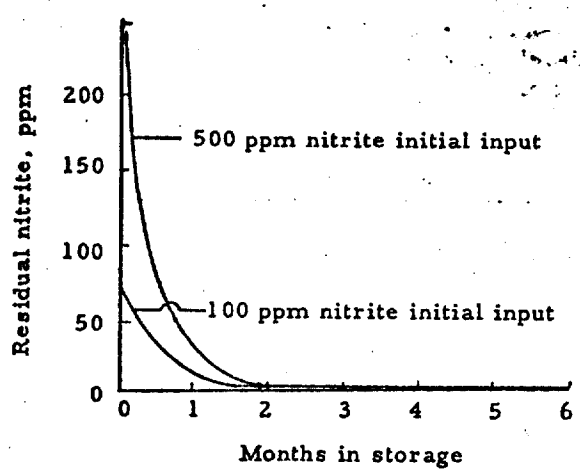

Figure 1. Residual nitrite during storage at 80°F.

There was no consistent change in nitrate concentration that could be associated with meat contact, cooking or storage.

Selected samples were analyzed for 14 volatile N-nitrosamines using the FDA multi-detection method[1] demonstrated to be sensitive to 10 ppb. No nitrosamines were found. Recoveries at the 10 ppb level ranged from 70-95%. Analyses were performed by the Division of Chemistry and Physics, FDA, Washington, D.C.

[1] To be published in Proceedings of Heidelberg Meeting, October 13-15, 1971 on Nitrosamines. Co-sponsored by IARC-WHO and German Cancer Research Center.

The number of confirmed botulinum toxic samples obtained during the study from product formulated with various levels of nitrite and the two levels of inoculum are in Tables 3 and 4. Increased levels of nitrite decreased the rate of toxin development and the total number of samples ultimately containing toxin. The level of nitrite necessary to completely inhibit toxin production was dependent upon initial spore levels. At the low inoculum level no toxin was confirmed in samples formulated with 200 or more ppm nitrite throughout the 6 months storage at 80°F. At the high inoculum level no botulinum toxin formation was confirmed at the 500 ppm nitrite level and relatively few samples were toxic at or above 200 ppm nitrite.

Table 3. Effect of Nitrite on Botulinum Toxin Formation in Canned Ham (Light Inoculum)

Nitrite added, ppm	Days until toxin first detected	Total no. toxic cans
0	7	45/80
50	7	32
100	28	16
150	68	2
200	>180	0
300	>180	0
400	>180	0
500	>180	0

Table 4. Effect of Nitrite on Botulinum Toxin Formation in Canned Ham (Heavy Inoculum)

Nitrite added, ppm	Days until toxin first detected	Total no. toxic cans
0	7	63/80
50	12	18
100	13	33
150	42	13
200	70	5
300	168	1
400	21	2
500	>180	0

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m Toxin Formation in Canned

...in ed	Total no. toxic cans
	45/80
	32
	16
	2
	0
	0
	0
	0

Toxin Formation in Canned

	Total no. toxic cans
	63/80
	18
	33
	13
	5
	1
	2
	0

The data were analyzed by multiple linear regression analysis to document the type and degree of relationship between toxin development and the test conditions. Observed toxic samples were assigned a probability of 1.0, while non-toxic samples were assigned a 0.0 (Figures 2 and 3). These binary response data were analyzed to develop prediction equations on the probability of toxicity for specified conditions. All the 80°F inoculated data were pooled for an analysis of toxicity as a function of time, nitrite, nitrate, and spore levels. Two sets of analyses were performed, one using the initial nitrite input, the other using the predicted residual nitrite associated with each sample. The effects of nitrite and time within the system were clearly dominant. While the results showed the effect of nitrate level to be significant in a statistical sense, it was extremely small relative to the nitrite effect.

Comparison of these two equations indicated that the toxicity can most accurately be predicted from initial nitrite formulation rather than the residual nitrite. This suggests that maintenance of a residual nitrite is not as critical to preventing toxin production as is the initial nitrite concentration.

Figures 2 and 3 represent the predicted probability of toxicity with the two spore levels after 1 month and 3 months storage at 80°F. These curves indicate that reducing currently approved nitrite levels in this product system will increase the probability of botulinal toxicity.

There was no botulinal toxin production in the samples stored up to 6 months at 45°F.

MPN assays for C. botulinum on 16 non-swollen samples (150 to 500 ppm nitrite) after 6 months did not detect (10 per gram) viable C. botulinum in the low inoculum portion of the study. Assays on 12 non-swollen samples from the high inoculum product (initial nitrites 200-500 ppm) showed counts at less than 10 (8 samples), 10 (2 samples) and one each at 100 and 10,000 C. botulinum per gram. Since no toxin was found in any of these samples, these results indicate the probability that no growth occurred.

Analysis of the numbers of swollen cans occurring at weekly intervals throughout the study showed that, if nitrite concentration increased, rate and consequently the number of swollen cans decreased. The results generally paralleled the toxicity data in that swelling was affected by nitrite and to a much lesser degree by nitrate.

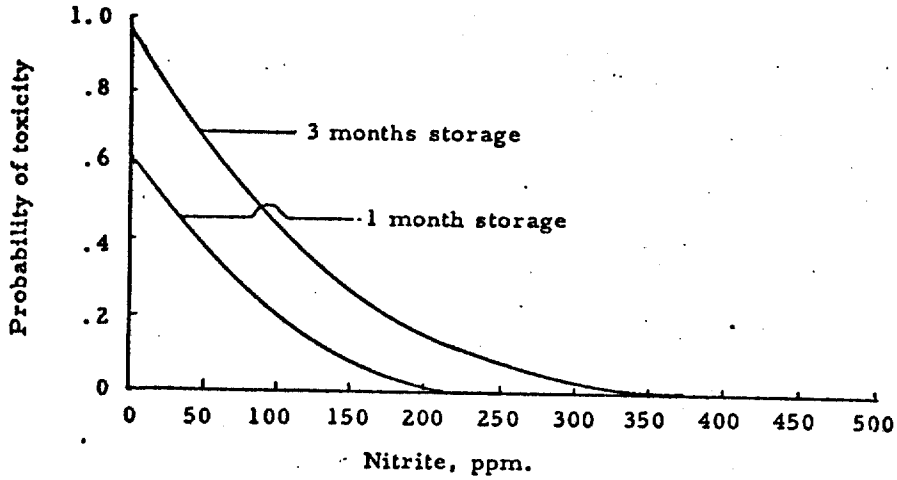


Figure 2. Probability of toxicity within 1 and 3 months (inoculum, 90 spores per gram; storage temperature, 80°F; average nitrate content, 875 ppm).

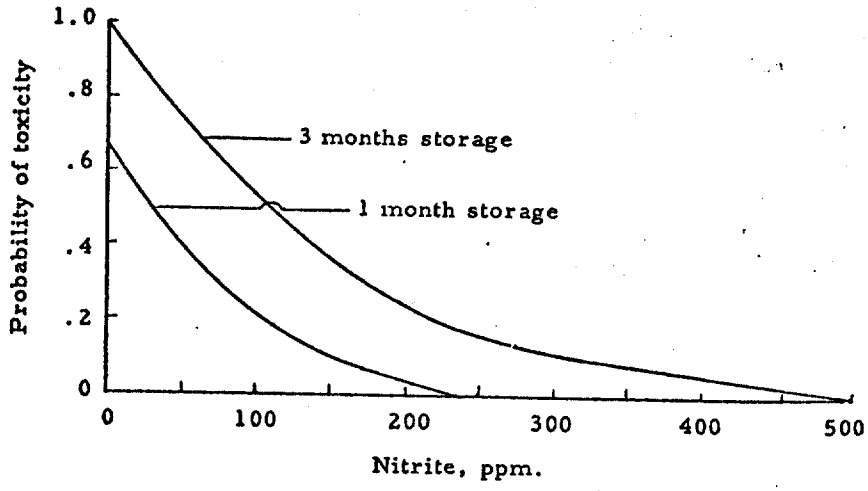
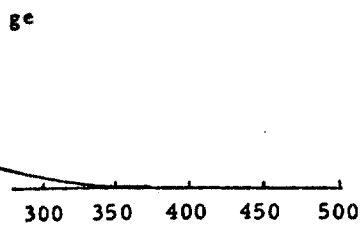


Figure 3. Probability of toxicity within 1 and 3 months (inoculum, 5000 spores per gram; storage temperature, 80°F; average nitrate content, 875 ppm).



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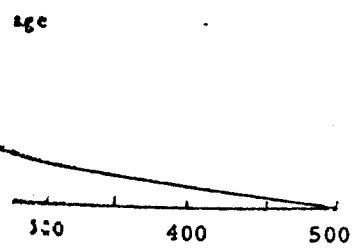

1 and 3 months (inoculum,
temperature, 80°F;
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Conclusions

The results show conclusively that sodium nitrite inhibits toxin production by C. botulinum in perishable canned cured meat. It is clear that the level of nitrite at the time of product manufacture, rather than the residual nitrite concentration, is the key protective factor. For example, within the low inoculum portion of the study, there were no confirmed toxic samples with initial nitrite levels of 200 or more ppm, even though virtually all samples were low in residual nitrite after a few weeks storage at 80°F. It would appear that the nitrite either reacts directly with the spores, or that in the reaction with the meat component a substance(s) is formed which inhibits germination and/or outgrowth of spores.

Sodium nitrate did not demonstrate practical anti-botulinal activity in the model system used. There was no evidence of mutual enhancement or synergism in samples containing combinations of nitrite and nitrate.

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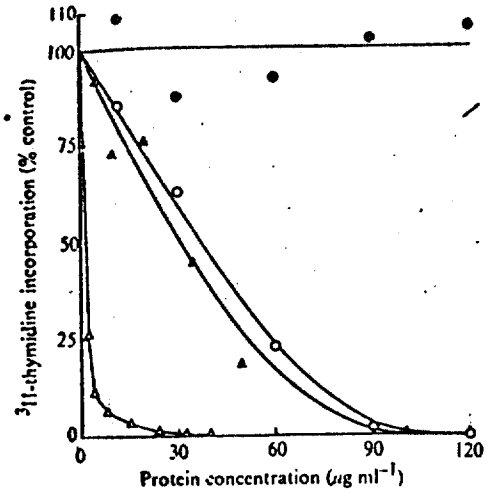

Fig. 3 Inhibition of concanavalin A-induced lymphocyte transformation by the fraction eluted from concanavalin A-'Sepharose' with methyl-α-D-glucopyranoside (△), pig lymphocyte plasma membrane (▲), the lipid-free membrane (○) and the plasma membrane lipid fraction (●). Increasing amounts of these materials were incubated at 25° C for 30 min with 1 μg of concanavalin A in 0.1 ml. of 0.9% NaCl, and 10^6 pig lymphocytes in 1 ml. of medium were then added. The degree of lymphocyte transformation was measured as described previously[2]. The lipid-free membrane and plasma membrane lipid fraction were prepared from lymphocyte plasma membrane by extraction with aqueous *n*-butanol (Table 1). Amounts of ^{3}H-thymidine incorporated in the presence of inhibitor are expressed as a percentage of that incorporated in the absence of inhibitor, and amounts of plasma membrane lipid fraction added are expressed in terms of the equivalent amounts of protein present in the whole membrane (see Table 3 in ref. 23).

glycoproteins would be obtained by eluting the column of concanavalin-'Sepharose' with various concentrations of different sugars. Also, it seems likely that the membrane glycoproteins can be fractionated by using phytohaemagglutinins with different specificities.

It has thus been shown that the specific interaction of cell membrane glycoproteins with concanavalin A is retained in the presence of the concentration of sodium deoxycholate (1%) used to solubilize the membrane and that the glycoproteins can be purified in a biologically active form by affinity chromatography. It seems likely that this method will prove to be generally applicable to the purification of those membrane receptors whose biological activities are retained after solubilization in detergent.

We thank Mr P. B. Gillett for the polyacrylamide-gel electrophoresis data.

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In vivo Conversion of Phenmetrazine into its N-Nitroso Derivative

THE possibility that nitrosamines, many of which are powerful carcinogens, can be formed in the stomach of mammals from ingested amines and nitrites[1] has led to speculation on the sources of relevant amines for man[2]. One such source is the large number of drugs that are secondary or tertiary amines, many being taken by an individual for a long time.

Studies have been made of the nitrosation of amines in gastric juice[3] and in animal stomachs[4], and a carcinogenic effect has been demonstrated in the simultaneous feeding of nitrites and amines to rats[5] and mice[6].

We have investigated the nitrosation of a drug commonly taken for appetite control, phenmetrazine (3-methyl-2-phenylmorpholine). The corresponding tertiary amine, phendimetrazine (3,4-dimethyl-2-phenylmorpholine) is used for the same purpose and would be expected to give rise to the identical nitrosamine, 3-methyl-2-phenyl-N-nitrosomorpholine, by nitrosative dealkylation[7].

Phenmetrazine as tablets of 'Preludin' (Geigy) was finely ground and suspended in water containing sodium nitrite. The molar proportion of phenmetrazine to sodium nitrite was 1 to 4. A 10 ml. sample of suspension, containing 1 mmol (177 mg) of phenmetrazine, was injected by syringe through the stomach wall of a rabbit which had been fasted for 24 h. Immediately before the injection, an occluding ligature had been placed at the pylorus; intravenous pentobarbital sodium was used for anaesthesia. After 5 h the animal was killed by injection of 10 ml. air into the ear vein. The stomach contents was washed into a beaker, the *p*H was measured and the solution was made strongly alkaline (*p*H 10) by addition of sodium hydroxide, to stop the nitrosation reaction during the subsequent extraction procedure.

The liquid was shaken with an approximately equal volume of methylene chloride and the lower layer was allowed to separate (due to emulsion formation this frequently took a long time). A second extraction with methylene chloride was carried out and the combined lower layers were back extracted with about one tenth the volume of dilute HCl, then with a few ml. of water, dried with anhydrous sodium sulphate and evaporated to dryness in a stream of nitrogen at room temperature. The nitrosamine in the residue was dissolved in a small volume, 2.0 ml. or 5.0 ml., of methanol (there was considerable undissolved brown material) and the concentration of nitrosophenmetrazine (NPM) was determined by gas chromatography on a 6 foot × 1/8 inch column packed with 8.4% diethyleneglycol succinate (DEGS) on 'Chromosorb W', using a flame ionization detector. NPM was estimated by comparison with a standard solution of the compound prepared from phenmetrazine; this had a melting point of 63–64°

C. Calculated for $C_{11}H_{14}N_2O_2$: C, 64.06; H, 6.84; N, 13.58 and analysis found: C, 63.66; H, 6.86; N, 13.20.

The yields of NPM in two rabbits were 26.5 mg and 37.5 mg (respectively 13% and 18% of theoretical). No NPM was detected in the stomachs of either of two rabbits given a corresponding dose (1 mmol) of phendimetrazine ['Plegine' (Ayerst)] and 4 mmol nitrite. No material with the chromatographic retention time of NPM was found in animals treated with phenmetrazine alone or with nitrite alone. The nitrite remaining in the rabbit stomach after 5 h was no more than 200 to 300 μg, showing that most had reacted or been absorbed in this time.

In another experiment, two rabbits were treated with phenmetrazine and nitrite exactly as before, and an indwelling intragastric catheter was placed through an incision in the stomach wall. At 1 h intervals after the treatment, 10 ml. samples were withdrawn from each stomach with a syringe and analysed as previously described for NPM. The remaining nitrite in the aqueous portion was estimated colorimetrically. The results from one rabbit showed 0.64 mg NPM/ml. and 0.12 mg nitrite/ml. after 1 h, 0.47 and 0.04 after 2 h, 0.33 and 0.02 after 3 h, 0.26 and 0.02 after 4 h and, after 5 h, in the total 130 ml. of gastric contents, 0.3 mg/ml. NPM and no nitrite. The presence of a great deal of mucus in the stomach of the second rabbit made the sampling and analysis so difficult that they were abandoned.

This experiment shows that nitrosation of phenmetrazine takes place quite rapidly in the rabbit stomach, the *p*H of which was about 3.5 throughout the experiment. The decline in concentration of NPM probably reflects a combination of continued secretion of gastric juice during the incubation and absorption of NPM from the stomach, which we have shown to occur with other nitrosamines. Nitrite seemed to disappear quite rapidly, although some was detectable after 4 h.

To establish the influence of change of the ratio of nitrite to amine on the rate of nitrosation, 1 mmol of phenmetrazine was given to each of three rabbits together with, respectively, 1 mmol nitrite, 2 mmol nitrite and 4 mmol nitrite, all in 10 ml. of suspension. The stomach contents of each rabbit was examined for NPM after 3 h. The *p*H of each of the stomach contents was 3.5 when the animal was killed. The respective yields of NPM were 10 mg (5% theoretical), 30 mg (15%) and 65 mg (31%), showing a definite increase in yield of nitrosamine with increasing nitrite concentration.

The experiments were repeated and amplified using rats, which are more easily anaesthetized and whose stomachs contain less mucus, which interfered with the extractions. Each rat was anaesthetized with sodium pentobarbital, the pylorus was tied off and 0.2 mmol of phenmetrazine (35 mg) together with 0.8 mmol sodium nitrite in 1 ml. water was injected into the stomach. The rats were killed after 3 h (with pentobarbital), the stomach contents were washed into a beaker (the *p*H varied from 2.5 to 3.5) and the NPM was extracted and estimated as previously described. The yields of NPM were 9.2 mg (23% theoretical), 19.4 mg (48%) and 18.6 mg (46%) in three rats, showing that nitrosation of phenmetrazine *in vivo* proceeded as efficiently in rats as in rabbits.

It has been suggested that nitrate can be reduced in the stomach and can therefore provide nitrite ions for interaction with ingested amines[4]. To examine this possibility, phenmetrazine (0.2 mmol), together with 400 mg sodium nitrate, in 1 ml. water was injected into the stomach of each of five rats. Two were killed after 3 h and the remaining three after 5 h. The stomach contents (*p*H range 3.5 to 5.5) was analysed for NPM as described. No NPM was detected in either rat killed at 3 h, and a trace (about 1 μg) in those killed at 5 h. The residual nitrate in the stomach contents was 330 mg and 340 mg at 3 h, 185 mg, 270 mg and 285 mg at 5 h, showing much slower elimination of nitrate than of nitrite from the stomach.

Although it is not known whether NPM is carcinogenic, it is acutely toxic to mice (LD_{50} = 120 mg/kg). Because nitrosomorpholine is a potent liver carcinogen in rats[8], it would be unwise to assume that the derivative NPM is noncarcinogenic. Therefore, our finding that NPM is readily formed in the stomach from nitrite and phenmetrazine suggests a possible hazard in taking this drug when food containing nitrite might be simultaneously present in the stomach. Our experiments with rats did not show significant formation of nitrosamine when nitrate, rather than nitrite, was present in the stomach. Although the tertiary amine, phendimetrazine, did not seem to interact with nitrite in the stomach to give NPM, this does not preclude the possibility that other tertiary amines might interact with nitrite under these conditions to form a nitrosamine.

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Increased Activity of Choline Acetyltransferase in Sympathetic Ganglia after Prolonged Administration of Nerve Growth Factor

A PROLONGED increase in the activity of preganglionic sympathetic nerves produces characteristic changes in the enzyme pattern of the postganglionic adrenergic neurones in adult rats[1-4]. In newborn mice the normal development of adrenergic neurones depends materially on the intactness of the preganglionic cholinergic nerves[5]. These two findings contribute to the definition of the so far vague term "trophic response to neuronal activity" and the question arises whether these trophic actions are unidirectional only, or whether there is also a retrograde effect, that is, a dependence of the preganglionic cholinergic nerves on the function of the postjunctional adrenergic neurones. Transection experiments in the brain have shown that after lesioning of particular areas, for example, optic nerve tract, cingulate or visual cortex, there occurs not only orthograde but also retrograde trans-synaptic degeneration[6]. It seemed of interest to study this possible retrograde trophic effect in a less complex system and to investigate whether there are not only negative (degeneration) but also positive effects. The administration of nerve growth factor (NGF) to newborn rats produces a marked growth and enhanced differentiation of the adrenergic neurones[7]. The biochemical correlate to these morphological changes is a selective induction of tyrosine hydroxylase and dopamine β-hydroxylase[8]. We have investigated whether these effects are also reflected by changes in the preganglionic nerves.

Newborn rats of either sex of a closed randomized colony

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Nitrosamine Studies: Induction of Lung Adenomas by Concurrent Administration of Sodium Nitrite and Secondary Amines in Swiss Mice [1,2]

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SUMMARY—Piperazine (6.25 g/kg of food), morpholine (6.33 g/kg of food), and *N*-methylaniline (1.95 g/kg of food) given together with sodium nitrite (1.0 g/liter of drinking water) to Swiss mice resulted in a highly significant increase in lung adenoma incidence. This increase was more pronounced in males than in females. Dimethylamine hydrochloride (5.9 g/kg of food) was ineffective when given with nitrite, probably because of the strong basicity of the amine. Dinitrosopiperazine given as a 40 mg/liter solution in the drinking water, nitrosomorpholine (80 mg/liter), dimethylnitrosamine (10 mg/liter), and *N*-nitroso-*N*-methylaniline (70 mg/liter) increased the number of lung adenomas per mouse tenfold to thirtyfold over control untreated mice. Treatment with sodium nitrite alone or with the secondary amines alone produced no effect. The data strongly suggest that the induction of lung adenomas in animals treated with these secondary amines and nitrite is due to *in vivo* nitrosation (presumably in the stomach) with the formation of carcinogenic nitrosamines.—J Nat Cancer Inst 46: 1029–1034, 1971.

THE RELEVANCE of nitrosamines to human carcinogenesis was broadened by the suggestion of Druckrey *et al.* (*1*) and Sander (*2*) that nitrosamines might be formed from nitrite and secondary amines under the acidic conditions of the human stomach. An experimental basis for this hypothesis was provided by the work of Sander *et al.* (*3*) and Sen *et al.* (*4*) and by the kinetic studies of Mirvish (*5*). Lijinsky and Epstein (*6*) and others (*7*, *8*) emphasized the public health implications of *in vivo* formation of nitrosamines. However, only three studies report the effect of chronic administration of secondary amines and nitrite to intact animals. Druckrey *et al.* (*1*) reported negative results on feeding diethylamine and sodium nitrite to rats. Sender *et al.* (*3*), in relatively short-term experiments, found that diethylamine and *N*-methylaniline were without carcinogenic effect when fed with nitrite to rats. Sander and

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Bürkle (*9*) later reported the occurrence of esophageal and hepatic tumors in rats fed *N*-methylbenzylamine or morpholine mixed with sodium nitrite in the diet.

Using lung adenoma induction as an index of carcinogenic effect, we tested the effect of concurrent administration of sodium nitrite in the drinking water and four secondary amines (dimethylamine, piperazine, morpholine, and *N*-methylaniline) given in powdered food to Swiss mice.

MATERIALS AND METHODS

Sodium nitrite (analytical grade) was obtained from J. T. Baker Chemical Company, Phillipsburg, New Jersey; dimethylamine hydrochloride, piperazine (practical grade, mp 108–109°), and dimethylnitrosamine from Eastman Organic Chemicals, Rochester, New York; morpholine (purified) from Fisher Scientific Company, Fairlawn, New Jersey; and *N*-methylaniline from Aldrich Chemical Company, Milwaukee, Wisconsin. Dinitrosopiperazine (mp 159°) and *N*-nitroso-*N*-methylaniline (bp 135°/15 mm) were synthesized by us, and nitrosomorpholine (bp 113°/15 mm) was synthesized by Dr. L. Keefer of this Institute, using the methods of Druckrey *et al.* (*10*). The food was Rockland Powdered Rat and Mouse Diet (Allied Mills Inc., Chicago, Ill.).

Randombred Swiss mice from our own closed animal colony were housed 10 per polycarbonate cage measuring 8 × 7 × 5 inches. Ground corncob (Sanicel) was used as bedding. Mice were separated according to sex at weaning and pooled. They were allocated to experimental groups by random number tables and were 6–11 weeks old at the beginning of the experiment. Stock solutions in distilled water were prepared of all compounds at 20 × the final concentrations. The dimethylamine. HCl, piperazine, and morpholine solutions were brought to *p*H 7 with HCl or NaOH, with use of a *p*H meter. The *N*-methylaniline solution was brought to *p*H 3, since it did not fully dissolve at *p*H 7. Stock amine solutions were added to a portion of powdered food twice weekly, ground with a mortar and pestle, mixed with the remainder of the powdered food in a paint-mixing machine (final concentration, 1 ml stock solution/20 g food), and fed *ad libitum*. The $NaNO_2$ and nitrosamine solutions were diluted with distilled water (final concentration, 1 ml stock/20 ml water) and given as drinking water in brown bottles, placed at some distance from the food. Stock solutions and amine-containing food were stored at 4°C, and working solutions were prepared freshly from stock every 2 days. The *p*H of the control and amine-containing food, as measured in a slurry with water, varied from 5.2 to 5.8.

The powdered food or water containing the compounds was administered continuously, except that $NaNO_2$ was given only 5 days per week. Mice not receiving special food were given food pellets, and mice not receiving special solutions were given tap water. One control group was given food pellets and tap water, and a second control group was given powdered food and tap water. These control groups did not differ in tumor yield, and they were combined for statistical purposes. All groups were treated for 28 weeks and then returned to standard laboratory maintenance (food pellets and tap water) for 12 weeks.

At 40 weeks all survivors were killed and autopsied. The number of lung adenomas larger than 0.5 mm on the surface of the lung was scored in all mice surviving 4 months from the start of the experiment. Mice having more than 10 adenomas were recorded as 10. One of every five autopsied animals received a complete microscopic examination, which in all cases confirmed the gross diagnosis of lung adenoma. Experimental results were analyzed in part by an automatic data processing system previously described (*11*).

RESULTS

The amines were given in the food and the sodium nitrite was given in the drinking water, to avoid any chemical reaction before ingestion. The concentration of $NaNO_2$ (0.1%) was the maximum tolerated chronic dose. Higher concentrations produced cyanosis, presumably due to methemoglobinemia. Cyanosis was particularly pronounced in animals receiving sodium nitrite and *N*-methylaniline. The concentration of the secondary amine in the food was chosen to provide a fivefold excess of amine-to-nitrite intake on a molar basis (assuming equal intakes of food and water). With *N*-methylaniline, the dose of amine

was decreased because the compound alone produced severe cyanosis at the higher level. The results are recorded in table 1.

Treatment with sodium nitrite alone or the secondary amines alone produced no effect in the mice when compared with untreated controls, as judged by the three parameters measured: percent adenoma-bearing mice, adenomas per adenoma-bearing mouse, and adenomas per mouse. All positive control groups (animals treated with the corresponding nitrosamine) showed a pronounced increase in the frequency of lung adenomas, in agreement with previous reports (*12*, *13*). These are apparently the first reported results for nitrosomethylaniline in mice.

Animals treated with the secondary amines—piperazine, morpholine, or *N*-methylaniline—in the food and given sodium nitrite in the drinking water had a highly significant increase in percent adenoma-bearing mice ($P<0.0005$) and in lung adenomas per mouse ($P<0.001$) compared with untreated controls. The number of adenomas per mouse was increased at least tenfold over that for the controls, and the number of adenomas per adenoma-bearing mouse was about twice that of the controls. The percent of lung adenoma-bearing mice was approximately the same as in the corresponding nitrosamine-treated controls.

Tumors other than lung adenomas consisted mainly of malignant lymphomas and showed no significant relationship to treatment. Liver tumors, both biliary adenocarcinomas and hepatocellular carcinomas, were found only in nitrosamine-treated animals. These data are presented in table 2.

Animals treated with amines plus nitrite showed weight increases similar to those of the untreated controls and the animals fed the amines or sodium nitrite alone. Feeding the nitrosamines produced a decrease in weight only in females given nitrosomorpholine and dinitrosopiperazine. Males in all groups consistently weighed 6–8 g more than the females. Survival curves were comparable for all groups except for dimethylnitrosamine-treated animals, most of which died by the 30th week of the experiment. Nitrite solution was consumed at an average daily rate of 6.4 (6.0–6.9) ml per mouse. Food consumption averaged 8 g per mouse daily, but this figure is probably too high because some powdered food was scattered.

The *p*H of mouse gastric contents determined in untreated animals varied from 1.9–5.8. The higher *p*H values tended to occur in animals with larger amounts of food in the stomach. No sex difference was apparent.

DISCUSSION

Our data strongly support the concept that *in vivo* nitrosation of the tested amines occurred, presumably under the acidic conditions of the stomach contents. The relative ease of nitrosation should increase as the basicity of the secondary amines is decreased, since nitrous acid reacts with the nonionized species of the amine molecule (*5*). The pK_a values of the amines used (*i.e.*, the *p*H at which the bases are half neutralized) decrease with decreasing basicity of the amine and are as follows: dimethylamine, 10.7; morpholine, 8.7; piperazine, 9.8 and 5.6 for the two amine groups; and *N*-methylaniline, 4.7. Hence it is not surprising that the only negative result was obtained with the strongly basic dimethylamine, despite the fact that dimethylnitrosamine is an extremely potent carcinogen in the lungs of mice (*14*, *15*). Diethylamine (pK_a 11.0) should react similarly to dimethylamine, which probably explains the negative results of Druckrey *et al.* (*1*) and Sander *et al.* (*3*) with diethylamine plus sodium nitrite.

A rough estimate of the amount of nitrosamine formed from the secondary amine and nitrite administered can be made on the basis of the lung adenoma yield per mouse. Nitrosomorpholine (0.69 mmoles/liter water) induced 4.3 adenomas/mouse more than the controls, and morpholine plus nitrite induced 1.2 adenomas/mouse more than the controls. Assuming that the lung adenoma yield is proportional to the dose of nitrosamine in this dosage range and that there is equal consumption of food and water, nitrosomorpholine production in the morpholine plus nitrite experiment would be $(1.2/4.3)\times 0.69 = 0.193$ mmoles/kg food. This corresponds to $(0.193/72.5)\times 100 = 0.27\%$ nitrosation of the 72.5 mmoles morpholine/kg food. Similarly, it was estimated that 2.0% of the *N*-methylaniline was nitrosated. In the piperazine experiment, dinitrosopiperazine was tested,

TABLE 1.—Lung adenoma induction in Swiss mice

Compound	Dose*	Initial No. of mice (females/males)	Effective No. of mice† (females/males)	Total adenoma-bearing mice		Total No. of lung adenomas	Adenomas per adenoma-bearing mouse	Adenomas per mouse‡
				Number	Percent			
None	—	80/80	71/73	20	14	26	1.3	0.18 ± 0.43
$NaNO_2$	1.0 g/liter (14.5)	40/40	38/36	14	19	16	1.1	0.2 ± 0.5
Dimethylamine·HCl	5.90 g/kg (72.5)	20/20	19/19	5	13	5	1.0	0.1 ± 0.3
Piperazine	6.25 g/kg (72.5)	40/40	36/32	10	15	14	1.4	0.2 ± 0.5
Morpholine	6.33 g/kg (72.5)	20/20	19/19	5	13	5	1.0	0.1 ± 0.3
Methylaniline	1.95 g/kg (18.1)	20/20	19/17	6	17	8	1.3	0.2 ± 0.6
Dimethylnitrosamine	0.010 g/liter (0.135)	20/20	17/14	29	94§	197	6.8	6.4 ± 3.9§
Dinitrosopiperazine	0.040 g/liter (0.278)	40/40	37/38	49	63§	319	6.5	4.9 ± 4.2§
Nitrosomorpholine	0.080 g/liter (0.69)	20/20	14/8	16	73§	106	6.6	4.5 ± 4.3§
Nitrosomethylaniline	0.070 g/liter (0.515)	20/20	20/16	22	61§	72	3.3	2.0 ± 2.4§
Dimethylamine·HCl + $NaNO_2$	5.90 g/kg; 1.0 g/liter	20/20	19/19	6	21	8	1.3	0.3 ± 0.6
Piperazine + $NaNO_2$	6.25 g/kg; 1.0 g/liter	40/40	39/36	48	64§	137	2.9	1.8 ± 2.2§
Morpholine + $NaNO_2$	6.33 g/kg; 1.0 g/liter	20/20	16/19	20	57§	41	2.0	1.4 ± 1.4§
Methylaniline + $NaNO_2$	1.95 g/kg; 1.0 g/liter	20/20	19/19	23	61§	55	2.4	1.5 ± 1.8§

*g/kg food or g/liter water; parentheses show concentration in mmoles/kg or mmoles/liter.
†Mice surviving more than 16 weeks from beginning of experiment.
‡Mean ± standard deviation.
§Significantly greater than control group ($P < 0.001$).

TABLE 2.—Tumors other than lung adenomas

Compound	Total effective No. of mice	Malignant lymphomas		Other tumors	
		Number	Percent	Number	Histologic type
None	144	10	7	1	Mammary tumor
$NaNO_2$	74	1	1	0	
Dimethylamine·HCl	38	1	3	1	Mammary tumor
Piperazine	68	2	3	0	
Morpholine	38	5	13	0	
Methylaniline	36	5	14	0	
Dimethylnitrosamine	31	1	3	3	2 ovarian carcinomas and 1 hemangiosarcoma
Dinitrosopiperazine	75	4	5	1	Mammary tumor
Nitrosomorpholine	22	0	0	5	4 liver carcinomas and 1 papillary adenoma of bile duct
Nitrosomethylaniline	36	5	14	1	Biliary adenocarcinoma
Dimethylamine·HCl + $NaNO_2$	38	0	0	0	
Piperazine + $NaNO_2$	75	4	5	0	
Morpholine + $NaNO_2$	35	2	6	0	
Methylaniline + $NaNO_2$	38	5	13	0	

but it is more likely that mononitrosopiperazine was formed, so that similar estimates are less reliable. The more extensive nitrosation of *N*-methylaniline might be predicted from its low pK_a value and has been demonstrated *in vitro* by Sander *et al.* (*3*).

In the dimethylamine plus nitrite experiment, 0.29 lung adenomas/mouse was observed, which was not significantly greater than the control value of 0.18 adenomas/mouse. It was estimated that a hypothetical yield of 1.4 adenomas/mouse (as in the morpholine plus nitrite experiment) would have corresponded to only 0.04% conversion of the dimethylamine, or a daily dose of 14 μg dimethylnitrosamine for an intake of 7 g food per day. Thus any actual nitrosation of dimethylamine must have been much less than 0.04%.

The two nitrosamines, dimethylnitrosamine and diethylnitrosamine, with which most laboratory experience has accumulated, are the least likely to be formed by *in vivo* nitrosation. An obvious corollary is that, in the evaluation of the possible significance of nitrosation of secondary amines in food processing or natural products, determinations of the amount of dimethylnitrosamine or diethylnitrosamine formed or present should not be used as the only index of possible biologically significant nitrosation.

Morpholine as the free base or as the fatty acid salt is permitted for use as a fungicidal surfactant on foods (*16*, *17*). Morpholine acetylsalicylates are apparently not used in the United States as analgesics, though Sander and Bürkle in Germany mention this application (*9*). Piperazine is widely used to treat intestinal roundworms, particularly in children.

A sex difference was noted in the response for mice fed amines plus nitrite, but not for the mice treated with nitrosamines. This difference was significant ($P<0.05$) in the piperazine plus nitrite group, where 36 males showed 2.55 ± 2.85 and 39 females showed 1.15 ± 1.20 adenomas/mouse (mean ± standard deviation), and was only suggestive in the smaller groups with morpholine plus nitrite and *N*-methylaniline plus nitrite. If the results from these 3 amine plus nitrite groups are combined, the overall difference becomes highly significant ($P<0.01$), 74 males showing 2.06 ± 2.40 and 74 females showing 1.08 ± 1.28 adenomas/mouse. The reason for this difference is unclear.

With respect to the lymphoma incidence in the various experimental groups, the relatively short period of observation (40 weeks) would have diminished differences that would have been apparent in animals held for their lifetime. Our animals were killed at 40 weeks to avoid coalescence of lung adenomas and the occurrence of other pulmonary lesions which would make quantitative data less reliable.

The relevance of our findings to problems of human carcinogenesis would be better understood if these studies were extended to other species and additional tumor models. It appears particularly important to 1) identify secondary amines in food or used in food processing or as drugs, 2) evaluate the likelihood of significant nitrosation by chemical kinetic studies, 3) test the carcinogenicity of the amines fed together with nitrite, 4) evaluate the the carcinogenicity of the corresponding nitrosamines, and 5) evaluate physiological conditions in the stomach that may affect the rate of *in vivo* nitrosation.

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Behavior of the Nitrate Ion in the Dog[1]

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THERE are two major aspects of homeostasis of extracellular body fluids, the control of composition and the control of volume. The osmotic composition of the extracellular fluids is rather closely regulated by the thirst mechanism and the antidiuretic hormone, regulating respectively the intake and output of water (1). The mechanism by which the total volume of extracellular fluid is controlled is not as well understood. A basic part of the control of volume is the regulation of the total body content of sodium chloride, the principal osmotic constituent of the extracellular fluids. There seems to be little control of intake of salt but the output is adjusted, somewhat sluggishly, to the intake in such a fashion as to maintain a fairly constant total content of salt in normal individuals, provided there is no extrarenal loss of salty fluid. The mechanism by which the excretion of salt is adjusted to the intake is obscure but it is agreed that the final effector cells are those of the renal tubules. It has been tacitly assumed that the control is exerted on the tubular reabsorption of sodium with chloride following passively by electrolytic association (1, 2). It is impossible to study the renal reabsorption of sodium chloride in the same way as other compounds have been studied because its concentration in body fluids cannot be significantly altered without drastic changes in osmotic concentration. Furthermore, it is not possible to say whether control of regulation is exerted on the anion or the cation or on both unless they can be varied independently. While there is no practical substitute for sodium, other ions can replace chloride at least temporarily. For our studies, the nitrate ion was selected as the least toxic anion which closely resembles the chloride ion in size and valency and which can to a large degree replace chloride in body fluids and tissues (3–5).

Our primary purpose was to study the mechanism of electrolyte excretion using the nitrate ion as a tool, but it was necessary first to determine the absorption, distribution, rate of metabolic alteration, toxicity and gross effect on body fluid balance of nitrate as a sodium salt. The results of these studies are described in this paper. A quantitative analysis of the excretion of the nitrate and its effect on electrolyte balace is reported elsewhere (6).

METHODS

Female dogs, weighing 9–18 kg, were used as subjects. Procedures were modified according to the particular study involved and are described below. The data include 30 experiments on 13 dogs.

Single-Dose Experiments. In this series, only the short-term effects of the administration of sodium nitrate were measured. The dog was loosely restrained, catheterized, and made as comfortable as possible. Creatinine and para-aminohippurate (PAH) were injected subcutaneously after an intravenous priming dose. Occasionally these solutions were given by continuous intravenous infusion. Control observations were made after a sufficient interval had elapsed to allow the plasma concentrations to stabilize. Sodium and chloride excretion under these conditions were also measured. After control periods, an infusion of sodium nitrate ($NaNO_3$) was begun using either isotonic (1.3%) or hypertonic (2.6%) solutions. The volume of solution infused varied from 500 ml to 4 liters. The rate of infusion was constant in some experiments at around 5 ml/min.; in others it was increased several times during the infusion period to a maximum of 10–16 ml/min. Urine and plasma samples were collected at intervals during the infusion period and for a short time afterwards. In some experiments, the salt was administered orally, mixed with ground beef, with blood and urine samples being collected over a 6–8-hour period. Blood pressure was measured on some occasions using a Sanborn electromanometer.

Balance Experiments. In this group, the dogs were fasted with access to water on the day preceding the experiment. After being given a dose of sodium nitrate, they were placed in a metabolism cage so that urine could be collected. Food was withheld during the first day. In some, the effects of daily doses of nitrate were

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studied, the animals being being maintained on a low salt diet consisting of ground beef washed with tap water. These balance experiments were designed to measure chloride loss and the percentage of administered sodium and nitrate that could be recovered in the urine in a given interval. In each of two dogs, equivalent doses of $NaNO_3$ and NaCl were given, both orally and intravenously on separate occasions in order to compare the rapidity of excretion of these two salts.

Volume of Distribution and Rate of Metabolic Alteration. Eight experiments were designed to determine the volume of distribution of nitrate. Measured doses of sodium nitrate were injected intravenously and plasma concentrations determined at intervals for several hours. In five dogs, bilateral nephrectomy was performed prior to the injection in order to avoid urinary loss. The volume of distribution was compared in one animal before and after nephrectomy.

The plasma concentration of nitrate at zero time was estimated by plotting the plasma levels against time after the injection and extrapolating the straight portion of this time-concentration curve to the zero time ordinate. (This theoretically represents a concentration after complete distribution corrected for urinary or metabolic loss.) Distribution of nitrate was calculated by dividing the total mEq of nitrate injected by the mEq/unit of plasma at zero time. In an animal which was given a larger dose, the urinary loss was measured at intervals and subtracted from the total nitrate injected to determine the amount remaining in the body. This quantity divided by the plasma concentration at the time of urine collection was taken to be the volume of distribution.

The rate of metabolic alteration was estimated in the nephrectomized animals from the slope of the plasma nitrate curve after equilibration in body fluids. It has been reported that loss of nitrate in the feces is negligible (7); therefore, the slope of the plasma curve was assumed to represent metabolic changes in the body. The decrease in the plasma nitrate concentration over a timed interval multiplied by the volume of distribution indicated the metabolic loss of nitrate during this period.

Nitrate was determined by the method recently described by Straughn, Travis and Hiatt (8). It involves the quantitative reduction of nitrate to nitrite by bacterial nitratase followed by the colorimetric determination of nitrite using the diazonium reaction. This procedure was found to be quite satisfactory and more suitable for our work than other methods.

Chloride was analyzed according to the iodometric method of Sendroy as modified by Van Slyke and Hiller (9).

Sodium was estimated using a Perkin-Elmer internal standard flame photometer.

The renal clearance of creatinine was taken to be a measure of glomerular filtration rate. Creatinine was determined by the alkaline picrate method of Folin and Wu (10).

The renal clearance of sodium para-aminohippurate (PAH) was used as a measure of effective renal plasma flow. PAH concentrations were determined by the method of Smith *et al.* (11).

TABLE 1. SUMMARY OF EXPERIMENTS ON DISTRIBUTION AND RATE OF METABOLIC ALTERATION OF NITRATE IN DOGS

Exper.	Wt., kg	Dose $NaNO_3$, mEq/kg	Duration, hr.	Volume of Distribution		Rate of Metabolic Alteration, mEq/kg/hr.
				ml	Body Wt., %	
Injected Nitrate/Plasma (zero time)						
6	12.1	0.51	5	3,580	29.6	
8*	15.0	0.77	10	3,360	22.6	0.017
7*	12.8	0.93	5	4,230	33.0	0.015
12*	9.7	1.10	31	2,462	25.4	0.013
29† (a)	9.2	1.10	4	3,120	33.0	
(b)*	8.6	1.20	4	2,600	30.2	0.024
15*	17.0	1.40	10	5,110	30.0	0.014
Injected — excreted NO_3/Plasma						
11‡	13.3	0.00	1‡	3,580	26.8	
			2	3,870	28.4	
			3	3,770	28.3	
			6	3,400	25.8	
			10	4,060	29.9	

* Nephrectomized. † Very thin animal. ‡ No correction for metabolic loss. All doses were administered intravenously as an isotonic (1.3%) solution of $NaNO_3$.

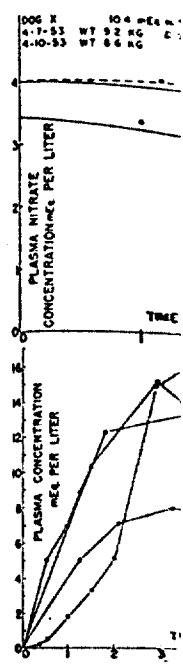
RESULTS

Toxicity. No signs of toxicity were observed with administration of $NaNO_3$, either orally or intravenously. The largest total dose in one day was 41 mEq/kg intravenously and 20 mEq/kg orally. The highest plasma concentration of nitrate attained was 44 mEq/l. This was achieved with the above mentioned intravenous dose. The highest level observed after oral dosage was 24 mEq/l.

It has been suggested that, through the action of intestinal bacteria, nitrate might be reduced to nitrite which on absorption would cause methemoglobinemia (12). There was no evidence of methemoglobinemia in our experiments. (In separate studies, none was found by analysis in spite of efforts to produce it experimentally by infusing sodium nitrate into the large intestine, or by introducing nitrate-reducing bacteria with nitrate salts into the small gut (13).)

Rapid infusion of hypertonic $NaNO_3$ occasionally produced retching or vomiting which is not uncommon when any saline solution is given too rapidly. Repeated daily doses of nitrate to animals on a low salt diet caused signs of dehydration as mentioned below.

Hemodynamics. In four experiments (including one in which 4 liters of isotonic $NaNO_3$ was infused) there was no appreciable change in heart rate or blood pressure. There was usually a slight increase in glomerular filtra-



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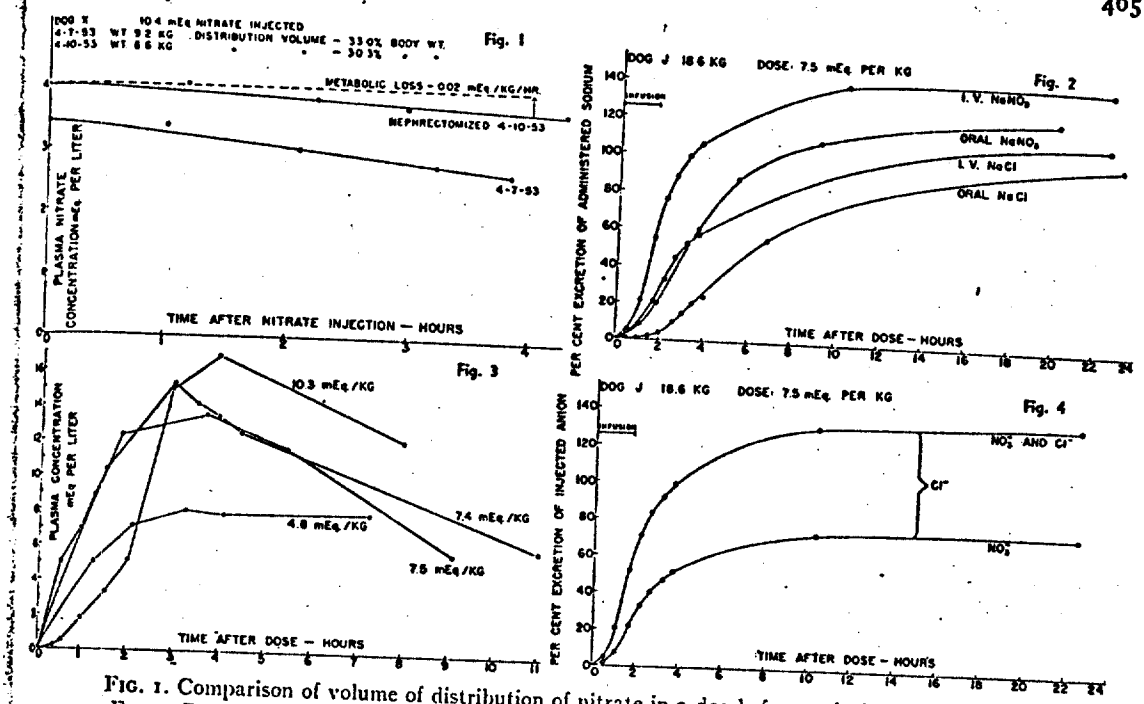

FIG. 1. Comparison of volume of distribution of nitrate in a dog before and after nephrectomy.
FIG. 2. Percentage of sodium excreted after equivalent doses of $NaNO_3$ and NaCl by oral and intravenous routes.
FIG. 3. Plasma concentrations of nitrate after oral $NaNO_3$.
FIG. 4. Percentage of injected anion excreted as NO^-_3 and Cl^- after infusion of hypertonic $NaNO_3$ (2.6%).

tion rate and in renal plasma flow during the infusion of nitrate, similar to that which has been observed with NaCl infusions (1).

Some of the animals which were maintained on a low salt diet with repeated injections of nitrate showed evidences of dehydration, consisting of dry nose, eyes and mouth, rapid pulse, anorexia and listlessness. In one 14-kg dog which was given a daily dose of 500 ml of hypertonic nitrate for 8 days, there was a 36% decrease in plasma chloride and a 14% decrease in plasma sodium with a 1.2-kg loss of weight. In spite of a 35% fall in mean blood pressure, the renal plasma flow and glomerular filtration rate were well maintained. In confirmation of previous work (3), it was noted that this dog refused to drink isotonic NaCl solution. When this solution was given intravenously as a partial correction of the salt and water loss, the above signs disappeared promptly. After a week on normal diet, plasma chloride and sodium, blood pressure and renal clearance had returned to control values.

Volume of Distribution and Rate of Metabolic Alteration. The results are summarized in table 1, and a typical experiment is graphed in figure 1. It can be seen that the volume of distribution was somewhat larger than the 16–20% of body weight which is now taken to be the volume of the extracellular fluid (14). The volumes were of the same order of magnitude as the volume of chloride distribution reported by Winkler *et al.* (15). Variation in different individuals is probably a reflection of corresponding variation in body water percentage due to different body composition, especially the fat content (16). It is recognized that there is an error involved in measurements of distribution due to the 'urinary dead space' (17) which may contain nitrate at a higher concentration, but the essential agreement of our data on nephrectomized dogs with that on the other dogs indicates that this error was not large. Figure 1 illustrates the method of determining the volume of distribution in the same dog before and after nephrectomy. This was an emaciated dog which may account for its large volume of distribution when expressed as percentage of body weight. The smaller volume after nephrectomy probably reflects the removal of the 'renal dead space.'

The rate of metabolic alteration of nitrate

TABLE 2. PERCENTAGE OF TOTAL DOSE OF NITRATE ($NaNO_3$) RECOVERED IN URINE

Exper.	Dose, mEq/kg	Mode of Administration	Time after Dose, hr.	Recovered, %
6	0.5	i.v. 1.3% (isotonic)	24	27
			48	31
21	4.8	i.v. 1.3%	24	69
	4.8	oral (in food)	24	67
13	7.4	oral 2% (stomach tube)	24	66
18	7.5	i.v. 2.6%	22	74
			36	75
	7.5	oral (in food)	20	90
11	9.0	i.v. 1.3%	29	70
5	10.3	i.v. 2.6%	24	60
26	10.3	oral (in food)	23	81
17	14.4	i.v. 2.6%	19	87
			44	91
19	23.0	i.v. 1.3% (150 ml/day for 5 days)	48 (after final injection)	92
10	41.4	i.v. 1.3%	24	83
			72	86

was rather slow so that most of an administered dose was recovered in the urine, regardless of whether it was given orally or intravenously. The percentage recovered in the urine decreased with small doses, but with larger doses it ranged up to 92% (table 2). The rate of metabolic alteration was also reflected in the slope of the plasma curve after a dose of nitrate was given to a nephrectomized dog, as illustrated in figure 1. In table 1 the results of five determinations of the rate of metabolic alteration of nitrate are shown, expressed as mEq/kg/hr. It is recognized that what we have termed metabolic alteration could be a sequestration of nitrate somewhere in the body, but for lack of evidence of this we have assumed the more likely possibility.

Comparison of Rates of Excretion of $NaNO_3$ and NaCl. In two dogs a direct comparison was made of the rates of excretion of sodium after equivalent doses of the nitrate and chloride salts given on separate occasions. Both oral and intravenous routes of administration were employed. Regardless of the manner of administration, the sodium was recovered much more rapidly in the urine when administered with nitrate than with chloride.

In figure 2 are plotted the results of two comparison experiments on one dog, one with oral and the other with intravenous doses. The intravenous solutions were twice isotonic (1.7% NaCl, 2.16% $NaNO_3$) and were administered at the rate of 4.42 ml/min. An amount of sodium equivalent to the amount injected was excreted in about 3 hours after the start of the $NaNO_3$ infusion, whereas with NaCl, only 50% of the sodium had been excreted at that time. When $NaNO_3$ was administered, 140% of the amount of sodium injected was excreted in 10 hours, but after NaCl was given only 109% of the sodium was excreted in 24 hours. With oral administration of the salts, the same differences were observed though the rate of excretion was somewhat slower, presumably because of the time required for intestinal absorption. (The plasma concentrations of nitrate obtained with oral doses, shown in figure 3, indicate that maximum absorption of nitrate did not occur until approximately 4 hours after the dose was given. These results were taken from four experiments in three dogs.)

Figure 4 illustrates the anion excretion after the $NaNO_3$ infusion mentioned above. Although only about 70% of the nitrate was recovered during the experiment, the combined excretion of nitrate and chloride was considerably in excess of the amount of anion injected. In 4 hours, the percentage of anion excretion was about 100% of the anion injected. In 10 hours, the anion excretion was about 33% above the anion dose.

These results were essentially duplicated in the other dog with a smaller dose of the salt. Although paired NaCl and $NaNO_3$ excretion studies were carried out on only two dogs, our other nitrate experiments verify the fact that $NaNO_3$ administration by either route causes a net loss of electrolyte.

DISCUSSION

Although nitrate can displace a large fraction of the chloride, via the urine, without toxic effect (3), it is not a physiologic substitute for the chloride ion because nitrate is not so effectively conserved by the kidneys as is chloride. Consequently, the ultimate effect of nitrate administration is a reduction in extracellular fluid volume if chloride intake is restricted.

The fact that sodium excretion is also enhanced, resulting in a net loss of sodium from the body reflects the interdependence of the anions and cations in the renal tubular reabsorptive mechanism. Rapoport and West investigated the capacity of a number of

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anions to promote chloride excretion and found nitrate second only to the thiocyanate ion in this regard (4).

Nitrate salts have been used as diuretic agents for centuries (11) though in recent decades they have largely been displaced by the other diuretic agents, particularly the mercurials. In 1930 Keith, Whelan and Bannick published the results of extensive studies in dogs and man of the diuretic effect of nitrate salts, particularly NH_4NO_3. They found a diuretic effect greater than that due to the acidifying action of ammonium salts indicating a specific action of the nitrate ion. They also reported these salts to be effective diuretics in edema of renal and cardiac disease (7). Fear of nitrite formation in the gut with the methemoglobinemia consequent to its absorption has contributed to the disuse of the nitrate salts. This fear has been based on experiences with bismuth subnitrate and observations on infants taking formulae made up with nitrate-containing water from contaminated wells (12). In our experience, it has been impossible to demonstrate methemoglobinemia as a result of nitrate administration to dogs even when special efforts were made to cause it. Whether the development of methemoglobin by certain individuals represents a susceptibility by reason of special conditions in their gut or is due to the occasional contamination of nitrates with nitrites is still uncertain.

Though no practical application of these studies is yet evident, any approach which can increase our knowledge of the control of sodium and chloride excretion or affect salt balance is of potential importance.

SUMMARY

The absorption, distribution, fate, toxicity and effect on electrolyte balance of nitrate salts were studied in 30 experiments on 13 dogs. The nitrate was administered as the sodium salt either intravenously or orally, the mode of administration making no appreciable difference in the effects obtained. By using the sodium salt, it was possible to vary the ion composition of body fluids without changing the cation. The following observations were made: At doses as high as 41 mEq/kg intravenously and 20 mEq/kg orally, there were no evidences of toxicity, nor were there marked changes in blood pressure or renal hemodynamics. A slight increase in glomerular filtration rate and renal plasma flow was usually observed. The volume of distribution of nitrate was found to average 28% of body weight in eight experiments on seven dogs (five nephrectomized). The average rate of metabolic alteration as determined from the slope of the plasma concentration curves in the nephrectomized dogs was 0.016 mEq/kg/hr. The amount of administered nitrate recovered in the urine varied directly with the dose, reaching 80–90% with doses of 10 mEq/kg or more. In two dogs, a comparison was made of the rapidity of sodium excretion after equivalent doses of $NaNO_3$ and NaCl. The rate of excretion of sodium administered with nitrate was much greater than its rate of excretion when given with chloride. With sodium nitrate administration, as much as 40% more sodium was excreted than was injected, bringing about loss of extracellular fluid.

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THE ACTION OF NITROUS ACID UPON CREATININE AND SOME OF ITS DERIVATIVES

ISIDOR GREENWALD and IRVING LEVY[1]

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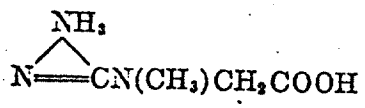
The work discussed in the present paper is an attempt to determine the reactivity of nitrous acid with creatinine and several of its derivatives and to consider the significance of the results for the formulation of the constitution of these substances.

Wilson (1) and Plimmer (2) found that creatine, for which the usual formula indicates an amino group, did not react with nitrous acid or did so only slowly and incompletely; whereas creatinine, for which the usual formula does not indicate an amino group, reacted quite rapidly.

Plimmer (2) added the observation that, in the presence of hydrochloric acid, creatine did liberate gas, whereas the yield from creatinine was diminished. He, therefore, proposed the alternative formulas I and II for creatine and creatinine, respectively, as expressing the state of the substance in aqueous solution and suggested that the usual formulas express the structures existing in solutions containing hydrochloric acid.



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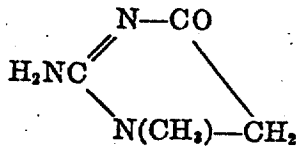

II

We confirmed Plimmer's report as to the effect of hydrochloric acid upon the reactivity of creatinine and nitrous acid. We mixed the creatinine with hydrochloric acid before adding it to the reaction mixture and used such small quantities of hydrochloric acid that they could not have affected the *p*H of the reaction mixture appreciably. We are, therefore, of the opinion that there are two tautomeric forms of creatinine, one of which is stable at low *p*H and does not react with nitrous acid.

However, if Plimmer's formulation were correct, methylcreatinine and N-benzylcreatinine should not react with nitrous acid or do so but slowly. Actually, they do (Table I, entries 6 to 12, 14 and 15), and rapidly and without apparent interference by small amounts of hydrochloric acid.

Since the *p*H of a solution of creatinine picrate is about 3.9 (glass electrode) and since that of the reaction mixture of acetic acid and nitrous acid cannot be far removed from this, it would appear that about ten per cent of the creatinine exists in the non-reactive form at this *p*H (entry 4). Moreover, since some reconversion to the reactive form may be expected to have occurred in the reaction

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TABLE I

ERATION OF NITROGEN BY ACETIC ACID AND SODIUM NITRITE FROM CREATININE AND ITS DERIVATIVES

c figures represent percentages of calculated yield of one atom per molecule. Only one value is reported if duplicate analyses yielded concordant results.)

	SUBSTANCE	SOLVENT	NITROGEN LIBERATED IN, MINUTES		
			3	15	30
			Molar percntage		
	Creatine	H_2O	0.4	7.2	7.5
	Creatinine	H_2O	22–45	88	101
3	Creatinine HCl	0.04 *M* HCl [a]	16–23	46	51
	Creatinine picrate	H_2O	21	80	90
	Creatinine	0.1 *M* NaOH [b]	19–28	83–91	97
6	Methylcreatinine HI	H_2O	33	88	99
7	Methylcreatinine HCl [c]	H_2O	50	96	100
	Methylcreatinine picrate	H_2O	39	87	107
	Methylcreatinine picrate	0.04 *M* HCl [a]	45		
10	Methylcreatinine HCl [c]	0.9 *M* HCl [a]		90	
	Methylcreatinine HI	0.04 *M* HCl [a]	41		
	Methylcreatinine H_2SO_4	H_2O	51	94	94
15	Methylcreatinine HI	0.04 *M* NaOH [a, b]			14
14	N-benzylcreatinine	H_2O		90	103
	N-benzylcreatinine HCl	H_2O	62	92	111
	Creatinine oxime	0.04 *M* NaOH [a]	81–97	104	
17	Creatinine oxime	0.44 *M* NaOH [a]	56		102
	Methylhydantoin oxime	0.065 *M* NaOH [a]			10–30
	Dimethylhydantoin oxime	0.068 *M* NaOH [a]		85	95
20	Methylhydantoin	H_2O			2
21	Acetylcreatinine	H_2O	13	24	32
	Benzoylcreatinine [e]	0.96 *M* NaOH [a]	4	10	10 [f]
	Benzalcreatinine, 2 HCl	H_2O	36	108	136
24	Benzalcreatinine	10% Acetic acid	24	83	99
	O-Benzylcreatinine	H_2O		90	103
	Dimethylcreatinine HCl [c]	H_2O		52	58
	Dimethylcreatinine HI	H_2O	22–36	34–45	46–53
28	Dimethylcreatinine HI	0.07 *M* HCl [a]		34	
	Dimethylcreatinine HI	0.07 *M* HCl [a, d]		41	
	Glycocyamine	H_2O	5	13	26
31	Glycocyamidine HCl	H_2O	37	77	90
	3-Methylglycocyamidine picrate	H_2O	38	66	70
	Dicreatinine picrate HBr	H_2O	47–68	110	125

[a] The concentration of hydrochloric acid and of sodium hydroxide are those calculated maining after displacement of picric acid or of hydroiodic acid from the salts or of one on from benzoylcreatinine or any of the oximes; or the addition of one proton to creatinine.

[b] The mixtures were prepared one hour before use.

[c] Prepared from the hydriodide by treatment with freshly precipitated silver chloride.

[d] After standing eight days.

[e] In a previous paper (10) it had been assumed, because of the different crystal forms, the benzoylcreatinine made from creatinine and benzoyl chloride (plates) was an er of that obtained by heating creatinine with benzoic anhydride (needles). Ing (7) med that the supposed isomer was a mixture of benzoylcreatinine and tribenzoyl creatinine. Our preparations did not contain tribenzoylcreatinine. However, upon recrystallization from alcohol, particularly in the presence of a few needles, the plates changed edles. Determinations of the solubility of the two materials, separately or together icated that they were different crystal forms of the same substance.

[f] The low yield of gas when treated with nitrous acid was probably due to precipitation nzoylcreatinine in the reaction chamber.

vessel in the experiments summarized in entry 3, it would appear that more than half of the creatinine at *p*H 1.4 was in this non-reactive form. At the lower *p*H prevailing in Plimmer's mixtures containing 5, 6, 7, and 8 ml. of concentrated hydrochloric acid, still more of the creatinine would have been in this inactive form and the approximately 0.5 atom of nitrogen liberated might well have been formed during the first few minutes (as Plimmer recognized), before all the acid had been added. Whatever the change may be, it does not seem to occur if the two hydrogens in position 5 are replaced by a benzal group (entries 23 and 24).

The removal of one atom of nitrogen from creatinine should result in the formation of methylhydantoin. Indeed, Schmidt (3), thirteen years before Plimmer, had shown that creatinine in approximately 23% nitric acid, to which an excess of sodium nitrite was added, deposited a mixture of a small quantity of creatinine oxime and a large quantity of methylhydantoin oxime.

Experiment showed that gas was liberated from creatinine oxime more rapidly than from creatinine, indicating that the reaction might be creatinine oxime → methylhydantoin oxime (entries 16 and 17). However, methylhydantoin oxime, dissolved in NaOH, in order to get it into the apparatus, also liberated gas (entry 18). Dimethylhydantoin oxime yielded even more (entry 19). Apparently, the addition of alkali to either of these oximes produces a change in configuration which is not readily reversed by the acetic acid in the reaction mixture. The postulated isomers are acted upon by nitrous acid, with the liberation of gas. Methylhydantoin, itself, dissolved in water, gave only traces of gas (entry 20).

It seems, therefore, quite likely that the reaction is creatinine → methylhydantoin in the dilute solution employed in the determination, and to creatinine oxime and then to methylhydantoin oxime in the more concentrated mixtures. The methylhydantoin oxime isolated could scarcely have been formed from methylhydantoin because the latter, when added to a mixture of sodium nitrite and acetic acid, yielded no oxime but was recovered unchanged upon extraction with chloroform.

When the same preparative technic was applied to methylcreatinine hydrochloride, no methylcreatinine oxime was obtained. Instead, 60% of the material employed was accounted for as dimethylhydantoin oxime, identified by analysis and by conversion to dimethylparabanic acid. However, Zeile and Meyer (4) have obtained and described the hydrochloride of methylcreatinine oxime. This was formed in the presence of an excess of hydrochloric acid; when an excess of sodium nitrite was present, the product was dimethylhydantoin oxime.

The fact that methylcreatinine reacted so readily indicated that the second methyl group is in position 3 and not in 2. Zeile and Meyer (4), came to a similar conclusion in spite of the opposite view held by Nicolet and Campbell (5) and by Cornthwaite (6).

Ing (7) has presented evidence for regarding acetylcreatinine and benzoylcreatinine as being substituted in position 2. Since neither of these (entries 21, 22) reacted rapidly and since, in large-scale experiments, benzoylcreatinine yielded only benzoylcreatinine oxime, Ing's views would seem to have been

confirmed. Such liberation of nitrogen as did occur might be considered as having taken place after hydrolysis.

However, this interpretation is contradicted by the fact that dimethylcreatinine (entries 26 to 29) reacted to a greater extent than did acetylcreatinine. In dimethylcreatinine, all the nitrogen atoms are methylated and there can be no question of hydrolysis.

Other compounds related to creatinine were also tested with nitrous acid (entries 30–33). Glycocyamine reacted to a greater extent than did creatine; glycocyamidine hydrochloride almost as much as did creatinine; methylglycocyamidine picrate to a greater extent than did creatinine picrate. The compound of two molecules of creatinine and one of picric acid, in which the creatinine is present in a form that does not give Jaffe's reaction (8) yielded more gas than did the equivalent amount of creatinine, added as the free base, the picrate or the hydrochloride.

In contrast to the apparent stability of creatinine to 0.1 *M* sodium hydroxide for one hour, is the great lability of methylcreatinine to an even lower concentration (entry 13). Colorimetric analysis, using Jaffe's reaction and creatinine as a standard, of some of the same mixture as was used for the gasometric determinations, showed it to contain 23% of the expected amount of methylcreatinine. However, at the end of an hour, when the diluted alkaline picrate solutions were again compared with one another, the readings indicated the presence of 37% of the calculated amount of methylcreatinine. In another experiment, in which the undiluted mixtures of creatinine, or methylcreatinine, picric acid and sodium hydroxide were allowed to stand an hour before dilution and comparison, the readings indicated the presence of 79% of the calculated amount.

Apparently, the ring in methylcreatinine is more readily opened by hydroxyl ions than is that in creatinine but, as enolization takes place in more concentrated alkali and as complex formation with picrate occurs, it tends to close again.

The same ready opening of the ring in methylcreatinine was observed by Zeile and Meyer (4) when they attempted to prepare methylcreatinine by Cornthwaite's method. We succeeded once in the preparation by Cornthwaite's method but, thereafter, always encountered opening of the ring, whether we used that method or Korndorfer's (9), which had previously given good results. Finally a satisfactory product was obtained by treating methylcreatinine sulfate with ammonia, as described in the experimental part.

Attempts were made to isolate the products of the reaction between nitrous acid and creatinine and some of its derivatives. Under the conditions chosen, which except for time and the concentration of the "creatinine", were similar to those obtaining in the Van Slyke analysis, the following substances were isolated and identified: from creatinine, the oximes of creatinine and methylhydantoin; from methylcreatinine, dimethylhydantoin oxime; from N-benzylcreatinine, benzylmethylhydantoin oxime; from benzoylcreatinine, benzoylcreatinine oxime.

Dimethylcreatinine yielded a mixture of substances, which were not separated satisfactorily. In one experiment, what appeared to be two different substances, of widely different melting points, were obtained. The empirical constitution

of both was that of dimethylhydantoin oxime but neither yielded dimethylparabanic acid upon hydrolysis. In another experiment, the bulk of the product had a melting point which was different from that of either of the other two materials or from that of pure dimethylhydantoin oxime but it yielded dimethylparabanic acid upon hydrolysis.

We wish to thank Mr. D. Rigakos for the determinations of carbon, hydrogen, and nitrogen.

EXPERIMENTAL

Except as mentioned below, all the derivatives of creatinine were prepared by the methods used in previous work (10).

Benzalcreatinine and its dihydrochloride were prepared by the method of Nicolet and Campbell (5).

Methylcreatinine. To 18 g. of methylcreatinine hydrogen sulfate (6), dissolved in 200 ml. of hot absolute alcohol, 12 ml. of concentrated NH_4OH was added with constant shaking. The mixture was cooled in ice, filtered, and the precipitate was washed with absolute alcohol. The filtrate and washings were evaporated, under diminished pressure, with a bath temperature of 35°, to about 15 ml. Some crystals had separated. Fourteen milliliters of anhydrous ether was added and, after a few hours, the mixture was filtered and the precipitate was dried over H_2SO_4 and then over P_2O_5. The yield was practically the theoretical. The crystals melted at 70°, and recrystallization either from alcohol (9) or from acetone (6) raised the m.p. only to about 73°, not the previously reported 80°. However, even the crude product could be used for the preparation of dimethylcreatinine hydriodide with perfectly satisfactory results.

Creatinine oxime and methylhydantoin oxime. A suspension of 5 g. of creatinine in 10 ml. of water was added in small quantities with constant stirring to a mixture of 8.4 g. of sodium nitrite, 25 ml. of H_2O and 7 ml. of glacial acetic acid. After standing at 10° for two days, the precipitate was filtered off, washed, and dried over KOH. It weighed 4.4 g. Extraction with hot alcohol yielded 2.3 g. of methylhydantoin oxime (m.p. 196°; diacetyl deriv., m.p. 187°). The material insoluble in alcohol was dissolved in 0.25 *M* NaOH, reprecipitated with acetic acid, washed and dried. It weighed 1.5 g., decomposed above 252° and yielded a diacetyl derivative of m.p. 208°.

Dimethylhydantoin oxime. Similar experiments in which methylcreatinine hydrochloride or hydrogen sulfate were used yielded no material insoluble in alcohol but only an alcohol-soluble material, of m.p. 236°.

Anal. Calc'd for $C_5H_7N_3O_3$: C, 38.19; H, 4.49; N, 26.76.
Found: C, 38.66; H, 4.2; N, 25.90.

Upon hydrolysis with HCl (4) it yielded dimethylparabanic acid; crystals, from alcohol, of m.p. 150–151°.

Oxime of 1-methyl-3-benzylhydantoin. N-benzylcreatinine hydrochloride yielded only a small amount of material that was insoluble in alcohol. This dissolved in NaOH and was reprecipitated by acetic acid. It darkened above 262° but did not melt even at 355°. The alcohol-soluble material was crystallized twice from 50% alcohol, and then melted at 158°.

Anal. Calc'd for $C_{11}H_{11}N_3O_3$: C, 56.64; H, 4.75; N, 18.01.
Found: C, 56.79; H, 4.34; N, 17.97.

Oximes from dimethylcreatinine. A solution of dimethylcreatinine hydriodide was treated with freshly precipitated silver chloride. The mixture was filtered and the filtrate and washings were evaporated, under diminished pressure, to small volume. Upon treatment with sodium nitrite and acetic acid, a crystalline material was obtained. This was fractionated into two portions, one less soluble in alcohol (A) of m.p. 206° and one more soluble (B) of m.p. 140–142°.

(A) Found: C, 37.98; H, 4.43; N, 26.25.
(B) Found: C, 38.28; H, 4.54; N, 26.48.

These figures agree very well with those calculated for dimethylhydantoin oxime. Some ach fraction was mixed with pure dimethylhydantoin oxime. The m.p. of the mixture itn A was 208–210°; of that of the mixture with B was 185–195°. Neither A nor B yielded imethylparabanic acid upon treatment with HCl.

a another experiment, the bulk of the material melted above 175°. This was not ana- d but, upon hydrolysis, yielded typical crystals of dimethylparabanic acid, m.p. 150°, nchanged by mixture with material obtained from pure dimethylhydantoin oxime.

Benzoylcreatinine oxime. Benzoylcreatinine (1.76 g.) was ground with 30 ml. of 0.04 *M* um hydroxide. There were then added 10 ml. of 30% sodium nitrite and 50 ml. of 20% cetic acid. After standing 24 hours, the mixture was filtered and the precipitate was ashed, air-dried, and extracted with benzene to remove a small quantity of unchanged oylcreatinine. The residue was dissolved in 0.1 *M* sodium hydroxide and reprecipi- d with acetic acid. After being filtered, washed, and dried, it melted at 129–130°; yield 79 g., or 90% of that calculated. Four-tenths gram was dissolved in 5 ml. of warm con- rated hydrochloric acid and the solution was then placed in a vacuum desiccator con- ing potassium hydroxide. The dry residue was extracted with ether. The ether was aporated and the residue was recrystallized from water. It melted at 120° and 0.0786 g. quired 0.61 ml. of 0.1014 *M* sodium hydroxide for a titration to *p*H 8.5; calculated for oic acid, 0.636 ml. The aqueous mother liquids from the benzoic acid were evaporated yness. The residue, after crystallization from alcohol, melted at 152–153°, unchanged pon mixing with pure methylparabanic acid. The material insoluble in ether weighed g., melted at 147° and contained 48.4% chlorine and 19.0% nitrogen. It was, apparently, oxylamine hydrochloride, containing 51% chlorine and 20% nitrogen, contaminated i... some material of lower chlorine and nitrogen content.

SUMMARY

The reaction between nitrous acid and creatinine and several of its derivatives as s ed. The results indicate the existence of a tautomer of creatinine is stable at low *p*H and that does not react with nitrous acid. This type automerism does not occur with methylcreatinine, which reacts readily to rm dimethylhydantoin oxime. Dimethylcreatinine is less reactive than hylcreatinine but also forms a compound of the composition of dimethylhy- ntoin oxime.

A method for the preparation of free methylcreatinine is described.

ttention is directed to the ready opening of the ring in methylcreatinine.

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Health Aspects of Nitrates in Drinking Water*

Nachman Gruener and Hillel I. Shuval

INTRODUCTION

The increasing intervention by man in his environment has greatly increased the danger to public health. The rapid technological advances both in agriculture and industry are apt to cause harmful environmental changes of diverse origin, e.g., biological, chemical or radioactive. Such changing conditions often call for a re-evaluation of the existing and accepted health standards.

In this article we will deal with the danger of water contamination by nitrate salts. These salts may accumulate in surface and groundwater as a result of contamination by industrial or organic waste products and chemical fertilizers. Rapid urban growth with the intensification of agriculture and industry, on the one hand, and the increasing utilization of groundwater, on the other, are in some areas greatly accelerating the danger to public health from nitrates in drinking water.

In Israel the problem of nitrates in drinking water has become in recent years a cause for considerable concern among water supply agencies and public health authorities alike.

* This study has been supported in part by a research grant from The Mekorot Water Co.

Surveys of the nitrate levels in groundwater have indicated that there is a definite upward trend with time, which has now reached serious proportions (see Figure 1).* Approximately 180 water supply wells in the densely populated central and southern coastal plain area show nitrate concentrations equal to or greater than the limit recommended by the health authorities (45 mg/l of NO_3). Fifty other wells still supplying water with permissible nitrate concentrations have shown a nitrate increase of 2 mg/l or more per year.

Figure 2** gives the nitrate levels in the water supply wells of the town Nes Ziona for 1968, revealing that most of the town's wells have exceeded the recommended maximum concentration although they were at acceptable levels a few years ago.

A number of clinical cases of infant methemoglobinemia were detected in this town in recent years, and the possible association with the high nitrate concentrations in the water supply cannot be overlooked. A few years ago several cases of the disease were definitely traced to drinking water in the nearby town of Ramleh (WAGO, 1956).

The nitrate problem has become of even greater concern of late due to a major project for wastewater reuse by recharge into the aquifer. The Dan Region Water Reclamation project will infiltrate tens of thousands of cubic meters of treated wastewater high in nitrate content into the groundwater daily and will in that way further increase the nitrate burden on Israel's limited water resources (SHUVAL, 1962).

These developments have created the need to embark on a study to determine if present nitrate concentrations in drinking water are having any detrimental effects on the population exposed. In addition, it was felt that a basic re-evaluation of the nitrate standard in drinking water was called for, since this standard was originally based on very limited toxicological and epidemiological information and has been subject to criticism for some years. Of particular concern has been our suspicion that a form of subclinical chronic methemoglobinemia might exist in infants in those areas with moderately high nitrate concentrations in drinking water. First let us review the present state of knowledge concerning nitrate methemoglobinemia.

* The survey of nitrates in well water shown in Figure 1 was provided by Mrs. E. Foa of the Israel Hydrological Service and is based on data supplied by the Ministry of Health and Mekorot Water Co.
** Data based on report of Central District Health Office.

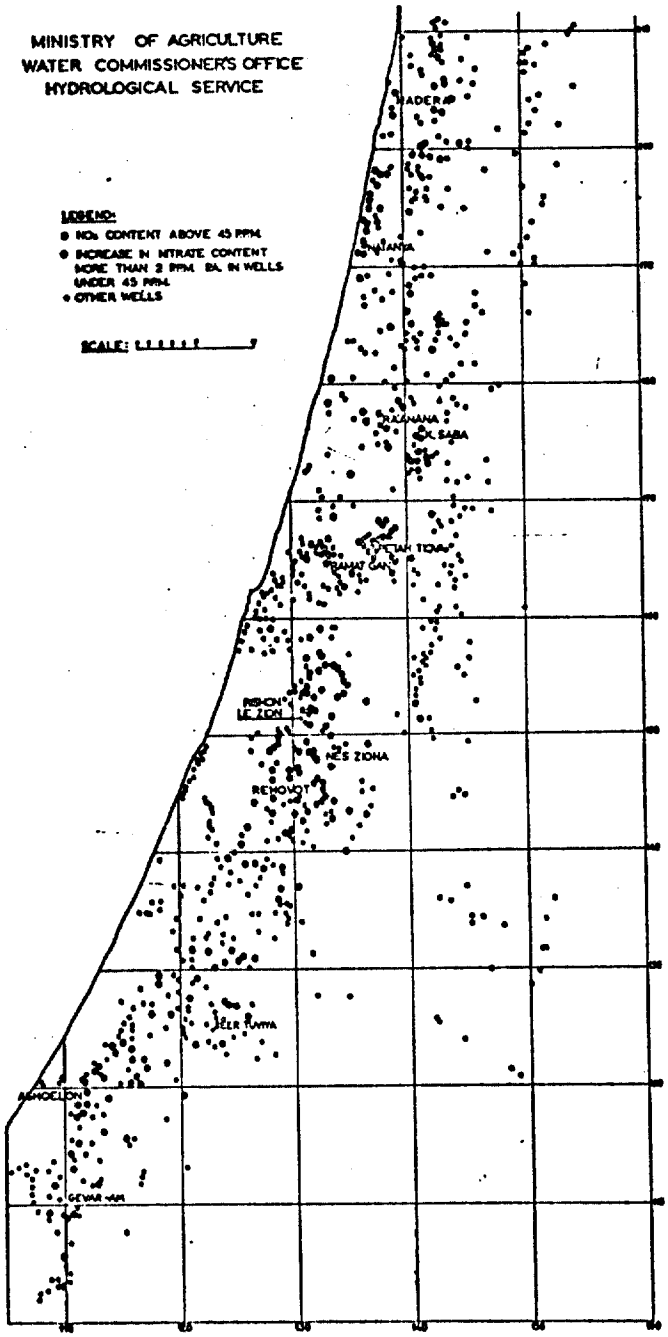

Figure 1

Nitrate radical (NO_3) content of pumped water in Central Israel as of 1968

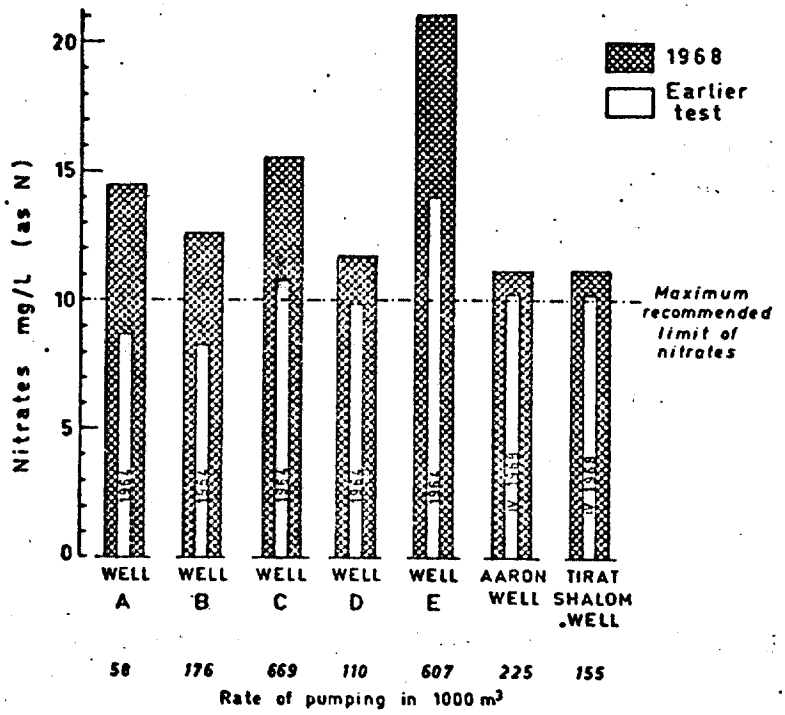

Figure 2
Concentration of nitrates (NO_3) in wells in Nes Ziona in 1968 in relation to earlier tests

NITRATE STANDARDS IN DRINKING WATER

Infant methemoglobinemia resulting from the consumption of water with high nitrate concentrations was first recognized clinically in 1945 (COMLY, 1945). Since then about 2,000 cases, including many fatal poisonings in infants resulting from ingestion of water containing nitrates, have been reported in various countries of the world.

This led the United States and later the World Health Organization to establish maximum permissible limits for nitrates in water used for drinking purposes at 45 mg/l of NO_3 (10 mg/l of N). However, there is as yet no consensus of opinion on the safety limits of nitrates in water. These limits vary in different countries from 0.5–228 mg/l of NO_3 (CABALLERO, 1950), most of them lying in the range of 20–100 mg/l NO_3 (U.S.P.H.S., 1962; RUMMEL, 1965).

Where nitrate limits in water are exceeded, it is generally recommended that infants up to the age of one year be supplied with low-nitrate water from other sources. These recommendations carry with them the tacit assumption that nitrates in water, irrespective of their concentration, are not harmful to children over one year of age or to adults.

In some areas where the water contains nitrate concentrations as high as 90 mg/l no clinical cases of the disease have been reported. This has led to pressure to relax the standard. On the other hand, clinical cases of the disease have more recently been reported in areas with less than 45 mg/l of NO_3 in the water (KNOTEK and SCHMIDT, 1964).

The existing standards are based on limited imperical evidence and leave many basic questions unanswered.

GENERAL ASPECTS OF METHEMOGLOBINEMIA

Methemoglobinemia can be caused by several chemicals such as nitrates, perchlorates, aminophenols, anilin, sulfonamides and others. Various endogenous forms of the disease are known. Nitrates do not directly convert hemoglobin to methemoglobin but can be converted to nitrites by intestinal microflora, with subsequent formation of methemoglobin. Hemoglobin (Hb) is the oxygen carrier of the blood. It is a protein of molecular weight 68,000, consisting of four identical sub-units, each containing a polypeptide chain (globin) and a heme group. Every heme contains at its center an atom of iron which, in the oxygenated form of Hb, oxyhemoglobin (HbO_2), is in the bivalent state (Fe^{2+} ferrous). Methemoglobin (MetHb) is the oxidized product of Hb in which the iron is in the trivalent state (ferric Fe^{3+}).

On transition from the ferrous to the ferric state the Hb loses its ability to combine with O_2. The exact molecular mechanism of the conversion of Hb to MetHb is still obscure.

Conversion of Hb to MetHb takes place all the time in the body, but the quantity of the latter is maintained at a low, steady-state level mainly by the action of an enzymatic system (JAFFE, 1964). Several enzymes with MetHb reduction capability have been purified, but the exact mechanism by which they operate *in vivo* has not yet been resolved. A direct, nonenzymatic reduction of methemoglobin is carried out by glutathion or ascorbic acid (LEMBERG and LEGGE, 1941). The normal concentration of methemoglobin is still a matter of dispute.

Methemoglobinemia constitutes a potential impairment of the proper supply

of oxygen to the tissues. For example, it was found that in trained subjects who underwent work tests, 10–20% MetHb resulted in impaired oxygenation of muscles (TEPPERMAN et al., 1946). This phenomenon is a result of both less hemoglobin and the greater affinity of the residual Hb for oxygen.

PATHOGENESIS OF METHEMOGLOBINEMIA

It is accepted by many workers that under normal circumstances less than 2% of the total hemoglobin exists as MetHb (FERRANT, 1946; GROSS, 1964). No external signs or symptoms are generally noted under 5%. The first signs of cyanosis can be seen between 5 and 10% (KNOTEK and SCHMIDT, 1964).

The presence of high concentrations of nitrates in water is the principal determinant of the occurrence of methemoglobinemia in infants; however, it is not the only one. Other factors important in the pathogenesis of the disease are:

Age: Most of the cases of nitrate methemoglobinemia occur in infants below one year of age. In a review of 146 cases of methemoglobinemia in Minnesota, 90% of them were found to have occurred by the age of eight weeks. The youngest case was seven days and the oldest five months (ROSENFIELD and HUSTON, 1950). SCHMIDT and KNOTEK (1966) reported on a survey carried out in Czechoslovakia, in which 52% of the infants, 0–3 months old, in a high-nitrate area had elevated methemoglobin levels; in the age group from 3–12 months old the percentage reached 13. There are also reports on elevated MetHb levels in older children and adults who had consumed water with high nitrate concentrations but showed no symptoms of the disease (TEERHAAG and EYER, 1958). In a Russian study (SUBBOTIN, 1961) of 800 children in day nurseries, it was found that 92.2% of them who ingested water which contained 20–40 mg NO_3/l had elevated MetHb levels. In 50% of the cases the level was higher than 5%. Previously it was found that 8 mg/l nitrate did not raise the methemoglobin level (SUBBOTIN, 1958).

Presence of bacteria: CORNBLATH and HARTMAN (1948) emphasized the important role of bacteria in the production of methemoglobinemia. They studied the gastrointestinal flora with regard to nitrate reduction, gastric acidity, age and the level of intestinal absorption of nitrate. It was found that all microorganisms isolated from the mouth and from the gastrointestinal tract were capable of reducing nitrate to nitrite and grew in media of pH 5–7.

The invading bacteria must adapt themselves to nitrite formation if they

were not previously in contact with nitrate. Consequently, it may take from four to five days after the first ingestion of nitrate until the full nitrate-reducing efficiency is reached. This may explain the commonly observed lag period of 1–3 weeks before the onset of the illness (TEERHAAG and EYER, 1958).

Gastric acidity: Examinations of gastric juice of infants who developed appreciable levels of MetHb revealed that the pH was usually higher than 4. It was found (MARRIOT et al., 1933) that the normal pH of infant gastric juice varied between 2–5, but with unspecific diarrhea, gastric pH increased and ranged between 4.6 and 6.5. MUCHA et al. (1965) examined the pH and bacterial flora of gastric juice from children. They found the gastric juice was sterile at pH < 4.6.

