A monograph on **Nitrates** & **Nitrites**-Volume 2 6/59

NITRATES/NITRITES

HARD COPY - CITED PAPERS

REFERENCE NUMBERS 12 - 589

SET 2 OF 5

Poultry Science - Vol 45 (1966)

SUMMARY

The effect of different template sizes, media and incubation temperatures on bacteria counts from poultry carcasses stored 0, 4 and 8 days were determined. Samples were taken by using a cotton swab rubbed over 2, 5 or 10 sq. cm. areas of skin outlined by templates. Although a 2 sq. cm. template gave the highest counts/sq. cm. in 29 out of 36 samples the differences were not significant ($p < .05$). No significant differences in counts ($p < .05$) were observed among the several media (Tryptone-Glucose Agar, Heart Infusion Agar, Eugon Agar and Trypticase Soy Agar) or among three incubation temperatures 10, 20 and 30°C., respectively.

REFERENCES

American Public Health Association, 1960. Standard Methods for the Examination of Dairy Products. New York.

Baltimore Biological Laboratories, 1956. Products for the Microbiological Laboratory. Baltimore, Md.

Difco Laboratories, 1953. Difco Manual of Dehydrated Culture Media and Reagents for Microbiological and Clinical Laboratory Procedures. Detroit, Mich.

Ayres, J. C., W. S. Ogilvy and G. F. Stewart, 1950. Post-mortem changes in stored meats. I. Microorganisms associated with development of slime on eviscerated cut-up poultry. Food Technol. 4: 199–205.

Gunderson, M. F., H. W. McFadden and T. S. Kyle, 1954. The Bacteriology of Commercial Poultry Processing. Burgess Publishing Co., Minneapolis, Minn.

Fromm, D., 1959. An evaluation of techniques commonly used to quantitatively determine the bacterial population on chicken carcasses. Poultry Sci. 38: 887–893.

Malimann, W. L., L. E. Dawson, D. M. Sultser and H. S. Wright, 1958. Studies on microbiological methods for predicting shelf-life of dressed poultry. Food Technol. 12: 122–126.

Straka, R. P., and J. L. Stokes, 1957. Rapid destruction of bacteria in commonly used diluents and its elimination. App. Microbiol. 5: 21-25.

12

Effects of Nitrate and Nitrite in the Drinking Water on Chicks, Poults and Laying Hens[1]

A. W. ADAMS[2] R. J. EMERICK[3] AND C. W. CARLSON[4]

Departments of Poultry Science and Station Biochemistry, South Dakota State University, Brookings, South Dakota

(Received for publication March 4, 1966)

INTRODUCTION

THE effect of high levels of dietary nitrate and nitrite on various mammalian species has been the subject of numerous investigations. In several of these studies dietary nitrate and nitrite have been reported to depress growth in swine, Tollet *et al.* (1960); rats, Smith *et al.* (1961); and cattle, Weichenthal *et al.* (1963). On the other hand, Seerley *et al.* (1965) found that drinking water containing up to 100 p.p.m. sodium nitrite nitrogen and 300 p.p.m. of sodium nitrate nitrogen had no significant effect on the performance of growing and finishing swine. They also found in these same studies that 1000 p.p.m. sodium nitrate nitrogen had no effect on the daily gain of fattening lambs.

Interference with normal vitamin A me-

[1] Approved for publication by the Director of the South Dakota Agric. Exp. Sta. as paper No. 709 of the Journal Series.

[2] Graduate assistant, Dept. of Poultry Science, present address, Kansas State Univ., Manhattan.

[3] Professor, Station Biochemistry Dept.

[4] Professor, Dept of Poultry Science.

tabolism by either nitrate or nitrite has been reported with cattle, Muhrer *et al.* (1955); rats, Emerick and Olson (1962); and sheep, Goodrich *et al.* (1964).

Several workers, among them Bloomfield *et al.* (1961), have reported that dietary nitrate interferes with normal thyroid function in rats.

Investigations on the possible deleterious effects of dietary sources of these materials on the avian species are limited. Sell and Roberts (1963) demonstrated that 0.4% dietary potassium nitrite depressed growth, lowered liver vitamin A stores and caused hypertrophy of the thyroid gland in chicks. Sunde (1964) reported that day-old turkey poults fed practical-type starting diets containing 400 p.p.m. of nitrate, as the potassium salt, exhibited high mortality and depressed growth. The addition of 20,000 I.U. of vitamin A per kilogram of diet prevented excess mortality and allowed normal growth.

Nitrate deposition in feedstuffs occurs primarily in the leaves and stems of non-leguminous plants such as oats, corn, barley, wheat and soghum (Whitehead, 1956). Since these plant materials make up a very small portion of modern poultry rations, it would appear that water represents the greatest potential nitrate hazard for poultry.

The data reported herein describes the effects of various levels of nitrate and nitrite, administered continuously in the drinking water, on chicks, poults and laying hens.

EXPERIMENTAL

Chicks. Two experiments were conducted with day-old Single Comb White Leghorn (S.C.W.L.) cockerels. The chicks were wingbanded, weighed and randomly distributed into electrically heated battery brooders. Duplicate groups of chicks were given Brookings tap water to which various levels of either sodium nitrate or nitrite were added. The levels used were as follows: experiment 1—0, 75, 150 and 300 p.p.m. of nitrate-nitrogen ($NO_3^- - N$), and 25 and 50 p.p.m. of nitrite-nitrogen ($NO_2^- - N$), and experiment 2—0, 50, 100 and 200 p.p.m. of nitrite nitrogen.

The experiments were conducted for 28 days. In each experiment a standard chick starter (Bonzer *et al.*, 1962) and the water were provided *ad libitum*. The ration contained approximately 9500 I.U. of vitamin A activity/kilogram, supplied as 5300 and 4200 I.U. of vitamin A and β-carotene, respectively. Stock solutions of sodium nitrate and nitrite were mixed as needed and stored in plastic containers. Each afternoon the solutions that were not consumed during the previous day were removed and measured, the zinc-coated galvanized tin troughs washed in a detergent-sanitizer solution and rinsed, and the troughs refilled with freshly mixed solutions. Additional amounts of the various solutions were added the following morning as needed. Records were maintained on mortality, feed and water consumption and body weight.

Total blood hemoglobin and methemoglobin values were determined approximately midway during the first chick experiment by the method of Evelyn and Malloy (1938).

At the end of each experiment, 20 chicks were randomly selected from each treatment and sacrificed. Their livers were removed and immediately frozen. Liver vitamin A and β-carotene values were determined later by the method of Johnson and Baumann (1947).

The nitrite-nitrogen content of a sample of each solution after sixteen hours in the drinking trough was determined once during each experiment by the American Waterworks Association method (1955) to de-

termine the degree of destruction of nitrate and/or nitrite.

Poults. An experiment with Broad Breasted White turkey poults was conducted under essentially the same conditions and procedures as those used in the chick experiments. For this experiment, sodium nitrite was added to the drinking water at the levels of 0, 50, 100 and 200 p.p.m. of nitrite-nitrogen. A standard turkey starter (Carlson and Bonzer, 1962) calculated to contain 14,000 I.U. of vitamin A activity/kilogram, supplied as 10,500 and 3,500 I.U. of vitamin A and β-carotene, respectively, was fed. Records were kept on mortality, feed and water consumption and body weight. Liver vitamin A and β-carotene determinations were made on 16 poults from each treatment according to the procedures described in the previous section.

Laying Hens. Two experiments were conducted with egg production-type pullets housed in a windowless, insulated and fan-ventilated building.

For the first experiment the pullets were housed in two types of cages. Ten S.C.W.L. pullets per treatment in individual cages and 15 egg production-type crossbred pullets per treatment in colony cages were used. The pullets in each type of cage received Brookings tap water for an eight-week period to which various levels of either sodium nitrate or nitrite were added. The levels used were as follows: 0, 75, 150 and 300 p.p.m. nitrate-nitrogen and 25 and 50 p.p.m. of nitrite-nitrogen.

The water was supplied to the pullets in zinc-coated galvanized cup waterers of 946 ml. capacity. One cup was provided for every two hens in individual cages and three cups for each colony cage.

For the second experiment, eight crossbred pullets were selected at random from each of the six colony-cage pens that were used in the first experiment and equally distributed (two per group) among four groups of individual cages. This made a total of 12 birds per treatment. Starting one week after transfer, the four groups of hens were administered Brookings tap water to which 0, 50, 100 and 200 p.p.m. levels of nitrite-nitrogen were added for a six-week period.

In both experiments a standard all-mash laying ration (Bonzer *et al.*, 1962) and water were provided *ad libitum*. The laying ration contained approximately 9850 I.U. vitamin A/kilogram supplied as 5300 and 4550 I.U. vitamin A and β-carotene, respectively.

Except for the second experiment, the procedures used in preparing and handling the solutions for this experiment were similar to those used in the chick and poult experiments. For the second experiment, sufficient amounts of the solutions were added to each cup to last for 24 hours, at about 4 P.M. each day.

During the experiments records were maintained on mortality, feed and water consumption, egg production and body weight. Egg quality measurements were made on all eggs laid on three successive days during the latter part of each experiment. At the termination of the last experiment, liver vitamin A and β-carotene values were determined on six hens from each treatment. Once during the second experiment a sample of water was taken from each treatment and analyzed for nitrite-nitrogen by the American Waterworks Association method (1955).

Where calculations appeared to be warranted, data were analyzed for significance according to Steele and Torrie (1960).

RESULTS AND DISCUSSION

Chicks. The data presented in Table 1 show that the levels of nitrate or nitrite-nitrogen used in these experiments had very little effect on feed and water consumption

TABLE 1.—*Effect of nitrate or nitrite administered in the water on chick weight, water and feed consumption and mortality*

Added nitrate or nitrite	Average 4-week weight	Feed cons.	Mortality	Water cons.
	gms.	gms./chick/day	%	ml./chick/day
Experiment 1				
0	262	23	0.0	40
75 p.p.m. NO_3^--N	271	22	0.0	42
150 p.p.m. NO_3^--N	281	23	0.0	44
300 p.p.m. NO_3^--N	262	24	2.6	44
25 p.p.m. NO_2^--N	252	23	7.5	39
50 p.p.m. NO_2^--N	251	23	5.0	38
Experiment 2				
0	263	22	2.5	40
50 p.p.m. NO_2^--N	261	25	0.0	39
100 p.p.m. NO_2^--N	263	24	2.5	39
200 p.p.m. NO_2^--N	237*	24	2.5	36

* L.S.D. = 18 gm. (P = .05)

of the chicks. A significant reduction in weight occurred with the 200 p.p.m. nitrite-nitrogen level.

Mortality in both experiments was attributed to weak chicks and starve-outs since the losses in all groups occurred primarily during the first six days of the experiments.

The data presented in Table 2 show that in experiment 1 neither nitrate nor nitrite had a consistent effect on liver vitamin A and β-carotene levels. Variation between determinations and replicates was high. On the other hand, the chicks in experiment 2, which received 200 p.p.m. nitrite-nitrogen had a significantly lower level of vitamin A than either the chicks receiving 50 p.p.m. nitrite-nitrogen or the controls. The 100 and 200 p.p.m. levels of nitrite-nitrogen appeared to depress liver β-carotene; however, the differences were not significant due to considerable variation between determinations.

The percent of total hemoglobin which

TABLE 2.—*Blood hemoglobin and methemoglobin, and liver vitamin A and β-carotene levels of chicks which received nitrate or nitrite in the drinking water*

Added nitrate or nitrite	Methemoglobin[1]	Total hemoglobin[1]	Vitamin A[2]	β-carotene[2]
	gm./100 ml. blood		mcg./gm. liver	
Experiment 1				
0	0.4	9.5	54	1,378
75 p.p.m. NO_3^--N	0.7	10.0	54	1,676
150 p.p.m. NO_3^--N	0.6	9.7	62	1,444
300 p.p.m. NO_3^--N	0.9	9.4	59	1,336
25 p.p.m. NO_2^--N	0.5	9.8	47	1,290
50 p.p.m. NO_2^--N	0.7	9.2	51	1,278
Experiment 2				
0	(No data obtained)		38[3]	1,075
50 p.p.m. NO_2^--N			37	1,078
100 p.p.m. NO_2^--N			30	944
200 p.p.m. NO_2^--N			22	753

[1] Based on six determinations per treatment.
[2] Based on four determinations per treatment, five livers were pooled for each determination.
[3] L.S.D. = 12 mcg. (P = .05).

TABLE 3.—*Effects of nitrite in the drinking water for poults on weight, water and feed consumption, and mortality*

Added Nitrite	Average 4-week weight	Feed cons.	Mortality	Water cons.
	gms.	gms./poult/day	%	ml./poult/day
0	643[1]	35	6.2	88
50 p.p.m. NO_2^--N	603	31	6.2	83
100 p.p.m. NO_2^--N	582	31	3.1	80
200 p.p.m. NO_2^--N	506	29	12.5	70

[1] L.S.D.—88 gms. (P = .05)

appeared as methemoglobin was low for all treatments in experiment 1 (Table 2). The results indicate that the levels of nitrate or nitrite nitrogen used in this experiment had very little effect on the rate of hemoglobin formation and its reduction to methemoglobin.

Poults. The data in Table 3 show that as the level of nitrite-nitrogen in the drinking water was increased there was a corresponding decrease in the growth rate of the poults. Accompanying this reduction in growth was a drop in water and feed consumption. Whether this reduced intake was the result of a smaller body size or of a physiological effect of nitrite on the poult cannot be determined from the data obtained. Sell and Roberts (1963) observed that 0.4 percent dietary nitrite administered in the ration depressed chick growth under *ad libitum* feeding conditions. However, when nitrite and non-nitrite fed chicks were pair-fed, no depression in growth was observed. Therefore they concluded that dietary nitrite depressed growth of the chicks primarily by reducing feed consumption.

There was a significant decrease in liver vitamin A and β-carotene with the intake of the water containing 200 p.p.m. nitrite-nitrogen (Table 4). These results indicate that the nitrite in some manner decreased the utilization of these materials. A similar effect of dietary nitrite on liver vitamin A and β-carotene was observed with rats by Emerick and Olson (1962) and, when vitamin A and β-carotene were administered orally with chicks, by Sell and Roberts (1963).

The data in Tables 1–4 suggest that the poult is more susceptible to nitrite toxicity than the chick. Krista *et al.* (1961) reported that poults were more susceptible to high levels of sodium chloride in the water than chicks or ducklings.

Layers. The data in Table 5 show that the addition to the drinking water of neither nitrate nor nitrite affected egg production and egg quality. Although marked differences were obtained in the rate of egg production among the various treatments in experiment 1, no consistent relationship between level of either nitrate or nitrite and rate of egg production was observed.

Egg weight, Haugh unit values and shell thickness were not affected by the levels of nitrate or nitrite-nitrogen used in these experiments.

There appeared to be a drop in liver vi-

TABLE 4.—*Effects of nitrite in the drinking water for poults on liver vitamin A and β-carotene*

Added nitrite	Vitamin A[1]	β-carotene[1]
	mcg./gm. liver	mcg./gm. liver
0	43[2]	131[3]
50 p.p.m. NO_2^--N	47	156
100 p.p.m. NO_2^--N	39	166
200 p.p.m. NO_2^--N	23	99

[1] Based on four determinations per treatment, four livers pooled for each determination.
[2] L.S.D. = 8 mcg. (P = .05)
[3] L.S.D. = 30 mcg. (P = .05)

TABLE 5.—*The effect of added nitrate or nitrite in the drinking water on egg production and egg quality*

Added nitrate or nitrite	Hen day egg production		Haugh unit[1]		Egg weight		Shell thickness	
	I[2]	C[3]	I	C	I	C	I	C
	%	%			gm.	gm.	mm.	mm.
Experiment 1								
0	53.8	61.5	83	83	60	59	0.386	0.396
75 p.p.m. NO_3^--N	58.5	45.5	83	78	59	61	0.379	0.386
150 p.p.m. NO_3^--N	58.7	60.5	81	85	59	60	0.368	0.399
300 p.p.m. NO_3^--N	44.3	74.0	81	83	57	58	0.391	0.379
25 p.p.m. NO_2^--N	59.5	58.4	83	85	58	58	0.383	0.376
50 p.p.m. NO_2^--N	56.0	60.1	84	84	58	55	0.376	0.376
Experiment 2								
0	65.1		82		57		0.371	
50 p.p.m. NO_2^--N	69.4		83		60		0.373	
100 p.p.m. NO_2^--N	69.5		80		62		0.376	
200 p.p.m. NO_2^--N	65.7		82		62		0.384	

[1] Interior egg quality measurement, as described by Haugh, 1937.
[2] Individual cages.
[3] Colony cages.

tamin A and β-carotene at the 200 p.p.m. level of added nitrite-nitrogen; however, this effect was not significant (Table 6).

Water consumption and body weight were not consistently affected by the added sodium nitrate or nitrite. The general appearance of the hens was good although during the first part of the experiment, the hens receiving 150 and 300 p.p.m. nitrate-nitrogen produced a rather fluid feces. The hens apparently adjusted to these levels since this condition was corrected after the third week of the experiment.