Gastrointestinal disturbances: DAUBNER (1962) has determined that all the members of the family Enterobacteriaceae are able to reduce nitrate to nitrite. Such organisms can gain access to the upper intestine during gastrointestinal disturbances (HORN, 1958). However, their ability to become established in the stomach is dependent on the pH, as mentioned in the preceding section. In the absence of nitrate-reducing bacteria in the stomach or upper intestine, most of the nitrate is probably absorbed as nitrate before reaching the colon in which the nitrate-reducing bacteria are normally found.

Type of powdered milk product: Studies in Czechoslovakia (KNOTEK and SCHMIDT, 1964; SCHMIDT and KNOTEK, 1966) indicated that the use of certain types of milk preparations has been suspected as the main cause for the development of methemoglobinemia in areas with high levels of nitrates in water. They reported on cases of methemoglobinemia in infants due to feeding with various brands of regular powdered milk which contained spores of *B. subtilis*, a nitrate-reducing bacteria; acidified milk powders which are often prepared by fermentation with *St. lactis* did not cause any disease. The acidified milk preparation can contain an antibiotic substance called nisin which inhibits the growth of nitrate-reducing bacteria. On the other hand, MUCHA et al. (1965) claimed that the main source of nitrate-reducing microorganisms could be eliminated by providing bacteria-free food to infants; however, under certain conditions, the ascent of bacteria from the colon to the duodenum and stomach cannot be prevented. *B. subtilis* spores are, however, not destroyed by normal milk pasteurization and drying processes. Little nitrate was found in the milk of cows drinking water with up to 800 mg NO_3/l (DAVISON, 1964). Many hold the opinion that mother's milk or cow's milk cannot be a cause of methemoglobinemia.

High fluid intake: Infants with an average fluid intake would ingest several times more nitrate per gram of hemoglobin than an adult due to their higher fluid intake per unit of body weight (GROSS, 1964). BURDON (1961), assuming that all nitrate is reduced to nitrite, sets the permissible level of nitrate for adults at 1,056 mg/l in England and 198 mg/l in the tropics; for infants the permissible levels would be 88 mg/l and 26 mg/l, respectively. His estimates are based on 13.2 mg NO_3/kg as the maximum daily amount which can be tolerated without giving rise to toxic symptoms. The governing factor would be the relative daily fluid intake which for infants would be 0.5 liter in England and 2.0 liters in the tropics.

Effect of nutrition: Food composition can also affect the severity of the illness. On the one hand, there are certain nutrients such as vitamin C that can cure or prevent methemoglobinemia. High vitamin C intake among infants in some areas may explain the scarcity of the disease even when waters rich in nitrates are consumed. On the other hand, certain vegetables such as spinach, rhubarb, etc. contain considerable amounts of nitrates. Several cases of nitrate poisoning in infants after eating spinach were reported (SINOIS and WODSAK, 1965). The nitrate content of selected food samples tested in Jerusalem are presented in Table 1 (SHUVAL and RAM, 1968). From this limited survey it can be seen for example that samples of spinach can contain as much as 1,330 ppm of nitrates. Such concentrations in food can become particularly toxic if exogenic bacterial activity converts the NO_3 to NO_2.

Fetal hemoglobin: Hemoglobin F is oxidized more readily to methemoglobin (BOI-DOKU and PICK, 1966). The fact that blood of newborn babies consists of more than 80% hemoglobin F might explain their increased tendency to develop methemoglobinemia.

Methemoglobin reduction: Methemoglobin reduction velocity in the presence of lactate or glucose is lower in cord blood erythrocytes than in adult blood. The lower methemoglobin reduction velocity in cord blood is explained by a temporary deficiency of DPNH—the methemoglobin reductase cofactor (ROSS and DESFORGES, 1959), or by low activity of the enzyme itself (BARTOS and DESFORGES, 1966). A positive correlation between DPNH diaphorase activity and methemoglobin reduction was shown in adult blood but not detected in cord blood (KANAZAWA et al., 1968).

Table 1. The concentration of nitrates [NO_3] in food samples in Jerusalem*

Type of Food	Concentration of NO_3 (ppm)
Butter	220
Onions, green	260
Orange drink	185
Tomato	140
Rhubarb	1050
Cabbage	600
Cauliflower	390
Strawberries	450
Spinach	1330
Artichoke	130
Spinach-potato (strained baby food)	400
Turnip	550
Asparagus	146
Cucumber	148
Carrot	500
Apple	380
Avocado	45
Potato	230
Tea	104
Coffee	540
Cocoa	100
Milk (from cows consuming water containing 90 mg/l NO_3)	17

* These analyses were carried out by Dr. E. Wiesenberg, the Institute for Control and Standardization of Pharmaceuticals, Ministry of Health, Jerusalem

RESEARCH PROGRAM IN ISRAEL

Our research activities concerned with the problem of the health effects of nitrates in drinking water were initiated in 1967 and are being carried out both in the field and in the laboratory.

Field Studies

The first question investigated was the possible health effects of medium to high levels of nitrates in drinking water (45–90 mg/l) on the infant populations exposed to such water.

Since only a few clinical cases of methemoglobinemia have been reported in the study areas, it was our intention to attempt to determine whether cases of chronic subclinical disease could be detected in such infant populations. This required a sensitive test for MetHb that had a high degree of precision capable of detecting small differences between normal and slightly raised levels of MetHb. It also had to be a micromethod simple enough to be used under field conditions.

Since blood samples in such surveys often have to be taken in communities distant from the laboratory, it is essential that the blood sample drawn be preserved for periods of up to 24 hours. MetHb is known to be unstable and it is generally recommended that blood be examined promptly (BODANSKY, 1951).

Previous reports have indicated the stability of MetHb in blood samples stored in buffers which contained a special detergent, even at room temperature (PASSANNANTE and GERARDE, 1966). In our initial testing of various methods of preservation of blood samples we found that with this method methemoglobin levels increased considerably on storage and that it was not reliable for the conditions of our study. Freezing of blood samples also proved unreliable.

The commonly accepted clinical methods are also not sufficiently accurate for this purpose (EVELYN and MALLOY, 1938) or call for the use of large volumes of blood, a requirement considered impractical for a survey of this kind (CRUZ, 1951). Therefore, an effort was made to develop a micromethod using samples of finger blood which would meet all the above-mentioned criteria.

The method developed in conjunction with Dr. E. Hegesh and his group at Kaplan Hospital requires only 0.2 ml of finger blood and is sensitive enough to detect 10 mg% of MetHb. Blood samples taken in the field are immediately hemolized in water and stored on ice. It has been shown that MetHb varies by no more than 10% of its initial concentration within 24 hours when treated by this method. Full details will be published elsewhere.

A field study of MetHb levels in infants up to one year of age has been initiated in two areas: Nes Ziona-Rehovot coastal plain area, having nitrate concentrations of between 45–90 mg/l; Jerusalem area, as a control, with a negligible nitrate content in the drinking water.

A questionnaire providing background information on milk and water consumption, nutrition and disease history is filled out for each infant tested. The

results of this survey are as yet incomplete and it is too early to be able to draw any conclusions.

Toxicology of Nitrites

In the laboratory phase of our studies we decided to concentrate on the acute and chronic effects of nitrites on experimental animals. We were particularly interested in investigating the possible physiological significance of low levels of methemoglobinemia such as might exist in cases of chronic subclinical intoxication. As mentioned previously, it is the nitrite form rather than the nitrate form that causes the formation of MetHb. *In vivo* conversion of NO_3 to NO_2 apparently is an essential step in the pathogenesis of the disease. Since we were not yet in a position to regulate or control the conversion of nitrates to nitrites in experimental animals, we felt that as a first step it would be best to work directly with nitrites. The need to establish permissible limits for nitrites in water has also been suggested (NICHOLS, 1965); however, nitrites usually occur in water in amounts too small to be of physiological importance.

Initially we studied lethal doses and the kinetics of MetHb formation in Hebrew University albino sabra rats. Lethal doses were found to be approximately 200 mg/kg. When a single sublethal dose of nitrite is administered to a rat by oral intubation, the MetHb level reaches a peak in about 45 minutes to one hour and returns to normal within about 3–4 hours, indicating the effectiveness of the MetHb reductase system (see Figure 3). We also found that the rats' body temperature is depressed as the MetHb levels increase and returns to normal as the MetHb disappears.

A preliminary chronic test of rats (initially weighing about 110 g) supplied with water containing 4.5 g/l of $NaNO_2$ (Group A) and 3.0 g/l of $NaNO_2$ (Group B) was carried out over a period of 56 days. The controls received no nitrites (Group C). The amount of water consumed daily was recorded and it was found during this chronic exposure study that rats showed some degree of rejection of water containing such high concentrations of NO_2.

On the basis of the concentration of nitrites in the water and average daily water intake, it was calculated that the nitrite dose of Group A ranged from 610–1,066 mg/kg/day while that of Group B ranged from 450–831 mg/kg/day. The MetHb blood levels were taken at various intervals during the study. Previous studies of rats given nitrites in drinking water throughout their life span did not reveal significant changes in MetHb levels. In pretests we also at first did not detect significantly elevated MetHb levels in blood samples taken during

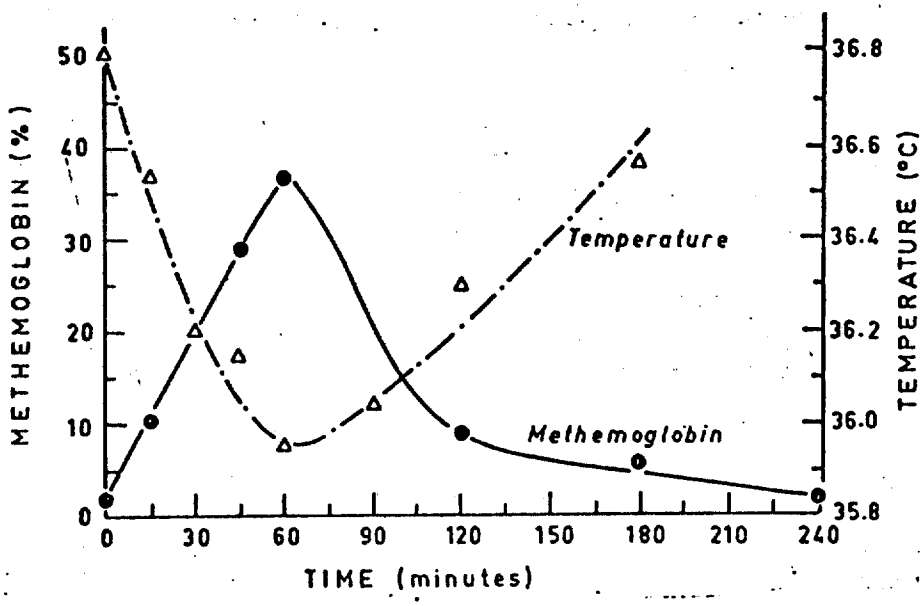

Figure 3
The effect of a single dose of 75 mg/kg $NaNO_2$ on methemoglobin development and body temperature in rats

normal laboratory working hours, despite the very high nitrite doses. However, it was decided to take blood samples at various times during the night on the assumption that the rats' water consumption would be greatest during those hours due to their nocturnal habits. Our measurements indicated that about 80% of the daily water intake is consumed during the night. We have previously shown that MetHb levels return to normal within a few hours after exposure to a dose of nitrites. It was thus hypothesized that rats would have low MetHb levels during the day, and higher levels at night. Our assumptions proved to be correct and we have shown that MetHb levels in most rats under study reach a peak during the night hours and might often return to normal during the day.

In Figure 4 it can be seen that the peak MetHb levels in Group A were about 20% while those for Group B were about 14%. Maximum MetHb values are those for blood samples taken at about 4:30 a.m. The lower MetHb figures represent samples drawn during various hours of the day. The MetHb of the controls, Group C, ranged between 1 and 2%.

In Figure 5 the body weights of the rats consuming nitrites in their water are given for the 56-day period. While the controls (Group C) increased in body

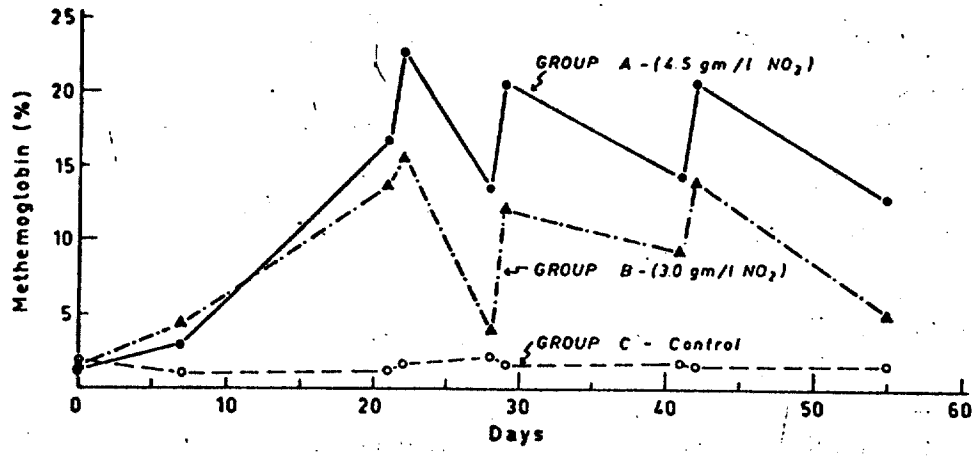

Figure 4
Methemoglobin levels in rats consuming nitrites in water

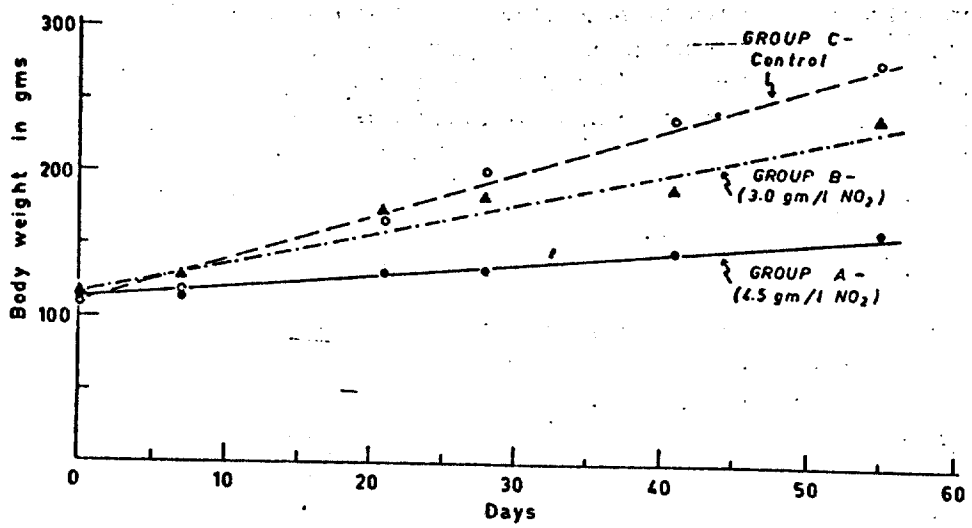

Figure 5
Body weight of rats consuming nitrites in water

weight from about 110 g at the beginning of the experiments to 270 g at the end, Group A increased to only 150 g and Group B to 235 g.

Further studies of the chronic effects of still lower nitrite levels on rats are being carried out in an effort to determine the possible long-term physiological impact of a chronic subclinical form of the disease.

Effects on Animal Behavior

Since the object of these experimental studies is to develop methods for evaluating the possible implications of chronic subclinical methemoglobinemia, it was felt that highly sensitive parameters should be studied. It was hypothesized that one of the effects of subclinical methemoglobinemia would be the reduction of the oxygen-carrying capacity of the blood system, which in turn would affect tissues particularly sensitive to reduced oxygen tension, such as the brain and muscles. This might lead to detectable behavioral changes in laboratory animals with induced chronic methemoglobinemia. A similar approach has been developed for the study of small doses of CO and found to be an effective toxicological tool (BEARD and WERTHEIM, 1967).

To study this possibility we carried out psychological experiments in a barrier activity box (LIEBLICH and GUTTMAN, 1965) using mice treated with nitrites. Preliminary experiments indicate the utility and sensitivity of this test. The treated animals showed significantly reduced activity patterns compared to the controls. Further experiments along these lines are being carried out. Following a similar line of approach, laboratory animals with implanted electrodes will be exposed to nitrites in their water for extended periods and checked regularly for changes in EEG patterns.

The Effects of Nitrites on Newborn and Suckling Rats

As noted previously, present nitrate standards for drinking water imply that the restrictions apply only to infants and that there is no need to limit the nitrate intake of adults. However, Donahoe reported what he considered to be a case of methemoglobinemia in a nursing infant who received no water. In this case the mother was consuming water high in nitrates and it was hypothesized that the nitrates may have been transferred through the mothers' milk (DONAHOE, 1949).

Such a possibility has been considered unlikely by other researchers. In order to study the possible transfer of nitrites in milk as well as possible direct transplacental transfer of nitrites to the fetus during pregnancy, we supplied pregnant rats with water containing 3.0 mg/l of $NaNO_2$ during the last week of pregnancy and during a period of three weeks after giving birth. In another test the same nitrite water was supplied to mother rats for a period of three weeks immediately after the birth of the litter.

In the former experiment it was found that the newborn rats showed MetHb levels of 5–10% at birth, while the mothers receiving nitrites in their water had

MetHb levels of 45%. However, after a few days the suckling rats showed normal MetHb levels while the mothers still had high levels. The birth weights and litter size of the controls and the experimental groups were equal.

In Figure 6 the body weights of the suckling rats of mothers consuming nitrites are compared with those of the controls. While the controls increased in weight from about 5 g at birth to 32 g after three weeks, the weight of the experimental group increased only to 22 g. Even more indicative of the possible negative effects of the nitrites supplied to the mothers before and after birth was the fact that of the 45 newborn rats in the experimental group only two remained alive at the end of the experimental period as against 17 in the control group of an original 38. Sixteen rats from each group were sacrificed at different times during the experiment for MetHb determination.

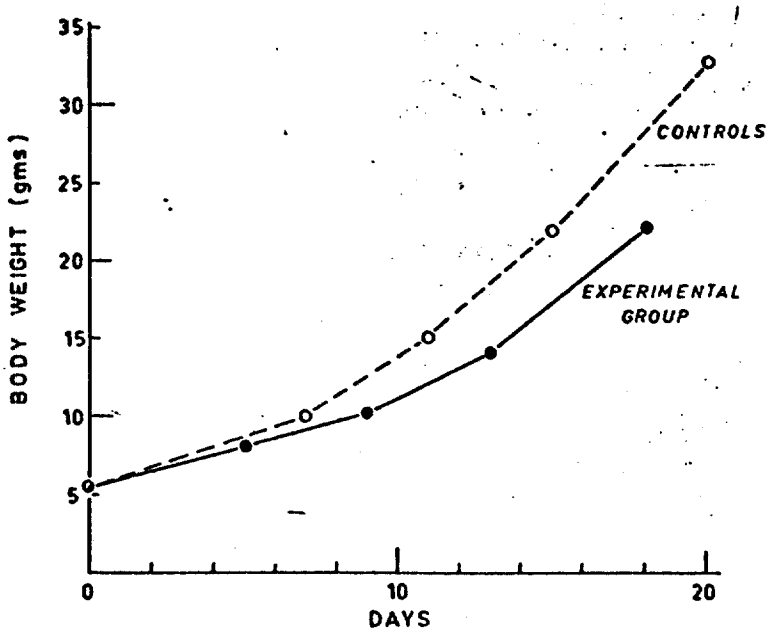

Figure 6
Body weight of suckling rats from mothers consuming nitrites in water before and after birth

In the latter experiment mother rats were given water containing 3.0 mg/l of $NaNO_2$ only after the birth of the litter. In this case MetHb developed in the mothers but not in the suckling rats, whose body weights paralleled the controls during the three-week period of the test. The results of these two experiments

suggest that there is transplacental transfer of nitrites to the fetus, leading to the development of methemoglobinemia in the fetus, which was detected in the newborn rats on the day of birth. These same newborn rats showed very poor development and a high death rate, although their MetHb levels returned to normal a few days after birth. Suckling rats whose mothers were exposed to nitrite in water only after birth showed no detrimental effects, thus indicating that the nitrites are not transferred in the milk. A possible inference from these very preliminary experiments is that there is some form of irreversible damage to the fetuses of mother rats exposed to nitrites during pregnancy. Increased abortion rates in cows exposed to nitrites in feed have been reported (MURHRER et al., 1956).

Surprisingly, it is only in recent years that toxicologists have begun to pay serious attention to possible fetal damage. Only since 1966 has the United States Food and Drug Administration required that fetal toxicity tests be included in all drug evaluation studies (GOLDENTHAL, 1966). Our preliminary findings concerning possible fetal damage in cases where mothers are exposed to methemoglobinemia-causing agents point to the urgent need for a most careful evaluation of this phenomenon.

CONCLUSIONS

It has been shown that the problem of nitrates in drinking water is one of increasing concern in many areas. This is already the case in Israel. There is no certainty that the current level of knowledge of the pathogenesis of nitrate methemoglobinemia or of the toxic effects of nitrates on adults and infants is adequate to support the present maximum permissible concentration of nitrates recommended for drinking water.

What is the public health significance of chronic subclinical methemoglobinemia which has been reported to exist in some areas? What is the effect on the fetus when mothers ingest methemoglobinemia-causing agents? The research program described may in time help to answer some of the basic questions left open on this subject.

ACKNOWLEDGEMENTS

Mr. K. Behroozi and Mrs. S. Cohen participated actively in both the laboratory and field phases of the study and their valuable contribution to the

success of this work is appreciated. The authors are also indebted to Professor G. Izak for his assistance in launching this study, and to Prof. I. Nir for his helpful advice.

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CLINICAL AND CASE REPORT

(Practitioners and others are invited to contribute to this department reports of unusual and interesting cases which may be helpful to others in the profession.)

POTASSIUM NITRATE POISONING IN CHICKENS, WITH A NOTE ON ITS TOXICITY

By John E. Guberlet

Parasitologist, Veterinary Department, Oklahoma Agricultural Experiment Station, Stillwater, Oklahoma

ON the morning of June 7, 1922, the writer had occasion to observe a flock of chickens suffering from poisoning, the symptoms of which were first observed by the owner on the preceding evening. Upon arrival several birds were found dead and 35 or 40 others were seriously affected, of which some were prostrate and others comatose. Those that were in a serious condition had a severe diarrhea with marked diuresis. The droppings were watery and contained large amounts of urate materials. The combs and wattles ranged in color from dark red to purple, or even black. Respiration was disturbed. The sick birds displayed great thirst and, if at all able to stand, were drinking water or trying to do so. Almost the entire flock of 225 birds showed discolored combs and displayed a great desire for water. There was a marked diarrhea and diuresis in the whole flock. Most of the birds showed no other ill effects and were taking food.

After inquiring of the poultryman as to what had been fed to the birds on the preceding day it was learned that they had been given a dose of Epsom salts (magnesium sulphate). The "salts," according to the poultryman, were given at the rate of three-fourths pound per 100 birds. It had been dissolved in water which was used in mixing bran mash. This was taken fairly readily by the birds. The owner insisted that the "salts" could not be responsible for the trouble, as he had treated the flock similarly on former occasions from the same box of salt, but "they didn't eat it as readily before."

Autopsy.—Skin very dark, subcutaneous blood vessels congested, flesh dark pink to red, blood almost inky black. The

lungs in most cases were extremely free from blood, while in some of the others they were hepatized. Liver severely congested and almost black. The veins of the liver were much enlarged and gorged with blood clots, while the arteries were extremely contracted. The kidneys were decidedly enlarged and highly inflamed. Crop and proventriculus severely congested; congestion beneath the lining of gizzard; enteritis and congestion throughout the entire length of the intestine. The mesenteric blood vessels were gorged with blood.

Further inquiry as to where the salt was procured revealed the fact that this was a consignment which had been purchased "at a bargain." This particular lot had been wet at the drug store and had been on hand for some time. The poultryman bought it for Epsom salts. A sample was taken to the laboratory for examination where it was discovered that the "salts" were not magnesium sulphate, but potassium nitrate (saltpeter).

Later conversation with the poultryman disclosed the information that at former administrations the salt was dissolved in the drinking water and given in that way instead of in a mash. On those occasions the birds would not drink much of the water. That accounts for the fact that no trouble was experienced at former treatments.

About 25 of the birds died during the days of June 7, 8 and 9. Some of the birds that were nearly prostrate had recovered by the morning of June 8, except that they were very weak. Some of the affected individuals lingered for two or three days before they died, and death in those cases was due to acute enteritis and nephritis. The entire flock apparently suffered to some extent from enteritis and nephritis for several days. This condition, however, did not interfere to any great extent with the activity of the birds, except that they displayed an unusual thirst and showed a decrease in appetite. Egg production was reduced.

Little is known concerning the toxic effects of various drugs upon fowls. The most important work along this line is that of Gallagher (1919), who conducted a number of very valuable experiments on chickens to determine the toxicity of some of the more important and commonly used medicinal agents and of poisonous substances to which fowls not infrequently have access. Kaupp (1917) and others give the dosage for nearly all of the

common drugs used in the treatment of fowl diseases but say little concerning their toxicity.

Saltpeter poisoning in fowls is not of uncommon occurrence, although not on as large a scale as shown in this instance. This salt is an important constituent of certain fertilizers, and chickens have been poisoned from that source. Many people often use this salt in the preservation and curing of meats, and poisoning from this source some times accidently occurs in fowls. It has been taken as a purgative by persons, being mistaken for magnesium sulphate (Holland, 1917, p. 218).

Potassium nitrate was formerly used rather extensively in medicine, as a diuretic, diaphoretic and febrifuge; also in acute cases of rheumatism. At present its use as a medicament is very limited, it being used principally as a local treatment.

Some experiments were conducted by the writer to determine what would be a toxic and a lethal dose of potassium nitrate for fowls. This was done because of the observance of such poisoning in a flock and on account of the interest this incident has incurred. Twenty birds, weighing 3 to 4½ pounds, were used in these experiments. Doses ranging from 1 to 90 grains were given by mouth and the results noted.

Very small doses, 1 to 2 grains, produced diuretic action with diarrhea in two to three hours provided water was near the birds constantly so that they could drink of it at any moment. This was especially true if the saltpeter was given in solution. In doses of 5 to 10 grains the same results were noted, except that the diarrhea and diuresis were more severe. Doses of 45 grains produced diuretic action in 40 to 45 minutes when the birds had constant access to water. Under these conditions doses containing 45 to 50 grains were toxic, and it required 80 to 90 grains for a lethal dose for fowls weighing 4 to 4½ pounds.

Chickens under range conditions, such as those in which the poisoning occurred, go considerable distances from their supply of water, and consequently can not drink at any moment they feel the desire. Therefore it seemed desirable that other experiments be conducted in which the birds would be given water about as often as they would drink under range conditions. Under this arrangement small doses produced no apparent external symptoms. Doses of 15 to 20 grains produced diarrhea and a diuretic condition after 2 or 3 hours, and then as a rule only after the birds had taken water. Fowls taking 25 to 30 grains under these conditions showed toxic symptoms, while 60 grains was a lethal dose for a 3½ pound hen and 65 to 70 grains were fatal to birds weighing 4 to 4½ pounds.

Toxic doses of potassium nitrate produce symptoms of gastroenteritis, nephritis, muscular weakness, slow, weak pulse, depression of the circulation, disturbed respiration and slight hemolysis of the blood. Along with these symptoms occurs the congestion of the peripheral blood vessels which results in the darkness of the skin and discolored comb. This is followed by a subnormal temperature, paralysis, collapse, and coma, followed by death. In nontoxic doses it causes diuresis and diarrhea. The toxic effect of the salt is lessened by keeping the system flushed with water. Therefore in the experiments the birds which consumed large quantities of water threw off from their systems a large amount of the poisonous substance. Birds which had been previously fed on a highly concentrated protein diet, whose excretory organs had already been taxed, suffered more acutely and showed more prominent symptoms than birds previously fed on a low protein diet.

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Dr. A. K. Merriman, formerly of Latham, Ill., is now located at Sullivan, Ill., where he has purchased the practice of Dr. W. C. Bateman.

Dr. Joseph Hawkins, the oldest practitioner in the city of Detroit, has gone to California, where he will spend the winter with relatives.

Dr. J. G. Jervis, of Milner, B. C., has been appointed lecturer in veterinary science at the University of British Columbia, to succeed Dr. T. H. Jagger, who resigned.

Dr. A. W. Lehman has moved from Duncans, Vancouver Island, to Penticton, B. C.

Proc Soc Exptl Biol. Med V.4 09 (1962) 800

Effect of Energy Level and Nitrate on Hepatic Vitamin A and Performance of Fattening Steers.* (27181)

W. H. Hale, Farris Hubbert, Jr., and R. E. Taylor
(Introduced by A. R. Kemmerer)
Department of Animal Science, University of Arizona, Tucson

While the necessity of a dietary source of Vit. A for fattening steers is well recognized, little information is available on ration factors that may affect the requirement. Symptoms of Vit. A deficiency have been observed with ruminants grazing high-nitrate forage during drought seasons(1). Recent field observations suggest that Vit. A deficiency in feedlot steers is somewhat more common than previously believed. The present day ration for fattening steers is considerably higher in grain than formerly and the roughages are often harvested from fields which have been highly fertilized with nitrogen. This fertilization practice may result in a higher level of forage nitrate(2). Studies by O'Dell, *et al.* (3) have shown that ration nitrate increased liver Vit. A losses in rats. Nitrate is reduced to nitrite by rumen fermentation(4).

This study was designed to determine the effect of ration energy level (as estimated by total digestible nutrients, "TDN") and nitrate on liver Vit. A depletion in fattening steers fed a low carotene ration. Performance of the animals also was studied.

Procedure. Twelve steers were allotted to a 2×2 factorial experiment in which the factors were energy level of the ration (54.3 and 71.3% TDN) and KNO_3 addition to the ration (none and 1% KNO_3). The steers were individually penned and fed. The composition of the rations is given in Table I. All rations contained 14% crude protein. Milo grain was used at a high level to formulate the high TDN ration and was replaced with cottonseed hulls in the low TDN ration. The steers were fed *ad libitum*. Liver biopsies were taken at the initiation of experiment and at 28-day intervals by the method of Erwin, *et al.*(5). Allotment of the animals was on the basis of initial liver Vit. A values. Liver Vit. A was determined by the method of Gallup and Hoefer(6).

TABLE I. Experimental Rations.

	54.3% TDN	71.3% TDN
Cottonseed hulls	55	10
Molasses	7	7
Dry rolled milo	11	63
Cottonseed meal	25	18
Salt	1	1
Dicalcium phosphate	.5	—
Ground limestone	.5	1

Results. Feedlot performance of the animals and liver Vit. A losses for the 56-day experiment are presented in Table II. While there was no treatment effect upon average daily gains, there was a significant interaction ($P<0.05$) between TDN level and nitrate addition. This was due to the reduction in daily gains when nitrate was fed with the low TDN ration (3.18 lb. *vs* 2.01 lb) and an increase in gains due to nitrate with the high TDN ration (2.02 lb. *vs* 2.29 lb.). Both high TDN and nitrate reduced daily feed intake. The TDN effect was significant at the 5% confidence level and the nitrate addition significant at the 1% confidence level.

Initial liver values had a range of 24.9 to 59.7 μg of Vit. A/g of wet liver. These values are considered within normal range. Table III presents the liver Vit. A loss for each of the 4 treatments at the 28- and 56-day periods. This Table shows the effect of nitrate on the 2 TDN levels as well as the TDN effect independent of the nitrate addition. The lower liver Vit. A loss during the second 28 days can be explained on the basis that the loss of Vit. A at any time during dietary deprivation is proportional to the magnitude of the reserves remaining at that time(7). The 56-day effects are combined for statistical analysis and presented in Table II. The high TDN ration caused a significantly ($P<0.05$) larger depletion of liver Vit. A than did the low TDN ration. The same trend occurred with the nitrate addi-

* Arizona Agricultural Experiment Station Technical Paper No. 687.

TABLE II. Effect of Ration TDN Level and Nitrate on Liver Vit. A Depletion, Performance, and Feed Intake of Fattening Steers (56 Days).

	TDN level		Nitrate addition	
	54.3%	71.3%	No KNO_3	KNO_3
Avg initial wt, lb	699.4	716.7	725.4	690.7
Avg daily gain, lb	2.60	2.16	2.60	2.15
Avg daily feed, lb	26.60*	19.69*	25.14†	21.16†
Feed/lb gain, lb	10.50	9.19	9.75	9.94
Initial liver Vit. A, μg/g wet liver	35.4	39.7	34.3	40.8
Final liver A value, μg/g wet liver	7.9	4.8	6.8	5.9
Liver Vit. A loss, %	78.6*	88.0*	80.7	86.0

* Differences due to TDN level, significant $P < 0.05$.
† Differences due to nitrate addition, significant $P < 0.01$.

tion; however, the difference was not significant. Fifty-six-day liver values ranged from 1.6 to 14.4 μg Vit. A/g of wet liver. Liver Vit. A losses appeared to be negatively correlated with gains.

Discussion. These results indicate that a high TDN beef cattle fattening ration (71.3% TDN) in which milo grain supplied the high TDN component caused a more rapid depletion of liver Vit. A stores than a low TDN (54.3% TDN) ration. Thus, higher dietary Vit. A requirements would be expected with high TDN rations. This finding, no doubt, aids in explaining the frequent occurrence of Vit. A deficiency in steers fed high TDN rations.

TABLE III. Effect of Ration TDN Level and Nitrate Addition on Liver Vit. A Loss in Fattening Steers (3 Steers/Treatment).

	Treatments			
	54.3% TDN	54.3% TDN + 1% KNO_3	71.3% TDN	71.3% TDN + 1% KNO_3
28-day liver Vit. A loss, %	45.3	58.4	64.5	65.2
56-day liver Vit. A loss, %	74.7	82.5	86.6	89.4

The results show that the ration nitrate level used in this experiment reduced feed intake but that average daily gain was little affected when the ration contained high levels of TDN. With high TDN rations the reduction of nitrate to ammonia by rumen microorganisms is probably complete but with high-roughage rations a large portion of the nitrate may be absorbed as nitrite. Forages containing more than 1.5% KNO_3 are generally considered toxic for ruminants(8). Information from this study suggests that even lower levels have an effect upon performance. The nitrate effect on liver Vit. A depletion was greater for the low TDN ration.

Summary. (1) With fattening steers, a high TDN ration resulted in a significantly larger liver Vit. A depletion than did a low TDN ration. The addition of 1% KNO_3 reduced liver Vit. A stores but the reduction was not significant. (2) Neither TDN level nor nitrate addition had a significant effect on gain, but there was a significant interaction between TDN level and nitrate addition. (3) Feed intake was significantly affected by both TDN level and nitrate addition.

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The Effect of the Nitrite Ion on Intact Human Erythrocytes

By John D. Harley and Helen Robin

THE CHANGES which result when intact erythrocytes are exposed to the nitrite ion have been studied for almost a century.[1-6] Such exposure has been found to cause the oxidation of oxyhemoglobin to methemoglobin,[2-6] and of reduced glutathione (GSH) to oxidized glutathione (GSSG).[3,6] In addition, erythrocytes treated with nitrite have been widely used to investigate the factors involved in the reduction of methemoglobin.

Despite the breadth of these investigations, certain inconsistencies are still apparent. The results of experiments with erythrocytes from various species have been interpreted to indicate that GSH protects against enzyme inactivation,[7] spontaneous hemolysis,[8] choleglobin formation[9] and Heinz body formation.[10] Nitrite-induced GSH depletion might therefore be expected to lead to these changes in vitro, and to excessive hemolysis in vivo. However, the rate of spontaneous hemolysis has been found to be unaffected by the addition of nitrite,[6] the evidence as to Heinz body formation is conflicting,[4,5] and erythrocyte survival in normal rats has been shown to be unchanged by the administration of nitrite.[11]

Previous investigations[3,6] as to the effect of nitrite on the GSH content of intact erythrocytes have been made in the absence of glucose or other source of cellular energy. The present work was designed to study the mode of action of the nitrite ion on intact human erythrocytes, both in the presence and absence of adequate substrate and of a normal pentose phosphate pathway.

Materials and Methods

Fresh, heparinized, venous blood from normal and glucose-6-phosphate dehydrogenase (G-6-PD)-deficient, adult, Caucasian males was used as a source of erythrocytes. The G-6-PD activity in the enzyme-deficient subjects, assayed by Zinkham's modification[12] of the method of Glaser and Brown,[13] was less than 5 units per 100 ml. of erythrocytes. The blood was centrifuged for 10 minutes at 2100 g and the supernatant plasma and the buffy coat discarded. The erythrocytes were then washed three times with 30 volumes of the appropriate buffer, and resuspended to a packed cell volume of approximately 50 in this buffer.

Depending on the substrate requirements, Krebs-Ringer phosphate buffer, pH 7.4, isotonic glucose-buffer or isotonic lactate-buffer was used. The latter buffers were made up of Krebs-Ringer phosphate buffer, pH 7.4, in which isotonicity had been re-established after the addition of glucose or lactate to a final concentration of 12 mM per liter. In each experiment, the erythrocytes were washed with the appropriate buffer, which was then used throughout.

Test and control incubation mixtures were prepared as described previously,[14] with the following exceptions; that sodium nitrite was used as the test substance, that the

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buffers described above were used, that larger volumes of the mixtures were prepared, and that the incubation flasks were only shaken at hourly intervals. The initial concentration of heme in the incubation mixtures was always between 0.45 and 0.50 mM per liter. As required, samples were removed from the test and control mixtures and the following determinations made.

Concentrations of methemoglobin, oxyhemoglobin and "intact" hemoglobin (methemoglobin plus oxyhemoglobin) were determined and expressed as previously described,[14] with the variation that the erythrocytes were washed three times with 30 volumes of Krebs-Ringer phosphate buffer, pH 7.4, before being hemolyzed.

The GSH content was determined by the method of Grunert and Phillips,[15] with the precautions recommended by Flanagan et al.[16] At the commencement, and as required during the period of incubation, 15 ml. samples were taken from the test and control incubation mixtures, centrifuged, and the supernatant fluid aspirated. The cells were then washed four times with 30 volumes of Krebs-Ringer phosphate buffer, pH 7.4, hemolyzed by the addition of distilled water, and the GSH content determined as described above. The decrease in the GSH content of the test and control incubation mixtures was expressed as a per cent of the GSH content of the control incubation mixture at the commencement of the period of incubation.

The G-6-PD activity was determined by Zinkham's modification[12] of the method of Glaser and Brown.[13] As required, 10 ml. samples were removed from the test and control incubation mixtures, and the supernatant fluid aspirated after centrifugation at 4 C. The cells were then washed three times with 30 volumes of cold isotonic saline, hemolyzed, and assayed for G-6-PD activity. Other than centrifugation as recommended by Zinkham,[12] no precautions for the complete removal of stromata from hemolysates were employed. The G-6-PD activity in the test mixture was expressed as a per cent of the G-6-PD activity in a sample simultaneously assayed from the control incubation mixture.

The fragility to hypotonic saline was determined as described previously.[10] The per cent spontaneous hemolysis was calculated by expressing the optical density at wave length 540 mμ of the supernatant fluid as a per cent of the optical density at wave length 540 mμ of the hemolyzed whole sample. Erythrocytes were stained and examined for the presence of Heinz bodies as already described.[10]

Results

Preliminary experiments showed that the technics used for washing erythrocytes after incubation with nitrite were adequate, no significant difference in results being produced by further washing. Of the various determinations, the GSH content was found to be most sensitive to the presence of residual traces of nitrite; an experiment illustrating this sensitivity will be presented in the course of the results.

Effect of Brief Incubation with Nitrite

Methemoglobin formation and GSH depletion: Normal erythrocytes suspended in glucose-buffer were incubated for 4 hours with nitrite, in molar ratios to heme ranging from 64:1 to 1:4. At intervals, the degrees of methemoglobin formation and of GSH depletion were measured. From figure 1 it may be seen that increasing concentrations of nitrite caused increasing degrees of methemoglobin formation, culminating in virtually complete conversion of oxyhemoglobin to methemoglobin with a molar ratio of nitrite to heme of 16:1. In addition, methemoglobin production was found to be almost maximal after 15 minutes with the higher concentrations, but increased slowly over the succeeding period of incubation with the lower concentrations of

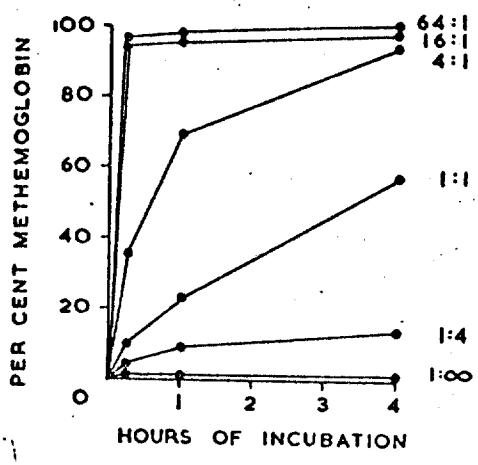

Fig. 1.—The degrees of methemoglobin formation in normal erythrocytes, suspended in glucose-buffer and incubated for 4 hours with varying concentrations of nitrite. The ratios opposite each curve represent the initial molar ratios of nitrite:heme.

nitrite. After 4 hours, the amount of methemoglobin formed by each lower concentration of nitrite was considerably less than would be expected to result from a stoichiometric reaction between nitrite and heme.

Despite the high degrees of methemoglobin formation, no significant decrease was found in the GSH content of either test or control incubation mixtures, even after 4 hours incubation with nitrite in a molar ratio to heme of 64:1.

To investigate the effect of impaired activity of the pentose phosphate pathway, normal and G-6-PD-deficient erythrocytes were incubated for 1 hour, under varying experimental conditions, with different concentrations of nitrite (fig. 2). From figure 2A it may be seen again that increasing concentrations of nitrite caused increasing degrees of methemoglobin formation, without GSH depletion, in normal cells with glucose as substrate. In the absence of substrate, however, increasing concentrations of nitrite were found to produce increasing degrees of GSH depletion (fig. 2B). The addition of lactate failed to prevent loss of GSH in erythrocytes exposed to nitrite (fig. 2C). Lastly, figure 2D shows that nitrite caused depletion of GSH in G-6-PD-deficient erythrocytes, even in the presence of glucose as substrate. Despite this varying effect on GSH depletion, no striking differences were observed between the degrees of methemoglobin formation produced by each concentration of nitrite under these four experimental conditions (fig. 2).

Even in the absence of nitrite, it may be seen from figures 2B, 2C and 2D that some loss of GSH occurred under circumstances which led to impaired activity of the pentose phosphate pathway. To investigate the effect of time of incubation, these experiments were repeated, with the modification that two concentrations of nitrite were used over a period of 4 hours (table 1). Firstly, it was observed that nitrite in high concentration caused rapid loss of GSH, with more than 85 per cent depletion after 15 minutes incubation. Secondly, in the absence of nitrite, the GSH concentration decreased progressively in normal cells incubated without glucose, but remained stable after an initial fall in G-6-PD-deficient cells with glucose as substrate. Thirdly, nitrite in low concentration appeared to exert a continuing effect in accelerating the proc-

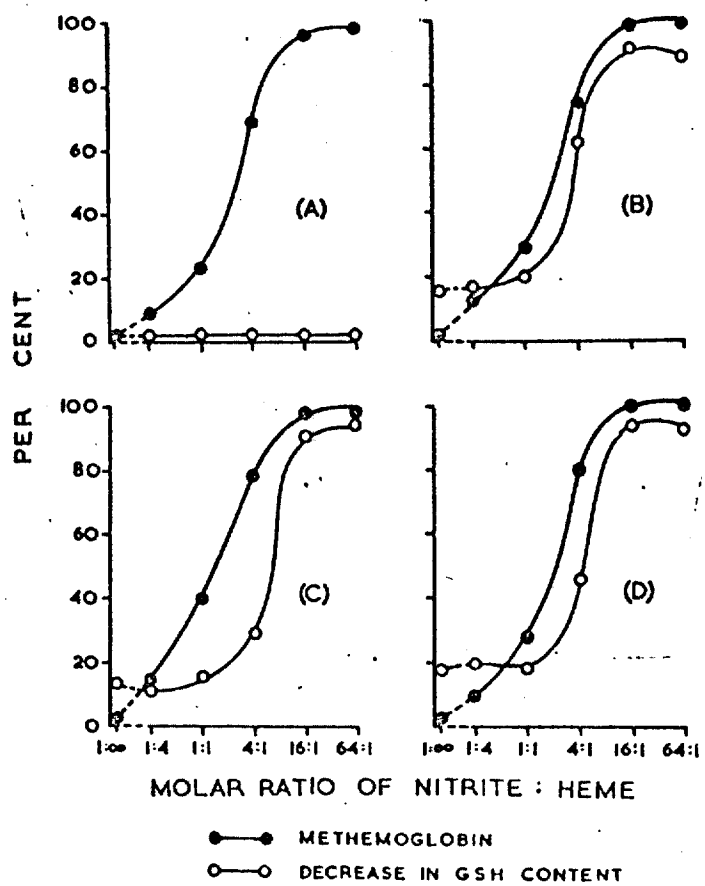

Fig. 2.—Results of four separate experiments as to the degrees of methemoglobin formation and of GSH depletion in erythrocytes incubated for 1 hour with varying concentrations of nitrite. (A) Normal cells, glucose substrate. (B) Normal cells, no substrate. (C) Normal cells, lactate substrate. (D) G-6-PD-deficient cells, glucose substrate.

ess of GSH depletion. Lastly, the degree of methemoglobin formation in the presence of nitrite consistently exceeded the degree of GSH depletion, even though loss of GSH occurred without accumulation of methemoglobin in the absence of nitrite.

It appeared from these various experiments, therefore, that nitrite oxidizes oxyhemoglobin in preference to GSH, which is depleted by nitrite only in situations which lead to impaired activity of the pentose phosphate pathway.

Effect of washing on the determined degree of GSH depletion in erythrocytes incubated with nitrite: The results of early experiments, with inadequate washing of erythrocytes after incubation with nitrite, had suggested that high degrees of GSH depletion may result from exposure to nitrite, even in the presence of glucose. To determine the effect of washing, the GSH content of erythrocytes incubated in glucose-buffer with a high concentration of nitrite was measured after from none to six washes with 30 volumes of Krebs-Ringer phosphate buffer. From table 2 it may be seen that evidence of significant

Table 1.—*Effect of Nitrite on the Degrees of GSH Depletion and of Methemoglobin Formation in Normal and G-6-PD-deficient Erythrocytes Incubated for 4 Hours in the Presence and Absence of Glucose or Lactate*

Erythrocytes	Substrate	Molar Ratio of Nitrite:Heme	GSH Depletion Per Cent			Methemoglobin Per Cent		
			15 min.	1 hr.	4 hr.	15 min.	1 hr.	4 hr.
Normal	—	64:1	92	94	100	100	100	100
		4:1	*	43	90	*	85	100
		1:∞	*	15	39	*	1	1
Normal	lactate	64:1	88	89	100	100	100	100
		4:1	*	25	78	*	83	96
		1:∞	*	19	45	*	1	1
G-6-PD-deficient	glucose	64:1	88	90	100	100	100	100
		4:1	*	48	86	*	80	100
		1:∞	*	19	20	*	1	1

*Not determined.

Table 2.—*Effect of Varying Numbers of Washes with 30 Volumes of Krebs-Ringer Phosphate Buffer on the Determined Degree of GSH Depletion in Erythrocytes Incubated for 1 Hour in Glucose-Buffer, with Nitrite in a Molar Ratio to Heme of 64:1*

No. of Washes	Determined GSH Depletion Per Cent
0	98
1	87
2	34
3	0
4	0
5	0
6	0

degrees of GSH depletion was observed with less than three, but not with three or more washes. These results were taken as evidence that residual nitrite resulting from inadequate washing may either oxidize GSH directly in hemolysates, or else interfere with the nitroprusside reaction for GSH. In either event, adequate washing would appear essential in any investigation as to the effect of the nitrite ion on intact erythrocytes.

Effect of nitrite on the G-6-PD activity of erythrocytes incubated in glucose-buffer: To investigate the effect of nitrite on one representative -SH enzyme, the G-6-PD activity of normal erythrocytes suspended in glucose-buffer was assayed at intervals during periods of incubation in the presence and absence of nitrite. Over a 4-hour incubation, slight decay in enzyme activity was found in the absence of nitrite, and no increase in this rate of decay was produced by nitrite in molar ratios to heme of 4:1 or less. With higher concentrations of nitrite, however, some evidence of an increased rate of G-6-PD inactivation was obtained. For example, in 10 experiments involving incubation of erythrocytes from normal subjects for 1 hour with nitrite in a molar ratio to heme of 16:1, the mean G-6-PD activity of the test mixtures was found to be 92 per cent of the mean activity of the control mixtures, with a range of 82 to 100

Table 3.—*Effect of Various Concentrations of Nitrite on Heinz Body Production, "Intact" Hemoglobin Destruction, and Osmotic Fragility in Normal Erythrocytes Incubated for 24 Hours, Both with and without Glucose as Substrate*

	No Substrate			Glucose Substrate		
Molar Ratio of Nitrite: Heme	Heinz body production	"Intact" hemoglobin destruction per cent	Hemolysis in 0.5 per cent saline per cent	Heinz body production	"Intact" hemoglobin destruction per cent	Hemolysis in 0.5 per cent saline per cent
64:1	+++	12	36	++	5	10
16:1	++	6	30	+	3	4
4:1	+	3	16	—	0	1
1:1	—	0	10	—	0	1
1:4	—	0	7	—	0	1
1:∞	—	0	6	—	0	1

The degree of Heinz body formation was assessed arbitrarily.

per cent and a standard deviation of 6 per cent. Five experiments, in which normal erythrocytes were incubated for 4 hours with nitrite in a molar ratio to heme of 64:1, yielded G-6-PD activities in the test mixtures of 68, 77, 83, 88 and 100 per cent of those simultaneously obtained in the control incubation mixtures.

Osmotic fragility and Heinz body studies: Normal erythrocytes were incubated for 4 hours in glucose-buffer with nitrite, in molar ratios to heme ranging from 64:1 to 1:4. Negligible degrees of hemolysis in 0.5 per cent saline, "intact" hemoglobin destruction or Heinz body production were observed.

This experiment was next repeated, using cells suspended in Krebs-Ringer buffer without added glucose. Again, insignificant hemolysis in 0.5 per cent saline was present after 4 hours incubation. However, quite appreciable degrees of Heinz body formation were found with the higher concentrations of nitrite. Thus, with molar ratios of nitrite to heme of 64:1 and 16:1, at least one small but clearly defined Heinz body was found in almost every cell, together with slight but appreciable "intact" hemoglobin destruction in hemolysates. These changes were not observed with lower concentrations of nitrite.

Effect of Prolonged Incubation with Nitrite

Studies were conducted to investigate the gross changes in erythrocytes exposed for long periods, under aseptic conditions, to high concentrations of the nitrite ion. In these prolonged experiments, the flasks were loosely plugged with cotton wool and shaken only at 12-hour intervals.

Initially, normal erythrocytes suspended in Krebs-Ringer buffer without glucose were incubated at 37 C. for 24 hours with varying concentrations of nitrite. From table 3 it may be seen that increased rates of Heinz body production, "intact" hemoglobin destruction, and development of osmotic hyperfragility were produced by the higher, but not by the lower, concentrations of nitrite. These experiments were repeated, using normal erythrocytes suspended in glucose-buffer. Table 3 shows that the higher concentrations of nitrite

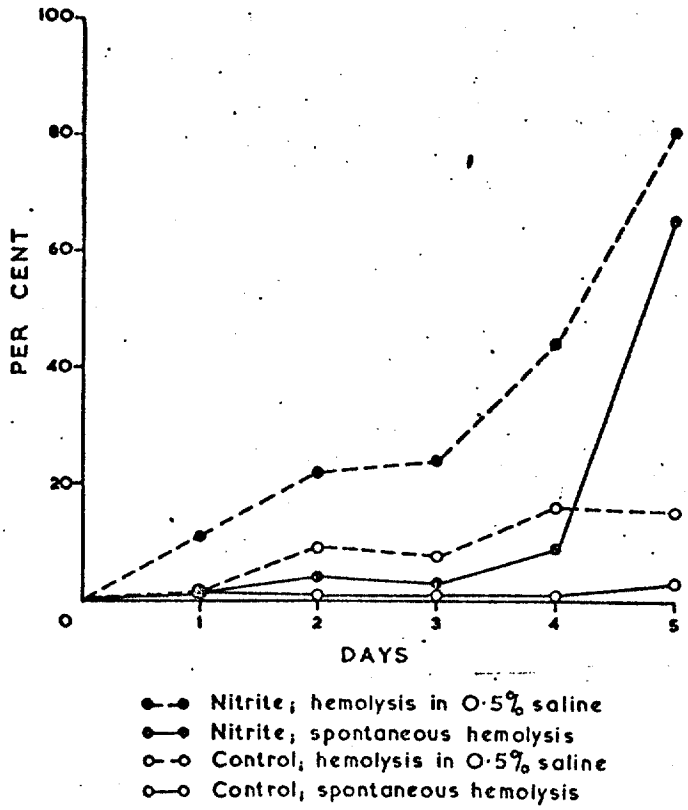

Fig. 3.—Per cent hemolysis in 0.5 per cent saline and per cent spontaneous hemolysis of erythrocytes incubated in glucose-buffer under aseptic conditions for 5 days, both in the presence and absence of nitrite. The initial molar ratio of nitrite: heme was 64:1.

again caused Heinz body production, "intact" hemoglobin destruction, and decreased osmotic resistance, but that these effects of nitrite were considerably less in degree than in the absence of glucose.

The result of more prolonged incubation was next investigated. Normal erythrocytes suspended in glucose-buffer were incubated at 37 C. for 5 days, both with and without nitrite in a molar ratio to heme of 64:1. Adequate substrate amounts of glucose were maintained in both the test and control incubation mixtures throughout the periods of incubation.

Results typical of the changes in osmotic fragility and spontaneous hemolysis are shown in figure 3. Hyperfragility to hypotonic saline occurred earlier and increased more rapidly in mixtures containing nitrite. Spontaneous hemolysis was slight in the absence of nitrite, even after 5 days incubation. In contrast, spontaneous hemolysis was apparent in mixtures containing nitrite after 3 days, and was found to increase rapidly after 4 days incubation.

Studies were also made as to Heinz body production and "intact" hemoglobin destruction. After 5 days, negligible changes were seen in cells incubated with glucose in the absence of nitrite. However, gradual changes occurred in those mixtures to which nitrite was added. After 24 hours, as noted

above, slight Heinz body formation and "intact" hemoglobin destruction were found. These changes then increased progressively, so that many Heinz bodies were present in each cell after 4 days. By the 5th day, large amounts of deeply staining material were seen, not only in relation to cells and cell ghosts, but in aggregates of spherical bodies in the extracellular medium. Concomitant with the formation of Heinz bodies, increasing turbidity and "intact" hemoglobin destruction were found in hemolysates. After prolonged centrifugation the hemolysates became clear; the proportional reduction in optical density at wave length 620 mμ, produced in these solutions by the addition of cyanide, decreased throughout the period of incubation. These findings were taken to indicate the formation, in addition to Heinz bodies, of those soluble degradation products of hemoglobin whose heightened absorption in red light is not abolished by cyanide.

The prolonged experiments would thus appear to provide evidence of a late, destructive effect exerted by the nitrite ion on hemoglobin and the cell membrane.

Discussion

The present work has shown that the nitrite ion, even in high concentration, does not cause loss of GSH in normal erythrocytes incubated with glucose as substrate. In the absence of either adequate substrate or a normal pentose phosphate pathway, however, incubation with nitrite in low concentration was found to result in significant degrees of GSH depletion, and in high concentration to produce rapid loss of GSH. It would appear, therefore, that nitrite acts directly to oxidize GSH, which is protected only in the presence of a normal and active pentose phosphate pathway.

The addition of lactate failed to prevent GSH depletion, either in the presence or absence of nitrite. Lactate is oxidized to pyruvate by lactic dehydrogenase, with the generation of reduced diphosphopyridine nucleotide (DPNH).[17] Despite previous observations that glutathione reductase in hemolysates may utilize DPNH,[18,19] the present results provide evidence that DPNH-dependent pathways play at most a minor part in the reduction of GSSG by the intact human erythrocyte.

Under experimental conditions involving differing levels of activity of the pentose phosphate pathway, no striking variation was observed in the rate of methemoglobin formation by a given concentration of nitrite. With all but the lowest concentration of nitrite, moreover, the degree of GSH depletion was found consistently to fall short of the degree of methemoglobin formation. In view of the molar preponderance of heme over GSH,[20] these results suggest that methemoglobin formation buffers the intact erythrocyte against nitrite-induced GSH depletion. They also indicate that an increased rate of GSH oxidation, rather than of methemoglobin formation, may be responsible for the evidence of increased activity of the pentose phosphate pathway which has been elicited in normal erythrocytes exposed to the nitrite ion.[21]

In support of these suggestions, reduced triphosphopyridine nucleotide (TPNH) is believed to reduce GSSG by a direct enzymatic mechanism,[22] but to mediate the reduction of methemoglobin only in the presence of an arti-

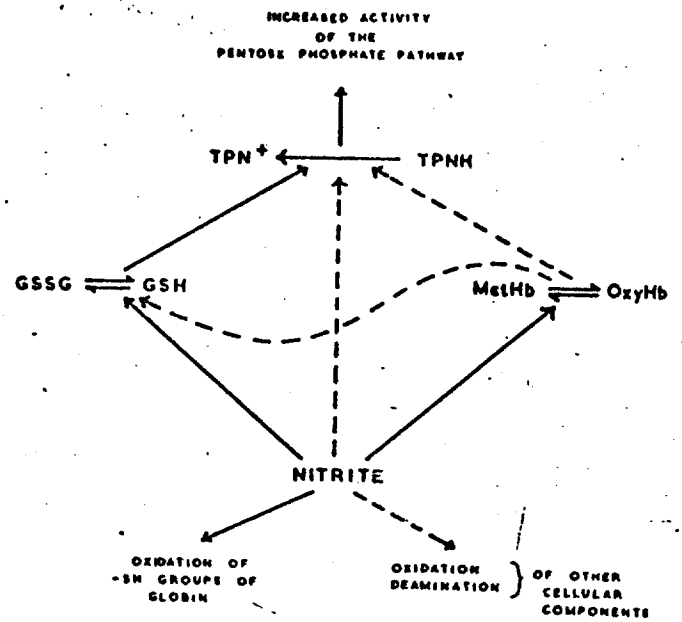

Fig. 4.—Diagrammatic representation of some mechanisms whereby the nitrite ion acts upon the intact erythrocyte. Continuous lines represent highly probable or certain mechanisms; interrupted lines, possible or minor mechanisms *(see text)*.

ficial electron carrier such as methylene blue.[17] The rate of oxidation of TPNH almost certainly determines the level of activity of the pentose phosphate pathway.[23] Nitrite might thus activate this pathway by increasing the rate of oxidation of TPNH, acting either indirectly through GSH or directly on TPNH. A metabolic hiatus between methemoglobin and TPNH would help to direct the full activity of the pentose phosphate pathway towards the reduction of GSSG, and the process of non-enzymatic oxidation of GSH by methemoglobin is relatively slow.[20,24] By absorbing a large part of the oxidation potential of nitrite, heme would thus appear to play a most significant part in the protection of intracellular GSH, and so presumably of the hemoglobin molecule[9,10] and other cellular components.[7,8] Some of these interrelations are depicted diagrammatically in figure 4.

The present investigation has also shown that nitrite exerts little effect on the activity in normal erythrocytes of the -SH enzyme, G-6-PD. However, the results do provide some evidence that nitrite in high concentration may cause a slight increase in the rate of decay of G-6-PD activity under conditions which do not lead to GSH depletion. Failing the use of methods[25] which ensure complete removal of stromata from hemolysates, it is possible that any effect of nitrite on G-6-PD activity may be secondary to a primary action on the stromal factor which activates this enzyme.[26]

In the absence of substrate, nitrite appeared to act as a "simple" oxidant, causing increased rates of methemoglobin formation, GSH depletion, oxidative destruction of hemoglobin with Heinz body formation, and loss of osmotic integrity. Even with adequate substrate, high concentrations of nitrite ultimately produced irreversible damage to hemoglobin and the cell membrane.

On the basis of previous investigations,[10,24,27] it seems reasonable to postulate that these effects of nitrite are mainly the result of direct oxidation of the -SH groups of globin and other erythrocytic components. However, deamination by nitrite[28] may well contribute to the denaturation of cellular proteins.

Despite the production of irreversible changes in vitro, the results suggest that the essentially reversible effect of nitrite on erythrocytes in vivo reflects the cellular capacity to maintain adequate levels of GSH. Presumably, the oxidation potential of sublethal doses of nitrite is largely expended in the formation of methemoglobin, which may then be reduced at leisure by DPNH and the Embden-Meyerhof pathway,[17] leaving the pentose phosphate pathway available for reduction of GSSG. At a cellular level it seems an interesting adaptation, that by temporarily relinquishing oxygen-carrying capacity the erythrocyte may maintain adequate concentrations of GSH and thus protect its various constituents from oxidative destruction. At an organismal level, these mechanisms may be important under evolutionary[29] or environmental[30] circumstances which involve intermittent exposure to high concentrations of the nitrite ion.

Summary

The mode of action of the nitrite ion on intact human erythrocytes has been investigated under varying experimental conditions. Nitrite appeared to act directly to cause methemoglobin formation and GSH depletion. Evidence was presented to show that such depletion does not occur in the presence of a normal and active pentose phosphate pathway, and to suggest that methemoglobin formation buffers the erythrocyte against nitrite-induced oxidation of GSH.

Both in the presence and absence of glucose, nitrite in high concentration was found to cause increased rates of oxidative destruction of hemoglobin with Heinz body formation, and of loss of osmotic integrity. It was suggested that nitrite acts by "simple" oxidation of the -SH groups of globin and other erythrocytic components, and possibly by causing deamination of cellular proteins.

The results were discussed, with reference to the significance of cellular protective mechanisms under evolutionary or environmental conditions which involve intermittent exposure to high concentrations of the nitrite ion.

Summario in Interlingua

Le modo de action de iones de nitrito in intacte erythrocytos human esseva investigate sub varie conditiones experimental. Il pareva que un effecto directe del action de nitrito esseva le causation del formation de methemoglobina e del depletion de reducite glutathiona (GSH). Es presentate datos e argumentos que monstra que un tal depletion non occurre in le presentia de un normal e active circuito de phosphato de pentosa e que suggere que le formation de methemoglobina age como tampon pro le erythrocytos contra le oxydation de GSH como effecto de nitrito.

Tanto in le presentia como etiam in le absentia de glucosa, il esseva trovate que nitrito in alte concentration causa un accelerate destruction

oxydative de hemoglobina in association con le formation de corpores de Heinz e un accelerate perdita del integritate osmotic. Es suggerite que nitrito age per simple oxydation del gruppos -SH de globina e de altere componentes erythrocytic e possibilemente per causar disamination de proteinas cellular.

Le resultatos del studio es discutite con referentia al signification de mechanismos de protection cellular sub conditiones evolutional e ambiental que porta con se intermittente expositiones a iones de nitrito in alte concentration.

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demonstrated by the effect of ovariectomy, which significantly decreased tryptophan oxygenase activity in females. Daily subcutaneous injections of 100μg of testosterone propionate administered for 14 days to intact female rats also resulted in decreased tryptophan oxygenase activity, not significantly different from that observed in intact male controls. Tyrosine aminotransferase activity remained unaltered.

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The Formation of Nitrosamines by Human Intestinal Bacteria

By Gabrielle Hawksworth and M. J. Hill (*Department of Bacteriology, St Mary's Hospital Medical School, London W.2, U.K.*)

The nitrosamines, a group of potent carcinogens that produce tumours in a range of sites in laboratory animals (Magee & Barnes, 1967), are produced by the action of nitrite on secondary amines under acid conditions. Sander (1968) demonstrated that four strains of nitrate-reducing enterobacteria could produce nitrosamines enzymically from nitrate and secondary amines at neutral pH values. We have confirmed this result using a number of strains of *Escherichia coli* isolated from the human intestinal tract, and have demonstrated that some non-nitrate-reducing strains of lactobacilli, group D streptococci, clostridia, bacteroides and bifidobacteria will nitrosate secondary amines with nitrite at neutral pH values. The nitrosamines were separated by t.l.c. and detected by spraying with sulphanilic acid and α-naphthylamine reagent after photolytic splitting to release the nitrite; quantitative assays were made by the polarographic method. The production of diphenylnitrosamine was confirmed from the i.r. spectra after purification by t.l.c.

The secondary amines nitrosated included dimethylamine, pyrrolidine and piperidine, all of which are present in normal human urine (Asatoor, 1960). The amount of nitrosamine formed is dependent on the basicity of the secondary amine, ranging from as much as 68% for diphenylamine to as little as 0.01% for dimethylamine. Nitrosamines are also degraded to secondary amines and nitrite, but the enzyme is of low activity; it is located in the cell cytoplasm.

Gut bacteria degrade lecithin to choline, which is then *N*-dealkylated to release dimethylamine and ethanolamine (which were characterized by chromatography of the DNP-amines; Asatoor & Kerr, 1961). This breakdown is carried out by strains of clostridia, bacteroides and bifidobacteria. By using a strain of *Clostridium bifermentans* it was demonstrated that optimum production of dimethylamine from choline occurred at pH 5.5–6.5. Another urinary amine, pyrrolidine, is produced by the bacterial decarboxylation of proline.

There are two sites of the body where bacteria might produce nitrosamines, (*a*) in the gut from ingested nitrate and amines produced from lecithin or proline, or (*b*) in the urine of people with urinary-tract infections (90% of which are due to *E. coli*, the nitrosating organism). We have not detected nitrosamine formation in broth cultures at nitrate concentration below 12μmol/ml, and although the concentration of amines is adequate the mean nitrate concentration in normal urine is only 1.5μmol/ml. However, in areas with a high nitrate content in their drinking water the urinary nitrate concentration is higher and approaches that necessary for the production of nitrosamines *in vitro*.

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Effect of Glucagon on Spheroplasts of *Escherichia coli*

By J. L. R-Candela, M. Carmen García and A. Nieto. (*Instituto 'G. Marañón', Velázquez 144, Madrid 6, Spain*)

Previously published results (R-Candela & García, 1969, 1970) show that actinomycin D inhibits glucose uptake by spheroplasts of *Escherichia coli*. This inhibition is completely reversed by glucagon, which enhances the glucose uptake. The present work was undertaken in order to throw further light on this effect.

Glucagon produces a stimulatory effect on the activity of β-galactosidase and enhances the incorporation into trichloroacetic acid insoluble protein of [1-^{3}H]leucine, [5-^{3}H]uridine and [2-^{14}C]-uracil in spheroplasts of *E. coli*. This effect has not been observed with other proteins (albumin, insulin, secretin and cal... cyclic AMP (10mM).

We are indebted to Dr S. ... suggestion and discussions, to ... E. Viñuela for advice and ... Miss M. Dolores Hermoso for ... ance and to Miss Carmela Gi... was supported in part by a gr... ceútica Lepetit and from Marg...

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R-Candela, J. L. & Garcia, M. ... 235.

A Catalytic Mechanism ... Benzylamine Oxidase

By Carole Taylor and P. ... ment of Biophysics, Univer... LS2 9JT, U.K.)

Pig plasma benzylamine ox... been purified to homogen... (Buffoni & Blaschko, 1964). ... specificity and some K_m val... (Buffoni & Blaschko, 1964; ... tailed steady-state kinetic ... enzymic mechanism has not ... kinetic study of the related ... amine oxidase (Oi, Inamasu ... suggests that this enzyme ... Ping Pong mechanism (Clel... hyde as the first product to ... tion of aldehyde under anaer... & Swindell, 1969) supports t...

In the present investigatio... product-inhibition studies o... amine oxidase from pig plas... The enzyme reaction was m... benzaldehyde production s... at 250nm; product-inhibition ... aldehyde was added were fo... oxygen disappearance (Estab...

Initial-velocity studies at ... concentrations with differe... centrations indicated a Pin... Product-inhibition studies and ... saturating conditions of each ... Ping Pong mechanism of the ... ammonia off, oxygen on, hy... benzaldehyde off. Failure to ... under anaerobic conditions ... benzyl alcohol to act as a subs... evidence in favour of this mec...

It is suggested that fundam... mechanism between appar... oxidases parallel differences in

appeared after precipitation of the S-RNA as quaternary ammonium salt and regeneration. This precipitation would thus constitute a method of purification of S-RNA, which is equivalent, so far as the elimination of inactive oligonucleotides is concerned, to chromatography on 'Ecteola'-cellulose.

In summary, after treatment with a quaternary ammonium salt, S-RNA can be dissolved in an organic solvent and regenerated from this solution as the sodium salt, without loss of its biological activity.

Experiments are in progress to take advantage of this dissolution in an organic solvent and attempt a fractionation of S-RNA.

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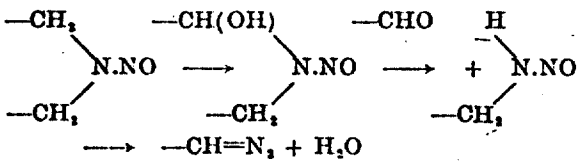
Mechanism of the Hepatoxic Action of Dialkylnitrosamines

DIMETHYLNITROSAMINE[1] and some other dialkylnitrosamines[2,3] cause centrilobular necrosis in rats. Schoental and Rose[4] suggested independently that centrilobular necrosis was caused by diazo-alkanes formed by the metabolic oxidation of the nitrosamines in the liver *in vivo*[5,6]. The sequence of reactions is presumed to be :



On this sequence (*n*-butyl)(methyl)nitrosamine (BuMeN.NO) should cause centrilobular necrosis, but (*tert*-butyl)(methyl)nitrosamine ($Me_3C.MeN.NO$) should not. α-Oxidation of the *n*-butyl compound can give either diazomethane or diazobutane, according to which alkyl group is attacked. In the *tert*-butyl compound the *tert*-butyl group cannot be attacked in the α position, and oxidation of the methyl group leaves a compound with no α-hydrogen atom, which cannot, therefore, yield a diazo-alkane.

The two compounds were synthesized and tested on female rats (Porton Wistar strain). Both compounds were given intraperitoneally as solutions in normal saline.

The *n*-butyl compound caused acute centrilobular necrosis in doses of 100–120 mgm./kgm. in 1–2 days. Doses of 60 mgm./kgm. were without obvious effect. This compound is about one-quarter as toxic as dimethylnitrosamine, and affects the liver in a very similar way.

Twenty rats were treated with the *tert*-butyl compound. Four died from the treatment they received, so the doses given were near the maximum tolerable. Three survived 1,600 mgm./kgm. given in divided doses over 4 days, and three others survived 1,400 mgm./kgm. given over the same period. The rest survived smaller doses given over shorter periods. The survivors were killed at various times after the last dose—from the highest doses at 1, 3 or 10 days after. Various lesions were observed on histological examination, but no centrilobular necrosis in any case. Thus the *n*-butyl compound caused the typical lesion, but the *tert*-butyl compound did not, in accordance with the diazo-alkane theory.

This evidence for the diazo-alkane theory is not, of course, conclusive. It is possible that still higher doses of the *tert*-butyl compound might cause centrilobular necrosis. Alternatively, perhaps the *tert*-butyl compound does not reach the sites in the liver at which metabolism takes place. This last possibility is being investigated with nitrosamines labelled with carbon-14.

I thank Dr. P. N. Magee for carrying out the histological examinations, Dr. A. R. Mattocks for preparing the specimens of nitrosamines, and Mr. J. Rose for technical assistance.

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PHYSIOLOGY

Cytotoxic Effect of Aminoacetonitrile on Fibroblast Cultures and its Prevention by Histidine

THE understanding of the ætiological factors involved in the curious poisoning arising from the ingestion of *Lathyrus odoratus* seeds has been greatly advanced by the identification of β-amino-propionitrile as the active moiety of the toxic principle and the recognition of the even more potent lathyrogenic property of aminoacetonitrile (AAN)[1,2]. However, the site and nature of the primary metabolic disturbance responsible for the characteristic mesenchymal lesions produced by aminonitriles have not yet been elucidated. The present investigation was designed primarily to explore the possibility of using tissue cultures as a prospective tool for studying under *in vitro* conditions the biochemical mechanism underlying the toxic action of AAN.

The experiments were performed regularly with chick embryo fibroblasts grown in a modified *M*–150 medium of Morgan *et al.*[3] consisting of Hanks's salt solution, a mixture of amino-acids and vitamins and fortified with 2 per cent calf serum. In most experiments 3–4 days-old cultures were used and AAN was added with fresh medium following the removal of the initial culture fluid. Under such conditions

Table 1. PREVENTIO

Aminoacetonitrile (moles per ml.)	L-Histidine (μmoles per ml.)	
0	0	
0	0·25–4	
0·7	0	
0·7	0·25	
0·7	0·50	
0·7	1·00	
0·7	2·00	
1·0	0	–
1·0	1·00	
1·0	2·00	
3·0	0	+
3·0	1·0	
3·0	2·0	
3·0	4·0	

Suitable dilutions of hydrochloride neutral along with the repo (at 37° C.) was contr scopically at the mo in 5-replicate tubes.

Key: — No effect; cell-sheet intact; + – pronounced fragmenta cells; + + + +, disin and cell debris.

AAN (100 μgm. sive alterations i rounding of the until the fibroi recognizable: co shoot became i complete disint the end of 2–3 d higher levels of the course of t ably accelerated

The noxious e young, actively to decrease with observation ma enhancement of blast cultures su ties of triiodoth shown previou metabolic rate soon to suggest tial for poisonin mention in this experimental la and declines s animals[5].

Mouse fibrob response to AA somewhat lower blasts. On the (kindly supplied kidney cells an cells, proved to threefold high blasts.

Of particular addition of an the culture me in a significant the toxic phen usual test con appears to be AAN, may be that the course in rats can be maintaining th

The protec duplicated by

Brit. J. industr. Med., 1962, 19, 276.

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TOXIC PROPERTIES OF DIALKYLNITROSAMINES AND SOME RELATED COMPOUNDS

BY

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The title compounds, which have some commercial applications, are described, and their toxic action is reviewed. Most results refer to rats.

Their main acute effect is hepatic centrilobular necrosis, though lung lesions may appear. The compounds also induce tumours in liver, lung, and kidney. One, dimethylnitrosamine, has been shown to cause kidney tumours after a single dose. The necrotic and carcinogenic doses of the compounds are closely related.

The relation between structure and toxicity is discussed. Analogous formamides are much less toxic (the LD_{50}'s in rats by intraperitoneal injection of dimethyl- and diethyl-formamides are 3,800 mg./kg. and 1,740 mg./kg.) and they do not cause centrilobular necrosis or tumours.

Nitrosamines are oxidized *in vivo* and by liver preparations *in vitro*. Their toxic action is due to the release of powerful alkylating agents in the liver. They also inhibit protein synthesis and alkylate liver protein and ribonucleic acid. In all cases the effective agent appears to be a metabolite.

The possible hazard to man in the uses of these compounds is emphasized.

This paper is mainly a review, but it also includes observations which have not been published elsewhere. Some dialkylnitrosamines are valuable chemical intermediates or solvents, and their use as insecticides has been suggested (Reilly, 1961). Our aim is therefore to increase awareness of the hazards in their use, as well as to summarize the properties of a group of compounds of toxicological interest.

The dialkylnitrosamines have the structure; RR'N·NO, where R and R' are alkyl groups. Closely related nitrosamines are also considered. These may contain substituents in the alkyl group, as in the 2-hydroxyethyl compounds (see Table 1); or one of the groups may be an aryl group, as in (methyl)(phenyl)nitrosamine; or the amino nitrogen may be in an aliphatic ring, as in N-nitrosomorpholine and N-nitrosopiperidine. Some of the corresponding dialkylformamides are also compared briefly. In these compounds a ·CHO group substitutes for the ·NO of the nitrosamines. Thus, dimethylformamide is $(CH_3)_2N{\cdot}CHO$

Methods

LD_{50}'s of the formamides were determined as described by Weil (1952) using solutions of the compounds in normal saline injected intraperitoneally. For the other compounds the original papers should be referred to.

Physicochemical Properties

The compounds are listed in Table 1, where some physical properties are also given. The simple dialkyl- and (alkyl)(aryl)-nitrosamines are all soluble in most organic solvents, and can be extracted from water by chloroform. The ease of extraction increases with the number of carbon atoms in the molecule in the usual way. Dimethylnitrosamine is miscible with water in all proportions. The rest have limited solubilities, depending on the shape and size of the alkyl groups. The distillation of aqueous solutions yields distillates which contain higher concentrations than the original solutions. This is the basis of their separation from animal tissues for analysis (Heath and Jarvis, 1955), and for some purification procedures (Dutton and Heath, 1956a; Heath and Mattocks, 1961). It follows that vapours above aqueous solutions may contain high concentrations, even though the compounds boil at higher temperatures than water. The 2-hydroxyethyl compounds are miscible with water, from which they are not

SOME N

Subst

dimethylnitrosamine
diethylnitrosamine
dipropylnitrosamine
dibutylnitrosamine
(*n*-butyl)(methyl)nitrosamine
(*tert*-butyl)(methyl)nitrosamine
(ethyl)(2-hydroxyethyl)-nitrosa
bis(2-hydroxyethyl)-nitrosamine
N-nitrosopiperidine
N-nitrosomorpholine
(methyl)(phenyl)nitrosamine, (N-

*Boiling points at pressures o

readily extracted by orga
less volatile, and cannot
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N-nitrosopiperidine res
rather closely.

Chemically they are
hydrolysed slowly by min
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The combination of hi
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acute LD_{50} in rats whet
intraperitoneally, or int
Magee, 1954; Heath, 1962
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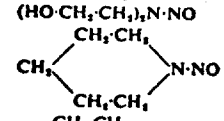
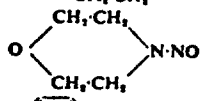
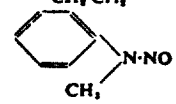
Toxic

The toxic effects fall
acute effects, which devel
and, after a lethal dose, us

TABLE 1

SOME NITROSAMINES AND THEIR BOILING POINTS AND SOLUBILITIES IN WATER

Substance	Structure	Boiling Point (°C.)	Water Solubility (mg./ml.)
dimethylnitrosamine	$(CH_3)_2N{\cdot}NO$	151	misc.
diethylnitrosamine	$(CH_3{\cdot}CH_2)_2N{\cdot}NO$	172	100
dipropylnitrosamine	$(CH_3{\cdot}CH_2{\cdot}CH_2)_2N{\cdot}NO$	196	10
dibutylnitrosamine	$(CH_3{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2)_2N{\cdot}NO$	234-7	1-2
(*n*-butyl)(methyl)nitrosamine	$CH_3{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{>}N{\cdot}NO$ (CH_3)	56/1·5 mm.*	20
(*tert*-butyl)(methyl)nitrosamine	$(CH_3)_3C{>}N{\cdot}NO$ (CH_3)	66/5 mm.*	70
(ethyl)(2-hydroxyethyl)-nitrosamine	$HO{\cdot}CH_2{\cdot}CH_2{>}N{\cdot}NO$ ($CH_3{\cdot}CH_2$)	103/3 mm.*	misc.
bis(2-hydroxyethyl)-nitrosamine	$(HO{\cdot}CH_2{\cdot}CH_2)_2N{\cdot}NO$	very high	misc.
N-nitrosopiperidine		214	70
N-nitrosomorpholine		219	misc.
(methyl)(phenyl)nitrosamine, (N-nitroso-N-methylaniline)		— 128/19 mm.*	very low

*Boiling points at pressures other than atmospheric are given as x°/y mm., *i.e.* x°C. under a pressure of y mm. of mercury.

readily extracted by organic solvents. They are much less volatile, and cannot be distilled from aqueous solutions. N-nitrosomorpholine is intermediate in physical properties between the two groups; and N-nitrosopiperidine resembles diethylnitrosamine rather closely.

Chemically they are stable. They are only hydrolysed slowly by mineral acids at room temperature (rapidly on heating), and too slowly to measure in water and alkaline solution. Bright light decomposes very dilute solutions rapidly, but in intermittent artificial light solutions containing about 50 mg./l. of several of the compounds have been stored for several months at 4°C. without detectable loss.

The combination of high stability with solubility in a wide range of solvents probably explains why the toxicities of several of the compounds are not much dependent on the route of injection. Dimethylnitrosamine, for example, shows the same acute LD_{50} in rats whether it is given by mouth, intraperitoneally, or intravenously (Barnes and Magee, 1954; Heath, 1962); it is not readily absorbed by the skin.

Toxic Effects

The toxic effects fall into two main groups: acute effects, which develop within one to two days and, after a lethal dose, usually kill the animal within a week; and carcinogenic effects. Some work has been done on several species (dog, hamster, mouse, rabbit, and rat), and the acute effects are similar in all, except for minor differences in susceptibility. The rat appears to be one of the more resistant species (Barnes and Magee, 1954). In studies on carcinogenity very much more attention has been paid to the rat than to other species, and in this paper observations always refer to the rat unless stated otherwise. There is some evidence of species difference (Magee and Barnes, 1956).

Three acute effects can be distinguished. Only one compound, (*n*-butyl)(methyl)nitrosamine, has been shown to cause all three.

1. Hepatic centrilobular necrosis. This has been described in several species, and the reader is referred to the original papers for a description (Barnes and Magee, 1954; O'Leary, Wills, Harrison, and Oikemus, 1957; Magee and Barnes, 1956). It is the most typical acute lesion. The doses needed to induce it are listed for several compounds in Table 2. The Table also contains results with three nitrosamines which do not cause this lesion, and with a fourth, bis(2-hydroxyethyl)-nitrosamine, where necrosis may be due to an impurity, as high doses were needed and the compound was hard to purify. As shown later, these exceptions throw some light on the structural requirements for activity in the series.

TABLE 2

TOXIC EFFECTS OF DIALKYLNITROSAMINES AND RELATED COMPOUNDS

The acute LD_{50}'s are given, and also the dose, ED, which, given singly, causes distinct hepatic necrosis. Under acute effects the lowest doses which have caused tumours in 50% of the rats treated are shown, and the organs in which tumours have been found. Note that carcinogenic doses are in g./kg., not mg./kg.

	Acute Effects		Carcinogenic Effects	
	LD_{50} (mg./kg.)	ED (mg./kg.)	g./kg.	Organs Affected
Nitrosamines				
dimethyl	27-41[1, 3]	15[2]	<0·25[3]	liver [3, 4, 8, 10, 11] kidney [3, 8, 10] lungs [3, 10]
diethyl	216[3]	130[3]	0·25[3]	liver [3, 4], kidney [3]
dipropyl		400	2[4]	liver [4]
dibutyl		800	10[5]	liver [4], bladder [5]
diamyl	1,750[4]	—	28[6]	liver [4], lungs [12]
(*n*-butyl)(methyl)	130[2, 7]	70[2, 7]	0·6	liver
(*tert*-butyl)(methyl)	700[2, 7]	none at 1,800[4, 7]	—	—
(ethyl)(2-hydroxyethyl)	—	none at 3,000[2]	—	—
bis(2-hydroxyethyl)	—	9,000[2]	160[4]	liver [4]
piperidine		200	2[4]	liver and oesophagus [4]
morpholine	282	200	0·7[4]	liver [4]
(methyl)(phenyl)	200	none at 200	none at high doses [4]	
Formamides				
dimethyl	3,800[2]	none	none at 13[4]	
diethyl	1,740[2]	none	—	
(*n*-butyl)(methyl)	c. 500	none	—	
(*tert*-butyl)(methyl)	c. 500	none	—	

References: where no reference is given, the figures have not been published elsewhere, and in acute tests the compounds were given intraperitoneally.

[1] Barnes and Magee, 1954. [2] Heath, 1962. [3] Argus and Hoch-Ligeti, 1961. [4] Druckrey *et al.*, 1961b. [5] Druckrey, Preussmann, Schmähl, and Müller, 1962. [6] Druckrey *et al.*, 1961c. [7] Heath, 1961. [8] Magee and Barnes, 1956. [9] Magee and Barnes, 1962. [10] Zak *et al.*, 1960. [11] Schmähl and Preussmann, 1959. [12] Druckrey and Preussmann, 1962.

2. Lesions in other organs, especially in the lung. These are generally observable, though after single doses of dimethyl- and diethylnitrosamine they are slight (Barnes and Magee, 1954; Magee, 1962). The (*n*-butyl)(methyl) compound causes haemorrhagic lesions in the lungs of rats at LD_{50} doses, and some rats die within a day before the liver lesion is fully developed. Others die two to four days after injection, and in these cases death appears to be more closely correlated with the liver lesion. Acute liver necrosis can, however, be caused by sublethal doses which cause only inconspicuous lung lesions.

3. After high doses of (*n*-butyl)(methyl)- and (*tert*-butyl)(methyl)nitrosamines rats suffer mild convulsions, followed by coma, and die quickly, usually within an hour. As the analogous dialkylformamides $(CH_3{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2)(CH_3)N{\cdot}CHO$ and $[(CH_3)_3C](CH_3)N{\cdot}CHO$, cause the same effects at similar doses, probably these effects should be attributed to some biophysical interaction with the nervous system, and will not be considered further.

The carcinogenic effects have been described by numerous authors, and the doses used and the organs affected in rats are listed in Table 2. Tumours have also been found in the trachea and lungs of golden hamsters fed dimethylnitrosamine (Dontenwill and Mohr, 1961). The compounds are remarkably consistent in their action, and it is easy to cause tumours with them in most of a group of animals tested.

Liver tumours are mainly liver cell carcinomas, with or without accompanying fibrosis and cystic bile-duct proliferation. All stages are seen from liver cell adenomas to highly anaplastic hepatocellular carcinomas. Some sarcomas are produced, and very occasionally carcinomas seem to arise from intrahepatic bile-ducts (Argus and Hoch-Ligeti, 1961; Magee and Barnes, 1962). Kidney tumours may be either well-differentiated renal adenocarcinomas, often papillary in structure, or undifferentiated. Magee and Barnes (1962) discuss the nature of the latter. In the lung, Zak, Holzner, Singer, and Popper (1960) observed epithelial tumours with alveolar or undifferentiated structures, while Argus and Hoch-Ligeti (1961) observed squamous carcinomas. Oesophageal, bladder, and stomach tumours are all squamous carcinomas.

The main feature in the liver between the necrotic lesion and tumour formation is the development of giant cells with grossly enlarged nuclei. These are conspicuous on histological examination one to two months after the initial dose. Their relation to the other lesions is uncertain (Magee and Barnes, 1956; Christie and Le Page, 1961).