Disappearance of nitrate and nitrite. The data in Table 7 indicate that the degree of destruction of nitrate and/or nitrite

TABLE 6.—*The effect of added nitrate or nitrite in the drinking water for layers on water consumption and liver vitamin A and β-carotene*

Added nitrate or nitrite	Water consumption		Vitamin A[3]	β-carotene[3]
	I[1]	C[2]		
	ml./hen/day	ml./hen/day	mcg./gm. liver	
Experiment 1				
0	199	245		
75 p.p.m. NO_3^--N	229	228		
150 p.p.m. NO_3^--N	206	268		
300 p.p.m. NO_3^--N	196	236		
25 p.p.m. NO_2^--N	213	215		
50 p.p.m. NO_2^--N	229	200		
Experiment 2				
0	243		316	2814
50 p.p.m. NO_2^--N	232		306	2564
100 p.p.m. NO_2^--N	226		346	3287
200 p.p.m. NO_2^--N	243		287	2409

[1] Individual cages.
[2] Colony cages.
[3] Based on two determinations per treatment, three livers pooled for each treatment.

was related to the environmental temperatures that were present in the rooms where the experiments were conducted.

Very little loss occurred in the layer experiment where the environmental temperatures on the building ranged between 4 and 16°C throughout most of the experiment. On the other hand, almost one-half of the nitrite was lost in the chick experiment. This experiment had been conducted in a heated battery room where the air temperature was maintained between 21–24°C. Although the data suggest some conversion of nitrate to nitrite in the chick experiment, the chick's tolerance to nitrite suggests that, under conditions of normal environmental temperatures, the hazard of nitrite toxicity due to bacterial reduction of nitrate to nitrite in water is limited. The data suggest that whereas the chick and hen are quite resistant to nitrite poisoning, the poult is less so. However under these conditions of vitamin A adequacy, the poult could still tolerate rather large amounts of nitrite nitrogen.

TABLE 7.—*Nitrite in the drinking water as influenced by treatments and environmental conditions*

Added nitrate or nitrite	Level of nitrite-nitrogen		
	Chick exp. No. 1[1]	Layer exp. No. 1	
		I[2]	C[3]
	p.p.m.	p.p.m.	p.p.m.
0	0.0	0.0	0.0
75 p.p.m. NO_3^--N	4.8	0.3	0.2
150 p.p.m. NO_3^--N	6.8	0.6	0.2
300 p.p.m. NO_3^--N	5.8	0.2	0.1
25 p.p.m. NO_2^--N	11.9	24.5	24.5
50 p.p.m. NO_2^--N	22.0	47.0	47.0

[1] Water in battery brooder room at about 21–24°C. for 16 hours.
[2] Individual cages.
[3] Colony cages.
[1,3] Water in cage layer house at about 4–16°C. for 16 hours.

SUMMARY

Various levels of either sodium nitrate or nitrite were administered continuously in Brookings tap water to day-old chicks or poults and laying hens. The stock was maintained in otherwise standard conditions and fed practical diets containing 9500, 14,000 and 9850 I.U. of vitamin A activity/kg., respectively.

Up to 200 and 300 p.p.m. of nitrite and nitrate nitrogen, respectively, had very little effect on blood methemoglobin, mortality, and feed and water consumption of chicks. A reduction in growth and liver vitamin A was observed with 200 p.p.m. nitrite-nitrogen.

With poults, as the level of nitrite-nitrogen was increased from 50 through 100 and 200 p.p.m. there were corresponding decreases in water and feed consumption and growth. The 200 p.p.m. level of nitrite-nitrogen significantly reduced liver β-carotene and liver vitamin A.

In the study with layers, levels up to 200 p.p.m. nitrite-nitrogen or 300 p.p.m. nitrate-nitrogen failed to consistently affect rate of lay, daily water consumption, mortality, egg quality, or liver vitamin A and β-carotene.

There was a greater disappearance of nitrate or nitrite in the water in the chick brooder room than of that in the layer house.

The results indicate that water containing up to 300 p.p.m. nitrate nitrogen or 100 p.p.m. nitrite nitrogen is satisfactory for poultry when used with a diet containing a good margin of vitamin A activity.

REFERENCES

American Waterworks Assoc., 1955. Standard Methods for Examination of Water, Sewage and Industrial Wastes, p. 153, 10th ed.

Bloomfield, R. A., C. W. Welsch, G. B. Garner and M. E. Muhrer, 1961. Effect of dietary nitrate on thyroid function. Science, 134: 1690.

Bonzer, B. J., C. W. Carlson and R. W. Peiper, 1962. Feeding chickens. Fact Sheet 136, Coop. Ext. Serv., South Dak. State Univ.

Carlson C. W., and B. J. Bonzer, 1962. Feeding turkeys. Fact Sheet 143, Coop. Ext. Serv., South Dak. State Univ.

Emerick, R. J., and O. E. Olson, 1962. Effect of nitrate and nitrite on vitamin A storage in the rat. J. Nutrition, 78: 73–77.

Evelyn, K. A., and H. T. Malloy, 1938. Microdetermination of oxyhemoglobin, methemoglobin and sulfhemoglobin in a single sample of blood. J. Biol. Chem. 126: 655.

Goodrich, R. D., R. J. Emerick and L. B. Embry, 1964. Effect of sodium nitrate on the vitamin A nutrition of sheep. J. Animal Sci. 23: 100–104.

Haugh, R. R., 1937. The Haugh Unit for measuring egg quality. U. S. Egg Poultry Mag. 43: 522–555, 572–573.

Johnson, R. M., and C. A. Baumann, 1947. Storage and distribution of vitamin A in rats fed certain isomers of carotene. Arch. Biochem. 14: 361.

Krista, L. M., C. W. Carlson and O. E. Olson, 1961. Some effects of saline waters on chicks, laying hens, poults and ducklings, Poultry Sci. 40: 938–944.

Muhrer, M. E., A. A. Case, G. B. Garner and W. H. Pfander, 1955. Toxic forage produced in a drought area. J. Animal Sci. 14: 1251.

Seerley, R. W., R. J. Emerick, L. B. Embry and O. E. Olson, 1965. Effect of nitrate or nitrite administered continously in drinking water for swine and sheep. J. Animal Sci. 24: 1014–1019.

Sell, J. L., and W. K. Roberts, 1963. Effects of dietary nitrate on the chick: Growth, liver vitamin A stores and thyroid weight. J. Nutrition, 79: 171–178.

Smith, G. S., A. L. Neumann and E. E. Hatfield, 1961. Carotene utilization and vitamin A nutrition as influenced by dietary nitrite and "high nitrate" silage: Laboratory studies. J. Animal Sci. 20: 683.

Steele, R. G. D., and J. H. Torrie, 1960. Principles and Procedures of Statistics, McGraw-Hill Book Co., Inc., N.Y., N.Y.

Sunde, M. L., 1964. The use of the turkey poult as a test animal for nitrite toxicity. Poultry Sci. 43: 1368.

Tollett, J. T., D. E. Becker, A. H. Jensen and S. W. Terrill, 1960. Effect of dietary nitrate on growth and reproductive performance of swine. J. Animal Sci. 19: 1297.

Weichenthal, B. A., L. B. Embry, R. J. Emerick and F. W. Whetzal, 1963. Influence of sodium nitrate, vitamin A and protein level on feedlot performance and vitamin A status of fattening cattle. J. Animal Sci. 22: 979–984.

Whitehead, E. I., 1956. Nitrate poisoning. South Dakota Farm and Home Res. 7: 70–73.

The Erythrocyte Sedimentation Rate Test in the Domestic Fowl (Chicken)[1]

R. R. BURTON, E. L. BESCH AND A. H. SMITH

Department of Animal Physiology, University of California at Davis, Davis, California, 95616

(Received for publication March 4, 1966)

INTRODUCTION

THE erythrocyte sedimentation rate (ESR) test was developed by Westergren (1920, 1926) as a prognostic clinical aid in medicine and its value to the veterinary profession, principally in the dog and cat, has been amply demonstrated (Schalm, 1961). The ESR of birds has been studied previously by Redmond *et al.* (1951), Ponder (1925), Grundboeck (1960), Swenson (1951), Gray *et al.* (1954), Gilbert (1962), Sturkie and Textor (1958, 1960), Goff *et al.* (1953), and Dittmer (1961). However, the value of the

[1] The research reported herein was supported by the Office of Naval Research, Project Number NR 102-448 and the National Aeronautics and Space Administration (Contract R-53) and U.S.P.H. Grant HE01920.

e gel pattern presented in Fig. 2 shows that material from -treated animals has two light bands, which are not in control or anthracene treated skin. The distribution ...y is shown in Fig. 3. The peaks correspond to n bands at positions 6, 12, 15, 20 and 24 of the gel. Most ounts were associated with protein components migrat- e upper half of the gel.

binding of carcinogens to water-soluble proteins has been sted[18,19] to be important in carcinogenesis. The speci- d cellular function of the protein binding have not yet entified. The hypothesis that chalones control mitotic ostasis[5,8,9] suggested that the carcinogen might bind to rowth regulating proteins.

ound that after mouse skin was painted with DMBA-^{14}C, ...est specific activity (c.p.m. per mg of protein) was ntered in the fraction of the water-soluble protein precipi- ith between 70 and 80% alcohol. This fraction has ported[2] to contain more than 80% of the epidermal ne. The binding to the 70–80% fraction seems to be ent, because no labelled material was released after pro- gestion or acid hydrolysis of the bound fraction, followed action with organic solvents and thin-layer chromato- y. Covalent binding has been reported to occur between gens and the soluble proteins of mouse skin[5,23,31].