The organs affected depend upon both the dose rates and the strain of rat. Magee and Barnes (1962) showed that when rats were given an LD_{50} dose of dimethylnitrosamine, 15% of the survivors developed kidney tumours, and that a total dose of about 200 mg./kg. given over a short period of a few

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Toxicity

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w... caused kidney tumours in over half the treated group. On the other hand, much lower dose rates continued for a period of several months caused liver tumours in nearly all the rats treated, and no kidney tumours (Magee and Barnes, 1956). The effect of dose rate was very marked in this instance. In other rat colonies low dose rates did not give liver tumours only, but about equal numbers of primary liver, lung, and kidney tumours (Argus and Hoch-Ligeti, 1961). These differences did not arise from differences in the total doses given, which were much the same throughout. Diethylnitrosamine has also been studied over a wide range of dose rates (Druckrey, Schmähl and Schildbach, 1961c), but with one exception (Zak *et al.*, 1960) has only been shown to cause liver tumours.

Tumours do not develop only in those organs in which either acute or chronic necrotic damage is most marked. For example, dimethylnitrosamine causes little acute damage to lungs or kidneys, but causes tumours there as readily as in the liver; and diethylnitrosamine caused haemorrhages in lungs, thymus, para-aortic lymph nodes, and gastric mucosa when given over long periods by mouth, but only caused liver tumours (Schmähl, Preussmann, and Hamperl, 1960). These haemorrhagic lesions were not observed in the rats studied by Argus and Hoch-Ligeti (1961).

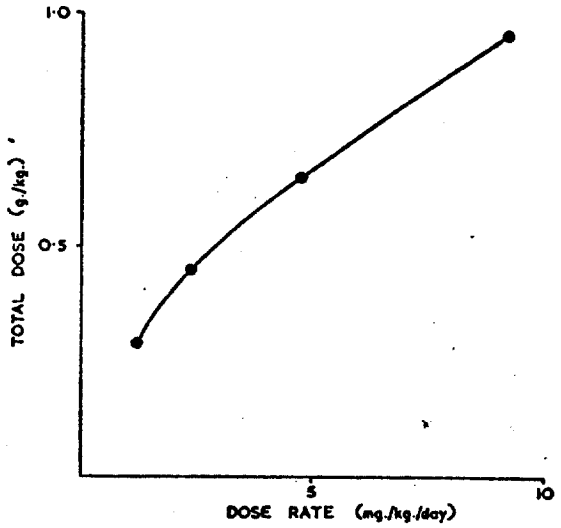


FIGURE Relation between dose rate and total median carcinogenic dose of diethylnitrosamine in rats. (The results are calculated from the Figure given by Druckrey *et al.*, 1961c.)

Toxicity Relationships

Two relationships are discussed: that between necrotic and carcinogenic activity; and that between toxicity of either kind and chemical structure.

No exact relation can be established between the acute and long-term effects of the compounds because of the difficulties in determining median carcinogenic doses. For example, Druckrey *et al.* (1961c) dosed rats continuously with various doses of diethylnitrosamine until tumours developed. The relation between total dose given and the dose rate, *i.e.* the dose given per day, is shown in the Figure. The total dose required increased rapidly with the dose rate. Much of this increase has an obvious explanation: a considerable time elapses between the time when enough damage has been done to make the production of a tumour inevitable and the time when the tumour is first observable as an entity, and all the compound given during this period is excess. The amount of the excess obviously increases with the dose rate. Although, given enough rats, this problem can be overcome, there are other less tractable difficulties. In the case of dimethylnitrosamine in rats, for instance, the relation between dose rate and effective dose is different according to whether kidney or liver tumours are studied. Bearing these factors in mind, the following may be stated.

1. In no case have tumours been induced in half of an experimental group by a total divided dose which will not kill more than half the animals by its acute effects if given singly.

2. When the single dose required to cause liver necrosis is under 200 mg./kg. tumours have been caused by total doses of under 1000 mg./kg.; when acute effects require 200 to 1000 mg./kg., tumour formation requires 1 to 10 g./kg.; and when acute effects require over 1000 mg./kg., tumour formation requires over 10 g./kg. It appears, therefore, that acute liver necrotic activity and general carcinogenic activity are related in this series of compounds, even though the organs in which tumours develop are not necessarily those in which the necrotic lesions are most pronounced.

It follows that in discussing structure-activity relationships the acute hepatic and long-term effects can be regarded alike; for any compound we can choose whichever effect has been most studied.

1. All the simple dialkylnitrosamines which have been studied are active, and also those with aliphatic nitrogen rings.

2. In those dialkylnitrosamines in which both alkyl groups are the same the activity falls rapidly as the chain length increases. The series has been studied up to diamyl (Druckrey, Preussmann, Schmähl, and Müller, 1961b).

3. Nitrosamines containing 2-hydroxyethyl groups are inactive. In fact, very high doses of the

bis(2-hydroxyethyl) compound produced tumours (see Table 2), but the authors thought that active impurities might have been present.

4. The introduction of a branched chain greatly decreases the activity. Thus di-isopropylnitrosamine did not show carcinogenic activity at the doses tested, and (methyl)(phenyl)nitrosamine, in which the phenyl group can be regarded as a particular case of a branched chain, was also without activity (Druckrey *et al.*, 1961b).* The effect is shown most clearly by (*tert*-butyl)(methyl)nitrosamine, which is without necrotic activity although it resembles diethylnitrosamine closely in physical properties (Heath, 1961), and is metabolized in the same way, as described later.

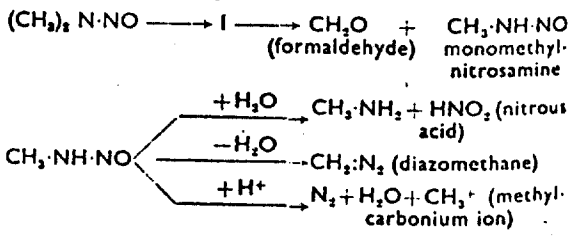
5. Analogues which do not contain the nitroso-group are without effect. Thus the reduction product of dimethylnitrosamine, dimethylhydrazine, $(CH_3)_2N{\cdot}NH_2$, is without carcinogenic effect (Argus and Hoch-Ligeti, 1961), and the only exception so far found is the oxidation product, dimethylnitramine $(CH_3)_2N{\cdot}NO_2$, which causes some kidney tumours and no liver tumours, in rats given a total dose of 40 g./kg. (Druckrey *et al.*, 1961b). The possibility that dimethylnitrosamine is formed by metabolic reduction has not been ruled out.

Of particular interest are the dialkylformamides. These are very similar in structure and physical properties to the analogous nitrosamines. Dimethylformamide is of very low toxicity to rats when given as a 50% solution in normal saline (see Table 2), and can be used as a vehicle for the injection of compounds insoluble in water. It is a little more toxic to mice (1560 mg./kg. intraperitoneally). These results contradict those of Massmann (1956), who claimed very much higher toxicities. Smyth and Carpenter (1948), however, found that dimethylformamide was less toxic orally in rats than Massmann did, and Davis and Jenner (1959) found its intraperitoneal LD_{50} in mice was 1122 mg./kg., not much less than our value. Diethylformamide is more toxic to rats, and (*n*-butyl)(methyl)- and (*tert*-butyl)(methyl)-formamides considerably more toxic. None, however, produces centrilobular necrosis at lethal doses. Dimethylformamide causes some periportal damage, which is easily distinguished.

Metabolism

Only dimethylnitrosamine has been studied *in vitro*. It is decomposed by liver slices and by liver microsomes plus cell sap. Nicotinamide-adenine dinucleotide phosphate (triphosphopyridine nucleotide) and oxygen are needed, and the rate of decomposition is reduced by SKF 525A, an inhibitor of microsomal oxidative enzymes. One of the products is formaldehyde (Magee and Vandekar, 1958, Brouwers and Emmelot, 1960). It is concluded that the compound is decomposed by enzymic oxidation, and that the enzymes are similar to those studied by Axelrod, Reichenthal, and Brodie (1954). Brouwers and Emmelot (1960) also showed that hepatic tumours had little capacity to oxidize the compound; and Magee and Vandekar (1958) that kidney, spleen, and lung preparations had also little or no capacity.

Much more extensive studies have been carried out *in vivo*, in attempts to explain the action of the compounds. Magee (1956) concluded from the high rate of disappearance of dimethylnitrosamine from rats and rabbits that dimethylnitrosamine was decomposed rapidly, as very little was excreted in the urine. This conclusion was confirmed by Dutton and Heath (1956b), who showed that 60% of the ^{14}C given as [^{14}C] dimethylnitrosamine to rats was expired as $^{14}CO_2$ in a few hours. Magee also showed that total hepatectomy reduced the rate of disappearance almost to zero in rats, while nephrectomy was without effect; and that in mice, SKF 525A also reduced the rate of disappearance. Thus, decomposition *in vivo* resembles decompostion *in vitro* closely, and is practically confined to the liver. As this is the only organ that undergoes severe necrosis, it was concluded that the toxic agent was a metabolite. This conclusion has been confirmed by a recent investigation (Heath, 1962). Dimethyl-, diethyl-, (*n*-butyl)(methyl)- and (*tert*-butyl)(methyl)-nitrosamines were prepared labelled in their methyl, ethyl, *n*-butyl, and *tert*-butyl groups (Heath and Mattocks, 1961). All four compounds were rapidly decomposed, as shown by $^{14}CO_2$ expiration, and were only excreted in the urine or in the expired air to a small extent. The action of inhibitors of oxidation was also studied, and this was correlated with their effects on acute toxicity. The only simple explanation of the results was provided by the following reaction scheme, in which dimethylnitrosamine is taken as an example.



In the first stage the nitrosamine is oxidized to formaldehyde and monomethylnitrosamine, via

*(Methyl)(phenyl)nitrosamine can cause oesophageal tumours when given orally in high doses (Druckrey, Preussmann, Schmahl, and Blum, 1961a). The local exposure may be very great in this case.

non-toxic intermediates. Monomethylnitrosamine then decomposes spontaneously and very rapidly, in part to nitrite and methylamine (Heath and Dutton, 1958), but also to diazomethane and/or carbonium ions. The toxic action must be attributed either to diazomethane, as suggested by Rose (1958) and R. Schoental (personal communication), or to monomethylnitrosamine acting through the release of carbonium ions. The toxic metabolites are both powerful alkylating agents. Either hypothesis can explain the lack of activity in (*tert*-butyl)(methyl)- and (methyl)(phenyl)-nitrosamines.

The work also suggested a reason for the lack of activity in (2-hydroxyethyl)nitrosamines. The two tested were only weak inhibitors of the oxidation of dimethylnitrosamine, and probably they are too insoluble in fat to be oxidized, but are excreted instead.

Biochemical Effects

Both *in vivo* (Emmelot and Mizrahi, 1961; Magee, 1958) and *in vitro* (Brouwers and Emmelot, 1960; Hultin, Arrhenius, Löw, and Magee, 1960) dimethylnitrosamine inhibits protein synthesis in the liver as measured by the incorporation of ^{14}C-labelled amino acids. Protein synthesis was also inhibited in microsomal preparations from poisoned rats (Hultin *et al.*, 1960). The inhibition appears to be due to a metabolite. Thus inhibition increased with the time the liver was exposed to the nitrosamine, and was marked after one hour's exposure *in vitro* and three hours' *in vivo*. Protein synthesis was very little inhibited in hepatic tumour tissue, which has little capacity to oxidize dimethylnitrosamine (Brouwers and Emmelot, 1960); and inhibition was slight in rats pretreated with high doses of cysteine, which may be expected to react with several of the possible metabolites, but not with dimethylnitrosamine itself (Emmelot and Mizrahi, 1961).

Dimethylnitrosamine also alters normal metabolism by reducing the incorporation of ^{32}P into RNA phosphorus (Magee, 1958) and the incorporation of adenine into nucleic acids (Hultin *et al.*, 1960). No other metabolic disturbances have been found until liver damage is marked (Magee, 1958; Bailie and Christie, 1959; Hultin *et al.*, 1960). At this stage they may be secondary.

Alkylation of Ribonucleic Acid and Proteins

In rats, both liver ribonucleic acid (Magee and Farber, 1962) and liver protein (Magee and Hultin, 1962) are methylated by dimethylnitrosamine. In ribonucleic acid, guanine residues are methylated in the 7-position, giving a ribonucleic acid which is not a normal constituent. Magee and Farber (1962) showed that ribonucleic acid is also methylated to some extent in the kidney (confirming that the kidney has a slight capacity to oxidize dimethylnitrosamine); and diethylnitrosamine probably also produces 7-ethylguanine in the liver (Magee, unpublished data).

Therapy

High and barely tolerable doses of cysteine given prophylactically over two days about doubled the resistance of rats to dimethylnitrosamine (Emmelot and Mizrahi, 1961). No other treatment has been found which has a marked effect on toxicity.

Any chemical therapy will probably have both to be applied very shortly after the uptake of the nitrosamine, as this is decomposed rapidly to toxic products; and also to protect the animal against the toxic products either by combining with them or in some other way. Inhibitors of decomposition have very little effect (Heath, 1962), and what there is can be wholly accounted for by the higher proportion of the nitrosamine excreted in urine and expired air when its persistence *in vivo* is increased.

Discussion

The high acute toxicities and carcinogenic activities of several nitrosamines are well established, and the main structural requirements are known, though later work may lead to some modifications. It is known that several compounds are mainly disposed of in the body by oxidation, and not by excretion, though the 2-hydroxyethyl compounds are probably exceptions to this rule.

The compounds causing acute necrosis are metabolites, and are probably alkylating agents, either diazoalkanes or carbonium ions. As diazomethane is carcinogenic (Schoental, 1960; Schoental and Magee, 1962) and mutagenic (Westergaard, 1957), it is tempting to assume that the carcinogenic action of the compounds should be attributed to the same metabolites. This fits in with the observed mutagenic effects of dimethyl sulphate on tobacco mosaic virus (Fraenkel-Conrat, 1961), and to the explanations of such effects discussed by Brookes and Lawley (1961 a and b). There are, however, three objections to this.

1. It is likely that nitrite is released, and this has been found to be more mutagenic on tobacco mosaic virus than any alkylating agent tried (Fraenkel-Conrat, 1961). Mutagenicity and carcinogenicity are not, of course, inevitably correlated.

2. Although median carcinogenic doses are not known accurately, dimethyl- and diethylnitrosamines have been investigated fully enough for it to be clear that their median carcinogenic

doses are fairly close; but diethylnitrosamine has only about one-tenth the necrotic action of dimethylnitrosamine. If it is allowed that most of the other carcinogenic doses given in Table 2 are too high, then there are probably other similar anomalies. This may not imply that the metabolite is different; the different effects may be caused in each case by the same metabolite acting on different biochemical systems.

3. The alkylating agents postulated are exceedingly unstable, and it seems unlikely that significant amounts could survive passage in the blood stream from organ to organ. Yet dimethylnitrosamine causes tumours in organs in which oxidation only takes place slowly, and which show little acute damage.

The carcinogenic compound is still, therefore, in doubt. It is therefore at least possible either that the toxic metabolite reacts with some normal liver constituent to give a more stable compound which is carcinogenic, or that the processes of repair in a damaged liver themselves produce a carcinogen.

The particular hazard from the nitrosamines is their "one-shot" carcinogenic effect. If man is like the rat in his response, not only long-term exposure but also a single serious incident may give rise to unpleasant sequelae years later. This feature of their toxic action should be considered if nitrosamines are to be used industrially.

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SPECIFIC ANALYTICAL METHOD OF DETECTION FOR NITROSAMINES THROUGH THE COMBINATION OF GAS CHROMATOGRAPHY AND MASS SPECTROMETRY

(Ein spezifisches analytisches Nachweisverfahren für Nitrosamine durch Kombination von Gaschromatographie mit der Massenspektrometrie)

Authors: K. Heyn and H. Röper

Source: Tetrahedron Letters No. 10, P. 737-740, 1970. Pergamon Press

Since Magee and Barnes (Ref. 1) detected the carcinogenic effect of dimethyl nitrosamine, N-nitrosamines have been at the center of cancer research. Several working groups studied their organ specific cancerogenic action. The presence of nitrosamines in natural products and in foodstuffs was described, thus, among others, in the fungus Clitocybe suavoelens, in streptomycins and solanacines, in wheat plants and in wheat flour, in tobacco and in tobacco smoke, in pasteurized milk and cheese, in salt meat and in herring meal (Ref. 2).

The analytical detection methods used so far have been film chromatography (Ref. 3), colorimetry (Ref. 4), UV (Ref. 4, 5), IR (Ref. 6) and fluorescence spectroscopy (Ref. 6), polarography (Ref. 7, 8) and gas chromatography (Ref. 9 - 13) with packed columns. These methods are by far insufficiently specific for the analysis of complex substance mixtures.

We developed the combination of capillary gas chromatography and mass spectrometry as a sensitive and substance-specific separation and detection method for nitrosamines. The following nitrosamines were presented in Table 1 in their order of increasing retention values and were examined by gas chromatography in two model mixtures.

Table 1: a. R-column (Mixture 1) 0.05 µl/per substance
b. PDEGS-column (Mixture 2) 0.05 µl/per substance

With the application of a temperature program, capillary columns proved suitable for the separation of the two mixtures. Because of its high temperature loadability, a 25 m PDEGS column gave satisfactory results (mixture 2) for the separation of aliphatic, cyclo-aliphatic and ar-aliphatic nitrosamines with a higher boiling point. A polypropylene glycol column (UCON LB 550 X) = R-column - alkalized by the Cieplinski method (Ref. 14) (with which the lower temperature loadability excluded the separation of substances with a higher boiling point) exhibited suitable separation ability (mixture 1). Alkalization occurred on account of the otherwise disturbing band sweeping effect (? "Bandenschweifung" band curving ?).

For all the nitrosamines, the mass spectra were taken on a double-focusing mass spectrometer (MAT SM 1) at 70 eV/300 μA (resolution about 20000) and at the same time exact mass determinations of typical fragment-ions were performed by the peak-match method. The individual nitrosamines exhibited characteristic and reproducible spectra, so that a definite correlation was possible.

The decomposition of nitrosamines in the mass spectrometer was investigated in detail through measurements of metastable ions and with the mass spectra of specifically deuterated nitrosamines. The governing functional relations found provide the possibility of a mass spectrometric analysis of unknown nitrosamines and preclude the presence of representative of other classes of substances.

A definite correlation of the individual peaks was made possible through the combination of capillary gas chromatography with a rapidly recording mass spectrometer (ATLAS CH 4), the column outlet leading directly through a connecting rod into the ion source of the mass spectrometer. This combination permits a safe separation and identification of different substances, the retention periods of which differ by a few seconds only.

Figure 1 exhibits the gas chromatogram of the gas chromatography - mass spectrometry combination of mixture 1; the mass spectrum related to each peak respectively permits a definite correlation. The same is true for mixture 2. Even complicated isomer mixtures of nitrosamines can thus be efficiently separated and definitely correlated.

Under the working conditions of the gas chromatographic separation of mixture 1 (Figure 1), the limit of determination is of the order of 0.2 - 0.5 γ/per substance. Through a change of the distribution ratio, the limit of determination could no doubt still be lowered, but at the expense of satisfactory separation. The limit of determination for the mass spectrometric detection is of the order of 0.01 γ/per substance in the ion source.

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EIN SPEZIFISCHES ANALYTISCHES NACHWEISVERFAHREN FÜR NITROSAMINE DURCH KOMBINATION VON GASCHROMATOGRAPHIE MIT DER MASSENSPEKTROMETRIE

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Seit der Entdeckung der krebserregenden Wirkung von Dimethylnitrosamin durch Magee und Barnes[1] stehen die N-Nitrosamine im Mittelpunkt der Krebsforschung. Mehrere Arbeitskreise beschäftigten sich mit ihren organspezifischen karzinogenen Wirkungen. Das Vorkommen von Nitrosaminen wurde in Naturprodukten und Nahrungsmitteln beschrieben, so u.a. im Pilz Clitocybe suavoelens, in Streptomyceen und Solanaceen, in Weizenpflanzen und Weizenmehl, in Tabak und Tabakrauch, in pasteurisierter Milch und Käse, in Pökelfleisch und Heringsmehl [2].

Als analytische Nachweisverfahren dienten bisher die Dünnschichtchromatographie[3], die Colorimetrie[4], die UV-[4,5], IR-[6] und Fluoreszenzspektroskopie[6], die Polarographie[7,8] und die Gaschromatographie [9-13] mit gepackten Säulen. Diese Verfahren sind für die Analytik komplizierter Stoffgemische überwiegend nicht genügend spezifisch.

Wir haben als empfindliches und substanzspezifisches kombiniertes Trenn- und Nachweisverfahren für Nitrosamine die Kopplung von Kapillargaschromatographie und Massenspektrometrie ausgearbeitet. Folgende Nitrosamine wurden dargestellt - in Tab. 1 in Reihenfolge steigender Retentionswerte aufgeführt - und in zwei Modellgemischen gaschromatographisch untersucht:

Tabelle 1

a.) R-Säule (Gemisch 1) 0.05 µl/pro Substanz	b.) PDEGS-Säule (Gemisch 2) 0.05 µl/pro Substanz
1. Dimethyl-N-NO	1. n-Pentyl-methyl-N-NO
2. Diäthyl-N-NO	2. N-Nitrosopiperidin
3. Di-isopropyl-N-NO	3. Phenyl-n-butyl-N-NO
4. n-Propyl-i-propyl-N-NO	4. N-Nitrosomorpholin
5. Di-n-propyl-N-NO	5. Cyclohexyl-methyl-N-NO
6. Äthyl-n-butyl-N-NO	6. Di-n-hexyl N-NO
7. Di-isobutyl-N-NO	7. Benzyl-methyl-N-NO
8. n-Pentyl-äthyl-N-NO	
9. sec-Butyl-n-butyl-N-NO	
10. n-Pentyl-i-propyl-N-NO	
11. Di-n-butyl-N-NO	
12. n-Pentyl-n-propyl-N-NO	
13. n-Pentyl-sec-butyl-N-NO	
14. Di-isopentyl-N-NO	
15. n-Pentyl-n-butyl-N-NO	
16. n-Pentyl-iso-pentyl-N-NO	
17. Di-n-Pentyl-N-NO	
18. Di-n-Hexyl-N-NO	

Kapillarsäulen unter Anwendung eines Temperaturprogramms erwiesen sich für die Trennung der beiden Gemische als geeignet. Eine 25 m PDEGS-Säule lieferte wegen ihrer hohen Temperaturbelastbarkeit für die Trennung höher siedender aliphatischer, cyclo-aliphatischer und ar-aliphatischer Nitrosamine gute Ergebnisse (Gemisch 2). Eine nach Cieplinski[14] alkalisierte Polypropylenglykol-Säule (UCON LB 550 X) = R-Säule , bei der die geringere Temperaturbelastbarkeit die Trennung höher siedender Substanzen ausschloß, zeigte gute Trennleistung (Gemisch 1). Die Alkalisierung erfolgte wegen der sonst störenden Bandenschweifung.

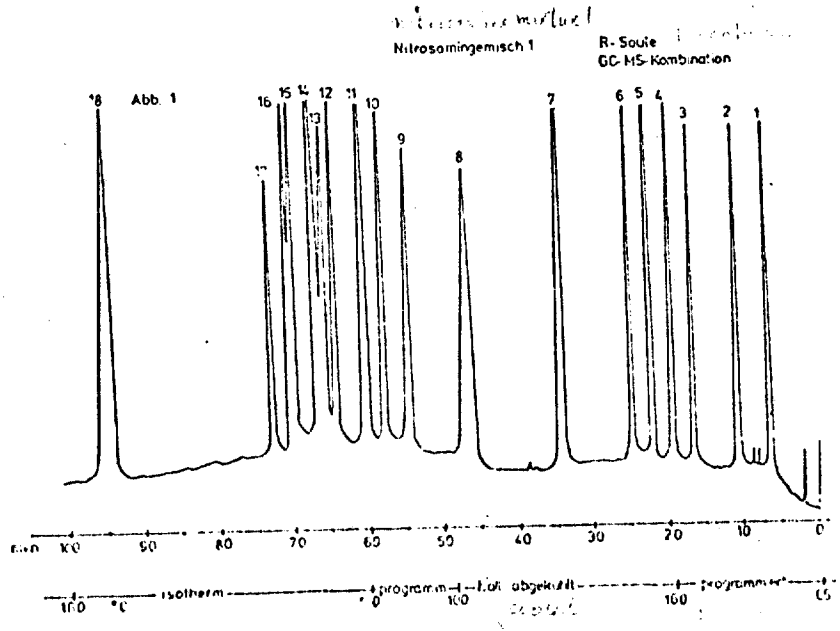
Von allen Nitrosaminen wurden die Massenspektren an einem doppeltfokussierenden Massenspektrometer (MAT SM 1) bei 70 eV/300µA (Auflösung ca 20000) aufgenommen und zugleich genaue Massenbestimmungen von typischen Fragment-Ionen nach der Peak-Match-Methode durchgeführt. Die einzelnen Nitrosamine zeigten charakteristische und reproduzierbare Spektren, die eine eindeutige Zuordnung erlaubten.

Der Zerfall von Nitrosaminen im Massenspektrometer wurde durch Messungen metastabiler Ionen und mit den Massenspektren spezifisch deuterierter Nitrosamine eingehend untersucht. Die aufgefundenen Gesetzmäßigkeiten geben die Möglichkeit einer massenspektrometrischen Analyse unbekannter Nitrosamine und ermöglichen, das Vorliegen von Vertretern anderer Substanzklassen auszuschließen.

Durch die Kombination der Kapillargaschromatographie mit einem schnell registrierenden Massenspektrometer (ATLAS CH 4), wobei der Säulenausgang direkt über eine Schubstange in die Ionenquelle des Massenspektrometers führt, wurde eine eindeutige Zuordnung der einzelnen Peaks ermöglicht. Diese Kombination erlaubt eine sichere Trennung und Identifizierung verschiedener Substanzen, deren Retentionszeiten nur um wenige Sekunden differieren.

Abb. 1 zeigt das Gaschromatogramm der Kombination Gaschromatographie-Massenspektrometrie von Gemisch 1, wobei das zu jedem Peak gehörende Massenspektrum die eindeutige Zuordnung ermöglicht. Gemisch 2 verhält sich analog. Auch komplizierte Isomerengemische von Nitrosaminen lassen sich damit einwandfrei trennen und eindeutig zuordnen.

Unter den Arbeitsbedingungen der gaschromatographischen Trennung von Gemisch 1 (Abb. 1) liegt die Erfassungsgrenze im Bereich 0.2 - 0.5 γ/pro Substanz. Durch eine Änderung des Teilungsverhältnisses könnte die Erfassungsgrenze zwar noch heruntergesetzt werden, allerdings nur auf Kosten der guten Trennleistung. Die Erfassungsgrenze für den massenspektrometrischen Nachweis liegt bei 0.01 γ/pro Substanz in der Ionenquelle.



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Tetrahedron Letters No. 10, pp. 737-740, 1970. Pergamon Press. Printed in Great Britain.

EIN SPEZIFISCHES ANALYTISCHES NACHWEISVERFAHREN FÜR NITROSAMINE DURCH KOMBINATION VON GASCHROMATOGRAPHIE MIT DER MASSENSPEKTROMETRIE

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(Received in Germany 29 December 1969; received in UK for publication 26 January 1970)

Seit der Entdeckung der krebserregenden Wirkung von Dimethylnitrosamin durch Magee und Barnes[1] stehen die N-Nitrosamine im Mittelpunkt der Krebsforschung. Mehrere Arbeitskreise beschäftigten sich mit ihren organspezifischen karzinogenen Wirkungen. Das Vorkommen von Nitrosaminen wurde in Naturprodukten und Nahrungsmitteln beschrieben, so u.a. im Pilz Clitocybe suavoelens, in Streptomyceen und Solanaceen, in Weizenpflanzen und Weizenmehl, in Tabak und Tabakrauch, in pasteurisierter Milch und Käse, in Pökelfleisch und Heringsmehl[2].

Als analytische Nachweisverfahren dienten bisher die Dünnschichtchromatographie[3], die Colorimetrie[4], die UV-[4,5], IR-[6] und Fluoreszenzspektroskopie[6], die Polarographie[7,8] und die Gaschromatographie[9-13] mit gepackten Säulen. Diese Verfahren sind für die Analytik komplizierter Stoffgemische überwiegend nicht genügend spezifisch.

Wir haben als empfindliches und substanzspezifisches kombiniertes Trenn- und Nachweisverfahren für Nitrosamine die Kopplung von Kapillargaschromatographie und Massenspektrometrie ausgearbeitet. Folgende Nitrosamine wurden dargestellt - in Tab. 1 in Reihenfolge steigender Retentionswerte aufgeführt - und in zwei Modellgemischen gaschromatographisch untersucht:

Der Zerfall von Nitrosaminen im Massenspektrometer wurde durch Messungen metastabiler Ionen und mit den Massenspektren spezifisch deuterierter Nitrosamine eingehend untersucht. Die aufgefundenen Gesetzmäßigkeiten geben die Möglichkeit einer massenspektrometrischen Analyse unbekannter Nitrosamine und ermöglichen, das Vorliegen von Vertretern anderer Substanzklassen auszuschließen.

Durch die Kombination der Kapillargaschromatographie mit einem schnell registrierenden Massenspektrometer (ATLAS CH 4), wobei der Säulenausgang direkt über eine Schubstange in die Ionenquelle des Massenspektrometers führt, wurde eine eindeutige Zuordnung der einzelnen Peaks ermöglicht. Diese Kombination erlaubt eine sichere Trennung und Identifizierung verschiedener Substanzen, deren Retentionszeiten nur um wenige Sekunden differieren.

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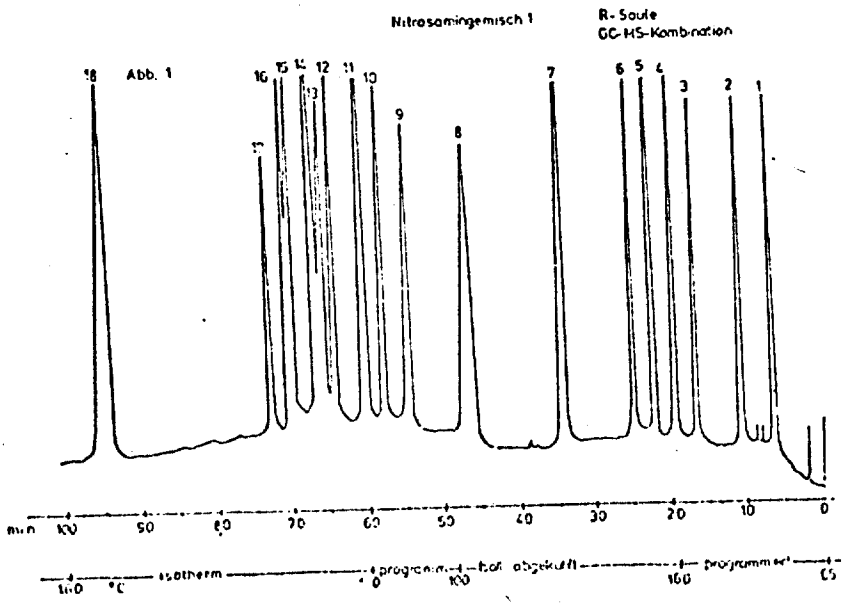

Tabelle 1

a.) R-Säule (Gemisch 1) 0.05 µl/pro Substanz	b.) PDEGS-Säule (Gemisch 2) 0.05 µl/pro Substanz
1. Dimethyl-N-NO	1. n-Pentyl-methyl-N-NO
2. Diäthyl-N-NO	2. N-Nitrosopiperidin
3. Di-isopropyl-N-NO	3. Phenyl-n-butyl-N-NO
4. n-Propyl-i-propyl-N-NO	4. N-Nitrosomorpholin
5. Di-n-propyl-N-NO	5. Cyclohexyl-methyl-N-NO
6. Äthyl-n-butyl-N-NO	6. Di-n-hexyl N-NO
7. Di-isobutyl-N-NO	7. Benzyl-methyl-N-NO
8. n-Pentyl-äthyl-N-NO	
9. sec-Butyl-n-butyl-N-NO	
10. n-Pentyl-i-propyl-N-NO	
11. Di-n-butyl-N-NO	
12. n-Pentyl-n-propyl-N-NO	
13. n-Pentyl-sec-butyl-N-NO	
14. Di-isopentyl-N-NO	
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COMMENTS ON THE DEVELOPMENT OF NITROSAMINES DURING THE REACTION OF MONOSACCHARIDES WITH AMINOACIDS (MAILLARD REACTION)

(Zur Frage der Entstehung von Nitrosaminen bei der Reaktion von Monosacchariden mit Aminosäuren (Maillard - Reaktion))

Authors: K. Heyns and H. Koch
Source: Tetrahedron Letters No. 10, P. 741-744, 1970. Pergamon Press.

Recently Devik (Ref. 1) published the alarming fact that nitrosamines, such as dimethyl-, diethyl-, dipropyl- and dibutyl-N-nitrosamine, which are known to be considerably cancerogenic (Ref. 2), develop during the Maillard reaction through a reaction that is widely distributed among natural substances and foodstuffs.

We conducted investigations of the Maillard reaction through the combination of capillary gas chromatography and mass spectrometry (Ref. 3) as well as by polarographic and thin layer chromatographic methods.

The reaction evaluations (? formulations "Ansätze") between glucose and amino acids were performed under standard conditions. The glucose/alanine-, glucose/lysine- and glucose/glutaminic acid- mixtures (molar ratio 1:2), absorbed on two potato starches, were adjusted to the required pH value, dry-frozen (exlusion of fungi), subjected for equal periods to a temperature treatment of 100-105 C and then disstilled by the method of Lydersen and Nagy (Ref. 4), with additional cooling of the rotation evaporator with ice-water. The distillate was extracted with methylene dichloride and was dried over Na_2SO_4. After careful evaporation of the solvent, yellow to brown liquid residues were obtained. As is known, during the Maillard reaction, the following classes of substances develop, among others (Ref. 5): pyrazines (Ref. 6, 7), pyrroles, furanes, lower alcohols, aldehydes, ketones, Schiff's bases. The low-boiling alcohols are distilled off during the thermal treatment, and the aldehydes, ketones and Schiff's bases

are destroyed during the treatment with 3n NaOH - as well as the furanes and pyrroles - beside the acetylated products. Thus the analytic problem is essentially reduced to the detection of pyrazines, acetylated pyrroles and furanes as well as of the nitrosamines indicated by Devik. Since the pyrazine and also nitrosamine mixtures, used for comparison, were separable on the same capillary columns and were identifiable without error with the help of the mass spectra (Ref. 8), it was possible also to investigate the yellow extracts of the above mentioned variants of the Maillard reaction through the already referred new method of the GC - MS combination respectively. Figure 1 exhibits a typical chromatogram. The other two chromatograms are similar.

The arrows designate the places where the nitrosamines should appear, when they existed.

Characterizing the fractions was performed through the mass spectra, and also through the gas chromatographically identical behavior. The efficiency of the working and detecting method used was demonstrated through test mixtures in which the nitrosamines were added in corresponding concentrations during the absorption of the aqueous glucose/aminoacid mixtures on the potato starch.

In order to verify the results obtained by Devik by the polarographic method, nitrosamines and nitrosamine mixtures as well as pyrazines and pyrazine mixtures were polarographied under identical conditions (Ref. 4). Figure 2 exhibits a typical polarogram of the easily volatile Maillard products of a glucose - alanine mixture. The other two polarograms are similar.

Comparing with a polarogram of the mixture of dimethyl-, diethyl-, dipropyl- and dibutyl- nitrosamines shows that here the splitting into individual steps cannot take place, because the half step potentials in each case differ only by 0.06 V. The polarogram - also shown in Figure 2 - of a mixture of pyrazine, 2-methyl- and 2.5 dimethyl pyrazine exhibit complete accord with the polarogram of the Maillard products. Thin layer chromatograpic investigations showed that with a coloration of the plates with diphenyl amine/$PdCl_2$ and with UV radiation two spots result, one of which resided between the purple-brown spots of

dimethyl- and diethyl nitrosamine and had a pink coloration. The second spot lay somewhat above the spot of dibutyl nitrosamines and had a grey-brown color. Through comparison chromatograms, it was possible to attributed the first spot to acetyl pyrrole. The second spot differred through its grey-brown color definitely from the color of the corresponding nitrosamine, and also lay somewhat higher.

The analytical results can be summarized as follows: With the help of the GC - MS combination, the presence of nitrosamines can be exluded. The polarographic results show that in steps II and III in Figure 2 4-electron processes in the reduction of nitrosamines cannot be involved, but rather non-diffusion controlled processes in the depolarization of methylated pyrazines (Ref. 10, 11). Step I can be attributed to a 2-electron process during the formation of the semiquinone from the pyrazines. The thin layer chromatographic investigations also show that in the spots that appeared, nitrosamines are not involved.

Tetrahedron Letters No.10, pp. 741-744, 1970. Pergamon Press. Printed in Great Britain.

ZUR FRAGE DER ENTSTEHUNG VON NITROSAMINEN BEI DER REAKTION VON MONOSACCHARIDEN MIT AMINOSÄUREN (MAILLARD - REAKTION)

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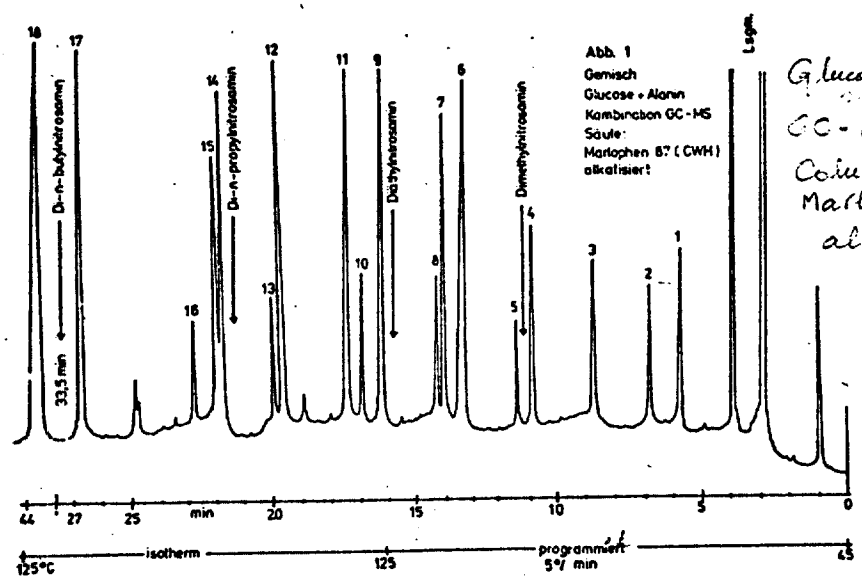
Vor kurzem wurde von Devik[1] die alarmierende Feststellung veröffentlicht, daß Nitrosamine wie Dimethyl-, Diäthyl-, Dipropyl- und Dibutyl-N-nitrosamin, die bekanntlich stark cancerogen sind [2], bei der Maillard-Reaktion durch einen in Naturstoffen und Nahrungsmitteln weitverbreiteten Reaktionsablauf entstehen.

Wir haben Untersuchungen an der Maillard-Reaktion mittels der Kombination von Kapillargaschromatographie und Massenspektrometrie[3] sowie mit polarographischen und dünnschichtchromatographischen Methoden durchgeführt.

Die Reaktionsansätze zwischen Glucose und Aminosäuren wurden unter Standardbedingungen durchgeführt. Die auf zwei Kartoffelstärken absorbierten Glucose/Alanin-, Glucose/Lysin- und Glucose/Glutaminsäure-Gemische (molares Verhältnis 1 : 2) wurden auf den erforderlichen pH-Wert eingestellt (Glaselektrode), gefriergetrocknet (Ausschluß von Pilzbefall), gleichen Zeiten einer Temperaturbehandlung von 100-105°C unterworfen und anschließend nach der Methode von Lydersen und Nagy[4] destilliert, bei zusätzlicher Kühlung des Rotationsverdampfers mit Eiswasser. Das Destillat wurde mit Methylendichlorid extrahiert und über Na_2SO_4 getrocknet. Nach vorsichtigem Abdampfen des Lösungsmittels wurden gelbe bis braune flüssige Rückstände erhalten. Es ist bekannt, daß bei der Maillard-Reaktion u.a. folgende Substanzklassen entstehen[5]: Pyrazine[6,7], Pyrrole, Furane, niedere Alkohole, Aldehyde, Ketone, Schiff'sche Basen. Die niedrig siedenden Alkohole werden bei der Temperaturbehandlung abdestilliert, die Aldehyde, Ketone und Schiff'schen Basen werden bei der Behandlung mit 3n NaOH zerstört - ebenso Furane und Pyrrole - außer den acetylierten Produkten. Damit reduziert sich das analytische Problem im wesentlichen auf die Fahndung nach Pyrazinen, acetylierten Pyrrolen und Furanen sowie den von Devik oben angegebenen Nitrosaminen. Da die zum Vergleich eingesetzten Pyrazin- und auch Nitrosamin-Gemische auf den gleichen Kapillarsäulen trennbar waren und mit Hilfe der Massenspektren eindeutig identifiziert werden konnten[8], ließen sich auch die gelben bis braunen Extrakte der oben

ausgeführten Variante der Maillard-Reaktion durch die bereits erwähnte neue Methode der Kombination GC - MS entsprechend untersuchen. Abb. 1 zeigt ein typisches Chromatogramm. Die beiden anderen Chromatogramme sind analog.



Glucose-alanine mixture.
GC-MS combination.
Column.
Marlophen 87 (CWH) alkalized.

Explanation of the numbers in Figure 1:

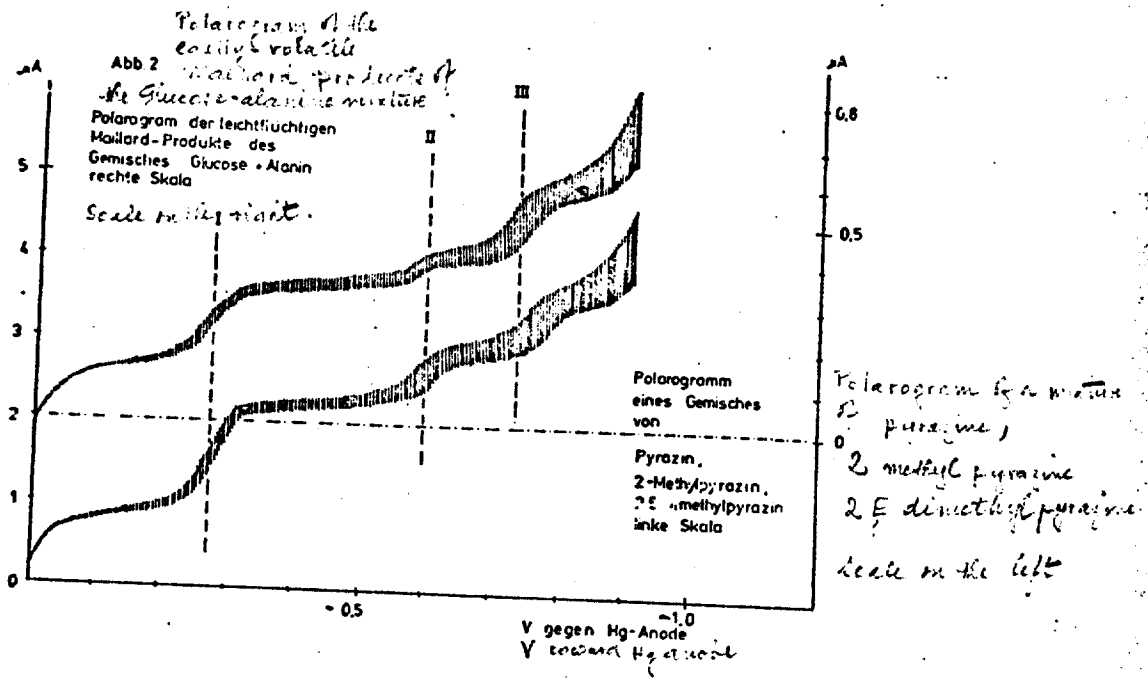
Erklärung der Zahlen in Abb. 1 :

1: MZ 86
2: Pyridin
3: MZ 85
4: Keton, MZ nicht bestimmbar
5: 2-Methylpyrazin
6: Alkohol, MZ nicht bestimmbar
7: MZ 100
8: 2,5-Dimethylpyrazin
9: Dichlorpropen
10: Methyl-äthylpyrazin
11: 2-Acetylfuran
12: 2,5-Dimethyl-3-Et-pyrazin
13: 2,6-Dimethyl-3-Et-pyrazin
14: iso-Propyl-Et-pyrazin + Furfurylalkohol
15: Alkylpyrazin MZ 150
16: Alkylpyrazin MZ 164
17: höher alkyliertes Pyrazin
18: 2-Acetylpyrrol

Die Pfeile markieren die Stellen, an denen Nitrosamine auftreten müßten, wenn sie vorhanden wären.

Die Charakterisierung der Fraktionen erfolgte durch die Massenspektren und daneben auch durch gaschromatographisch identisches Verhalten. Die Leistungsfähigkeit der angewandten Arbeits- und Nachweismethoden wurde durch Testgemische bewiesen, bei denen die Nitrosamine in entsprechenden Konzentrationen bei der Absorption der wäßrigen Glucose/Aminosäure-Gemische auf der Kartoffelstärke zugesetzt wurden.

Zur Überprüfung der von Devik auf polarographischem Wege erhaltenen Ergebnisse wurden sowohl reine Nitrosamine und Nitrosamingemische als auch Pyrazine und Pyrazingemische unter identischen Bedingungen polarographiert[4]. Abb. 2 zeigt ein typisches Polarogramm der leichtflüchtigen Maillard-Produkte von einem Glucose/Alanin-Gemisch. Die beiden anderen Polarogramme sind analog.



Der Vergleich mit einem Polarogramm des Gemisches von Dimethyl-, Diäthyl-, Dipropyl- und Dibutyl-nitrosamin zeigt, daß hier die Aufspaltung in einzelne Stufen nicht erfolgen kann, weil die Halbstufenpotentiale sich jeweils nur um 0,06 V unterscheiden. Das ebenfalls in Abb. 2 aufgeführte Polarogramm eines Gemisches von Pyrazin, 2-Methyl- und 2,5-Dimethylpyrazin zeigt völlige Übereinstimmung mit dem Polarogramm der Maillard-Produkte. Dünnschichtchromatographische Untersuchungen zeigten, daß sich beim Anfärben der Platten mit Diphenylamin/$PdCl_2$ und UV-Bestrahlung zwei Flecke ergeben, von denen einer zwischen den violett-braunen Flecken von Dimethyl- und Diäthylnitrosamin lag und eine rosa Färbung hatte. Der zweite Fleck lag etwas oberhalb des Fleckes von Dibutylnitrosamin und hatte eine grau-braune Färbung. Durch Vergleichschromatogramme konnte der erste Fleck dem Acetylpyrrol zugeordnet werden. Der zweite Fleck unterschied sich durch die grau-braune Färbung deutlich von der Färbung des entsprechenden Nitrosamins und lag zudem etwas höher.

Die analytischen Ergebnisse lassen sich wie folgt zusammenfassen: Mit Hilfe der GC-MS-Komb. ist das Vorhandensein von Nitrosaminen auszuschließen. Die polarographischen Ergebnisse zeigen, daß es sich bei den Stufen II und III in Abb. 2 nicht um 4-Elektronenprozesse bei der Reduktion von Nitrosaminen handeln kann, sondern um nicht diffusionskontrollierte Prozesse

bei der Depolarisation von methylierten Pyrazinen[10,11]. Die Stufe I kann einem 2-Elektronenprozeß bei der Bildung des Semichinons aus den Pyrazinen zugeordnet werden. Auch die dünnschichtchromatographischen Untersuchungen zeigen, daß es sich bei den auftretenden Flecken nicht um Nitrosamine handelt.

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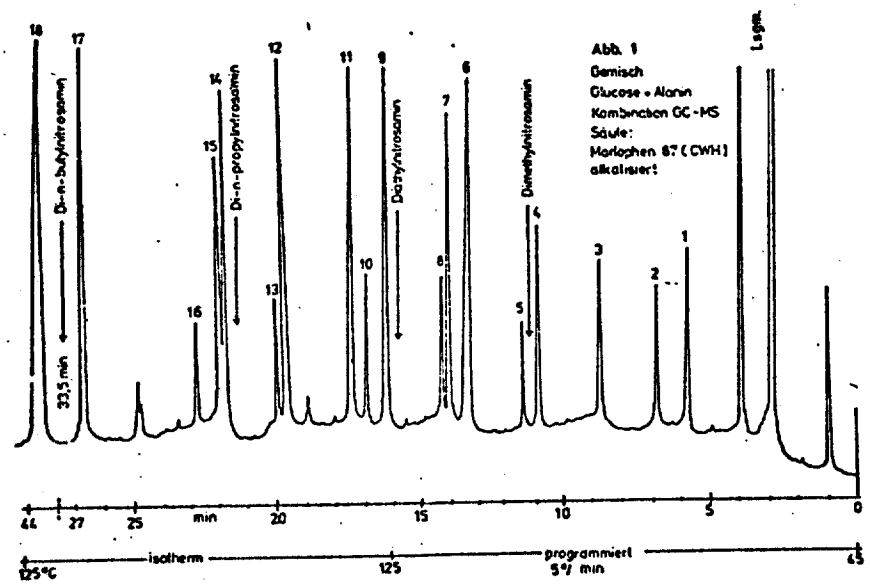
Vor kurzem wurde von Devik[1] die alarmierende Feststellung veröffentlicht, daß Nitrosamine wie Dimethyl-, Diäthyl-, Dipropyl- und Dibutyl-N-nitrosamin, die bekanntlich stark cancerogen sind [2], bei der Maillard-Reaktion durch einen in Naturstoffen und Nahrungsmitteln weitverbreiteten Reaktionsablauf entstehen.

Wir haben Untersuchungen an der Maillard-Reaktion mittels der Kombination von Kapillargaschromatographie und Massenspektrometrie[3] sowie mit polarographischen nd dünnschichtchromatographischen Methoden durchgeführt.

Die Reaktionsansätze zwischen Glucose und Aminosäuren wurden unter Standardbedingungen durchgeführt. Die auf zwei Kartoffelstärken absorbierten Glucose/Alanin-, Glucose/Lysin- und Glucose/Glutaminsäure-Gemische (molares Verhältnis 1 : 2) wurden auf den erforderlichen pH-Wert eingestellt (Glaselektrode), gefriergetrocknet (Ausschluß von Pilzbefall), gleichen Zeiten einer Temperaturbehandlung von 100-105°C unterworfen und anschließend nach der Methode von Lydersen und Nagy[4] destilliert, bei zusätzlicher Kühlung des Rotationsverdampfers mit Eiswasser. Das Destillat wurde mit Methylendichlorid extrahiert und über Na_2SO_4 getrocknet. Nach vorsichtigem Abdampfen des Lösungsmittels wurden gelbe bis braune flüssige Rückstände erhalten. Es ist bekannt, daß bei der Maillard-Reaktion u.a. folgende Substanzklassen entstehen[5]: Pyrazine[6,7], Pyrrole, Furane, niedere Alkohole, Aldehyde, Ketone, Schiff'sche Basen. Die niedrig siedenden Alkohole werden bei der Temperaturbehandlung abdestilliert, die Aldehyde, Ketone und Schiff'schen Basen werden bei der Behandlung mit 3n NaOH zerstört - ebenso Furane und Pyrrole - außer den acetylierten Produkten. Damit reduziert sich das analytische Problem im wesentlichen auf die Fahndung nach Pyrazinen, acetylierten Pyrrolen und Furanen sowie den von Devik oben angegebenen Nitrosaminen. Da die zum Vergleich eingesetzten Pyrazin- und auch Nitrosamin-Gemische auf den gleichen Kapillarsäulen trennbar waren und mit Hilfe der Massenspektren eindeutig identifiziert werden konnten[8], ließen sich auch die gelben bis braunen Extrakte der oben

ausgeführten Variante der Maillard-Reaktion durch die bereits erwähnte neue Methode der Kombination GC - MS entsprechend untersuchen. Abb. 1 zeigt ein typisches Chromatogramm. Die beiden anderen Chromatogramme sind analog.



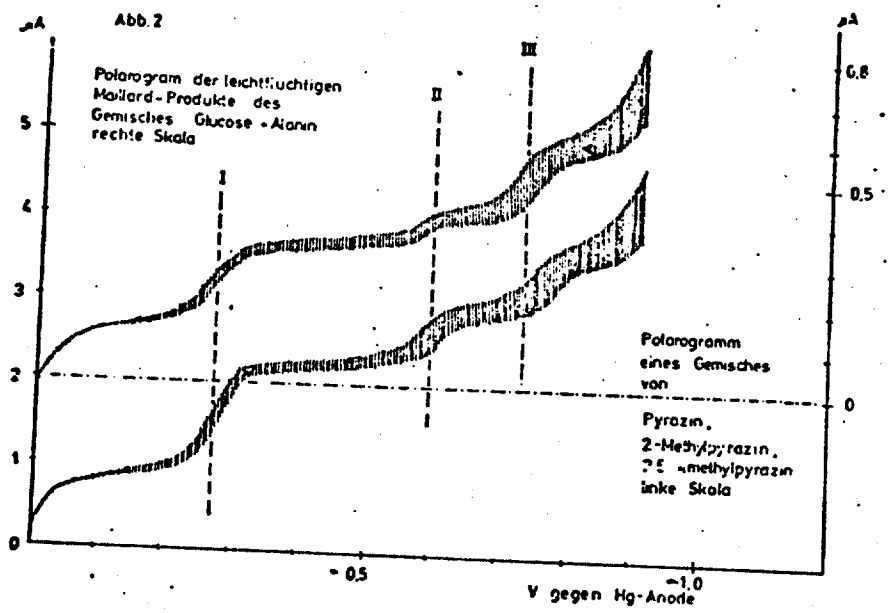
Erklärung der Zahlen in Abb. 1 :

1: MZ 86
2: Pyridin
3: MZ 85
4: Keton, MZ nicht bestimmbar
5: 2-Methylpyrazin
6: Alkohol, MZ nicht bestimmbar
7: MZ 100
8: 2,5-Dimethylpyrazin
9: Dichlorpropen
10: Methyl-äthylpyrazin
11: 2-Acetylfuran
12: 2,5-Dimethyl-3-Et-pyrazin
13: 2,6-Dimethyl-3-Et-pyrazin
14: iso-Propyl-Et-pyrazin + Furfurylalkohol
15: Alkylpyrazin MZ 150
16: Alkylpyrazin MZ 164
17: höher alkyliertes Pyrazin
18: 2-Acetylpyrrol

Die Pfeile markieren die Stellen, an denen Nitrosamine auftreten müßten, wenn sie vorhanden wären.

Die Charakterisierung der Fraktionen erfolgte durch die Massenspektren und daneben auch durch gaschromatographisch identisches Verhalten. Die Leistungsfähigkeit der angewandten Arbeits- und Nachweismethoden wurde durch Testgemische bewiesen, bei denen die Nitrosamine in entsprechenden Konzentrationen bei der Absorption der wäßrigen Glucose/Aminosäure-Gemische auf der Kartoffelstärke zugesetzt wurden.

Zur Überprüfung der von Devik auf polarographischem Wege erhaltenen Ergebnisse wurden sowohl reine Nitrosamine und Nitrosamingemische als auch Pyrazine und Pyrazingemische unter identischen Bedingungen polarographiert[4]. Abb. 2 zeigt ein typisches Polarogramm der leichtflüchtigen Maillard-Produkte von einem Glucose/Alanin-Gemisch. Die beiden anderen Polarogramme sind analog.



Der Vergleich mit einem Polarogramm des Gemisches von Dimethyl-, Diäthyl-, Dipropyl- und Dibutyl-nitrosamin zeigt, daß hier die Aufspaltung in einzelne Stufen nicht erfolgen kann, weil die Halbstufenpotentiale sich jeweils nur um 0,06 V unterscheiden. Das ebenfalls in Abb. 2 aufgeführte Polarogramm eines Gemisches von Pyrazin, 2-Methyl- und 2,5-Dimethylpyrazin zeigt völlige Übereinstimmung mit dem Polarogramm der Maillard-Produkte. Dünnschichtchromatographische Untersuchungen zeigten, daß sich beim Anfärben der Platten mit Diphenylamin/$PdCl_2$ und UV-Bestrahlung zwei Flecke ergeben, von denen einer zwischen den violett-braunen Flecken von Dimethyl- und Diäthylnitrosamin lag und eine rosa Färbung hatte. Der zweite Fleck lag etwas oberhalb des Fleckes von Dibutylnitrosamin und hatte eine grau-braune Färbung. Durch Vergleichschromatogramme konnte der erste Fleck dem Acetylpyrrol zugeordnet werden. Der zweite Fleck unterschied sich durch die grau-braune Färbung deutlich von der Färbung des entsprechenden Nitrosamins und lag zudem etwas höher.

Die analytischen Ergebnisse lassen sich wie folgt zusammenfassen: Mit Hilfe der GC-MS-Komb. ist das Vorhandensein von Nitrosaminen auszuschließen. Die polarographischen Ergebnisse zeigen, daß es sich bei den Stufen II und III in Abb. 2 nicht um 4-Elektronenprozesse bei der Reduktion von Nitrosaminen handeln kann, sondern um nicht diffusionskontrollierte Prozesse

bei der Depolarisation von methylierten Pyrazinen[10,11]. Die Stufe I kann einem 2-Elektronenprozeß bei der Bildung des Semichinons aus den Pyrazinen zugeordnet werden. Auch die dünnschichtchromatographischen Untersuchungen zeigen, daß es sich bei den auftretenden Flecken nicht um Nitrosamine handelt.

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SPECIFIC METHOD OF ANALYSIS FOR THE SEPARATION AND IDENTIFICATION OF NITROSAMINES BY COMBINING CAPILLARY GAS CHROMATOGRAPHY AND MASS SPECTROMETRY

(Ein spezifisches analytisches Trenn- und Nachweisverfahren für Nitrosamine durch Kombination von Capillargaschromatographie und Massenspektrometrie)

Authors: Kurt Heyns and Harald Röper

Source: Z. Levensmittel-Untersuch. u. -Forsch., Vol. 145, No. 2, 1971, P. 69-75

Introduction:

The detection of nitrosamines in biological material, in natural substances and in food, especially at the microscale, is rendered difficult by the presence of a large number of disturbing accompanying elements. A sure identification of nitrosamines, which are important because of the carcinogenic effect of these elements (References 1 - 4), presupposes an efficient specific method of separation and identification.

A combination of capillary gas chromatography and mass spectrometry proved to be suitable in detecting nitrosamines at the microscale. The gas fractions, issuing from the capillary column, can be identified unmistakably with the help of a high-speed recording mass spectrometer in the light of the pertinent mass spectra.

Thin-layer chromatography with special staining reagents (Ref. 5), colorimetry (Ref. 6), the UV- (Ref. 6, 7), IR- (Ref. 8) and fluorescence spectroscopy (Ref. 8), polarography (9, 10) and gas chromatography with packed columns (Ref. 11 - 16) were so far the methods used for the analytical detection of nitrosamines.

Model Substances:

For the gas chromatographic and mass spectrometric investigations, 24 different nitrosamines were used as model substances which proved to be pure gas chromatographically and mass spectrometrically (Table 1).

Table 1: Survey of the model substances. Abbreviations: MG = molar weight rounded off, Ausb = yield, output, ( ) = Values from the literature.

Gas Chromatographic Investigations:

Preliminary tests on capillary columns with a simple model mixture out of dimethyl-, diethyl-, Di-n-propyl- and Di-n-butylnitrosamine exhibited satisfactory separating efficiency (Ref. 17).

These were 1. a 26-m-PDEGS-column, 2. a 25 m alkalined polypropylene glycol column = R-column. The suitability of these column was investigated for the separation of very complex nitrosamine model mixtures with the use of a temperature program.

a Measurements on the R-column. On a 25-m-polypropylene glycol (Ucon LB 550 X) = R-column, alkalined according to Cieplinsky's instructions (Ref. 18) and at a maximum column temperature of 160 C, the retention periods of individual aliphatic nitrosamines were determined (Table 2).

Table 2: Retention periods (t_r) of individual substances: nitrosamine mixture 1. R-column, working conditions: preliminary pressure: 0.6 kp/cm^2, N_2-current: 1.7 ml/min, division ratio: 1:50

kp = kilopond = 980 665 dynes, or 7,866 65 newtons (1 m^3 of air has a weight of about 1.19 kilopond)

Discussion of the Analytical Results:

The alkaline 25-m-R-column (Figure 1) possesses a suitable separating efficiency for straight-chain and branched aliphatic nitrosamines with the use of a temperature program.

Figure 1: Gas chromatographic separation of a nitrosamine mixture (according to Table 1) with the help of a temperature program. R-column.
Figure 2: Gas chromatographic separation of a nitrosamine mixture (according to Table 2) with the help of a temperature program. PDEGS-column.

The 25-m-PDEGS-column is convenient on account of its higher temperature capacity (180 C) for the separation of the aliphatic, cycloaliphatic, araliphatic and heterocyclic nitrosamines, which have a rather high boiling point (Figure 2).

Mas Spectrometric Investigations:

Mas spectra of aliphatic nitrosamines (dimethyl-, diethyl-, di-n-propyl-, di-i-propyl- and di-n-butylnitrosamine) were published for the first time by Collins (Ref. 19). Thirteen years later, Schroll (Ref. 10) and collaborators, within the scope of a mass spectrometric investigation, processed through nitroso-compounds the above mentioned aliphatic nitrosamines and some additional araliphatic, aromatic and heterocyclic nitrosamines. The mass spectra, taken by us, are still published (Ref. 21).

Combination of Gas Chromatography and Mass Spectrometry:

After selection has been made of suitable gas chromatographic capillary columns, and optimum working conditions for the separation of nitrosamine mixtures rich of components have been determined, one obtains a microanalytic separation method which can resolve into their components amounts of specimens of the order of magnitude of a few 1/1000 mg with a very high separating efficiency. The substance specificity of this method is low, because a definite identification of the individual components is not possible on account of the similarity of their retention periods. Through mass spectrometry it is possible to unmistakably identify pure substances in the same order of magnitude because of its high substance specificity. The characteristic mass spectra of the nitrosamines - a prerequisite for their definite identification - exist. By associating directly together capillar gas chromatography and mass spectrometry, a suitable separating effect and a high substance specificity are combined. In this way, it is possible, even in the case of other separation problems, to identify unequivocally nitrosamines and to preclude the presence of representatives of other substance classes.

Table 3: Retention periods t_r of individual substances: nitrosamine mixture 2. PDEGS-column, working conditions: preliminary pressure: 0.6 kp/cm^2, N_2-current: 1.7 ml/min, division ratio: 1:50

The nitrosamine-model mixtures 1 and 2 (Table 2, 3) were separated with this combination and the corresponding nitrosamines were coordinated with the gas chromatographic peaks by taking the mass spectra with the help of a high-speed recording mass spectrometer (Figure 3).

Figure 3: Gas chromatographic separation of nitrosamine mixtures (1 + 2) in combination with mass spectrometer with the help of a temperature program
a. R-column b. PDEGS-column

The heatable column outlet of the capillary column leads through a connecting rod directly to the ion source of a high-speed recording mass spectrometer. The recording of the mass spectra is performed by means of a light spot recording device. The preliminary pressure of the carrier gas (He) was adjusted to mass spectrometer conditions.

Disscussion of the Analytical Results:

The gas chromatographic separation of the nitrosamine mixture 1 in the combination (Figure 3) is satisfactory and is comparable in quality with the separation obtained without combination with the mass spectrometer (Figure 1). The displacement in the retention periods is conditioned by the modified temperature program and by the changed pressure conditions. The separation of the nitrosamine mixture 2 without combination (Figure 2) is satisfactory, if one disregards the band curving (? = Bandschweifung ?) of the nitrosamine which boil at a higher temperature. In the combination, the nitrosamine mixture 2 (Figure 3) separates well up to peak No. 6.

All the gas chromatographic peaks of the nitrosamine mixture were definitely coordinated with the corresponding nitrosamines, through the taking of mass spectra.

Limit of Determination. Under the working conditions of the gas chromatographic separation of nitrosamine mixture 1 (Figure 1 and 3) the limit of determination extends from 0.2 to 0.5 μg/per substance. Through a change of the division ratio the limit of determination could no doubt be lowered, but at the expense of good separating efficiency. The determination limit for the mass spectrometric detection is by 0.01 μg/pro substance in the ion source.

Description of the Tests: The gas chromatographic investigations were conducted in a Perkin-Elmer F6 gas chromatograph with a flame ionisation detector and a temperature program.

Table P. 74: a. Working conditions b. Nitrosamine mixture 1 c. Nitrosamine mixture 2 d. Column e. T-program f. Preliminary pressure N_2-current h. Division ratio i. Sensitivity j. Peak numbering k. Image l. Injected volume.

The mass spectra were taken on a double-focusing mass spectrometer MAT SM 1 at a resolution of about 3000

Working conditions:

Electron energy	: 70 eV;	Inlet system	: 170 C
Electron current	: 300 µA;	Direct admission	: 190 C
Admission system	: 200 C	Ion source	: 200 C.

The optimal temperature conditions were determined through heating tests.

The measurements with the combination of gas chromatography and mass spectrometry were taken with a Perkin-Elmer-F6 gas chromatograph with a temperature program (carrier gas He) and an Atlas CH 4 mass spectrometer (electron energy: 70 eV) with a light spot recorder. Detector: pressure measuring ion source.

Table P. 75: a. Working conditions b. Nitrosamine mixture 1 c. Nitrosamine mixture 2 d. Column e. T-program f. Ballistic cooling g. T-program h. Isotherm i. Preliminary pressure j. He-current k. Division ratio l. Peak numbering m. Injected volume.

The preparation of the nitrosamines was performed by nitrositing the corresponding amines with $NaNO_2$ in HCl-acid solution (Ref. 21). The working up of the reaction mixture was obtained through extraction with CH_2Cl_2. The extract was dried over Na_2SO_4, and the solvent was distilled off under decreased pressure. The solid nitrosamines were purified by repeated recrystallisation. Liquid nitrosamines were purified through fractionated distillation over a packed column.

The preparation of the secondary amines, as far as they did not exist on the market, was performed by the reaction of primary amine - in excess - with alkyl halide in ethanol (Ref. 22). The resulting mixture of primary, secondary, tertiary and quarternary ammonium salts was nitrosited with $NaNO_2$ in HCl-acid solution. The separation of the nitrosamine was obtained through fractionated distillation over a packed column.

Z. Lebensmittel-Untersuch. u. -Forsch., Band 145, Heft 2, 1971

Ein spezifisches analytisches Trenn- und Nachweisverfahren für Nitrosamine durch Kombination von Capillargaschromatographie und Massenspektrometrie

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Eingegangen am 12. August 1970

A Specific Method of Analysis for Separation and Identification of Nitrosamines by Combination of Capillary Gas Chromatography and Mass Spectrometry

Summary. By coupling gas chromatography and mass-spectrometry using special prepared columns we succeeded in finding a specific separating and detecting procedure for nitrosamines in region of microgramms. This procedure was tested with various nitrosamine model-mixtures.

Zusammenfassung. Mit Hilfe der Kombination Gaschromatographie-Massenspektrometrie und speziell präparierter Capillarsäulen wurde ein spezifisches Trenn- und Nachweisverfahren für Nitrosamine im Mikrogramm-Bereich gefunden. Die Wirksamkeit des Verfahrens wurde an verschiedenen Nitrosamin-Modellgemischen erprobt.

Einleitung

Der Nachweis von Nitrosaminen in biologischem Material, in Naturstoffen und Nahrungsmitteln, insbesondere im Mikromaßstab, ist wegen der Anwesenheit von zahlreichen störenden Begleitsubstanzen schwierig. Eine sichere Identifizierung von Nitrosaminen, die wegen der carcinogenen Wirkung dieser Substanzen [1—4] von Wichtigkeit ist, setzt ein leistungsfähiges spezifisches Trenn- und Nachweisverfahren voraus.

Als geeignet für den Nachweis von Nitrosaminen im Mikromaßstab erwies sich eine Kombination von Capillargaschromatographie und Massenspektrometrie. Die aus der Capillarsäule austretenden Gasfraktionen können mit Hilfe eines schnell registrierenden Massenspektrometers anhand der zugehörigen Massenspektren eindeutig identifiziert werden.

Als analytische Nachweisverfahren für Nitrosamine dienten bisher die Dünnschichtchromatographie mit speziellen Anfärbereagentien [5], die Colorimetrie [6], die UV- [6, 7], IR- [8] und Fluorescenzspektroskopie [8], die Polarographie [9, 10] und die Gaschromatographie mit gepackten Säulen [11—16].

Modellsubstanzen

Für die gaschromatographischen und massenspektrometrischen Untersuchungen wurden 24 verschiedene Nitrosamine als Modellsubstanzen verwendet (Tab. 1), die sich gaschromatographisch und massenspektrometrisch als rein erwiesen.

Gaschromatographische Untersuchungen

Vorversuche an Capillarsäulen mit einem einfachen Modellgemisch aus Dimethyl-, Diäthyl-, Di-n-propyl- und Di-n-butylnitrosamin zeigten gute Trennleistungen [17].

Es waren dies eine: 1. 25-m-PDEGS-Säule, 2. 25 m alkalisierte Polypropylenglykol-Säule = R-Säule. Die Eignung dieser Säulen wurde für die Trennung sehr komplexer Nitrosaminmodellgemische unter Verwendung eines Temperaturprogramms untersucht.

a) Messungen an der R-Säule. An einer nach Cieplinsky [18] alkalisierten 25 m-Polypropylenglykol (Ucon LB 550X) = R-Säule wurden bei einer maximalen Säulentemperatur von 160° C die Retentionszeiten einzelner aliphatischer Nitrosamine ermittelt (Tab. 2).

Aus diesen 18 Nitrosaminen wurde durch Mischen gleicher Mengen ein Modellgemisch hergestellt. 1,0 µl des Nitrosamingemisches 1 wurden an der R-Säule getrennt. Dabei stellte sich heraus, daß eine wesentlich bessere Trennung durch Anwendung eines Temperaturprogramms erreicht wurde (Abb. 1).

b) Messungen an der PDEGS-Säule. An einer 25-m-PDEGS-Säule wurden bei einer maximalen Säulentemperatur von 180° C die Retentionszeiten weiterer Einzelsubstanzen ermittelt (Tab. 3).

Aus diesen 7 Einzelsubstanzen wurde wie unter a) beschrieben das Nitrosamingemisch 2 hergestellt. 1,0 µl wurden an der PDEGS-Säule unter Verwendung einer Temperaturprogramms getrennt (Abb. 2).

Tabelle 1. *Übersicht der Modellsubstanzen. Abkürzungen: MG = Mol.-Gew. abgerundet, Ausb. = Ausbeute, ( ) = Werte aus der Literatur*

Nr.	-Nitrosamine	Ausb. %	Smp °C	Sdp °C	n_D^{20}
1	Dimethyl- MG 74	85		153 (153)	1.4395 (1,4392)
2	Diäthyl- MG 102	90		177 (176,9)	1,4410 (1,4400)
3	Di-n-propyl- MG 130	56,4		101—104/25 mm (104—105/22 mm)	1,4487 (1.4478)
4	Di-i-propyl- MG 130	80	46 (46)		
5	i-Propyl-n-propyl- MG 130	56		39/0,12 T (—)	1,4469 (—)
6	Di-n-butyl- MG 158	69		115/17 mm (235)	1.4510 (—)
7	Di-i-butyl- MG 158	62		97/17 mm (123/37 mm)	1,4477 (1,4460)
8	sec-Butyl-n-butyl- MG 158	38		71/0,7 T (—)	1,4519 (—)
9	Di-n-pentyl- MG 186	42		99/0,18 T (—)	1,4540 (—)
10	Di-i-pentyl- MG 186	dargestellt durch Trennung eines Isomerengemisches am präparativen Gaschromatographen			
11	n-Pentyl-i-pentyl- MG 186				
12	Di-n-hexyl- MG 214	70,2		160/15 mm (—)	1,4565 (—)
13	Äthyl-n-butyl- MG 130	62		88/17 mm (—)	1,4468 (—)
14	n-Pentyl-methyl- MG 130	39,4		57/0,55 T (—)	1,4506 (—)
15	n-Pentyl-äthyl- MG 144	30,2		73/0,9 T (—)	1,4517 (—)
16	n-Pentyl-n-propyl- MG 158	82		97/1,4 T (—)	1,4536 (—)
17	n-Pentyl-i-propyl- MG 158	47		52/0,07 T (—)	1,4509 (—)
18	n-Pentyl-n-butyl- MG 172	32		67/0,06 T (—)	1,4540 (—)
19	n-Pentyl-sec-butyl- MG 172	38,2		64/0,08 T (—)	1,4538 (—)
20	Phenyl-n-butyl- MG 178	34,3		148/17 mm (—)	1,5425 (—)
21	Benzyl-methyl- MG 150	85		93/0,14 T (—)	1,5180 (—)
22	Cyclohexyl-methyl- MG 142	61,3		92/1 T (121/12 mm)	1,4909 (—)
23	N-Nitrosopiperidin MG 114	90		110/17 mm (102/13 mm)	1,4958 (1,4950)
24	N-Nitrosomorpholin MG 116	93	29	101/17 mm (224)	(—) (—)

Diskussion des analytischen Ergebnisses

Gute Trennleistung für geradkettige und verzweigte aliphatische Nitrosamine unter Anwendung eines Temperaturprogramms besitzt die alkalisierte 25-m-R-Säule (Abb. 1). Die 25-m-PDEGS-Säule eignet sich wegen ihrer höheren Temperaturbelastbarkeit (180° C) für die Trennung der höher siedenden aliphatischen, cycloaliphatischen, araliphatischen und heterocyclischen Nitrosamine (Abb. 2).

Tabelle 2. *Retentionszeiten (t_r) von Einzelsubstanzen: Nitrosamingemisch 1. R-Säule, Arbeitsbedingungen: Vordruck: 0,6 kp/cm², N_2-Strom: 1,7 ml/min, Teilungsverhältnis: 1:50*

Peak-Nr.	-Nitrosamin	t_r min	Subst. Nr.
1	Dimethylnitrosamin	2,55	1
2	Diäthyl-	3,36	2
3	Di-i-propyl-	4,44	4
4	n-Propyl-i-propyl-	4,90	5
5	Di-n-propyl-	5.50	3
6	Äthyl-n-butyl-	5,72	13
7	Di-i-butyl-	6,95	7
8	n-Pentyl-äthyl-	8,38	15
9	sec-Butyl-n-butyl-	9,53	8
10	n-Pentyl-i-propyl-	10,10	17
11	Di-n-butyl-	11,17	6
12	n-Pentyl-n-propyl-	11,72	16
13	n-Pentyl-sec-butyl-	13,92	19
14	Di-i-pentyl-	15.80	10
15	n-Pentyl-n-butyl-	16,70	18
16	n-Pentyl-i-pentyl-	20,57	11
17	Di-n-pentyl-	26,13	9
18	Di-n-hexyl-	>60	12

Tabelle 3. *Retentionszeiten t_r von Einzelsubstanzen: Nitrosamingemisch 2. PDEGS-Säule, Arbeitsbedingungen: Vordruck: 0,6 kp/cm², N_2-Strom: 1,7 ml/min, Teilungsverhältnis: 1:50*

Peak-Nr.	-Nitrosamin	t_r min	Subst. Nr.
1	n-Pentyl-methylnitrosamin	4,22	14
2	N-Nitrosopiperidin	5,88	23
3	Phenyl-n-butyl-	6,57	20
4	N-Nitrosomorpholin	7,82	24
5	Cyclohexyl-methyl-	8,58	22
6	Di-n-hexyl-	10,42	12
7	Benzyl-methyl-	17,85	21

Massenspektrometrische Untersuchungen

Massenspektren von aliphatischen Nitrosaminen (Dimethyl-, Diäthyl-, Di-n-propyl-, Di-i-propyl- und Di-n-butylnitrosamin) wurden erstmals von Collins [19] veröffentlicht. 13 Jahre später bearbeiteten Schroll [10] u. Mitarb. im Rahmen einer massenspektrometrischen Untersuchung über Nitrosoverbindungen die oben genannten aliphatischen Nitrosamine und einige zusätzliche araliphatische, aromatische und heterocyclische Nitrosamine. Die von uns außerdem aufgenommenen Massenspektren werden noch veröffentlicht [21].

Kopplung von Gaschromatographie und Massenspektrometrie

Nach Auswahl geeigneter gaschromatographischer Capillarsäulen und Erprobung optimaler Arbeitsbedingungen für die Trennung komponentenreicher Nitrosamingemische liegt ein mikroanalytisches Trennverfahren vor, das Probenmengen in der Größenordnung von wenigen $^1/_{1000}$ mg bei sehr hoher Trennwirkung in ihre Komponenten zerlegen kann. Die Stoffspezifität dieser Methode ist gering, weil eine eindeutige Identifizierung der Einzelbestandteile wegen der Ähnlichkeit ihrer Retentionszeiten oft nicht möglich ist. Die Massenspektrometrie gestattet reine Substanzen in der gleichen Größenordnung auf Grund ihrer großen Stoffspezifität eindeutig zu

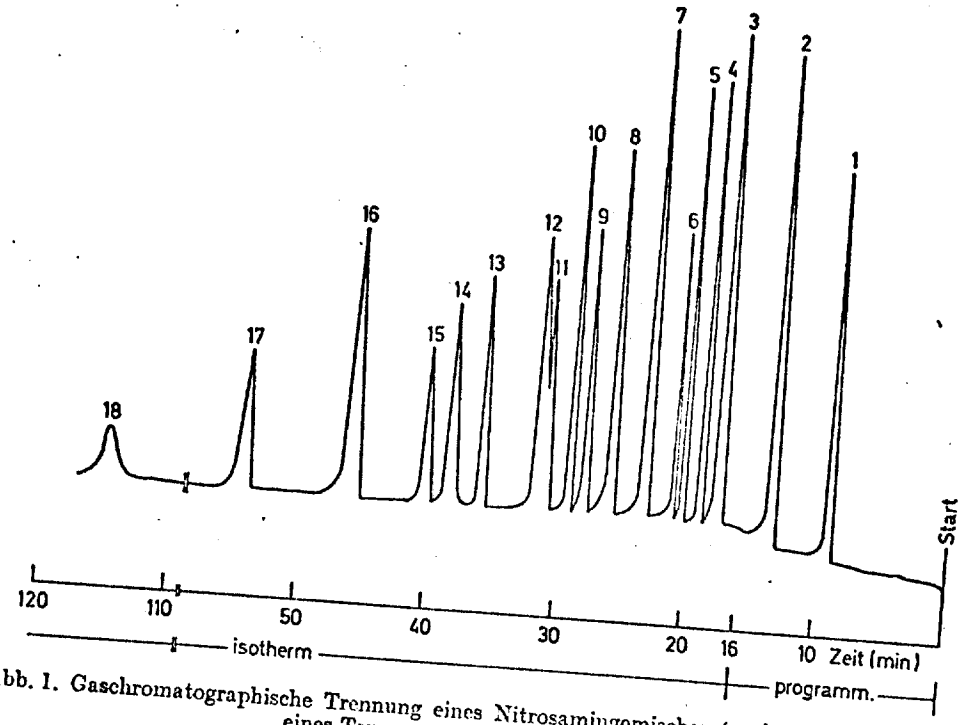

Abb. 1. Gaschromatographische Trennung eines Nitrosamingemisches (nach Tab. 1) mit Hilfe eines Temperaturprogramms. R-Säule

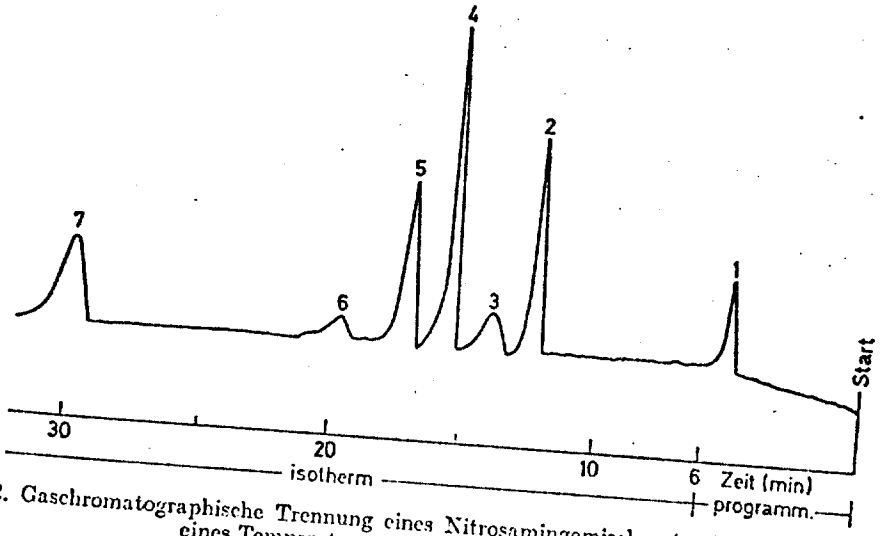

Abb. 2. Gaschromatographische Trennung eines Nitrosamingemisches (nach Tab. 2) mit Hilfe eines Temperaturprogrammes. PDEGS-Säule

identifizieren. Die charakteristischen Massenspektren der Nitrosamine – Voraussetzung für ihre eindeutige Identifizierung – liegen vor. Durch eine direkte Kopplung der Capillargaschromatographie und Massenspektrometrie werden gute Trennwirkung und große Substanzspezifität miteinander vereinigt. Auf diese Weise ist es auch bei

Ein spezif

a) Nitros

b) Nitrosam

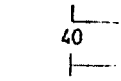

Abb. 3. Gaschromatogra
mit Massenspektrometer

anderen Trennproblem
Vorliegen von Vertre
Die Nitrosamin-M
tion getrennt und d

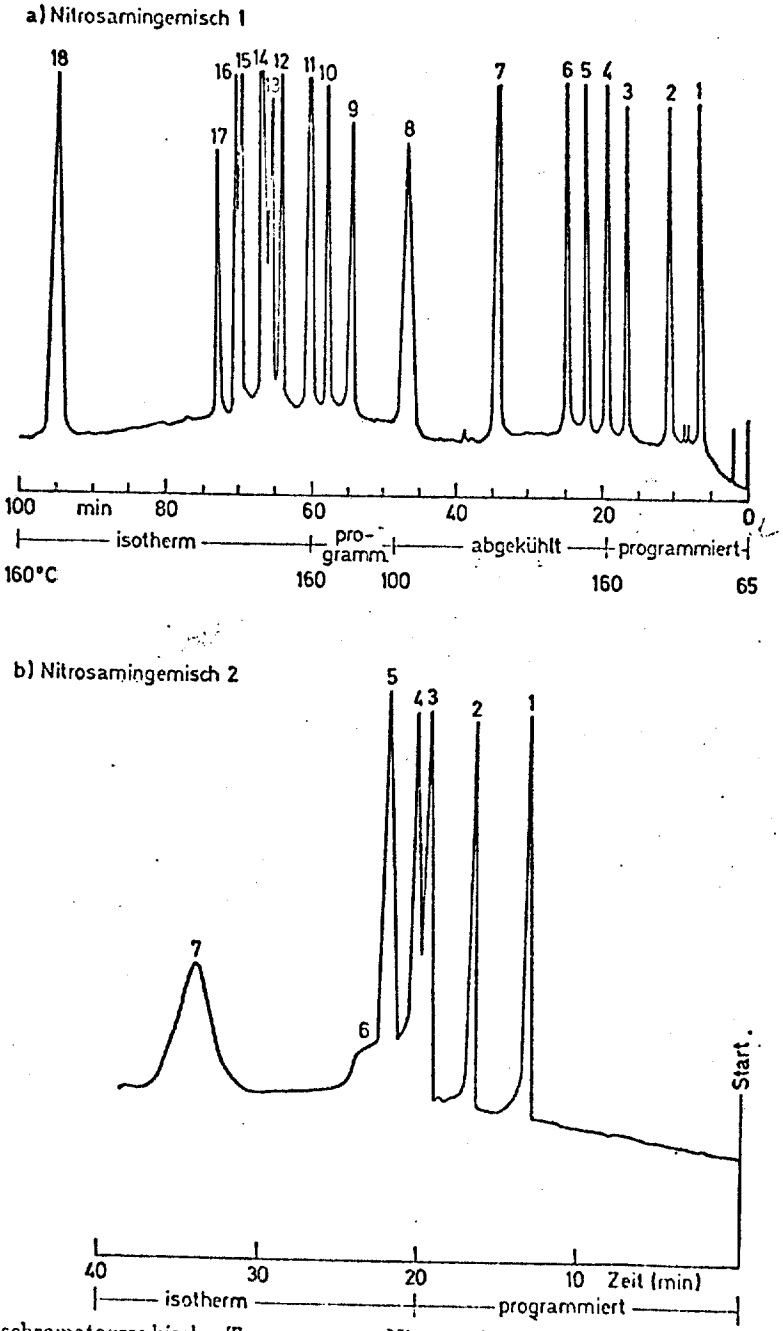

Abb. 3. Gaschromatographische Trennung von Nitrosamingemischen (1 + 2) in Kombination mit Massenspektrometer mit Hilfe eines Temperaturprogrammes. a) R-Säule, b) PDEGS-Säule

anderen Trennproblemen möglich, Nitrosamine eindeutig zu identifizieren und das Vorliegen von Vertretern anderer Substanzklassen auszuschließen.

Die Nitrosamin-Modellgemische 1 und 2 (Tab. 2, 3) wurden mit dieser Kombination getrennt und den gaschromatographischen Peaks durch Aufnahme der Massen-

spektren mit einem schnell registrierenden Massenspektrometer die entsprechenden Nitrosamine zugeordnet (Abb. 3).

Der heizbare Säulenausgang der Capillarsäule führt über eine Schubstange direkt in die Ionenquelle eines schnell registrierenden Massenspektrometers. Die Registrierung der Massenspektren erfolgte mit einem Lichtpunktschreiber. Der Vordruck des Trägergases (He) wurde auf Massenspektrometerbedingungen eingestellt.

Diskussion des analytischen Ergebnisses

Die gaschromatographische Trennung des Nitrosamingemisches 1 in der Kombination (Abb. 3) ist gut und in der Qualität mit der Trennung ohne Kombination mit dem Massenspektrometer vergleichbar (Abb. 1). Die Verschiebung in den Retentionszeiten ist durch das abgewandelte Temperaturprogramm und die veränderten Druckverhältnisse bedingt. Die Trennung des Nitrosamingemisches 2 ohne Kombination (Abb. 2) ist gut, wenn man von der Bandschweifung der höher siedenden Nitrosamine absieht. In der Kombination wird das Nitrosamingemisch 2 (Abb. 3) bis auf Peak Nr. 6 gut getrennt.

Alle gaschromatographischen Peaks der Nitrosamingemische konnten durch Aufnahme der Massenspektren eindeutig den entsprechenden Nitrosaminen zugeordnet werden.