70–80% fraction is very active in inhibiting the mitosis o and *in vivo*[5], and so it is possible that the protein to which arcinogen binds is the chalone and, if so, this action may onsiderable importance in carcinogenesis. The 70–80% fraction is not, however, a pure protein, as shown by lectrophoresis, which leaves the identity of the protein in doubt. Further purification of the alcohol fraction is . because of the very small quantity of chalone obtained final step[5]. Only if such a purified chalone is readily able testing of the present hypothesis, that the effect of cinogen is to inactivate intracellular chalones, thereby g mitosis to go unchecked, be possible.

I. S. M. BISHAI

ment of Pathology,
g Institute,
ersity of Toronto,
o, Ontario

M. A. MOSCARELLO

arch Institute,
Hospital for Sick Children,
o 101, Ontario

A. C. RITCHIE

rtment of Pathology,
sity of Toronto,
o, Ontario

d December 14, 1970; revised February 18, 1971.

...ough, W. S., and Laurence, E. B., *Exp. Cell Res.*, 24, 289 (1961).
...ough, W. S., Hewett, C. L., and Laurence, E. B., *Exp. Cell es.*, 36, 192 (1961).
sen, O. H., *Ciba Foundation Symp. Homeostatic Regulators* (edit. by Wolstenholme, G. E. W., and Knight, J.), 29 (Churchill, London, 1969).
...ough, W. S., and Laurence, E. B., in *Adv. Biol. Skin*, 7 (edit. by Montana, W., and Dobson, R. L.) (Pergamon, New York, 1965).
oldingh, W. H., and Laurence, E. B., *Europ. J. Biochem.*, 5, 91 (1968).
lou W. S., Laurence, E. B., Iversen, O. H., and Elgjo, K., *Nature*, 214 (1967).
ersen, O. H., Aandahl, E., and Elgjo, K., *Acta Pathol. Microbiol. Scand.*, 64, 506 (1965).
omaa, T., and Kiviniemi, K., *Cell Tissue Kinetics*, 1, 329 (1968).
ctren, H. A., *Exp. Cell Res.*, 11, 229 (1956).
mnett, J. D., and Chopra, D. P., *Nature*, 222, 1189 (1969).
den, M. J., and Leeson, S. J., *Exp. Eye Res.*, 9, 57 (1970).
m, M., Kinzel, V., Mohr, U., and Suss, R., *Experientia*, 25, 8 (1969).
Bullough, W. S., *The Evolution of Differentiation* (Academic Press, London, 1967).
Bullough, W. S., and Laurence, E. B., *Europ. J. Cancer*, 4 (1968).
Schmidt, O., *Naturwissenschaften*, 29, 146 (1941).
Heidelberger, C., *J. Cell Comp. Physiol.*, 64, Suppl. 1, 129 (1964).
Miller, E. C., *Cancer Res.*, 11, 100 (1951).
Miller, J. A., and Miller, E. C., *Adv. Cancer Res.*, 1, 339 (1953).
Miller, E. C., and Miller, J. A., *Cancer Res.*, 12, 547 (1952).
Sorof, S., Young, E. M., and Fetterman, P. L., *Exp. Cell Res.*, 20, 253 (1960).
Bullough, W. S., *Biol. Rev.*, 37, 307 (1962).
Bullough, W. S., and Laurence, E. B., *Proc. Roy. Soc.*, B, 151, 517 (1960).
Wiest, W. G., and Heidelberger, C., *Cancer Res.*, 13, 246 (1953).
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, 193, 265 (1951).
Patterson, M. S., and Greene, R. C., *Anal. Chem.*, 37, 854 (1965).
Matsushita, H., Suzuki, Y., and Shakabe, H., *Bull. Chem. Soc., Japan*, 36, 13711 (1963).
Panyim, S., and Chalkley, R., *Arch. Biochem. Biophys.*, 130, 337 (1969).
Reisfeld, R. A., Lewis, U. J., and Williams, D. E., *Nature*, 195, 281 (1962).
Clarke, J. T., *Ann. NY Acad. Sci.*, 121, 428 (1964).
Peter, V., Thishler, and Epstein, C. J., *Anal. Biochem.*, 22, 89 (1968).
Abell, C. W., and Heidelberger, C., *Cancer Res.*, 22, 931 (1962).
Mohr, U., Althoff, J., Kimzel, V., Suss, R., and Volm, M., *Nature*, 220, 138 (1968).

Formation of N-Nitrosopiperidine from Piperidine and Sodium Nitrite in the Stomach and the Isolated Intestinal Loop of the Rat

SINCE the first report of hepatic cancer in rats induced by nitrosodimethylamine[1], many nitrosamines have been shown to be carcinogenic in a wide range of organs of a wide variety of species[1–4]. Following the suggestion that dietary nitrite and secondary amines might interact in the human stomach to form nitrosamines[5], such synthesis has been demonstrated *in vitro* with animal and human gastric juice[6,7], and *in vivo* in laboratory animals[7] and man[8]; nitrosamine synthesis was *p*H-dependent[7–10]. Nitrosamines can also be synthesized *in vitro* at near neutral *p*H values in the presence of enteric bacteria[11]; yields of nitrosamines are also determined by the basicity of the secondary amine. Presumptive evidence for *in vivo* synthesis of nitrosamines has been demonstrated by the induction of tumours in rats following chronic feeding of nitrite and secondary amines[11]. We report here the synthesis of nitrosopiperidine in rats from nitrite and piperidine in gastric contents *in vitro*, and in the stomach and in the small intestine *in vivo*.

Male Sprague–Dawley rats (Charles River Laboratories) weighing 150–300 g were fed Purina chow and water *ad libitum* before the day of test. Each rat was anaesthetized with sodium pentobarbital, the abdominal wall incised and the small intestine pulled out and isolated between ligatures at the gastro-duodenal and ileo-caecal junctions. A polyethylene tube (Intramedic, PE No. 50) was inserted into the upper end of the isolated intestine and a third ligature tightened around it. The intestinal loop was then isolated by section above the ileo-caecal ligature, and flushed with saline through the polyethylene tubing. Finally, the open end of the washed loop was ligated. Solutions of various concentrations of sodium nitrite and of piperidine hydrochloride (5 ml.) (Table 1) were introduced into the intestinal loop through the tubing. The loop, with its vasculature intact, was then returned to the abdomen of the anaesthetized rat for 40 min; the abdominal incision was covered by a warmed saline pad. Intestinal contents were subsequently washed out with saline and collected. The *p*H of the intestinal contents was measured with a Beckman meter before and after incubation. *In vivo* experiments were conducted concurrently with intestinal loop

Table 1 *In vivo* Formation of Nitrosopiperidine from Piperidine Hydrochloride and Sodium Nitrite in the Isolated Intestinal Loop and the Isolated Stomach of the Rat

Replicate	Reactant concentration (mg) Piperidine HCl	Sodium nitrite	Total volume of reactants (ml.)	Nitroso-piperidine yield* (μg)	pH
Small intestine					
1	125	25	10	0	6.5
2	125	25	10	0	6.5
3	125	25	10	0	6.5
4	125	25	10	0	6.6
5	625	25	10	25	6.5
6	625	25	10	21	6.6
7	625	25	10	5	6.6
8	1,250	25	10	159†	6.5
9	1,250	25	10	157	6.5
Stomach					
1	625	25	10	12	3.0
2	625	25	10	24	4.0
3	312.5	12.5	5	11	5.4
4	250	10	4	22	3.6
5	500	10	4	19	4.2
6	500	10	4	46	3.6
7	500	10	4	23	4.2

* Estimated by GLC.
† Confirmed by mass spectroscopy.

studies in some rats. The stomach was isolated between ligatures at the oesophago-gastric and gastro-duodenal junctions. A polyethylene tube was inserted through the latter and a third ligature tightened around it. Solutions of various concentrations of nitrite and piperidine (Table 1) were then introduced through the polyethylene tube. The stomach, with its intact vasculature, was then returned to the abdomen of the rat for 40 min. The stomach contents were subsequently washed out with saline and collected. Intestinal washings and stomach contents, excluding coarse food particles, were incubated aerobically with constant shaking on a mechanical shaker, for 30 min with nitrite and piperidine at 37° C (Table 2).