Erfassungsgrenze. Unter den Arbeitsbedingungen der gaschromatographischen Trennung von Nitrosamingemisch 1 (Abb. 1 u. 3) liegt die Erfassungsgrenze im Bereich 0,2—0,5 μg/pro Substanz. Durch eine Änderung des Teilungsverhältnisses könnte die Erfassungsgrenze zwar noch heruntergesetzt werden, allerdings nur auf Kosten der guten Trennleistung. Die Erfassungsgrenze für den massenspektrometrischen Nachweis liegt bei 0,01 μg/pro Substanz in der Ionenquelle.

Beschreibung der Versuche. Die gaschromatographischen Untersuchungen wurden an einem Perkin-Elmer F6 Gaschromatographen mit Flammenionisationdetektor und Temperaturprogramm durchgeführt.

Table

Arbeitsbedingungen	Nitrosamingemisch 1	Nitrosamingemisch 2
Säule	25-m-R-Säule + KOH	25-m-PDEGS-Säule
T-Programm	80—160° (5° C/min)	150—180° C (5° C/min)
Vordruck	0,4 kp/cm²	0,4 kp/cm²
N_2-Strom	1 ml/min	1 ml/min
Teilungsverhältnis	1:50	1:50
Empfindlichkeit	8	8
Peakbezifferung	Tab. 2	Tab. 3
Abbildung	1	2
Eingespritztes Vol.	1,0 µl	0,8 µl

Die Massenspektren wurden an einem doppelt fokussierenden Massenspektrometer MAT SM 1 bei einer Auflösung von ca. 3000 aufgenommen.

Arbeitsbedingungen:

Elektronenenergie : 70 eV; Einlaßleitung : 170° C;
Elektronenstrom : 300 µA; Direkteinlaß : 190° C;
Einlaßsystem : 200° C; Ionenquelle : 200° C;

Die optimalen Temperaturbedingungen wurden durch Heizversuche ermittelt.

Die Messungen mit der Kombination Gaschromatographie-Massenspektrometrie wurden mit einem Perkin-Elmer-F6 Gaschromatographen mit Temperaturprogramm (Trägergas He) und einem Atlas CH 4 Massenspektrometer (Elektronenenergie: 70 eV) mit Lichtpunktschreiber durchgeführt. Detektor: Druckmeßionenquelle.

Arbeitsbed
Säule
T-Program
Ballistische
T-Program
Isotherm
Vordruck
He-Strom
Teilungsver
Peakbezifferung
Eingespritzt

Die präparative D
Amine mit $NaNO_2$ in
durch Extraktion mit
unter vermindertem D
mehrfaches Umkristall
eine Füllkörperkolonne
Handel nicht erhältlich
Alkylhalogenid in Äth
und quartären Ammo
trennung des Nitrosam

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14. Mayrhofer, O. L., M
15. Petrowitz, H. J.: A
16. Foreman, J. K., P
17. Koch, H.: s. folg. A
18. Cieplinsky, E. W.
19. Collins, J.: Bell, S.
20. Schroll, G., Cook, G
21. Mass Spectrometry
22. Houben-Weyl: 12/2
23. Houben-Weyl: 11, 1,

Arbeitsbedingungen	Nitrosamingemisch 1	Nitrosamingemisch 2
Säule	25-m-R-Säule + KOH	25-m-PDEGS-Säule
T-Programm	65–140° C (5° C/min) 0–15 min	80–180° C (5° C/min) 0–20 min
Ballistisches Abkühlen	140°–100° C 15–48 min	—
T-Programm	100–160° C (5° C/min) 48–60 min	—
Isotherm	160° C ab 60 min	180° C, ab 20 min
Vordruck	0,43 kp/cm²	0,42 kp/cm²
He-Strom	1 ml/min	1 ml/min
Teilungsverhältnis	1:50	1:50
Peakbezifferung	Tab. 2	Tab. 3; vgl. Abb. 3
Eingespritztes Vol	1,0 µl	1,0 µl

Die präparative Darstellung der Nitrosamine erfolgte durch Nitrosierung der entsprechenden Amine mit $NaNO_2$ in HCl-saurer Lösung [21]. Die Aufarbeitung des Reaktionsgemisches erfolgte durch Extraktion mit CH_2Cl_2. Der Extrakt wurde über Na_2SO_4 getrocknet und das Lösungsmittel unter vermindertem Druck abdestilliert. Die Reinigung der festen Nitrosamine erfolgte durch mehrfaches Umkristallisieren. Flüssige Nitrosamine wurden durch fraktionierte Destillation über eine Füllkörperkolonne gereinigt. Die präparative Darstellung der sekundären Amine, soweit im Handel nicht erhältlich, erfolgte durch Umsetzung von primärem Amin — im Überschuß — mit Alkylhalogenid in Äthanol [22]. Das entstandene Gemisch aus primären, sekundären, tertiären und quartären Ammoniumsalzen wurde mit $NaNO_2$ in HCl-saurer Lösung nitrosiert. Die Abtrennung des Nitrosamins erfolgte durch fraktionierte Destillation über eine Füllkörperkolonne.

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Comparison of the Diuretic Effects of Nitrate Salts With Other Diuretic Agents[1]

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ABSTRACT

HIATT, EDWIN P. (U. North Carolina School Med., Chapel Hill.) *Comparison of the diuretic effects of nitrate salts with other diuretic agents.* Am. J. Physiol. 189(1): 173–176. 1957.—A comparison of the diuretic effect of single large doses of NH_4NO_3, KNO_3, $NaNO_3$, NaCl, Diamox and Mercuhydrin was made in three dogs. Measurement was made of urinary volume, sodium, potassium and osmolar excretion during the 24-hour period following administration of each agent. The net sodium excretion was taken to be the best diuretic index for our purposes. By this index, Mercuhydrin, Diamox, and NH_4NO_3 were of the same order of effectiveness (*ca.* 3 mEq/kg/24 hr.), with KNO_3 next (*ca.* 2 mEq/kg/24 hr.) and $NaNO_3$ still less (*ca.* 0.5 mEq/kg), while NaCl was not completely excreted in 24 hours. The effect on urine volume was in the same order but with smaller differences. Net potassium excretion was significant only with the acidifying agents Diamox and NH_4NO_3, which caused about 2.5 mEq/kg loss in 24 hours, and $NaNO_3$, which caused about 0.7 mEq/kg loss. The excretion of osmotically active material as measured by freezing point depression of the urine was much less than would be predicted from the cation excretion.

IN STUDYING the behavior of the nitrate ion in dogs, it was observed that oral or intravenous administration of $NaNO_3$ resulted in the excretion of sodium in excess of the amount given and at a rate considerably more rapid than that following the administration of NaCl (1, 2). Until a few decades ago nitrates were commonly used as diuretics, particularly the potassium and ammonium salts (3). We were curious to know the relative efficacy of these three nitrate salts as diuretic agents, particularly in the restricted clinical sense of the word diuretic, i.e. promoting net loss of salt and water from the body via the urine. In addition, it seemed interesting to compare the effect of these salts with that of two commonly used diuretic agents: acetazoleamide (Diamox) which is a carbonic anhydrase inhibitor, and meralluride (Mercuhydrin sodium), an organic mercurial compound. The studies reported here are concerned with the effect of single doses of these various agents in dogs.

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METHODS

Three trained mongrel female dogs of approximately the same size (13.8–16.0 kg) served as subjects.

The salts were given orally in doses of 7.5 mM/kg of initial body weight dissolved in a vehicle consisting of 50 gm of Lonalac, a powdered food similar to whole milk in composition but almost free of sodium, in 300 ml of water. This quantity of salt was found to be close to the maximum tolerated without emesis. In fact, for KNO_3 and NH_4NO_3, it was sometimes necessary to divide the dose into two parts administered an hour apart to avoid emesis. Sometimes the dogs voluntarily ingested the dose but at other times it was necessary to use a stomach tube. For control experiments the vehicle was given without added salt.

Mercuhydrin was given in doses of 2 ml (78 mg Hg) by slow intravenous injection. Diamox was given by mouth in doses of 500 mg. In each case; the above mentioned Lonalac suspension was fed at approximately the same time.

The dogs were deprived of food and water for 24 hours before each experiment and, except for the vehicle, during the experimental period. The urine produced in the 24-hour period following the administration of agent and/or the vehicle was collected, the bladder being emptied by catheter at the beginning and at the end of this period. Two or three experiments on each dog with each agent were performed. The animals were not used more frequently than once a week and the observations were made during a period of more than a year. Urinary infection was largely avoided by

TABLE 1. CONTROL VALUES—VEHICLE ALONE

Excretion/kg/24 hr.

Measurement	Mean*	95% Confidence Interval
Volume, ml	23.45	16.8–30.1
Sodium, mEq	1.08	0.72–1.44
Potassium, mEq	1.34	1.02–1.66
Milliosmols	20.65	18.14–23.16

* Three measurements on each of three dogs.

administering a gram of a triple sulfonamide mixture at the termination of each experiment. A preliminary series of experiments using washed ground meat as a vehicle was in essential agreement with this series, but was omitted from this report for the sake of consistency.

The urine samples were analysed for sodium and potassium by the flame photometer. The osmolar content was determined by the lowering of the freezing point using a thermistor technique. Chloride was determined by the method of Sendroy as modified by Van Slyke and Hiller (4), and nitrate by the method of Straughn *et al.* (5).

Measurements of the creatinine clearances, taken as a measure of glomerular filtration rate, were made on each dog to determine the similarity of the dogs in this respect. The average of several clearance periods for *dog 1* was 54.4 ml/min., for *dog 2*, 54.9 and for *dog 3*, 52.6.

RESULTS

Control Values (Table 1). These values, obtained after the vehicle was given alone, were used to correct the values measured after the diuretics were administered in order to get the net effect of the diuretics separate from the basal or control rates of excretion. There was considerable variation among the three dogs and in different experiments on the same dog. This is reflected in the breadth of the 95% confidence intervals. Factors contributing to the variation among the dogs are the different ages and body types. The variation in different experiments on the same dog is probably due, in part, to the inclusion of estrus cycles in the period of observation.

Sodium Excretion (Table 2). Net sodium excretion for each experiment was obtained by subtracting the mean control rate of sodium excretion for each dog, plus the sodium administered, from the total sodium excreted. The net sodium excretion, expressed as milliequivalents per kilogram per 24 hours after administration of the various agents, is shown in table 2. Comparing the relative natriuretic effect of the several agents, it can be seen that Mercuhydrin, Diamox and NH_4NO_3 are of the same order of effectiveness under these conditions, with KNO_3 somewhat less effective. $NaNO_3$ has a still smaller but definite effect, while NaCl has no significant effect. The natriuretic effect of all these agents, except NaCl, is virtually complete within the first 12 hours.

Water Excretion (Table 3). The effectiveness of these agents in regard to the net excretion of water is in essentially the same order as their effectiveness in promoting sodium excretion, but the differences are not as great between the most effective (NH_4NO_3, Mercuhydrin and Diamox) and the other nitrate salts. NaCl has a small and extremely variable effect. The excretion of the nitrate salts in the urine osmotically obligates a certain volume of water which makes them appear to be more potent diuretics than they actually are, if one considers only urine volume as the index of diuresis.

Potassium Excretion (Table 4). The mean control rate of potassium excretion in the three dogs ranged from 1.1 to 1.5 mEq/kg/24 hr. The net excretion of potassium is highest after the acidifying agents, Diamox and NH_4NO_3, which have essentially equal effects, with $NaNO_3$ causing a small but significant net excretion. The other agents cause essentially no net loss of potassium.

Osmolar Excretion (Table 5). The osmolar excretion in the diuretic experiments is less than one would predict from the excretion of sodium and potassium. This deficit may be due to one or both of two factors. First, there may be an inhibition, under diuretic conditions, of the excretion of substances which

TABLE 2. NET SODIUM EXCRETION

Net = Na excreted − (control Na excretion + Na administered)

Agent	Mean* Net Na Excretion, mEq/kg/24 hr.	95% Confidence Interval, mEq/kg/24 hr.
NaCl	−0.59	−0.62–1.80
$NaNO_3$	0.52	0.22–0.82
KNO_3	1.99	1.23–2.75
NH_4NO_3	3.13	2.10–4.16
Diamox	2.85	2.21–3.49
Mercuhydrin	3.50	2.62–4.38

* Two or three measurements with each agent on each of three dogs.

contribute to the osmotic activity of the urine during the control experiments. Secondly, there may be preferential excretion of polyvalent anions in electrolytic association with the univalent cations thus reducing osmotic activity. We do not have the necessary experimental data to justify further speculation on this point.

As in our previous investigations, none of our animals showed evidence of the methemoglobinemia which has been a rare complication of nitrate administration (6), or of any other toxic effect.

DISCUSSION

The comparison of diuretic agents of widely different potency per unit of weight and of different modes and rates of action presents special difficulties. Since there is wide variation in the duration of action of the agents under investigation there is no value in comparing the peak or maximal effect of such agents. We have elected to observe the effect over an interval of time adequate for the complete action of all the agents employed.

The best index of diuretic effectiveness appears to be the net sodium loss. The usual practical purpose of the use of diuretics is to reduce edema, an excessive volume of extracellular fluid. Sodium is the principal ion in this fluid and, in general, water loss is proportional to sodium loss (7).

Ideally, agents of varying modes of action should be compared at maximally effective but nontoxic dosages. With the salts we have approached this by giving them in equivalent amounts in the largest dosages possible without promoting emesis. For Mercuhydrin and Diamox, we have arbitrarily selected amounts four or five times the usual clinical dosages for man on a body weight basis. Maren *et al.* reported that increasing the dose of Diamox above 5–10 mg/kg does not increase the intensity of the response in dogs but it does increase its duration (8). Our dose of 30–35 mg/kg has a duration of effect of about 10–12 hours, which is comparable to that of the nitrate salts. With regard to the maximal dose of a mercurial diuretic, Duggan and Pitts reported that in dogs of similar size which were pretreated with saline infusion Mercuhydrin had its maximal effect in doses containing 100–240 mg Hg, with the latter dose approaching the lethal point. However, in dogs not so pretreated doses of this magnitude depressed the glomerular filtration rate. Their measurements were made in the first 2 hours after intravenous injection of the mercurial (9). In the light of these observations our dosage of Mercuhydrin is presumed to be close to the maximal nontoxic dose.

There have been some previous attempts to compare the diuretic effectiveness of various nitrate salts with each other and with other

TABLE 3. NET VOLUME EXCRETION

Net = volume excreted − mean control volume excreted

Agent	Mean* Net Volume, ml/kg/24 hr.	95% Confidence Interval, ml/kg/24 hr.
NaCl	3.46	3.11–10.01
$NaNO_3$	11.03	8.26–13.80
KNO_3	15.70	12.08–19.32
NH_4NO_3	19.63	11.76–27.50
Diamox	17.07	12.80–21.34
Mercuhydrin	19.34	11.14–27.54

* Two or three measurements with each agent on each of three dogs.

TABLE 4. NET POTASSIUM EXCRETION

Net = K excreted − (control K excretion + K administered)

Agent	Mean* Net K Excretion, mEq/kg/24 hr.	95% Confidence Interval, mEq/kg/24 hr.
NaCl	0.02	−0.09–0.49
$NaNO_3$	0.71	0.48–0.94
KNO_3	0.07	−0.50–0.37
NH_4NO_3	2.30	1.79–2.81
Diamox	2.35	2.02–2.68
Mercuhydrin	0.03	−0.34–0.40

* Two or three measurements with each agent on each of three dogs.

TABLE 5. OSMOLAR EXCRETION

Total osmolar excretion minus mean control for each dog

Agent	Mean* Osmolar Excretion, mOs/kg/24 hr.	95% Confidence Interval, mOs/kg/24 hr.
NaCl	7.75	5.50–10.00
$NaNO_3$	11.39	7.35–15.43
KNO_3	12.44	6.24–18.64
NH_4NO_3	10.50	8.36–12.64
Diamox	3.50	1.82–5.18
Mercuhydrin	1.13	−2.17–4.43

* Two or three measurements with each agent on each of three dogs.

diuretic agents. Lipschitz *et al.* devised a bioassay of diuretics in which they measured the volume of urine in saline-loaded rats after administering various agents (10). By comparing dose-action curves, they arrived at an estimate of relative diuretic activities based essentially on the millimoles per kilogram of the various agents required to cause a given diuretic effect. Assigning a value of 1.0 to the effect of urea, they found that sodium nitrate has an activity of 2.9, KNO_3 3.9, the mercurial Salyrgan 400, with the xanthines in intermediate positions. This is a much greater difference between the nitrate salts and a mercurial diuretic than we found. Our dosages were similar but we point to the difference in species, a different period of observation, and, particularly, to a different base line, as contributing to this discrepancy. Their method of estimating diuretic potency does not measure the actual effectiveness of the diuretics, since it does not take into account the wide variation in the range of dosage over which different agents have their diuretic effect. Specifically, the mercurials and the xanthines are very active in small doses. However their range is limited by toxic effects whereas agents like the nitrate salts and urea, though ineffective except in relatively large doses, have a wider range of effectiveness and can cause the excretion of as much net salt and water.

In a carefully controlled experiment on one normal human subject, Keith, Whelan and Bannick demonstrated that NH_4NO_3 was about 50% more effective than $NaNO_3$ as a diuretic, as judged by the net chloride excretion over a period of 4 days with daily administration of 10 gm of nitrate salt (11). The urinary excretion of hydrogen ion and ammonia was increased after NH_4NO_3 administration but relatively unaffected by $NaNO_3$, an observation we have confirmed.

Ethridge, Myers and Fulton studied the diuretic effect of various salts alone and in combination with a mercurial (Salyrgan) in dogs (12). Unfortunately, their detailed observations were limited to a 3-hour period after the administration of the agent, which encompasses most of the action of Salyrgan but is too short for the full effect of the salts. In this short period, NH_4NO_3 in an amount of 2.5 mEq/kg promoted only one-third of the chloride excretion caused by 1 ml of Salyrgan, (39.6 mg Hg), but this is not an adequate comparison. They found that $Ca(NO_3)_2$ and NH_4NO_3, like other acidifying salts, greatly potentiated the effect of mercurial diuretics. Alkalinizing salts reduced the action and neutral salts had little effect.

Obviously, such experiments as we are reporting are only an indication of the relative order of potency of these agents in single doses in normal dogs. Before these agents could be compared for usefulness as diuretics further studies should be done on the effect of repeated doses, the questions of cumulative toxicity, electrolyte imbalance and the response of edematous subjects. After the completion of these studies the order of effectiveness may be quite different.

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NITRATE AND VITAMIN A INTERRELATIONSHIPS IN SHEEP[1]

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INTERRELATIONSHIPS between nitrate and vitamin A or carotene have been studied by several workers using ruminants and non-ruminants. Emerick and Olson (1962) and Phillips (1965) working with rats, and Sell and Roberts (1963) working with chicks concluded that the effect of nitrite, but not nitrate, in reducing liver storage of vitamin A in these species was due to the destruction of carotene or vitamin A in the digestive tract. Although several workers (Jordan *et al.*, 1961; Goodrich *et al.*, 1964; Newland and Deans, 1964) have demonstrated that dietary nitrate reduced liver stores of vitamin A in ruminants, it has not been demonstrated whether the effect is due to a more rapid depletion of existing vitamin A stores or to a lowered hepatic deposition of the vitamin.

The experiments described herein were conducted to determine the effect of nitrate on plasma vitamin A, hepatic vitamin A and performance of sheep during a growth period (experiment 1) and vitamin A depletion and repletion periods (experiment 2).

Experimental

Experiment 1. One hundred and twenty black-faced ewe lambs from Texas were used in this experiment to determine the effects of nitrate on feedlot performance and plasma and hepatic vitamin A levels. This was accomplished under conditions of vitamin A depletion and vitamin A or carotene supplementation. Treatments (table 1) used with control and 2.5% sodium nitrate diets were control (1,4), 4,000 I.U. vitamin A (2,5) and 8 mg. carotene per lamb daily (3,6). Treatments were replicated with 10 lambs per lot. Sodium nitrate, when used, was incorporated at a rate of 2.5% into a concentrate mixture that also included 75.0% rolled oats, 16.5% ground corn, 5.0% soybean meal and 1.0% limestone. This resulted in a diet that was low in carotene. The ground corn served as a carrier preventing excessive separation of the finer constituents including sodium nitrate. The 2.5% sodium nitrate used in these studies is the same level as that used previously by Goodrich *et al.* (1964) to lower liver vitamin A stores of sheep. In control diets, 2.5% sodium chloride was used to replace the sodium nitrate. In the absence of the sodium nitrate, these mixtures contained 13.1% protein (N × 6.25, air-dry basis).

Diet supplements shown in table 1 provided the vitamin A and carotene variations and contained 2.5% sodium nitrate or sodium chloride as did the concentrate mixes. The supplements were fed at a constant rate of 0.114 kg. per head daily to furnish the daily treatment levels of 4,000 I.U. of vitamin A or 8.0 mg. carotene from dehydrated alfalfa meal. In the absence of sodium nitrate, the crude protein content of the supplements was maintained at 17% (calculated). New batches of each supplement were mixed at 6-wk. intervals. The dehydrated alfalfa meal was analyzed (A.O.A.C. 1960) prior to each mixing, and the ratio of it to the corn-soybean meal mix was adjusted so that the treatment level (8 mg.) of carotene would be supplied in the daily allotment (0.114 kg.) of the supplement. The vitamin A source (see table 1) was analyzed initially using the Carr-Price reaction and was stored at −10° C until mixed into the supplements. The concentrate mix fed in addition to the supplements contained 0.73 mg. carotene per kg. by analysis.

During a 3-wk. pretreatment period, the lambs were brought to a full feed on the non-nitrate control diet while gradually eliminating alfalfa hay and corn silage fed previously. Feeding of experimental diets was then initiated and the lambs were fed these all-concentrate diets thereafter. Diets were fed once daily in amounts that would be almost completely consumed within 24 hours. Trace mineral salt and a mineral mix of two parts dicalcium phosphate, two parts ground limestone and one part trace mineral salt were fed *ad libitum*. The lambs were vaccinated against enterotoxemia and drenched with thiabenda-

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TABLE 1. COMPOSITION OF DIET SUPPLEMENTS—EXPERIMENT 1

Ingredient	Treatment 1 Control	2 Vit. A	3 Carotene	4 Nitrate	5 Nitrate and vit. A	6 Nitrate and carotene
Corn-soybean meal mix,[a] %	95.1	95.1	0–69.8	95.1	95.1	0–69.8
Dehydrated alfalfa meal, %[b]	....	....	25.3–95.1	....	....	25.3–95.1
Ground limestone, %	1.0	1.0	1.0	1.0	1.0	1.0
Sodium nitrate, %	....	....	....	2.5	2.5	2.5
Sodium chloride, %	2.5	2.5	2.5	....	....	....
Vitamin A,[c] I.U./kg.	....	35,200	....	....	35,200	....
Diethylstilbestrol,[d] mg./kg.	17.6	17.6	17.6	17.6	17.6	17.6
Chortetracycline,[e] mg./kg.	220	220	220	220	220	220

[a] Three parts ground corn and one part soybean meal.
[b] Commercial dehydrated alfalfa meal (17% crude protein) was added in amounts necessary to provide 70.4 mg. carotene/kg. supplement.
[c] Nopcay "30," Nopco Chemical Co., Newark, N. J.
[d] Stilbosol—2, Elanco Products Co., Indianapolis, Ind.
[e] Aureomycin—50, American Cyanamid Co., Agricultural Division, Princeton, N. J.

zole for internal parasite control prior to the experiment.

Blood samples were obtained by jugular puncture initially and at approximately 3-wk. intervals thereafter for lambs fed the vitamin A depletion rations and at about 6-wk. intervals for those fed rations supplemented with vitamin A or carotene. The individual blood samples were analyzed for total hemoglobin and methemoglobin by the method of Evelyn and Malloy (1938), and plasma carotene and vitamin A were determined by the method of Koehn and Sherman (1940).

One lobe of each liver was collected at slaughter after 136 days on experiment and stored frozen until analyzed. Carotene and vitamin A contents of the livers were determined by the method of Johnson and Baumann (1947).

Some losses occurred during the course of the experiment from causes unrelated to treatment, and weight gain data were calculated only for lambs completing the trial. Feed consumption data were adjusted by subtracting an average value for each loss. Statistical analysis was performed using the method of least squares (Steel and Torrie, 1960).

Experiment 2, Depletion Period. Concurrently with experiment 1, another 12 lots of ewe lambs were fed diets identical to those fed the four lots of lambs on treatments 1 (control) and 4 (2.5% sodium nitrate) in experiment 1. These were used to obtain more extensive data on vitamin A depletion as affected by nitrate and to provide lambs for subsequent use in the repletion phase of experiment 2 as described below. The 12 lots of lambs were divided equally between the control and nitrate treatments without vitamin A or carotene supplementation. These lambs were from the same source as those in experiment 1 and were allotted and fed in the same manner with 10 lambs per lot initially.

The lambs were bled initially and near the end of the 136-day depletion period. Analysis of plasma for vitamin A and statistical analyses of gain, feed and analytical data were performed as described for experiment 1.

Experiment 2, Repletion Period. Upon termination of the vitamin A depletion period, four lots of lambs from each of the control and nitrate treatments were used to determine the effects of nitrate on vitamin A repletion rates of lambs. Two lots from each of the control and nitrate treatments were supplemented with 10,000 I.U. vitamin A per lamb daily and the other two lots from each source were supplemented with 20 mg. daily of carotene from dehydrated alfalfa meal.

Vitamin A or carotene supplements (table 2) were fed at the rate of 0.227 kg. per lamb daily with a concentrate mixture full-fed to appetite. Composition of the concentrate mixture was 91.5% coarsely ground oats, 5.0% soybean meal, 1.0% ground limestone and 2.5% sodium nitrate or sodium chloride. Grinding the oats permitted the omission of ground corn used in the previous studies as a carrier for sodium nitrate and other fine constituents. The method of feeding, adjusting feed ingredients to obtain the proper level of carotene and other experimental procedures were as described for experiment 1. Blood samples were obtained initially, after 3 wk. and upon termination of the 43-day repletion period. The lambs were slaughtered and livers analyzed for vitamin A as described previously.

TABLE 2. COMPOSITION OF DIET SUPPLEMENTS—EXPERIMENT 2, REPLETION PERIOD

Ingredient	Treatment 1 Vit. A	2 Carotene	3 Nitrate and vit. A	4 Nitrate and carotene
Oats-soybean meal mix,[a] %	95.8	60.7–64.6	95.8	60.7–64.6
Dehydrated alfalfa meal,[b] %	...	30.5–36.6	...	30.5–36.6
Ground limestone, %	1.0	1.0	1.0	1.0
Sodium nitrate, %	...	...	2.5	2.5
Sodium chloride, %	2.5	2.5	...	...
Vitamin A,[c] I.U./kg.	44,000	...	44,000	...
Diethylstilbestrol,[d] mg./kg.	8.8	8.8	8.8	8.8
Chlortetracycline,[e] mg./kg.	110	110	110	110

[a] Four and one-half parts ground oats and one part soybean meal.
[b] Commercial dehydrated alfalfa meal (17% crude protein) was added in amounts necessary to provide 88 mg. carotene/kg. supplement.
[c] Nopcay "30," Nopco Chemical Co., Newark, N. J.
[d] Stilbosol—2, Elanco Products Co., Indianapolis, Ind.
[e] Aureomycin—50, American Cyanamid Co., Agricultural Division, Princeton, N. J.

Results and Discussion

Data from these studies are presented in tables 3 (experiment 1), 4 (experiment 2, depletion period) and 5 (experiment 2, repletion period), and mean squares from the analysis of variance are presented in table 6. No visible signs of nitrate toxicity were observed in these studies. Average methemoglobin values were low and never exceeded 0.6 gm. per 100 ml. of blood. Therefore, these data are not reported. Goodrich *et al.* (1964) reported similarly low methemoglobin levels in lambs fed 2.5% sodium nitrate. The presence of carotene in the liver or plasma of lambs was negligible in all instances and the values are not reported. Low plasma and liver carotene values have previously been reported to be characteristic of sheep (Maynard and Loosli, 1962).

Experiment 1. No particular problems were encountered from feeding the all-concentrate diets to lambs. For these ewe lambs, the average daily weight gain of 0.17 kg. and average daily feed consumption of 1.23 kg. was considered satisfactory. The control diet supplied about 1 mg. carotene daily per lamb, and supplementing it with 4,000 I.U. of vitamin A or 8 mg. of carotene from dehydrated alfalfa meal did not significantly affect weight gains, feed consumption or feed efficiency during the 136-day experiment.

The feeding of 2.5% sodium nitrate significantly ($P<.05$) reduced weight gains. In

TABLE 3. EFFECT OF 2.5% SODIUM NITRATE ON LAMBS—EXPERIMENT 1[a]

Item	Treatment 1 Control	2 Vit. A	3 Carotene	4 Nitrate	5 Nitrate and vit. A	6 Nitrate and carotene
No. of lambs	20	20	18	17	20	19
Av. initial wt., kg.	31.6	31.6	31.2	31.8	31.7	31.7
Av. daily gain, kg.	0.179	0.167	0.190	0.154	0.168	0.164
Av. daily feed, kg.						
Concentrate mix	1.13	1.10	1.25	1.10	1.04	1.06
Supplement	0.114	0.114	0.114	0.114	0.114	0.114
Total	1.244	1.214	1.364	1.214	1.154	1.174
Feed/kg. gain, kg.	6.96	7.26	7.20	8.04	6.85	7.20
Av. plasma vit. A, mcg./100 ml.						
Initial	28	27	29	27	31	28
26 days	35	..	..	36	..	..
46 days	36	35	36	37	43	35
67 days	37	..	..	37	..	..
88 days	36	39	35	36	42	35
110 days	32	..	..	28	..	..
132 days	27	36	37	19	41	31
Av. liver vit. A, mcg./gm.	9	22	14	3	18	9

[a] 136 days.

TABLE 4. EFFECT OF 2.5% SODIUM NITRATE ON PERFORMANCE AND PLASMA VITAMIN A OF LAMBS DURING VITAMIN A DEPLETION—EXPERIMENT 2, DEPLETION PERIOD[a]

Item	Treatment	
	Control	Nitrate
No. of lambs[b]	79	72
Av. initial wt., kg.	31.7	31.9
Av. daily gain, kg.	0.176	0.158**
Av. daily feed, kg.		
Concentrate mix	1.15	1.09
Supplement	0.114	0.114
Total	1.264	1.204**
Feed/kg. gain, kg.	7.18	7.64*
Av. plasma vit. A, mcg./100 ml.		
Initial	29	28
132 days	27	23*

[a] 136 days.
[b] Includes data from lambs on treatments 1 and 4 in experiment 1.
* Treatments differ significantly, P<.05.
** Treatments differ significantly, P<.01.

addition, significant nitrate × vitamin source and replication × vitamin source interactions were obtained (table 6). In the former instance, the interaction appeared to be due to the relatively poor performance of the nonnitrate vitamin A treatment group. Average weight gain for this group was 0.167 kg. daily and was below the value of 0.179 kg. for the control group. Taking this into consideration, we cannot conclude that the similarity in weight gain data (0.167 *vs.* 0.168 kg. daily) for the vitamin A treatments with and without nitrate indicates a protective effect for vitamin A, or that the nitrate × vitamin source interaction indicates a difference in the protective effect of vitamin A and carotene. Data previously reported by Weichenthal *et al.* (1963) have indicated that supplemental vitamin A is not effective in overcoming the weight gain depression caused by dietary nitrate in cattle.

Plasma vitamin A values after 26 days were higher than initial values for all treatments. Only small changes were observed thereafter until the 110-day bleeding. At that time and at 132 days, values declined for lambs not supplemented with vitamin A or carotene. This effect was more pronounced in the presence of nitrate. Lambs supplemented with vitamin A or carotene had significantly ($P<.05$) higher final plasma vitamin A values than the unsupplemented lambs. In addition, a significant ($P<.05$) nitrate × vitamin source interaction was obtained. In the absence of nitrate, there were only small differences in final plasma vitamin A values between vitamin A and carotene treatments, but in the presence of nitrate, plasma vitamin A values were higher for lambs fed vitamin A than for those fed carotene.

Average liver vitamin A levels at the end of the 136-day experimental period were lowest in each instance for the nitrate-fed lambs. However, individual values were highly variable and the effect of nitrate on hepatic vitamin A stores during this period was not statistically significant. This is in agreement with results reported by Hale *et al.* (1962) in which only a small nonsignificant reduction

TABLE 5. EFFECT OF 2.5% SODIUM NITRATE ON LAMBS DURING VITAMIN A REPLETION—EXPERIMENT 2, REPLETION PERIOD[a]

Item	Treatment			
	1 Vit. A	2 Carotene	3 Nitrate and vit. A	4 Nitrate and carotene
No. of lambs	20	20	19	18
Av. initial wt., kg.	55.1	56.2	53.1	54.0
Av. daily gain, kg.	0.094	0.100	0.109	0.106
Av. daily feed, kg.				
Concentrate mix	1.12	1.17	1.05	1.10
Supplement	0.227	0.227	0.227	0.227
Total	1.347	1.397	1.277	1.327
Feed/kg. gain, kg.	14.56	14.64	11.77	12.65
Av. plasma vit. A, mcg./100 ml.				
Initial	25	30	24	24
21 days	39	42	36	37
43 days	44	44	39	34
Av. liver vit. A, mcg./gm.	25	18	10	7

[a] 43 days.

TABLE 6. MEAN SQUARES FROM ANALYSIS OF VARIANCE OF PERFORMANCE AND ANALYTICAL DATA

Source of variation	df.	Mean squares: Av. daily gain	132-day plasma vit. A	Liver vit. A	Feed consumption	Feed efficiency
Experiment 1, 2 lots/treatment, 6 treatments						
Nitrate (N)	1	3.751*	16666	69053	0.101	1541
Vitamin source (V)	2	0.562	240106*	179552*	0.041	2026
Replication (R)	1	0.173	934	7580	0.016	85
N × V	2	10.652**	41094**	1706	0.041	5882
R × N	1	0.019	5538	7870	0.002	161
R × V	2	2.034*	5295	38851	0.032	1951
Residual	104[a]	0.559	7357	15531	0.002	453
Experiment 2, depletion phase, including lambs designated for later repletion, 8 lots/treatment, 2 treatments						
N	1	6.321**	68719*	.....	0.072**	8772*
R	7	0.335	10432	.....	0.011	924
R × N	7	0.271	10125	.....	.....	...
Residual	135[b]	0.581	9786	.....	0.006	736
Experiment 2, repletion phase, 2 lots/treatment, 4 treatments						
N	1	0.944	107933*	312715	0.048*	114003
V	1	0.021	11129	44738	0.026*	4560
R	1	1.816	3520	2878	0.026	29646
N × V	1	0.121	11285	10298	0.000	3160
R × N	1	0.206	320	2858	0.000	2850
R × V	1	3.58	6593	5740	0.000	146070
Residual	69[c]	0.915	6016	10703	0.026	84666

[a] Residual for feed consumption and feed efficiency is based on 2 degrees of freedom due to group feeding.
[b] Residual for feed consumption and feed efficiency is based on 7 degrees of freedom due to group feeding.
[c] Residual for feed consumption and feed efficiency is based on 1 degree of freedom due to group feeding.
* Significant, $P<.05$.
** Significant, $P<.01$.

of hepatic vitamin A stores was obtained in nitrate-fed cattle not receiving carotene or vitamin A. Vitamin A of carotene supplementation resulted in significantly ($P<.05$) higher liver vitamin A values. Assuming the vitamin A value or carotene from dehydrated alfalfa meal to be approximately 500 I.U./mg. for sheep (N.R.C., 1964), the two sources used would be expected to provide similar amounts of the vitamin. However, 8 mg. of carotene per lamb daily did not support liver vitamin A stores at as high levels as did 4,000 I.U. of vitamin A. Terminal liver vitamin A stores of lambs fed carotene with and without nitrate, respectively, were only 50 and 64% as high as for corresponding lambs fed vitamin A.

Analysis of the supplements containing dehydrated alfalfa meal showed that the carotene content had decreased an average of 11% over the 6-wk. storage periods. Assuming a uniform rate of loss, the average daily rate of supplementation during the experiment would have been reduced by only about 0.5 mg. carotene per head. The carotene content of the non-nitrate and nitrate supplements at the end of any 6-wk. period were similar. This indicates that nitrate had no effect on carotene stability in the supplements. The vitamin A content of the supplements was not determined after mixing, but analyses conducted in conjunction with other experiments (*unpublished data*) have indicated a high degree of stability for the product used. Davison and Seo (1963) have previously reported that nitrate does not hasten the loss of preformed vitamin A in prepared feeds.

Experiment 2, Depletion Period. Data for lambs on each of the two vitamin A depletion treatments are shown in table 4. A significant ($P<.01$) reduction in weight gains resulted from the feeding of 2.5% sodium nitrate. Reduction in weight gain attributed to the feeding of sodium nitrate in this instance amounted to 10.2%. The lower weight gain was accompanied by a 4.8% lower ($P<.01$) feed consumption and a 6.4% higher ($P<.05$) feed requirement per unit of gain.

Plasma vitamin A values for both groups tended to become lower during the depletion period, with lambs on the nitrate treatment yielding significantly ($P<.05$) lower values than those obtained for the controls after 132 days. Two lots of lambs receiving these vitamin A depletion diets were bled periodically during the experiment to follow plasma vita-

min A levels during depletion (table 3, treatments 1 and 4). Liver vitamin A values for these depleted lambs at slaughter are assumed to be representative of the other depleted lambs used in the later repletion studies.

Experiment 2, Repletion Period. Weight gains were low and there were only small differences among treatments during the 43-day vitamin A repletion period. However, this phase of the experiment was of short duration and lambs of this size (average, 54.6 kg.) would be expected to have small daily gains.

Plasma vitamin A increased after 21 days of supplementation with 10,000 I.U. of vitamin A or 20 mg. of carotene per lamb daily. After 43 days, there was no difference in plasma vitamin A between lambs supplemented with vitamin A or carotene in the absence of nitrate. However, the feeding of 2.5% sodium nitrate resulted in significantly ($P<.05$) lower plasma vitamin A at the 43-day sampling period, and this effect appeared to be more pronounced with the feeding of carotene than with vitamin A. These results differ from those obtained in experiment 1 for non-depleted lambs (table 3, treatments 2, 3, 5 and 6) in that the effect of nitrate in lowering plasma vitamin A was not evident in lambs fed preformed vitamin A in experiment 1. The vitamin A treatments in experiments 1 and 2 (repletion period) differed mainly in that the level of vitamin A supplementation was 2.5 times higher in the latter instance.

A comparison of repleted liver vitamin A values (table 5) and prior depleted values (table 3, treatments 1 and 4) indicates that some degree of hepatic vitamin A storage occurred during the repletion period. In the absence of nitrate, the increases in hepatic storage of vitamin A amounted to 16 and 9 mcg. per gm. with the feeding of vitamin A and carotene, respectively. Increases in hepatic vitamin A stores in nitrate-fed lambs during this period were only 44% as high as the increases observed in lambs on corresponding non-nitrate treatments. This effect of nitrate in lowering vitamin A storage approached significance at the 5% level of probability ($P<.10$). The effect of nitrate on vitamin A storage in lambs fed carotene and those fed vitamin A was similar. In contrast to these data, Cline *et al.* (1963) reported that 4% potassium nitrate had no effect on weight gains or carotene and vitamin A utilization in sheep given weekly injections of vitamin A and/or fed rations containing 10% alfalfa hay.

Based on increases in hepatic vitamin A stores during the 43-day repletion period, the 20 mg. carotene with and without nitrate was only 57 and 56%, respectively, as effective as corresponding 10,000 I.U. vitamin A treatments in contributing to hepatic vitamin A stores. This relative effectiveness of carotene *vs.* vitamin A is higher than was associated with lower levels of supplementation in experiment 1.

Olson *et al.*[1] (1963) and Pugh and Garner (1963) have demonstrated that nitrite, under acidic conditions typical of abomasum contents, can rapidly destroy carotene and vitamin A, respectively. The data reported herein indicate that a reduction of vitamin A storage, possibly through a destruction of dietary vitamin A or carotene, is of greater importance than an accelerated depletion of existing vitamin A stores in affecting the vitamin A status of nitrate-fed sheep. The extent that these results, obtained with sheep, may apply to cattle has not been demonstrated.

Summary

Experiments were conducted to determine the effect of 2.5% sodium nitrate on performance, plasma vitamin A levels and hepatic vitamin A stores of sheep during periods of modest (4,000 I.U. vitamin A or 8 mg. carotene per lamb daily) vitamin A supplementation, vitamin A depletion and vitamin A repletion (10,000 I.U. vitamin A or 20 mg. carotene per lamb daily). Nitrate significantly ($P<.05$) reduced weight gains in lambs being depleted or receiving modest supplements of vitamin A or carotene, but it did not have the same effect during a repletion period when the lambs were considerably larger and making smaller weight gains.

Carotene fed at a rate of 20 mg. per head daily from dehydrated alfalfa meal was only 56 to 57% as effective as 10,000 I.U. of vitamin A in the repletion of hepatic vitamin A stores of depleted lambs. This relative effectiveness of carotene *vs.* vitamin A was higher than was associated with lower levels of supplementation in non-depleted lambs. The feeding of 2.5% sodium nitrate did not appear to influence the relative effectiveness of carotene compared with vitamin A.

Significantly ($P<.05$) lower plasma vitamin A and lower ($P<.10$) liver vitamin A values accompanied the feeding of nitrate to lambs supplemented with either carotene or vitamin A following vitamin A depletion.

These effects of nitrate during vitamin A repletion were more pronounced than those observed during vitamin A depletion. It is concluded that dietary nitrate exerts its greatest influence on the vitamin A status of ruminants through a reduction in the amount of dietary vitamin A reaching hepatic stores rather than through an accelerated depletion of existing stores.

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Nitrite Poisoning in Sheep, with Special Reference to the Detoxification of Nitrite in the Rumen

An Experimental Study

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Introduction and Review of the Literature

Nitrite is known to be formed from nitrate by reduction under the influence of certain bacteria (QUASTEL & STEPHENSON 1925 and STEPHENSON & STICKLAND 1931). As the rumen flora is known to have this nitrate-reducing power (WHITEHEAD & MOXON 1952), oral administration of nitrate is dangerous for ruminants.

A disease of sheep designated tribulosis has been described in South Africa by QUIN (1930) and RIMINGTON & QUIN (1933). By administering an aqueous extract of plants belonging to the genus *Tribulus*, or by giving potassium nitrate, a similar disease could be produced. SEEKLES & SJOLLEMA (1932) experimentally produced nitrate poisoning in cattle by the oral administration of 100–200 g potassium nitrate. Oat hay poisoning of cattle, a disease causing great losses, has been described in USA by NEWSOM, STOUT, THORP, BARBER & GROTH (1937) and THORP (1938). These investigators did not succeed in demonstrating the toxic substance in oat hay. It was pointed out that only some of the animals which were fed oat hay died suddenly without showing signs of illness while others, although they consumed some of the same oat hay, remained healthy. BRADLEY, EPPSON & BEATH (1939, 1940) showed that oat hay contained large amounts of nitrate, 3.2–7.2 per cent of the dry material, and that an identical disease could be produced by administering potassium nitrate. Cases of oat hay poisoning have also been reported by DAVIDSON, DOUGHTY & BOLTON (1941) and OLSSON & MOXON (1942).

Like oat hay, sugar beet tops contain large amounts of nitrate and fatal cases of poisoning after feeding this material have been described by SAVAGE (1949), LINDNER (1950) and KENDRICK, TUCKER & PEOPLES

(1950). In addition to these, other plants may have such high nitrate levels that there is risk of poisoning as has been shown by BELLUE (1952), WEBB (1952), and KENDRICK & *al.* (1955).

CRAWFORD (1941) reports that sixteen cows died after being placed on a pasture recently fertilised with saltpetre. Cases of poisoning have also occurred as the result of high nitrate levels in drinking water (LINDNER, BRIESKORN & FRANKE 1948, LINDNER 1950 and CAMPBELL, DAVIS & MYHR 1954).

Spraying with the anti-weed preparation 2,4-dichlorophenoxy-acetic acid ("2,4-D") leads to an accumulation of nitrates in plants, especially sugar beets and wheat (STAHLER & WHITEHEAD 1950, BELLUE 1952 and KENDRICK & *al.* 1955).

The toxicity of nitrate depends upon its being reduced to nitrite in the rumen and absorbed in that form. Nitrite transforms haemoglobin to methaemoglobin and, if the methaemoglobin concentration is sufficiently great, death occurs, supposedly as the result of asphyxiation (WHITEHEAD & *al.* 1952). SAPIRO, HOFLUND, CLARK & QUIN (1949) have studied methaemoglobinaemia in sheep after the oral administration of nitrate. They also demonstrated the formation of nitrite after the addition of nitrate to rumen contents *in vitro*. The formation of methaemoglobin in sheep after dosing with nitrate and nitrite and the reduction of nitrate by washed suspensions of rumen microorganisms have been studied by LEWIS (1951 a, b).

Attention has been drawn to the effects of nitrates on ruminants by the cases of acute poisoning which have occurred. SAPIRO & *al.* (1949) and CLARK & QUIN (1951) have carried out a number of experimental investigations and proposed the use of small quantities of nitrate as a nitrogen supplement for protein-deficient feed.

Nitrate is the most important source of nitrogen for plants and, even when present in large quantities, does not injure them. Soil bacteria are able to form nitrate from other nitrogen-containing fertilisers and plant proteins (DAVIDSON & *al.* 1941 and STEPHENSON 1949). Plants may thus contain nitrates although these salts had not been applied to the soil. According to BURSTRÖM (1946), it is impossible to state normal values for the nitrate content of plants as the levels are extremely variable. Concentrations of up to 1 mmol per g dry material have, however, often been recorded. This would mean that 10 kg dry material, an amount which can easily be consumed by a cow in a day, could contain 620 g nitrate, or calculated as sodium nitrate, 850 g. The nitrate content of plants can be increased by dryness and, as DOUGHTY & WARDER (1942) have shown, by the application of nitrate

to the soil. As nitrate fertilisation, especially in the case of improved pastures, is commonly carried out, it appears that many animals at pasture consume nitrates in amounts which cannot be disregarded. The presence of nitrate in the rumen, therefore, must be considered as a natural phenomenon.

The object of the present investigations was to study experimental nitrite poisoning in sheep, and to investigate the factors influencing nitrite accumulation in the rumen after the administration of nitrate and nitrite detoxification in the rumen.

Chapter I

Signs and Course of Poisoning under Various Feeding Conditions after Oral Administration of Nitrate

The clinical picture of oat hay poisoning has been described by DAVIDSON & *al.* (1941) as follows: rapid pulse, 150 per minute, followed by short, accelerated respiratory movements, muscle tremor, weakness, a staggering gait, and, in some cases, blindness: The animals cease eating, sink to the ground with wide-open mouth, and lie on the brisket or roll over onto the side. Cyanosis of the tongue and sclerae can be observed, and death ensues with little or no struggling. The usual autopsy findings are petechial haemorrhages in the epicardium, mucous membranes of the respiratory organs, peritoneum, and the serosal membranes of the digestive canal. Hyperaemia of the mucous membrane of the abomasum is a constant finding. The blood has a reddish-brown colour which becomes lighter after exposure to air.

The lethal dose of potassium nitrate for cattle is 1 g per kg body weight according to WHITEHEAD & *al.* (1952). LEWIS (1951 a) has shown that 25 g sodium nitrate given orally to sheep weighing 60 kg results in 60 per cent of the haemoglobin being transformed to methaemoglobin. SAPIRO & *al.* (1949) found that oral administration of 30 g potassium nitrate per 100 lb body weight (i.e. 0.67 g/kg) could result in the death of sheep maintained on poor quality grass hay, while sheep fed lucerne hay and maize withstood larger amounts of nitrate. It seems difficult, therefore, to give reliable information about the amount of nitrate which ruminants can withstand without becoming seriously ill. Because of this, the formation of methaemoglobin and the mortality among sheep given nitrate and maintained under various conditions has been studied.

1. *Materials and Methods*

As experimental animals, twenty-two sheep weighing between 24 and 32 kg were used.

A Beckman B spectrophotometer has been used to demonstrate methaemoglobin. The method used in the present work was that of EVELYN & MALLOY (1938) and the per cent level of methaemoglobin has been determined by the modified method described by BODANSKY (1951). A mixture of 0.1 ml blood and 10 ml *M*/60 phosphate buffer, pH 6.6 was allowed to stand for five minutes. A portion of this mixture was taken for the reading of optical density at 635 mμ. One drop neutralised sodium cyanide solution was added, transforming the methaemoglobin to cyanomethaemoglobin. After two minutes, another reading at 635 mμ was made. The difference between the two readings is proportional to the methaemoglobin concentration. To another portion of the diluted blood was added 1 drop 20 per cent potassium ferricyanide solution in order to transform haemoglobin to methaemoglobin. Readings at 635 mμ were made before and after the addition of neutralised sodium cyanide and the difference between the two readings was determined. The methaemoglobin percentage has been calculated by dividing the difference between the first two readings by the difference between the second two. In every case duplicate determinations were made.

Experiment 1

This experiment was carried out using six sheep divided into three groups of two. Group I was maintained on oat straw alone, a ration deficient in protein and highly-soluble carbohydrates. Group II received an ordinary ration with free access to hay and a concentrate supplement consisting of 100 g crushed oats and 50 g wheat bran per animal per day. Group III, maintained on a somewhat more concentrated ration, received hay ad lib. and 100 g crushed oats, 50 g wheat bran, and 50 g pea meal per animal per day. Feeding took place twice daily, at 7 a.m. and 2 p.m. After an adjustment period of eight days, daily treatment of all animals with potassium nitrate by stomach tube at 1 p.m. was begun. The animals in groups I and III received 0.4 g/kg body weight, and in group II, one sheep received 0.4 g/kg body weight and the other, 0.55 g/kg body weight. Following the death of the animals in group I, two new animals were subjected to the same feeding and treatment with nitrate, but the adjustment period was lengthened to one month.

Experiment 2

For this experiment, fourteen sheep were used. One group of eight animals were maintained on 0.5 kg hay, 200 g crushed oats and 50 g molasses per animal per day with free access to oat straw. Another group of six sheep were fed only oat straw ad lib. The adjustment period before the experiment commenced was lenghtened to six weeks. Both these groups received single doses of sodium nitrate by stomach tube and the subsequent formation of methaemoglobin was followed in heparinised blood samples taken from the jugular vein. The nitrate was given at 10 a.m. The blood samples were examined immediately after being taken.

2. *Results*

Experiment 1

Daily nitrate-dosing of the three groups of sheep gave the following results. Both sheep in group I died after 19 and 21 days respectively. The animal in group II which received 0.55 g sodium nitrate per kg body weight died after 12 days. The other animal of that group and both sheep of group III showed no signs of illness although the experiment was carried on for three months. The two sheep which replaced the orginal group I died after 2 and 5 days respectively.

The fatalities occurred 5–7 hours following the administration of nitrate. At the time the nitrate was given, all the animals seemed to be completely healthy and no signs of illness were displayed during the experimental period before one half hour or so preceding death. In all cases the course was quite uniform. The first sign was dullness accompanied by a markedly increased pulse rate but with a normal rate of respiration. Subsequently, muscle tremors appeared, especially over the shoulder regions, the animals became uneasy, showed difficulties in co-ordination, sank to the ground and lay on their sides. At this time they scarcely reacted to such stimuli as blood sampling. Death usually occurred 15–20 minutes following the assumption of recumbency. If autopsy was performed immediately after death, the typical chocolate-brown methaemoglobin colour of the blood could be distinctly seen. If, on the other hand, autopsy was delayed for some hours after death, the blood had a normal appearance because of the breakdown of much of the methaemoglobin. The most usual autopsy findings were pulmonary hyperaemia and oedema, hyperaemia of the abomasum, intestine, thymus, and lymph nodes, haemorrhages in the subendocardial, subepicardial, subperitoneal tissues, and thymus, and hydrothorax and hydropericardium.

Experiment 2

The results of the methaemoglobin determinations made on the two groups which received single doses of sodium nitrate are shown in Table 1.

Haemoglobin determinations, made by SAHLI's method, revealed markedly reduced values.

Table 1.

Concentration of methaemoglobin as per cent total haemoglobin after single doses of sodium nitrate.

Dose $NaNO_2$ g/kg body weight	Time after administration, hours 4	5	6	7	8	9	10
Feeding regimen: Hay, crushed oats & molasses							
0.5	0	0			0		0
0.6	0	2	1			0	
0.5	2	8			8		1
0.5	0			0			
0.5		1		0			
0.6	3	14	10			5	
0.6	0	10	8			2	
0.5	4		4				0
Oat straw							
0.5		50					
0.4	65[1]	48		40			
0.25		50					
0.25		20					
0.25		35					
0.25		30					
0.25		25					
0.4		89[2]					
0.4	12	17	17	13			

[1] Blood sample taken after 4 hours, 30 minutes. At this time the animal lay comatose and was treated with 60 mg methylene blue i.v. Fifteen minutes after treatment, the sheep began to be active. The next blood sample was taken 20 minutes after treatment with methylene blue.

[2] The sheep died after 4 hours, 30 minutes. Blood sample taken after death.

3. *Discussion*

The results of these experiments make it clear that the risks of methaemoglobin formation and a fatal outcome after the consumption of nitrate depend to a large extent upon the feed composition. In

experiment 1, all four sheep maintained on straw died after receiving daily doses of 0.4 g/kg body weight sodium nitrate, while all three sheep which received similar doses but were maintained on a good ration did not become ill. Experiment 2 with single doses of sodium nitrate demonstrated a wide difference in the formation of methaemoglobin between the two groups receiving different rations. It was seen that the animals in group I receiving daily doses of similar amounts of sodium nitrate lived for 19 and 21 days respectively when the adjustment period was eight days. The animals receiving the same dose of sodium nitrate after a period of adjustment to the straw ration of one month died after two and five days respectively. This supports the hypothesis that the rumenal capacity to detoxify the nitrate administered, or the nitrite formed from it, is gradually reduced under conditions of poor feeding.

The concentration of methaemoglobin reached high levels five hours after the oral administration of nitrate, a result which agrees with the investigations of LEWIS (1951 a). After this, a reversal took place and the methaemoglobin gradually disappeared. As this reversal takes place even after death, there is little chance of being able to establish the diagnosis on the basis of the findings if autopsy is not carried out within the first few hours after death.

Chapter II

Investigations of Respiratory Exchange and the Effects of Vasoconstrictory Drugs in Nitrite Poisoning

In haemoglobin, the normal blood pigment, iron is present in the ferrous state (Fe++). Haemoglobin combines with oxygen to form oxyhaemoglobin and when the partial pressure is decreased, the oxygen is released ($HbO_2 \rightleftharpoons Hb + O_2$). Oxidising agents such as nitrites oxidise the iron in haemoglobin from the ferrous (Fe++) to the ferric (Fe+++) state and thus form methaemoglobin. This compound does contain oxygen but here it is so firmly bound that it is not easily released when the partial pressure is decreased.

As methaemoglobin has little or no ability to transport oxygen, the cause of death in cases of methaemoglobinaemia is generally considered to be asphyxiation (WHITEHEAD & *al.* 1952). In cases of methaemoglobinaemia experimentally produced in dogs by p-aminopropiophenone, death occurs with methaemoglobin levels aproaching 95 per cent (VANDENBELT, PFEIFFER, KAISER & SIBERT 1944).

Since sheep with nitrite poisoning seem to become ill with values for methaemoglobin much lower than these, there is reason to suppose that other factors than a decreased supply of oxygen to the body tissues may be of importance. Oxygen consumption and carbon dioxide elimination in poisoned sheep have, therefore, been studied with the aid of a respiration chamber.

The treatment of nitrite poisoning in both human beings and animals has, up to the present, been directed towards the transformation of methaemoglobin to haemoglobin. Methylene blue, injected intravenously, has been the drug most widely used for this purpose. For human beings, the dose is usually 1–2 mg/kg body weight in a 1 per cent solution (BODANSKY 1951) and clinical effects are produced within 30 minutes (MARCUS & JOFFE 1949). BRADLEY & *al.* (1940) used up to 8 mg/kg body weight for cattle. As there is reason to suppose that the vasodilatory effect of nitrite is of significance in the clinical course of poisoning (WEISS & ELLIS 1933 and HUEPER & LANDSBERG 1940), the effect of vasoconstrictory drugs has been tested.

1. *Materials and Methods*

For the experiments in the respiration chamber, five sheep weighing between 29–36 kg have been used, and for the experiments with vasoconstrictory drugs eight sheep weighing between 22–30 kg. A diagrammatic sketch of the respiration chamber, constructed and described by BLOMBÄCK & PAULSSON (1957), is shown in Figs. 1 a and 1 b. The chamber is constructed of welded iron plates, 1 mm thick, and is insulated by cork sheets, 2 cm thick, and porous and hard masonite.

The air entering the chamber was led through a roof intake from a park outside the building. Composition of the air was constant and was controlled by numerous analyses. During the experiments, the temperature within the chamber was held at a constant 16°C ± 0.2, the air pressure was held 2 mm Hg below the atmospheric pressure, the circulation of air was normally 900 l per hour, and the humidity was a constant 50 mm Hg. About 10 per cent of the total out-going air was led through three columns of potassium hydroxide for the collection of carbon dioxide for subsequent titration. During the experiments, 0.5 ml out-going air was automatically collected twice every minute by a sampling pump. These air samples were later analysed by HALDANE's method for carbon dioxide and oxygen.

Before the experiments, the sheep were fasted for 36 hours. BLOM-

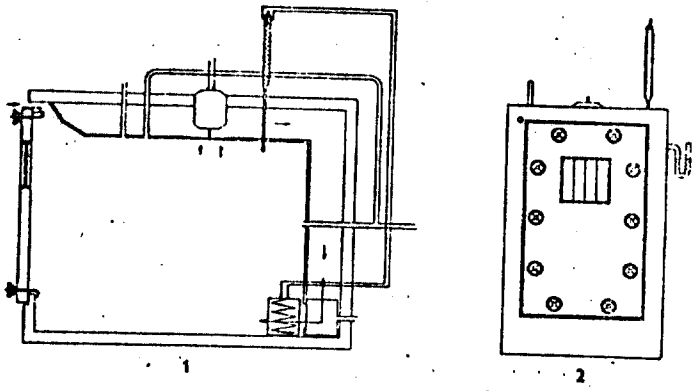


Fig. 1 a. Diagram of the respiration chamber. *1*, side view; *2*, front view.

BÄCK & *al.* (1957) have shown that after 30 hours fast, the variations between the respiratory quotients of normal sheep are very small. The elimination of carbon dioxide by eructation from the rumen is by that time so insignificant that no influence on the gas analyses can be demonstrated. The results of 36 determinations on six sheep gave an average value of 0.72 for the respiratory quotient (BLOMBÄCK & *al.* 1957). These values agree with the results of RITZMAN & BENEDICT (1938).

The experiments were not started until repeated gas samples taken from the chamber at ten-minute intervals gave consistent results for the carbon dioxide values. These were generally obtained after 1 ½ hours. The sheep within the chamber have always remained calm and still. A polyethylene catheter was inserted into the jugular vein by the method of HALLGREN & BJÖRCK (1953). This catheter was joined to a rubber tube led through the roof of the chamber. In order to prevent coagulation within the tubing, physiological salt solution was very slowly infused. These arrangements allowed intravenous injections to be carried out while the animal was enclosed within the chamber. Blood could be aspirated for methaemoglobin determinations and when necessary, treatment with methylene blue could be carried out without interrupting the respiration experiment.

Sodium nitrite, given intravenously, was selected as the means of producing poisoning. Nitrate poisoning actually depends upon the formation of toxic nitrites. In a number of experiments with intravenous injections of sodium nitrite, poisoning identical with that seen after oral administration was produced although much more quickly. Signs of illness and high methaemoglobin values occurred as early as 30

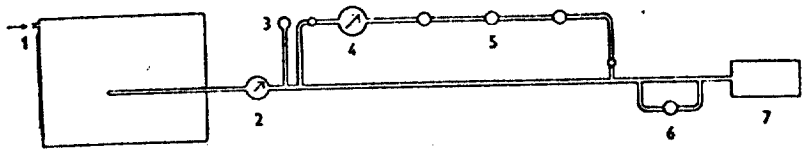


Fig. 1 b. Working diagram of the respiration chamber. *1*, air intake; *2*, gas meter; *3*, sampling pump; *4*, gas meter; *5*, potassium hydroxide containers; *6*, air-flow regulator; *7*, air pump.

minutes following injection. Doses of similar size per kg body weight produced similar effects on various animals regardless of the feeding regimen. In contrast to the oral administration of sodium nitrate, the intravenous injection of sodium nitrite while the animal was enclosed within the respiration chamber has permitted the selection of suitable doses and, because of the rapid effect, the course could be easily controlled. Thus, for one sheep under standard conditions, the normal values and subsequently the effects of both nitrite and nitrite and methylene blue together could be studied. Sodium nitrite was injected intravenously as a 5 per cent solution, and methylene blue as a 1 per cent solution.

Sodium nitrite as a 5 per cent solution given intravenously has also been used to produce nitrite poisoning in the experiments to test the vasoconstrictory agents. Preliminary experiments showed that 35 mg sodium nitrite per kg body weight invariably produced illness of such a degree that the animals sank to the floor and could not rise. The methaemoglobin determinations were carried out by the same method described earlier. Blood samples were taken when the animals collapsed, and in those cases in which prophylactic treatment was given, samples were taken 1 ½ hours after the injection of nitrite. Ephedrine hydrochloride (ACO) and in one case, oxedrine (ACO), were used in the experiments. These preparations were used for the treatment of severly poisoned animals (2 cases, intravenous administration), and as a prophylactic measure before the injection of nitrite (3 cases, subcutaneous injection 30 minutes before the injection of nitrite). Three cases were treated with methylene blue only, 4 mg/kg body weight. Treatment began after the animals had lain for 5 to 10 minutes.

2. *Results*

Results of the experiments in the respiration chamber have been summarised in Tables 2 and 3.

Table 2.

Respiratory exchange in nitrite poisoning.

	Normal			After nitrite injection				
Expt.	CO_2 production l/kg body weight per hour	O_2 consumption l/kg body weight per hour	RQ*	CO_2 production l/kg body weight per hour	O_2 consumption l/kg body weight per hour	RQ*	Methaemoglobin %	Dose $NaNO_2$ mg/kg body weight
1	0.286	0.354	0.81	0.307	0.387	0.79		20
2	0.297	0.398	0.75	0.297	0.371	0.80	40	20
				0.331	0.435	0.76	28	5
				0.328	0.406	0.81	35	10
3	0.217	0.329	0.66	0.266	0.377	0.70	54	30
				0.265	0.377	0.70	54	
4	0.242	0.349	0,69	0.299	0.381	0.78	65	35
				0.307	0.416	0.74	50 [1]	
				0.327	0.543	0.60	20	
5	0.273	0.399	0.68	0.292	0.428	0.68	50	30
				0.298	0.441	0.66	50	
				0.281	0.449	0.69	32 [2]	
				0.290	0.379	0.77	5	
6	0.244	0.437	0.56	0.279	0.439	0.64	62 [3]	35
				0.330	0.501	0.66		
				0.305	0.421	0.72		
				Values after the injection of nitrite but before treatment with methylene blue				
Average	0.260	0.378	0.69	0.296	0.404	0.73		
				All values following the injection of nitrite				
				0.300	0.422	0.71		

$$* \; RQ = \frac{\text{carbon dioxide production in litres per kg body weight per hour}}{\text{oxygen consumption in litres per kg body weight per hour}}.$$

Every value corresponds to an experimental period of one hour. In those cases, however, in which the animals became seriously ill, samples for analysis were taken immediately before treatment with methylene blue.

[1] Animal comatose. Treated with methylene blue.
[2] Treated with methylene blue.
[3] Animal comatose. Treated with methylene blue.

It can be seen from Table 2 that the nitrite caused a slight elevation of both oxygen consumption and carbon dioxide elimination. Nitrite in combination with methylene blue gave an additional slight elevation.

Table 3.

Respiratory exchange after the injection of methylene blue.

	Normal			After methylene blue injection			
Expt.	CO_2 production l/kg body weight per hour	O_2 consumption l/kg body weight per hour	RQ	CO_2 production l/kg body weight per hour	O_2 consumption l/kg body weight per hour	RQ	Methylene blue mg/kg body weight
7	0.242	0.347	0.70	0.236	0.380	0.62	6.5
				0.252	0.387	0.65	
				0.245	0.382	0.64	
				0.250	0.384	0.65	13
8	0.212	0.366	0.58	0.212	0.366	0.58	6
				0.213	0.377	0.56	
				0.240	0.387	0.62	

Methylene blue without nitrite also seems to have a slight tendency to increase oxygen consumption (Table 3).

Attempts have been made to devise a method of determining the oxygen consumption during shorter periods of time so that the oxygen consumption immediately before the death of an animal could be studied. For this purpose, a tightly-fitting respiration mask has been used. A three-way valve was attached to the mask so that the inspired and expired air followed different routes. The expired air was collected in a rubber bag with a capacity of about four litres. It was evident, however, that this method was unsuitable because of the difficulties of obtaining accurate values for the volume of expired air during the short duration of these experiments. The animals were disturbed by the application of the respiratory mask and breathed irregularly. Increased muscular activity certainly also contributed towards irregularity and inaccuracy in the values.

Results of the experiments with vasoconstrictory drugs are shown in Table 4.

3. *Discussion*

In methaemoglobinaemia, a reduced amount of haemoglobin is available for oxygen transport, and as this residual haemoglobin has an increased affinity for oxygen so that this can not be fully released in the tissues, the condition results in a decreased oxygen supply for

Table 4.

The effect of vasoconstrictory drugs on nitrite poisoning.

Expt.	Prophylactic treatment with ephedrine mg	Dose $NaNO_2$ mg/kg body weight	Clinical signs	Methaemoglobin %	Treatment	Effect of treatment
1		35	Lay apathetically 40 min. after injection	65	Methylene blue i.v.	Stood up 20 min. after treatment
2		35	Lay apathetically 50 min. after injection	62	Methylene blue i.v.	Stood up 15 min. after treatment
3		35	Lay apathetically 45 min. after injection	63	Methylene blue i.v.	Stood up 20 min. after treatment
4		35	Lay apathetically 35 min. after injection	68	40 mg. ephedrine (ACO) i.v.	Stood up immediately, displayed normal ability to move. Later showed signs of distress, grunted somewhat, and died suddenly 20 min. after treatment, with a methaemoglobin value of 71 %
5		35	Lay apathetically 65 min. after injection	62	30 mg oxedrine (ACO) i.v. and 30 mg i.m. Methylene blue 20 min. later	Stood up immediately after oxedrine treatment and displayed normal ability to move
6	50 [1]	35	Slight dullness. Normal ability to move. Did not lie down during the experiment	71	Methylene blue 3 hrs. after the injection of nitrite	
7	50 [1]	35 12 [2]	"	60 62	"	
8	60 [1]	35	"	64	"	

[1] An additional 30 mg ephedrine were given subcutaneously 3 hours after this injection.

[2] Second injection given 2 hours after the first, methaemoglobin determination 45 minutes later.

the tissues (BODANSKY 1951). To a certain extent this is compensated for by an increase in cardiac output but, according to the investigations carried out by CLARK, VAN LOON & ADAMS (1943) on dogs, cardiac output remains unchanged until the methaemoglobin level exceeds 40 per cent.

The experiments on the nitrite-poisoned sheep in the respiration chamber do not, however, indicate that methaemoglobin levels of up to 65 per cent of the total haemoglobin result in reduced oxygen transport to the tissues although the animals were clearly affected. On the contrary, a significant increase of both oxygen consumption and carbon dioxide elimination was obtained. Even in those cases in which the poisoning was of such a degree as to make the animals comatose, both the oxygen consumption and the carbon dioxide elimination were at higher levels than during the control experiments. This increase in oxygen consumption does not support the supposition that the clinical signs of nitrite poisoning are the result of internal asphyxiation. Increased muscular activity may possibly account for the slight increase in oxygen consumption, as the animals display light muscular tremors and difficulty in maintaining balance. It may be possible that a strong increase in the pulse rate compensates for the decreased amounts of haemoglobin for an extended period. It is not likely that a very rapid change-over to decreased oxygen consumption occurs shortly before death. This, however, can not be demonstrated by means of the respiration chamber as it takes about 30 minutes before such a change can influence the composition of the air in the chamber.

Although attempts to measure the oxygen consumption and carbon dioxide elimination during the period immediately before death were not successful, the experiments carried out in the respiration chamber support the hypothesis that the production of clinically-evident signs of nitrite poisoning does not primarily depend upon an oxygen deficiency. Animals lying in coma with a methaemoglobin level of 65 per cent have been shown to have an oxygen consumption greater than normal. It is difficult to see why an increase to 70 per cent methaemoglobin should lead to such a pronounced reduction of the oxygen supply that the animal dies in asphyxiation.

In experiments with methaemoglobinaemia produced by *p*-aminopropiophenone, VANDENBELT & *al.* (1944) found that in dogs death occurs with 95 per cent methaemoglobin and recovery can be seen with 87 per cent methaemoglobin. As this substance has no other toxic effects, the cause of death is evidently anoxia.

According to WEISS & *al.* (1933), nitrite reduces blood pressure through its strong vasodilatory effect. This decrease in blood pressure reflexly stimulates an increased cardiac activity. Following customary therapeutic doses, human beings show a reduction of 25–35 mm Hg in blood pressure and an increase in pulse rate to approximately 120 per minute. The cerebrospinal pressure rises markedly with increases from 150 mm H_2O to 350 mm H_2O having been observed in human beings (STEFAN 1934).

After studying chronic experimental nitrite poisoning in rats, HUEPER & *al.* (1940) have come to the conclusion that the changes observed are associated with circulatory disturbances caused by the vasodilatory effect of nitrite. The blood vessels which react first and for the lowest doses are those of the head, neck, brain, and meninges. Larger doses dilate the coronary vessels and then the visceral vessels.

As small doses of nitrite in human beings can influence the circulation apparatus so drastically, it is possible that the circulation may collapse after large doses. The clinical course of nitrite poisoning—collapse, loss of consciousness, relatively rapid death—support this hypothesis. Although LESTER & GREENBERG (1944) have been able to demonstrate disturbances in the oxygen-dissociation curve in experimental methaemoglobinaemia of cats and dogs, they believe that the other toxic effects of methaemoglobinogenic substances can be of greater significance. As an example is cited cerebral anaemia resulting from the reduction in blood pressure caused by nitrite.

In all those cases in which nitrite, 35 mg/kg body weight, was injected without preliminary treatment, the animals become seriously ill. They staggered, could not co-ordinate movements, and sank to the ground, 35–65 minutes after the injection. Following treatment with methylene blue, the animals were able to rise after 15–20 minutes, an effect similar to that observed by BRADLEY & *al.* (1940). The results of intravenous treatment with ephedrine were seen immediately; the animal rose and was able to move normally. This animal died, however, 20 minutes following treatment although probably not as the result of methaemoglobinaemia or other effects of nitrite as the clinical signs were quite different. The animal was conscious and could move but was uneasy, had colicky pains, and died suddenly. Death could possibly have been due to cardiac insufficiency. The intravenous administration of ephedrine leads to great irregularities in cardiac activity and therefore ought to be avoided. In this particular case a large dose was given, and as the heart was already greatly affected by the nitrite, it proved unable to withstand this additional stress.

In order to reduce the risk of heart failure, the milder drug oxedrine was used in place of ephedrine for the next experiment. An immediate effect was also obtained in this case. As the effect of oxedrine is of fairly short duration, methylene blue was administered 20 minutes later.

In experiments 6, 7, and 8 in which ephedrine was administered subcutaneously 30 minutes before the injection of nitrite, serious clinical signs were not evident although in experiment 6, the methaemoglobin level was 71 per cent, about 10 per cent higher than values previously encountered in standing sheep. As the animal had remained three hours in this condition, and as untreated animals become ill 35–65 minutes after the injection of nitrite, the results of the ephedrine treatment can safely be taken as positive. Methylene blue was administered three hours after the nitrite so that the animal would not be subjected to methaemoglobinaemia longer than was necessary. These experiments demonstrate that vasocontractory drugs show a very evident effect on nitrite poisoning. The hypothesis that the clinical picture is to a great extent dependent upon the effect of nitrite on the circulatory apparatus is supported not only by these experiments but also by those carried out in the respiration chamber. These results are also of interest for the treatment of clinical cases of nitrite poisoning; such cases ought to be treated with vasoconstrictory drugs in addition to the usual measures.

The question arises why methylene blue gives such good results in view of its well-known ability to transform methaemoglobin to haemoglobin and perhaps even to serve as an oxygen carrier. Nitrite first affects the blood vessels of the brain and meninges, and it is possible that the consequent cerebral anaemia can partially be compensated for by a reduction in the level of methaemoglobin even although the oxygen supply to the body as a whole is not markedly affected. As methylene blue has a reducing effect on nitrite, some of the effect may depend upon a decrease in the nitrite concentration in the blood.

Chapter III

Effect of Feed Composition on Nitrate and Nitrite Breakdown in the Rumen

As was shown by the experimental results in chapters I and II, there is an increased mortality and a greater methaemoglobin formation (Table 1) among animals on inadequate rations after the con-

sumption of nitrate. This is probably due to increased accumulation of nitrite in the rumen and consequently greater absorption. SAPIRO & *al.* (1949) found in this connection that added nitrate disappeared without the appearance of nitrite from the rumen contents *in vitro* obtained from sheep receiving protein-rich feed. On the other hand, nitrite was formed and a longer time was required to break down added nitrate in rumen contents obtained from sheep receiving a cellulose-rich, protein-deficient ration.

On the basis of these observations, it has been considered desirable to study nitrite breakdown in more detail, both qualitatively and quantitatively, after the administration of nitrate to animals receiving two different rations. The breakdown of nitrite in rumen contents has been studied by the direct addition of this substance. Every experiment has been carried out *in vitro* as well as *in vivo*.

1. *Materials and Methods*

Six sheep with permanent rumen fistulae and four without have been used in these experiments. Three of the sheep with fistulae and two without received a nutritionally adequate ration consisting of 0.5 kg hay, 200 g crushed oats, and 50 g molasses per animal per day as well as free access to oat straw. The other five sheep received an inadequate diet consisting of 0.2 kg hay per animal daily and free access to oat straw. The terms adequate and inadequate ration in the following refer to these rations. The animals were maintained on these rations for six weeks before the commencement of the experiments. Rumen samples were taken at 9 a.m. and feeding took place at 10 a.m. and 2 p.m. The rumen samples were examined immediately after collection. A water pump was used to obtain rumen contents from sheep with fistulae while for the others, a stomach tube attached to the water pump was used. This tube was constructed on the same principles as that for cattle described by GERLE (1954) and was made from a 1.5 m long rubber tube with an outside diameter of 15 mm. The flexible metal portion attached to the tube was 7 cm long and had a metal weight attached to its free end so that an angle of approximately 50° between the metal portion and the rubber tube was formed when the latter was held in a horizontal position. This arrangement directed the free end into the reticulum where the contents are more watery and easily aspirated.

For the *in vitro* experiments, test tubes containing 10 ml fresh rumen contents were placed in a water bath at 39°C. The experiments were

begun immediately and continued for two hours at the most so that the rumen contents could not undergo changes deviating too widely from physiological conditions. In every case duplicate determinations were performed.

Sulphanilic acid and α-naphthylamine were used for the qualitative demonstration of nitrite in rumen contents according to the method of FEIGL (1935). Quantitative determinations were carried out on a Beckman B spectrophotometer using a modification of the method described by WHITEHEAD (1943). Five ml rumen contents were mixed with 10 ml acetone and after shaking the mixture was filtered. The filtrate was then diluted with distilled water. The dilutions used ranged from 1/10 to 1/100 depending upon the concentration of nitrite, although 1/50 was found to be a suitable dilution for most cases. Perfectly clear solutions were obtained by these means. To 10 ml of the dilute solution was added 0.7 ml sulphanilic acid reagent (acid. sulphanil. 2 g, acid. acetic. 30 per cent ad 200 ml) and 0.7 ml α-naphthylamine reagent (α-naphthylamine 0.6 g, acid. acetic. conc. 16 ml, aq. dest. 140 ml).

Readings were made on the spectrophotometer at 520 mμ, 30 minutes after the addition of α-naphthylamine, and with distilled water as a control. The amount of nitrite was obtained from reading off a standard curve calculated from known amounts of sodium nitrite dissolved in distilled water.

Table 5.

Control of method for quantitative determination of nitrite in rumen contents.

Sodium nitrite added mg per 100 ml rumen contents	Sodium nitrite found mg per 100 ml rumen contents	Recovery %
2.0	2.0	100.0
2.0	1.9	95.0
4.0	3.8	95.0
4.0	3.9	97.5
8.0	7.5	93.8
8.0	7.8	97.5
15.0	14.1	94.0
15.0	14.6	97.3
45.0	44.6	99.1
45.0	43.8	97.3

As a control of the method, known amounts of sodium nitrite were added to rumen contents and determined by the same method. The results are shown in Table 5.

As can be seen from the table, between 93.8 and 100 per cent of the added amounts could be recovered.

The presence of nitrite following the addition of nitrate has been demonstrated as follows:

Test tubes containing 0.2, 0.7, and 1.0 ml 0.1 per cent sodium nitrate and 10 ml rumen contents were used for every *in vitro* experiment with rumen contents. One drop was taken every five minutes for two hours for the demonstration of nitrite. *In vivo,* 0.45 g sodium nitrate per kg body weight was given by stomach tube or through the rumen fistula. Repeated samples were subsequently taken for the quantitative determination of nitrite. When the nitrite had disappeared, or at the conclusion of the experiments, diphenylamine in concentrated sulphuric acid was used according to FEIGL (1935) to check that the nitrate had also disappeared in both the *in vitro* and *in vivo* experiments.

The ability to break down nitrite was studied *in vitro* by the addition of 0.2, 0.5, and 0.7 ml 0.025 per cent sodium nitrite to test tubes containing 10 ml rumen contents. One drop was taken every five minutes for two hours for the demonstration of nitrite. When the nitrite had disappeared, diphenylamine in concentrated sulphuric acid was used to check that oxidation to nitrate had not occurred.

The *in vivo* experiments were carried out on sheep with rumen fistulae. One gram sodium nitrite dissolved in 300 ml water was introduced into the rumen through the fistula and mixed with the rumen contents by stirring with a narrow metal rod. Rumen samples were taken every five minutes with a pipette and examined for nitrite ions. When a negative sample was obtained, several additional samples were immediately taken from various portions of the rumen and when all these were negative the experiment was concluded. The time required for the breakdown of the amount of nitrite added could be estimated by this means.

In every case duplicate determinations were made.

2. *Results*

Results of the *in vitro* experiments with nitrate are shown in Table 6. For these experiments 20 rumen samples have been taken from each of the two groups of animals. All three nitrate doses have been used for each sample.

Table 6.

The presence of nitrite following the addition of 0.1 per cent $NaNO_3$ solution to rumen contents *in vitro*.

The number of nitrite-positive samples is expressed as per cent of the total number of samples.

Nitrate dose ml	Time in minutes 0–15	15–30	30–45	45–60	60–75	75–90	90–105	105–120	Feeding regimen
1.0	25	20	15	5	0	0	0	0	Adequate
0.7	20	15	5	5	0	0	0	0	,,
0.2	5	0	0	0	0	0	0	0	,,
1.0	50	75	95	100	100	100	95	80	Inadequate
0.7	60	75	85	95	95	85	80	70	,,
0.2	65	60	40	30	20	15	15	5	,,

It can be seen from the table that, in rumen samples from animals maintained on an adequate ration,

(1) the added nitrate disappeared and nitrite could be demonstrated in only 25, 20, and 5 per cent of all cases after the addition of 1.0, 0.7, and 0.2 ml nitrate solution respectively;

(2) the number of nitrite-positive samples was largest in the first 15 minutes and after 60 minutes, both nitrate and nitrite had disappeared from all samples; and

(3) the number of nitrite-positive samples corresponded directly to the size of the nitrate dose.

It can also be seen from Table 6 that, for animals receiving an inadequate ration,

(1) the number of nitrite-positive samples was very high; in 100, 95, and 65 per cent of the cases after the addition of 1.0, 0.7, and 0.2 ml nitrate solution respectively nitrite was detected at some time during the experiment;

(2) the number of nitrite-positive samples was smallest during the first 15 minutes except in the case of smallest nitrate dose, while most were found between 45 and 90 minutes for both the larger doses; and

(3) the number of nitrite-positive samples corresponded directly to the size of the nitrate dose.

Results of the comparable *in vivo* experiments are shown in Figs. 2 a and 2 b.

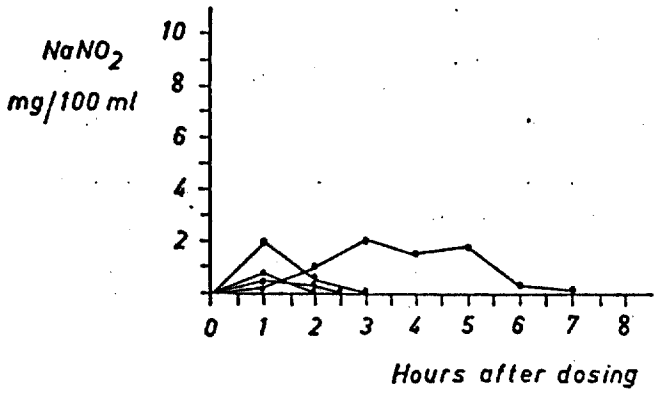


Fig. 2 a. Nitrite in the rumen following the administration of sodium nitrate, per kg body weight, to four sheep receiving an inadequate ration.

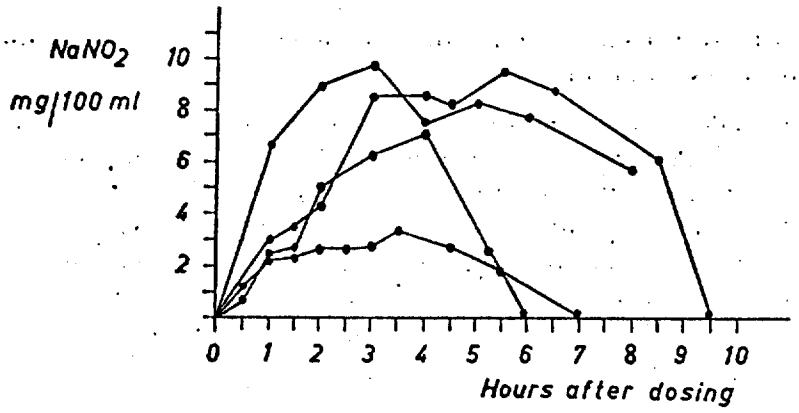


Fig. 2 b. Nitrite in the rumen following the administration of sodium nitrate, 0.45 g per kg body weight, to four sheep receiving an inadequate ration.

Small amounts of nitrite were demonstrated for a relatively short period in the rumen of the animals fed an adequate ration. The nitrite concentration was markedly higher, and nitrite persisted longer, in the animals with inadequate feeding. These results closely agree with those obtained for the *in vitro* experiments.

The experiments with nitrite added to rumen contents *in vitro* are considered in Table 7. For these experiments 20 rumen samples have been taken from each of the two groups of animals. All three nitrite doses have been used for each sample.

The table shows that the added nitrite had disappeared after 75 minutes from every rumen sample obtained from the adequately-fed

Table 7.

The disappearance of nitrite following the addition of 0.025 per cent $NaNO_2$ solution to rumen contents *in vitro*.

The number of nitrite-positive samples is expressed as per cent of the total number of samples.

Nitrite dose ml	Time in minutes 15	30	45	60	75	90	105	120	Feeding regimen
0.7	70	45	25	10	0	0	0	0	Adequate
0.5	55	30	5	0	0	0	0	0	,,
0.2	10	0	0	0	0	0	0	0	,,
0.7	100	100	100	100	100	100	95	90	Inadequate
0.5	100	100	95	90	90	75	75	70	,,
0.2	100	80	70	45	45	30	30	10	,,

animals. The time required for the breakdown of nitrite increases directly as the dose of nitrite.

Few nitrite-free samples were obtained from the inadequately-fed animals after 75 minutes. At the conclusion of the experiment after 120 minutes, none of the groups was completely nitrite-free, not even the animals receiving the smallest doses of nitrite. The time required for the breakdown of nitrite also increased directly as the dose.

Results of the experiments with nitrite added *in vivo* are shown in Table 8.

It can be seen from the table that animals which received an adequate ration had a distinctly better ability to break down nitrite in the rumen than those which received an inadequate ration. The results agree closely with those obtained for the comparable experiment *in vitro*.

Table 8.

Time in minutes for the breakdown of 1 g sodium nitrite in the rumen for two groups of sheep fed different rations.

Feeding regimen	Number of animals	Number of determinations	Time in minutes Average	Minimum value	Maximum value
Adequate	4	16	46	20	75
Inadequate	4	14	99	80	140

3. *Discussion*

It may be concluded from the experiments described above that the composition of the feed is of very great significance for controlling the accumulation of nitrite in the rumen after the administration of nitrate and for the breakdown of introduced nitrite.

Nitrite is an intermediate stage in the breakdown of nitrate in the rumen (SAPIRO & *al.* 1949 and LEWIS 1951 a). This may be confirmed by introducing nitrate to the rumen as it is invariably possible to demonstrate nitrite if sufficiently large doses are given. Following the introduction of nitrate, LEWIS (1951 a) has shown that the amount of ammonia in the rumen increases as the amount of nitrate decreases. If the various, more or less unknown, intermediate stages are disregarded and if the reaction is assumed to be reduction to NH_3 as LEWIS (1951 b) has shown can occur, the sequence can be written in a simplified fashion as

$$NO_3^- \xrightarrow[1]{} NO_2^- \xrightarrow[2]{} NH_3$$

As accumulations of nitrite are always obtained if sufficiently large doses of nitrate are given, reaction 1 must take place more readily than reaction 2. On an inadequate diet, the breakdown of both nitrate and nitrite is inhibited, but reaction 2 is inhibited to a much greater degree than reaction 1 with the result that nitrite accumulates in greater amounts for longer periods than it does in animals receiving an adequate ration. This leads to larger amounts of nitrite being absorbed from the rumen and explains the greater concentration of methaemoglobin seen in the inadequately-fed groups described in chapter I.

Chapter IV

Effect of the pH Value on the Breakdown of Nitrate and Nitrite in Rumen Contents

As in the case of other microbiological and enzymatic processes, those occuring in the rumen are dependent upon the pH value. An investigation of the degree to which changes in the pH values can influence nitrate metabolism and whether abnormal values result in dangerous accumulations of nitrite seems to be justified.

Values for pH in the rumen are greatly influenced by the feeding regimen. Following the ingestion of highly-soluble carbohydrates, liberal amounts of acids, especially volatile fatty acids, are formed with a consequent depression of pH. The production of these acids decreases and the pH values rise following periods of fast. Normal variations in pH are not great because of the continuous absorption of the acids and mixing of saliva with the ingesta. (REID & HUFFMAN 1949 and CLARK & LOMBARD 1951.) Information on rumen pH determinations can be obtained from many sources (PHILLIPSON 1942, MYBURGH & QUIN 1943 and BROBERG 1956).

In vitro estimations give pH values lying between 6 and 7 while somewhat lower values are obtained *in vivo*. Disturbances in rumen function can lead to pH values markedly higher or lower than normal.

1. *Materials and Methods*

Eight sheep were used for these investigations; six were fed the adequate and two the inadequate ration described in chapter III. Test tubes, each containing 10 ml rumen contents obtained from these sheep, were placed in a water bath at 39°C. In every case duplicate determinations were performed. By the addition of *N*/10 HCl and *N*/5 NH_3 respectively, the pH values in the various tubes were altered to give readings of 5.7, 6.6, and 7.8. Measurements were made with a pH-meter, type 22. To each tube was added 0.2 ml 0.1 per cent sodium nitrate solution. Tests for the presence of nitrite were subsequently made by taking one drop every five minutes as was described earlier in chapter III. The experiments were continued until both nitrate and nitrite had disappeared.

In order to study *the breakdown of nitrite* in more detail at various pH levels, 125 μg sodium nitrite in a 0.025 per cent solution were added to tubes containing rumen contents at pH 5.7, 6.1, 6.5, 6.9, 7.3, and 8.0, and the time required for the breakdown of the added nitrite was recorded. At the conclusion of the experiment, the pH values in the various tubes were checked again with the usual finding that these had undergone only insignificant changes.

The study of nitrate and nitrite breakdown *in vivo* at various pH levels has not been attempted because of the difficulties which would arise through the activity of the normal buffering mechanisms and the effects of changes in pH on rumen motility. These sources of error are eliminated with *in vitro* experiments.

2. *Results*

The results of the experiment of adding nitrate to rumen contents at various pH levels *in vitro* are shown in Table 9. For these experiments 20 rumen samples have been taken.

Table 9.

The number of nitrite-positive samples at various intervals following the addition of 0.2 ml 0.1 per cent $NaNO_3$, expressed as per cent of the total number of samples.

pH	Time in minutes 0–45	45–90	90–135	135–180	180–225
5.7	75	85	50	30	0
6.6	55	50	20	0	0
7.8	90	70	30	10	0

It can be seen from the table that at these three pH values, nitrite was present in the smallest number of samples and for the shortest time at pH 6.6. Reduction to 5.7 or elevation to 7.8 resulted in a markedly increased number of samples showing nitrite for a longer time. The rate of disappearance of nitrite is somewhat slower at pH 5.7 than at pH 7.8 with the largest number of nitrite-positive samples occurring later and the total number of positive samples decreasing more slowly.

The average values for 16 different investigations of the breakdown of nitrite in rumen contents *in vitro* at various pH levels are sum-

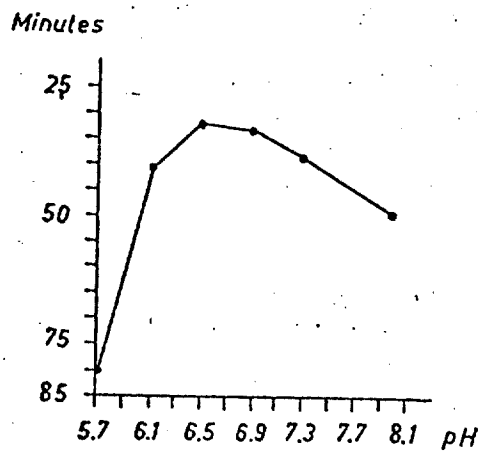


Fig. 3. Nitrite breakdown at different pH levels.

marised in Fig. 3. Good breakdown of nitrite was obtained between pH 6.1 and 7.3, and the best at 6.5–6.9. Reduction of the pH to 5.7 resulted in a strong inhibition of the ability to break down nitrite. Elevation to 8.0 showed a relatively weak inhibition.

3. *Discussion*

The optimum pH for the reduction of nitrate in an atmosphere of hydrogen in the presence of washed suspensions of rumen microorganisms was found by LEWIS (1951 b) to be 6.5, and that for nitrite reduction, 5.7.

The present experiments with rumen contents showed that the breakdown of both nitrate and nitrite occurred at an optimum pH of approximately 6.5. As these chemical processes are both normal and common in the rumen, it is to be expected that they take place most readily under normal rumen conditions.

Acetic acid and sodium bicarbonate were used in a few experiments in order to ascertain that the inhibition seen in conjunction with changes in the pH did not depend upon specific effects of hydrochloric acid or ammonia. Both sets of experiments were in complete agreement.

As the reduction of nitrate to nitrite apparently occurs within a wider pH range than the further reduction of nitrite, an abnormal pH value in the rumen increases the risks of nitrite accumulation (see Table 9).

Chapter V

Effect of Certain Growth-Promoting Substances on Nitrite Breakdown in Rumen Contents and in Cultures of Rumen Microorganisms

The ability of bacteria to reduce nitrate has long been recognised, and cases of poisoning among cattle have also been associated with the reduction of nitrate to nitrite by rumen flora. The reduction of nitrate forms a part of nitrogen metabolism in the rumen. It has been shown by several investigators that the rumen microorganisms can utilise inorganic nitrogenous compounds for protein synthesis. Urea has been most widely used as a source of nitrogen in the experimental investigations which have been carried out (PEARSON & SMITH, 1943, and BURROUGHS, ARIAS, DEPAUL, GERLAUGH & BETHKE 1951).

KAEMMERER (1956) has shown that protein synthesis in rumen contents *in vitro* was increased by the addition of penicillin or β-indolylacetic acid. This latter substance, occurring in plants and stimulating their growth, is considered to be a heteroauxin. KAEMMERER (1956) has even postulated that the growth-stimulating effect of antibiotics on animals is comparable with that of heteroauxin on plants.

HOFLUND, NIELSEN, ANDERSSON, BROBERG & HÄSSLER (1957) have shown that antibiotics are formed in the rumen and that this formation is depressed or ceases in certain disturbances of rumen function. Both antibiotics and β-indolylacetic acid may thus occur normally in the rumen.

Much has been written about bacteria which have the ability to reduce nitrate to nitrite (QUASTEL & *al.* 1925, STEPHENSON & *al.* 1931 and AUBEL 1938) all working with *Escherichia coli*. WOODS (1938) has studied two strains of *Escherichia coli* and one of *Clostridium welchii* and considers it likely that nitrite and hydroxylamine are intermediate stages in the reduction of nitrate.

Twenty-two strains of the genus *Bacillus* and twenty strains of coliform bacteria, isolated from the rumens of sheep, were tested by MÅNSSON & ANDERSSON (1956) for their capacity to break down added nitrite. Of eleven strains of *Selenomonas ruminantium*, three were able to reduce nitrate (BRYANT 1956). HOLTENIUS & NIELSEN (1957) have shown that fungi from the rumen of sheep, *Absidia ramosa* and *Fusarium* species, have the ability to reduce nitrate and nitrite.

There is apparently no information about other rumen organisms which can reduce nitrate or nitrite.

Since penicillin and β-indolylacetic acid stimulate protein synthesis in rumen contents, it may be possible that this depends upon a better utilisation of inorganic nitrogenous compounds. In order to test this hypothesis, the effect of both these substances on nitrite breakdown in rumen contents and in cultures of certain organisms has been investigated.

Bacteria-free infusoria cultures were obtained by SUGDEN (1953) by the addition of streptomycin to rumen contents.

In order to investigate whether the infusoria have the ability to break down nitrite, the activity of the fungi must also be blocked. For this reason, the effect of this antibiotic on the breakdown of nitrite by cultures of *Absidia ramosa* isolated from the rumen has been studied.

1. *Materials and Methods*

Four sheep with permanent rumen fistulae and four without have been used in these experiments. Rumen samples for the preparation of bacterial and fungal cultures were obtained from the sheep without fistulae by aspiration of 2–5 ml into a 20 ml sterile syringe attached to a 10 cm long needle, 2 mm in diameter, inserted through the abdominal wall into the rumen. The needle was inserted on the left side, a few centimetres posterior to the last rib and about 5 cm ventral to the ridge formed by the lateral processes of the lumbar vertebrae. Precautions were taken to disinfect the skin adequately and sterile apparatus was used. For the other experiments rumen samples were obtained from the sheep with fistulae.

Bacteria cultures were made by the method described by MÅNSSON & *al.* (1956), and fungi cultures by the technique of HOLTENIUS & *al.* (1957). Fungi cultures which had been cultivated submerged on a rotary shaker and bacteria cultures in bouillon were placed in test tubes and incubated at 39°C in a water bath. Sodium nitrite, usually 50 μg, was added and the rate of disappearance of the added nitrite was checked by sulphanilic acid and α-naphthylamine. Control tubes containing identical amounts of nitrite and medium but free from bacteria or fungi were examined at the same time.

The effect of procaine penicillin and β-indolylacetic acid on the breakdown of nitrite was studied in fungi and bacteria cultures as well as on rumen samples. The experiments were set up so that four test tubes containing identical amounts, usually 10 ml, of culture or rumen contents were incubated at 39°C. Identical amounts of sodium nitrite were added to all four tubes, and to two of these, 500 I.U. procaine penicillin, 5 mg dihydrostreptomycin, or 10 μg β-indolylacetic acid were added per ml.

These doses were chosen on the basis of preliminary experiments with rumen contents and fungi cultures which showed an effect for penicillin and β-indolylacetic acid which was largely independent of minor variations in the size of dose. The dose of streptomycin was based upon SUGDEN's (1953) investigations using 560 μg streptomycin per ml, and as that dose was sufficient to stop bacterial activity, a much stronger dose was taken for testing on cultures of *Absidia ramosa*. The time required for the breakdown of the added nitrite in the various tubes was determined in the same way as has been described earlier.

2. *Results*

The results are summarised in Tables 10, 11, and 12.

Table 10.

Effect of added penicillin on the breakdown of nitrite.

Micro-organism	Number of determinations	Dose $NaNO_2$ µg	Time in minutes		Difference		
			Control	Penicillin 500 I.U. per ml	Average	Minimum value	Maximum value
Rumen contents	21	125	16	12	4	2	10
Absidia ramosa	12	100	125	13	112	55	180
Fusarium	7	50	480	490	−10	−30	20
Coliform bacteria	16	50	91	97	−6	−35	15
Genus *Bacillus*	10	50	227	348	−121	−210	20

Table 11.

Effect of added β-indolylacetic acid on the breakdown of nitrite.

Micro-organism	Number of determinations	Dose $NaNO_2$ µg	Time in minutes		Difference		
			Control	β-indolylacetic acid 10 µg/ml	Average	Minimum value	Maximum value
Rumen contents	16	125	18	18	0	−2	2
Absidia ramosa	13	50	22	11	11	5	25
Fusarium	6	50	480	115	365	190	400
Coliform bacteria	16	50	91	84	7	−10	40
Genus *Bacillus*	10	50	227	191	36	−20	100

Table 12.

Effect of added streptomycin on the breakdown of nitrite.

Micro-organism	Number of determinations	Dose $NaNO_2$ μg	Time in minutes		Difference		
			Control	Streptomycin 5 mg/ml	Average	Minimum value	Maximum value
Absidia ramosa	9	75	81	78	3	0	15

In no instance could nitrite breakdown be demonstrated in medium in the absence of microorganisms although samples were taken up to 48 hours following the commencement of the experiment.

It can be seen from the Tables 10 and 11 that the *Bacillus* species, the coliform bacteria, and both fungi species have the capacity to break down nitrite. Of the fungi, *Absidia ramosa* was much more effective than *Fusarium*. It would serve no purpose to try to compare the effectivity of the various species as it is difficult to establish identical conditions such as equal numbers of organisms and so forth.

Penicillin added to rumen contents in the doses mentioned above distinctly stimulated the breakdown of nitrite. The addition of penicillin to cultures of *Absidia ramosa* gave a very strong effect although cultures of *Fusarium* was not affected at all. Penicillin in these doses and especially in the case of *Bacillus* had an inhibiting effect upon the breakdown of nitrite by the bacteria.

Addition of β-indolylacetic acid to rumen contents did not influence nitrite breakdown. A pronounced effect, on the other hand, was observed with cultures of *Absidia ramosa* and *Fusarium*. A slight but not significant stimulating effect was produced on the bacteria.

The addition of streptomycin in fairly large doses to cultures of *Absidia ramosa* did not affect the breakdown of nitrite.

3. *Discussion*

The microorganisms tested are of frequent occurrence in the rumen. The degree to which the differences between the ability of different animals to break down nitrite depend upon variations in the microbe population has not been demonstrated up to the present time.

Beta-indolylacetic acid is a bacterial breakdown product of protein. The effect of this substance in promoting plant growth is well known (Kögl 1938) and as it is present in plants, there is reason to suppose that it can also be found in the rumen. How this substance affects the breakdown of nitrite in fungi cultures is not clear.

Supplementing the feed of calves with penicillin gives an increased rate of growth and better utilisation of feed (Brüggeman 1955). In what way this effect is obtained is still unknown. Kaemmerer (1956), however, has shown that both β-indolylacetic acid and penicillin increase protein synthesis in the rumen.

On the basis of the experiments with these growth-promoting substances on nitrite breakdown, the question arises whether or not a more effective utilisation of inorganic nitrogenous compounds for protein synthesis takes place.

The inhibiting effect of penicillin upon the breakdown of nitrite by bacteria may depend upon too large a dose having been used. There may be an optimum dose which would have a stimulating effect instead.

It has not proved possible to investigate whether or not the rumen infusoria which are especially numerous during feeding with protein-rich rations (van der Wath & Myburgh 1941) have the ability to break down nitrite. There appear to be no means by which to obtain these organisms in pure culture.

Feeding with highly-soluble carbohydrates supports luxurious growth of *Selenomonas ruminantium*. This organism has been described by, among others, McGaughey & Sellers (1948), and Ingram & McGaughey (1948), and is probably identical with the *Selenomastix ruminantium* described by Woodcock & Lapage (1913). Bryant (1956) has obtained these organisms in pure culture, described them as being anaerobic, Gram-negative, motile bacteria, and found that three of eleven strains could reduce nitrate. Although these organisms are not of great significance in the direct reduction of nitrate, they may be of indirect importance through their ability to split sugars.

Chapter VI

Effect of Finely-Divided Plant Particles in Rumen Contents on Nitrite Breakdown

During investigations into the ability of rumen contents from different sheep to break down nitrite, the observation was made that in those cases in which the breakdown was rapid, the rumen contents had a somewhat viscous consistency and a greenish colour. In those cases, on the other hand, in which the nitrite breakdown proceeded at a slower pace, the rumen contents were more watery, the colour was more of brownish shade, and the solid particles rapidly settled out. In order to establish whether the viscous mass *per se* could influence the rate of nitrite breakdown, or whether the effect depended upon a concentration of microorganisms within this mass, nitrite breakdown was studied following the removal of most of the microorganisms by washing.

This mass in the rumen contents has, as far as the present writer is aware, not been the subject of any earlier study.

Quantitative determinations of the sediment in the rumen contents of animals on different rations and with different capacities for breaking down nitrite have also been carried out.

1. *Materials and Methods*

Eight sheep with permanent rumen fistulae were used for these investigations. Four were held on adequate and four on inadequate rations. Rumen samples were obtained before the morning feeding.

Rumen contents were filtered through four thicknesses of gauze to remove solid particles and then centrifuged for one minute at 1000 r.p.m. Most of the bacteria remain in the supernatant as a distinctly greater speed for a longer time, 3000 r.p.m. for thirty minutes (LEWIS 1951 b), is required for their sedimentation. The sediment consisted of protozoa and finely-divided plant particles as well as a few microorganisms which had been thrown down. After centrifugation, the sediment was suspended in sterile 0.05 molar phosphate buffer solution at pH 6.5 and recentrifuged for one minute at 1000 r.p.m. The material was washed an additional two times. After washing, the sediment was resuspended in the sterile buffer solution and test tubes containing 10 ml of the suspension were placed in a water bath at

39°C. Test tubes containing 10 ml of the supernatant from the first centrifugation were also placed in the water bath. To each of the tubes was added 125 μg $NaNO_2$ in 0.025 per cent solution and the time required for the breakdown of the added nitrite was determined.

The ability to break down nitrite was also studied following warming of the washed suspension to 50°C for thirty minutes before incubating at 39°C. Cooling to 1°C for five hours has also been carried out to determine whether nitrite breakdown can occur at that temperature. These tubes were subsequently incubated at 39°C and the breakdown of nitrite followed.

Quantitative measurements of the sediment in rumen contents were made by filtering rumen contents through four thicknesses of gauze, and then centrifuging 2 ml of the filtrate in graduated sedimentation tubes for one minute at 1000 r.p.m. In all experiments duplicate determinations were made.

The washed suspensions of finely-divided plant particles were examined microscopically.

2. *Results*

Comparisons between the ability to break down nitrite shown by untreated rumen contents, washed suspensions of finely-divided plant particles, and by rumen contents from which infusoria and plant particles were removed are shown in Table 13.

Table 13.

Time in minutes for the breakdown of 125 μg $NaNO_2$ in 10 ml of different fractions from rumen contents. Average for 20 experiments.

Untreated rumen contents	Washed suspensions	Supernatant; first centrifugation
17	3	41

The experiments of cooling washed suspensions of finely-divided plant particles from rumen contents gave the results shown in Table 14.

Suspensions incubated at 39°C after being heated at 50°C for 30 minutes showed no ability at all to break down nitrite.

Results of the quantitative determinations of sediment are given in Table 15.

Microscopic examination revealed that the plant particles were so finely divided that the cell membranes to a very large extent were ruptured.

Table 14.

Time required for the breakdown of 1 mg $NaNo_2$ in 10 ml suspension. Average for 7 experiments.

At 39°C	At 1°C	At 39°C directly after being held at 1°C for 5 hours
34 minutes	> 5 hours	41 minutes

Table 15.

Ml sediment per 2 ml rumen contents. Number of samples from every group = 20.

Feeding regimen	Average	Minimum value	Maximum value
Adequate	0.10	0.04	0.20
Inadequate	0.03	0.01	0.08

3. *Discussion*

The washed suspensions of finely-divided plant cells were not free from bacteria as a number of them were intimately associated with the plant cells although most of the microorganisms had been removed by repeated centrifugation for short periods at a low speed. The good capacity for breaking down nitrite shown by this fraction could not depend upon an increase in the number of microorganisms as this was, in fact, greatly reduced. The effect is probably also independent of the presence of the large protozoa which can be found in this fraction. Cooling to 1°C kills at least most of these organisms judging by microscopic examinations. As the breakdown of nitrite was essentially unchanged when the suspensions were subsequently incubated at 39°C, it is unlikely that the infusoria were of much significance in this connection. It seems as though the finely-divided plant particles were capable of rapidly removing the free nitrite ions. It may be debated whether or not bacteria must also be present. It has not been possible to study nitrite breakdown in sterilised suspensions as the ability to

break down nitrite is lost before the bacteria are killed. Heating to 50°C completely inhibits the effect. Reduction of the temperature to 1°C also inhibits the process which, however, takes place normally following rewarming to 39°C. These results are compatible with the effect being the result of enzymatic reactions, greatly dependent upon temperature. The origin of these enzymes, whether from microorganisms or plants, has not been determined. It has also proved impossible to determine the nature of the active substance in the plant particles and how it acts. As was shown by microscopic examination, the plant particles were so finely divided that the cell membranes were ruptured. Cellulose particles were largely eliminated by filtration and the soluble substances were removed by washing.

Once the nitrite had disappeared, it could never be shown to reappear although samples were taken over a period of several hours.

Quantitative determinations of the sediment in filtered rumen contents showed that animals which received the adequate ration and which had a better ability to break down nitrite also displayed a significantly greater amount of sediment consisting of finely-divided plant particles than the animals held on inadequate ration. It thus appears that the presence of sufficient amounts of partially-digested plant cells is a very significant prerequisite for the removal of nitrite.

Chapter VII

The Metabolism of Nitrate and Nitrite in the Normally Functioning Rumen

Ruminants break down a portion of the protein in the feed to lower nitrogenous compounds which may subsequently be used for the synthesis of microbial protein (SYNGE 1952). As these lower nitrogenous compounds are often toxic, their removal by the rumen flora is of great importance for the health of the animal.

Investigations of the utilisation of non-protein nitrogen for protein synthesis in the rumen have made it clear that this occurs only in the presence of added carbohydrates (WEGNER, BOOTH, BOHSTEDT & HART 1940, JOHNSON, HAMILTON, MITCHELL & ROBINSON 1942 and MCCLYMONT 1946). Similar results have been obtained with rumen contents *in vitro* and by feeding experiments.

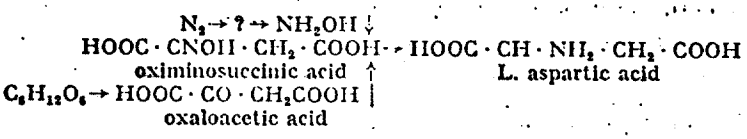
According to BURSTRÖM (1946), fixation of nitrate-nitrogen in plants is always associated with carbohydrate metabolism since carbohydrates

supply the carbon structure for the nitrogenous compounds which are to be formed. Under conditions of carbohydrate deficiency, nitrite and ammonia are accumulated. Addition of nitrates leads to a reduction in the amounts of all the carbohydrates.

Using nitrate as a source of nitrogen, the formation of amino acids in green plants has been studied by VIRTANEN & ARHIMO (1939), and details of the process of nitrogen-fixation carried out by bacteria in the root nodules of legumes have been investigated by VIRTANEN & LAINE (1939). It was shown that the mechanism was the same in both instances. The first stage is the formation of hydroxylamine by the reduction of nitrate through nitrite.

$$NO_3^- \longrightarrow NO_2^- \longrightarrow NH_2OH$$

Hydroxylamine subsequently reacts with oxaloacetic acid and probably with ketoglutaric acid to produce the corresponding oximes from which aspartic and glutamic acid are formed after reduction. The following formula has been proposed by VIRTANEN & LAINE (1939) for the fixation of nitrogen in legume roots:



STEWARD & THOMPSON (1950) consider that the formation of oximes from hydroxylamine and ketoacids are very important reactions in the fixation of nitrate-nitrogen in plants.

A more rapid disappearance of added nitrate and less tendency towards nitrite accumulation was observed by SAPIRO & *al.* (1949) upon the addition of glucose to rumen contents. BARNETT & BOWMAN (1956) have found that the addition of glucose to rumen contents hindered the appearance of nitrite after the administration of nitrate.

The only chemical reaction which seems to have been described for the metabolism of nitrate in the rumen is reduction to ammonia. LEWIS (1951 b) has shown in this connection that washed suspensions of rumen organisms in the presence of gaseous hydrogen reduce nitrate, nitrite, and hydroxylamine to ammonia. Other hydrogen donors exist, but they are not as effective as gaseous hydrogen. There is, however, reason to suppose that this is not the sole route for nitrate metabolism in the rumen and that fixation of nitrate-nitrogen takes place under the same circumstances as in other natural systems. Striking similarities between conditions required for nitrogen-fixation

in plants and in the rumen can be adduced. In plants, the fixation of nitrogen can take place with the aid of a bacterial system in the root nodules of legumes and in the plant itself with the aid of carbohydrates formed by photosynthesis. In the rumen can be found a bacterial system and, supplied by the feed, portions of plants in various phases of digestion together with carbohydrates.

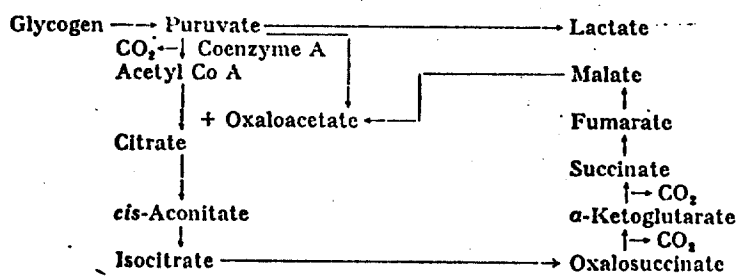
The use of isotopic tracers has, in recent years, helped to clarify many details of the carbohydrate breakdown which occurs in rumen metabolism. Glucose can be built up into glycogen, and when required, glycogen is broken down to pyruvic acid.

Pyruvic acid can be decarboxylated by oxidation to acetyl coenzyme A, active acetate (JOHNSON 1953).

Much of the energy requirement of the ruminant is satisfied by oxidation of the volatile fatty acids, especially acetic acid, which are formed in the rumen in large amounts. This oxidation can take place via KREBS' citric acid cycle.

In this cycle, active acetate and oxaloacetic acid are combined to form citric acid, and the various stages in the cycle are passed through until oxaloacetic acid is again formed.

The scheme depicted below is given by HARROW & MAZUR (1954).



If metabolism of the ruminant follows these principles, it is possible for the keto-compounds formed to take part in the fixation of nitrogen.

It can be seen from the scheme depicted above that, carbon dioxide is released during the formation of acetyl coenzyme A from pyruvic acid. Carbon dioxide is also released during oxidation in KREBS' citric acid cycle. If pyruvic acid, oxaloacetic acid, and α-ketoglutaric acid take part in nitrogen fixation, they are removed from the above cycle, and a change in the chemical reactions takes place. This may lead to a reduction of the products which are obtained from the breakdown of carbohydrate in the rumen, i.e., carbon dioxide and volatile fatty acids.

To test this hypothesis, studies were made of the effect of nitrite

on the production of carbon dioxide and volatile fatty acids in rumen contents.

To study whether protein or protein breakdown products could influence nitrite breakdown, investigations were carried out to see whether the amino acid glycine had any effect upon the removal of nitrite from rumen contents or washed suspensions of finely-divided plant particles obtained from the rumen. Glycine was chosen as a simple and easily-obtainable amino acid.

1. *Materials and Methods*

Six sheep with permanent rumen fistulae were used for these investigations. Five were held on an adequate and one on an inadequate ration.

Studies were made of *the effect in vitro of various substances on the breakdown of nitrite* in rumen contents and in washed suspensions of finely-divided plant particles prepared by the method described earlier. Duplicate series of test tubes containing 10 ml rumen contents or particle suspension were incubated at 39°C. Two tubes served as controls, and to two additional tubes were added 25 mg of the substances which were to be tested. For the experiments using rumen contents, 125 μg sodium nitrite in 0.025 per cent solution were added, and for those with the washed suspension, 1 mg sodium nitrite in 0.25 per cent solution. The time required for breakdown of the added nitrite was determined by the same methods as before.

The substances tested were glucose, fructose, sodium lactate, sodium pyruvate, and glycine.

The effect of nitrite on gas production by rumen contents was studied in graduated fermentation saccharometers. Rumen contents were obtained from sheep receiving the adequate ration described in chapter III. Each saccharometer contained 10 ml rumen contents, filtered through gauze, and 25 mg glucose. In each experiment, two tubes were used as controls and sodium nitrite was added to the other two. The size of the nitrite doses varied somewhat but were usually 50, 80, and 120 μg per ml rumen contents. These doses were so small that they could not be considered toxic for the bacteria (GREEN & WILSON 1953). The saccharometers were placed in a water bath at 39°C and the amount of gas formed was recorded every 30 minutes for four hours. Tests with sulphanilic acid and α-naphthylamine were carried out at the same intervals to see whether the nitrite had disappeared.

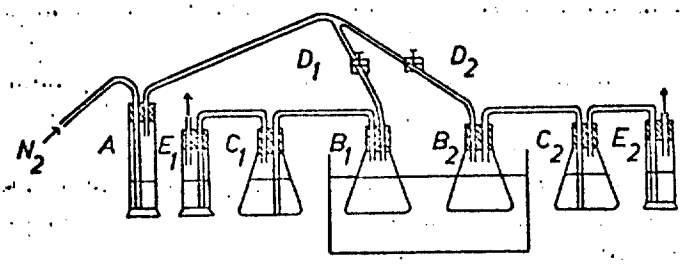


Fig. 4. **Apparatus for the quantitative determination of carbon dioxide production by rumen contents.**

Quantitative determinations of carbon dioxide production in rumen contents with and without added nitrite were carried out in the apparatus depicted in Fig. 4.

In each of two flasks (B_1 and B_2) were placed 200 ml fresh rumen contents and 500 mg glucose, and to one of these flasks, 20 mg sodium nitrite was added. The flasks were then placed in a water bath at 39°C. A slow stream of gaseous nitrogen was passed through potassium hydroxide in the container A in order to remove possible carbon dioxide before being passed through the two flasks, B_1 and B_2. The gases released from the rumen contents were carried in the nitrogen stream to flasks C_1 and C_2, containing known amounts of potassium hydroxide for the absorption of carbon dioxide. Clamps D_1 and D_2 were regulated so that the rate of flow of the gaseous stream through flasks C_1 and C_2 was the same. From flasks C_1 and C_2, the gases were led through lime-water in the containers, E_1 and E_2 to check that no carbon dioxide remained. Each experiment was continued for an hour at which time barium chloride was added to flasks C_1 and C_2 to precipitate the carbonate. The amount of carbon dioxide was determined by titration of the excess potassium hydroxide with hydrochloric acid.

The effect of nitrite on the production of volatile fatty acids in rumen contents was studied in the following manner. Fresh rumen contents were filtered through gauze and centrifuged thirty minutes at 3000 r.p.m. The supernatant was discarded and the sediment suspended in sterile 0.05 molar phosphate buffer at pH 6.5. The suspension was recentrifuged thirty minutes at 3000 r.p.m. and the sediment resuspended in buffer solution. By these means the fatty acids were practically completely removed. Two test tubes were set up containing equal amounts, usually 10 ml, of suspension together with 150 mg glucose. To one of these test tubes was added 100 μg sodium nitrite per ml and both tubes were incubated at 39°C for one hour.

The amount of volatile fatty acids was determined by SCHAMBYE's (1951) method at both the beginning and the conclusion of the experiments.

2. *Results*

The results of adding various substances to rumen contents are summarised in Table 16, and the corresponding experiments with the washed suspensions in Table 17. The stimulating effect on nitrite breakdown of the added substances occurs in rumen contents from both adequate and inadequate fed animals.

The results of the fermentation experiments have been grouped so that those cases in which the nitrite disappeared within one hour are

Table 16.

Effect on nitrite breakdown of adding various substances to rumen contents.

Substance added	Number of determinations	Time in minutes for the breakdown of 125 μg $NaNO_2$		Difference		
		Control	With substance	Average	Minimum value	Maximum value
Glucose	17	52	25	27	5	60
Fructose	18	67	39	28	0	70
Lactate	26	61	27	34	10	65
Pyruvate	20	66	28	38	10	70
Glycine	20	65	29	36	5	75

Table 17.

Effect on nitrite breakdown in washed suspensions of finely-divided plant cells from rumen contents after the addition of various substances.

Substance added	Number of determinations	Time in minutes for the breakdown of 1 mg $NaNO_2$		Difference		
		Control	With substance	Average	Minimum value	Maximum value
Glucose	30	30	120	−90	−150	−40
Fructose	12	30	104	−74	−130	−30
Lactate	22	40	26	14	5	35
Pyruvate	26	40	20	20	5	45
Glycine	16	35	35	0	−9	4

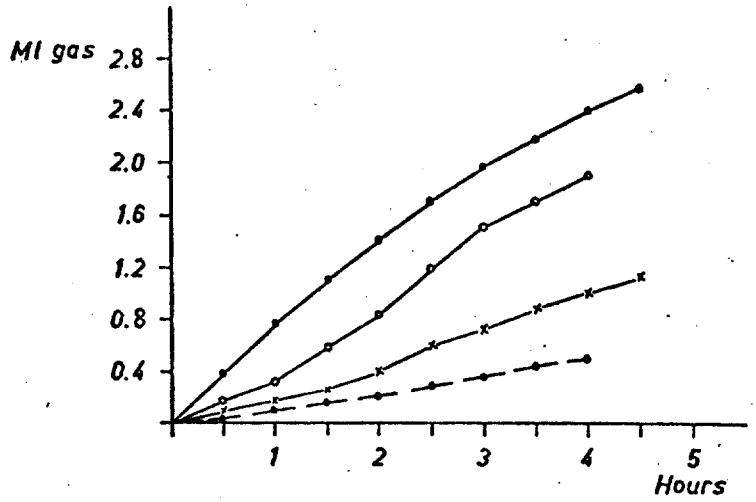


Fig. 5. The effect of nitrite on gas production by rumen contents.
—●—●— control.
—○—○— nitrite disappearing within one hour.
—×—×— nitrite disappearing between one and 2½ hours.
–●– –●– nitrite present after 2½ hours.

considered as the first group. The second group comprises the cases in which the nitrite disappeared after one hour but in less than 2 ½ hours. Those cases in which nitrite could be demonstrated after 2 ½ hours form the third group. Average values for these groups are given in Fig. 5. The number of experiments falling into each group was 13, 9, and 10 for the first, second, and third groups respectively.

Fig. 5 shows that nitrite has a strong inhibitory effect on the development of gas in rumen contents. As nitrite disappears, the inhibiting effect is reduced and gas release increases.

Table 18.

Effect of added nitrite on the carbon dioxide production in 200 ml rumen contents per hour expressed as ml at 0°C and 760 mm Hg.

Expt.	Control	With added nitrite
1	52	21
2	44	21
3	19	10
4	38	17
5	72	16
Average	45	17

Table 19.

Effect of nitrite on the production of volatile fatty acids after the addition of glucose. The amount of acid is expressed as mEq/100 ml.

Expt.	Control	With added nitrite
1	1.54	0.40
2	1.40	0.28
3	0.88	0.24
4	2.04	1.40
5	1.04	0.28
6	1.70	0.98
7	1.52	0.54
8	0.84	0.34
Average	1.37	0.56

Results of the quantitative determinations of carbon dioxide are shown in Table 18.

The table shows the strong inhibitory effect of nitrite on the production of carbon dioxide in rumen contents.

The results of the investigations into the effect of nitrite on the formation of volatile fatty acids in the rumen after the addition of glucose are shown in Table 19. Corrections have been made for the small amounts present at the commencement of the experiments.

3. *Discussion*

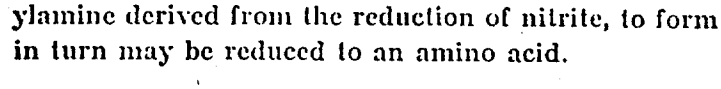
The only chemical reaction described for the metabolism of nitrate in the rumen is reduction to ammonia (LEWIS 1951 b). It is, however, most likely that conditions are much more complicated and that other reactions may be expected to take place. The very distinct effect obtained after the addition of glycine may possibly be an example of the general reaction for nitrite and an amino acid,

$$RCHNH_2COOH + HONO \rightarrow RCHOHCOOH + N_2 + H_2O$$

The same principle forms the basis of VAN SLYKES' method for determining the degree of protein hydrolysis in which nitrous acid is used to release nitrogen from free amino groups. Another possible explanation for the accelerating effect shown by glycine may be deamination of the amino acid with the resulting compound taking part in nitrite breakdown. Deamination of glycine, however, was not

observed by SIROTNAK, DOETSCH, BROWN & SHAW (1953) in their investigations of the breakdown of various amino acids in the rumen.

The observed stimulating effect of pyruvate may possibly be accounted for by its serving as a keto-acid which can react with hydroxylamine derived from the reduction of nitrite, to form an oxime which in turn may be reduced to an amino acid.



$$H_2NOH + RCOCOOH \rightarrow RCNOHCOOH$$
$$RCNOHCOOH \rightarrow RCHNH_2COOH$$

Since hydroxylamine is difficult to demonstrate, its occurrence as an intermediate stage of nitrate reduction has been debated.

Nitrite and hydroxylamine have been demonstrated by VIRTANEN & ARHIMO (1939) following the administration of potassium nitrate to plants. Hydroxylamine subsequently reacts with oxaloacetic acid and probably with keto-glutaric acid. Only when keto-acids are absent is hydroxylamine reduced to ammonia.

In investigations of the root-nodule bacteria of leguminous plants, VIRTANEN & LAINE (1939) demonstrated that oxaloacetic acid reacts strongly with hydroxylamine, especially in neutral or slightly acidic solutions. For this reason, hydroxylamine can never be found if oxaloacetic acid is simultaneously present. DE LA HABA (1950) has developed some mutations of *Neurospora* which can utilise nitrate and nitrite, but not ammonia. In this case, nitrate and nitrite do not require to be reduced to ammonia in order to be utilised.

The effect obtained following the addition of glucose, fructose, and lactate may depend partly upon these substances serving as hydrogen donors accelerating reduction, and partly upon their being easily broken down in the rumen with the keto-compounds formed as intermediate products taking part in the reaction as has been described above.

Addition of glucose, fructose, lactate, pyruvate, and glycine to washed suspensions of finely-divided plant particles found in rumen contents did not give results similar to those obtained by adding these substances to uncentrifuged rumen contents. Only pyruvate and lactate have an accelerating effect, equally effective in both cases, while glucose and fructose had a strong inhibitory effect. Glycine had apparently no effect at all. The lack of accelerating effect shown by glucose and fructose may be due to the absence of sugar-splitting bacteria or other factors necessary for sugar breakdown with no keto-acids being formed as a consequence. It has not been possible to clarify the reasons for the inhibitory effect observed. In order to check

the possibility that this inhibition could be attributed to the effects of the phosphate buffer used, a number of control experiments were made using physiological saline solution or distilled water in its place. Results identical with those using phosphate buffer were obtained. Lactic acid can be oxidised to pyruvic acid, and it is possible that this explains why equally good results were obtained with this substance as with pyruvate.

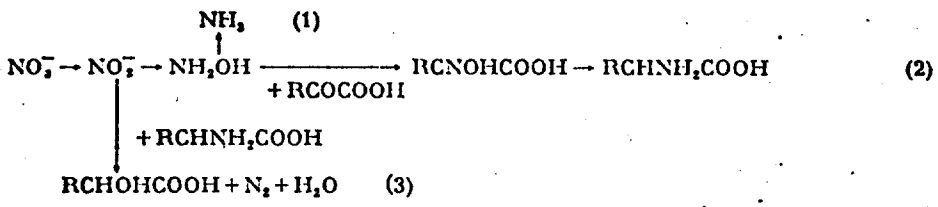
Since both amino acids and keto-acids are normally found in the rumen, they can take part in nitrate metabolism. It may be possible, therefore, that in the presence of abundant amounts of amino acids, added nitrate-nitrogen could be released. In the presence of liberal amounts of keto-acids, nitrate-nitrogen may be fixed for the synthesis of amino acids. Nitrate may thus be a factor which contributes towards maintaining the balance between protein and carbohydrate metabolism.

Even relatively small doses of nitrite strongly inhibit gas production by rumen contents. As the nitrite is removed, the inhibitory effect is reduced. These results may indicate that the effect of nitrite is purely chemical. Quantitative determinations of carbon dioxide production show that the inhibition of gas formation is due to a large extent to reduced formation of carbon dioxide. This and the reduced production of volatile fatty acids (Table 19), may be the result of the disappearance of keto-acids.

Volatile fatty acids can be formed from pyruvic acid (JOHNSON 1953) and from intermediate products from the citric acid cycle. Several writers, among them JOHNS (1951) SIJPESTEIJN & ELSDEN (1952), DOETSCH, ROBINSON, BROWN & SHAW (1953), and DOETSCH, ROBINSON & SHAW (1953), have shown that succinic acid can be decarboxylated to propionic acid under the influence of rumen bacteria. As VIRTANEN & LAINE (1939) consider that hydroxylamine reacts much more easily with oxaloacetic acid than with pyruvic acid, it is possible that the inhibition of the production of volatile fatty acids by nitrite also comprises acids formed from intermediate products of the citric acid cycle.

It is difficult to estimate the significance which the disturbance in carbohydrate metabolism caused by nitrites may have for an individual animal. On a ration containing liberal amounts of protein but deficient in easily-soluble carbohydrates, the presence of nitrite and the consequent risk of inhibition of the citric acid cycle would most likely have a drastic effect, especially in the case of highly-producing cows in which the metabolism is already subject to extreme stress.

Various possibilities for the metabolism of nitrate in the rumen are summarised in the following scheme:



1. Reduction to ammonia, as shown by LEWIS (1951 b).
2. Reaction with keto-acids to form amino acids.
   Support for this possibility:
   (a) the effect of pyruvic acid and lactate on both rumen contents and washed suspensions of finely-divided plant particles,
   (b) the lack of effect of glucose and fructose on washed suspensions of finely-divided plant particles from which sugar-splitting microorganisms are presumably missing, and
   (c) the inhibition of carbon dioxide and volatile fatty acid production in the presence of nitrite.
3. Reaction with amino acids. The effect of glycine in rumen contents serves as support for this hypothesis.

Summary

The purpose of the studies reported here has been to investigate experimental nitrite poisoning in sheep, and to study the factors influencing nitrite accumulation in the rumen after the administration of nitrate and nitrite detoxification in the rumen.

Chapter I describes experiments on the oral administration of nitrate to animals maintained on different rations. The clinical picture of nitrite poisoning in sheep is described. It was observed that animals receiving an adequate ration were not affected after doses of nitrate which were large enough to cause the death of animals receiving an inadequate ration. Methaemoglobin determinations carried out on the blood showed that little or no methaemoglobin was present in adequately-fed animals while very high values were obtained in inadequately-fed after similar doses of nitrate.

Chapter II. Studies on oxygen consumption and carbon dioxide elimination were carried out on animals in a respiratory chamber before and after the intravenous injection of sodium nitrite and after treatment with methylene blue. Up to 65 per cent of the total haemoglobin could be in the form of methaemoglobin and the animals lying in coma without any decrease in oxygen consumption being seen. Actually, both oxygen consumption and carbon dioxide elimination were somewhat increased. This supports the hypothesis that methaemoglobinaemia was not responsible for the first signs

of illness but rather that the vasodilatory effect of nitrite with the associated decrease in blood pressure was of primary importance. Good prophylactic and therapeutic results in nitrite poisoning were obtained by the use of vasoconstrictory drugs.

Chapter III. The formation of nitrite following the addition of nitrate to rumen contents was studied in sheep receiving an adequate diet and animals receiving an inadequate ration. The ability to break down added nitrite was also studied in both these groups. All experiments were carried out *in vitro* as well as *in vivo*. Following the administration of nitrate to the animals receiving an inadequate ration, it was possible to demonstrate significantly greater amounts of nitrite for a longer time than was the case for the animals receiving an adequate ration. Much more time was required by this group to break down added nitrite.

Chapter IV. A decrease or increase in the pH value of rumen contents outside the normal range led to an increased accumulation of nitrite following the administration of nitrate. Strong inhibition of nitrite breakdown could be observed, especially at lower pH values. The breakdown of nitrite added to rumen contents occurred best at pH values normally found in the rumen.

Chapter V deals with studies of the effect of certain growth-promoting substances on nitrite breakdown in rumen contents and in cultures of microorganisms from the rumen. Addition of procaine penicillin to rumen contents stimulated the breakdown of nitrite. Nitrite breakdown by *Absidia ramosa* cultures could be strongly accelerated by penicillin but a similar dose gave no effect on the *Fusarium* species or on the strains of *Escherichia coli* and *Bacillus* spp. examined. Addition of β-indolylacetic acid to the fungi cultures strongly stimulated nitrite breakdown but it did not appear to have a similar effect on rumen contents or bacterial cultures.

Chapter VI. A fraction consisting of finely-divided plant particles was freed from most of the rumen bacteria by washing. This suspension displayed a good capacity to break down nitrite. Quantitative determinations showed that sheep receiving an adequate ration and with good ability to break down nitrite also had liberal amounts of this fraction in the rumen contents. Very small amounts of this fraction were present in the rumen contents of the sheep receiving an inadequate ration and with a poor ability to break down nitrite. It seems likely that the differences between the capacity of different animals for nitrite breakdown depend to a large extent upon the amount of this partially-digested plant fraction present in the rumen.

Chapter VII deals with more detailed studies of nitrite breakdown in the rumen. Addition of glucose, fructose, lactate, pyruvate, and glycine stimulates the nitrite breakdown in rumen contents. Addition of small amounts of nitrite inhibits carbon dioxide production in rumen contents. The production of volatile fatty acids in rumen contents is inhibited by small amounts of nitrite.

This inhibition of carbohydrate metabolism may possibly serve as a potential hazard for maintenance of the normal metabolism.

Acknowledgements

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Professor SVEN HOFLUND, my chief, rightly deserves my warm thanks for the great interest he has shown in my work and for the good advice and continuous support I have received. He also placed the resources of the department at my disposal and, as far as possible, reduced my routine work at the cost of greatly increasing his own load.

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principles (especially distillations) and because the diffusion and spectrophotometric steps used may be considered as more exact than corresponding steps involved in the standard procedure, it may be concluded that results of the fluoride electrode method agree with those of the official method. Moreover, the described method is simpler, faster, and very suitable for precise routine analyses. The method should also be applicable to various mineral mixed feeds, because materials analyzed in this study are commonly used as mineral feed ingredients.

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FOOD ADDITIVES

Extraction and Gas Chromatographic Determination of N-Nitrosodimethylamine in Smoked Fish: Application to Smoked Nitrite-Treated Chub

By JOHN W. HOWARD, THOMAS FAZIO, and JAMES O. WATTS (Division of Food Chemistry and Technology, Food and Drug Administration, Washington, D.C. 20204)

N-Nitrosodimethylamine can be determined in smoked nitrite-treated chub fish as follows: The fish sample is digested in methanolic potassium hydroxide; the nitrosamine is distilled from alkaline solution, further isolated by extraction and column chromatographic techniques, and determined by GLC with a modified thermionic detector. Recoveries of N-nitrosodimethylamine added at levels of 10 ppb ranged from 70 to 86%. The application of the procedure to the analysis of 5 experimental lots of smoked nitrite-treated chubs indicates that N-nitrosodimethylamine is not produced in the flesh under the conditions of the processing treatment used.

Nitrites and nitrates may be utilized as preservatives and color fixatives in various smoked fish (sable, shad, salmon, and tuna fish) and in smoked meats under prescribed limitations in food additive regulations, *CFR* 121.1063 and *CFR* 121.1064 (1). More recently another regulation, *CFR* 121.1230 (2), concerns the use of sodium nitrite in combination with salt to aid in inhibiting the outgrowth and toxin formation from *Clostridium botulinum*, type E, in the commercial processing of smoked chub fish.

Recently, the safety of the use of nitrites and nitrates in smoked fish has been questioned, mainly in regard to the possible formation of carcinogenic N-nitrosamines after treatment of fish with nitrite and/or nitrate and their reaction with the naturally occurring amines. The carcinogenic properties of N-nitrosodimethylamine (dimethylnitrosamine, DMNA) were first described by Magee and Barnes (3) and later verified by other workers (4, 5). Since publication of these findings, there has been increasing evidence that such N-nitroso compounds may occur in the human environment (6-10).

Herring meal produced from nitrite-treated fish

has been reported to cause a malignant liver disease in animals (11–13). DMNA was isolated from a toxic meal produced on a laboratory scale from nitrite-treated herring (6). This meal was produced by adding about three times the quantity of nitrite (630 g/100 kg fish) employed by the Norwegian industry to the fish which, contrary to industrial practice, were immediately boiled. The production of the whole meal was then carried out in conformity with the conventional procedure except that higher than normal temperatures were used for the drying process. The identity of the N-nitrosodimethylamine isolated from the toxic herring was confirmed by various gas chromatographic and spectrophotometric techniques (14). In addition, the oral administration to mink of a single dose of 17 mg of either the material isolated from the herring meal or of pure DMNA resulted in death after 24 hr. In both cases the same pathological-anatomical picture existed and was similar to that noted in field cases of herring meal intoxication. In contrast, studies on meal products obtained from a fresh water fish claimed to contain only traces of methylamines showed no detectable toxicity when tested on mink in large doses over a long period.

Ender *et al.* (14) also conducted model experiments in which monomethylamine, dimethylamine, trimethylamine, and trimethylamine oxide were demonstrated to react with sodium nitrite to yield N-nitrosodimethylamine under varying conditions. The strongest contributor to nitrosamine formation was dimethylamine followed by trimethylamine, whereas monomethylamine and trimethylamine oxide did not contribute significant quantities. Although such studies are not directly comparable to herring meal production, they do indicate the potential for possible reactions.

Various polarographic (15, 16), colorimetric (17–20), thin layer (8, 21–24), and GLC techniques (8, 19, 22, 25, 26) have been utilized for the detection and estimation of nitrosamines in foodstuffs, tobacco, and smoke condensates. However, the majority of these methods are lacking in sensitivity and/or specificity. Eisenbrand *et al.* (27) have indicated that there are several analytical procedures that can be used for the detection and estimation of N-nitroso compounds in simple mixtures; however, analysis of complicated products is very difficult at the present time.

With respect to foodstuffs most of the aforementioned work has been carried out in Europe, and there is little available information on products processed in the United States. Recently Sen *et al.* (28) in Canada published a thin layer chromatographic (TLC) procedure for N-nitrosamines; the sensitivities reported ranged from 0.05 to 0.15 ppm, depending upon the nitrosamines analyzed. The Griess and ninhydrin spray reagents were used for detection and estimation. The procedure was applied to the analysis of various samples of cheese, spinach, flour, and herring; however, no nitrosamines were detected.

During the past 6 months, this laboratory has been developing an analytical method with a sensitivity of 10 ppb to determine if the use of nitrite in smoked fresh water or marine fish may result in the formation of DMNA. In the procedure developed, the fish is digested in methanolic potassium hydroxide and the nitrosamine is distilled from the resultant solution. The distillate is extracted with methylene chloride which is concentrated and placed on a Celite chromatographic column impregnated with hydrochloric acid. GLC was used as the determinative technique. To attain the desired sensitivity, the thermionic detector was modified to allow the detection of 4 ng DMNA.

Initially, the procedure was applied to the analysis of experimental lots of nitrite-preserved smoked chub. Since 1960, three major outbreaks of botulism have been recorded as the result of the consumption of this species of smoked fish of Great Lakes origin. The use of nitrite in the processing of the fish has been demonstrated under specified conditions to assist in inhibiting the growth of *C. botulinum*, type E; however, before a regulation was issued it was deemed of the utmost importance to determine whether DMNA was formed under the conditions of the proposed treatment.

METHOD

Apparatus

(*Note:* Glassware catalogue numbers refer to Kontes Glass Co., Vineland, N.J. 08360, unless otherwise noted.)

(*Caution:* All glassware must be scrupulously cleaned (acid-washed, thoroughly rinsed with distilled water, dried, and rinsed with CH_2Cl_2).)

(a) *Evaporative concentrator.*—Kuderna-Danish 250 ml capacity with 24/40 column connection and 19/22 lower joint (K-570001).

(b) *Concentrator tube.*—Size 125, 19/22 joint, 1.5

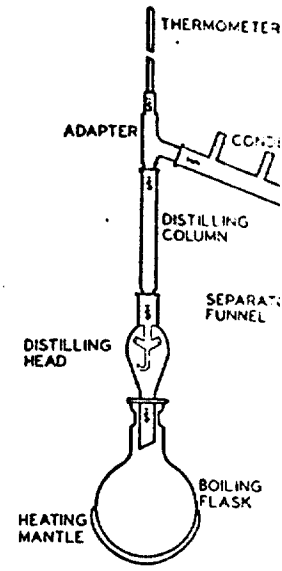

FIG. 1—Distillation ap

capacity, with 0.1 ml subdivi- K-570050) and 19/22 penny 50500). Calibrate before use.

(c) *Distilling column.*—Snyd sections (6630-02, Ace Glass, I or equivalent).

(d) *Funnel.*—Büchner, 60 ml porosity disk (K-955000).

(e) *Chromatographic column.* C-3, 22 mm id × 300 mm, with K-420540).

(f) *Distillation apparatus.*—S flasks: round bottom, 2 L capaci K-601000); (2) *Adapter:* connec joints (K-167000); (3) *Conden* with 24/40 joint (K-871000) and with 24/40 joint (K-437000); (4 with 24/40 joint (K-157000); (5 110°C with 10/30 joint (K-87 column: plain 450 mm × 25 m joints (K-50100); (7) *Distilling* joints (K-517000).

(g) *Heating mantles.*—Hemi-sp L round-bottom flasks (use w former heat control).

(h) *Microliter syringes.*—10 µl Lok (Liquid Syringe Precision Baton Rouge, La., or equivalent

(i) *Platinum-iridium wire.*—N Thomas Co., Philadelphia, P

(j) *Columns.*—Glass, coiled, Lawshe Instrument Co., Inc. equivalent).

(k) *Gas chromatograph.* Bar

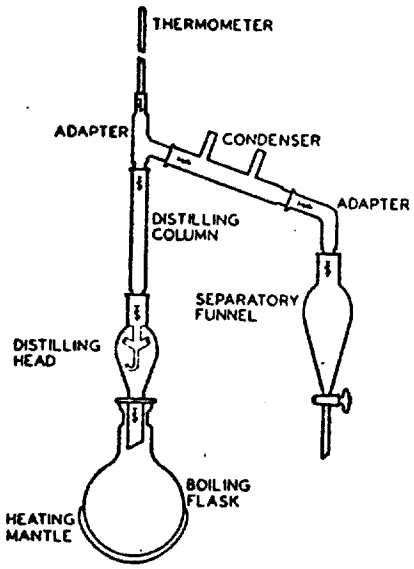

FIG. 1—Distillation apparatus.

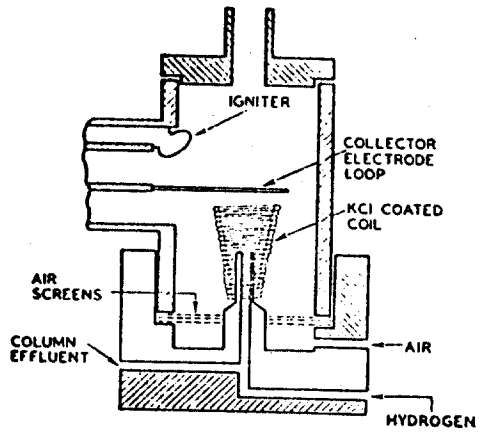

FIG. 2—Modified thermionic detector.

capacity, with 0.1 ml subdivisions from 0 to 2 (K-570050) and 19/22 pennyhead stopper (K-550500). Calibrate before use.

(c) *Distilling column.*—Snyder, 24/40 joint, 3 sections (6630-02, Ace Glass, Inc., Vineland, N.J., or equivalent).

(d) *Funnel.*—Büchner, 60 ml capacity with coarse porosity disk (K-955000).

(e) *Chromatographic column.*—Chromaflex, size 123, 22 mm id × 300 mm, with coarse fritted disk (K-420540).

(f) *Distillation apparatus.*—*See* Fig. 1. (*1*) *Boiling flasks:* round bottom, 2 L capacity with 24/40 joint (K-601000); (*2*) *Adapter:* connecting type with 24/40 joints (K-167000); (*3*) *Condensers:* Liebig, 200 mm with 24/40 joint (K-871000) and Friedrich, 325 mm with 24/40 joint (K-437000); (*4*) *Adapter:* bent 105° with 24/40 joint (K-157000); (*5*) *Thermometer:* −10 to 110°C with 10/30 joint (K-871000); (*6*) *Distilling column:* plain 450 mm × 25 mm od with 24/40 joints (K-50100); (*7*) *Distilling head:* with 24/40 joints (K-517000).

(g) *Heating mantles.*—Hemispherical, for 1 and 2 L round-bottom flasks (use with variable transformer heat control).

(h) *Microliter syringes.*—10 μl capacity, Pressure-Lok (Liquid Syringe Precision Sampling Corp., Baton Rouge, La., or equivalent).

(i) *Platinum-iridium wire.*—No. 26 gauge (Arthur H. Thomas Co., Philadelphia, Pa., or equivalent).

(j) *Columns.*—Glass, coiled, 9′ × 4 mm id (JOW-Lawshe Instrument Co., Inc., Bethesda, Md., or equivalent).

(k) *Gas chromatograph.*—Barber-Colman Model 5000, with following: Model 5000 electrometer, Model 8000 stripchart recorder 0–5 mv, ⅓″/min chart speed, Model 5077 coiled glass column cabinet, Model 5103-1 injector-detector temperature controller (SCR's—2 units), Model 5084 temperature programmer, Model 5003 rear chamber detector bath, and modified KCl thermionic detector (l).

GLC conditions: 9′ × 4 mm id coiled glass column packed with 10% Carbowax 1540 + 3% KOH on 100–120 mesh Gas Chrom P support. Condition column 24 hr at 180°C, while maintaining carrier gas (argon) flow of 50 ml/min. Maintain following parameters throughout analyses—temperatures (°C): injector 185 and detector 220; flow rates (ml/min)—carrier gas (argon) 43, hydrogen 40 (producing a standing current of 3×10^{-9} amp), and air 300; electrometer setting 100 (2×) equivalent to 2×10^{-9} amp FSD; and temperature programmed from 80 to 120°C at 10°/min after initial 3 min hold at 80°C.

(l) *Thermionic detector.*—*See* Fig. 2. Form continuous platinum-iridium helix by coiling one end of wire (26 gauge) on 12-24 thread 2″ bolt for 10 windings or loops; remove bolt and continue winding with 5 additional smaller diameter loops made on 8–32 thread 2″ bolt. Heat coil over burner until metal glows and tightly compress top 5 larger diameter loops with metal tweezers. Maintain heat and compress entire coil with tweezers to 1/16″. Coat top 5 larger diameter loops on inside with KCl by heating the 5 loops to a glow and then add saturated KCl solution dropwise on inside of wire, followed by evaporation and fusion. Place coated coil on jet tip of detector in vertical position, using tweezers, so that the smaller diameter windings surround jet tip.

Reagents

Reagents purified by distillation were distilled with air-cooled reflux condenser (ca 300 mm long) between reservoir and water-cooled condenser. The

solvents were distilled in 2 L lots; the first 200 ml distillate was discarded and the next 1400 ml collected for use.

(a) *Methylene chloride* (CH_2Cl_2).—Purify as follows: Place 500 ml CH_2Cl_2 into 1 L separatory funnel. Add 20 g anhydrous $CaCl_2$, stopper, and shake vigorously 10 min. Let $CaCl_2$ settle in bottom of funnel. Filter CH_2Cl_2 through 60 ml coarse fritted-glass funnel and collect filtrate in suitable glass container previously rinsed with small portions of purified filtrate. Check before use as described below.

Place 180 ml CH_2Cl_2 (volume used in actual extraction procedure) into 250 ml Kuderna-Danish concentrator fitted with 4 ml concentrator tube and concentrate to 0.5 ml as specified in method. Inject 8 µl into gas chromatograph. Solvent *must* be free of interfering peaks.

(b) *Sodium sulfate.*—Analytical reagent, anhydrous, granular.

(c) *Hydrochloric acid.*—About 6*N*. Transfer 50 ml concentrated HCl (ACS reagent grade) to 100 ml volumetric flask and dilute to volume with distilled water.

(d) *Potassium hydroxide.*—Analyzed reagent grade.

(e) *Methanol.*—ACS reagent grade. Purify as follows: Reflux 2 L alcohol with 10 g KOH and 25 g zinc dust 3 hr. Distill, using air-cooled condenser. Add drying tube to collection flask to protect distilled solvent from moisture.

(f) *Pentane.*—Technical grade, redistilled (Eastman Organic Chemicals, Rochester, N.Y., or equivalent).

(g) *Celite 545.*—Not acid-washed (Fisher Scientific Co., Fairlawn, N.J., or equivalent). Heat in muffle furnace overnight at 700°C. Prepare acid-Celite column as follows: Add 10 ml 6*N* HCl in small increments to 6 g Celite with vigorous stirring; pack mixture (fluffy in consistency) into chromatographic column on top of glass wool plug and compress to ca 2". Then place small glass wool plug on top of adsorbent to prevent channeling. Test prepared column before use: Pass 200 ml pentane through column and discard eluate. Add 50 ml CH_2Cl_2, collect eluate in Kuderna-Danish apparatus, and concentrate solvent to 0.5 ml. Inject 8 µl into gas chromatograph. Solvent *must* be free of interfering peaks.

(h) *Nitrogen.*—Ultra high purity.

(i) *N-Nitrosodimethylamine* (*DMNA*). — About 99% purity, 150–152°C boiling range (Chemical Procurement Laboratories, Inc., College Point, N.J., or equivalent).

(j) *DMNA standard solution.*—Weigh 250 mg DMNA into 250 ml volumetric flask and dilute to volume with CH_2Cl_2. Transfer 1 ml of this stock solution to 100 ml volumetric flask and dilute to volume with CH_2Cl_2. Transfer 5 ml of this solution to 100 ml volumetric flask and dilute to volume with CH_2Cl_2. Standard solution contains 0.5 µg DMNA/ml.

Procedures

Weigh 400 g comminuted and thoroughly mixed fish fillets (skins and bones removed) into 2 L boiling flask. Add 50 g KOH dissolved in 200 ml methanol and boiling chips. Insert Friedrich condenser into boiling flask-fitting and reflux mixture 8 hr. At end of digestion, remove condenser and filter contents of boiling flask while warm through glass wool plug into glass-stoppered Erlenmeyer flask.

Transfer 37.5 ml filtrate (equivalent to 25 g sample) into 2 L boiling flask. Add 125 ml distilled water, 17 g KOH, and boiling chips. Connect distillation apparatus to boiling flask. Collect 50 ml distillate in 250 ml separatory funnel. Shake with 50 ml CH_2Cl_2 3 min. (To facilitate separation of aqueous and CH_2Cl_2 layers add small portion of NaCl.) Filter CH_2Cl_2 layer through 35 g anhydrous Na_2SO_4 (held in 60 ml coarse fritted-glass funnel and prewetted with 25 ml CH_2Cl_2) into Kuderna-Danish evaporator with 4 ml concentrator tube attached. Repeat extraction 2 additional times and pass CH_2Cl_2 layer through anhydrous Na_2SO_4. Wash Na_2SO_4 with 25 ml CH_2Cl_2. Place carborundum grains in concentrator tube and insert distilling column into fitting of Kuderna-Danish evaporative concentrator and carefully concentrate solvent to ca 4 ml in steam bath. Remove apparatus from bath; let cool and drain any remaining solvent in distilling column and concentrator into concentrator tube. Concentrate solvent in concentrator tube to 1.0 ml under stream of nitrogen.

Quantitatively transfer 1 ml concentrated CH_2Cl_2 extract to acid-Celite column, using small aliquots of pentane (20 ml total). After column has drained, add 180 ml pentane to column, let column drain completely, and discard pentane eluate. Add 50 ml CH_2Cl_2 and collect eluate in Kuderna-Danish apparatus. Concentrate CH_2Cl_2 as described before to 0.5 ml.

Inject 8 µl standard solution containing 0.5 µg DMNA/ml into gas chromatograph. Record retention time and peak heights (mm). Using same parameters, inject 8 µl concentrated sample in CH_2Cl_2. Record retention time and peak heights (mm) and calculate amount of DMNA present.

Results and Discussion

All of the chub fish utilized in this study were prepared by the Bureau of Commercial Fisheries (BCF) and the National Fishery Institute (NFI). Both smoked and nitrite-preserved smoked fish processed as described below, were used in the

recovery studies. Before recovery studies, the individual sample lots of fish were comminuted and analyzed for DMNA. Appropriate aliquots of standard solutions of DMNA in water were then added directly to the boiling flask before sample digestion. Recoveries of DMNA added at levels of 10 ppb to 400 g of various lots of chub fish ranged from 70 to 86% (Table 1). All reported values were corrected for apparent nitrosamine content. To investigate the possibility of protein binding of DMNA (29), the amine was added to untreated smoked chub meat at the 10 ppb level (2 μg/200 g), allowed to remain in contact overnight, and digested as described under the method. The results indicated that DMNA was not bound to the proteins and could be successfully recovered.

Although the reaction of nitrites and amines under alkaline conditions was not considered possible, studies with both dimethylamine hydrochloride and trimethylamine (80% tri- and 20% mono- and di-forms) were conducted to demonstrate that DMNA was not formed during the analysis. The individual amine was added at a level of 250 ppm to the boiling flask which contained 200 ppm sodium nitrite in 200 ml methanol and 400 ml water. The solutions were adjusted to 3V alkalinity and the analyses were carried out as described above. No DMNA was detected.

Five different experimental lots, 1–5, of smoked chubs preserved with sodium nitrite have been analyzed by the above procedure. The fish were processed as follows: The frozen chub were thawed at about 36°F for 2 days and then immersed in a brine tank containing NaCl (30° Baumé reading) and 1000 ppm sodium nitrite. After 16 hr of brining, the fish were removed and smoked with the maximum temperature about 180°F maintained 30 min. The total heating time for the fish was 4 hr. Each lot of chubs was prepared in the above manner on different days and therefore, representative of a separate process sequence. The estimated values obtained for apparent DMNA, range 1–2 ppb, in these lots are far below the demonstrated sensitivity of the procedure, 10 ppb; *see* Table 1. Values of the same magnitude were also found in the smoked chubs (X samples) without the nitrite treatment. Also included in Table 1 are the results of previous studies in which an attempt was made to increase the amine content of the fish and, therefore, present the potential for nitrosamine formation. Lot 6 was an aged sample held at 36°F for 8 days, then subjected to the nitrite (1000 ppm) and smoking processes described above. There was no increase in apparent nitrosamine content. Lots 7, 8, and 9 were fortified at levels of 300, 700, and 1500 ppm trimethylamine hydrochloride, respectively, and then treated with 2500 ppm sodium nitrite and smoked to 180°F 30 min. In these cases the values ranged from 3 to 5 ppb for apparent DMNA, which would appear to indicate that the presence of the trimethylamine may tend to cause an increase in the nitrosamine content or in extraneous components appearing as the amine. No attempt was made to confirm the identity of apparent DMNA contents reported above, since the levels were below the sensitivity of the procedure.

Table 1. Analysis of experimental lots of smoked nitrite-treated chubs for N-nitrosodimethylamine (DMNA) and recoveries of DMNA added to samples at 10 ppb levels

Lot	Apparent DMNA Content, ppb[a]	% Recovery of 10 ppb Added[b]
1	1	70, 78
2	1	70
3	1	70
4	1	70
5	2	83
6	1	—
7	3	86
8	5	83
9	5	70

[a] All reported values of apparent DMNA content were adjusted to one significant figure and were too low for practical confirmation of identity.

[b] Recovery values were corrected for the apparent DMNA content.

Typical gas chromatograms of commercially processed smoked nitrite-treated chubs without and with DMNA fortification are shown in Fig. 3.

Studies of other species of nitrite-preserved smoked fish covered under the present regulations are in progress and the results of these analyses will be reported in a subsequent communication. Thus far, investigations have shown that the identity of DMNA can be confirmed at levels as low as 10 ppb by mass spectrometry. This was accomplished by combining multiple distillates, redistilling, and proceeding as described. The mass spectral analysis was obtained on the GLC effluent from the gas chromatograph through a single-stage Llewellyn molecular separator into the ion source of the mass spectrometer. Investigations of other confirmatory techniques more

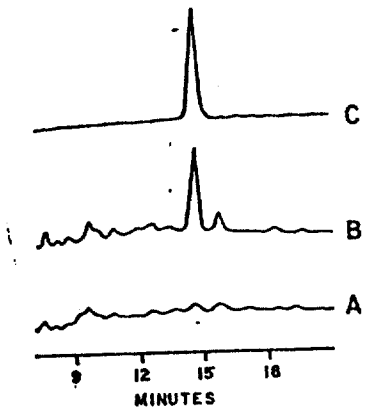

FIG. 3—Gas chromatograms of: A, commercially processed nitrite-treated smoked chub; B, commercially processed nitrite-treated smoked chub fortified with 10 ppb DMNA; and C, reference DMNA standard (0.5 μg/ml).

practical for general laboratory use, i.e., TLC and/or polarography, are being explored.

In summary, a GLC method with a demonstrated sensitivity of 10 ppb for the determination of DMNA in fish has been developed. The thermionic detector has been modified to yield increased sensitivity for detection of DMNA. Linearity studies up to 50 ng have indicated that a straight line relationship (peak height response to weight) can be achieved.

The results of the analysis of five experimental lots of smoked chubs indicate that DMNA is not produced in the flesh under the conditions of the processing treatment used.

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EXPERIMENTAL STUDIES IN CARDIOVASCULAR PATHOLOGY

I. PATHOLOGIC CHANGES IN THE ORGANS OF RATS PRODUCED BY CHRONIC NITRITE POISONING

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An urgent need exists for additional information concerning the etiologic factors and the causative mechanism of the noninfectious degenerative and sclerosing lesions of the arterial walls and their degenerative parenchymatous sequelae in the organs involved, especially the heart, kidney and brain. Among the various endogenous factors which have been incriminated in the production of arteriosclerosis, some are of general chemical or hormonal nature (elevation of the cholesterol and calcium levels of the blood, as in diabetes and hyperparathyroidism, respectively; hyperadrenalism; renal ischemia, causing hypertension), while others are of local type (locally restricted special mechanical strain; vibrations, as at bifurcations). The experimental production of arteriosclerosis in animals by prolonged administration of cholesterol or epinephrine and by restriction of the blood flow in the renal arteries, respectively, has lent support to the contention that these factors play a causative role.

On the premise that the fundamental and general direct causative mechanism active in the development of atheromatous, arterionecrotic and arteriosclerotic lesions is represented by an impairment of the nutrition of the inner portions of the vascular walls (which are not supplied with blood by the vasa vasorum) and that this condition may be brought about by a great variety of factors acting in different ways, the postulate is advanced that hypotensive agents administered over a sufficiently long period and in adequate amounts should exert the same effect on the vascular walls as have been observed in connection with hypertensive agents. To test this hypothesis, rats were placed under the prolonged influence of a hypotension-producing chemical, erythrol tetranitrate.

This communication embraces a report on the vascular and organic changes produced by such a procedure.

Warner Institute for Therapeutic Research.

CONSIDERATIONS IN SELECTION OF EXPERIMENTAL MATERIAL

In selecting erythrol tetranitrate from the large group of nitrites and nitrates producing nitrite effects (sodium nitrite, amyl nitrite, nitroglycerin, mannitol pentanitrate, ethylene glycol dinitrate, erythrol tetranitrate and others) special consideration was given to the fact that this chemical causes not only an appreciable but a prolonged lowering of the blood pressure, in contrast to other nitrites, which have a more transitory effect in this respect. By employing this chemical, it was thought, animals could be kept for the greater part of the day under the influence of nitrite action.

Rats were chosen as test animals since they spontaneously acquire arteriosclerotic lesions in vessels of various organs (heart, lungs, kidney, testis) with old age, thus displaying a vascular reactivity similar to that observed in man, with whom, moreover, they have in common an exposure to nutritive influences of the same type, both being omnivores. The selection of rats was further influenced by the fact that recent pathologic and histologic investigations (Hueper [1]; Wilens and Sproul [2]; Hummel and Barnes [3]) have provided detailed knowledge concerning the incidence as to age, the character and the extent of the arteriosclerotic lesions occurring in rats. These animals have been used successfully, moreover, for the production of experimental hypertension by the method of renal ischemia developed by Goldblatt.[4]

EXPERIMENTAL PROCEDURE

Three experiments were conducted. In the first, 30 male rats, approximately 2 months old and weighing between 52 and 110 Gm. (average, 77 Gm.), were placed on a stock diet adequate in all respects. Increasing amounts of erythrol tetranitrate were added to the food. The chemical was obtained in tablets (tetranitrol, Merck) containing 0.015 Gm. each. As erythrol tetranitrate is sparingly soluble in water (1:20,000) but readily soluble in alcohol, a suitable number of tablets were dissolved in 95 per cent alcohol, leaving an insoluble mineral residue. This solution was shaken vigorously, after which approximately 5.0 cc. was removed and added to the food in each cage, containing 6 rats. The initial daily dose per rat was 0.00035 Gm. of erythrol tetranitrate. The dose was increased every week by 25 per cent of the amount given during the preceding week. At the end of the experimental period of thirty-two weeks the daily amount of erythrol tetranitrate administered with the food per rat had reached 0.064 Gm., an increase of approximately twenty times.

Inasmuch as the conditions prevailing in the experiment with chronic oral administration neither insured exact control of the dose consumed by the individual animal nor permitted determination of the quantity of nitrite actually resorbed into the organism, a series of 12 female rats was given subcutaneous

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injections of a saline suspension of erythrol tetranitrate over a period of one hundred and forty days (twenty weeks) on five days of the week (a total of eighty injections). The dose administered daily was 0.014 Gm. during the first week and 0.028 Gm. thereafter for the remainder of the experiment. The injections were interrupted on four occasions for the duration of one week, to permit healing of ulcerative lesions formed as the result of the subcutaneous introduction of the suspended solid material. The rats in this experiment were approximately 3 months old at the start and weighed from 118 to 158 Gm. (average, 124 Gm.).

The third experiment was devised to permit study of the organic and especially the cardiovascular effect of massive doses of an inorganic nitrite, sodium nitrite, for purposes of comparison. Six rats were used, weighing between 160 and 236 Gm. at the start of the experiment. They were placed on a meat diet, to which 0.4 Gm. of sodium nitrite per rat was added daily. After two weeks the daily dose was decreased to 0.20 Gm., as the rats did not consume their entire ration. After another week it was doubled again and three weeks later was increased to 0.5 Gm. The rats were maintained on this diet until the last survivors of the series were killed, after being in the experiment for eighteen weeks.

BIOLOGIC OBSERVATIONS

Sixteen of the 30 rats placed on chronic oral administration of erythrol tetranitrate survived for thirty-two weeks. They were then killed by bleeding from the jugular vein. During the experimental period a constant rise in weight was recorded, ranging at the time of death (when they were approximately 9 months old) from 200 to 376 Gm. (average, 262 Gm.). Nine rats of this series died or were killed during the first three months of the experiment because of an apparent inflammatory infectious condition of the middle ear, while 5 additional rats died during a later part.

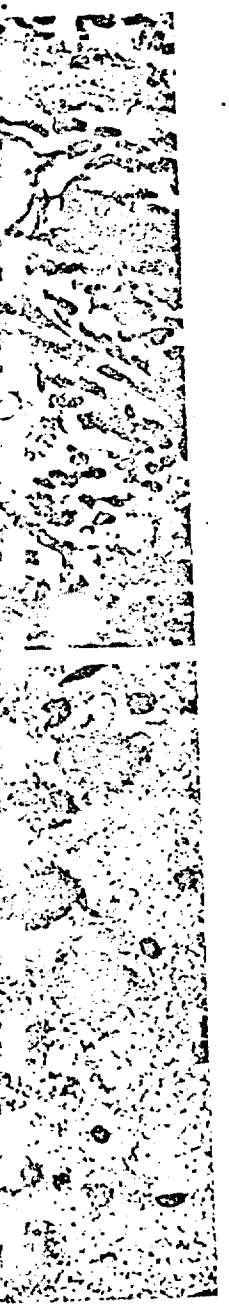
Of the rats given subcutaneous injections, 10 were alive at the end of the experiment. The animals gained only slowly, their weights remaining stationary during the first two months. There was a subsequent gain which brought the weights at the end of the experiment to between 175 and 212 Gm. (average, 199 Gm.). In the course of the experiment several rats became aggressive and vicious, engaging in frequent and violent fights, especially after the injection of the nitrate.

The rats of the sodium nitrite series were listless and exhibited a cyanotic color of skin, which was especially prominent in the tails, ears and feet. All rats except one showed a more or less marked loss of weight. Two rats of this series died during the first two weeks of the experiment, and 2 additional ones were killed after being in the experiment for four weeks.

OBSERVATIONS AT AUTOPSY

Macroscopic Changes.—The rats belonging to the two erythrol tetranitrate series had relatively few macroscopically demonstrable organic lesions. In the oral series several rats which died in the course of the experiment had hyperemic meninges, small flabby brown livers, enlarged, greenish brown kidneys and small soft testes. In 7 other rats there were yellowish to greenish yellow abscesses involving the occipital region of the brain, adjacent to the region of the middle ear. The rats belonging to the sodium nitrite series showed, on the other hand, meningeal vessels filled to the point of congestion with brownish blood, flattened cerebral gyri, collapsed brownish lungs, a soft brown liver, dark red kidneys, small testes and coffee ground-colored mucosal erosions in the stomach, which contained similarly colored material. The intestine was congested and in places was filled with hemorrhagic material.

Fig. 1.—*A*, perivascular fibroblastic proliferation and lymphocytic infiltration with marked edema of the surrounding nerve tissue; × 450. *B*, extensive vacuolar degeneration of ganglion cells in a center of the brain stem; × 450.



nphocytic infiltration
B, extensive vacuolar
× 450.

Microscopic Changes.—Inasmuch as the histologic changes observed in the organs of rats of all three series were essentially identical, varying mainly in degree and extent, it appears appropriate to present them together, especially as they supplement each other in certain respects. Sections were prepared from the following organs: brain, heart, lung, aorta, thyroid, submaxillary gland, stomach, intestine, pancreas, liver, spleen, lymph nodes, adrenal, kidney, bladder, prostate, seminal vesicles, epididymis, testis and bone marrow of the sternum and femur. The sections were stained with hematoxylin-eosin; sections of the brain were stained in addition with toluidine blue; sections of the kidney and heart were prepared with Van Gieson's connective tissue stain; sections of the spleen and kidney were stained by the prussian blue method for iron-containing pigments.

(*a*) Brain: If the brains of 8 rats containing abscesses in the occipital region are excluded from further consideration, as these lesions could be ascribed to infections of the middle ear, the following changes were observed in the majority of animals examined: The meningeal membranes were often edematous and cellular and contained engorged vessels. Intrameningeal hemorrhages were seen occasionally. Focal or more or less diffuse lymphocytic infiltrations of the meninges were rather common. The intracerebral vessels were often congested and surrounded either by perivascular edema or small hemorrhages. Perivascular glia cell accumulations and fibroblastic proliferations were found not infrequently (fig. 1 *A*). Endothelial proliferation of the precapillary and capillary vessels was marked in some rats, causing obliteration of the lumens. In a considerable proportion of the rats the nervous substance contained either local or more or less diffuse glial cell infiltrations, involving mainly the region of the brain stem and of the cerebellum. There were small focal necroses with ameboid gliosis in several rats. Edema of the brain, sometimes associated with marked vacuolation (Swiss cheese-like picture), was observed in several animals. These lesions were often complicated by acute and chronic degenerative changes of the ganglion cells, especially those composing the centers of the brain stem (fig. 1 *B*).

(*b*) Heart: While the myocardium and coronary vessels were normal in some of the rats, many of the animals exhibited degenerative myocardial as well as coronary vascular lesions. In a few animals these changes consisted of marked vascular congestion and multiple small hemorrhagic or plasmatic extravasations. In addition to myocardial edema, there was usually an increase of interstitial histiocytic cells. Hyaline and vacuolar myocardial degenerations were found in a moderate number of rats, whereas focal fibroblastic scars existed in several (fig. 2 *A*). The lesions most frequently found in the hearts of these animals, however, involved the walls of the coronary myocardial vessels. The media was swollen and completely or partially hyalinized. The nuclei were much enlarged and irregularly round and were sometimes surrounded by a vacuolar zone. In addition to a frequent reduction in the number of nuclei, there was often a localized complete loss of these elements. Histiocytic cells were occasionally seen within the thickened, hyalinized media. The lumens of some of these pathologic vessels were narrowed or showed a compressed oblong shape (figs. 2 *A* and 3 *A*). Perivascular edema was seen in a moderate number of animals.

(*c*) Lung: An appreciable number of rats had numerous polypous as well as plaquelike calcifications in the subendothelial spaces as well as in the media of large and small branches of the pulmonary artery. The base of the calcified spurs was usually located within a break of the media. In some small arterioles the calcified projections were so large that they occluded the greater part of the lumen. The nuclei of the smooth muscle cells located in the stumps of the media were irregularly arranged in small clusters, and fibroblastic cells were interspersed with the muscle cells. The pulmonary parenchyma was normal in general.

Fig. 2.—*A*, fibroblastic myocardial scar; × 345. *B*, myocardial arteriole with thickened, homogeneous wall and perivascular edema. The nuclei are reduced in number, markedly swollen and distorted. The localized crowding of nuclei is caused by an invasion of histiocytes into the degenerated and vacuolated media; × 625.

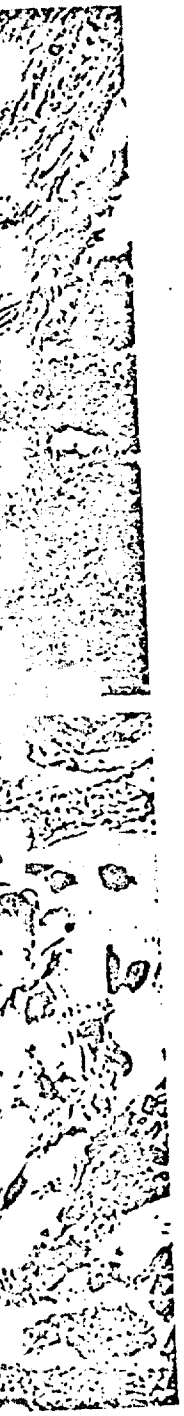


Fig. 3.—*A*, medium-sized myocardial artery with collapsed lumen and markedly thickened and hyalinized wall; × 450. *B*, large renal artery near the hilus with localized thickening and hyalinization of the wall, × 230. A few leukocytes and histiocytes are present in the marginal zone of the necrosis.

(*d*) Stomach: The gastric mucosa was normal in the rats of the two erythrol tetranitrate series, whereas it was congested and contained small hemorrhagic defects in the animals of the sodium nitrite series.

(*e*) Liver: The liver cells of most of the rats showed a peculiar fine granular or vacuolar degeneration of the cytoplasm. The nuclei often seemed to lie in empty spaces or to be located in a cytoplasm consisting of a loose accumulation of coarse granules.

(*f*) Spleen: The more or less markedly congested pulp contained in most instances a moderate to large amount of amorphous brown pigment located intracellularly and extracellularly. The brown granules stained greenish blue with the prussian blue method, while the delicate reticulum was impregnated with a dark blue iron-containing compound.

(*g*) Kidney: Frequently the tubular epithelium showed hyaline, granular degeneration and contained brownish globules which gave a negative reaction by the prussian blue method. Several of the large renal arterial branches at the hilus had a markedly thickened and locally hyalinized wall, the hyalinized area being demarcated from the intact smooth muscle cells of the media by small accumulations of mononuclear and lymphoid cells (fig. 3 *B*). The walls of the renal arterioles were not infrequently markedly thickened and contained vacuolated and swollen smooth muscle cells and subendothelial and medial hyalinizations. The lumens were narrowed (fig. 4 *A*). Several kidneys contained wedge-shaped areas in the cortex, consisting of a thickened interstitial tissue infiltrated with lymphocytes, atrophic and cystic tubules partly filled with a homogeneous, faintly calcified material and congested or atrophic and fibrotic glomeruli (fig. 4 *B*).

(*h*) Testes: In the testicular arteries of an appreciable number of rats calcified plates involved the media, occupying the entire circumference of the vessel. Some of these rats as well as some without the vascular changes showed more or less marked degenerative lesions of spermatogenic epithelium of the tubules. Spermatid giant cells and calcium incrustations in the tubular debris were seen in several instances.

(*i*) Bone Marrow: The marrow of the sternum and femur was often congested and was composed in the majority of cases of a dense myeloid tissue, in which the mature myeloid elements predominated. The erythropoiesis was usually moderate to marked. An immature marrow was seen in some instances.

(*j*) Other Organs: These were essentially normal with the exception of varying degrees of congestion.

The recorded observations demonstrate that an appreciable number of the rats treated for varying periods with erythrol tetranitrate showed degenerative lesions in the myocardial arterioles. These were associated sometimes with regressive or histiocytic-fibroblastic changes in the myocardium. Occasionally, capillary hemorrhagic extravasations were seen in a congested and edematous myocardial interstitial tissue. A considerable proportion of the rats also exhibited calcifications of the subendothelial spaces and media of the arterial vessels of the lungs and testes. Arteriolosclerotic lesions were not infrequently found in the kidneys. Regressive cellular changes were present in a large number of livers, especially among those from rats which survived for the entire experimental period. The spleens often showed evidence of chronic

passive congestion associated with destruction of erythrocytes, indicated by the large amount of iron-containing brown pigment found in many of these organs. In some of the testes extensive regressive changes

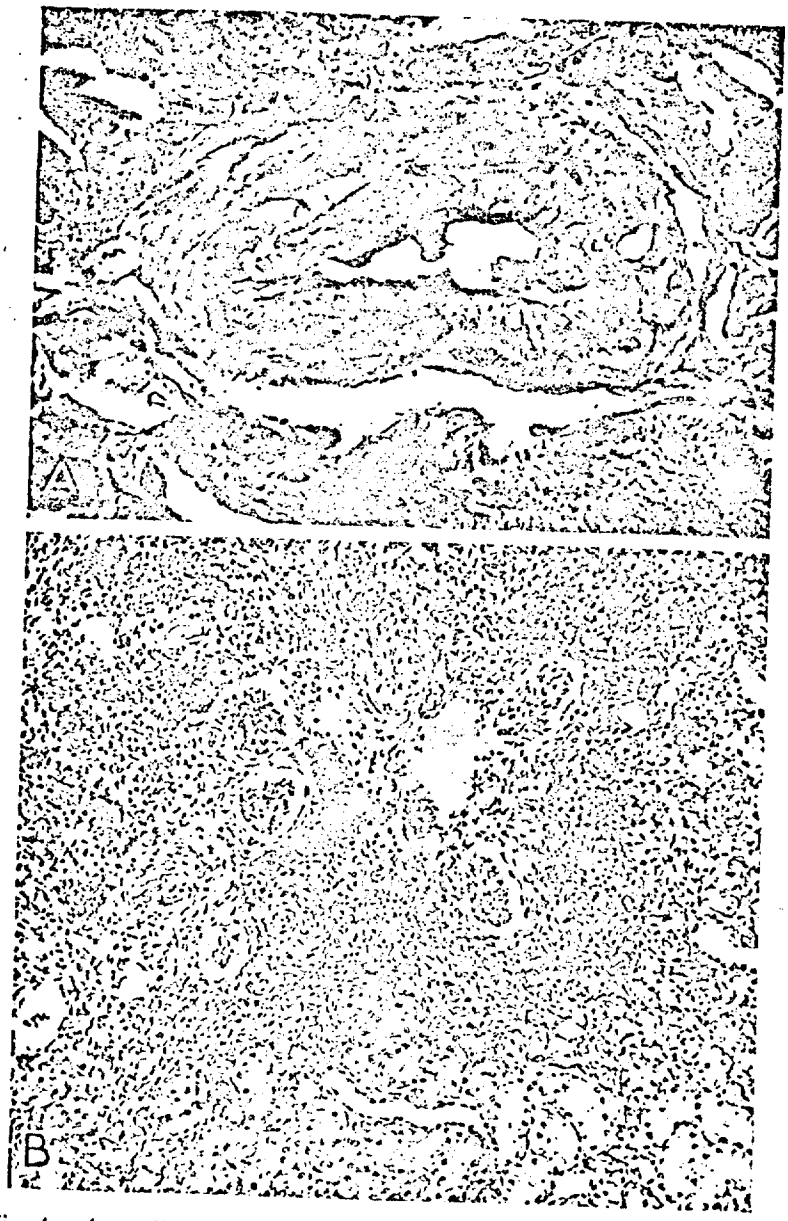


Fig. 4.—*A*, small renal arteriole with markedly thickened wall and narrowing of the lumen; × 625. *B*, area of renal scarring with atrophic, fibrotic glomeruli, lymphocytic interstitial infiltration, fibroblastic proliferation, thickening of arteriolar walls, cystic distention of tubular lumens; × 110.

of the spermatogenic epithelium were noted. The bone marrow in general displayed active erythropoiesis in the presence of an often rather mature but dense myeloid tissue. Hemorrhages and edema observed in a few cases, as well as hemosiderin in one instance, pointed to disturbances affecting both the circulation and the erythrocytes. The circulatory, vascular, glial and ganglionic changes found in the brains of an appreciable number of rats were a striking feature of the various pathologic lesions noted in these rats after the chronic introduction of erythrol tetranitrate by mouth. An analysis of these data in relation to the duration of exposure to this agent brought out the fact that the various pathologic lesions recorded increased in frequency and severity with the time of exposure.