Nitrosopiperidine was extracted by described methods earlier[7]. The intestinal or stomach contents along with the washings, in volumes of 60–80 ml., were filtered through Whatman No. 1 paper to remove coarse particles. The filtrate was passed through anion ('Amberlite IRA-400', chloride form) and cation ('Rexyn 101', hydrogen form) exchange columns; columns were then washed with distilled water. The combined washings and effluent were extracted twice with 150–200 ml. aliquots of methylene chloride; emulsions forming during solvent extraction were broken by centrifugation. The extract was dried over anhydrous sodium sulphate, and the solvent was distilled off at 37° C until 15–20 ml. of the extract remained. This was transferred to a centrifuge tube and evaporated to dryness under nitrogen. The residue was dissolved in 0.1–0.2 ml. methylene chloride. An aliquot of this solution was used for the estimation of nitrosopiperidine by gas liquid chromatography and for its identification by thin-layer chromatography (TLC)[12]. An F and M 500 gas chromatograph equipped with 1609 hydrogen flame ionization detector and 1 mV recorder was used. Gas liquid chromatography (GLC) conditions were: 6% coating of 'Reoplax' on 60–80 mesh chromosorb W, in stainless steel column ¼" inner diameter; helium flow rate at delivery pressure of 26–30 pounds/inch² and rotameter setting at 6.2–6.5, column temperature 100°–115° C, range setting at 100 and attenuation 2; 1–2 μl. samples were injected into the GLC column. The limit of detection of nitrosopiperidine in 0.1–0.2 ml. of total extract was 1 μg. The mean zero time recoveries of 5–10 mg of nitrosopiperidine added to saline, and to intestinal contents *in vitro*, were 23% and 22%, respectively. The mean recoveries of 10 mg nitrosopiperidine at zero time and after 40 min in the small intestine were 10% and 3%, respectively.

Nitrosopiperidine was synthesized *in vivo* in the small intestine of rats from nitrite and piperidine (Table 1). With a constant nitrite concentration, the yield of nitrosopiperidine was dependent on the piperidine concentration, and varied from 5 to 159 μg. The identity of nitrosopiperidine was established by a combination of techniques. After TLC separation the spot with the same R_F value as standard nitrosopiperidine was extracted and injected into a GLC column. A single peak with the same retention time as authentic nitrosopiperidine was obtained. The presence of nitrosopiperidine in an extract was confirmed by mass spectrometry, using an AEI-MS-9 instrument. A peak at m/e 114 was obtained; accurate mass measurement showed it to be 114.0794; $C_5H_{10}N_2O$ (nitrosopiperidine) = 114.0793. Nitrosopiperidine was also synthesized *in vivo* in the rat stomach from piperidine and nitrite in concentration ratios of 25/1 and 50/1 (Table 1); yields of nitrosopiperidine ranged from 11–40 μg. Higher yields of nitrosopiperidine, ranging from 327–785 μg, were, however, found *in vitro* in gastric juice (Table 2). Nitrosopiperidine was not, however, synthesized *in vitro* from its precursors in small intestinal washings (Table 2).

Table 2 *In vitro* Formation of Nitrosopiperidine from Piperidine Hydrochloride and Sodium Nitrite in Small Intestine Washing and in Gastric Juice of the Rat

Replicate	Reactant concentration (mg) Piperidine HCl	Sodium nitrite	Total volume of reactants (ml.)	Nitroso-piperidine yield (μg)	pH
Small intestine washing					
1	1,250	25	10	0	6.5
2	1,250	25	10	0	6.6
3	1,250	25	10	0	6.7
Gastric juice					
1	1,250	25	10	327	4.0
2	1,250	25	10	346	4.0
3	1,250	25	10	785	4.0

The yields of nitrosopiperidine reported here are probably underestimates in view of the low recoveries. Furthermore, no effort was made to correct for nitrosopiperidine absorbed during 40 min *in vivo*. This may also account for the relatively lower yields of nitrosopiperidine from the stomach than from gastric contents *in vitro*. The failure to detect nitrosopiperidine in intestinal contents *in vitro* may reflect the unfavourable condition of incubation. Nitrosopiperidine is a potent carcinogen producing tumours of the oesophagus in rats, as well as at other sites, after oral, intravenous or subcutaneous administration[3]. The oral carcinogenic dose of nitrosopiperidine in rats is 5 mg/kg for 9–12 months, equivalent to a total lifetime dose of 2,000 mg/kg[3]; but no reports of dose response studies are available on the carcinogenicity of nitrosopiperidine. Our studies show the synthesis of nitrosopiperidine in the rat from nitrite and piperidine in gastric juice *in vitro* and in the stomach and small intestine *in vivo*. The *in vivo* synthesis of nitrosopiperidine from a single dose of nitrite and piperidine, in yields ranging from 5–159 μg and 11–40 μg in small intestine and stomach, respectively, is noteworthy especially because these yields are underestimates as a result of low recovery.

Piperidine may be ingested by man through some foods and spices. It can also be synthesized from the dietary lysine by intestinal bacteria[13]. The average amounts of piperidine in urine of normal healthy subjects were found to be 1,236 μg/day; tetracycline administration decreases urinary piperidine[14]

This demonstration of synthesis of a nitrosamine *in vivo* in the small intestine is unique and consistent with previous reports on the role of bacteria in biosynthesis of nitrosamines *in vitro*[9]. The role of acid *p*H conditions in nitrosamine biosynthesis is thus probably less critical and less limiting than has hitherto appeared. Our findings thus confirm and extend previous reports on the synthesis of other nitrosamines from their nitrite and secondary amine precursors, both *in vitro*[6-10,15] and *in vivo*[7,8].

We thank Drs W. Lijinsky and R. Preussman for comments, and Dr Lijinsky for mass spectrometry determinations. This work was supported by a grant from the US Public Health Service, and a contract from the National Air Pollution Control Administration.

B. S. ALAM
I. B. SAPOROSCHETZ
S. S. EPSTEIN

Laboratories of Environmental Toxicology and Carcinogenesis, Children's Cancer Research Foundation, Inc., and the Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

Received July 9, 1970.

1 Magee, P. N., and Barnes, J. M., *Brit. J. Cancer*, 10, 114 (1956).
2 Magee, P. N., and Barnes, J. M., *Adv. Cancer Res.*, 10, 163 (1967).
3 Druckrey, H., Preussman, R., Ivankovics, S., and Schmahl, D., *Z. Krebsforsch.*, 69, 103 (1967).
4 Schmahl, D., and Osswald, H., *Experientia*, 23, 497 (1967).
5 Druckrey, H., Steinhoff, D., Beuthner, H., Schneider, H. V., and Klarner, P., *Arzneimitt-Forsch.*, 13, 320 (1963).
6 Sander, V. J., *Arch. Hyg. Bakt.*, 151, 22 (1967).
7 Sen, N. P., Smith, D. C., and Schwinghamer, L., *Food Cosmet. Toxicol.*, 7, 301 (1969).
8 Sander, V. J., and Seif, F., *Arzneimittel-Forsch.*, 19, 1091 (1969).
9 Sander, V. J., *Z. Physiol. Chem.*, 349, 429 (1968).
10 Mirvish, S. S., *J. Nat. Cancer Inst.*, 44, 633 (1970).
11 Sander, V. J., Schweinsberg, F., and Menz, H. P., *Z. Physiol. Chem.*, 349, 1691 (1968).
12 Sen, N. P., Smith, D. C., Schwinghamer, L., and Marleau, J. J., *J. Assoc. Anal. Chem.*, 52, 47 (1969).
13 Blau, K., *Biochem. J.*, 80, 193 (1961).
14 Kasé, Y., Kataoka, M., and Miyota, T., *Jap. J. Pharmacol.*, 19, 354 (1969).
15 Lijinsky, W., and Epstein, S. S., *Nature*, 225, 21 (1970).

Dechlorination of DDT in Solution by Ionizing Radiation

THERE have been several studies of the effects of ultraviolet light on DDT[1-5] but little is known of the chemical effects of ionizing radiation on the pesticides. In the light of the availability of long lived radioisotopes such as ^{60}Co and ^{137}Cs, by-products of the nuclear power industry, and of the current concern about pollution of lakes and rivers by persistent pesticides, we undertook to study the feasibility of dechlorinating DDT solutions by exposure to ^{60}Co γ-rays. Because of the low efficiency with which ionizing radiation absorbed by materials is converted into chemical energy[6], however, it is evident that to be of economic importance, any radiation-initiated process must involve a chemical chain mechanism.

In contrast to its high solubility in lipids and other organic materials, DDT is almost insoluble in pure water[7]. The bulk of DDT carried in contaminated water is probably in an organic environment, dissolved in suspended liquid fats, in soap and in detergent micelles, or adsorbed on microparticulates. We therefore turned first to the study of radiation dechlorination of DDT in organic solvent. 2-Propanol was chosen because it had proved to be a suitable solvent in previous work on the mechanism of chain dechlorination of carbon tetrachloride[8], which may be considered a structural model for DDT.