TABLE 1.—*Summation of Averaged Findings on the Blood of Rats After Chronic Oral Administration of Erythrol Tetranitrate*

Days of Experiment	Erythrocytes, Millions	Hemoglobin, Gm. in 100 Cc.	Mean Corpuscular Hemoglobin, Gm. in 100 Cc.	Leukocytes, Thousands
1	6.74	10.4	15	18.7
15	7.41	14.9	20	16.3
35	6.64	17.5	28	18.5
61	7.15	15.3	21	15.9
77	6.95	12.8	19	19.9
106	8.57	13.5	15	24.0
119	8.83	13.3	16	31.7
127	7.85	14.2	19	28.4
152	8.43	15.3	18	30.1
190	7.76	11.9	16	25.1
213	7.75	11.1	17	26.0
222	8.31	12.8	16	16.6

HEMATOLOGIC DATA

During the course of the experiment, studies were made on the blood of some of the rats of the two series which received erythrol tetranitrate. The hematologic examinations were started with 10 rats of the oral series (only 6 survived the experimental period) and an equal number of the subcutaneous series. The examinations were made at rather irregular intervals, which in general became longer with the duration of the investigation. The blood for the examination was obtained by cutting the tails. The blood constituents studied were the leukocytes (including the differential count), erythrocytes and hemoglobin. From the figures obtained for the two last mentioned elements, the value of corpuscular hemoglobin was calculated.

The results of the hematologic studies are presented in tables 1 and 2. The tables are constructed on the basis of averaged values obtained from the 6 and 10 rats, respectively, which could be followed through the entire period of nitrite treatment. This procedure was adopted because all the animals in a particular series showed the same trend. An analysis of the hematologic data shows the following facts:

Oral Series.—The hemoglobin started to rise on the fifteenth day of the experiment and reached its peak of 17.5 Gm. on the thirty-fifth day. The values receded gradually during the rest of the experiment but always remained above the original level.

During the early period the erythrocyte counts fluctuated about a range within that given for normal rats.[5] This was followed by a marked elevation, which reached the highest peak on the one hundred and nineteenth day with 8,830,000 cells. Although the total red cell count dropped somewhat afterward, it fluctuated and maintained a mean definitely higher than the mean established for normal rats.

The mean value of the corpuscular hemoglobin showed changes similar to those in the values of hemoglobin. Following a rapid rise, which not only paralleled but surpassed the upward movement of the values of hemoglobin, there occurred a rather rapid drop in the amount of the corpuscular hemoglobin, which reached its lowest point on the one hundred and sixth day, when it was somewhat below the normal range. The latter part of the experimental period was thus characterized by the fact that after marked fluctuations in the hemoglobin and the corpuscular hemoglobin values during the first part and considerable increases of

TABLE 2.—*Summation of the Averaged Findings on the Blood After Subcutaneous Injection of Erythrol Tetranitrate*

Days of Experiment	Erythrocytes, Millions	Hemoglobin, Gm. in 100 Cc.	Mean Corpuscular Hemoglobin, Gm. in 100 Cc.	Leukocytes, Thousands
1	7.23	16.2	22	18.7
3	6.67	14.7	22	14.4
5	6.53	13.2	19	14.5
9	6.03	12.5	21	13.8
14	5.52	11.2	20	20.4
20	4.91	11.5	24	15.6
36	6.16	9.7	18	30.3
57	6.53	11.8	16	29.0
73	6.07	13.0	16	38.9
79	6.56	12.9	20	34.1
86	7.12	9.0	13	35.7
107	6.72	11.1	17	34.5
126	7.47	9.9	13	42.5
133	7.31	11.0	15	22.1

the erythrocytes during the middle part, the three constituents became more or less stabilized toward the end, remaining somewhat above the original levels in a parallel movement.

The total leukocyte count, after relatively minor fluctuations during the first two months of the experiment, showed a definite increase, which continued for the remaining five months. The peak was reached on the one hundred and nineteenth day, when a maximum of 31,730 leukocytes was recorded. From this period until almost the end of the experiment the total white cell count maintained itself at a level high above the normal range. On the day that the rats were killed, however, the white cell count had dropped to 16,640 (twelve days after the discontinuation of the nitrite treatment).

The differential count did not show any significant shifts from the normal in spite of the considerably increased number of leukocytes present during the latter half of the experimental period.

It was observed that in most instances the number of reticulocytes was increased definitely after the administration of erythrol tetranitrate, while the number of platelets exhibited a tendency toward lower values, within the normal range, during the latter part of the experimental period.

5. Wintrobe, cited by Cushny.[6]

Subcutaneous Series.—The values of hemoglobin dropped almost steadily from the start of the experiment, reaching their lowest figure (9.76 Gm.) at the end of the fifth week. Following a transitory rise, the values dropped again and reached their lowest figure (9 Gm.) at the end of the third month. There was subsequently a moderate rise with values somewhat below the normal range (i. e., between 9.9 and 12.9 Gm.).

The erythrocyte count exhibited a similar initial reduction, which was also followed by a gradual rise. This, however, was more prolonged than that of the values of hemoglobin. After having reached a figure slightly above the original one, the erythrocyte count decreased again and remained for the rest of the experimental period within normal limits.

Following a slight initial drop and a subsequent steep rise to somewhat above the normal level, the corpuscular hemoglobin decreased severely and persistently and remained at a reduced level for the last part of the experiment.

The leukocytes remained stationary as to number and type during the first quarter of the experimental period, then rose constantly until they reached their peak before the end of the experiment, when 42,470 white cells represented the average leukocyte count. There was at this time a moderate increase in the proportion of the neutrophilic and especially of the juvenile leukocytes.

COMMENT

The significance of the recorded vascular lesions and of the parenchymatous organic alterations depends on the demonstration of causative interrelations between the morphologic phenomena and the administration of erythrol tetranitrate and sodium nitrite, respectively. Inasmuch as Hueper,[1] Wilens and Sproul[2] and Hummel and Barnes[3] reported the spontaneous occurrence of similar cardiovascular lesions in rats, it is essential that satisfactory evidence be supplied which will exclude a possibly senile character of the organic changes observed in the rats treated with nitrites. The observations made by Wilens and Sproul[2] on the age distribution of these pathologic changes of the circulatory tissue of rats are therefore of great importance. These investigators found that rats less than 400 days old are practically free from degenerative cardial lesions, while the coronary sclerotic changes noted in 23 of 487 rats examined showed in their age incidence a direct relation to the senile period. Similar observations were made by Hummel and Barnes, who noted that sclerotic cardiovascular lesions were found in only 2 of 111 rats dying at an age of 261 to 374 days. As the animals comprising the nitrite series were in no instance older than 300 days, i. e., they belonged to an age period during which normally degenerative cardiovascular changes are rare or absent, it becomes obvious that the organic lesions observed cannot be considered as the physiologic accompaniments of old age but must be regarded as the results of the nitrite treatment. This conception is supported by the fact that they occurred in the rats not only with marked severity and high incidence but with increasing frequency corresponding to the duration of exposure to the nitrites.

A possible senile nature of the lesions observed in the treated rats having been excluded, it becomes pertinent to demonstrate whether the symptomatic and morphologic manifestations noted in the experimental animals resulted from the pharmacologic and toxicologic effects known to be exerted by nitrites and whether these reactions were similar to or identical with those seen in chronic nitrite poisoning of man.

The functional effects which inorganic nitrites as well as inorganic and alkyl nitrates exert on the vascular walls and the circulation of the blood are expressed in a relaxation of the walls of the peripheral arterioles with a resultant drop in blood pressure. The most susceptible vessels, which react first and to the smallest doses, are those of the head, neck, brain and meninges. Larger doses cause relaxation of the coronary vessels, while still larger ones produce dilatation of the vessels of the splanchnic region. It is this widening of the vascular bed which causes the drop in blood pressure. The slowing of the blood flow results in congestion, which in turn is followed by development of hypoxemic states, especially in those organs in which the demand for oxygen is high or which are highly susceptible to even minor deficiencies in the oxygen supply (brain and heart). It has been noted that the degree of vasodepression elicited depends not only on the particular type of nitrite used and the dose given but to a great extent on the subject as well as on his age (Cushny [6]; Löwy [7]; Prodger and Ayman [8]; Sprague and White [9]).

The ensuing subjective symptoms of vasculocirculatory disturbances observed in chronic nitrite poisoning are: transitory or more or less persistent low blood pressure, which may drop below 80 mm. of mercury; a weak, soft pulse; dilatation of the left side of the heart; accentuation of the first heart sound; exaggeration of the apical beat; murmurs similar to those found with anemia, aortic regurgitation or mitral insufficiency; irregularity of the heart beat, and other signs of myocardial impairment. Tachycardia or bradycardia is often present, the latter condition, however, changing readily into tachycardia on slight physical exertion, excessive change in environmental temperature and humidity or consumption of alcoholic beverages or other agents which exert an additional vasodepressant effect. Precordial pain and anginoid attacks are not infrequently recorded. During more acute episodes the color

6. Cushny, A. R.: Die Nitritgruppe, in Heffter, A.: Handbuch der experimentellen Pharmakologie, Berlin, Julius Springer, 1923, vol. 1, p. 833; A Text Book of Pharmacology and Therapeutics, ed. 11, Philadelphia, Lea & Febiger, 1936, p. 630.

7. Löwy, J.: Die Klinik der Berufskrankheiten, Vienna, Emil Haim & Co., 1924, p. 392.

8. Prodger, S. H., and Ayman, D.: Am. J. M. Sc. **184**:480, 1932.

9. Sprague, H. B., and White, P. B.: M. Clin. North America **16**:895, 1933.

of the skin is purplish livid, most noticeable in the nail beds, lips, ears and nose. As the result of the direct or indirect action of the nitrites on the cerebral circulation there occur, in addition to headache, diverse symptoms such as insomnia, restlessness, maniacal episodes, states of drowsiness and mental dulness. The vasodepressant effect on the splanchnic system causes the appearance of symptoms of indigestion and gastroenteritis (colic, bloody diarrhea) resulting from chronic congestion and hemorrhages of the gastric and intestinal mucosa. Sudden death may ensue with the symptoms of cerebral paresis (Meixner and Mayrhofer[10]) or of coronary thrombosis (International Labor Office Report[11]; Löwy[7]; Laws[12]; Ebright[13]; Robert[14]; Schulz,[15] and others).

The following macroscopic and microscopic lesions have been recorded in connection with deaths from nitrite poisoning: meningeal and cerebral edema and congestion with perivascular edema and accumulation of lymphoid and glia cells and brown pigment; multiple cerebral hemorrhages affecting mainly the region of the brain stem; intimal proliferation, medial hyalinization, swelling and calcification of the cerebral vessels; perivascular fibrous scars with capillary proliferation, gliosis and blanching of the nerve substance; glial scars; acute and chronic degeneration of ganglion cells, involving chiefly the centers of the brain stem; coronary sclerosis; swelling and hyalinization of the walls of the myocardial arterioles; enlargement of the heart with hypertrophy of the left ventricular wall; myocardial degeneration, fibrosis and cicatrization; chronic passive congestion of the liver with pericentral fatty degeneration; hemorrhagic gastritis and enteritis; nephrosis; arteriosclerosis of the renal vessels, and pancreatic hemorrhages (Solis-Cohen and Githens[16]; Löwy[7]; von Jaksch[17]; Binz[18]; Meixner and Mayrhofer[10]; Fischer-Wasels,[19] and others).

During the course of the experiment several of the listed symptomatic manifestations of chronic nitrite poisoning, such as cold livid skin, mental depression, aggressiveness, as well as fainting spells, and attacks of tachycardia, were observed following the injection of nitrite. The

10. Meixner, K., and Mayrhofer, A.: Vrtljschr. f. gerichtl. Med. **61**:228, 1921.
11. International Labor Office Reports, vol. 2, no. 281, Geneva, Switzerland, International Labour Office.
12. Laws, G. C.: J. A. M. A. **31**:793, 1898.
13. Ebright, G. E.: J. A. M. A. **62**:264, 1914.
14. Robert, E.: J. de méd. de Bordeaux **112**:10, 1935.
15. Schulz, O.: Samml. f. Vergiftungsfälle (B) **6**:59, 1935.
16. Solis-Cohen, S., and Githens, T. S.: Pharmacotherapeutics, New York, D. Appleton and Company, 1928, p. 370.
17. von Jaksch, cited by Löwy.[7]
18. Binz, C.: Arch. f. exper. Path. u. Pharmakol. **13**:133, 1881.
19. Fischer-Wasels, B.: Frankfurt. Ztschr. f. Path. **45**:1, 1933.

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hematologic findings reflected the changes produced in the circulation and oxygenation of the blood. The hematologic effects were especially striking in the oral group. Following a primary compensatory phase of hemoglobinosis, there developed an appreciable and relatively continuous erythrocytosis in response to the persistent and possibly intensified circulatory hypoxemia produced by the administration of nitrites. Confirmatory evidence of the presence of marked circulatory embarrassment during the latter part of the experimental period in both series is furnished by the behavior of the leukocytes. The considerable leukocytosis without the usually occurring shift toward a higher proportion of immature cell forms and with the presence of a predominantly mature or resting myeloid marrow characterizes the leukocytotic condition as a distributory phenomenon representing leukocytostasis in the distal, peripheral vessels, such as those of the tip of the tail, caused by excessive slowing of the blood flow.

The anatomic studies of the organs of the rats treated with nitrites revealed that the vascular and parenchymatous lesions produced in these young animals were identical with those observed in human subjects who had been exposed and subsequently succumbed to a chronic exposure to chemically related nitrites and similarly acting nitrates. It is obvious that the existence of causal relations between the regressive and sclerotic vascular alterations and some of the related parenchymatous degenerations (especially those in the brain, heart, kidney and testis) and the hypotensive and hypoxemic effect exerted by the nitrite has not been recognized in the past because the anatomic manifestations are very similar to those considered representative of, and resulting from, or accompanying, chronic hypertension. In the absence of adequate etiologic and symptomatic data these pathologic changes may have been misinterpreted or may have been considered as coincidental.

It is believed that the experimental data presented offer satisfactory evidence in support of the actual existence of such interrelations and thus furnish experimental proof of the original premise that arteriosclerotic lesions may develop on a hypotensive as well as on a hypertensive basis, provided the hypotensive condition is sufficiently severe and prolonged. While an inordinate hypertensive vasoconstriction interferes with proper nutrition of the vascular wall, especially of its inner portion, not supplied with vasa vasorum, by a reduction of the quantity of blood which can penetrate these more or less compressed vessels (ischemic hypoxemia), the same effect is produced by the stagnant hypoxemia resulting from the excessive slowing of the blood flow accompanying hypotensive vasodilation, which also affects mainly those parts of the vascular walls in which vital activity depends on nutritive exchange and discharge of waste products through the process of diffusion.

SUMMARY

Oral and subcutaneous administration of erythrol tetranitrate and sodium nitrite, respectively, to young, immature rats over a period of several months results in the production of degenerative vascular and parenchymatous lesions in the heart, lung, brain, kidney and testis.

The chief causative factor in the development of these changes is represented by a stagnant hypoxemia resulting from the slowing of the blood flow caused by the hypotensive vasodilatation elicited by the nitrite.

These experimentally produced organic changes are identical with those seen in man following chronic nitrite poisoning and are very similar to those observed in spontaneous or experimental hypertension as found in man and animals, respectively.

Inasmuch as vasodilating agents as well as vasoconstricting agents cause a nutritive disturbance of the vascular walls, prolonged vasodilation or vasoconstriction (hypotension or hypertension) results in the appearance of degenerative and reactive proliferative phenomena in the vascular walls, which are considered as characteristic of arteriosclerosis and arteriolosclerosis, angionecrosis and atheromatosis.

The same causative mechanisms produce, by hypoxemia and associated nutritive disturbances, regressive changes in the parenchymatous organs (heart, brain, kidney, testis), which are accentuated by the progressive circulatory impairment resulting from the vascular changes.

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ENZYME STUDIES OF THE KIDNEY AND SERUM OF RATS DURING CARCINOGENESIS BY LEAD ACETATE AND DIMETHYLNITROSAMINE

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ABSTRACT

In male Wistar strain rats, weighing about 100 g, which received dimethylnitrosamine (DMNA) in an oral dose of 500 ppm for 14 consecutive days or were placed on a schedule of 10 per cent basic lead acetate in feed daily with a view to inducing formation of renal tumors, assays of the serum and renal cortex for the changes of lactic dehydrogenase (LDH), alkaline phosphatase and total acid phosphatase activity and isoenzyme patterns of LDH were conducted monthly over a 12 month period. The results were as follows: (i) Rats given DMNA displayed marked elevation of LDH activity in both serum and renal cortex after a period of 9 months over that of the control group; (ii) among those given DMNA, there was also evidence of a predominance of LDH-111 fraction and concomitant decrease in LDH-H fraction of the renal tissue during the latter half of the experiment; (iii) in both experimental groups alkaline phosphatase activity in the renal cortex was lowered; (iv) in rats treated with lead acetate, leucine aminopeptidase activity in the renal tissue was lowered, whereas in the serum of rats given DMNA a slight increase was seen after a period of 10 months; and (v) neither rats on lead acetate nor those given DMNA displayed significant alterations in total acid phosphatase activity both in the serum and renal cortex during the 12 month period.

Numerous studies (1–5) are published concerning chemical agents which are implicated in the genesis of malignant neoplasms to elucidate the tumorigenic mechanism.

It is known in certain diseases, particularly with tumors, that the serum enzyme levels are correlated with the biologic attributes of malignancy (6–8). This is caused by either the increased production of enzymes by the tumorous tissue, increased release of enzymes by the disintegrated tissue, hyperpermeability of the membrane of the tumor cells, or the delay in dissipation of enzymes from the blood (9).

The the field of urology, pursuit on the changes of enzyme activity in urine, serum, or tissue of patients with malignant neoplasm of the urinary tract has also been made (5–8, 10). Thus, the present report deals with the alterations of the kidney and serum enzyme level in rats with experimentally produced renal tumors.

MATERIALS AND METHODS

Four hundred ten Wistar strain male rats, weighing about 100 g, were divided into three groups: Group I (control)—a series of 90 rats was supplied

with a standard diet (powdered Clea CE-2 food, Nippon Clea Inc., Tokyo); Group II (lead acetate treated)—160 rats were placed on a schedule of a standard diet with 10 per cent basic lead acetate ((LA) Wako Junyaku, Osaka, reagent grade); Group III (dimethylnitrosamine-treated)—a group of 160 rats was supplied with a standard diet containing 500 ppm of dimethylnitrosamine ((DMNA) Tokyo Kasei, Tokyo, chemical grade), for the first 2 weeks, followed by standard diet only. The rats were housed in wire cages, in groups of five, and were placed in an air conditioned room with a relatively constant temperature and optimal humidity. Food and water were given ad libitum. The duration of the experiments was 12 months for all groups. From each of the three groups, three rats were killed by decapitation at 1 month intervals, and serum and renal cortex were assayed for such enzymes as lactic dehydrogenase (LDH, Iatron, Tokyo), alkaline phosphatase (ALP, Ishizu, Osaka), leucine aminopeptidase (LAP, Ishizu, Osaka), total acid phosphatase (TAP, Ishizu, Osaka) and isoenzymes of lactic dehydrogenase (agar gel electrophoresis by Wieme (11)). The activity is expressed as units per milliliter of the serum or 1 g wet weight of the tissue. Rats which died during the course of the study for enzyme determination were excluded from this study. The decapitation of animals was in all instances performed between 8 and 9 AM. All of the determinations for enzyme activity and electrophoresis were carried out simultaneously. Spectrophotometric assays of enzyme activity were performed by QV-50 spectrophotometer (Shimadzu, Kyoto). Isoenzyme zymograms of LDH were prepared using a Lumicon P-2 autorecording densitometer (Kayagaki, Tokyo) with the absorption peak at 340 mμ.

RESULTS

LDH

Renal cortex (*Fig. 1*). The LDH activity in the control group ranged from 3.05×10^5 to 5.11×10^5 Wroblewski units (WU) (average 4.17×10^5 WU)

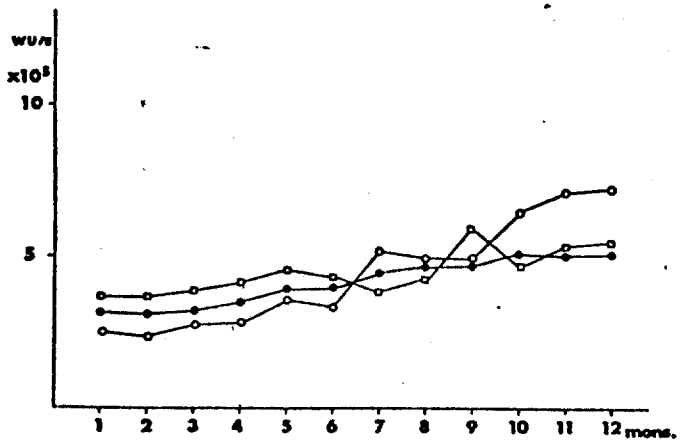

FIG. 1. LDH activity of the renal tissue in rats during carcinogenesis of the kidney. ●, control group; ○, dimethylnitrosamine treated group; □, lead acetate treated group.

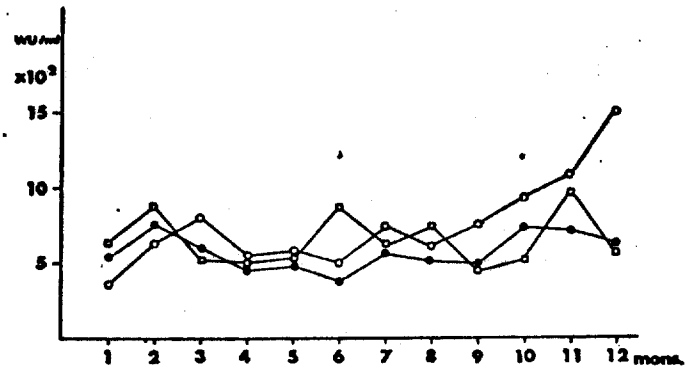

FIG. 2. LDH activity of the serum in rats during carcinogenesis of the kidney

and an elevation with progress of experiment was evident. In the DMNA treated group it ranged from 2.53×10^5 to 7.42×10^5 WU (average 4.54×10^5 WU), whereas in the LA treated group, it ranged from 3.75×10^5 to 5.46×10^5 WU (average 4.50×10^5 WU). In these two experimental groups, as well as in the control, evidence of elevation of LDH activity with the duration of experiment could be observed. However, in the DMNA treated group increase in LDH activity became prominent only 10 months after the initiation of medication with DMNA.

Serum (*Fig. 2*). In the control group LDH activity ranged from 3.8×10^2 to 7.6×10^2 WU (average 5.8×10^2 WU). There were no definite alterations during the experiment. The LDH activity ranged from 3.8×10^2 to

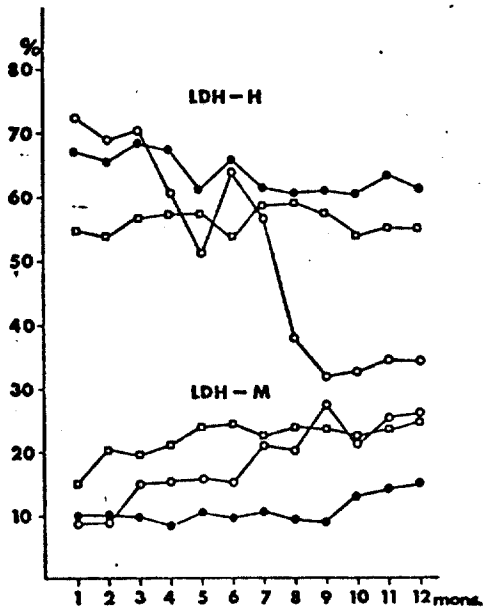

FIG. 3. LDH-M and -H distribution of the renal tissue in rats during carcinogenesis of the kidney.

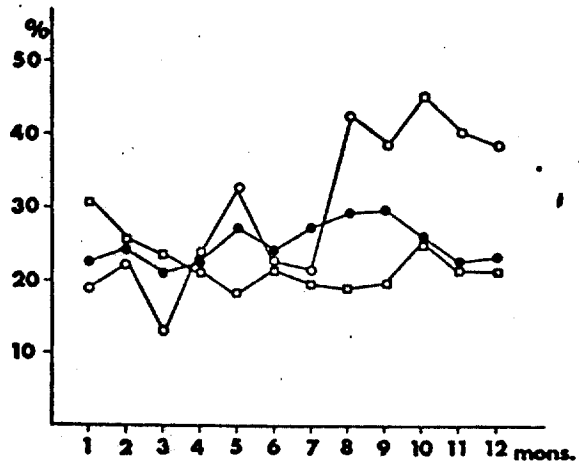

FIG. 4. LDH-III fraction of the renal tissue in rats during carcinogenesis of the kidney

15.3 × 10^2 WU (average 7.5 × 10^2 WU) in the DMNA treated rats and from 4.5 × 10^2 to 9.7 × 10^2 WU (average 6.6 × 10^2 WU) in the LA treated rats, respectively. The results ascertained obvious elevation of LDH activity in the DMNA treated animals, particularly marked in the last 3 months of the experiment.

Isoenzymes of LDH

Renal cortex (Figs. 3 and 4). In the control group LDH-M fraction showed an increase, the value ranging from 9.4 to 14.9 per cent (average 11.1 per cent). In the case of rats given DMNA, LDH-M fraction was increased from 8.9 to 27.6 per cent (average 18.7 per cent). LDH-M fraction in the LA treated animals ranged from 14.9 to 24.5 per cent (average 21.8 per cent). This was fairly great even at the initial period but no significant increase was observed during the progress of the experiment.

In the control group LDH-H fraction slightly diminished from 68.7 to 61.0 per cent (average 64.0 per cent), whereas in the DMNA treated group this fraction was fairly widely distributed from 72.4 to 32.2 per cent (average 51.3 per cent), showing a marked decrease in the latter half of the experiment. The rats given LA showed a distribution of values from 58.2 to 54.1 per cent (average 56.1 per cent), being smaller than those of the control through the early stage of the experiment and practically no evidence of further alterations thereafter.

LDH-III fraction in the control group ranged from 21.5 to 29.4 per cent (average 24.9 per cent), in which alterations with increase of age were slight. It ranged from 13.2 to 45.1 per cent (average 30.0 per cent) in rats given DMNA, with a marked increase in the latter half of the experiment. In contrast, the LA treated rats displayed a slight increase in this fraction from 30.6 to 18.0 per cent (average 22.1 per cent).

Serum (Figs. 5 and 6). During the experiment there were no significant

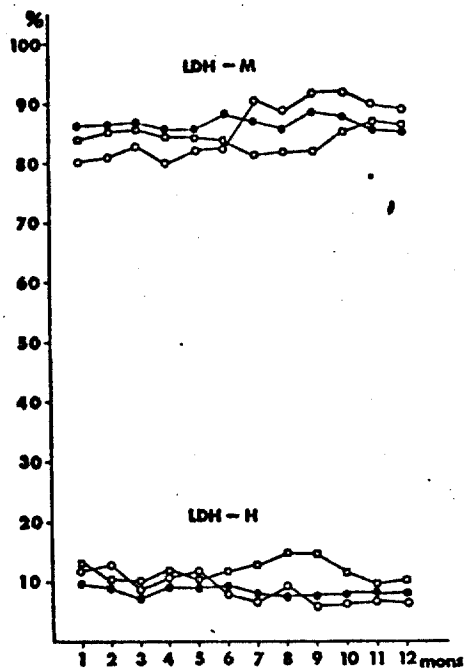

FIG. 5. LDH-M and -H distribution of the serum in rats during carcinogenesis of the kidney.

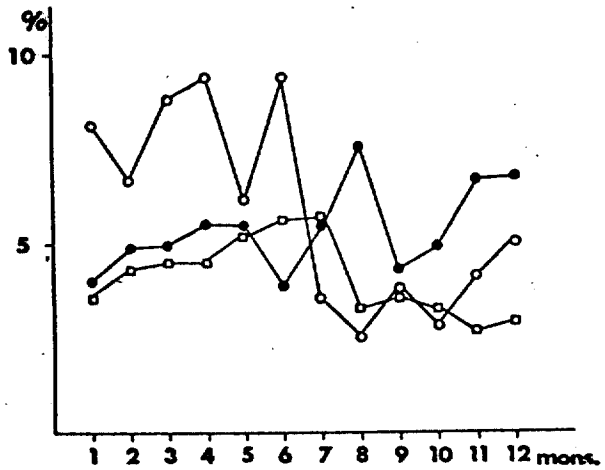

FIG. 6. LDH-III fraction of the serum in rats during carcinogenesis of the kidney.

alterations of LDH-M fraction in the control group which was distributed from 85.3 to 87.2 per cent (average 86.3 per cent). Such was also the case with the remaining two experimental groups: in the DMNA treated group the range was from 79.7 to 90.7 per cent (average 85.5 per cent), and in the LA treated group from 81.3 to 86.9 per cent (average 84.1 per cent), respectively.

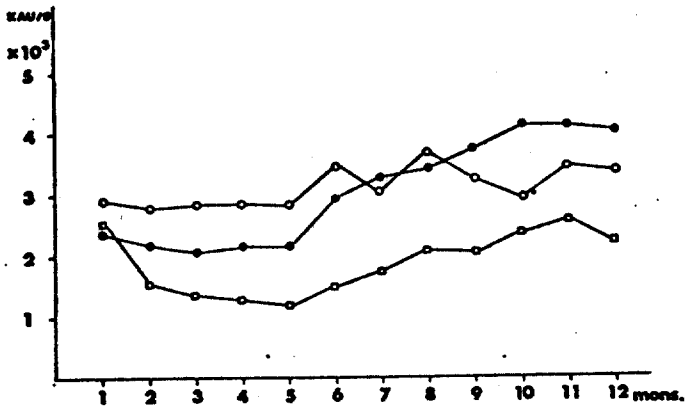

FIG. 7. ALP activity of the renal tissue in rats during carcinogenesis of the kidney

With increase of age there were also no significant changes of LDH-H fraction in the control group which ranged from 9.6 to 7.1 per cent (average 8.3 per cent). In the DMNA treated group, measurements ranged from 12.4 to 5.5 per cent (average 8.6 per cent), diminishing slightly as the experiment progressed, whereas in the rats given LA it ranged from 15.1 to 10.0 per cent (average 11.8 per cent), which surpassed the control to some extent.

In all three groups a fairly great dispersal in the distribution of values for LDH-111 fraction was observed. In the control group this fraction ranged from 4.0 to 7.6 per cent (average 5.4 per cent), slightly rising with increase in age, whereas in the DMNA treated group this fraction displayed a decrease from 9.4 to 2.6 per cent (average 5.9 per cent). In the LA treated group the value ranged from 5.7 to 2.7 per cent (average 4.1 per cent).

ALP

Renal cortex (*Fig. 7*). In the control group, tissue ALP activity ranged from 2.08×10^3 to 4.13×10^3 King-Armstrong units (KAU) (average 3.06×10^3 KAU), and showed an elevation with increase in age. Activity for the DMNA treated group was distributed within the range of 2.76×10^3 to 3.51×10^3 KAU (average 3.12×10^3 KAU), being greater than the control in the first half, but followed by no definite alterations along with the duration of the experiment. Conversely, the LA treated group clearly displayed a decrease in ALP activity of the renal cortex, namely from 1.15×10^3 to 2.25×10^3 KAU (average 1.88×10^3 KAU).

Serum (*Fig. 8*). As shown in Figure 8, the control group serum ALP activity ranged from 2.9 to 6.3 KAU (average 4.9 KAU), whereas in the DMNA treated group the activity ranged from 2.8 to 9.5 KAU (average 5.1 KAU), and in the LA treated group from 1.3 to 7.8 KAU (average 4.4 KAU), respectively. Although a fairly great difference in the distribution can be noted at all occasions, in all three groups of animals a tendency toward a decrease in serum ALP activity was observed.

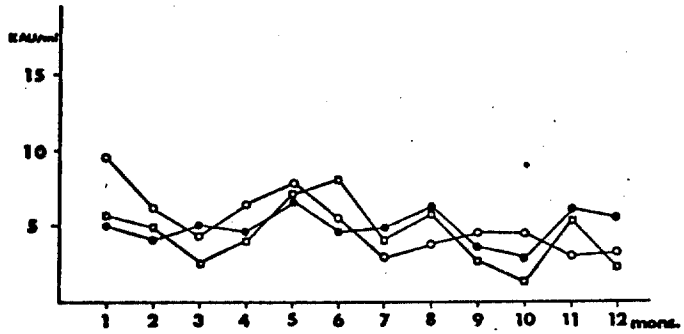

FIG. 8. ALP activity of the serum in rats during carcinogenesis of the kidney

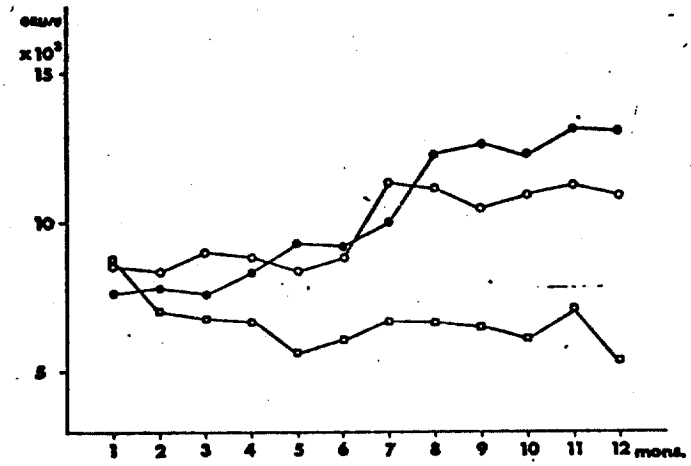

FIG. 9. LAP activity of the renal tissue in rats during carcinogenesis of the kidney

LAP

Renal cortex (*Fig. 9*). In the control group, there was evidence of a decrease in tissue LAP activity with increase in age, which ranged from 7.61×10^3 to 13.14×10^3 Goldberg-Rutenberg units (GRU) (average 10.27×10^3 GRU). This increased activity along with the duration of the experiment was also the case with the DMNA treated group in which the tissue LAP activity ranged from 8.37×10^3 to 11.32×10^3 GRU (average 9.82×10^3 GRU). On the other hand, in the LA treated group the activity ranged from 5.37×10^3 to 8.47×10^3 GRU (average 6.5×10^3 GRU), which conversely tended to diminish.

Serum (*Fig. 10*). Evidence of a slight increase in serum LAP activity with increase of age in the control group, which ranged from 1.6×10^2 to 2.9×10^2 GRU (average 2.3×10^2), was observed. After a period of about 6 months, increase of serum LAP activity among the rats was noted. It ranged from 2.1×10^2 to 5.9×10^2 GRU (average 3.5×10^2 GRU). In the LA treated group, the activity ranged from 0.8×10^2 to 4.1×10^2 GRU (average 2.6×10^2 GRU).

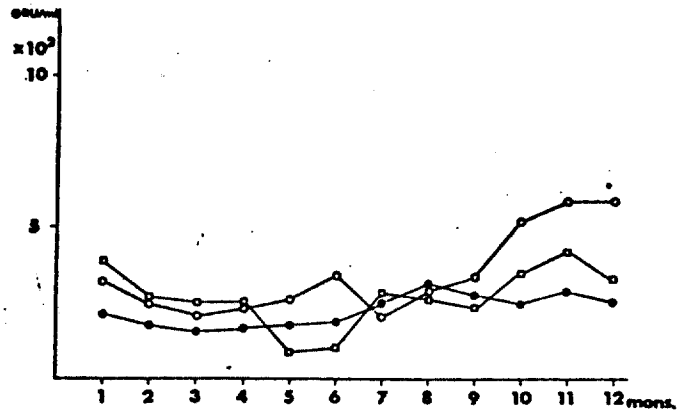

FIG. 10. LAP activity of the serum in rats during carcinogenesis of the kidney

TAP

Renal cortex (*Fig. 11*). In the control group tissue TAP activity ranged from 1.09 × 10^3 to 1.64 × 10^3 KAU (average 1.39 × 10^3 KAU), but no definite tendency was noted in TAP activity with increase in age. In the DMNA treated group the activity ranged from 0.84 × 10^3 to 1.65 × 10^3 KAU (average 1.34 × 10^3 KAU), and in the LA treated group from 0.84 × 10^3 to 1.46 × 10^3 KAU (average 1.15 × 10^3 KAU), respectively.

Serum (*Fig. 12*). In the control group serum TAP activity ranged from 9.1 to 13.3 KAU (average 11.0 KAU); there was no definite change in the activity with increase in age. There were also no significant alterations in the serum TAP activity with the duration of the experiment in both DMNA and LA treated groups, although the activity was relatively wide in distribution.

DISCUSSION

The carcinogenic agents such as DMNA, methylcholanthrene, thoratrast, LA, and female sex hormone are now well recognized, and a number of stud-

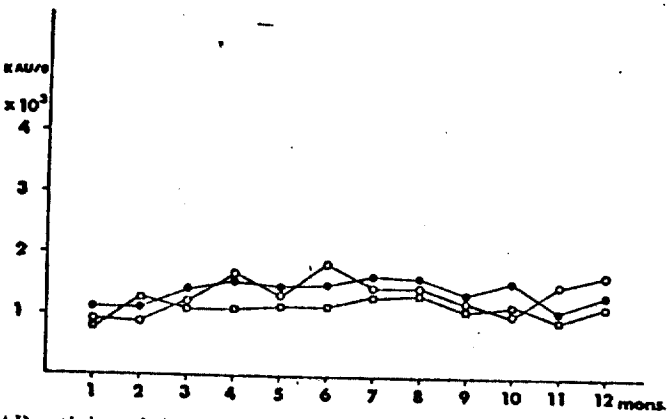

FIG. 11. TAP activity of the renal tissue in rats during carcinogenesis of the kidney

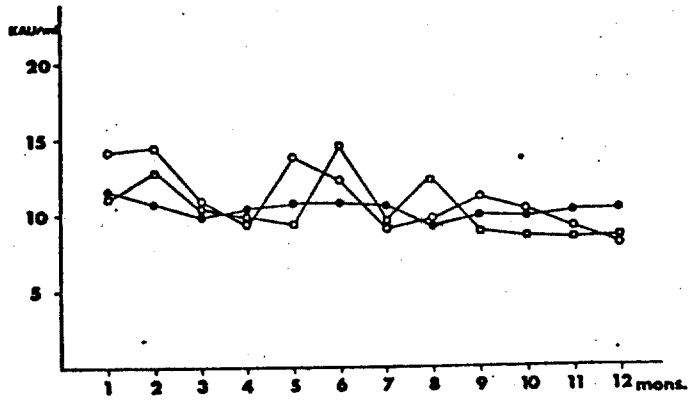

FIG. 12. TAP activity of the serum in rats during carcinogenesis of the kidney

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ies have been published on the production of kidney tumors by the use of these agents in laboratory animals (1, 3–5, 12, 13). In a careful examination of the long term effects of DMNA, Zak, Holzner, Singer, and Popper (12) observed malignant changes of the kidney in rat at an early stage of 80 days after the initiation of the experiment. However, the incidence of renal tumor developed with DMNA treatment greatly varies with the dose and mode of administration as stated by Magee and Barnes (3). In connection with the present experimental condition, Itoh et al. (2) described that in rats receiving medication with a dosage of 500 ppm of DMNA given for 14 consecutive days renal tumors arose in as many as 79.5 per cent of the rats examined after 6 months. Histologically, there are two types of DMNA induced renal tumors (3, 14). The views of Magee and Barnes (3) as to the degree of malignancy and developmental time of the renal neoplasm differ considerably with those described by Zak et al. (12).

Renal tumors produced experimentally by the use of LA were first described by Zollinger (13). As was shown experimentally by Boyland et al. (1), among 16 rats which survived 320 days, 15 were found to have either adenoma or adenocarcinoma of the kidney. It seems that the occurrence of the kidney tumor by the application of LA was great and that in the histopathologic findings the tumors were found to be rather uniform, although the development of the tumor was delayed.

Concerning enzymologic studies on the development of these renal tumors, Murphy et al. (5) carried out a determination of various enzymes in the urine following medication of DMNA in rats, but reports referring to the changes of enzyme activity of experimentally induced renal neoplasms are few.

Both Stolk (14) and Ikoma and Nagahara (10) have shown histochemically a rise in LDH activity in the tumorous tissue of human renal neoplasma. Isoenzyme patterns of LDH in the renal tissue have been studied by a number of investigators (7, 8, 15), and evidence has indicated that one part of the kidney contains a predominance of one type of LDH, and another part a

predominance of the other type of LDH, and this has been attributed to the adaptation of the enzyme to tissue metabolisms (15). In the present study it was clearly observed that the elevation of LDH in both serum and renal cortex of rats given DMNA was predominant in the latter half of the experiment, and in the tissue an increase in LDH-III and LDH-M fraction occurred with a concomitant decrease in LDH-H fraction with lapse of time. If these findings should reflect a regression in isoenzyme patterns of LDH to the embryonal form with increase in malignancy (6), the results obtained provide evidence in support of the opinion by Zak et al. (12) that the renal tumor induced by DMNA gains malignancy with lapse of time in the experiment, but do not support the opinion postulated by Magee and Barnes (3). Meanwhile, with the renal tissue of rats given LA, an increase in LDH-M fraction and a concurrent decrease in LDH-H fraction were observed in the initial course of the experiment, although these alterations were rather slight in the latter half of the experiment. As far as the LDH isoenzymes are concerned, these results suggest that there exists no common feature between the tumorigenic process after the application of DMNA and that after LA application.

Phosphatase displays some connection with the reabsorption of glucose as well as the reabsorption of H_2CO_3 and the formation of NH_4. In the kidney, these are found predominantly in the proximal tubules. Mori et al. (16) described the elevation of ALP in inflamed epithelium of the experimentally induced tumor through a series of their histochemical studies. In contrast, however, the present investigation showed that the ALP level of the renal cortex was lower in both rats given DMNA and those given LA than in the control, and this was particularly marked with the group of rats on LA. These findings have attributed to a greater predominance of the renal cell type in the LA treated rats than in those given DMNA (10). The decreased level of ALP was also associated with the serum of the rats belonging to these two groups although the values showed a wide dispersal in distribution. These results altogether may suggest that a diminution of ALP activity takes place in both serum and renal tissue of animals with renal neoplasm.

TAP is slightly elevated in clinical renal neoplasms (10), but, because of the lack of localization of the enzyme in the kidney, its value in the diagnosis of renal tumors is considered to be practically very small. In both the serum and tissue no evidence of alterations of this enzyme was observed during the course of the present investigation.

Inasmuch as the proximal renal tubules are particularly rich in LAP as in the localization of ALP, it is probable that this enzyme is found to rise in the urine of patients with disorders of the proximal renal tubules. Indeed, Gomori (17) described elevations of LAP in carcinomatous tissues of the kidney and Glenner, Burstone, and Meyer (18) showed the correlation of the LAP level with the degree of malignancy in kidney tumors, whereas Burstone (19) and Monis, Nachalas, and Seligman (20) showed greater LAP activity in the adjoining connective tissue than in the tumor cells themselves. The results of the present study indicate lowered levels of LAP activity in the renal tissue

of both experimental groups, especially in those given LA, and, conversely, in the serum of these rats stress elevation of the LAP activity was observed. Thus, rise in serum LAP activity may well be expected to be associated with the development of renal neoplasms, although many studies remain to be pursued in this respect.

In the present investigation no attempt was made to describe the concomitant formation of the carcinoma which may be inherent to the medication of DMNA or LA. Boyland et al. (1) found no mammary carcinoma during the 12 months after LA medication. On the other hand, Magee and Barnes (3) observed hepatic tumors with DMNA treatment at a relatively early stage. Therefore, in this study rats with macroscopic evidence of concomitant formation of carcinoma were precluded from the experiment. Possibilities of the reticuloendothelial system that might have influence upon enzyme levels (17) are now under progress.

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NITRATE TOXICITY IN DAIRY HEIFERS. 2. ERYTHROPOIETIC RESPONSES TO NITRATE INGESTION DURING PREGNANCY[1]

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SUMMARY

The effects of feeding sublethal levels of nitrate were studied under conditions of management and feeding practices recommended for dairy cattle. Heifers ingesting nitrate at levels of 440 and 660 mg/kg body weight for a long period of time were capable of adapting to a deficiency of oxygen in their blood. This compensatory mechanism consisted of a marked increase in the number of circulating erythrocytes, and consequently increased hemoglobin concentration and blood volume. These increases were not due to a reduced plasma volume because plasma volumes of nitrate-fed heifers were normal.

Nitrate poisoning occurs in many species of farm animals, but it has been reported most frequently in cattle (2, 9, 22, 28). Results of some studies have been interpreted as indicating that the consumption by cattle of sublethal levels of nitrate causes abortion (4, 29, 32), retarded growth (17, 35), lowered milk production (4, 30), and vitamin A deficiency (4).

Bradley et al. (3) postulated that maternal hypoxia resulting from methemoglobinemia caused fetal death and abortion in cattle ingesting nitrate. Results of recent studies (7, [illegible]) indicated that cattle could tolerate very high levels of nitrate or nitrate-induced methemoglobinemia without fetal death. Retarded growth, lowered milk production, or disturbed vitamin A metabolism did not occur.

Results of an exact replicate of this investigation (11) suggested that an adaptation was occurring in the erythropoietic system of cows fed nitrate to compensate for the ensuing hypoxia. Efforts were, therefore, intensified to determine the nature of the adaptations made to hypoxia caused by prolonged nitrate ingestion by pregnant heifers, and their fetuses, during the course of gestation.

EXPERIMENTAL PROCEDURE

Twenty Holstein heifers about 16 months old and weighing 550 to 650 lb were randomly assigned to four equal groups as follows: (1) controls, (2) fed nitrate beginning three estrous cycles before breeding and arbitrarily referred to as Day Zero in figures and tables, (3) fed nitrate beginning the 40th day of pregnancy, and (4) fed nitrate beginning the 150th day of pregnancy. Groups 2, 3, and 4 were subdivided so that two heifers in each group were fed 440 mg nitrate ion/kilogram body weight per day and three heifers were fed 660 mg.

All heifers were confined to stanchions and fed chopped alfalfa-grass hay to appetite and 4 lb of concentrate daily. Sodium nitrate was dissolved in hot water and mixed with the hay just before each of two equal daily feedings. The amounts of nitrate fed were adjusted every 28 days for changes in body weight. All heifers were bred on the fourth estrus after beginning the experiment and rebred at recurrent estrus until all were pregnant. Other details concerning management were given in a previous paper (10).

Jugular blood was collected 4 hr post-feeding at biweekly intervals into 10- or 50-ml vials containing about 2 mg disodium ethylenediaminetetraacetic acid per milliliter of blood to prevent clotting. Erythrocytes were enumerated using a hemocytometer. Packed cell volume was determined by centrifugation for 45 min in Wintrobe tubes at 2,800 rpm in a centrifuge with a head diameter of 18 cm. Hemoglobin and methemoglobin were determined by the procedure of Evelyn and Malloy (13), modified by adding 0.02% saponin to the M/60 buffer (1).

At 4-wk intervals, leukocytes were enumerated using the hemocytometer, and differentiated using Giemsa's stain. Plasma was analyzed for protein by the gradient tube method (20) and phosphorus by the aminonaphthol sulfonic acid method (14).

Plasma and blood volumes, and oxygen-carrying capacity, oxygen saturation, and carbon dioxide content of whole blood were determined initially, and at 90, 120, 150, 180, 210, 240, and 270 days of pregnancy. Plasma and blood volumes were estimated 3 hr after the morning feeding by the T-1824 dye-hematocrit method as adapted for the cow (26, 27). Blood samples for gas analyses were collected 4 hr after feeding as follows: 45 ml jugular blood was drawn into a syringe well-oiled with paraffin and containing 5 ml of an oxalate-fluoride mixture (2.0% $K_2C_2O_4$ and 0.6% NaF). The syringe was then capped and immersed in water. A few drops of mercury were added to facilitate mixing of the blood and the syringe. Gas analyses were completed within 2 hr of collection by the method of Van Slyke and Neill (34). The oxygen content of the blood sample rotated for 30 min in an [illegible]ed tonometer was used as the oxygen capacity after correcting the results of the [illegible] for physically dissolved oxygen (8).

Data were evaluated statistically by analysis of covariance. Differences among treatment means were tested with Duncan's multiple [illegible] test when significant F ratios were obtained.

RESULTS

Data reported in Figure 1 were obtained from three pregnant cows receiving 660 mg nitrate/kilogram body weight in their ration. Methemoglobin concentrations and oxygen capacity of the blood were determined 0, 3, 4, and 5 hr after the morning feeding of one-half the daily dose of nitrate. Maximum levels of methemoglobin occurred about 3 hr after feeding and coincided with the greatest reduction in the oxygen-carrying capacity. This was the basis for selecting the fourth hour after feeding for making methemoglobin and blood gas measurements.

Results of the blood analyses are given in Table 1. The oxygen-carrying capacity

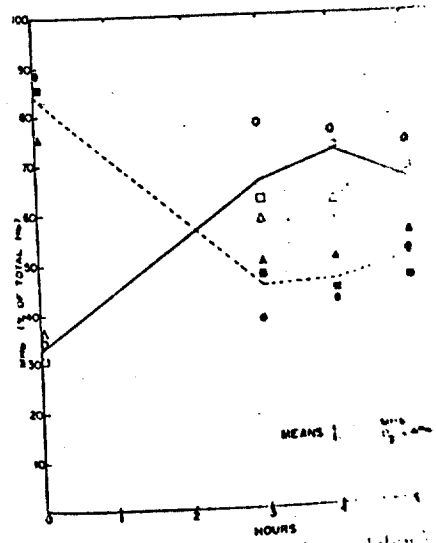

FIG. 1. Formation of methemoglobin and reduction in O_2 capacity in the blood of three heifers fed 660 mg nitrate/kilogram [illegible]

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Results of the blood analyses are given in Table 1. The oxygen-carrying capacity of the blood was reduced significantly by addition of 440 and 660 mg nitrate/kilogram body weight to the diet. Heifers ingesting 660 mg nitrate had lower ($P < 0.05$) oxygen capacities than those fed 440 mg nitrate. At the higher level of nitrate, oxygen capacities of cows fed nitrate beginning before breeding were not significantly different from those of heifers fed nitrate from the 40th day of pregnancy, but they were lower ($P < 0.05$) than those of heifers fed nitrate from the 150th day of pregnancy. Changes in oxygen capacity are illustrated in Figure 2 for heifers fed the 660-mg level of nitrate. Changes were less obvious for heifers fed the 440-mg level of nitrate. Although there was a significant reduction in the oxygen-carrying capacity of the blood, nitrate-fed cows did not exhibit signs of hypoxia.

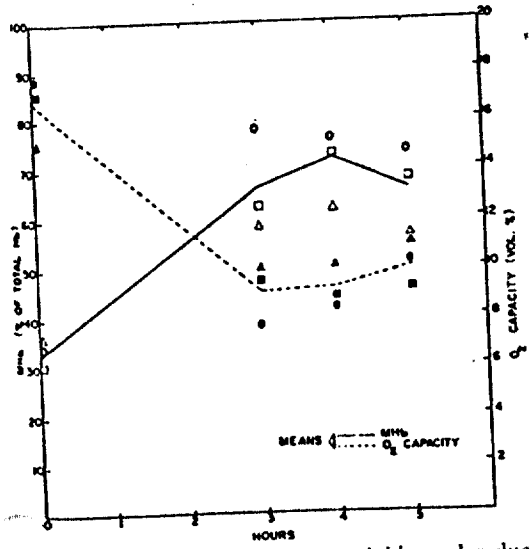

FIG. 1. Formation of methemoglobin and reduction in O_2 capacity in the blood of three pregnant heifers fed 660 mg nitrate/kilogram body weight.

The percentage oxygen saturation and the carbon dioxide content of the blood were not significantly affected by nitrate.

Total blood volume was increased by feeding nitrate, and it was higher in heifers fed the higher level of nitrate than in those fed the lower level ($P < 0.05$). Heifers fed 660 mg nitrate from the beginning of the experiment had higher blood volumes than heifers fed nitrate from 40 and 150 days of pregnancy. Changes in blood volumes for heifers fed the higher level of nitrate are plotted in Figure 3.

The plasma volumes of the control and nitrate-fed heifers were similar. The packed cell volume was increased by feeding nitrate, which explains the higher total blood volume. The packed cell volume was higher in heifers fed the 660-mg level of nitrate than in those fed the 440-mg level, and heifers fed 660 mg nitrate beginning before breeding or at 40 days of pregnancy had higher hematocrit readings than those fed nitrate beginning at the 150th day of pregnancy ($P < 0.05$).

The concentration of total hemoglobin and the number of erythrocytes followed the same pattern as the packed cell volume. These values increased with the quantity of nitrate fed and with length of time it was fed. The changes in packed cell volumes, erythrocyte numbers, and hemoglobin concentrations as they occurred during the course of the feeding period for heifers fed the 660-mg level of nitrate are given in Figures 4, 5, and 6. Mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration were not increased by feeding nitrate.

Methemoglobin concentrations, expressed as a percentage of hemoglobin, were increased with each level of nitrate. The level of methemoglobin did not differ with the time of feed-

TABLE 1

Effects of feeding two levels of nitrate at three different stages of pregnancy on blood constituents of dairy heifers[a]

Level of nitrate *(mg/kg)*		440			660		
Stage of pregnancy when nitrate feeding was begun *(days)*	Control	0	40	150	0	40	150
Number of cows	5	2	2	2	3	3	3
Gases							
O_2 capacity, *(ml/100 ml)***	16.3	14.6	15.1	14.5	11.4	13.1	14.4
O_2 saturation *(%)*	55	52	48	50	49	48	54
CO_2 whole blood *(mM/liter)*	22.4	22.9	23.2	24.6	22.8	22.9	22.3
Volume							
Whole blood, 1**	28.1	33.0	29.1	29.7	38.7	33.6	30.2
Plasma, 1	18.3	19.9	17.1	18.8	19.6	17.8	17.4
Packed cell volume *(%)***	36	41	40	38	51	47	42
Hemoglobin							
Total hemoglobin *(g/100 ml)***	10.9	12.2	12.7	11.2	15.6	14.3	12.6
Methemoglobin *(% of Hb)***	4.6	26.6	25.2	24.0	57.3	44.6	29.7
Erythrocytic indices							
Total, $10^3/mm^3$ **	7.5	8.6	8.2	8.3	10.4	9.5	9.2
M. C. H.[b] *(μμg)*	14.6	14.3	15.7	13.6	15.0	15.2	13.5
M. C. H. C.[c] *(g/100 ml)*	29.9	30.0	31.9	29.8	30.8	30.6	30.2
Plasma							
Phosphorus *(mg/100 ml)*	6.6	6.7	6.5	6.0	7.0	7.5	7.0
Protein *(g/100 ml)*	7.3	7.4	7.6	7.7	7.7	7.3	7.4
Leukocytic indices							
Total *($10^3/mm^3$)*	9.9	9.7	11.1	9.0	9.9	12.0	12.1
Lymphocytes *(%)*	63	58	61	56	60	55	60
Monocytes *(%)*	4	4	4	5	5	4	4
Eosinophils *(%)*	8	5	6	12	8	9	8
Neutrophils *(%)*	25	33	29	27	27	32	28
Basophils *(%)*	0	0	0	0	0	0	0

** Significant ($P < 0.01$) difference between control and nitrate-fed heifers.
[a] Covariance adjustment for pregnancy; values in table are adjusted means.
[b] Mean corpuscular hemoglobin.
[c] Mean corpuscular hemoglobin concentration.

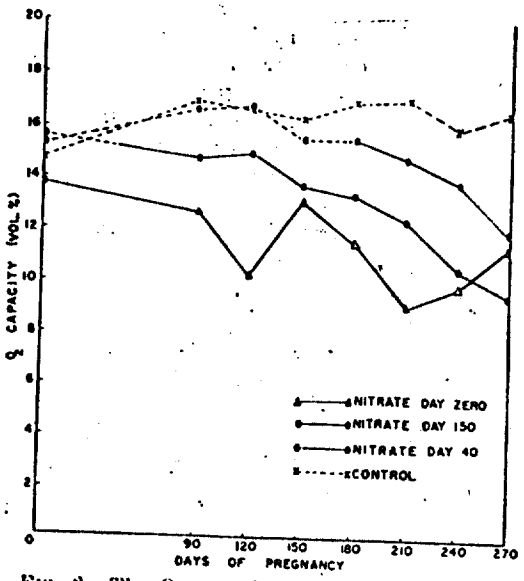

FIG. 2. The O_2 capacity of blood collected 4-5 hr post-feeding from heifers fed 660 mg nitrate/kilogram body weight.

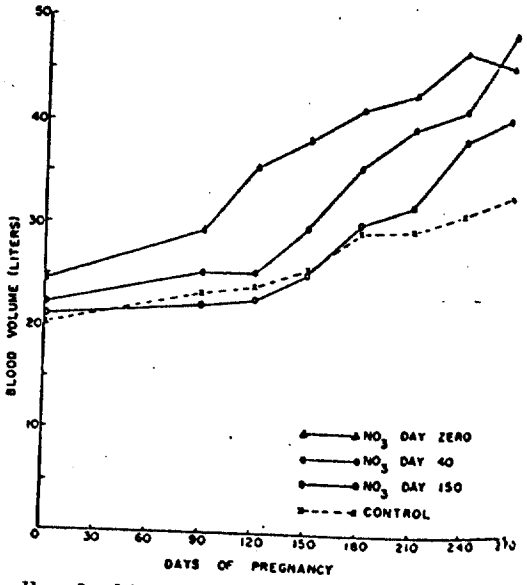

FIG. 3. Blood volume response of heifers fed 660 mg nitrate/kilogram body weight.

ing for heifers fed 440 mg nitrate/kilogram body weight, but it became greater (Figure 7) in heifers fed 660 mg nitrate as the length of the feeding period increased ($P < 0.05$). Examination of individual data revealed day-to-day fluctuations in methemoglobin levels within

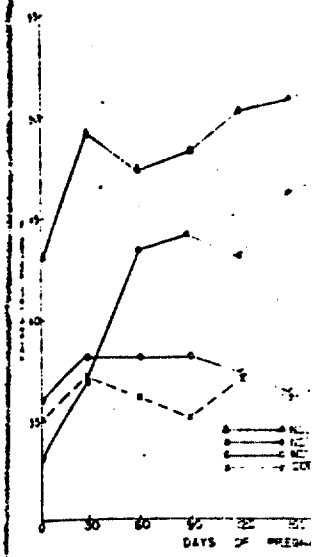

FIG. 4. Packed cell volume ... fed 660 mg nitrate/kilogram ...

the same cow and among ... level of nitrate, depending ... trate-treated hay was consu... ing. The blood of heifers ... of nitrate was dark-brown ... the nonpigmented skin and ... to appear brownish to a bl... sions the methemoglobin le... but signs of respiratory di... served. Respiratory distre... one heifer in the first repl... and two of the heifers in t...

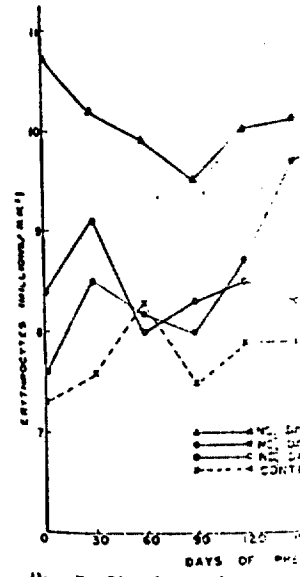

FIG. 5. Erythrocytic resp... ...g nitrate/kilogram body ...

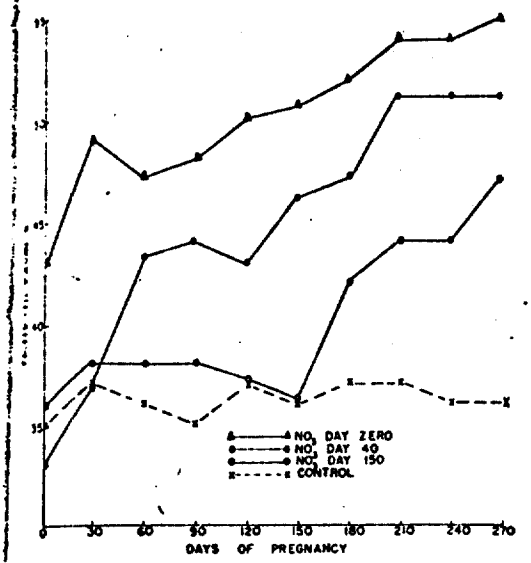

FIG. 4. Packed cell volume response of heifers fed 660 mg nitrate/kilogram body weight.

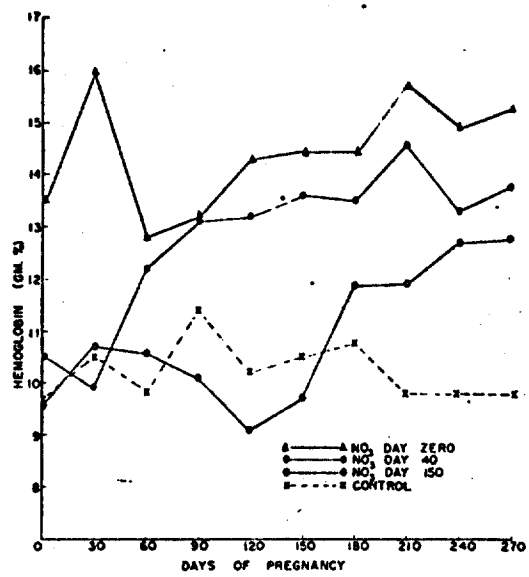

FIG. 6. Hemoglobin response of heifers fed 660 mg nitrate/kilogram body weight.

the same cow and among cows fed the same level of nitrate, depending on the rate the nitrate-treated hay was consumed at each feeding. The blood of heifers fed the higher level of nitrate was dark-brown in color causing the nonpigmented skin and mucous membranes to appear brownish to a bluish-gray. On occasions the methemoglobin levels exceeded 70%, but signs of respiratory distress were not observed. Respiratory distress was observed in one heifer in the first replicate of this study, and two of the heifers in this second replicate died of nitrate poisoning and three aborted [see Davison et al. (10)].

Plasma phosphorus and protein concentrations were not changed by feeding nitrate. The feeding of nitrate caused no changes in the total number of leukocytes or in the proportion of the different leukocytes.

DISCUSSION

A study of the literature shows that very divergent results have been obtained from feeding nitrate, depending upon the experimental

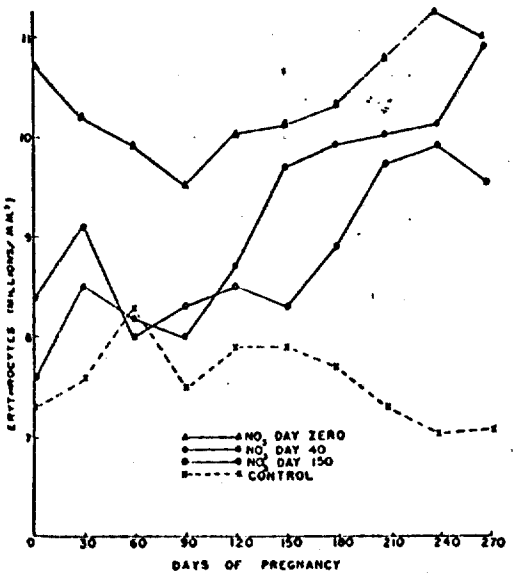

FIG. 5. Erythrocytic response of heifers fed 660 mg nitrate/kilogram body weight.

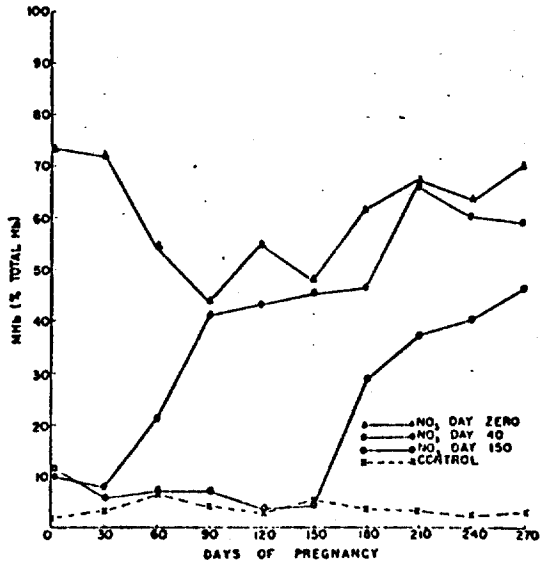

FIG. 7. Methemoglobin response of heifers fed 660 mg nitrate/kilogram body weight.

conditions. This study was conducted under feeding and management practices recommended for dairy cattle. Since it was not possible to obtain forages containing nitrate in the desired quantities, a predetermined amount of nitrate was added twice daily to the hay of each animal. The effects of nitrate added to hay and the nature of the signs thus produced are presumably similar to those produced by nitrate accumulated in the forage (7).

One of the most difficult problems in this study was the large variability in response among animals fed the same level of nitrate. The degree of methemoglobinemia was unpredictable, because it depended on the quantity and rate of feed consumption by the animals at each feeding. Results in the present investigation confirm earlier observations (29, 36) suggesting variations in the methemoglobin response with a given quantity of nitrate. The maximum level of methemoglobinemia occurred approximately 4 hr after feeding, findings in agreement with observations of Stormorken (31) and Prewitt and Merilan (24).

The data showed that the heifers fed at the 660-mg rate of nitrate beginning early in pregnancy attained higher methemoglobin levels and lower oxygen capacities than those fed the nitrate later in pregnancy. The physiological events which account for these differences are not entirely clear. For some unknown reason, it was more difficult to get the heifers to consume the nitrate-treated hay when feeding was begun at the 150th day of pregnancy than when feeding was begun earlier. The resultant slower rate of consumption may account for most of the differences.

The erythropoietic responses to nitrate feeding were dramatic. Heifers fed 660 mg nitrate/kilogram body weight adapted markedly by increasing the number of circulating erythrocytes, with parallel increases in their hemoglobin concentrations, packed cell volumes, and blood volumes (Figures 3, 4, 5, and 6). These increases were not caused by diuresis and dehydration, because plasma volume was the same as in control animals. Plasma phosphorus and protein remained unchanged, further indicating a normal plasma volume. Similar responses, but to a lesser degree, were observed in the heifers fed the 440-mg level of nitrate.

In an extensive review, Grant and Root (16) considered hypoxia to be the fundamental stimulus for erythropoiesis; the trigger mechanism being either hypoxic hypoxia or anemic hypoxia. Although the heifers did not appear to be suffering from hypoxia, the hypoxia must have been severe enough to stimulate the production of more erythrocytes. The increased number of erythrocytes enabled the blood of nitrate-fed heifers to carry sufficient, and nearly normal, amounts of oxygen despite high levels of methemoglobin.

Since feeding nitrate causes a reduction in the oxygen-carrying capacity of the blood, nitrate-fed heifers responded to this hypoxic stimulus by increasing the number of circulating erythrocytes, with consequent increases in hemoglobin concentration, packed cell volume, and blood volume. Other observers have noted similar increases in hemoglobin concentrations (6, 18, 21), number of erythrocytes (16, 2[illegible] 33), packed cell volumes (5, 6), and blood volumes (19) in response to hypoxia. According to Reisman (25) increases in blood volume are caused by a temporary increase in the production of erythrocytes, and not by a decrease in their rate of destruction. Beutler and Mikus (1) reported that treatment with the nitrite ion would prolong the life of the erythrocyte in humans with sickle cell disease. However, these sickled cells have a shorter lifespan than normal cells. Future experiments should consider in greater detail the action of nitrite on erythrocyte survival to further elucidate the mechanisms responsible for the increased number of erythrocytes when nitrate is fed.

Evidence has accumulated in recent years to indicate that the erythropoietic response to hypoxia is mediated by a humoral erythropoietic stimulating factor (ESF or erythropoietin) named originally hemopoietine (15). Apparently, erythropoietin acts upon the stem cells stimulating differentiation into the erythroblastic series (12). Results of this investigation have shown that the anemic hypoxia resulting from prolonged feeding of nitrate exerts a marked erythropoietic response. Assuming that erythropoietin mediates erythropoiesis, it seems interesting to determine if plasma of nitrate-fed heifers possesses increased erythropoietin activity.

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NITRATE TOXICITY IN DAIRY HEIFERS. 3. ENDOCRINE RESPONSES TO NITRATE INGESTION DURING PREGNANCY[1]

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ABSTRACT

An investigation was conducted to gain information concerning the effects of nitrate on anterior pituitary and thyroid function in dairy heifers fed 0, 440, and 660 mg nitrate ion/kg body weight under conditions of management and feeding practices recommended for dairy cattle. Pituitaries from nitrate-fed heifers were heavier and contained a slightly higher concentration of growth hormone than those from control heifers. Nitrate did not adversely affect the thyrotropic, adrenocorticotropic, or gonadotropic activity of the pituitary glands. Barely sublethal levels of nitrate did not increase thyroid gland weights or acinar cell heights. Continuous feeding of sublethal levels of nitrate to dairy heifers, under the conditions of this study, did not produce a syndrome of chronic nitrate toxicosis characterized by thyroid dysfunction.

Nitrate poisoning, as seen clinically, has been divided broadly into the acute and chronic forms (4). The acute form manifests itself primarily as methemoglobinemia, a blood disorder leading to hypoxia. Chronic nitrate poisoning is less specific. It has been blamed for a group of reproductive and nutritional disorders in cattle, characterized by abortion, lowered milk production, retarded growth, and vitamin A deficiency.

Many compounds may inhibit thyroid function and induce the formation of goiters in experimental animals. Rats treated with perchlorates developed hyperplastic, colloid-depleted, low-iodine goiters; whereas, those treated with nitrate developed only minor hyperplastic changes and a small reduction of iodine in the thyroid gland (17). This goitrogenic effect of nitrate has led a few investigators to state that hypothyroidism exists in cattle and sheep consuming forages containing nitrate (2). The data of Bloomfield et al. (2) indicated that dietary nitrate caused a reduction in plasma protein-bound iodine in the sheep, and these authors later speculated that it caused an increased secretion of thyroid-stimulating hormone (3). However, there is no evidence at present to show that dietary nitrate produces a thyroid deficiency in cattle.

Most studies of the relationship of nitrate ingestion to endocrine function have been done with rats. The present studies were undertaken to determine the effects of feeding nitrate over a long period of time to dairy heifers at different stages of pregnancy, and fed according to the recommendations of the National Research Council (13). The investigation was conducted to gain information concerning the effects of nitrate on anterior pituitary and thyroid gland functions, and to relate the endocrine status to such factors as growth, vitamin A nutrition, and reproduction. An erythropoietic response to nitrate feeding was reported previously (10).

MATERIALS AND METHODS

Forty Holstein heifers, 1 to 2 yr of age, were randomly allotted to four equal groups as follows: 1) controls, 2) fed nitrate beginning three estrous cycles before breeding, 3) fed nitrate beginning the 40th day of pregnancy, and 4) fed nitrate beginning the 150th day of pregnancy. Five heifers in each treatment group received 440 mg nitrate ion/kg body weight, and the remaining five received 660 mg/kg. The heifers were confined to stanchions and fed a ration of chopped alfalfa-grass hay to appetite and 4 lb concentrate of the following composition: ground shelled corn 80%, soybean meal 18%, trace-mineralized salt 1%, and dicalcium phosphate 1%. Nitrate, as sodium nitrate, was dissolved in water and sprayed on hay twice daily to provide the daily

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intake. The quantity of nitrate was adjusted every 4 wk for changes in body weight. Other details concerning management have been reported previously (5).

All cows were necropsied 30 days after calving. The thyroids, adrenals, and pituitaries were dissected free of adipose and connective tissue and weighed. Approximately half of each pituitary gland was weighed and frozen within 1 hr after slaughter. This portion of the gland was used for pituitary hormone assays. The thyroid glands were fixed in Bouin's fluid for 24 hr, washed, and stored in 70% alcohol. They were later embedded in paraffin, sectioned at 6 μ, stained with hematoxylin and eosin, and their acinar cell heights determined as outlined in the rat assay below.

Extracts of the pituitary glands were made by tissue homogenization, as described by Armstrong and Hansel (1). Female albino rats, hypophysectomized at 26-28 days of age,[3] were placed on a 10-12 day weighing schedule to confirm the effectiveness of hypophysectomy. Rats gaining more than 7 g during the period were discarded. Five rats were used for assaying each pituitary gland. They were injected intraperitoneally with 0.5 ml of the pituitary extracts daily for four days, and were euthanized on the fifth day. At necropsy the sella turcica of each rat was examined for pituitary fragments.

Growth hormone (STH). The rat tibia test (8) was used to assay growth hormone in the pituitary extracts. The growth hormone contents of the pituitary glands were estimated by a log-dose response curve, established by using a standard growth hormone preparation.[4]

Thyrotropic hormone (TSH). After fixing, embedding, sectioning, and staining, acinar cell heights were measured at a magnification of 550× with a calibrated micrometer eyepiece. A total of 30 cell heights was measured on each rat as follows: two cells approximately opposite in position per follicle, three follicles per slide, five slides per rat; or, 150 cells per pituitary gland assayed. A log-dose response curve was established using a purified preparation of thyrotropic-stimulating hormone.[5] The TSH content of each pituitary gland was estimated by referring the thyroid acinar cell heights of rats receiving pituitary extracts to the response curve.

Gonadotropins. The uteri and ovaries of rats receiving the pituitary extracts were weighed with a torsion balance to provide a measure of the total gonadotropic activity of the pituitary extracts. No attempt was made to measure follicular stimulating hormone and luteinizing hormone separately.

Adrenocorticotropins (ACTH). Adrenal glands of the rats were weighed with a torsion balance to provide an estimate of the adrenocorticotropic potency of the pituitary extracts.

All data were analyzed statistically by analysis of variance according to the outline in Table 1.

TABLE 1

Outline of analysis of variance used for statistical analysis

Source	Degrees of freedom
Control vs. nitrate	1
Among nitrate	5
Level	(1)
Days of nitrate feeding	(2)
Level × days	(2)
Error	33

RESULTS

The average weights of the pituitary glands, their hormonal contents, and their weights and contents expressed relative to body weights of the heifers, are given in Table 2. Heifers fed nitrate had significantly ($P < 0.05$) heavier pituitaries than the controls. The pituitaries tended to be heavier ($P < 0.10$) in heifers fed 660 mg nitrate/kg than in those fed 440 mg. When the weight of the pituitaries was expressed on a body weight basis, the nitrate-fed heifers still tended to have heavier pituitaries than the control heifers ($P < 0.10$).

The STH and TSH dose response curves obtained while assaying the pituitary glands are shown in Figures 1 and 2. Each point on the STH dose response curve is an average of eight rats, and each point on the TSH curve is an average of five rats. The indices of precision, Gaddum's lambda (6), were 0.1718 and 0.1376 for the STH and TSH curves, respectively.

Growth hormone. Nitrate feeding may have increased the total growth hormone content of the pituitary glands, and their contents proportional to body weight of the heifers. These increases approached significance ($P < 0.10$).

[3] Purchased from the Hormone Assay Inc., Chicago, Illinois.

[4] USP Growth Hormone Reference Standard (1 mg/USP Unit) obtained from USP Reference Standards, 46 Park Avenue, New York 16, New York.

[5] Thyrotropic-Stimulating Hormone (TSH) Lyophilized (bovine) C grade, California Corporation for Biochemical Research, Los Angeles 63, California.

TABLE 2

Effects of feeding two levels of nitrate beginning at three different stages of pregnancy on pituitary and thyroid function of dairy heifers[a]

Level of nitrate *(mg/kg)*		440			660			Error mean squares
Stage of pregnancy when nitrate feeding was begun *(days)*	Control	Before	40	150	Before	40	150	
No. of animals	10	5	5	5	5	5	5	
			Pituitary	glands				
Total weight *(g)*	2.46	2.84	2.55	2.59	3.14	2.99	2.60	0.1557
mg/kg body weight	5.5	5.9	6.1	5.7	6.8	6.6	6.1	0.0017
Somatotropin content								
Total *(mg)*	2.91	5.50	8.60	7.06	4.88	6.47	2.98	24.0690
meg/kg body weight	6.6	11.2	19.1	16.7	10.8	14.3	6.8	0.2367
Thyrotropin content								
Total *(IU)*	14.57	18.38	16.74	16.58	15.24	15.60	15.90	21.2470
IU/100 kg body weight	3.3	4.6	4.0	3.7	3.3	3.5	3.7	0.2532
Gonadotropins[b]	24.4	27.1	22.4	24.3	29.7	22.7	25.2	
Adrenocorticotropin[c]	13.1	14.2	13.0	12.7	14.0	12.0	13.4	
			Thyroid	glands				
Total weight *(g)*	25.9	24.9	30.5	26.2	28.2	31.9	27.2	21.1648
mg/kg body weight	58.5	52.8	72.6	57.2	60.7	71.7	62.9	0.2242
Acinar cell height *(μ)*	9.3	8.8	8.1	8.4	9.2	9.3	9.4	1.9797

[a] Tabular values are means.
[b] Average uterine weights (mg) of hypophysectomized rats injected with pituitary extracts.
[c] Average total adrenal weights (mg) of hypophysectomized rats injected with pituitary extracts.

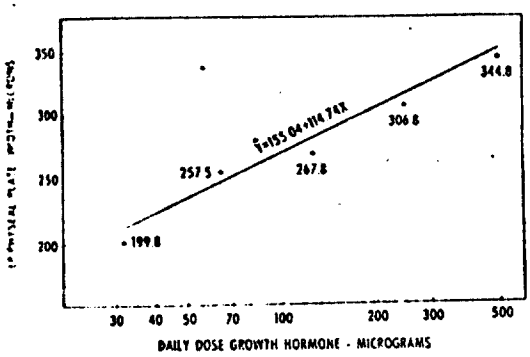

FIG. 1. Response of the proximal tibial epiphyseal plate of hypophysectomized rats to USP Growth Hormone Standard.

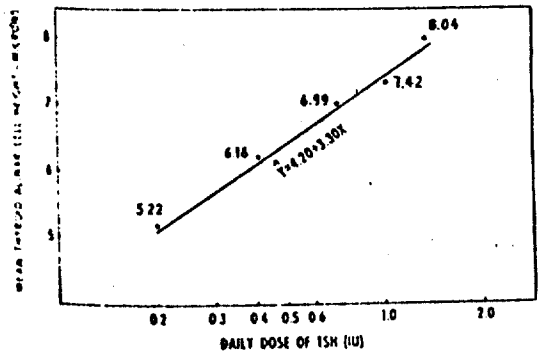

FIG. 2. Response of the thyroid acinar cell of hypophysectomized rats to Thyrotropic-Stimulating Hormone.

despite the low growth hormone content of the group fed 660 mg nitrate/kg body weight beginning at 150 days of pregnancy.

Gonadotropins. Increases in uterine weights of hypophysectomized rats injected with pituitary extracts indicate increases in gonadotropin contents of the pituitary glands. Nitrate feeding did not significantly influence the gonadotropin activity of the pituitary glands.

Adrenocorticotropin. An increase in the adrenal weights of hypophysectomized rats injected with pituitary extracts reflects an increase in the amount of adrenocorticotropin injected. The adrenocorticotropic activities of the pituitary glands from the heifers in this experiment were not altered by the ingestion of nitrate.

Thyrotropic hormone. The thyrotropic hormone content of the pituitary glands was not significantly affected by the feeding of nitrate.

The functioning of the thyroid glands of the cattle was also assessed on the basis of their weights, and the apparent secretory activity of the acinar cells. The total thyroid weights, relative weights expressed in proportion to body weight, and thyroid acinar cell heights are presented in Table 2. Apparently, nitrate did not interfere with the functioning of the thyroid glands of the heifers. There were no significant differences among total thyroid weights, or relative weights, of control and nitrate-fed heifers. In addition, thyroid acinar cell heights

were not significantly different among the nitrate-fed and control heifers.

DISCUSSION

Pituitary gland function. In view of the functions ascribed to anterior pituitary hormones, it is surprising that, although much has been written about the effects of nitrate on growth, thyroid function, and reproduction, no information is available concerning the role of these hormones in nitrate toxicity.

The quantity of hormones detected in pituitary extracts may or may not represent the secretory activity of the pituitary gland. Armstrong and Hansel (1), using techniques similar to those employed in this study, found that growth hormone and TSH contents of anterior pituitary extracts were positively correlated with growth rates and thyroid weights of growing heifers. These workers concluded that the amounts of hormones detected were a reflection of the secretion rates of these hormones under the conditions of their experiment.

The significance of heavier pituitary glands in nitrate-fed heifers cannot be attributed to the size of the animals. When the pituitary weights were expressed per unit of body weight, the heavier pituitaries of nitrate-fed heifers were still apparent. No logical explanation for this effect can be given at present.

These data also suggest that the nitrate-fed heifers had higher concentrations of growth hormone in their pituitaries than control heifers. This may or may not be a real response to nitrate ingestion since, as reported earlier (5), the nitrate-fed heifers grew at a rate nearly identical to that of the control heifers. However, these differences were significant at the 10% level of probability, and approached significance at the 5% level. The possibility exists that nitrate feeding increases the requirement for growth hormone, and that the pituitary gland responded to this demand by a compensatory increase in the production of the hormone. The increased pituitary weights may have reflected this compensatory hypertrophy. Further investigations of the STH contents of pituitaries of nitrate-fed cattle are clearly indicated.

Continued ingestion of nitrate did not cause an increase in the TSH content of the pituitary gland under the conditions of this experiment. In contrast, Bloomfield et al. (3), studying the effects of nitrate on I^{131} metabolism in sheep, suggested that nitrate may induce an increase in pituitary TSH or cause a decrease in circulating plasma protein-bound iodine. In the latter case, pituitary TSH was not measured.

These findings also show that no obvious changes occurred in the pituitary gonadotropin and adrenocorticotropin contents of heifers fed nitrate. The normal growth rates and estrous cycles of these heifers, reported previously (5), confirm this observation.

Thyroid function. This study was conducted in an area where goiter in livestock is observed occasionally and it is a common recommendation in this area that cattle receive 1% trace-mineralized salt (in this case containing 0.007% iodine) in the grain portion of their ration.

In this study, sublethal levels of nitrate in the rations of dairy heifers had no effect on thyroid gland weights, acinar cell heights, and pituitary TSH content. In contrast, other studies have shown that dietary nitrate interfered with iodine metabolism causing increased thyroid weights, with histological evidence of hyperplasia and hypertrophy in rats (15, 16), and possibly increased TSH secretion in sheep (3). More recently, Jordan et al. (11) were unable to find definite evidence that high nitrate silages produced a thyroid deficiency when fed to steers during the winter.

Iodine supplementation has been shown to overcome the inhibitory effects of nitrate on thyroid gland function in rats (16, 17). However, there is no real evidence to suggest that nitrate in forages adversely affects thyroid gland function in cattle and there is, therefore, no justification for inferring that iodine in the diet prevented the thyroid-depressing effects of nitrate in this study.

Bloomfield et al. (2) postulated that the goitrogenic effect of nitrate may possibly be responsible for the vitamin A deficiency previously observed in cattle by Muhrer et al. (12), since the thyroid hormone is probably involved in vitamin A synthesis from carotene. Results of experiments with fattening steers (9, 11, 14) indicate that dietary nitrate does not deplete liver stores of vitamin A. The results of vitamin A nutrition studies carried out on the animals used in the present experiments confirm these observations (5). In the light of these observations, it seems unlikely that nitrate-induced thyroid insufficiency explains the vitamin A deficiency observed in cattle in field outbreaks of chronic nitrate toxicity (4, 7). This syndrome may have been related to poor management, true nutrient deficiencies, or subclinical diseases, or to some other factor in the nitrate-containing rations.

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The offspring of the *bWr* immune or normal mothers, even when nursed by a normal Wistar female, may resist tumour growth in varying degrees, usually depending on the dose of tumour cell used. Their resistance may be explained most plausibly by the relatively long lag in tumour growth with small doses, so that the young *bWr* rats have time to produce tumour antibodies of their own. In the offspring of immune mothers, some passive transfer of antitumour factors by way of placenta, or intake of immune milk (colostrum) in the short interval before the exchange cannot be excluded.

Another experiment, on 'double passive transfer', was undertaken. The offspring, all born within an interval of 36 hr., of three normal Wistar females, were inoculated simultaneously with a dose of about 200,000 live tumour cells each, and left to nurse with their own mothers. One of these mothers was injected intraperitoneally, after delivery, with relatively large doses of immune rat serum (4 ml. every third day, about 20 ml. altogether); the second mother rat was injected with the same dose of normal rat serum, and the third was left untreated. The immune serum was taken, this time, not from *bWr* immune rats, but from resistant Wistar rats, which can be selected from our commercial stock.

On the eighteenth day after tumour inoculation, the following proportions of palpable tumours appeared. (*a*) Mother treated with immune serum: 3/9. (*b*) Mother treated with normal serum: 9/9. (*c*) Untreated mother: 8/9.

It appears here that the antitumour factors from the immune serum injections, though diluted by the metabolic stream of the recipient mother, are still effective in tumour inhibition in the sucklings. But conditions were different: the tumour dose was relatively high; the last serum injection to the mother was done on the ninth day after tumour inoculation, so that by the eighteenth day its effect may have been exhausted; lastly, the milk proteins and antibodies are more thoroughly digested by the maturing enzymes of the sucklings (*a*). Therefore, during the following 10 days, tumours also appeared in all but one of the sucklings in the protected litter, so that the protection was manifested here only by a postponement of tumour growth.

Besides these, a 'feeding experiment' was also undertaken. Sucklings nursed by their own normal Wistar mother were given daily, by means of a pipette, some drops of either immune or normal rat serum by mouth. Here, too, there was an indication of protection against tumour growth.

In the experiments on 'milk protection' it was noted repeatedly that a normal (non-immune) *bWr* nurse cannot protect sucklings against the tumour growth.

The results of these experiments agree remarkably with results of passive transfer of antitumour factors by serum injections[2,3], or by injections of culture media from tumour-immune spleens[4]. They are very persuasive evidence that these antitumour factors circulate, very much as do the antibodies of current immunology.

Participation of a general transplantation immunity component in these results cannot be wholly excluded, but the relative share of this factor may be rather small: Wistar sucklings nursed by a *bWr* female, immunized by a tumour of Wistar origin, grew normally, and only their tumour growths were inhibited. The influence of this anti-strain effect of immune serum may be even less in the double transfer experiment, where immune serum of Wistar rats was injected into Wistar females nursing their own offspring.

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Adverse Effect of Nitrate Metabolic Products on Sheep Growth

It has been found that the metabolism of nitrate in the rumen of the sheep on pasture can induce marked changes in the molecular percentage structure of the rumen volatile fatty acids. These changes consisted in a lowering of the percentage of acetic acid with a compensating increase in those of propionic and butyric acids. The composition of the volatile fatty acids of the rumen was determined by gas-liquid chromatography[1].

Rumen samples of similar volatile fatty-acid composition had been frequently recorded from sheep on ryegrass – clover pasture. Animals showing these low percentages of acetic acid tended to be making poor growth about the time of sampling. The nitrate content of the pasture was variable, and covered the range 0·18–9·28 gm. potassium nitrate/100 gm. dry matter.

The investigations of Lewis[2] suggest that the sequence, nitrate → nitrite → hydroxylamine → ammonia, long postulated in the chemistry of nitrate assimilation on the part of plants and micro-organisms[3], occurs in the rumen. These nitrate-reduction products have been examined for their influence on the volatile fatty acids of the rumen and for their effect on sheep growth.

Table 1 shows the effect of dosing nitrate, nitrite and hydroxylamine on the molecular percentage structure of the rumen volatile fatty acids of the sheep on grass. The change obtained with nitrate persisted over the period of dosing, whereas that associated with nitrite was transient and a return to the original percentage of acetic acid occurred during dosing. Hydroxylamine produced no significant effect.

An additional finding of considerable interest was the fact that hydroxylamine and certain oximes in comparatively small amounts caused a severe hæmolytic anæmia in sheep after oral or intravenous administration.

Oral doses of 40–80 mgm./kgm., and intravenous doses of 1–7 mgm./kgm. body-weight of hydroxylamine hydrochloride brought about a lowering of the hæmatocrit readings within twelve to fifteen days from the normal range of 30–40 per cent to values as low as 10–20 per cent. The serum contained hæmoglobin at the third to fifth day after dosing, and some animals exhibited hæmoglobinuria just after this stage. These results extend to the sheep the finding of Emerson, Hunn and Castle[4] of hæmolytic activity on the part of hydroxylamine in the cat and rabbit. An apparently similar hæmolytic anæmia in sheep has been found to occur spontaneously in

Table 1. CHANGES PRODUCED IN THE VOLATILE FATTY ACID COMPOSITION OF RUMEN LIQUOR FROM SHEEP ON PASTURE DURING THE METAB[illegible] OF NITRATE AND ITS REDUCTION PRODUCTS

Condition of sheep	pH of rumen contents	Total volatile fatty acids (m.mole/100 ml.)	Molecular percentages of individual fatty acids							
			Formic	Acetic	Propionic	*iso*Butyric	*n*-Butyric	*iso*Valeric	*n*-Methyl-butyric	*n*-V[illegible]
Control sheep	6·7	7·5	0	57·5	22·5	2·5	13	1·5	1·5	1[illegible]
Nitrate dosed	6·5	7·1	0·5	41·5	35·5	3	15·5	1	1	[illegible]
Nitrite dosed	6·6	6·9	1·0	42	36·5	2·5	14	1	1	[illegible]
Hydroxylamine dosed	6·8	7·7	0	57	23	2	13·5	2	1·5	1

normal untreated experimental animals grazing ryegrass – clover pasture.

The presence of hydroxylamine in the rumen of the normal sheep on pasture, and at higher concentrations up to 300 μgm./100 ml. in the rumen metabolizing added nitrite, has been detected and estimated by the Endres method as used by Novak and Wilson[5].

It has been possible to show that if a hæmolytic anæmia induced by hydroxylamine in the sheep was prolonged by repeated intravenous doses, a highly significant decrease in growth relative to non-anæmic control animals grazing the same pasture occurred. From an initial mean live-weight of 70 lb., the anæmic animals in 49 days lost 2·25 lb., while the control sheep gained 11·75 lb.

The production of a hæmolytic anæmia in sheep after sustained dosing of nitrite, the recognized primary reduction product of nitrate, has been demonstrated. Three sheep were dosed twice daily with a total daily oral dose of 8 gm. of sodium nitrite/100 lb. live-weight over a period of 19–23 days. This dose was very close to the lethal limit and the animals were methæmoglobinæmic for a considerable portion of each day; nevertheless, they continued to graze in the normal way, made moderately satisfactory gains in weight and their rumen contents and rumen flora appeared to be normal. Three to five days after stopping the nitrite dosing, the serum showed the presence of hæmoglobin; one animal had very slight hæmoglobinuria on the seventh day, and by the eleventh day the packed red-cell volume of all three sheep had fallen from 38–40 per cent to about 25 per cent and continued to fall in two sheep until the twenty-first day to levels of 17 and 20 per cent, recovering slowly over the next eleven days. This effect could be attributed to a cumulative toxicity, but at the present stage of the investigation is considered to be due rather to the sudden withdrawal of substrate from the rumen adapted to handling nearly the maximal amount of nitrite. Under these conditions, there is a possibility of the release of a hæmolytic dose of hydroxylamine within 24–72 hr. of the cessation of nitrite additions to the rumen, conceivably arising from a reversal of the hydroxylamine → ammonia stage of the series of enzyme reactions.

Nitrate, which is a normal rumen metabolite for the grazing sheep, can vary markedly in its concentration in pasture, and there is now reason to believe that such variation in the diet of the animals can cause adverse effects. The present work strongly supports the view that hydroxylamine is an intermediate in the reduction of nitrate and nitrite to ammonia in the rumen. This compound or its oximes have hæmolytic activity if present in the blood-stream in sufficient concentration. The fact that toxic manifestations apparently identical with those due to hydroxylamine can be produced in the ruminant by interrupting an established nitrite metabolism gives a possible explanation of some of the metabolic disorders besetting sheep grazing pasture of variable nitrate content.

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Goitrogen of Milk produced on Kale

IN a recent report Clements and Wishart[1] ha[illegible] shown that when cows are grazed on kale th[illegible] produce milk with goitrogenic properties. The[illegible] evidence for this consists of the discharge of accum[illegible]lated radioiodine from the thyroid glands of sch[illegible] children on drinking the milk.

It is generally accepted (see review by Berson[1], among others) that goitrogenic substances fall in[illegible] two classes: those which, like thiocyanate, block th[illegible] iodide 'trap' of the thyroid and those, like thiourea, which do not affect the absorption of iodide [illegible] prevent its binding into thyroglobulin. In addition, thiocyanate causes the discharge of the accumulated inorganic iodide from glands treated with the second type of goitrogen. I have not seen a report of an experiment designed to test the possibility that goitrogens of the thiouracil type behave similarly but in view of the lack of interference of such substances with radioiodine accumulation, it is most unlikely that they do. In addition, I have treated sheep with thiouracil daily before and during radioiodine measurements and have seen no such effect, whereas

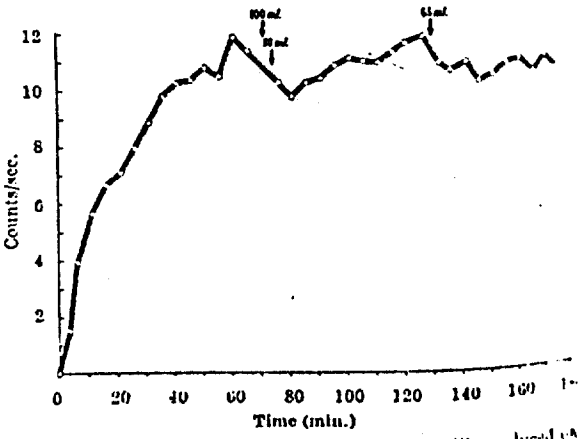

Fig. 1. The partial discharge of radioiodine by milk produced on kale in animals pre-treated with propyl-thiouracil

Inhibition of Clostridium Botulinum by Sodium Nitrite as Affected by Bacteriological Media and Meat Suspensions

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Abstract

The inhibitory effect of sodium nitrite on the outgrowth of spores of Clostridium botulinum 13983B was studied in various bacteriological media and meat suspensions. The media and meat suspensions were adjusted to pH 6.2 and heated in the presence of nitrite at 110°C for 20 min. Perigo factor was produced in Perigo-type medium (PT) and Reinforced Clostridial Medium (RCM) when heated with 20 ppm of nitrite, but not in Liver Veal Medium (LVM) and Wynne Fluid Medium (WFM) even when heated with 100 ppm of nitrite. Suspensions of 50% meat in water, prepared from under-process, normal-process and over-process meats, required 500 ppm, 150 ppm and 50 ppm of nitrite, respectively, for inhibition of outgrowth.

Dialysis removed the inhibitory activity from two of the bacteriological media (LVM and WFM) and from all meat suspensions, but not from PT and RCM media. The residual nitrite concentrations were approximately the same in all seven heated preparation (100 ppm added before heating), but the redox potential varied considerably among them. The addition of reducing agents to meat suspensions decreased the redox potential and increased the inhibitory activity but did not induce the formation of the Perigo factor.

Résumé

L'effet inhibiteur du nitrite de sodium sur la croissance des spores de Clostridium botulinum 13983B a été étudié dans différents milieux de culture et suspensions de viande. Les milieux et suspensions de suspensions de viande ont été ajustés à pH 6.2 et chauffés 20 minutes à 110°C en présence de nitrite de sodium. Le facteur Périgo a été produit dans le milieux de type Périgo (TP) et dans le milieu renforcé pour clostridiales (MRC), chauffés avec 20 ppm de nitrite; le facteur n'a pas été produit dans le milieu au foie de veau (MFV) et dans le milieu liquide de Wynne (MFV) même lorsqu'ils étaient chauffés avec 100 ppm de nitrite. Pour les suspensions aqueuses de viande à la concentration de 50%, préparées à partir de viandes ayant subi un traitement léger, normal et intensif, il a fallu respectivement 500 ppm, 150 ppm et 50 ppm de nitrite pour inhiber la croissance des spores.

La dialyse a fait disparaître l'activité inhibitrice de deux des milieux de culture (MFV et MFW) et de toutes les suspensions de viande mais a été sans action sur les milieux TP et MRC. Les concentrations résiduelles de nitrite étaient à peu près les mêmes dans les sept préparations chauffées (avec addition de 100 ppm de nitrite avant chauffage) mais le potentiel redox variait considérablement d'une préparation à l'autre. L'addition d'agents réducteurs aux suspensions de viande a diminué le potentiel redox et augmenté l'activité inhibitrice mais elle n'a pas provoqué la formation du facteur Périgo.

Introduction

The inhibitory effect of sodium nitrite on the growth of bacteria has been studied in bacteriological media and in meat preparations. Results from these studies with media of various compositions have been used to explain the role of nitrite in the safety of commercially-produced cured meats. For example, Tanner and Evans (1931) showed that the composition of the growth medium influenced the toxicity of sodium nitrite to *Clostridium botulinum*: a pork infusion medium was less inhibitory than bouillon medium, although both media had the same concentration of residual nitrite after autoclaving. Tarr (1942) found that the growth of facultative anaerobes was inhibited by sodium nitrite to a greater extent in nutrient broth than in fish digest broth. The initial pH of the media to which nitrite was added after autoclaving was less critical in nutrient broth than in fish digest broth. More recent work by Perigo *et al.* (1967) has shown that an antimicrobial compound (Perigo factor) can be formed in a bacteriological medium if nitrite is present during autoclaving. The Perigo factor has not been demonstrated in commercially-produced meat (Johnston *et al.*, 1969).

The addition of certain compounds to bacteriological media and meat preparations can influence the toxic effect of nitrite on bacterial growth. Castellani and Niven (1955) found that glucose and sulfhydryl compounds, especially cysteine, increased the inhibitory effect of nitrite on food poisoning staphylococci in a synthetic medium under anaerobic conditions. Ascorbic acid has been used successfully to extend the shelf-life of cured meats. Schack and Taylor (1963) showed that meat previously treated with ascorbic acid and nitrite required less heat treatment than normally cured meats.

The concentration of sodium nitrite required to prevent outgrowth of spores in canned cured meat is dependent on such factors as the concentrations of sodium chloride, pH, amount of thermal processing, and the number of spores (Pivnik *et al.*, 1969). In this report, we have investigated the effect of the components of various media on the inhibition of spore outgrowth by sodium nitrite.

Materials and methods

Spore suspension. Spores of *C. botulinum* 13983B were prepared according to the method of Schmidt *et al.*, (1962). The spores were enumerated in Prickett tubes containing Wynne Agar (Difco) with 0.1% filter sterilized sodium bicarbonate added after autoclaving. The concentrated spore suspension containing 4.7×10^6 spores/ml was stored at 5°C and diluted to approximately 1×10^6 spores per ml before being used.

Media. The following bacteriological broth media were used: Perigo-type (PT) (Johnston *et al.*, 1969), Reinforced Clostridial Medium (RCM; BBL), Liver Veal (LV; Difco), and Wynne Fluid Medium (WFM; Difco). Raw, frozen canned pork luncheon meat (donated by Canada Packers, Toronto) used to prepare the meat suspensions was sterilized in 300 x 308 cans by 2 megarads of gamma radiation (Gamma cell 220, Atomic Energy of Canada Ltd., Ottawa). Radiation reduced the bacterial count of the meat from 2.2 x

10^4/g to <1 in 5 g but did not affect outgrowth of spores of *C. botulinum* added to the meat after irradiation. The cans of irradiated meat were then treated as follows: not heated (under-process), heated 115°C for 90 min plus 4 days at 80°C (over-process).

Preparation and inoculation of media. The bacteriological media were adjusted to pH 6.2 after autoclaving. Filter-sterilized sodium nitrite was added to each medium to obtain concentrations of nitrite from 0 to 500 ppm. The medium was dispensed into test tubes and heated at either 100°C for 5 min to remove dissolved oxygen or at 110°C for 20 min to obtain the optimum conditions for the production of the Perigo factor. After cooling to 80°C, the medium was inoculated with 10^5 spores per tube, capped with vaspar and incubated at 35°C for 14 days.

Fifty percent meat-water (w/v) mixtures, prepared from the 3 meat samples, were adjusted to pH 6.2 before the addition of various concentrations of nitrite. The meat suspensions were dispensed, heated and inoculated in the same manner as the bacteriological media. Uninoculated tubes were used as controls in each experiment. Gas production was used as the criterion for growth.

Nitrite analysis. Nitrite was determined in bacteriological media by the AOAC method (AOAC, 1960), and in meat suspensions by the method of Kamm *et al.* (1965). Although we have used the terms nitrite and sodium nitrite synonymously, we have expressed all concentrations as ppm of sodium nitrite.

Eh measurement. The oxidation-reduction potential of each medium was determined with a combination platinum-calomel electrode (Radiometer) connected to a pH meter (Corning, Model 7). The working standard half-cell was a saturated solution of quinhydrone in M/20 KH phthalate (Knight, 1930). The observed EMF (215-217 millivolts) of this working standard was checked at room temperature between all determinations.

To determine the Eh of media or meat, the vaspar in each test tube was melted by carefully heating the vaspar-containing area of the tube with a Bunsen burner. The electrode, containing a protective plastic cap, was immersed into the test medium just before the vaspar solidified. Then the protective cap was pushed off the tip with a needle. The EMF was recorded 15 to 20 min after the electrode was inserted.

Dialysis. Medium or meat suspension containing sodium nitrite was heated at 110°C for 20 min. When the temperature cooled to 80°C, volumes of 25 ml were introduced to dialysis bags which were then put into stoppered flasks containing 225 ml of the same medium that had undergone the same heating without nitrite. Thereafter, dialysis was for 6 hours at room temperature. The dialyzed medium was then dispensed into test tubes, inoculated with spores, heated for 5 min at 100°C to remove dissolved oxygen and capped with vaspar.

Results

Inhibition of growth. Table 1 shows the outgrowth of spores in various media heated in the presence of nitrite. PT, RCM and WFM media inhibited the outgrowth of 1 x 10^5 spores per tube when heated at 100°C for 5 min in the presence of 150 ppm of nitrite. More nitrite (150-250 ppm) was required to prevent growth in LV medium. Heating the bacteriological media at 110°C for 20 min resulted in complete inhibition with 20 ppm of nitrite in PT and RCM media but had no effect on nitrite inhibition in LV or WFM media.

Both normal-process and over-process meat suspensions heated at 100°C for 5 min required about the same amount of nitrite to inhibit spore outgrowth as LV medium. In under-process meat, about 250 to 500 ppm of nitrite was required. Heating at 110°C for 20 min had little effect on nitrite inhibition in under-process or normal-process meats, but prevented outgrowth in over-process meat containing 50 ppm of nitrite.

Omission of thioglycolate from PT medium and cysteine from RCM medium had little or no effect on

Table 1. Effect of nitrite and heat treatment on outgrowth of spores of Clostridium botulinum in various media.

Media	Heat treatment [a]	Number of tubes showing growth — Sodium nitrite (ppm) 0	20	50	100	150	250	500
Bacteriological:								
Perigo-type (PT)	+	6/6	0/9	0/9	0/9	0/9	0/9	0/9
	—	6/6	9/9	9/9	6/9	0/9	0/9	0/9
Reinforced Clostridial Medium (RCM)	+	6/6	0/9	0/9	0/9	0/9	0/9	0/9
	—	6/6	9/9	9/9	7/9	0/9	0/9	0/9
Liver Veal (LV)	+	6/6	9/9	9/9	9/9	9/9	0/9	0/9
	—	6/6	9/9	9/9	9/9	9/9	0/9	0/9
Wynne Fluid Medium (WFM)	+	6/6	9/9	9/9	6/9	0/9	0/9	0/9
	—	6/6	9/9	9/9	6/9	0/9	0/9	0/9
Meat suspensions:								
Under-process	+	6/6	9/9	9/9	9/9	8/9	5/9	1/9
	—	6/6	9/9	9/9	9/9	9/9	3/9	0/9
Normal-process	+	6/6	9/9	9/9	9/9	1/9	0/9	0/9
	—	6/6	9/9	9/9	8/9	3/9	0/9	0/9
Over-process	+	10/10	13/13	0/31	0/31	0/31	0/13	0/13
	—	10/10	13/13	31/31	30/31	6/31	0/13	0/13

[a] Heat treatment: + = 110°C for 20 min, and — = 100°C for 5 min.

Table 2. Effect of dialysing bacteriological media and meat suspensions heated in the presence of nitrite on subsequent spore outgrowth.

Sodium nitrite added (ppm)	Dialysis (a)	Number of tubes showing growth						
		Bacteriological media			Meat suspensions			
		Liver Veal (LV)	Perigo-type (PT)	Reinforced Clostridial Medium (RCM)	Under-process	Under-process + cysteine (1000 ppm)	Normal-process	Over-process
0	—	6/6	6/6	6/6	6/6	6/6	6/6	6/6
	+	6/6	6/6	6/6	6/6	6/6	6/6	6/6
50	—	6/6	0/6	0/6	6/6	0/6	6/6	12/12
	+	6/6	0/6	0/6	6/6	6/6	6/6	12/12
250	—	0/6	0/6	0/6	0/6	1/6	0/6	0/6
	+	6/6	0/6	0/6	6/6	6/6	6/6	6/6

[a] Dialysis: + = dialysis, and — = no dialysis.

inhibition of outgrowth when these media were heated at 110°C for 20 min with nitrite. However, the addition of these reducing agents to under-process meat suspensions greatly reduced the amount of nitrite required to inhibit spore outgrowth (Fig. 1). For example with meat suspensions containing 100 ppm of nitrite the amount of reducing agents to prevent the growth of spores were 850 ppm of ascorbate, 1050 ppm of cysteine and 3500 ppm of thioglycolate. In the absence of nitrite, the reducing agents prevented outgrowth only at high concentrations (3500 ppm ascorbate, 2800 ppm cysteine and 17,500 ppm thioglycolate).

Dialysis. Inhibition due to the Perigo factor was distinguished from inhibition of residual nitrite by dialysis; dialysis removes nitrite but not the Perigo factor (Johnston *et al.*, 1969). PT and RCM media, heated with 50 and 250 ppm of nitrite at 110°C for 20 min and then dialyzed against the same medium without nitrite, did not support the growth of *C. botulinum* spores (Table 2). Under the same experimental conditions inhibitory activity was lost in LV medium and in all meat suspensions, even in suspensions containing cysteine.

Residual nitrite. In order to determine a possible relationship between inhibitory activity and residual nitrite, the nitrite concentrations of the media were analyzed immediately after heating, and during the 14 day incubation period. The amount of residual nitrite recovered from the 4 bacteriological media varied between 85 and 91 ppm when the media were heated at 100°C for 5 min, and between 48 and 78 ppm when heated at 110°C for 20 min (Table 3). Nitrite disappeared faster in the PT (91 to 25 ppm) and RCM (90 to 18 ppm) media than in LV (88 to 50 ppm) and WFM (85 to 43 ppm) media during the 14 day incubation period at 35°C. The residual nitrite concentration of all 3 meat suspensions varied between 90 and 93 ppm when the suspensions were heated at 100°C for 5 min and between 60 and 67 ppm when heated at 110°C for 20 min. After 14 days of incubation, normal-process and over-process meat suspensions contained approximately one-half the residual nitrite that was found in under-process meat suspensions.

Reducing agents lowered the residual nitrite concentration of under-process meat suspensions (Fig. 2). The addition of about 800-850 ppm of cysteine or

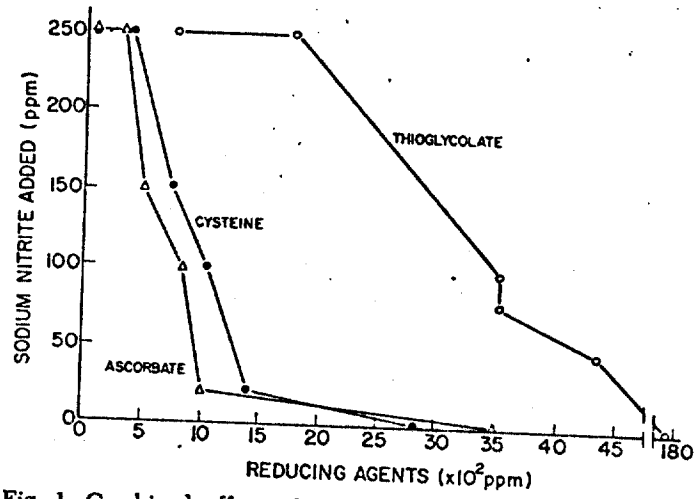

Fig. 1 Combined effect of reducing agents and nitrite on the inhibition of Clostridium botulinum in 50 percent under-process meat suspensions.

ascorbate to meat suspensions containing 100 ppm of nitrite reduced the nitrite after heating to 40 ppm as compared to 90 ppm without reducing agents. A higher concentration of thioglycolate (1750 ppm) was required to give approximately the same residual nitrite concentration.

Eh measurement. The data in Table 4 show the Eh values in different media heated at 100°C for 5 min and at 110°C for 20 min with 100 ppm of nitrite. The values among the 4 bacteriological media determined after heating at 110°C for 20 min varied from −45 mv to +175 mv, while the values of the 3 meat suspensions were similar (+10 mv to +33 mv). All preparations heated at this higher temperature have Eh values 5 to 54 mv lower than when heated at 100°C for 5 min. The values did not vary by more than 50 mv for each preparation during the 14 day period. Sulfydryl compounds (cysteine and thioglycolate) added to under-process meat suspensions containing 100 ppm of nitrite lowered the Eh from 65 mv to 0 mv with 800-1000 ppm of each compound, while ascorbate did not significantly affect Eh (Fig. 3).

Table 3. Recovery of sodium nitrite from various media heated in the presence of 100 ppm of nitrite.

Media	Days of Incubation	Sodium nitrite recovered (ppm) Heat treatment 100°C for 5 min.	110°C for 20 min.
Bacteriological: Perigo-type (PT)	0	91	48
	1	71	37
	14	25	15
Reinforced Clostridial Medium (RCM)	0	90	76
	1	75	67
	14	18	9
Liver Veal (LV)	0	88	78
	1	72	60
	14	50	24
Wynne Fluid Medium (WFM)	0	85	75
	1	68	62
	14	43	32
Meat suspensions: Under-process	0	92	67
	1	85	60
	14	58	41
Normal-process	0	93	64
	1	88	60
	14	29	19
Over-process	0	90	60
	1	70	55
	14	30	22

Table 4. Oxidation-reduction potential of various media heated in the presence of 100 ppm of nitrite.

Media	Days of Incubation	Eh (mv) Heat treatment 100°C for 5 min.	110°C for 20 min.
Bacteriological: Perigo-type (PT)	0	22	—32
	1	32	—22
	14	50	—10
Reinforced Clostridial Medium (RCM)	0	85	38
	1	105	40
	14	125	36
Liver Veal (LV)	0	190	175
	1	185	135
	14	160	145
Wynne Fluid Medium (WFM)	0	—40	—45
	1	—23	—42
	14	—10	—35
Meat suspensions: Under-process	0	55	33
	1	22	25
	14	82	30
Normal-process	0	30	10
	1	40	15
	14	40	60
Over-process	0	30	15
	1	15	20
	14	60	55

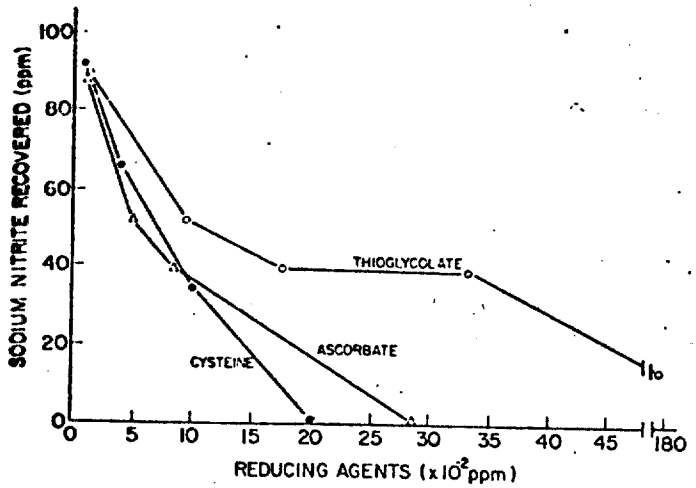

Fig. 2 Effect of reducing agents on the disappearance of nitrite in 50 percent under-process meat suspensions.

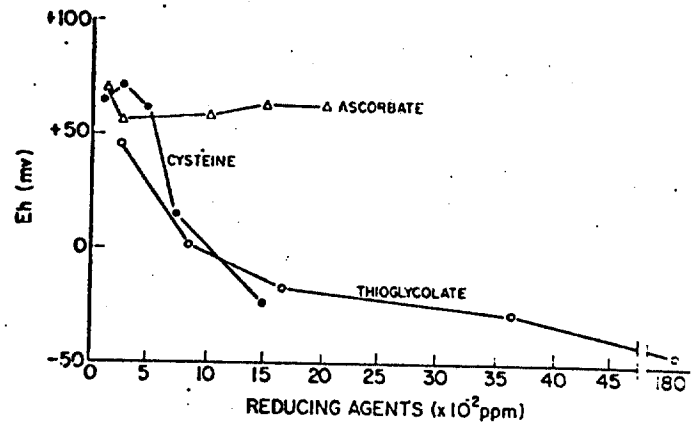

Fig. 3 Effect of reducing agents on the oxidation-reduction potential of 50 percent under-process meat suspensions.

Discussion

The inhibitory effect of sodium nitrite on the outgrowth of spores of *C. botulinum* was influenced by components of the growth media. Toxicity has been attributed to an antimicrobial compound (Perigo factor) (Perigo *et al.*, 1967) formed between components of media and nitrite, and to the direct action of nitrous acid (Castellani and Niven, 1955).

The bacteriological media, PT and RCM, and the over-process meat suspensions are much more toxic to spore outgrowth when heated with nitrite at 110°C for 20 min than after heating at 100°C for 5 min. However, formation of the Perigo factor could be demonstrated in the PT and RCM media only. Toxicity in over-process meat suspensions, and its loss during dialysis may be due to any or all of the following: (a) an increase in the nitrous acid concentration; (b) formation of a low-molecular weight antimicrobial compound that could be removed by dialysis; (c) destruction of the unstable "Perigo-type" factor during manipulation of the thick meat suspension; sufficient exposure to the air may have occurred, immediately before and after dialysis, to inactivate the factor. The Perigo factor can be destroyed in dialyzed PT medium by bubbling air into the medium (unpublished results).

The inhibitory effect of nitrite can be increased in media by the addition of reducing agents. We have found that the amount of nitrite required to prevent outgrowth in under-process meat could be lowered by adding reducing agents such as cysteine, thioglycolate and ascorbate. These agents are known to aid in the reduction of nitrite (Burton, 1959; Castellani and Niven, 1955; Kelley and Watts, 1957), and may affect the formation of nitroso-reductants (Foy and Acherman, 1968). These intermediate carriers may transfer the nitroso group directly to components of the bacterial cells or release nitric oxide. The variation in the inhibitory activity found between LV (150 ppm of nitrite) and RCM (100 ppm of nitrite) media when heated at 100°C for 5 min may be due to the presence of cysteine in RCM medium.

An examination of the data on residual nitrite concentration in the bacteriological media showed that only a small amount of the nitrite that disappeared is involved in the formation of the Perigo factor. Although the Perigo factor could not be demonstrated in over-process meat by dialysis experiments, the over-process meat was much more inhibitory when heated at 110°C than either under-process or normal-process meat even though nitrite disappeared at approximately the same rate for all 3 meat preparations. Thus, it is not possible to distinguish between the two types of nitrite toxicity on the basis of the residual nitrite concentrations.

Since reducing agents were found to affect nitrite toxicity, we examined the oxidation-reduction potential of the various media under conditions which were optimum for inhibition by the Perigo factor and by nitrous acid. The Eh values obtained for all media and meat suspensions heated at 110°C were lower than for the same media heated at 100°C. This observation suggests that a low potential is needed for Perigo factor formation. However, a comparison of the various media showed that a wide variation in Eh occurred under conditions which allowed formation of this factor. The reasons for failure of ascorbate to reduce the Eh of the under-process meat suspensions is not readily explained (Fig. 3). Ascorbate, not destroyed by the meat itself during heating, may be unable to lower the Eh below the value obtained through heating.

Conclusion

This study has shown that the concentration of sodium nitrite required to prevent outgrowth of spores of *C. botulinum* varies in different media. Nitrite can inhibit growth by forming a Perigo factor or by the direct action of nitrous acid. From a practical point of view, these findings indicate that great care must be taken in selecting a medium to study the mechanism of action of sodium nitrite in commercially produced cured meats.

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Inhibition of Clostridium Botulinum by Sodium Nitrite as Affected by Bacteriological Media and Meat Suspensions

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Abstract

The inhibitory effect of sodium nitrite on the outgrowth of spores of Clostridium botulinum 13983B was studied in various bacteriological media and meat suspensions. The media and meat suspensions were adjusted to pH 6.2 and heated in the presence of nitrite at 110°C for 20 min. Perigo factor was produced in Perigo-type medium (PT) and Reinforced Clostridial Medium (RCM) when heated with 20 ppm of nitrite, but not in Liver Veal Medium (LVM) and Wynne Fluid Medium (WFM) even when heated with 100 ppm of nitrite. Suspensions of 50% meat in water, prepared from under-process, normal-process and over-process meats, required 500 ppm, 150 ppm and 50 ppm of nitrite, respectively, for inhibition of outgrowth.

Dialysis removed the inhibitory activity from two of the bacteriological media (LVM and WFM) and from all meat suspensions, but not from PT and RCM media. The residual nitrite concentrations were approximately the same in all seven heated preparation (100 ppm added before heating), but the redox potential varied considerably among them. The addition of reducing agents to meat suspensions decreased the redox potential and increased the inhibitory activity but did not induce the formation of the Perigo factor.

Résumé

L'effet inhibiteur du nitrite de sodium sur la croissance des spores de Clostridium botulinum 13983B a été étudié dans différents milieux de culture et suspensions de viande. Les milieux et suspensions de suspensions de viande ont été ajustés à pH 6.2 et chauffés 20 minutes à 110°C en présence de nitrite de sodium. Le facteur Périgo a été produit dans le milieux de type Périgo (TP) et dans le milieu renforcé pour clostridiales (MRC), chauffés avec 20 ppm de nitrite; le facteur n'a pas été produit dans le milieu au foie de veau (MFV) et dans le milieu liquide de Wynne (MFV) même lorsqu'ils étaient chauffés avec 100 ppm de nitrite. Pour les suspensions aqueuses de viande à la concentration de 50%, préparées à partir de viandes ayant subi un traitement léger, normal et intensif, il a fallu respectivement 500 ppm, 150 ppm et 50 ppm de nitrite pour inhiber la croissance des spores.

La dialyse a fait disparaître l'activité inhibitrice de deux des milieux de culture (MFV et MFW) et de toutes les suspensions de viande mais a été sans action sur les milieux TP et MRC. Les concentrations résiduelles de nitrite étaient à peu près les mêmes dans les sept préparations chauffées (avec addition de 100 ppm de nitrite avant chauffage) mais le potentiel redox variait considérablement d'une préparation à l'autre. L'addition d'agents réducteurs aux suspensions de viande a diminué le potentiel redox et augmenté l'activité inhibitrice mais elle n'a pas provoqué la formation du facteur Périgo.

Introduction

The inhibitory effect of sodium nitrite on the growth of bacteria has been studied in bacteriological media and in meat preparations. Results from these studies with media of various compositions have been used to explain the role of nitrite in the safety of commercially-produced cured meats. For example, Tanner and Evans (1934) showed that the composition of the growth medium influenced the toxicity of sodium nitrite to *Clostridium botulinum*: a pork infusion medium was less inhibitory than bouillon medium, although both media had the same concentration of residual nitrite after autoclaving. Tarr (1942) found that the growth of facultative anaerobes was inhibited by sodium nitrite to a greater extent in nutrient broth than in fish digest broth. The initial pH of the media to which nitrite was added after autoclaving was less critical in nutrient broth than in fish digest broth. More recent work by Perigo *et al.* (1967) has shown that an antimicrobial compound (Perigo factor) can be formed in a bacteriological medium if nitrite is present during autoclaving. The Perigo factor has not been demonstrated in commercially-produced meat (Johnston *et al.*, 1969).

The addition of certain compounds to bacteriological media and meat preparations can influence the toxic effect of nitrite on bacterial growth. Castellani and Niven (1955) found that glucose and sulfhydryl compounds, especially cysteine, increased the inhibitory effect of nitrite on food poisoning staphylococci in a synthetic medium under anaerobic conditions. Ascorbic acid has been used successfully to extend the shelf-life of cured meats. Schack and Taylor (1963) showed that meat previously treated with ascorbic acid and nitrite required less heat treatment than normally cured meats.

The concentration of sodium nitrite required to prevent outgrowth of spores in canned cured meat is dependent on such factors as the concentrations of sodium chloride, pH, amount of thermal processing, and the number of spores (Pivnik *et al.*, 1969). In this report, we have investigated the effect of the components of various media on the inhibition of spore outgrowth by sodium nitrite.

Materials and methods

Spore suspension. Spores of *C. botulinum* 13983B were prepared according to the method of Schmidt *et al.*, (1962). The spores were enumerated in Prickett tubes containing Wynne Agar (Difco) with 0.1% filter sterilized sodium bicarbonate added after autoclaving. The concentrated spore suspension containing 4.7×10^8 spores/ml was stored at 5°C and diluted to approximately 1×10^6 spores per ml before being used.

Media. The following bacteriological broth media were used: Perigo-type (PT) (Johnston *et al.*, 1969), Reinforced Clostridial Medium (RCM; BBL), Liver Veal (LV; Difco), and Wynne Fluid Medium (WFM; Difco). Raw, frozen canned pork luncheon meat (donated by Canada Packers, Toronto) used to prepare the meat suspensions was sterilized in 300 x 308 cans by 2 megarads of gamma radiation (Gamma cell 220, Atomic Energy of Canada Ltd., Ottawa). Radiation reduced the bacterial count of the meat from 2.2 x

^{4}/g to <1 in 5 g but did not affect outgrowth of spores of *C. botulinum* added to the meat after irradiation. The cans of irradiated meat were then treated as follows: not heated (under-process), heated 115°C for 90 min plus 4 days at 80°C (over-process).

Preparation and inoculation of media. The bacteriological media were adjusted to pH 6.2 after autoclaving. Filter-sterilized sodium nitrite was added to each medium to obtain concentrations of nitrite from 0 to 500 ppm. The medium was dispensed into test tubes and heated at either 100°C for 5 min to remove dissolved oxygen or at 110°C for 20 min to obtain the optimum conditions for the production of the Perigo factor. After cooling to 80°C, the medium was inoculated with 10^5 spores per tube, capped with vaspar and incubated at 35°C for 14 days.

Fifty percent meat-water (w/v) mixtures, prepared from the 3 meat samples, were adjusted to pH 6.2 before the addition of various concentrations of nitrite. The meat suspensions were dispensed, heated and inoculated in the same manner as the bacteriological media. Uninoculated tubes were used as controls in each experiment. Gas production was used as the criterion for growth.

Nitrite analysis. Nitrite was determined in bacteriological media by the AOAC method (AOAC, 1960), and in meat suspensions by the method of Kamm *et al.* (1965). Although we have used the terms nitrite and sodium nitrite synonymously, we have expressed all concentrations as ppm of sodium nitrite.

Eh measurement. The oxidation-reduction potential of each medium was determined with a combination platinum-calomel electrode (Radiometer) connected to a pH meter (Corning, Model 7). The working standard half-cell was a saturated solution of quinhydrone in M/20 KH phthalate (Knight, 1930). The observed EMF (215-217 millivolts) of this working standard was checked at room temperature between all determinations.

To determine the Eh of media or meat, the vaspar in each test tube was melted by carefully heating the vaspar-containing area of the tube with a Bunsen burner. The electrode, containing a protective plastic cap, was immersed into the test medium just before the vaspar solidified. Then the protective cap was pushed off the tip with a needle. The EMF was recorded 15 to 20 min after the electrode was inserted.

Dialysis. Medium or meat suspension containing sodium nitrite was heated at 110°C for 20 min. When the temperature cooled to 80°C, volumes of 25 ml were introduced to dialysis bags which were then put into stoppered flasks containing 225 ml of the same medium that had undergone the same heating without nitrite. Thereafter, dialysis was for 6 hours at room temperature. The dialyzed medium was then dispensed into test tubes, inoculated with spores, heated for 5 min at 100°C to remove dissolved oxygen and capped with vaspar.

Results

Inhibition of growth. Table 1 shows the outgrowth of spores in various media heated in the presence of nitrite. PT, RCM and WFM media inhibited the outgrowth of 1 x 10^5 spores per tube when heated at 100°C for 5 min in the presence of 150 ppm of nitrite. More nitrite (150-250 ppm) was required to prevent growth in LV medium. Heating the bacteriological media at 110°C for 20 min resulted in complete inhibition with 20 ppm of nitrite in PT and RCM media but had no effect on nitrite inhibition in LV or WFM media.

Both normal-process and over-process meat suspensions heated at 100°C for 5 min required about the same amount of nitrite to inhibit spore outgrowth as LV medium. In under-process meat, about 250 to 500 ppm of nitrite was required. Heating at 110°C for 20 min had little effect on nitrite inhibition in under-process or normal-process meats, but prevented outgrowth in over-process meat containing 50 ppm of nitrite.

Omission of thioglycolate from PT medium and cysteine from RCM medium had little or no effect on

Table 1. Effect of nitrite and heat treatment on outgrowth of spores of Clostridium botulinum in various media.

Media	Heat treatment (a)	Number of tubes showing growth: Sodium nitrite (ppm) 0	20	50	100	150	250	500
Bacteriological:								
Perigo-type (PT)	+	6/6	0/9	0/9	0/9	0/9	0/9	0/9
	—	6/6	9/9	9/9	6/9	0/9	0/9	0/9
Reinforced Clostridial Medium (RCM)	+	6/6	0/9	0/9	0/9	0/9	0/9	0/9
	—	6/6	9/9	9/9	7/9	0/9	0/9	0/9
Liver Veal (LV)	+	6/6	9/9	9/9	9/9	9/9	0/9	0/9
	—	6/6	9/9	9/9	9/9	9/9	0/9	0/9
Wynne Fluid Medium (WFM)	+	6/6	9/9	9/9	6/9	0/9	0/9	0/9
	—	6/6	9/9	9/9	6/9	0/9	0/9	0/9
Meat suspensions:								
Under-process	+	6/6	9/9	9/9	9/9	8/9	5/9	1/9
	—	6/6	9/9	9/9	9/9	9/9	3/9	0/9
Normal-process	+	6/6	9/9	9/9	9/9	1/9	0/9	0/9
	—	6/6	9/9	9/9	8/9	3/9	0/9	0/9
Over-process	+	10/10	13/13	0/31	0/31	0/31	0/13	0/13
	—	10/10	13/13	31/31	30/31	6/31	0/13	0/13

(a) Heat treatment: + = 110°C for 20 min, and — = 100°C for 5 min.

Table 2. Effect of dialysing bacteriological media and meat suspensions heated in the presence of nitrite on subsequent spore outgrowth.

Sodium nitrite added (ppm)	Dialysis (a)	Number of tubes showing growth						
		Bacteriological media			Meat suspensions			
		Liver Veal (LV)	Perigo-type (PT)	Reinforced Clostridial Medium (RCM)	Under-process	Under-process + cysteine (1000 ppm)	Normal-process	Over-process
0	—	6/6	6/6	6/6	6/6	6/6	6/6	6/6
	+	6/6	6/6	6/6	6/6	6/6	6/6	6/6
50	—	6/6	0/6	0/6	6/6	0/6	6/6	12/12
	+	6/6	0/6	0/6	6/6	6/6	6/6	12/12
250	—	0/6	0/6	0/6	0/6	1/6	0/6	0/6
	+	6/6	0/6	0/6	6/6	6/6	6/6	6/6

(a) Dialysis: + = dialysis, and — = no dialysis.

hibition of outgrowth when these media were heated 110°C for 20 min with nitrite. However, the addition of these reducing agents to under-process meat spensions greatly reduced the amount of nitrite quired to inhibit spore outgrowth (Fig. 1). For xample with meat suspensions containing 100 ppm f nitrite the amount of reducing agents to prevent growth of spores were 850 ppm of ascorbate, 1050 m of cysteine and 3500 ppm of thioglycolate. In the bsence of nitrite, the reducing agents prevented outwth only at high concentrations (3500 ppm ascorte, 2800 ppm cysteine and 17,500 ppm thioglycolate).

Dialysis. Inhibition due to the Perigo factor was tinguished from inhibition of residual nitrite by lysis: dialysis removes nitrite but not the Perigo cto (Johnston *et al.*, 1969). PT and RCM media, eated with 50 and 250 ppm of nitrite at 110°C for min and then dialyzed against the same medium hout nitrite, did not support the growth of *C. otulinum* spores (Table 2). Under the same experital conditions inhibitory activity was lost in LV lium and in all meat suspensions, even in susensions containing cysteine.

Residual nitrite. In order to determine a possible tionship between inhibitory activity and residual rite, the nitrite concentrations of the media were alyzed immediately after heating, and during the lay incubation period. The amount of residual nire recovered from the 4 bacteriological media varied tween 85 and 91 ppm when the media were heated 00°C for 5 min, and between 48 and 78 ppm when ed at 110°C for 20 min (Table 3). Nitrite disapared faster in the PT (91 to 25 ppm) and RCM (90 8 ppm) media than in LV (88 to 50 ppm) and M (85 to 43 ppm) media during the 14 day ination period at 35°C. The residual nitrite concention of all 3 meat suspensions varied between 90 93 ppm when the suspensions were heated at C for 5 min and between 60 and 67 ppm when ted at 110°C for 20 min. After 14 days of incubanormal-process and over-process meat suspens contained approximately one-half the residual rite that was found in under-process meat suspens.

Reducing agents lowered the residual nitrite conation of under-process meat suspensions (Fig. The addition of about 800-850 ppm of cysteine or

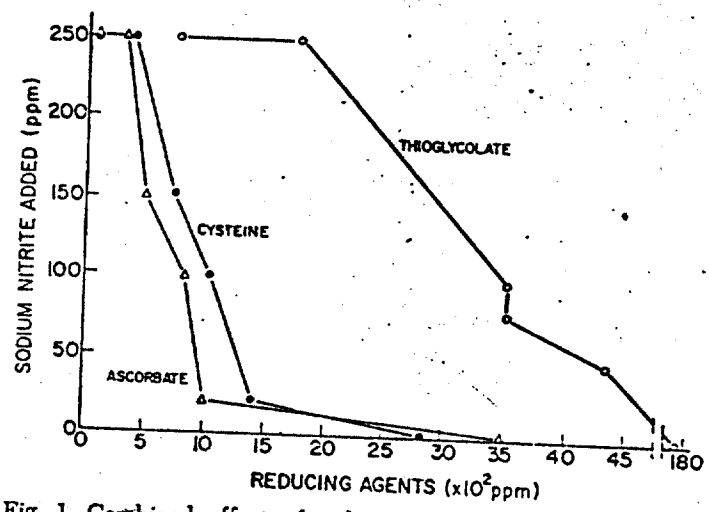

Fig. 1 Combined effect of reducing agents and nitrite on the inhibition of Clostridium botulinum in 50 percent under-process meat suspensions.

ascorbate to meat suspensions containing 100 ppm of nitrite reduced the nitrite after heating to 40 ppm as compared to 90 ppm without reducing agents. A higher concentration of thioglycolate (1750 ppm) was required to give approximately the same residual nitrite concentration.

Eh measurement. The data in Table 4 show the Eh values in different media heated at 100°C for 5 min and at 110°C for 20 min with 100 ppm of nitrite. The values among the 4 bacteriological media determined after heating at 110°C for 20 min varied from −45 mv to +175 mv, while the values of the 3 meat suspensions were similar (+10 mv to +33 mv). All preparations heated at this higher temperature have Eh values 5 to 54 mv lower than when heated at 100°C for 5 min. The values did not vary by more than 50 mv for each preparation during the 14 day period. Sulfydryl compounds (cysteine and thioglycolate) added to under-process meat suspensions containing 100 ppm of nitrite lowered the Eh from 65 mv to 0 mv with 800-1000 ppm of each compound, while ascorbate did not significantly affect Eh (Fig. 3).

Table 3. Recovery of sodium nitrite from various media heated in the presence of 100 ppm of nitrite.

Media	Days of Incubation	Sodium nitrite recovered (ppm) Heat treatment 100°C for 5 min.	110°C for 20 min.
Bacteriological: Perigo-type (PT)	0	91	48
	1	71	37
	14	25	15
Reinforced Clostridial Medium (RCM)	0	90	76
	1	75	67
	14	18	9
Liver Veal (LV)	0	88	78
	1	72	60
	14	50	24
Wynne Fluid Medium (WFM)	0	85	75
	1	68	62
	14	43	32
Meat suspensions: Under-process	0	92	67
	1	85	60
	14	58	41
Normal-process	0	93	64
	1	88	60
	14	29	19
Over-process	0	90	60
	1	70	55
	14	30	22

Table 4. Oxidation-reduction potential of various media heated in the presence of 100 ppm of nitrite.

Media	Days of Incubation	Eh (mv) Heat treatment 100°C for 5 min.	110°C for 20 min.
Bacteriological: Perigo-type (PT)	0	22	—32
	1	32	—22
	14	50	—10
Reinforced Clostridial Medium (RCM)	0	85	38
	1	105	40
	14	125	36
Liver Veal (LV)	0	190	175
	1	185	135
	14	160	145
Wynne Fluid Medium (WFM)	0	—40	—45
	1	—23	—42
	14	—10	—35
Meat suspensions: Under-process	0	55	33
	1	22	25
	14	82	30
Normal-process	0	30	10
	1	40	15
	14	40	60
Over-process	0	30	15
	1	15	20
	14	60	55

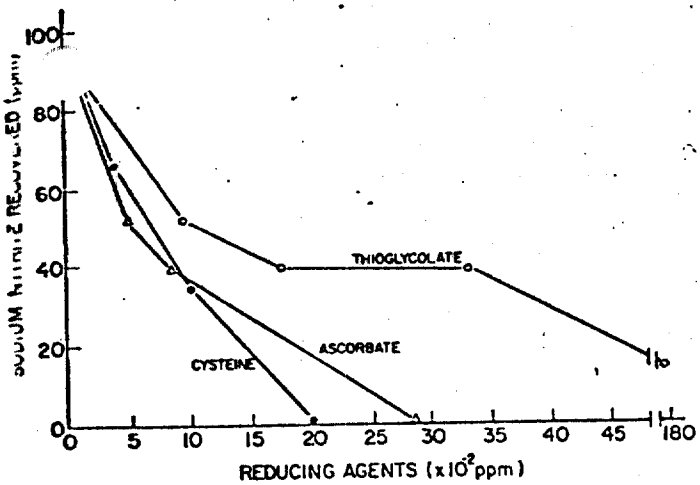

Fig. 2 Effect of reducing agents on the disappearance of nitrite in 50 percent under-process meat suspensions.

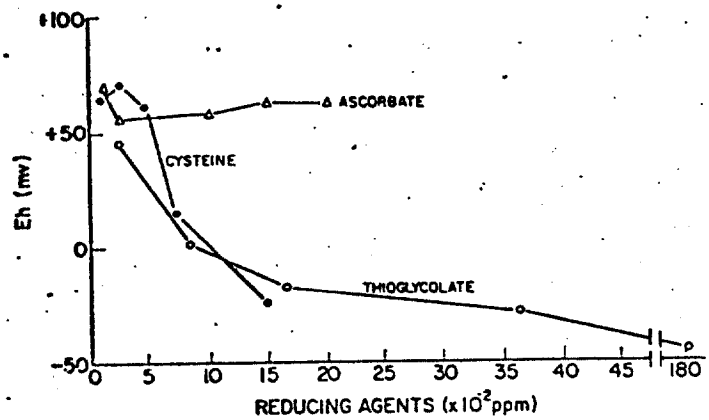

Fig. 3 Effect of reducing agents on the oxidation-reduction potential of 50 percent under-process meat suspensions.

Discussion

The inhibitory effect of sodium nitrite on the outgrowth of spores of *C. botulinum* was influenced by components of the growth media. Toxicity has been attributed to an antimicrobial compound (Perigo factor) (Perigo *et al.*, 1967) formed between components of media and nitrite, and to the direct action of nitrous acid (Castellani and Niven, 1955).

The bacteriological media, PT and RCM, and the over-process meat suspensions are much more toxic to spore outgrowth when heated with nitrite at 110°C for 20 min than after heating at 100°C for 5 min. However, formation of the Perigo factor could be demonstrated in the PT and RCM media only. Toxicity in over-process meat suspensions, and its loss during dialysis may be due to any or all of the following: (a) an increase in the nitrous acid concentration; (b) formation of a low-molecular weight antimicrobial compound that could be removed by dialysis; (c) destruction of the unstable "Perigo-type" factor during manipulation of the thick meat suspension; sufficient exposure to the air may have occurred, immediately before and after dialysis, to inactivate the factor. The Perigo factor can be destroyed in dialyzed PT medium by bubbling air into the medium (unpublished results).

The inhibitory effect of nitrite can be increased in media by the addition of reducing agents. We have found that the amount of nitrite required to prevent outgrowth in under-process meat could be lowered by adding reducing agents such as cysteine, thioglycolate and ascorbate. These agents are known to aid in the reduction of nitrite (Burton, 1959; Castellani and Niven, 1955; Kelley and Watts, 1957), and may affect the formation of nitroso-reductants (Foy and Ackerman, 1968). These intermediate carriers may transfer the nitroso group directly to components of the bacterial cells or release nitric oxide. The variation in the inhibitory activity found between LV (150 ppm of nitrite) and RCM (100 ppm of nitrite) media when heated at 100°C for 5 min may be due to the presence of cysteine in RCM medium.

An examination of the data on residual nitrite concentration in the bacteriological media showed that only a small amount of the nitrite that disappeared is involved in the formation of the Perigo factor. Although the Perigo factor could not be demonstrated in over-process meat by dialysis experiments, the over-process meat was much more inhibitory when heated at 110°C than either under-process or normal-process meat even though nitrite disappeared at approximately the same rate for all 3 meat preparations. Thus, it is not possible to distinguish between the two types of nitrite toxicity on the basis of the residual nitrite concentrations.

Since reducing agents were found to affect nitrite toxicity, we examined the oxidation-reduction potential of the various media under conditions which were optimum for inhibition by the Perigo factor and by nitrous acid. The Eh values obtained for all media and meat suspensions heated at 110°C were lower than for the same media heated at 100°C. This observation suggests that a low potential is needed for Perigo factor formation. However, a comparison of the various media showed that a wide variation in Eh occurred under conditions which allowed formation of this factor. The reasons for failure of ascorbate to reduce the Eh of the under-process meat suspensions is not readily explained (Fig. 3). Ascorbate, not destroyed by the meat itself during heating, may be unable to lower the Eh below the value obtained through heating.

Conclusion

This study has shown that the concentration of sodium nitrite required to prevent outgrowth of spores of *C. botulinum* varies in different media. Nitrite can inhibit growth by forming a Perigo factor or by the direct action of nitrous acid. From a practical point of view, these findings indicate that great care must be taken in selecting a medium to study the mechanism of action of sodium nitrite in commercially produced cured meats.

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Inhibition of Clostridium Botulinum by Sodium Nitrite in a Bacteriological Medium and in Meat

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Abstract

The inhibitory effect of sodium nitrite on the growth of Clostridium botulinum was studied in a bacteriological medium and in meat suspension. We found that a bacteriological medium heated at 110°C for 20 min with as little as 20 ppm of sodium nitrite became inhibitory to C. botulinum and the inhibitor was non-dialyzable. Meat suspension heated with 150 ppm of nitrite was inhibitory to C. botulinum, but the inhibitory activity was probably due to a residuum of greater than 100 ppm of nitrite: dialysis removed the inhibitory activity. Inhibitor formed in medium could be inactivated by non-fat meat solids but not by fat or water soluble meat extracts. It appears that the inhibitor produced in media is of little or no consequence in explaining the role of nitrite in the safety of commercially-produced canned cured meat.

Résumé

L'action bactériostatique du nitrite de sodium sur la croissance du clostridium botulinum fût étudiée en milieu bactériologique et en suspension de viande. Nous avons trouvé que le milieu bactériologique chauffé à 110°C pendant 20 minutes avec aussi peu que 20 ppm de nitrite de sodium devint bactériostatique au C. Botulinum sans que la substance inhibitrice ne soit dialysée.

Une suspension de viande chauffée avec 150 ppm de nitrite [illegible] inhibitrice au C. botulinum mais cette action inhibitrice fût probablement due à un résidu de nitrite de plus de 100 ppm: la dialyse enleva l'action inhibitrice.

L'inhibiteur formé dns le milieu pouvait être inactivé par les solides de viande dégraissés mais non par les extraits de viande solubles dans le gras ou l'eau. Il semble que la substance inhibitrice formée dans le milieu a peu ou pas de correlation avec le rôle du nitrite sur la sécurité d'une viande saumurée commercialement mise en boite.

Introduction

The stability of canned cured meats is dependent on a number of factors such as the concentrations of sodium chloride and sodium nitrite, the degree of thermal processing, and the number of spores. The excellent public health record of commercially canned meats is thought to be due to death of some spores by heating and failure of the viable, but injured spores to grow in the presence of sodium chloride and sodium nitrite. The addition of at least 78 ppm of sodium nitrite seems to be necessary for obtaining a stable product with present day processing conditions (Silliker *et al*, 1958). During processing the concentration of nitrite may fall to 20 ppm or lower. After processing the residual nitrite disappears at a rate which depends on the storage time and temperature (Nordin *et al*, 1966). At 30°C the half-life of nitrite in canned ham at pH 6.0 is 5 days (Nordin *et al*, 1966). [illegible]spite the loss of nitrite the stability of the product usually maintained.

The role of nitrite in maintaining stability is not fully understood. Some possible roles of nitrite are: a) to enhance the destruction of spores by heat; b) to increase the rate of spore germination during thermal processing, with subsequent killing of the germinated spore by heat; c) to prevent growth of the germinated spores which survive thermal processing; d) to react with some component of the meat to form an antimicrobial compound. The latter hypothesis has been suggested by a number of investigators (Castellani and Niven, 1955; Inklaar, 1967; Perigo *et al*, 1967; Perigo and Roberts, 1968). Recently Perigo *et al* (1967) reported that such a bacterial inhibitor can be formed in a bacteriological medium containing concentrations of sodium nitrite as low as 5 – 10 ppm. Maximum inhibitor formation occurred when the medium was heated with sodium nitrite at 109°C for 20 min. Unlike nitrite (Castellani and Niven, 1955; Silliker *et al*, 1958; Roberts and Ingram, 1966), the inhibitory activity was not pH dependent. The inhibitor was formed with concentrations of nitrite that were too low to be themselves inhibitory; for brevity this will be called Perigo inhibitor.

Viable spores of *Clostridium botulinum* have been isolated from cured meats processed by heating after the nitrite concentration has decreased to less than 5 ppm (Pivnick *et al*, unpublished data). Thus, the absence of growth cannot be explained solely on the basis of residual nitrite. An attractive hypothesis to explain the failure of these spores to grow would be the formation in meat of an inhibitor similar to that found by Perigo *et al* (1967) in bacteriological medium. This investigation was undertaken to determine if nitrite can interact with meat to form a compound which prevents the growth of spores of *C. botulinum*.

Materials and Methods

Spore suspensions. Spore suspensions of *C. botulinum* strain 13983B were prepared and stored as described by Schmidt *et al* (1962).

Meat samples. Comminuted pork luncheon meat was thermally processed in 300 x 308 cans by Canada Packers, Ltd., Toronto. The meat, prior to processing, contained various levels of sodium nitrite as described later.

Media. A medium (PT medium) similar to that described by Perigo *et al* (1967) was made with 2% tryptone (Difco), 1% peptone (Difco), 1% beef extract (B.D.H.), 0.5% yeast extract (Difco), 0.5% sodium chloride, 0.25% dibasic potassium phosphate, 0.2 glucose, 0.1% soluble starch, 0.1% sodium thioglycollate and 0.008% bromo-cresol purple. The pH of the medium was adjusted to 6.0 and sodium nitrite was added to the medium prior to autoclaving for 20 min at 110°C. In some cases, filter-sterilized nitrite was added to

Table 1. Inhibition of growth of Clostridium botulinum spores by sodium nitrite in PT medium and in meat suspended in water.

Sodium nitrite added (ppm)	Number of tubes showing growth[a]			
	PT medium	Percent meat in suspension[b]		
		10%	20%	50%
0	6	6	6	6
20	0 (7)[c]	6 (19)	6 (15)	6 (5)
50	0 (23)	6 (46)	6 (39)	6 (22)
150	0 (70)	0 (129)	0 (107)	0 (102)

[a] Number of tubes out of 6 showing growth within 14 days.
[b] Meat devoid of nitrite was heat processed in cans for 90 min at 115.6°C, ground with an equal amount of water to obtain a 50% suspension, and further diluted with water.
[c] Figures in brackets refer to ppm of nitrite immediately after heating at 110°C for 20 min.

previously autoclaved medium.

Spores were enumerated in Prickett tubes containing Wynne's agar (Difco) with 0.1% filter-sterilized sodium bicarbonate added after autoclaving.

Nitrite analysis. Nitrite was determined by the method of Kamm *et al* (1965) and expressed as ppm of sodium nitrite.

Preparation of meat suspension. Fifty percent meat suspensions were obtained by blending 340 gm of previously heat processed canned meat with either 170 ml of medium or water. Further dilution (v/v) of the meat was made, as required, with medium or water. The pH of these suspensions was 6.0 — 6.2. Filter sterilized sodium nitrite (maximum volume 1 ml per 100 ml of meat suspension) was mixed with the meat suspension which was then dispensed into 16 mm test tubes in 10 - 15 ml quantities. The tubes of meat suspension were subjected to 2 types of heating: a) 5 min at 100°C to drive off oxygen or b) 20 min at 110°C in an attempt to produce a Perigo inhibitor.

Inoculation. After heating, each tube of medium was centrally inoculated with 6 - 8 spores contained in 0.1 ml of suspension. After inoculation, media were layered with Vaspar and incubated at 30°C. Gas production was used at the criterion for growth and tubes were observed after 4, 7 and 14 days. Uninoculated tubes were used as controls in each experiment.

Results and Discussion

Table 1 shows the results of an experiment designed to determine if an inhibitor could be formed in meat suspension similar to that obtained by Perigo *et al* (1967) in laboratory medium. More nitrite was required to prevent growth of *C. botulinum* in meat suspension than in PT medium (both heated at 110°C for 20 min). The addition of 20 ppm of nitrite (7 ppm remained after autoclaving) prevented growth in PT medium. In contrast meat suspensions autoclaved with 50 ppm did not prevent growth despite a residuum of 22 - 46 ppm of nitrite. Meat suspensions autoclaved with 150 ppm of nitrite, and containing a residuum of over 100 ppm after autoclaving, prevented growth. Although nitrite disappeared during heating in direct proportion to the concentration of meat there was no indication that the concentration of meat or the amount of nitrite that had disappeared influenced inhibition of growth of the inoculum. It appeared that inhibition of growth in meat suspension was due to 102 - 129 ppm of residual sodium nitrite.

This finding was further examined by aseptically dialyzing PT medium and 50% meat suspension, both previously heated with nitrite at 110°C for 20 min, against nitrite-free PT medium and meat suspension respectively. After dialysis, the media and meat suspensions from the dialysis sacs were tubed. The tubes were then heated at 100°C for 5 min to remove oxygen and inoculated. Dialysis of PT medium heated with as little as 20 ppm of nitrite (Table 2) did not remove the inhibitor while dialysis of the meat suspension heated with 150 ppm of nitrite at 110°C for 20 min caused it to become non-inhibitory. Thus, the 2 types of inhibition shown in Table 1 were due in one case (PT medium) to non-dialyzable inhibitory agent and in the other (meat suspension) to a dialyzable agent. It is probable that the dialyzable agent was nitrite: at pH 6.0, 100 ppm of sodium nitrite inhibits growth of some *Clostridium* species (Duncan and Foster, 1968a,b; Roberts and Ingram, 1966).

Perigo *et al* (1967) showed that the amount of inhibitor formed in their medium was directly proportional to the amount of nitrite present at the time of heating. To determine if a Perigo inhibitor was formed in meat during commercial processing, meat containing 0 — 200 ppm of sodium nitrite was pro-

Table 2. Effect of dialysis on removal of inhibitory activity from PT medium and meat suspension heated with nitrite.

Sodium nitrite added (ppm)	Number of tubes showing growth[a]			
	PT medium		50% meat suspension[b]	
	Before dialysis	After dialysis	Before dialysis	After dialysis
0	6	6	6	6
20	0	0	6	6
50	0	0	6	6
150	0	0	0	6

[a] Six tubes inoculated.
[b] Meat devoid of nitrite was heat processed in cans for 90 min at 115.6°C, ground with an equal amount of water to obtain a 50% suspension, and further diluted with water.

Table 3. Effect of supplemental sodium nitrite on the growth of spores of Clostridium botulinum in meat suspension.

Nitrite (ppm) in canned meat[a]			Number of tubes showing growth[b]		
Added before canning	Found after thermal process	Days of incubation	Adjusted nitrite (ppm in 50% meat suspension) 80	100	120
0	0	0	(72)[c]	(82)	(107)
		4	20(33)	19(59)	20(70)
		7	20(28)	20(35)	20(60)
		14	20(21)	20(29)	20(43)
75	6	0	(81)	(95)	(106)
		4	19(50)	13(68)	6(76)
		7	19(28)	13(32)	6(60)
		14	19(30)	13(40)	6(56)
150	15	0	(74)	(98)	(114)
		4	20(62)	14(66)	6(78)
		7	20(30)	16(42)	7(64)
		14	20(30)	17(40)	7(55)
200	21	0	(75)	(94)	(106)
		4	17(50)	13(70)	6(78)
		7	18(30)	14(38)	6(55)
		14	18(32)	14(40)	6(58)

[a] Commercially processed at 110°C for 90 min.
[b] Number of tubes showing growth of 20 tubes inoculated.
[c] Figures in brackets refer to ppm of nitrite found immediately after heating at 100°C for 5 min.

cessed at 110°C for 90 min. Regardless of the original concentration, the nitrite was reduced by about 90% during the thermal process (Table 3). When the various lots of canned meat were made into 50% suspensions with water, tubed, heated at 100°C for 5 min, and inoculated, all supported growth. This showed that if a Perigo inhibitor was present, it was not detectable by this method.

However, even sub-detectable amounts of a Perigo inhibitor, if present, might augment the inhibitory activity of residual sodium nitrite. This supposition was tested in the following experiment: the 50% suspensions of meat were supplemented with sufficent sterile nitrite to obtain 80, 100, and 120 ppm, tubed, and inoculated as above. Table 3 shows that in the suspensions supplemented to obtain 80 ppm of nitrite, there was little or no inhibition of growth. When the residual nitrite was supplemented to obtain 100 and 120 ppm of nitrite, there was no inhibition in meat suspension made from canned meat commercially produced without nitrite. Canned meat which contained nitrite during commercial processing, however, gave suspensions that were inhibitory: but in these suspensions the amount of inhibition was related only to the concentration of nitrite at time of inoculation and not to the concentration of nitrite originally present in the raw meat prior to commercial processing. For example, canned meat made with 75 and 200 ppm of nitrite gave suspensions which, when supplemented to obtain 100 ppm of nitrite, allowed growth in 13 to 14 of 20 inoculated tubes. The same suspensions supplemented to 120 ppm of nitrite allowed growth in only 6 of 20 tubes. Increasing the supplemental nitrite in these suspensions to obtain 150 ppm (not shown in Table 3) prevented growth in all suspensions, even those made from canned meat devoid of nitrite at time of processing.

An explanation for the above observations is not readily available. Possibly some Perigo inhibitor was formed when meat was processed with nitrite but if this were so, one would expect increased inhibitory activity with increasing concentrations of nitrite present at time of thermal processing (Perigo *et al.* 1967). We did not find this: 75, 150, and 200 ppm of nitrite added before processing all had the same effect. A second possibility is that thermal processing of meat with nitrite changed the meat in some way to enhance the antimicrobial activity of the supplemental nitrite. We have found (unpublished experiments) that 50% suspensions, made from commercially canned meat additionally heated at 80°C for 4 days, required less supplemental nitrite to inhibit *C. botulinum* than suspensions made from meat that did not receive the additional heating. A third possibility is that suspensions made from meat commercially processed without nitrite lose the supplemental nitrite more rapidly than

Table 4. Effect of concentration of meat and sodium nitrite on Perigo inhibitor activity in PT medium.

Sodium nitrite added (ppm)	Number of tubes showing growth[a]					
	0	0.1	1.0	10.0	20.0	50.0
0	6	6	6	6	6	6
20	0	0	4	6	6	6
50	0	0	0	1	6	6
150	0	0	0	0	0	0

[a] Six tubes were inoculated.
[b] Perigo medium containing nitrite and meat was heated at 110°C for 20 min.

Table 5. Effect of components of canned thermally processed meat on Perigo inhibitor activity.

Sodium nitrite added (ppm)	Number of tubes showing growth[a] PT medium with: No addition (A)	Meat (B)	Fat (C)	Meat extract (D)	Non-fat solids (E)
0	6	6	6	6	6
20	0	6	0	0	6
50	0	6	0	0	6
150	0	0	0	0	0

[a] Six tubes inoculated.

spensions made from meat which contained nitrite at e of processing. However, the residual nitrites own in Table 3 negate the latter hypothesis.

Additional attempts to study the possible forma- n of a Perigo inhibitor in meat were made by heat- PT medium containing various levels of nitrite and eat at 110°C or 20 min (Table 4). As little as 1% meat in PT medium interfered with the expression the inhibitor and with 20% or more of meat there no inhibitory activity despite the addition of 50 pm of nitrite to the PT medium. It is likely that the dia containing 150 ppm of nitrite were inhibitory cause of the residual nitrite (> 100 ppm) after ating at 110°C for 20 min.

Similar experiments were done to determine ether the failure to obtain inhibition in the pre- nce meat (Table 4) was due to prevention of the rma a of an inhibitor or due to prevention of tivity of an inhibitor. For these experiments, in ntrast to those shown in Table 4, we added sterile eat suspension to PT medium previously heated with trite at 110°C to form the Perigo inhibitor. The final ncentrations of meat and nitrite were identical with se shown in Table 4, and the final concentration of T medium was also the same. The results were i- ntical with those shown in Table 4, indicating that at prevented activity rather than formation of the hibitor.

In an attempt to determine which component of at was responsible for interfering with the Perigo hibitor, the following experiment was performed. A % suspension was made from meat processed without trite; then the suspension was centrifuged, refrige- ted, and the hardened fat removed. Meat extract pernatant was obtained by centrifuging the defatted maindar at 34,000 x G for 20 min and the non-fat id pellet was washed twice in distilled water.

Five media (A-E) were prepared (Table 5) and ch was supplemented with nitrite as shown. PT dium (A) was prepared as described previously. remaining 4 media were prepared by adding 50 ml double strength PT medium to the following: (B) al of 50% meat suspension; (C) fat; (D) meat tract; and (E) washed non-fat solids. Fat, meat ex- and solids, each from 50 ml of 50% meat suspen- e diluted with water to obtain a total volume ml. All media were dispensed in tubes, heated at C for 20 min, and inoculated as before.

Neither fat nor the water soluble meat extract vented the activity of Perigo inhibitor (Table 5). ditional experiments with commercially available lard (25%) and gelatin (10%) in PT medium showed that these materials also did not prevent the activity of Perigo inhibitor. The only component of canned meat which prevented activity of the inhibitor was the non-fat, non-water soluble portion.

Conclusions

An inhibitor of the type found by Perigo *et al* (1967) in bacteriological medium could not be demonstrated in meat, and appears to be of little importance in explaining the role of nitrite in the safety of canned meat produced under commercial conditions. The nitrite that disappears during normal thermal processing of meat does not form a potent antimicrobial agent although under special conditions (small numbers of spores and high concentrations of supplemental nitrite) some inhibitory activity could be demonstrated. The mechanism by which nitrite acts as a preservative in canned meat still remains unexplained.

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Effect of High-Nitrate Consumption on Lactation and Vitamin A Nutrition of Dairy Cows [1]

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Abstract

Groups of dairy cows obtaining about one-half of their total digestible nutrients (TDN) from high nitrate corn silage 0.78% KNO_3, dry basis) or from regular corn silage (0.40% KNO_3, dry basis) with either 1.25% or 0.75% KNO_3 added to their diet dry matter continued to produce normal outputs of milk with normal fat, protein, and vitamin A content during a nine-wk period. There was no demonstrable effect of nitrate supplementation on hemoglobin, on plasma carotene and vitamin A, or on liver carotene and vitamin A. Cows of different milking levels secreted in milk 1% to 2% of the two to more than three million I. U. of vitamin A consumed as carotene with no demonstrable effect of the nitrate levels being consumed. The 24 cows were quite consistent in having 55 I. U. to 65 I. U. of vitamin A per gram of milk fat produced. There was no observed effect of the nitrate feeding on the reproductive performance of the cows. Milk cows fed good rations showed no evidence of adverse effects to sublethal nitrate intakes even early in the experiment.

Experimental Procedure

Following the ensiling of a fall-seeded crop of vetch and oats, a 4.86 ha field was plowed, disked, and harrowed for corn planting in June, 1962. Previous to planting, ammonium/nitrate at the rate of 280 kg/ha and superphosphate to supply 112 kg P_2O_5/ha were applied with a spreader and harrowed into the soil. The field was divided into three areas and the two outside areas planted with round seed-corn (Oregon 150 variety) about 15 cm apart, in rows 71 cm apart. The inside 1.62 ha was thickly seeded with a regular grain drill, using 112 kg/ha of the same variety of small, round kernels of corn. The field was sprayed with Atrazine following planting on June 20 and was sprinkler-irrigated with 10.2 cm of water. The corn received two additional irrigations at 3 and 6 wk of about 7.6 cm of water per irrigation.

The corn on the thickly seeded area grew 1.8 to 2.4 m tall without ear development and lodged considerably in late August. It was cut with a flail harvester and ensiled on September 11 and 12 in a 3.7- by 9.1-m wood-stave silo. The forage yielding 56.8 mt/ha averaged only 13.55% dry matter, or 7.68 mt dry matter per ha. The normally seeded corn stood erect and was cut with a corn forage harvester on September 26 and 27 and ensiled in a companion 3.7- by 9.1-m wood-stave silo. The corn yielded 43.9 mt of green forage per ha and averaged 18.51% dry matter, or 8.12 mt dry matter per ha.

A composite obtained by sampling each load of chopped corn at the time of ensiling contained 1.68% KNO_3 on a dry basis for the thick-seeded forage and 0.81% KNO_3 for the corn seeded normally. Samples of the silages analyzed four months later, when the feeding studies were being started, showed a large decrease in KNO_3 during ensiling. The thick-seeded silage had almost double the KNO_3 content of the corn normally seeded, respective values being 0.93 and 0.53% on a dry basis. Based upon the experimental reports referred to, feeding of large amounts of the high-nitrate silage might be expected to prove detrimental to the performance, or even dangerous to the health, of milk cows.

Effects of feeding large amounts of these corn silages to lactating cows were studied according to the two following procedures:

Procedure 1. Eight cows were paired into four groups and fed a basal ration of the normally seeded corn silage, a small amount of alfalfa-hay wafers, and concentrates according to production. After 4 wk on the basal ration, the groups were fed for the 9-wk experimental period as follows:

Group A. Continuation of feeding the basal ration to an early-lactation, high-producing Holstein and to a high-producing, early-lactation identical Jersey twin.

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Group B. Same basal ration supplemented with 1.25% KNO_3 based on the daily dry matter intake, calculated weekly, fed to a high-producing Holstein cow and to the identical-twin Jersey mate of the cow in Group A.

Group C. Same basal ration fed to mid-lactation pregnant Holstein and Jersey cows.

Group D. Same basal ration supplemented with 0.75% KNO_3, based on daily dry matter consumption, fed to mid-lactation pregnant cows paired with those in Group C.

Procedure 2. Sixteen high-producing cows in early lactation were grouped as closely as possible in milk production into four groups consisting of three Holsteins and a Jersey, and were fed the following rations:

Group E. Normally seeded corn silage and concentrate pellets.

Group F. Thickly seeded high-nitrate corn silage and concentrate pellets.

Group G. Same as Group F with limited alfalfa-hay wafers.

Group H. Same as Group F with injections of vitamins A, D, and E.

During both procedures the cows were individually fed ad libitum twice daily weighed amounts of corn silage, and concentrate pellets according to production. The alfalfa wafers were fed once daily. Silage or any other feed refused by individual cows was weighed back and recorded daily. The cows were milked twice daily, with the amount of milk recorded. Individual night and morning milk samples were taken weekly and tested for fat, protein, solids-not-fat, carotene, and vitamin A. Milk protein was determined by a slight modification of the Dolby (9) Amido Black dye-binding procedure, solids-not-fat by the Golding (14) bead test, and vitamin A and carotene in the milk by the method of Boyer et al. (2). Carotene and vitamin A values were calculated in IU per 100 ml milk and per gram of fat, using 0.6 γ carotene and 0.3 γ vitamin A as 1 IU. The same factors were used in converting blood plasma and liver carotene and vitamin A determinations to total IU.

The cows in Procedure 1 were bled at the start of the experiment and at 3-wk intervals thereafter. Blood was analyzed for hemoglobin, carotene, and vitamin A. Hemoglobin was determined using a Spencer hemoglobinometer and Evelyn's (11) method for methemoglobin. A modification (milk saponification) of Kimble's (24) method was used for analyses of blood samples for carotene and vitamin A. On the day of bleeding, liver biopsy samples, using the technique of Bone (1), were obtained for carotene and vitamin A determinations. The procedure for the analysis of the liver for vitamin A and carotene was that of Gallup and Hoefer (12).

Each cow in Group H was given an intramuscular injection of 5 ml of a mixed vitamin preparation at the end of the 4-wk preliminary period and every 3 wk thereafter. Each milliliter contained 100,000 IU vitamin A palmitate, 100,000 IU calciferol, and 10 mg *dl*-alpha tocopherol acetate in a 2% benzyl alcohol, sesame oil solution.

Results

Samples of the normally seeded and thickly seeded corn silage were taken daily, dried, composited, and analyzed (results given in Table 1). While the normally seeded silage averaged 0.40% KNO_3 on a dry basis, the thickly seeded silage contained 0.78% KNO_3, an amount that

TABLE 1

Composition of feeds as fed

	Dry matter	Crude protein	TDN [a]	KNO_3	Carotene
	(%)				(mg/kg)
Corn silage, thick seeding [b]	18.03	1.88	9.6	0.141	32.65
Corn silage, normal seeding [c]	17.23	1.76	10.4	0.069	46.62
Alfalfa wafers [d]	90.50	15.30	50.7	.....	33.00
Concentrate pellets	90.00	18.00	73.0	.....	.....

[a] Digestible nutrients in thick-seeded corn silage were determined in a total-collection digestion trial with four bred dairy heifers. TDN values of other feeds were calculated from Morrison's (28) tables of digestion coefficients.

[b] Composite of chopped corn forage, thick seeding, at time of ensiling analyzed 1.68% KNO_3 on a dry basis.

[c] Composite of chopped corn forage, normal seeding, at time of ensiling analyzed 0.81% KNO_3 on a dry basis.

[d] Two-year-old wafers, hence carotene-low.

might be expected to affect the performance of the cows (13).

A summary of the average daily feed consumption of the individual cows by groups is given in Table 2. It was found that the KNO_3 sprinkled on top of the silage reduced its palatability. To insure the consumption of all of the KNO_3 supplement, the daily allowance of each cow was divided into two portions for twice-daily feeding and thoroughly mixed with 6.8 kg of the corn silage. Additional silage was fed after this was eaten. However, there was about 25% less silage consumed with the 1.25% KNO_3 supplement, and it was necessary to feed more concentrates, to maintain production. Silage consumption was correspondingly less with the groups fed a small alfalfa-wafer allowance. As seen in Table 2, the dry matter and calculated digestible protein and TDN intakes were quite similar for all groups of cows, except for the lower-producers in Groups C and D. All of the rations were highly digestible, ranging from about 66% calculated TDN for Groups C and D to over 70% TDN for Groups B, E, and F.

The average daily KNO_3 intake from silage and supplement of cows in Groups A, B, C, and D is shown graphically by weeks in Figure 1 and during the entire feeding period for each group in Table 2. No determinations or estimates were made of the nitrate consumed in the alfalfa wafers, concentrates, and water. The variation in KNO_3 intake varied from 0.16% of the dry matter for cows in Group A to 1.47% in Group B. The Holstein cow of Group B, weighing 655 kg, consumed an average of 254 g

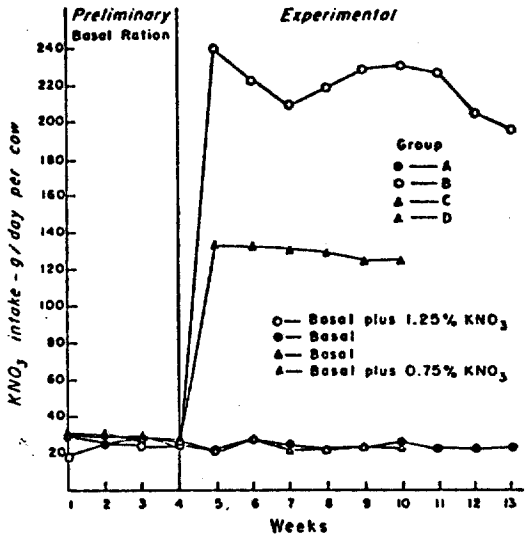

FIG. 1. Average nitrate intake per cow daily. Procedure 1.

of potassium nitrate daily during the 9-wk feeding trial and the 455-kg identical-twin Jersey in this group had an average intake of 186 g daily. As seen in Table 2, cows in Groups F and H obtained about 70 g of KNO_3 daily from the corn silage. This amounted to an average intake of 126 mg/kg of body weight for cows in Groups F and H, whereas the KNO_3-supplemented cows in Groups B and D consumed, respectively, 404 and 236 mg/kg. The KNO_3 intake of control cows in Groups A, C, and E, respectively, amounted to 45, 45, and 61 mg/kg of body weight.

The milk production, protein, and fat in the

TABLE 2

Feed ingredients consumed per cow daily by groups

Group	Corn silage	Alfalfa wafers	Concentrates	Dry matter	Digestible protein	TDN	KNO_3	KNO_3 in dry matter
	(kg)						(g)	(%)
			Procedure 1					
A	34.47[a]	3.58	6.99	15.42	2.40	10.52	24	0.16
B	26.30[a]	3.37	8.71	14.97	2.46	10.48	220[c]	1.47
C	34.02[a]	3.36	4.85	13.25	1.94	8.80	23	0.17
D	35.83[a]	3.27	4.08	12.79	1.86	8.39	128[d]	1.00
			Procedure 2					
E	48.86[a]	.....	8.20	15.80	2.34	11.12	34	0.22
F	48.49[b]	.....	8.12	16.00	2.36	11.37	70	0.44
G	41.78[b]	3.87	7.64	17.74	2.73	12.04	59	0.33
H[e]	47.78[b]	.....	7.86	15.78	2.31	10.86	68	0.43

[a] Normal seeding, 280 kg nitrogen per ha.
[b] Thick seeding, 280 kg nitrogen per ha.
[c] Including 202 g KNO_3 supplement.
[d] Including 103 g KNO_3 supplement.
[e] Also 500,000 IU vitamin A palmitate, 500,000 IU calciferol, and 50 mg *dl*-alpha tocopherol acetate injection every week.

milk of cows in Groups A, B, C, and D are shown in Figures 2 and 3. The high-producing, early-lactation cows in Group B, fed the basal ration plus a supplement of KNO_3 calculated as 1.25% of their dry matter intake, maintained production over a 9-wk feeding period, as well as Group A cows fed the basal ration. Likewise, the mid-lactation, pregnant cows in Group D fed 0.75% supplementary KNO_3 produced as well as control cows in Group C. As seen in Figure 3, nitrate feeding did not affect milk protein or milk fat.

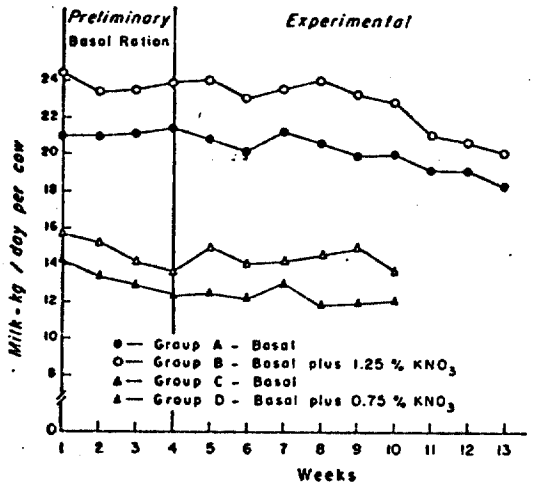

FIG. 2. Average milk yield per cow daily. Procedure 1.

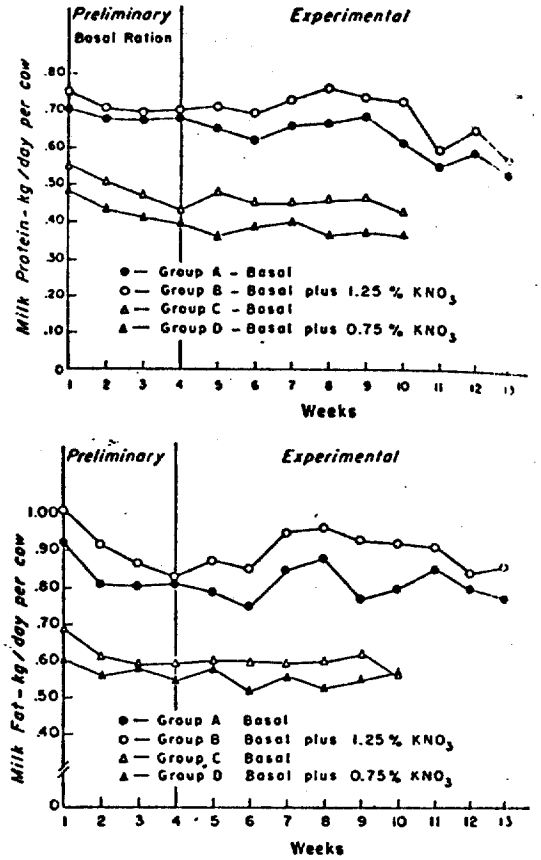

FIG. 3. Milk protein and milk fat per cow daily. Procedure 1.

The milk yield of cows in Groups E, F, G, and H is presented graphically in Figure 4, and the milk constituents by groups in Table 3. The lowest average milk production was with cows in Group F, obtaining more than half of their dry matter from thick-seeded, 0.78% KNO_3 corn silage. As seen in Figure 4, cows in Group G, fed 3 to 4 kg of alfalfa wafers daily, maintained a more uniform level of milk flow than cows in Group F, but the average differences among groups shown in Table 3 were not statistically significant. Likewise, there was no significant improvement with the intramuscular injection of vitamins A, D, and E with cows in Group H.

The total IU of vitamin A in milk produced per cow daily by Groups A, B, C, and D is shown in Figure 5. Addition of either 1.25 or 0.75% KNO_3 to the dry matter did not affect the vitamin A content of the milk. The conversion of carotene and vitamin A consumed, mostly corn silage carotene, to carotene and vitamin A in milk is summarized in IU of vitamin A per cow daily by groups in Table 4. Cows in Group E, consuming large amounts of the normally seeded corn silage with its high carotene content, produced the largest amount of vitamin A in their milk. The proportion of the total vitamin A value of the milk occurring as carotene varied from a low of 19.5% for a Holstein cow to 40.8% for a Jersey. Jerseys and Holsteins produced milks averaging 61.5 and 76.0%, respectively, of their total vitamin A values in the form of vitamin A. From the 199 milk samples listed in Table 4, about one-third of the vitamin A activity occurred in the form of carotene and about two-thirds as preformed vitamin A. The percentage recovery in milk of the consumed vitamin A varied from about 1% for the cows in the lower-producing Groups C and D to about 2% for the higher producers. As seen in Table 4, there was a remarkably constant 55 to 60 IU of vitamin A per gram of milk fat for cows in all groups. For all samples, the mean IU vitamin A value was 59.1 ± 0.95 per gram of fat. Calculated in terms of a kilogram of 81% fat in butter, the average potency would be 47,870 IU kg.