Table 1 Dependence of Yield on Radiation Time in the γ Radiolysis of De-aerated Solutions of DDT in 2-Propanol*

Dose rate† (10^{17} eV ml.$^{-1}$ min^{-1})	Time (min)	HCl (mM)	Acetone (mM)	DDD (mM)	DDT consumed (mM)
13.6	5	3.0	2.9	—	—
13.6	10	5.8	5.0	4.6	5.7
13.6	20	12	10	5.4	11
13.6	30	18	18	9.9	15
0.49	30	2.0	—	—	—
0.49	45	3.5	—	—	—
0.49	90	5.8	5.2	5.5	6.5
0.49	150	9.7	9.2	9.2	9.2
0.49	270	16	15	14	15

* Initial DDT concentration, 20 mM.
† Measured by Fricke dosimetry, with correction for the lower electron density of 2-propanol.

Solutions of DDT in 'Pyrex' tubes were irradiated in anaerobic conditions. Products identified were hydrochloric acid, acetone and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane (DDD). A comparison of the data for the lowest and highest radiation dose rates used (Table 1) shows that at the low dose rate these products conform to the stoichiometry

$$DDT + (CH_3)_2CHOH \rightarrow DDD + (CH_3)_2CO + HCl$$

while at the high dose rate the DDD yield is consistently smaller than the DDT consumption. Inspection of the results for the three other dose rates used (7.50, 4.58 and 2.14×10^{17} eV ml.$^{-1}$ min^{-1}) shows that while the lowest dose rate data conform with the above stoichiometry, there is a progressive disparity between DDD formation and DDT consumption in the two sets of higher dose rate data. At all dose rates HCl, acetone and −DDT yields were essentially equal, with a linear dependence on dose up to approximately 100% consumption of DDT (Fig. 1). Curves I–V represent dechlorination (*G*) values (molecules per 100 eV) of 25, 29, 34, 43 and 81, respectively.

To determine whether dechlorination of DDT involves free radical intermediates, radiolysis was carried out in the presence of small concentrations ($\sim 10^{-2}$ M) of free radical scavengers such as O_2, I_2, DPPH, nitrobenzene and phenyl disulphide. In all cases dechlorination yields were reduced significantly. If it is assumed that in irradiated 2-propanol the primary yield of radicals which are amenable for reaction with a solute is

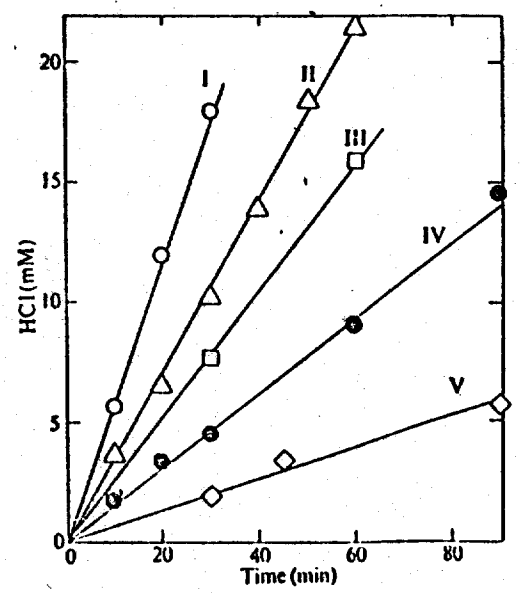


Fig. 1 Effect of dose rate on the dechlorination of DDT in 2-propanol. Dose rates for curves I, II, III, IV and V are respectively 1.36, 0.75, 0.46, 0.21 and 0.049×10^{18} eV ml.$^{-1}$ min^{-1}.

and who was on a maintenance dose of prednisolone (5 mg/day) when the experiment was initiated, was tested for the presence of iso-antibodies against leukaemic cells from other patients. He was then immunized for 7 weeks with chemically tagged O group WBCs as described below.

Blood was periodically withdrawn to check the iso-antibody levels. Because the patient had a history of short remission periods, 6-mercaptopurine (150 mg/day) was administered in addition to the prednisolone during the immunization period. The patient was discharged from the hospital after seven weeks of the immunization schedule and a blood sample was obtained a month later in order to check the antibody levels.

O group WBCs from normal individuals were tagged with 1-fluoro-2,4-dinitrobenzene (FDNB)[2]. A sample (1 ml.) containing 10^7 FDNB-tagged cells was injected subcutaneously into the patient each week. The site of injection was varied. The patient experienced no discomfort or ill effects as a result of injections and the schedule was continued for 7 weeks. Blood (10 ml.) was withdrawn before the immunization schedule began to find out whether there were any inherent iso-antibodies present. This was repeated each week to check the production of iso-antibodies against leukaemic cells. The iso-antibody titre was determined by measuring the degree of agglutination with leukaemic cells from other patients, and also by studying the agar-gel precipitin reaction with leukaemic cells. Both procedures have been described before[2].

The immunological responses of patients with acute lymphatic leukaemia are believed to be comparatively poor. In addition, the patient was being given prednisolone, an immunosuppressive drug[3], as a maintenance dose. Yet the iso-antibody titre increased to 256 after 4 weeks (Fig. 1). The agar-gel diffusion test also revealed a single, clear precipitin line (Fig. 2). During treatment, when the patient was dosed with 6-mercaptopurine (150 mg/day), another potent immunosuppressive drug[3], the iso-antibody titre did not increase but remained at 128. The iso-antibody titre remained at that level even after the immunization schedule had been discontinued. This investigation suggests that it is possible to elicit a specific autoimmune response to leukaemic cells by using non-malignant antigens. Further data on use of active immunization in patients and their clinical response will be published later.

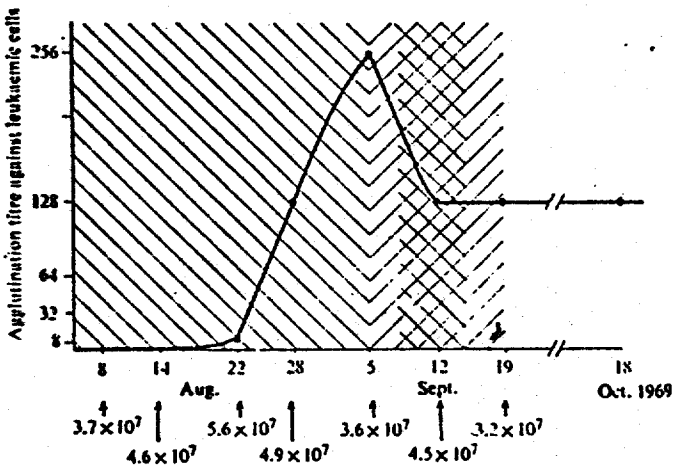


Fig. 1 Development of antibodies specific to leukaemic cells in human acute lymphatic leukaemia patient. Arrows indicate the schedule of immunization. ▨, Prednisolone administration; ▨, 6-mercaptopurine administration.

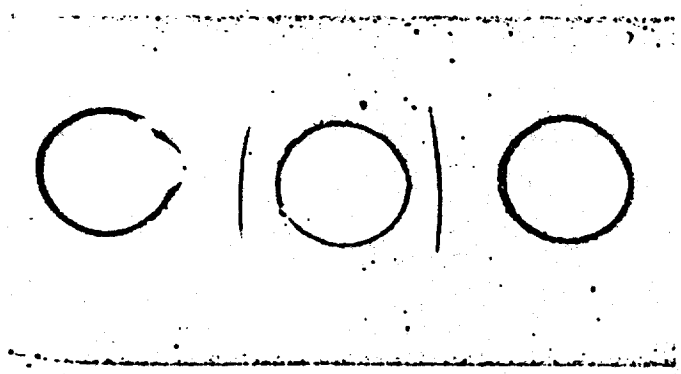


Fig. 2 Demonstration of specific precipitation reaction with the patient's serum against leukaemic cells by Ouchterlony gel diffusion method. Clear-cut precipitin lines were seen on either side of the central well. Central well: patient's serum after four immunizations; side wells: extracts of leukaemic cell membranes.

M. B. SAHASRABUDHE
K. R. MADYASTHA
S. PREMA

Cancer Research Institute,
Parel,
Bombay-12

R. D. LELE

Grant Medical College,
and J. J. Group of Hospitals,
Byculla,
Bombay-8

M. P. GOLLERKERI

Tata Memorial Hospital,
Parel,
Bombay-12

S. S. RAO

Haffkine Institute,
Parel,
Bombay-12

Received December 5, 1969; revised August 12, 1970.

1 Sahasrabudhe, M. B., *Indian J. Cancer*, 5, 217 (1968).
2 Sahasrabudhe, M. B., Prema, S., Madyastha, K. R., Gollerkeri, M. P., and Rao, S. S., *Nature*, 232, 197 (1971).
3 Berenbaum, M. C., in *Immunity, Cancer and Chemotherapy* (edit. by Mihich, E.), 217 (Academic Press; 1967).

Synthesis of Nitrosopiperidine from Nitrate and Piperidine in the Gastro-intestinal Tract of the Rat

MANY nitrosamines are carcinogenic to a wide range of animal species[1-4]. The synthesis of nitrosamines from nitrite and secondary amines has been demonstrated *in vitro* with animal and human gastric juice[5,6] and *in vivo* in animals[6]; nitrosamine synthesis was *p*H-dependent[6-9]. The synthesis of the non-carcinogenic diphenylnitrosamine has been reported in the human stomach after oral administration of nitrate and diphenylamine[7]. Nitrosamines can also be synthesized from nitrate and secondary amines *in vitro* at near neutral *p*H in the presence of enteric enteria[10]. We have demonstrated *in vivo* synthesis of nitrosopiperidine from nitrite and piperidine in the stomach and small intestine of the rat[11]. We report here *in vivo* synthesis of the carcinogenic nitrosopiperidine from nitrate and piperidine in the rat stomach and small intestine, and also with rat gastric contents *in vitro*.

Male Sprague-Dawley rats weighing 200-275 g were used. Methods used for *in vitro* studies with the gastric contents and for *in vivo* studies using the stomach or small intestine have been described before[11]. The ranges of *p*H values for stomach and small intestine were 3.4-4.3 and 6.3-6.8, respectively. For *in vivo* studies, solutions of sodium nitrate and piperidine were incubated for 30 min in the isolated intestine and/or in the

stomach of the same rat or of different rats (Table 1). After incubation, intestinal and stomach contents were removed and extracted with dichloromethane as described before[11]; the solvent extract was dried overnight with sodium sulphate using a mechanical shaker. Nitrosopiperidine in extracts was quantitatively determined by gas–liquid chromatography[11]. The identity of nitrosopiperidine was confirmed by thin layer chromatography[12] and by additional use of another column of 3% QF-1 (Dow Corning fluoroalkyl silicone) on silanized gas chrom p. Using these techniques, the recovery from saline of a standard sample of pure nitrosopiperidine was 35%.

Table 1 Formation of Nitrosopiperidine from Sodium Nitrate and Piperidine Hydrochloride

Replicate*	Reactant concentrations (mg) Piperidine HCl	$NaNO_3$	Nitrosopiperidine yield† (μg) Gastric juice *in vitro*	In different rats *in vivo* Stomach	Small intestine	In the same rat *in vivo* Stomach	Small intestine
1	1,250	25	25	44	54	39	34
2	1,250	25	27	40	20	30	40
3	1,250	25	24	41	8	38	8
4	1,250	25	13	8	2	32	1

* Each replicate is based on four rats.
† Estimated by gas–liquid chromatography.

Nitrosopiperidine was synthesized from nitrate and piperidine *in vivo* in the stomach and small intestine and from gastric juice *in vitro* (Table 1). Yields of nitrosopiperidine *in vivo* were generally somewhat higher in the stomach than in the small intestine, following incubation in the same or in different rats. Yields *in vitro* in the gastric juice were comparable with *in vivo* yields. These yields of nitrosopiperidine are underestimates in view of the relatively low recoveries of standard samples of nitrosopiperidine. Additionally, no effort was made to correct for nitrosopiperidine possibly absorbed during *in vivo* incubation.

The yields of nitrosopiperidine following *in vitro* and *in vivo* synthesis from nitrite and piperidine[11] were greater than for corresponding yields from comparable concentrations of nitrate and piperidine, as reported here. The synthesis of nitrosopiperidine even in small amounts, however, may have cumulative toxic effects, for nitrosopiperidine is a potent carcinogen[13]. Our results are in accord with human data on *in vivo* gastric synthesis of diphenylnitrosamine from nitrate and diphenylamine[7]; bacterial reduction of nitrate and nitrosamine synthesis in these studies was *p*H-dependent. The synthesis *in vivo* of the carcinogenic nitrosamine nitrosopiperidine from nitrate and piperidine is of practical interest in view of the common dietary occurrence of these precursors. Piperidine is a urinary constituent of normal human subjects, presumably formed by intestinal bacterial decarboxylation from dietary lysine[14]. Piperidine may also be ingested by man through some foods and spices; some bread flavouring agents contain piperidine[15]. Sodium nitrate and nitrite are used in the United States and elsewhere as preservatives and colour fixatives in cured meats and fish at permissible levels of 500 and 200 p.p.m. respectively[16]. Nitrite is also used alone at levels below 10 p.p.m. in smoked and cured tunafish. The use of nitrate or nitrite in certain types of cheese is permitted in some European countries. Nitrates are also widely distributed in many plants. Certain plants such as beets and spinach accumulate nitrates, especially when overfertilized[17] and in conditions of molybdenum deficiency[18–20]. Further, nitrate levels in drinking water, particularly in certain agricultural areas, may be high enough to induce acute methaemoglobinaemia in infants[21–25]. The transplacental transmission of nitrates and the development of methaemoglobinaemia in the foetuses of rats drinking water with high concentrations of nitrates has been recently demonstrated[26]. The implication of these findings to human cancer and the propriety of the continued use of nitrate and nitrite as food additives, and of drinking water with a high nitrate content, clearly merits critical consideration.

These studies were supported by grants from the US National Institutes of Health and National Air Pollution Control Administration.

B. S. ALAM
I. B. SAPOROSCHETZ
S. S. EPSTEIN

Laboratories of Environmental Toxicology and Carcinogenesis, Children's Cancer Research Foundation, Inc., and the Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

Received January 22, 1971.

1 Magee, P. N., and Barnes, J. M., *Brit. J. Cancer*, 10, 114 (1956).
2 Magee, P. N. and Barnes, J. M., *Adv. Cancer Res.*, 10, 163 (1967).
3 Schmahl, D., and Osswald, H., *Experientia*, 23, 497 (1967).
4 Lijinsky, W., and Epstein, S. S., *Nature*, 225, 21 (1970).
5 Sander, V. J., *Arch. Hyg. Bakt.*, 151, 22 (1967).
6 Sen, N. P., Smith, D. C., and Schwinghamer, L., *Food Cosmet. Toxicol.*, 7, 301 (1969).
7 Sander, V. J., and Sief, F., *Arzneimittel-Forschung*, 19, 1091 (1969).
8 Sander, V. J., Schweinsberg, F., and Menz, H. P., *Z. Physiol. Chem.*, 349, 1691 (1968).
9 Mirvish, S. S., *J. Nat. Cancer Inst.*, 44, 633 (1970).
10 Sander, V. J., *Z. Physiol. Chem.*, 349, 429 (1968).
11 Alam, B. S., Saporoschetz, I. B., and Epstein, S. S., *Nature* (in the press).
12 Sen, N. P., Smith, D. S., Schwinghamer, L., and Marleau, J. J., *J. Assoc. Off. Analyt. Chem.*, 52, 47 (1969).
13 Druckrey, H., Preussman, R., Ivankovics, S., and Schmahl, D., *Z. Krebsforsch.*, 69, 103 (1967).
14 Blau, K., *Biochem. J.*, 80, 193 (1961).
15 US Patent No. 3,336,140: CA 67,899,046 (1967).
16 *Code of Federal Regulations*, 121 (1963–1964).
17 Schupan, W., and Ernahr, Z., *Wissenschaften*, 6, 207 (1965).
18 Hewitt, E. S.; and Jones, E. W., *J. Pomol. Hort. Sci.*, 23, 254 (1947).
19 Vlitos, A. J., in *Chemical and Biological Hazards in Food* (edit. by Ayers, J. C., Kraft, A. A., Snyder, H. F., and Walker, H. W.), 89 (Iowa State University Press, 1962).
20 Brown, J. R., and Smith, G. E., *Res. Bull. University Missouri College of Agric.*, No. 920 (1967).
21 Knotech, Z., and Schmidt, P., *Pediatrics*, 34, 78 (1964).
22 Steyn, D. G., Publ. No. 11, Univ. Pretoria, Union of South Africa (1960).
23 Werner, V., and Thal, W., *German Med. Monthly*, 10, 142 (1965).
24 *Cyanosis of Infants Produced by High Concentration in Rural Wells* (*WHO/MCH*/13) (1949).
25 Simon, C., Mazke, M., Kay, H., and Mrowitz, G., *Z. Kinderheilk*, 91, 124 (1964).
26 Nachman, G., and Shuval, H. I., in *Water Quality Research*, 89 (Ann Arbor–Humphrey Science publication, 1969).