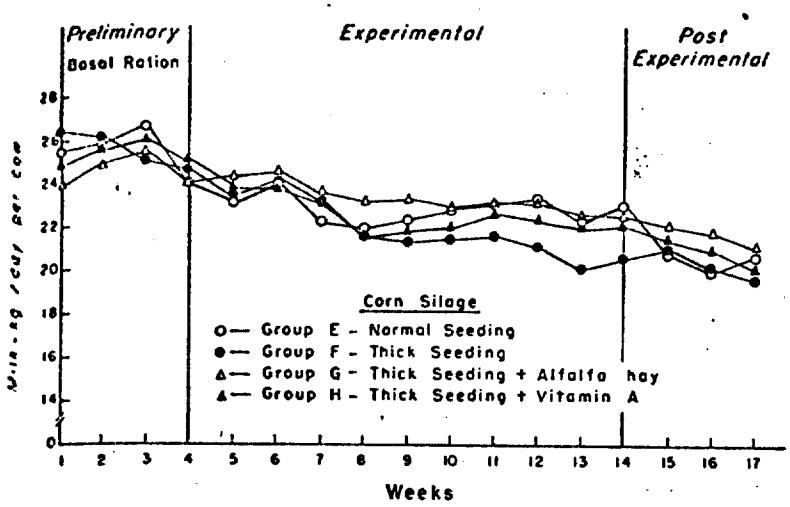

FIG. 4. Average milk yield per cow daily. Procedure 2.

TABLE 3

Milk and its constituents produced per cow daily by groups

Procedure 2

Group	Milk [a]	Fat [a]	Solids-not-fat [a]	Protein [a]	4% ECM [b]
	(kg)				
E	22.8	0.85	1.93	0.68	20.7
F	21.9	0.84	1.94	0.67	20.2
G	23.2	0.81	2.00	0.70	20.2
H	22.7	0.78	1.89	0.66	19.6

[a] Differences among groups are not statistically significant.
[b] Energy-Corrected Milk (30) = 15.5 F + 0.33 M.

higher than the reported values of 40,180 IU/kg for Oregon June butter (38).

The plasma carotene and vitamin A values for cows in Groups A, B, C, and D at the start of the feeding and at 3-wk intervals are shown in Figure 6. Initial plasma carotene and vitamin A values reflected different stages of lactation and different levels of milk production. The variations continued throughout the feeding, with the higher values tending to reflect the higher intakes of corn silage. There was no effect of nitrate supplementation on the plasma carotene and vitamin A values.

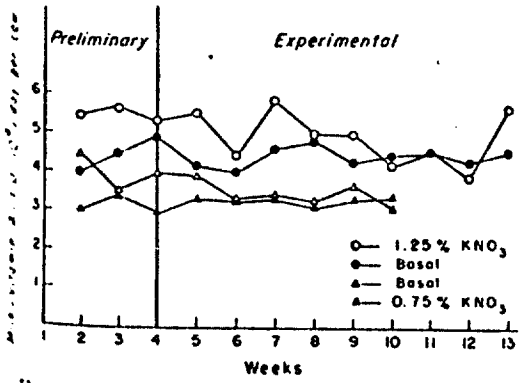

FIG. 5. Milk vitamin A per cow daily. Procedure 1.

The liver carotene and liver vitamin A values for cows receiving the basal ration for 4 wk and then either continued on the basal ration or fed KNO_3 during three biopsy periods of 3 wk each are presented in Figure 7. The initial liver carotene values averaging about 11 γ/g of fresh liver agree well with earlier reported values from this station, but liver vitamin A values of about 80 γ/g were somewhat lower (19). The feeding of KNO_3 over the 9-wk period did not interfere with liver carotene, but liver vitamin A tended to decrease. Milk vitamin A per kilogram of fat tended to increase with advancing lactation of the cow.

Results of blood hemoglobin determinations are given in Figure 8. While there was considerable individual cow variation, with cows having high or low initial values tending to remain high or low over the sampling period, the average of values at the initial sampling of 11.2 g/100 ml agrees with expected hemoglobin values (5). There was no indication of nitrate supplementation affecting the hemoglobin level

TABLE 4

Conversion of carotene and vitamin A consumed to carotene and vitamin A in milk produced per cow daily and vitamin A per gram of milk fat

Group	Vitamin A intake IU	Vitamin A activity in milk IU	Vitamin A recovery	IU vitamin A/g milk fat		
				Samples	Mean	S.D.M.
	(1,000)	(1,000)	(%)	(no.)		
			Procedure 1			
A	2,859	44.7	1.6	18	54.5	1.18
B	2,225	48.9	2.2	18	54.9	1.82
C	2,817	33.6	1.2	15	56.0	1.52
D	2,955	31.2	1.1	12	57.7	3.69
			Procedure 2			
E	3,430	54.6	1.6	36	65.8	1.77
F	2,596	44.8	1.7	30	56.7	3.02
G	2,617	47.3	1.8	35	60.6	2.97
H	2,321	48.0	2.1	35	59.2	2.47

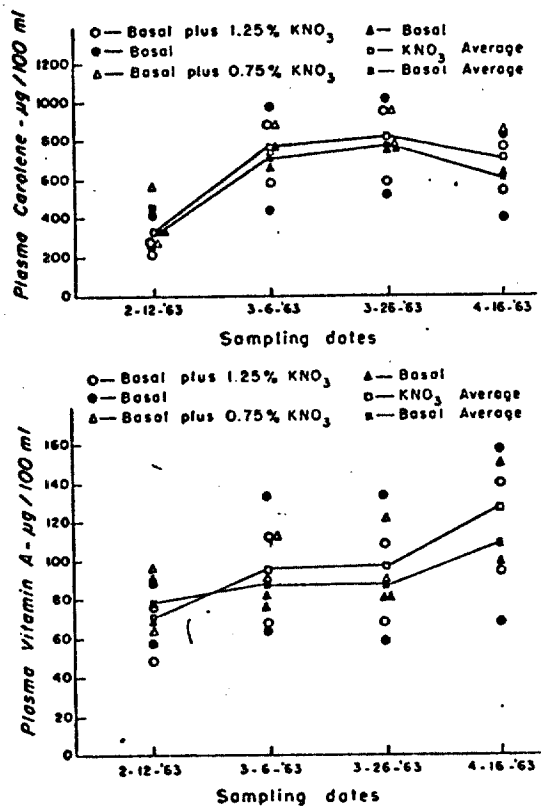

FIG. 6. Plasma carotene and plasma vitamin A at 3-wk intervals. Procedure 1.

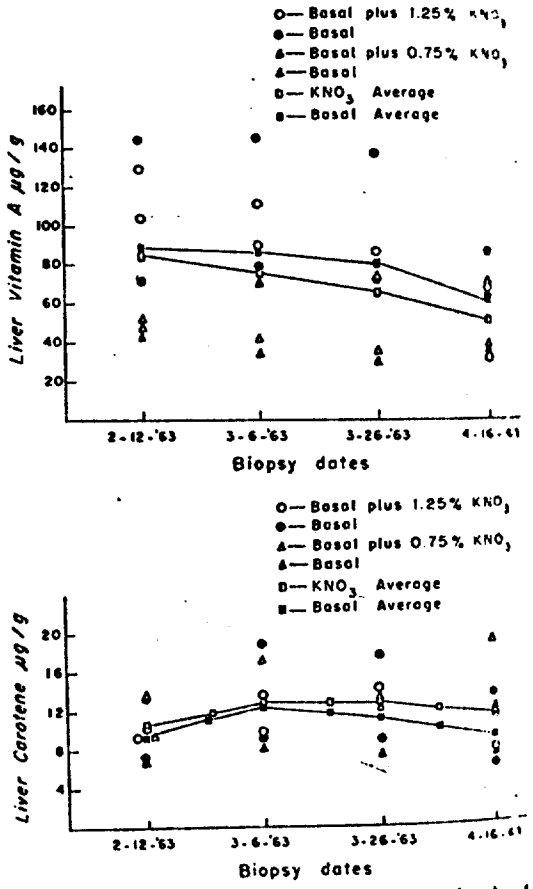

FIG. 7. Liver carotene and liver vitamin A at 3-wk intervals. Procedure 1.

of the blood during the first 6 wk, but at 9 wk two of the nitrate-supplemented cows had rather low hemoglobin values. At this 9-wk sampling period the average hemoglobin and methemoglobin values per 100 ml of plasma for the basal group were, respectively, 11.8 and 0.31, and for the nitrate-fed cows 10.6 and 0.18.

There was no indication of the high nitrate intake affecting the health of the cows. No cow went off-feed and, in addition to producing normal milk at a high level, all cows gained weight, the average daily gain for Groups A through H, respectively, being 204, 136, 22[illegible]

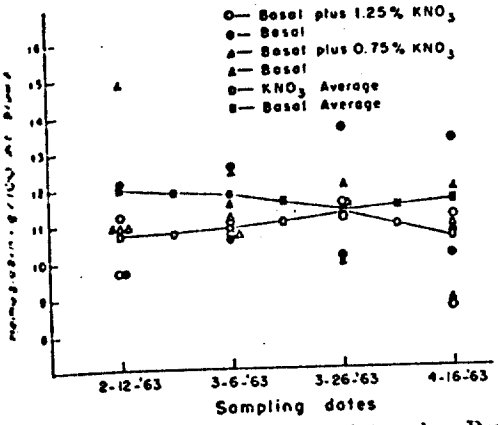

FIG. 8. Hemoglobin at 3-wk intervals. Procedure 1.

95, 326, 198, 261, and 242 g. All except one of the nitrate-fed cows were pregnant during the experimental period and continued for normal gestations. Reproductive behavior was likewise normal for cows in Groups F, G, and H, fed the thick-seeded silage with 0.78% KNO_3 in the dry matter.

Discussion

The primary objective of this study was to determine if so-called sublethal levels of nitrate, 0.4 to 1.5% KNO_3 in the dry matter consumed, would affect the performance of high-producing dairy cows as measured by milk and its constituents, and their vitamin A nutritional status. The effect on milk composition, particularly the vitamin A value of milk, could be of considerable significance in human nutrition, because milk provides from 15 to 30% of the requirement of vitamin A if consumed in amounts recommended by health authorities (25).

Our results showed no deleterious effect on the milk yield of high-producing cows fed a normal basal ration with the addition of either 0.75 or 1.25% KNO_3 based on their dry matter intake (Figures 1 and 2), or when corn silage with 0.78% KNO_3 on a dry basis provided about half of the dry matter consumed (Figure 4). This finding corroborates results of Davison et al. (7), in a controlled experiment in which dairy heifers fed sodium nitrate at levels of 110 and 660 mg NO_3-N per kilogram body weight produced milk for about one month following calving as well as control heifers receiving the same ration without nitrate supplementation. A similar conclusion was reached by Morris et al. (27) after feeding lactating cows high-nitrate roughage over a 4-wk period. Davison et al. (7) found an increase in the nitrate content of milk, but the percentages of milk fat and protein were not affected, which agrees with our studies (Figure 3 and Table 3). Davison et al. (7) did not report on the vitamin A and carotene content of the milk which our studies show not to have been affected by the ration fed (Figure 5 and Table 5).

A decreased consumption of hay with nitrate additions was reported by Davison et al. (7). In our studies, a decreased consumption of corn silage with nitrate additions is shown in Table 2 for Group B cows. These cows were fed increased concentrates so that the TDN consumption was equal to that of the control cows in Group A. A decreased feed intake was reported with steers (17, 37) following nitrate additions to the rations.

Plasma carotene and vitamin A values are often variable, but are useful in indicating whether the day-by-day intake is adequate. Plasma values shown in Figure 6 were in the range (21) for lactating cows on normal rations of grass or corn silage and sun-cured alfalfa hay, and were not affected by the nitrate additions.

The liver vitamin A values of 40 to 140 γ/g are about as expected from earlier reports (4, 21, 39) with cows fed high-carotene rations and agree quite well with fattening cattle results in Michigan (30), where a vitamin A supplement of 20,000 to 30,000 IU per head daily was added to a basal ration with and without nitrate additions, but were much higher than Illinois values (23) for steers wintered on corn silage having a carotene content of 11 mg/kg. The rather low liver vitamin A values of 8 to 40 γ/g with dairy heifers reported by Davison et al. (7) also reflected the low carotene intake, with hay having 2 to 35 mg/kg. In one report (16) in which sheep were fed a low-carotene ration containing 3% sodium nitrate, vitamin A stores in the liver were significantly reduced, but in another study (34) addition of 4% KNO_3 to the dry matter increased liver vitamin A.

The blood for hemoglobin values was taken at the same time of day about 3 hr after feeding. Values tended to remain high or low for individual cows, but showed the variation often found between collections (5, 19). Methemoglobin levels remained low (1.5 to 2.5%) with the 1.5% KNO_3 ration, in agreement with reports in which nitrate was added to rations of dairy cattle (27), steers (37), and sheep (16, 34). When high levels of nitrate (equivalent to about 1 g KNO_3/kg body weight) were fed dairy heifers (19) over long periods of time, or when even larger levels were given in single or multiple doses to calves in capsules or in

milk (32), variable degrees of methemoglobinemia occurred. Jainudeen et al. (19) have reported that dairy heifers compensate for the reduction in oxygen-carrying capacity of the blood with nitrate feeding by an increase in the number of erythrocytes and in hemoglobin concentration. In our studies, lactating cows fed up to 400 mg KNO_3/kg body weight in high TDN rations were able to continue normal milk production, indicating that the rumen flora were breaking down the nitrates to ammonia or that blood changes were rapidly compensating for nitrate being absorbed.

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fection of the beans, provided their specificity is established.

Preliminary tests with beans infected with *X. phaseoli* have indicated that this method holds promise in the diagnosis of common blight as well. This procedure may be applicable to the rapid detection of both plant and animal pathogens or saprophytic organisms in various complex substrates or in simple mixtures of closely related forms.

Details of the experiments discussed above will be published elsewhere.

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The Potentiation of Muscular Contraction by the Nitrate-Ion[1]

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It has long been known that frog skeletal muscles that have been exposed to various anions of the lyotropic series yield potentiated contractions when stimulated by such agents as cold, potassium, or electric shock (*1–5*). There is general agreement in this earlier work that the ions cause a "sensitization" of the excitatory membranes of the muscle fibers, which is then effective in increasing the mechanical response of the whole muscle. Thus, in work most directly pertinent to our own research, Chao (*4*) studied the behavior of frog sartorii exposed to a Ringer's solution normal with respect to potassium and calcium chlorides, but having all the NaCl replaced by an equivalent amount, e.g., of $NaNO_3$. Such muscles were found to quickly develop a reduction in rheobase for electric stimulation; and, furthermore, if they were stimulated with twitch shocks of a *submaximal* intensity, as determined for these muscles when previously equilibrated to ordinary Ringer's solution, their isotonic contractions became greatly augmented after only a few minutes' contact with the nitrate medium. Chao attributed the mechanical potentiation to recruitment of fibers; i.e., the lowering in threshold of the fibers of the nitrate-treated muscle permitted response in those fibers that were not excited by the submaximal shock in normal Ringer's solution, and thus, by adding their outputs to the total twitch response, caused the observed potentiated contractions. In this investigation the effects of other anions were similarly tested, and it was found that the degree of lowering of the rheobase and of the associated enhancement of shortening by submaximal stimulation was a function of the particular anion, the relative effects falling into the usual lyotropic series, $Cl < Br < NO_3 < I < CNS$.

In our research, which is reported here in preliminary form, we have used a procedure rather similar to Chao's, with the important exception that stimulation was effected by originally slightly *supermaximal* shocks. Under these conditions, irrespective of the lowered threshold of the NO_3-treated muscle, no recruitment of fibers is possible; yet such muscles produce greatly potentiated twitches approaching, or even equalling, the strength characteristic of full tetanus output. Full analysis of our results proves that the nitrate ion not only results in an increase in excitability but also causes, among other things to be discussed below, augmentation of the maximal mechanical response of which each fiber is capable.

We have studied the isometric twitch responses of excised curarized[2] frog sartorii when supermaximally shocked by the massive, transverse stimulation procedure (*6*). Records have been made by piezoelectric, cathode-ray methods (*7*) of the latent period changes and, using optical myography, of the associated developed peak tension outputs. All experiments have been done at 25°C. After 1 hr of equilibration in oxygenated phosphate buffered (pH 7.2) normal Ringer's solution, the behavior of the muscle was recorded for 2 responses separated by an interval of 1 min. This chloride-Ringer's solution was then withdrawn from the muscle chamber and replaced by a similarly buffered nitrate-Ringer's solution, through which oxygen was bubbled. In our test medium *all* the chloride was replaced by nitrate, and it is in this sense that we shall refer to our experimental medium as a nitrate-Ringer's solution. In this new environment the muscle was tested at intervals, the stimuli being again massive, transverse electric shocks of the same supermaximal strength as those applied to the muscle in normal Ringer's.

The results of a representative experiment are presented in Fig. 1. The inset at the top of this figure is a diagrammatic sketch of a typical photographic record obtained with our apparatus. The curved line, corresponding to the deflection of the cathode-ray beam in a single sweep, gives the mechanical changes that appear during the twitch latent period. The arrow at the left indicates the instant of stimulation. Following this moment 2 time intervals are presented: L_R, which measures the duration of a mechanically quiescent period —i.e., the latency of the immediately following latency relaxation (*LR*); and L, which measures the time interval of the mechanical latent period for positive tension development. After the termination of L, the record shows in the upward deflection the very beginning of the contraction period. Also on this sketch are indicated 2 tension variables: R, measuring the depth of the *LR*;

[1] Aided by a contract between the Office of Naval Research, Department of the Navy, and the New York University (NR113-300).

[2] We are very grateful to E. R. Squibb & Sons for generously supplying us with the *d*-tubocurarine chloride used in these experiments.

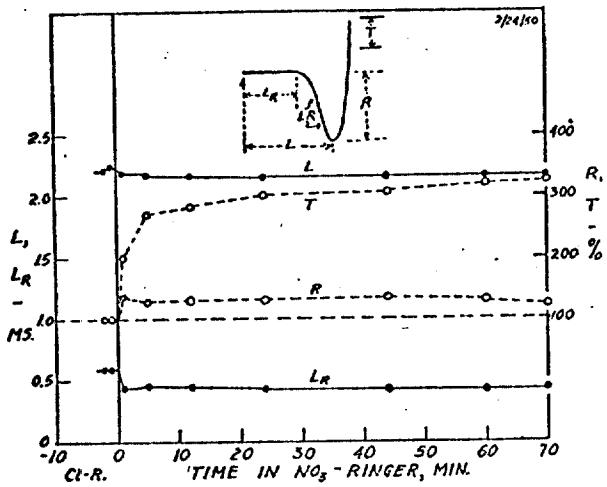

Fig. 1. Effect of nitrate-Ringer's solution on the latent period and peak-developed tension responses of the isometric twitch of the frog sartorius muscle.

and T (upper right corner), which measures the peak tension developed by the contraction. (The upper horizontal line represents the light beam of the optical myograph at a level representing the muscle's resting tension, and the lower line corresponds to the displaced position of the beam at peak of contraction.)

The solid graphed lines give the changes in L_R and L, with the time scaling indicated in ms on the left, vertical axis. Both these time intervals decrease by 0.1–0.2 ms immediately upon immersion of the muscle in the NO_3-Ringer's, and these decreases are maintained throughout the remainder of the 70-min exposure to the experimental medium. These are very small diminutions, but they are definite and have been repeatedly obtained in every experiment performed. The variations in R and T (dashed lines), with their scaling (right vertical axis) in per cent of the chloride-Ringer's outputs, also occur very soon after the muscle is put into the nitrate-Ringer's; the increase in R is about 50%, whereas T is maximally potentiated to about 325%—i.e., to a value about as large as the maximum tetanus tension. Thus, as compared to the chloride-Ringer's behavior, the muscle, when placed in the modified salt solution, quickly develops a small but certain speeding up of the latent period events, a considerable deepening of the latency relaxation, and a quite enormous increase in developed tension. Furthermore, although the data are not shown in the figure, the nitrate-induced changes are all rapidly reversed when the muscle is restored to a normal Ringer's bath.

The highly potentiated peak tensions of the nitrate-Ringer's muscles cannot be accounted for by recruitment of fibers, since the supermaximal shocks we used throughout ensured that even in the chloride-Ringer's all the available fibers of the muscle were stimulated. Nor can these potentiations be a consequence of a sort of tetanization that would occur if the single shock we used evoked a series of action potentials, as occurs, for example, in a veratrinized muscle. For a study of the electrical response of the muscle after exposure to the nitrate-Ringer's (using wire-electrode stimulation and conventional recording technique, with the muscle in a moist chamber) proves that only a single, evidently normal, action potential occurs. This result is shown in Fig. 2, which presents the action potential response of the chloride-Ringer's muscle in the upper record, and of the nitrate-Ringer's muscle in the lower. The much greater tension output of the experimental muscle is incidentally demonstrated also by comparing the tension beam deflections of the 2 muscles in the upper right portion of each record. Our results thus prove that the responses in the nitrate-Ringer's solution are potentiated maximal twitches in which each fiber produces a peak tension of the order of its maximal tetanus strength.

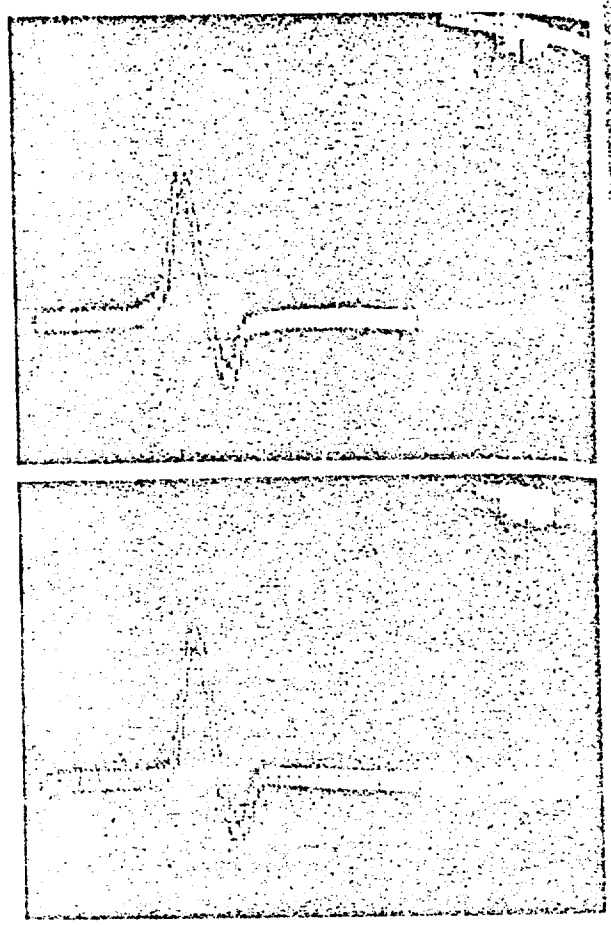

Fig. 2. Records of action-potential and peak-developed tension responses in isometric twitch of frog sartorius muscle: in chloride-Ringer's (upper photograph), and in nitrate-Ringer's (lower photograph). A 5,000 c/sec timing wave is superimposed on each action potential record.

Following Chao's procedure, we have also studied the effects of submaximal shocks (50% of maximal), as determined in chloride-Ringer's, on muscular responses in the nitrate-Ringer's. Here, again, all electrical responses consist of a single action potential, which, however, is much larger in the nitrate medium, thus indicating recruitment of fibers made possible by the nitrate-induced increase in excitability. But the mechanical response of these muscles is very much larger than would be expected if only recruitment of mechanically normal fibers were

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... the muscle in a moist ...gle, evidently normal. ...esult is shown in Fig. 2, ...ential response of the ...per record, and of the ...er. The much greater ...tal muscle is incidentally ...he tension beam deflec... ...r right portion of each ...that the responses in the ...tiated maximal twitches ...k tension of the order

...we have also studied the ...% of maximal), as de... ...muscular responses i... ...n, all electrical respons... ...al, which, however, is ...n, thus indicating re... ...by the nitrate-induced ...ical response of ...uld be expected ...normal fibers were

...volved. Hence, our results in general prove that the ...trate ion does not merely reduce the threshold, but—and ...is is the essential new finding of our research—it also ...an somehow modify the events of the latent period and ...crease the contractile strength of each excited fiber.

It is significant that the effect of nitrate on the ...echanical behavior, both of the latent period and of the ...eak tension development of the contraction period, ...curs so rapidly after immersion of the muscle in the ...itrate-Ringer's solution. This indicates that the nitrate ...on could not have had sufficient time to penetrate into ...the muscle fibers and thus modify the mechanical response ...by a direct action on the contractile system. We therefore infer that this ion directly affects only the surface—i.e., the excitatory membrane of the fibers—and that this ...alteration leads, in turn, to the observed changes in the ...echanical response by a modification of the mechanism ...by which excitation of the membrane by the electric ...shock is coupled to the inner contractile system.

That the membrane is altered is clear. This is proved by the increase in excitability. But it is also demonstrated by the fact that the nitrate ion slightly increases the resting potential (*5*) and considerably enhances the permeability (*8*) of muscle cells. Probably, as Chao suggests (*2, 3*), the increased excitability is a consequence of the greater dispersibility of the membrane under the influence of nitrate, and the possibility that this structural change may underlie the other differences in membrane properties we have mentioned is not excluded.

Our principal interest, however, lies in the influence of nitrate on the mechanism of excitation-contraction (*E-C*) coupling. Our results indicate that this ion has its initial effect on the excitation part of this process. This influence, however, is not on the reactions responsible for the action potential, since the electrical response of the excitatory membrane of each fiber is unaffected. It would therefore seem that some other feature of the excited membrane is altered by the nitrate ion, and that this occurs in such a fashion as to change the subsequent course of the coupling mechanism. The time course of *E-C* coupling extends from the instant of stimulation to the onset of tension change—i.e., it is coincident with the events of the latent period, or at least with the earlier phases of this interval. Hence, it is not surprising that under the influence of nitrate we record latency alterations—the shortening of L_R and L, and the deepening of the latency relaxation. Previous work (*7*) has shown that the *LR* is an expression of the contractile material undergoing activation for its positive tension development. It is therefore evident that these latency mechanical changes may reflect the modified course of the latter portion of *E-C* coupling, during which the chain of events initiated by the action of the stimulus on the membrane becomes linked to the contractile material, and in such manner, enables the fiber-tension output to be so greatly potentiated.

Needless to say, the full elucidation of the mechanisms discussed above requires the performance of much more research. We plan to study the effects of other anions and of a variety of other agents (e.g., of the cholinesterase system) that are believed to play a role in, or to affect, excitability mechanisms. Some insight into the complexity of the processes is indicated in already reported results (*9, 10*) concerned with the effects of the potassium ion. This agent in relatively small concentrations acting for prolonged periods, or in larger concentrations acting for short durations, increases *R* up to 300% or more, potentiates *T* about 25%—effects that are irreversible—and reversibly depresses the action potential. Here, as in the NO_3 effects, the various changes are due to the direct action of K on the excitatory membrane. Yet, as can be seen by comparing the noted actions of these two ions, considerable differences exist regarding the quantitative alterations in *R* and *T*, the action potential behavior, and the possibility of reversal of the induced changes. It is felt, however, that a systematic study of these phenomena will shed some light on the fundamental mechanisms of excitation-contraction coupling, and, since this process activates contraction, it is hoped that any knowledge gained concerning it may also help in furthering comprehension of the contraction process itself.

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The Mechanism of Action of Organic Mercury Compounds on Cytochrome Oxidase

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Several organic mercury compounds (phenylmercuric nitrate [PMN], phenylmercuric hydroxide [PMOH], and *p*-chloromercuribenzoic acid [benzoate]), known to react with the –SH group, have been shown to inhibit the cytochrome c-cytochrome oxidase system at the oxidase portion of the chain (*1*). Since Barron (*2*) and others have considered this oxidase not to be sulfhydryl-dependent, it was suggested that the inhibition of the oxidase (a crude rat heart preparation) was nonspecific and might be due to the particulate nature of the preparation.

Investigations presented in this note, in which a solubilized sodium desoxycholate preparation of cytochrome oxidase (*3*)[1] was used, show that this partially purified enzyme is also inhibited by the mercurials. The in-

[1] The cytochrome oxidase was generously supplied by S. J. Cooperstein, of Western Reserve University.

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FOOD ADDITIVES

New Colorimetric Method for the Determination of the Nitrate and Nitrite Content of Baby Foods

By LEN KAMM, G. G. MCKEOWN[1] and D. MORISON SMITH (Food Chemistry Section, Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Ontario, Canada)

A new method for the determination of nitrite and nitrate in foods will accurately determine concentrations as low as 1 ppm. 1-Naphthylamine is diazotized by nitrite and coupled with excess amine to give 4-(1-naphthylazo)-1-naphthylamine. The compound is extracted into $CHCl_3$, the extract is acidified with methanol-hydrochloric acid, and absorbance is measured at 555 mμ. Nitrate is quantitatively reduced by passage through a cadmium column and determined as nitrite; nitrite passes through the column unaltered. Therefore, in samples containing both forms of nitrogen, nitrate is determined by difference. The method was applied to 194 samples of baby foods. Spinach, beets, wax beans, squash, "garden vegetables," green beans, carrots, and "mixed vegetables" were found to contain, on the average, more than 20 ppm nitrate nitrogen.

In 1962 the World Health Organization (1) reviewed the literature on the toxicity of nitrates and nitrites. Rosenfield and Huston (2) recorded numerous cases of poisoning in small children and infants from the use of well water containing more than 93 ppm of nitrate (21 ppm nitrate nitrogen). Tap water containing slightly more than 50 ppm of nitrate (11 ppm nitrate nitrogen) produced 72% methemoglobinemia in a dyspeptic child (3). Municipal water supplies in Canada contain varying amounts of nitrate nitrogen, many from 18 to 36 ppm of nitrate (4–8 ppm nitrate nitrogen) and one as high as 54 ppm of nitrate (12 ppm nitrate nitrogen) (4). No mention is made in these toxicity studies of the nitrate content of foods consumed with the water. Certain foods, especially some vegetables (5, 6), contain relatively high concentrations of nitrate nitrogen (Table 1). In many instances, the concentration is more than 100 ppm and may be as high as 1820 ppm (6). Since these foods may constitute a significant portion of the diet of infants, they may have a harmful effect, particularly if the child is in a weakened condition or the water used in the formulation contains an appreciable quantity of nitrate. In such instances, it may become necessary to lower the nitrate concentration in the total diet by including foods relatively free of this substance. Thus, a method which will measure concentrations of 1 ppm or less nitrate nitrogen in foods is necessary.

The accurate determination of nitrate in foods is difficult (7). Analysis is complicated by the nearly colloidal nature of strained baby foods, the high color of certain vegetable extracts, and the complicated formulas of some preparations. A literature

Table 1. Nitrate nitrogen content (ppm) of food as reported in the literature

Food	Wilson (5)	Richardson (6)
Beets	300	210–1820
Broccoli	175–870	—
Cabbage	500	8–110
Carrots	100	9–20
Cauliflower	450	6–90
Celery	270	180–650
Lettuce	200–280	90–800
Rhubarb	1230	—
Egg plant	—	80–4000
Spinach	—	70–860
Squash	—	70–310
String beans	—	10–150
Sweet potato	—	6–29
Fruits	—	0–15
Ham	—	10–480
Bacon	—	7–420

[1] Deceased.

search showed that there was no method that would accurately determine nitrate nitrogen in foods at levels below 10 ppm.

Follett and Rateliff (8) reported a sensitive method based on the formation of the water-soluble diazo color Orange I. The method is applicable to nitrate or nitrite determinations in meats down to 10 ppm.

The method described in this paper is based on the formation of an oil-soluble diazo color which is extracted into chloroform. The procedure is capable of accurately determining nitrate nitrogen in food at the 1 ppm level.

METHOD

Reagents

(a) *Buffer solution.*—To 500 ml distilled water add 20 ml conc'd HCl and 50 ml conc'd aqueous NH_3. Dilute to 1 L; pH should be 9.6–9.7.

(b) *Reduction column.*—Prepare metallic cadmium by placing 4 zinc rods in 500 ml 20% solution of cadmium sulfate. Let the reaction proceed for 3–4 hours. Pour off solution and remove moss-like growth of cadmium from the zinc rods. Place the cadmium and 500 ml distilled H_2O in a Waring Blendor and blend for 1 or 2 seconds. Wash with distilled H_2O, sieve, and retain 20–40 mesh particles. This should yield enough cadmium to prepare 4 reduction columns. Fill a column (Fig. 1) with distilled H_2O and add wetted glass wool, pressing out trapped air with a glass rod. Add wetted Ottawa sand (Ottawa Silica Co., Ottawa, Ill.), and then the cadmium, tapping the sides of the column to lightly pack the column. Attach the reservoir and run in 25 ml 0.10N HCl followed by two 25 ml portions distilled H_2O and finally 25 ml buffer solution diluted 1:10. (The columns should be useful for an indefinite number of runs. However, check column efficiency periodically by passing through a known quantity of nitrate.)

(c) *1-Naphthylamine solution.* — Reagent grade (British Drug House "Analar" or Anachemia, Montreal). Dissolve 1 g 1-naphthylamine in 50 ml glacial acetic acid and add 150 ml distilled H_2O. If solution is colored, add ca 1 g powdered zinc and shake for 3 min. Filter solution through a No. 41 Whatman paper into a dark glass bottle and store under refrigeration. This reagent should be prepared fresh daily for a high degree of accuracy. In normal use, the reagent is stable for 1 week, after which a blank reading equivalent to about 0.05 μg nitrogen is observed.

(d) *Alumina cream.*—Add conc'd aqueous NH_3 to 4 L saturated aqueous solution of aluminum potassium sulfate with constant stirring until the mixture is alkaline to litmus paper (ca 255 ml NH_3). Then add saturated aqueous aluminum potassium sulfate (ca 340 ml) until the solution is just acid to litmus. Filter off insoluble product and wash with distilled H_2O until the washings are sulfate-free. Transfer precipitate to a 2 L beaker and add ca equal volume of distilled H_2O. Stir the mixture until it is homogeneous and pour into a stoppered glass container. If the alumina cream settles out, resuspend it by shaking before use.

(e) *Methanol-HCl solution.*—Add 17 ml conc'd HCl to 2 L MeOH.

Procedure

(Caution: All glassware must be carefully rinsed with distilled H_2O and cleaned with live steam to prevent nitrate contamination.)

Preparation of standard nitrate curve.—Weigh 0.6068 g analytical grade sodium nitrate and make to 1 L with distilled H_2O; dilute a 10 ml aliquot to 1 L (1 μg nitrate nitrogen/1 ml). Dilute another 10 ml aliquot to 100 ml (10 μg/1 ml). Into a series of 30 ml beakers, pipet 0, 1, 2, 5, 10, 15, and 20 ml of the 1 μg/ml solution and 5, 10, 15, and 20 ml of the 10 μg/ml solution. Add distilled H_2O until each beaker contains about 20 ml.

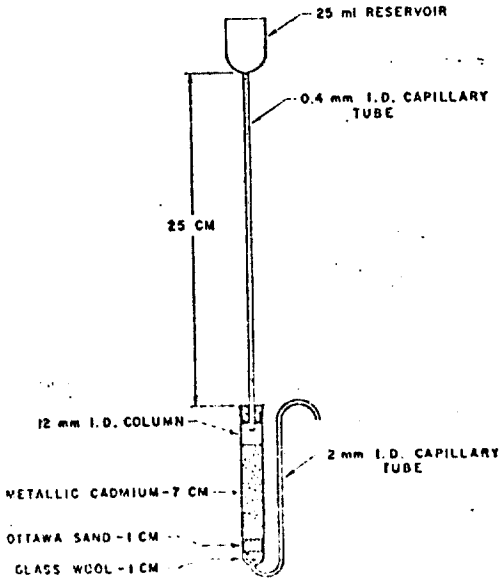

Fig. 1—Cadmium column (from Follett and Rateliff (8)).

To each solution add 5 ml buffer solution and pour into the reservoir of a prepared reduction column. As the reservoir empties, add four successive 20 ml portions of distilled H_2O. Collect the entire eluate in a 200 ml Erlenmeyer flask, add 2 ml of the 1-naphthylamine solution, and mix. Place the flask in the dark for 2 hours (9, 10) for color development. Transfer the solution into a 250 ml separatory funnel. Rinse the flask with ca 5 ml $CHCl_3$ and pour rinsings into the same separatory funnel. Shake until most of the color is transferred to the $CHCl_3$ and draw off the lower layer into a 50 ml volumetric flask. Extract with small volumes of the solvent until the washings are colorless. The volume of $CHCl_3$ used in extracting the color should not exceed 40 ml. Add 10 ml methanol-HCl solution and dilute to volume with $CHCl_3$. The color changes from yellow-orange to deep purple. Dilute solutions with more than 20 μg nitrate nitrogen further before measuring absorbance. Determine absorbance at 555 mμ against a blank, using 10 mm cells. A plot of absorbance vs. nitrate nitrogen is shown in Fig. 2.

Preparation of standard nitrite curve.—Weigh 0.4925 g analytical grade sodium nitrite, add 5 ml buffer solution, and make to 1 L with distilled H_2O. Dilute solutions as before. Proceed as for standard nitrate curve, beginning "dilute a 10 ml aliquot . . ." through ". . . of the 10 μg solution." Make up to 100 ml with distilled water. Pipet 5 ml buffer solution and 2 ml 1-naphthylamine solution into each flask. Place the flask in the dark for 2 hr; then extract and determine absorbance of the colored solution formed as outlined previously.

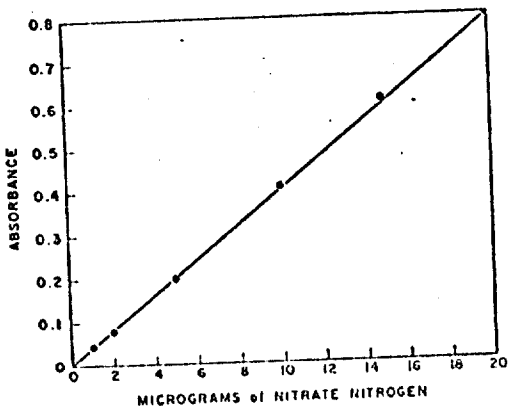

Fig. 2—Plot of absorbance against weight of nitrate nitrogen.

Extraction of food sample.—Weigh 2.00 g baby food in a 200 ml phosphoric acid flask. Add 5 ml buffer solution and ca 50 ml alumina cream. Wash down the sides of the flask with about 50 ml distilled H_2O. Place a clean rubber stopper on the flask and shake for 10 min. on a mechanical shaker. Make to volume with distilled H_2O, shake, and filter the solution through Whatman No. 41 paper, collecting the eluate in a steam-cleaned dry flask.

Determination of nitrite content.—Pipet 20 ml dilute food extract into a 125 ml Erlenmeyer flask. Proceed as for *Preparation of standard nitrite curve*, beginning "Make up to 100 ml . . ."

Determination of nitrate and nitrite content—Pipet 20 ml food extract into a 30 ml beaker, add 5 ml buffer solution, and continue as for *Preparation of standard nitrate curve*, beginning ". . . pour into the reservoir of a prepared reduction column." Calculate the nitrate nitrogen level by difference.

Calculations.—The 20 ml aliquot taken for testing represents 200 mg food. Determine μg nitrate or nitrite nitrogen present in the aliquot by reference to the appropriate standard curve, and multiply by 5 to obtain the ppm of nitrate or nitrite nitrogen in the sample.

Results and Discussion

Various dye intermediates were examined singly and in combination as diazotizable amines or coupling compounds for their suitability in producing a colored compound soluble in $CHCl_3$. 1-Naphthylamine was found to be most satisfactory, since it diazotized rapidly and coupled with excess amine to give a stable, easily measured color. The major colored product was identified as 4-(1-naphthylazo)-1-naphthylamine by paper chromatographic and spectrophotometric comparisons with an authentic sample. Under the conditions described it was as the hydrochloride salt, which gave a maximum absorbance at a longer wavelength than the free base.

A pure sample of 4-(naphthylazo)-1-naphthylamine, m.p. 179°C, was prepared and its absorption spectrum measured in the $CHCl_3$-MeOH-HCl system used in the determination. Maximum absorption occurred at 555 mμ with an absorptivity of 96.2. The absorptivity found for nitrate and nitrite calculated as nitrogen for the method was found to be 2000.

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Multiplying by 1/21.2, which is the ratio of the molecular weight of nitrogen to that of 4-(1-naphthylazo)-1-naphthylamine, gave a value of 94.4 for the colored compound formed. This agrees with the absorptivity value found for the pure color. Therefore, the conversion of nitrate to nitrite to diazo color appears to be quantitative.

A blank prepared by adding fresh 1-naphthylamine solution to 100 ml of distilled water yielded no color. The addition of 1-naphthylamine solution to 100 ml of distilled water which had been passed through a cadmium column showed a color development equivalent to 0.001 ppm of nitrate nitrogen.

Quantities of nitrate, nitrite, and mixtures of the two containing equal quantities of nitrogen were run through cadmium columns. Each solution gave identical readings. Thus, nitrite was not altered by the metallic cadmium, and the determination of nitrate by difference was accurate.

Both the nitrate and nitrite standard curves followed Beer's Law. The coefficient of variation was found to be 5% over the range 1–200 μg nitrate and nitrite nitrogen. There was no statistically significant difference between absorbances obtained for equivalent quantities of nitrite nitrogen and nitrate nitrogen by cadmium reduction, indicating complete conversion. In contrast, reduction of nitrate by zinc (11) gave only 91% of the absorbances of an equivalent amount of nitrite nitrogen.

None of the prepared food extracts yielded $CHCl_3$-soluble pigments. Moreover, any interference of this nature should be eliminated by a preliminary washing of the food extract with chloroform.

Alumina cream (12) was superior to potassium aluminum sulfate (8) as a clarifying agent; filtration was more rapid and the resulting solution less turbid. A standard nitrite solution to which 50 ml of alumina cream was added gave the same reading as a similar solution without alumina cream.

The method as described is accurate at concentrations of 1 ppm of nitrate or nitrite nitrogen in food. Lower concentrations might be determined by using larger aliquots of the food extract and/or by utilizing a smaller total volume of $CHCl_3$ for extracting the colored compound produced. However, increasing the sensitivity lessens the accuracy of the method. A greater ratio of food to extractant could be used with certain foods (see Tables 2 and 3).

Table 2. Recovery of nitrite added to various infant foods[a]

Food	Recovery, %		
	2 g[b]	10 g[b]	50 g[b]
Strained prunes	90	71	3
Strained applesauce	96	69	12
Tomato soup	98	87	87
Strained mixed vegetables	100	104	64
Strained carrots	98	91	59
Precooked wheat cereal	98	85	0
Canned milk	95	97	83
Strained egg yolk	107	95	104
Strained ham	100	98	99
Strained beef liver	87	84	66
Strained chocolate custard	98	91	59
Strained butterscotch pudding	98	92	84

[a] 5 μg nitrite nitrogen added to each extract. The difference in μg between treated and untreated samples divided by 5 μg and multiplied by 100 gives the per cent recovery.
[b] Weight of sample taken for dilution to 200 ml.

Table 3. Recovery of nitrate added to various infant foods[a]

Food	Recovery, %		
	2 g[b]	10 g[b]	50 g[b]
Strained prunes	91	67	8
Strained applesauce	96	76	5
Tomato soup	105	100	104
Strained mixed vegetables	102	99	80
Strained carrots	97	101	98
Precooked wheat cereal	99	95	103
Canned milk	100	91	100
Strained egg yolk	98	105	133
Strained ham	104	103	off color
Strained beef liver	93	62	off color
Strained chocolate custard	97	101	98
Strained butterscotch pudding	104	103	90

[a] 5 μg nitrate nitrogen added to each extract. The difference in μg between treated and untreated samples divided by 5 μg and multiplied by 100 gives the per cent recovery.
[b] Weight of sample taken for dilution to 200 ml.

Table 4. Survey of the nitrite and nitrate nitrogen content of 194 prepared infant foods

Food	No. of Samples	Nitrite, ppm Range	Nitrite, ppm Av.	Nitrate, ppm Range	Nitrate, ppm Av.
Fruits	22	0–2	0.5	0–7	2
Dairy products	9	0.4–4	2	0–7	3
Puddings	5	0–2	0.6	0.8–11	4
Egg products (including custards)	13	0–2	0.9	0.9–11	4
Meats	15	0–0.6	0.3	1–11	4
Meat dinners	20	0–2	0.5	0.9–16	7
Dry and concentrated food supplements[a]	27	1–10	3	4–49	17
Precooked cereal products	22	2–8	4	0.4–48	13
Vegetables	61	0–2	0.5	0.2–492	68

[a] Purchased from pharmacies. Modified milks, artificial milks (soybean base), carbohydrate supplements, casein supplements, and prepared formulas are included. The nitrate and nitrite figures refer to the product in its concentrated or dried form.

Table 5. Prepared infant foods containing more than 20 ppm of nitrate nitrogen

Food	No. of Samples	Nitrite N, ppm Range	Nitrite N, ppm Av.	Nitrate N, ppm Range	Nitrate N, ppm Av.
Mixed vegetables	2	0–2	0.8	21–24	22
Carrots	8	0–2	0.7	15–38	23
Green beans	3	0–1	0.8	16–71	37
Garden vegetables	5	0–2	0.7	19–62	41
Graham crackers	1		2		48
Squash	5	0–1	0.7	10–93	64
Wax beans	2	1–2	2	73–129	101
Beets	6	0–2	0.8	144–192	222
Spinach	5	0.3–1	0.5	244–379	312

Table 2 shows the percentage recovery when 5 μg of nitrite nitrogen was added to three different concentrations of food extracts. Good recoveries were obtained for all samples for the 1% food extract (2 g/200 ml). Ten of the 12 food samples gave recoveries within the 5% coefficient of variation determined for the standard curves. At the 5% concentration, recoveries of 69% or better were obtained for all samples. At the 25% concentration, only a few samples gave satisfactory results.

Table 3 shows the percentage recovery when 5 μg of nitrate nitrogen was added to the same three concentrations of food extract used above. The results were similar to those obtained for the nitrite recoveries.

Table 4 lists the nitrate and nitrite nitrogen levels of 194 samples of baby foods. The baby foods that contained more than 20 ppm of nitrate nitrogen are listed in Table 5. Spinach and beets contain exceptionally large quantities of nitrate, followed, in decreasing order by wax beans, squash, Graham crackers, "garden vegetables," green beans, carrots, and "mixed vegetables."

Quantitative conversion of the nitrate or nitrite to a $CHCl_3$-soluble diazo colored compound rather than a water-soluble one offered a number of advantages. The $CHCl_3$ extract could be kept to a small volume for a high sensitivity, and there was no problem of interference from the water-soluble and colloidal substances found in infant foods. The method was applicable over a range of 1–200 ppm of nitrite plus nitrate nitrogen. The standard curves demonstrate that if the color formed in the range of 20–200 μg of nitrate and nitrite nitrogen was diluted, it would fall in line with the linear relationship between concentration and absorbance established in 0–20 μg range.

Acknowledgments

The authors wish to thank Mr. R. Fortin for technical assistance. Thanks are also due to Dr. W. P. McKinley and Dr. E. Coffin for suggestions made in reviewing the paper, and to Mr. J. M. Airth for the statistical analysis.

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OILS, FATS, and WAXES

Detection of Peanuts in Adulterated Tehina and Halva

By ARIEH LETAN, BERTA TURZYNSKI, and ARIELA RAVEH (Department of Food and Biotechnology, Technion, Israel Institute of Technology, Haifa, Israel)

A proposed method for detection and approximate estimation of peanut admixtures in adulterated tehina and halva (local sesame products) at a minimum level of 3–7% is presented which is based on the difference in behenic acid content of oils extracted from peanuts and sesame seeds. Long-chain saturated fatty acid esters from the analyzed mixture of methyl esters were isolated through urea complexing, and the gas chromatographic technique of analysis was used. Behenic acid was found to be present not only in peanut oil (as reported in the literature) but also in sesame oil in amounts not exceeding 0.3%.

Tehina is an oily product prepared from ground roasted sesame seeds. Halva is made from tehina and contains a number of nonoily ingredients. Both of these local sesame products are often adulterated by substitution (partial or complete) of peanuts, which are much cheaper than sesame seed.

Over 50% of the tehina and over 30% of the halva consist of triglycerides; it was natural, therefore, that the present study should be prefaced by analysis of this fraction.

According to Hilditch (1), Goddard (2), Gunstone (3), and Iverson, *et al.* (4), peanut oil invariably contains a considerable amount (3.2–8.0%) of higher saturated fatty acids: arachidic (20:0), behenic (22:0), and lignoceric (24:0).

Iverson, *et al.* (4) studied 22 samples and 3 varieties of peanuts and found 2.7–3.5% behenic acid in the oil fraction and an estimated 1.7–3.8% content in 5 samples of refined peanut oil. They also reported that roasting the peanuts had no effect on the composition of the oil.

The project described in this paper was sponsored by the Food Division of the Israel Ministry of Commerce and Industry, Jerusalem.

Table 3. Percentage of sodium cyclamate in diets before and after rats have eaten from containers

Nominal % Cyclamate	Analyzed % Cyclamate: Original Diet	Analyzed % Cyclamate: Remaining Diet
1.0	0.98	1.14
		1.16
		1.11
		1.06
	Av.	1.12 (14.4% increase)
5.0	5.09	5.77
		5.48
		5.74
		5.46
	Av.	5.61 (10.2% increase)
10.0	10.72	13.44
		13.48
		13.68
		13.31
	Av.	13.48 (25.7% increase)

concentration than expected. The cyclamate ingested is therefore less than calculated, giving a low recovery in the balance studies. This difference in cyclamate concentration in the diets was also observed in the stock diet preparations, as demonstrated by the fact that the concentration of the original 10% Na cyclamate had increased to 10.72% (Table 3).

The results clearly demonstrate the usefulness of the GLC procedure in the determination of cyclamate as confirmed by congruent ^{14}C-cyclamate studies. In addition, both ^{14}C and GLC show the nearly quantitative elimination of cyclamate in the rat independent of whether the dose of cyclamate is high or low, or whether the administration is chronic (22 weeks) or acute.

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Assessment of the Naphthylazo-naphthylamine Method for the Determination of Nitrate and Nitrite

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The 1-naphthylazo-naphthylamine method for nitrate and nitrite, reported in 1965, was subjected to specific experiments statistically designed to determine more precisely the precision of the method. Experimental designs included the following: standard nitrite curve, stability of 1-naphthylazo-naphthylamino dye, reduction of nitrate to nitrite by the cadmium column, reproducibility of results comparing natural foodstuffs with pure solutions, and collaborative study on food samples. The statistical data obtained indicate that the method is consistent and precise.

In 1965 the 1-naphthylazo-naphthylamine method (1) for nitrate and nitrite was published. A limited amount of data were presented, illustrating the probable sensitivity and accuracy of the method. The present paper utilizes specific statistical designs to determine more precisely the precision of the method. Progressing from the analysis of pure nitrite solutions to the analysis of food samples for nitrite and nitrate in a collaborative study, each step in the procedure is isolated and its contribution to variation is assessed.

METHODS

Experiment 1: Standard Curve

Source of reagent standard, preparation of standard solution, nitrite concentration, type of spectrophotometer, and time interval between production of the naphthylazo-naphthylamine and measurement of color intensity were incorporated in a single experimental design. Duplicate samples of reagent grade sodium nitrite were taken from each of two brands (brand or bottle variation[1]). The samples were dissolved in distilled water containing 5% HCl-NH_4OH (v/v) buffer (1) to yield solutions containing 1 μg nitrite nitrogen/ml (solution variation). Each of these four solutions was assigned a set of volumetric pipets, one 5 ml and one 15 ml. Duplicate 5 ml and duplicate 15 ml aliquots were taken from each of the four solutions previously described (aliquot variation). The aliquots were diluted to ca 100 ml; the 1-naphthylazo-naphthylamine dye was formed, extracted, acidified, and diluted to volume as previously described (1). Two aliquots were removed from each of these solutions for measurement of optical density using two different spectrophotometers, one line-operated and the other battery-powered (spectrophotometric variation). Using different aliquots of these same solutions, optical density readings were repeated at 2, 4, and 24 hr (time variation).

The diagrammatic representation of experiment 1 is shown in Fig. 1.

Experiment 2: Stability of 1-Naphthylazo-naphthylamine Dye

Analytical grade sodium nitrite (0.5 g) was dissolved in 1 L distilled water containing 5 ml HCl-NH_4OH buffer solution; 10 ml of this solution was pipetted into a 1 L glass-stoppered Erlenmeyer flask containing 900 ml distilled water, 50 ml HCl-NH_4OH buffer solution, and 20 ml 1-naphthylamine solution. The flask was stoppered, shaken, and stored in the dark for 2 hr. The flask was then inverted several times to insure uniformity and five 10 ml aliquots were pipetted into 25 ml glass-stoppered Erlenmeyer flasks (aqueous suspension). Two 10 ml aliquots were pipetted into 200 ml separatory funnels, 90 ml distilled water was added, and the solution was extracted with chloroform. The extracts were collected in 25 ml volumetric flasks; 5 ml methanol-HCl was added and the solutions were diluted to volume with chloroform. Four 100 ml aliquots of the aqueous 1-naphthylamine solution were pipetted into individual 250 ml separatory funnels and extracted with chloroform into 100 ml volumetric flasks. Two of these chloroform extracts were diluted to volume with chloroform (basic form of the dye) and 10 ml of the methanol-HCl solution was added to the other two chloroform extracts before diluting to volume (acidic form of the dye). The samples were inverted several times until the color was uniform throughout. Ten ml aliquots of these four solutions were pipetted into individual 25 ml volumetric flasks, 5 ml methanol-HCl solution was added, and the aliquot was diluted to volume with chloroform. A total of six 25 ml portions of dye solution were prepared and examined between 350–700 mμ range on a Cary 14 recording spectrophotometer; identical curves were obtained for each. Four of the eight 25 ml Erlenmeyer flasks containing the aqueous suspension of the dye and two of the 100 ml volumetric flasks, one containing the basic form of 1-naphthylazo-naphthylamine and the other the acidic form, were placed on a window ledge for exposure to daylight. A similar set of samples was stored in a dark cupboard at room temperature. After 2, 4, 24, and 48 hr, acidic dye solutions were prepared from each solution and their spectral curves were determined on the Cary 14.

Experiment 3: Reduction of Nitrate to Nitrite by the Cadmium Column

Part A.—Continuous levels of nitrate: A total of thirty-six 20 ml aliquots, 18 containing 5 μg and 18 containing 15 μg nitrate nitrogen, were prepared, using steam-cleaned glassware. Three columns were used for each dilution, thus permitting triplicate analyses of each nitrate concentration in a series consisting of six successive runs through each of the cadmium columns in a single day. The color was developed as described (1) and the optical densities were recorded on a Beckman B spectrophotometer.

Part B.—Discontinuous levels of nitrate: Eighteen 20 ml aliquots containing 5 μg (*L*) and 18 containing 15 μg (*H*) of nitrate nitro-

[1] Brand or bottle: Since only a single bottle of each brand was examined it was impossible to establish that variation is due to difference in brand or bottles of a single brand.

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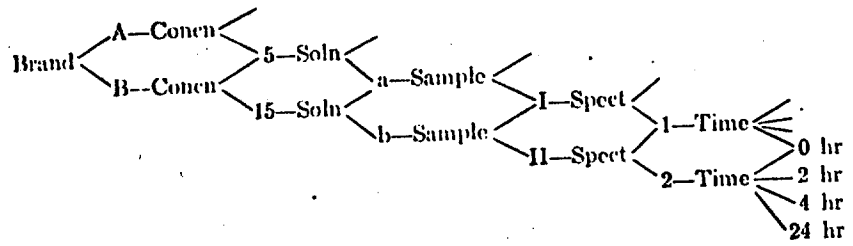

Fig. 1—Diagrammatic scheme for experiment 1.

gen were prepared, using steam-cleaned glassware. The aliquots were passed through six cadmium columns, alternating the concentration according to the following patterns:

3 columns—*L, H, H, L, L, H*
3 columns—*H, L, L, H, H, L*

Optical densities of the 1-naphthylazo-naphthylamine dye produced were measured as in Part A.

Experiment 4: Reproducibility of Results Comparing Natural Foodstuffs with Pure Solutions

The experimental pattern outlined in Experiment 3B was applied to foods arranged to pair a food containing little nitrate with one containing an appreciable quantity of nitrate. Four different vegetable baby foods were chosen on the basis of previous analysis (1) for their high nitrate content. These were arbitrarily paired with four low nitrate foods: one fruit, one dairy, one egg, and one meat product.

High Nitrate Paired With Low Nitrate

Squash	Mixed fruit
Waxed beans	Canned evaporated milk
Beets	Egg yolk
Spinach	Beef and farina

Three samples of each member of a pair of foods were analyzed on one column, using the patterns described in Experiment 3B. As before, 3 columns were used for each pattern for a total of 6 columns for each food pair.

Experiment 5: Collaborative Study

Six cans of the same lot number from each of three baby foods (beets, squash, and beef in broth) were purchased from retail sources. The contents of each set of six were pooled and twenty-four 20 g aliquots were sealed in 1 oz screw-cap vials and immediately frozen. Four samples of each food were sent to six cooperating laboratories. Each laboratory was requested to assign two analysts each to perform two analyses on each of the three samples. A variety of spectrophotometers were used, depending on what was available in the cooperating laboratory. All results were reported as optical density and the nitrate level calculated by the authors.

Statistical Methods

Both simple linear regression and analysis of variance techniques were used. The linear regression tests were used to look for the time trend effects, and the usual assumption of homoscedasticity was made. The usual assumptions regarding homogeneous variances, normal distributions, and additivity of effects were made when analyses of variance were carried out. The models will be introduced at the appropriate time in the text, and any interactions which were considered will be indicated in the model but not described in detail for each model. Y, with appropriate subscripts, will denote the observed variable, μ the overall mean, and E the random error term. R, when used, denotes residual variation and will include any interaction effects which may not be zero, in addition to the random error. (*See* reference (2), for example.)

A distinction was made between linear time trends and time effects. No attempts were made to fit curves. A time trend was thought of as being repeatable while a time effect was identified as an uncontrolled source of variation which could not be predicted. When such time effects were found, it is implied that an explanation as to what caused the effect could be offered.

Results and Discussion

Experiment 1: Standard Curve

The optical density readings recorded on the Spectronic 20 were consistently higher than those on the Beckman B at all time intervals following color development (0, 2, 4, and 24 hr). The Beckman B gave progressively lower readings at 0, 2, and 4 hr with a slightly larger drop at 24 hr. The data suggest that the rate of decline was independent of dye concentration, but directly related to time. Thus, the decrease in optical density at 15 μg nitrite nitrogen was three times the drop at the 5 μg level. The dye appears to be sufficiently stable under normal operating conditions since the decrease in optical density was about 1% after 4 hr standing and about 3% after 24 hr.

Within any given time period the line-operated spectrophotometer gave as consistent results as the battery-operated instrument. However, the line-operated Spectronic 20 gave more variable results at different time intervals without time trend. This suggests that the line-operated instrument gives accurate results when the unknown sample is compared to a standard curve run at the same time, whereas optical density readings on the battery-operated machine may be converted to nitrite nitrogen values by comparison with a previously established standard curve.

The data from each instrument at each time were analyzed statistically, assuming the following model to be suitable:

$$Y_{ijkl} = u + B_i + S_{j(i)} + D_k + BD_{ik} + SD_{jk(i)} + E_{l(ijk)}$$

where B and S were random effects of brands (or bottles) and samples, respectively, while D was a fixed dilution effect.

The major source of variation was dilution, as intended. The slight difference ($P < 0.10$) between brands and between samples within brands indicates that the color reaction, NO_2^- + 1-naphthylamine → 1-naphthylazonaphthylamine, is sufficiently consistent to detect small differences in the purity of the standard nitrite (brand or bottle), and slight errors in analytical techniques (samples within brands).

Experiment 2: Stability of 1-Naphthylazonaphthylamine Dye

Figure 2 shows that the acidic form of the dye dissolved in chloroform is the more stable, whether stored in the dark or in daylight. There is no discernable drop in optical density up to a 4 hr period in the presence or absence of light and only a 2.3% drop after 24 hr in the dark. The rate of deterioration was apparently less than in Experiment 1, where there was a 1% drop in optical density after 4 hr and 3% after 24 hr. The dye is much more photosensitive in the basic form whether in aqueous suspension or in chloroform solution. There is a slight rise in optical density after 2 hr with the basic form of the dye suspended in aqueous solution and stored in the dark. This same solution when exposed to sunlight shows no change in optical density after 2 hr. Apparently the reaction is not complete after 2 hr; it reaches a maximum at 3 hr, after which there is a rapid decline in optical density. With the sample stored in daylight it appears that photodegradation keeps pace with the continuing dye formation for a 2 hr period, followed by a sharp drop in optical density. The basic form of the dye dissolved in chloroform is the most stable of all forms after 48 hr when stored in the dark (Fig. 2). However, there is an early drop in optical density between 2 and 4 hr. Consequently it is advisable to partition the dye into chloroform and convert it to the acid form immediately after the 2 hr reaction period. In this form the dye is relatively stable in the presence or absence of light for 4 hr.

Spectral scans over the range of 350–700 mμ of the fresh and degraded dye are shown in Fig. 3. Compared to the spectral curve for the fresh dye, the degraded form shows a decrease in optical density at 555 mμ and an increase at 440 mμ. As a result, the ratio of optical density at these two wavelengths provides a means of detecting dye deterioration.

Experiment 3: Reduction of Nitrate to Nitrite by the Cadmium Column

Since the precise nature of the reaction of nitrite with 1-naphthylamine was established, we could test the efficiency of the cadmium column for the reduction of nitrate to nitrite.

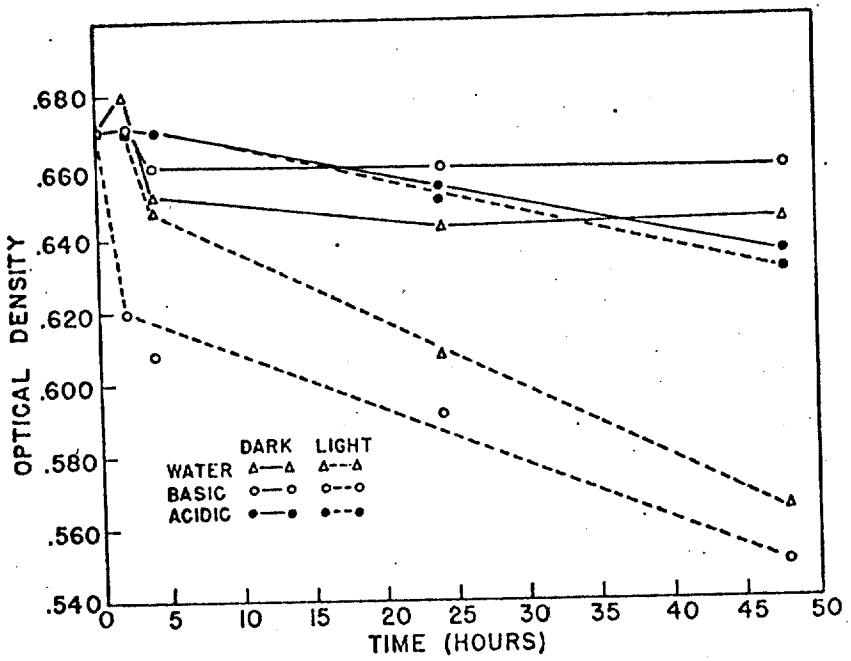

Fig. 2—Stability of 1-naphthylazo-naphthylamine in daylight and in the dark.

In both Part A and Part B of the experiment equal amounts of nitrogen, whether in the form of nitrite (Experiment 1) or reduced nitrate, gave the same values for optical density. As reported earlier (1), the conversion of nitrate to nitrite by use of the cadmium column is virtually 100%.

Part A.—A simple linear regression analysis of the results from each column showed no evidence of drift with repeated use of the same cadmium column over a short period of time (8 hr day) to analyze successive samples of nitrate nitrogen, all at the same level. The data were further examined according to the following model

$$Y_{ij} = u + C_i + T_j + R_{ij}$$

where C and T represented the effects of column and times of observation.

Column effect was absent, but a time effect ($P < 0.05$) was found in the three columns through which the higher concentrations were processed.

Part B.—The experimental design permitted a direct check on repeatability by two specific statistical analyses to look for biases associated with changing concentrations in successive samples. The first was based on the expectation that each adjacent low-high or high-low concentration grouping should give the same measure of an absolute difference. Three such differences can be computed for each column and the following model was appropriate:

$$D_{ij} = u + T_i + C_j + TC_{ij}$$

where T and C again represented time and column effects, and D was a difference, $H - L$ or $L - H$.

The second specific statistical analysis was based on the expectation that two adjacent measures of the same dilution should give similar results, and hence their difference should be close to zero. Again a difference model was appropriate where D was a difference computed as $H - H$ or $L - L$. Analysis of the differences confirmed the presence of a time effect and the absence of between column variation in the magnitude of the differences. Examination of the differences indicated no consistent bias due to having used the column previously to process a different dilution. This suggested that, although the activity of the cadmium columns was not completed by continuous use, there was an unknown variable that was influencing the

efficiency of all columns during any specific time period in a series of runs in a single day. The lack of bias due to previous use of the column to process a different dilution (Part B) suggested that the prescribed washing regime removed essentially all of the nitrate prior to the introduction of the succeeding sample.

Experiment 4: Foods Subjected to the Experimental Conditions of Experiment 3B

The method of experiment 3B was used to evaluate the effect of foods expected to contain different levels of nitrate nitrogen. The data gave no apparent indication of drift and since in the previous experiments a lack of drift had been demonstrated using pure nitrite and nitrate solutions, regression analyses were not carried out.[2] Analyses of differences indicated that significant effects attributable to both times and columns sometimes occur. However, differences do not lend themselves to the direct estimation of standard deviations associated with individual observations. The following model was therefore employed for this purpose:

$$Y_{ijk} = u + F_i + T_{j(i)} + C_k + R_{ijk}$$

where F represented a fixed food effect, and T and C represented random time and column effects.

This model was used to analyze the data from columns 1–3 (*HLLHHL*) and 4–6 (*LHHLLH*) separately (Table 1). Times within foods was highly significant in those cases where residual values were low. It is clear that the variance may be expected to differ from food pair to food pair. There seems less reason to expect it to vary with the order in which the samples were analysed. The mean squares were therefore pooled across sequences for each food pair separately and used to obtain estimates of variance components and the standard deviations (Table 2). It is readily apparent that these estimates cover a fairly wide range (ρ^2_{Total} beets and eggs = 3 ρ^2_{Total} squash and mixed fruits) suggesting that the variability expressed may be dependent on the food. Taking 3 times the standard deviation it may be seen that a single observation could have an error of as little as ± 0.06 or as much as ± 0.11 units of optical density.

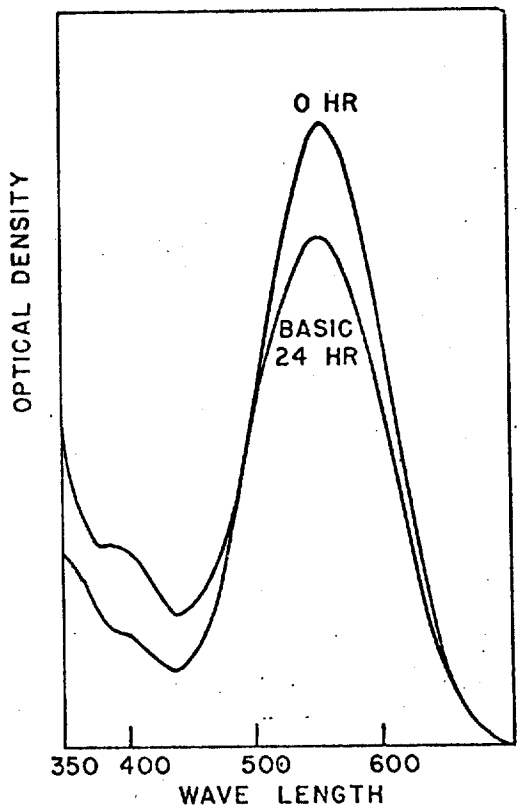

Fig. 3—Spectral curve of fresh and degraded forms of 1-naphthylazo-naphthylamine.

The trivial contribution of columns to the total variance in three of the four cases suggested that it is a negligible source of variation. Even in the case where columns were a significant source of variation (beets vs. eggs), they contributed less than 10% to the total variation. If a column effect is present when the method is applied to foodstuffs it is of such low magnitude that it does not normally interfere. It is apparent that the magnitude of the time effect is dependent on the food. Regardless of the variation attributable to the time effect it is obvious from the standard deviations shown in the experiment (Table 2) that the method is sufficiently accurate for general usage.

[2] The lowest value obtained (0.180) was considerably [illegible] the remaining ones (0.290–0.318). The analyses [illegible] done including this "outlier" and then using [illegible] value in place of it. The second analysis yielded [illegible] consistent estimates of variability and are those [illegible]. The authors believe this analysis to be more [illegible] correct.

Table 1. Mean squares of data from Experiment 4

Parameters	D.F.	Beets vs. Egg	Spinach vs. Beef	Squash vs. Fruit	Beans vs. Milk
		Columns 1-3: HLLHHL			
Foods	1	1.750944	1.053184	0.008669	0.043414
Times/foods	4	0.001939[a]	0.003910[a]	0.001516[a]	0.002562[a]
Columns	2	0.000958[b]	0.000372	0.000070	0.000222
Residual	10	0.000129	0.000286	0.000049	0.000117
		Columns 4-6: LHHLLH			
Foods	1	1.785420	0.866367	0.002914	0.025614
Times/foods	4	0.002474	0.001288[c]	0.000627[a]	0.001639[d]
Columns	2	0.001482	0.000363	0.000114	0.000232
Residual	10	0.001085	0.000398	0.000058	0.000384

[a] $P < 0.001$
[b] $P < 0.01$
[c] $P < 0.10$
[d] $P < 0.05$

Table 2. Variance components and standard deviations of data from Experiment 4

Source of Variance	Beets vs. Eggs	Spinach vs. Beef	Squash vs. Fruit	Beans vs. Milk
Error	0.000607	0.000342	0.000053	0.000251
Columns	0.000102	0.000005	0.000006	−0.0
Time/food	0.000533	0.000752	0.000339	0.000617
Total	0.001242	0.001099	0.000398	0.000868
Std dev.	0.0353	0.0332	0.0199	0.0295

Experiment 5: Collaborative Study

The initial plan was modified when we discovered that the various cooperating laboratories were using distilled water with nitrate levels varying from about 0.0004 to 0.004 ppm. This in combination with the fact that a few of the analysts used a chloroform-HCl-methanol blank, rather than one of distilled water passed through the column, affected the results in the sample containing only a trace of nitrate. The ratio of food to distilled water passed through the column is about 1 to 500 (200 mg food, 100 ml distilled water). Thus, percentagewise, traces of nitrate in the distilled water may contribute appreciably to the apparent nitrate content in samples containing low nitrate levels. Therefore we send out another sample of beef in broth with special instructions regarding the blank.

Since the samples were sent out at different times and because examination of the data suggested that the amount of variation expressed differed with the sample, separate statistical analyses were done for each food. The model employed was as follows:

$$Y_{ijk} = u + L_i + A_{j(i)} + E_{k(ij)}$$

where L and A represent laboratories and analysts within laboratories, respectively.

The mean squares and standard deviations are shown in Tables 3 and 4. Analysts were a highly significant source of variation in Samples A (beef with broth) and C (beets) but not in Sample B (squash). Analysts also contributed relatively more to the total variation in Samples A and C than in Sample B. These results and those of experiment 4 suggest that the degree of variation is related to the food being analyzed. If this is the case the variation reported for analyst/laboratory may be exaggerated by the degree of influence of this or these unknown factors in various laboratories.

The standard deviation for the samples of squash and beets was about ± 10% of the nitrate level which is twice the 5% coefficient of variation for a single laboratory reported in the previous paper (1). The sample of beef in broth showed a much

Table 3. Analysis of variance of collaborative study data

Source of Variation	D.F.	Mean Squares for Sample: A—Beef	B—Squash	C—Beets
Approximate nitrate nitrogen level (ppm)		1	100	300
Laboratories	5	0.8096[a]	138.97	1135.0
Analyst/laboratories (A/L)	6	0.2275[b]	78.50[a]	1622.3[b]
Determinations: A/L	12	0.0100	62.00	89.8

[a] $P < 0.10$
[b] $P < 0.01$

Table 4. Variance components of collaborative study data

Component	(A) Beef	(B) Squash	(C) Beets
Error	0.0100	62.00	89.8
Analysts	0.1088	8.25	766.2
Laboratories	0.1455	15.12	—
Total variance	0.2643	85.37	856.0
Std dev.	0.5141	9.24	29.26

higher standard deviation (± 50%); this suggests that the method is being extended when accuracy at the 1 ppm level is required in a collaborative study.

Conclusions

Working with pure solutions, we have shown that nitrite reacts with 1-naphthylamine to produce a quantity of colored material linearly related to the nitrite content. In addition the results exhibit consistency since eight consecutive determinations, each of two nitrite levels, show no evidence of statistically discernable variation. The 1-naphthylazo-naphthylamine dye has been shown to be relatively stable up to 4 hr when dissolved in chloroform and converted to its acid salt. The cadmium columns effectively reduce (virtually 100%) nitrate to nitrite without tendency towards inefficiency due to consistent usage over an 8 hr period. It is also shown that previous use of a column to process a specific nitrate solution did not affect the results of a subsequent sample of a different nitrate level. The appearance of a statistically significant time effect is evidence of the presence of an unknown variable affecting all columns at the time of processing a solution of nitrate. There is a suggestion that the degree of variation may be dependent upon the food. A collaborative study shows interlaboratory results were comparable within a ± 10% level of standard deviation when the nitrate level was 100 ppm or greater in the food. The results at the 1 ppm level were much less precise but still in the correct range. The variation within a single laboratory was far less than that for interlaboratory comparisons. Since there was a ten-fold difference in the nitrate content of distilled water among laboratories, slight differences in the washing volumes may be responsible for variation at the ppm level (0.2 μg).

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SHORT PAPERS

An Investigation into the Possible Presence of Nitrosamines in Nitrite-Bearing Spinach

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Summary—Within the limits of detection of the analysis used (0·1–0·5 ppm), no nitrosamines were demonstrable in nitrite-bearing spinach. It was, however, possible to demonstrate minute amounts of diethylnitrosamine under very extreme experimental conditions, for example, extremely high diethylamine concentrations. The effects of temperature, duration of reaction and pH on nitrosamine synthesis were also studied. Both the weakly alkaline reaction of spinach and the low concentration of secondary amines are important factors limiting the synthesis of nitrosamines, even in spinach with a high nitrite concentration.

Introduction

Since Barnes & Magee (1954 & 1956) discovered that dimethylnitrosamine has carcinogenic properties, many animal studies have been carried out on the toxic and carcinogenic properties of different nitrosamines (Druckrey & Preussmann, 1962; Heath, 1962; Heath & Magee, 1962; Sakshaug, Sögnen, Hansen & Koppang, 1965). These investigations have shown that dimethylnitrosamine is the most toxic and carcinogenic substance in this category of compounds. In rats, for example, the acute toxic LD_{50} for dimethylnitrosamine is reported to be between 27 and 41 mg/kg, and that for diethylnitrosamine 261 mg/kg body weight (Heath, 1962). According to Argus & Hoch-Ligeti (1961), the carcinogenic dose for rats is 250 mg/kg for diethylnitrosamine and less for dimethylnitrosamine. Druckrey (1963) conducted long-term experiments in rats, demonstrating that amounts under 70 μg diethylnitrosamine/kg body weight/day cause no acute toxicity and need not be expected to have any carcinogenic effect. The toxic and carcinogenic doses for man remain to be established.

Nitrosamines can be produced by reactions between secondary (and, under dealkylating circumstances, also tertiary) amines and nitrous acid or nitrous gases:

$$\begin{matrix}R_1\\R\end{matrix} > NH + HNO_2 \rightarrow \begin{matrix}R_1\\R\end{matrix} > N - N = O + H_2O$$

$$2\begin{matrix}R_1\\R\end{matrix} > NH + NO + NO_2 \rightarrow 2\begin{matrix}R_1\\R\end{matrix} > N - N = O + H_2O$$

Research into the possible presence of nitrosamines in foods (and tobacco smoke) was prompted by events involving animal feeds.

In 1960 certain Norwegian herring meal plants introduced a new method of preservation employing sodium nitrite. This served to preserve herring which in peak supply periods could not immediately be processed to herring meal. At that time, toxic effects (mainly

hepatic lesions) were observed in mink fed on feeds containing fish preserved with sodium nitrite (Böhler, 1960 & 1962) and in ruminants whose fodder contained fishmeal prepared from fish similarly preserved (Hansen, 1964; Koppang, 1964). Finally Ender, Havre, Helgebostad, Koppang, Madsen & Ceh (1964) isolated the toxic dimethylnitrosamine from herring meal prepared from herring preserved with sodium nitrite. Apparently the conditions favoured nitrosamine production, the fact that fish is rich in amines and that much nitrite was used in preservation having possibly contributed to this.

The elaboration of sensitive and specific reactions to nitrosamines and their quantitative determination became important when it was realized, especially in Germany, that the presence of these substances was not *a priori* ruled out in foods and drinks in which secondary amines were present if nitrite had been added or could be formed from nitrate by means of nitrate reducing bacteria, or if exposure to the effect of nitrous gases had been possible.

Marquardt & Hedler (1966) demonstrated the presence of nitrosamines as well as nitrite in samples of German wheat meal, and found that heated meals contained more nitrosamines. In their report they stated that no exact nitrosamine concentrations could be given, but remarked that in heated wheat meal amounts of over 50 mg nitrosamine/kg could occur. In samples of English wheat flour, however, Thewlis (1967) could find neither nitrite nor nitrosamines. In a sample of bleached wheat meal, Kröller (1967) found minute traces of diethylnitrosamine, and he also reported traces of nitrosamines in a few samples of cheese. He ascribed these to abnormal processes taking place during maturation. A recent publication by Hedler & Marquardt (1968) reports the presence of diethylnitrosamine in wheat plants (all nine samples examined), wheat (both samples), wheat flour (all three samples), heated wheat flour (22 out of 30 samples), pasteurized milk (one sample) and cheese (both samples). These authors again mention that quantitative nitrosamine determinations were impossible, a fact which in our opinion greatly reduces the value of their publication.

Möhler & Mayrhofer (1968) maintain, on the grounds of the toxicity studies of Druckrey (1963) already mentioned, that it is a waste of effort to develop methods of nitrosamine determination capable of demonstrating less than 1 mg/kg food. In a study of samples of Tilsit cheese, salt meat and meal they found no indications of the presence of nitrosamines in quantities in excess of that level.

Spinach normally contains no nitrite, but when the raw vegetable is kept too long, particularly in hot damp weather, or when prepared spinach is left too long at room temperature, nitrate-reducing bacteria may form nitrite from the nitrate which is present (Sinios & Wodsak, 1965; Groot, 1967; van Burg, Groot, Keller & Rauw, 1968). Secondary amines occur ubiquitously in nature as intermediate products in the protein metabolism of plants, animals and micro-organism (Guggenheim, 1951). In principle, therefore, it might be possible for nitrosamines to be formed from these substances. Efforts were made to establish whether nitrosamines could indeed be demonstrated (and estimated) in nitrite-bearing spinach, and to identify the conditions (reagent concentrations, pH and time) influencing the production of these compounds.

Experimental

Material. Commercially bought spinach, in which no nitrite was demonstrable, was divided into two lots and stored either at 20–23°C for 1 wk, during which the nitrite concentration rose to 251 ppm, or at 4°C for 2 wk, the nitrite concentration in this case rising to 78 ppm.

Extraction of nitrosamines. Homogenization of 50-g samples of spinach with 100 ml 0·5 M caustic soda was carried out with a polytron homogenizer. The homogenized mass was boiled for 30 min under reflux (with antifoam) and then introduced to a steam distillation plant for distillation of 100 ml in about 20–30 min. The distillate was extracted four times with 50 ml dichloromethane. To remove most of the water from the extract, it was kept overnight at −10°C so as to freeze the water out. The dichloromethane extract was then cautiously concentrated by evaporation of the ice-cold fluid in a rotating film evaporator *in vacuo*, at a waterbath temperature of about 20°C, to a volume of 0·5–1 ml. Evaporation to dryness was effected in an air current. The residue was collected immediately in 0·5 ml chloroform.

The dichloromethane extract must be concentrated with great care in view of the considerable losses of volatile dialkylnitrosamines that may occur, as has been reported by Möhler & Mayrhofer (1968). The efficacy of extraction increases with an increase in the number of carbon atoms in the series of dialkyl and alicyclic nitrosamines. The loss of dimethylnitrosamine due to extraction and evaporation is 70–80% while that of diethylnitrosamine is 30–40%.

Analysis of extracts. Like other investigators, we used the method of Preussmann, Neurath, Wulf-Lorentzen, Daiber & Hengy (1964), which separates the nitrosamines by thin-layer chromatography on silica gel G, using a mixture of hexane–diethyl ether–dichloromethane (4:3:2, by vol.) as the developing solvent. On two silica gel plates (250 nm thick), up to 100 μl of the concentrated extract was applied per spot. Pure nitrosamines were simultaneously chromatographed as controls. After development, one plate was sprayed with a diphenylamine–palladium chloride reagent and then exposed for a few minutes to shortwave (240 mμ) ultraviolet irradiation (Daiber & Preussmann, 1964), under which conditions nitrosamines give blue-to-violet spots. The other plate was first exposed to ultraviolet light and then immediately sprayed with Griess reagent (sulphanilic acid–naphthylamine) (Preussmann, Daiber & Hengy, 1964). With this reagent, alkylnitrosamines give reddish-violet spots, while aromatic nitrosamines give green-to-blue spots with a red border. Neither staining reaction is entirely specific, but the two methods supplement each other in specificity so that nitrosamines can be considered to have been demonstrated only when both reactions are positive. For identification, the R_{ST} values of the spots on the chromatograms were used, these values being defined as follows:

$$R_{ST} = \frac{\text{distance between spot to be determined and start}}{\text{distance between standard spot and start}}$$

The R_{ST} values are determined by applying to the plates spots of the concentrated extract with an internal standard. The reproducibility of R_{ST} values is superior to that of R_F values (Stahl, 1962). The amounts of nitrosamines in the extracts can be estimated by semi-quantitative approximation, by visual comparison of the spot diameters on the chromatograms.

The sensitivity of this reaction was determined by adding pure dimethyl- or diethylnitrosamine to the spinach. It was found that 0·5 ppm dimethyl- and 0·1 ppm diethylnitrosamine were demonstrable in the spinach.

Experiments with addition of diethylamine to spinach. Taylor & Price (1929) demonstrated that the rate of reaction in the production of nitrosamine from amine and nitrite is proportional to the concentration of the amine ion, the NO_2-ion and the undissociated nitrous acid molecule. It is evident that the rate of reaction is influenced, not only by temperature

and the concentration of the reagents, but also by the pH. Consequently, the hydrogen ion concentration and the reaction temperature were varied in a number of experiments using a high amine concentration. For this purpose, samples of 50 g nitrite-bearing spinach were treated by addition and admixture of a 100-fold excess of diethylamine (calculated on the nitrite) dissolved in 2 M-HCl, and then left to stand at room temperature for several days or heated for 1 hr at 70–80°C.

Results and Discussion

Thin-layer chromatography of the extracts of the stored spinach samples revealed no nitrosamines within the limits of detection of the analyses. Neither could nitrosamines be demonstrated by the extraction and analysis of nitrite-bearing spinach samples treated with excess diethylamine and stored at room temperature for several days or heated for 1 hr at 70–80°C. However, when the pH was lowered from 10·1 to 3·1 and the spinach was kept for a week, it was possible to demonstrate the presence of a small amount of diethylnitrosamine, equivalent to 0·45% of the theoretically possible amount.

In spinach with a higher nitrite concentration (580 ppm), at pH 7·6, it was possible after storage for 27 hr at room temperature or after heating for 2·5 hr at 70–100°C, to demonstrate more than 0·7 and 0·8% respectively of the theoretically possible amount of nitrosamine.

The influence of the pH on the production of diethylnitrosamine is in agreement with the equation formulated by Taylor & Price (1929). A low pH will mainly cause the amine-ion concentration to rise and suppress the dissociation of nitrous acid.

Sander (1968) focused attention on the bacterial production of nitrosamines. Certain nitrate-reducing Enterobacteriaceae are capable of synthesizing nitrosamines from nitrates and secondary amines. For the synthesis of dimethyl- and diethylnitrosamine in spinach, however, these processes are of less importance because these micro-organisms show only a limited ability to synthesis aliphatic secondary amines into the corresponding nitrosamines. These aliphatic secondary amines are more strongly basic than aromatic or semi-aromatic ones. The lower the pK_b value, or the greater the basicity, the more difficult it is to nitrose a secondary amine in a bacteriological or purely chemical process.

According to Sander, Schweinsberg & Menz (1968), synthesis of nitrosamines as a purely chemical reaction, without bacterial activity, is possible below pH 4·5; but Sander (personal communication in 1969) also believes that nitrosamines can be formed in a neutral or even a weakly alkaline medium, provided the nitrite concentration is high and the reaction time long. The results of our study confirm this. At pH 7·6, traces of diethylnitrosamine were demonstrable after 27 hr at room temperature or 2·5 hr at 70–100°C.

The data in the literature and the results obtained in our own experiments warrant the conclusion that production of nitrosamine in nitrite-bearing spinach takes place only under extreme conditions, such as the presence of high or very high concentrations of secondary amines and nitrite. Even with a 100-fold excess of amine, analyses disclosed less than 1% of the theoretically possible amount of nitrosamine. This is consistent with the results found in flour under similar conditions by Thewlis (1967), who found about 2% of the theoretically possible amount of nitrosamine in flour treated with secondary amine at pH 3·3.

Such extreme conditions are ruled out completely in the normal storage of spinach. Nitrosamines can be formed in nitrite-bearing spinach, albeit in amounts that appear to be relatively small, the nitrosamine production being limited by the low concentration of secondary amines and the pH (weakly alkaline to neutral). In practice it is also important

to remember that spinach above a certain nitrite concentration (some 250 ppm) always looks more or less wilted and consequently would generally be rejected for consumption.

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