Distribution and Concentration of Mercury in Autopsy Specimens of Human Brain

THE dangers of mercury, a pollutant of natural bodies of water and a metal with a strong tendency to accumulate in the nervous tissues, have recently caused considerable concern. We have studied the distribution and concentration of mercury in the brain in order to determine the levels normally present.

Eight autopsy specimens were selected at random for the study. The subjects (Table 1) had lived, at least briefly, in an area where mercurial contamination of the regional inland waters and their fish has been demonstrated, but there was otherwise no known pathological exposure to the metal. The area includes Buffalo, New York, and the adjacent Great Lakes. Six brains were taken from the subjects 6–30 h after death; Nos. 1 and 2 were fixed in formalin before dissection. The tissues were analysed for mercury by neutron activation

G-1673-1
German Trsl
DKD

25

TERATOGENIC ACTION OF ETHYL UREA AND NITRITE IN RATS

(Die teratogene Wirkung von Äthylharnstoff und Nitrit bei Ratten)

Authors: V.A. Alexandrov and W. Jänisch

Source: Experientia 27/5, 1971, P. 538-539

Many nitrosamides are teratogenic (Reference 1). Recently the possibility of the endogenous development of nitroso compounds in the gastro-entestinal tract was pointed out (Reference 2). The endogenous synthesis of ethyl nitroso urea from nitrite and ethyl urea can be detected through its tumor-producing effect on rats (Reference 3). We investigated on gravid Sprague-Dawley rats whether the amount of the endogenously developed ethyl nitroso urea sufficed in order to exert an embryotoxic and teratogenic action.

Ethyl urea (melting point 92 C, Ferak Berlin) and Na nitrite were administered enterally to rats on the 9th and 10th day of pregnancy. These days were selected because the rat embryo exhibits on the 9th day a high embryotoxic sensitivity and on the 10th day the maximum teratogenic sensitivity. The mother animals were killed on the 21st day post coitum, and the number of embryos that died and of the fetuses with malformations was determined by examination and microsection.

The addition of 1% Na nitrite and of 0.5% ethyl urea to the food or to the drinking water, and the supplementary single administration of 200 mg/kg of ethyl urea in aqueous solution through a stomach probe result in the death of 60% of the embryos and in the malformation of 76% of the surviving fetuses (Table, Group I). Addition of 1% Na nitrite to the food alone (Group VII) causes poisoning of the mother animals, which was mortal in 6 out of 10 bearing rats. No embryotoxic or teratogenic effect on the progeny of the surviving mother animals was detectable, however. With decreasing concentration of ethyl urea and nitrite in the food or in the drinking water, the embryotoxic action disappears, since the number of the fetuses that dies does not significantly differ from the values of the control groups. On the other hand, the teratogenous activity remains recognizable (Groups II and III).

The results show that the amount of the endogenously developed ethyl nitroso urea suffices for a teratogenic effect, whereas the prestages alone causes no malformations. It is possible that in the human organism also an endogenous synthesis of substances of this type occurs from food constituents. This gives indications regarding a lowering of the teratogenous risk by eliminating the prestages of teratogenous substances from the food of pregnant individuals.

Table: Malformations in rat fetuses after simultaneous administration of ethyl urea and nitrite to pregnant mother animals.

bekamen an der linken Rückenseite ein schwarzes Homotransplantat und an der rechten Seite ein Autotransplantat; schwarze Mäuse erhielten weisses Homotransplantat. Bei allen überlebenden, operierten Tieren war das Homotransplantat am 25. Tag gut erhalten. Es ist bekannt, dass bei nicht behandelten Tieren das Homotransplantat ca. am 8. Tag (± 2) abgestossen wird[5]. Makroskopische Untersuchung zeigte vollkommenes Verschwinden des Grenzstranges[7].

Aus diesen Versuchen können wir nicht sagen, ob 6-HD, welches zur Zerstörung des sympathischen Grenzstranges geführt hat, auch Veränderungen der immunologischen Prozesse, die für Abstossung des Homotransplantates verantwortlich sind, hervorruft. Vielleicht handelte es sich nur um verbesserte Durchblutung der transplantierten Haut. Weitere Versuche, die im Gange sind, sollen zeigen, ob für die Homotransplantation Sympathikus-Zerstörung nur des Donators oder nur des Empfängers notwendig ist und ob man durch lokale Applikation des 6-HD in die Haut bei erwachsenen Mäusen auch denselben Effekt erreichen kann.

Summary. As is known, 6-HD destroys the sympathetic trunc when given to new-born mice. In these mice (weighing 18 g) skin homo-transplantations were made and it was found that the homografts strikingly survived.

P. STERN

Institut für Pharmakologie der Medizinischen Fakultät, Sarajevo (Jugoslawien), 23. November 1970.

1 P. U. ANGELETTI und R. LEVI-MONTALCINI, Archo ital. Biol. *108*, 213 (1970).
2 A. BERTELLI und G. FRONTINO, Nature, Lond. *197*, 510 (1963).
3 P. STERN und E. VAJS, Vojno-sanit. Pregl. *20*, 141 (1963).
4 P. STERN und E. VAJS, in *Pharmacological Treatment in Organ and Tissue Transplantation* (Eds. A. BERTELLI und A. P. MONACO; Excerpta Medica Foundation, Amsterdam 1970), p. 130.
5 TH. C. MOORE, Nature, Lond. *215*, 871 (1967).
6 N. E. ANDÉN, M. HENNING und H. OBIANWU, Acta pharmac. Copenh. *26*, 113 (1968).
7 Herrn Doz. Dr. M. ČUŠ, Anatomisches Institut der Medizinischen Fakultät, Sarajevo, danken wir für diese Kontrolle.

Die teratogene Wirkung von Äthylharnstoff und Nitrit bei Ratten

Zahlreiche Nitrosamide sind teratogen[1]. Kürzlich wurde auf die Möglichkeit der endogenen Entstehung von Nitrosoverbindungen im Magen-Darm-Trakt hingewiesen[2]. Die endogene Synthese des Äthylnitrosoharnstoffs (ÄNH) aus Nitrit und Äthylharnstoff lässt sich durch dessen geschwulsterzeugende Wirkung an Ratten nachweisen[3]. Von uns wurde an tragenden Sprague-Dawley-Ratten untersucht, ob die Menge des endogen entstehenden ÄNH ausreicht, um embryotoxisch und teratogen zu wirken.

Äthylharnstoff (Schmelzpunkt 92°C, Ferak Berlin) und Natriumnitrit wurden bei Ratten am 9. und 10. Schwangerschaftstag enteral verabreicht. Diese Termine wurden gewählt, da der Rattenembryo am 9. Tag eine hohe embryotoxische und am 10. Tag die maximale teratogene Empfindlichkeit aufweist (ALEXANDROV, unveröffentlicht). Die Muttertiere wurden am 21. Tag post coitum getötet und die Zahl der abgestorbenen Embryonen sowie der Feten mit Missbildungen durch Inspektion und Mikrosektion bestimmt.

Der Zusatz von 1% Natriumnitrit und 0,5% Äthylharnstoff zum Futter bzw. Trinkwasser und die zusätzliche einmalige Gabe von 200 mg/kg Äthylharnstoff in wässriger Lösung durch eine Magensonde führen zum Absterben von 60% der Embryonen und zu Missbildungen bei 76% der überlebenden Feten (Tabelle, Gruppe I)

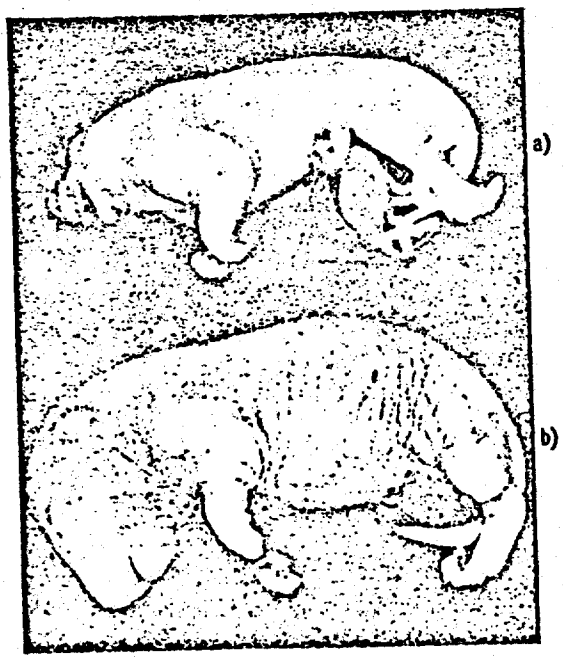


Fig. 1. a) Anenzephalie und Eventeration der Bauchorgane bei einem Rattenfetus der Gruppe III. b) Gleichaltriger Fetus der Kontrollgruppe IX.

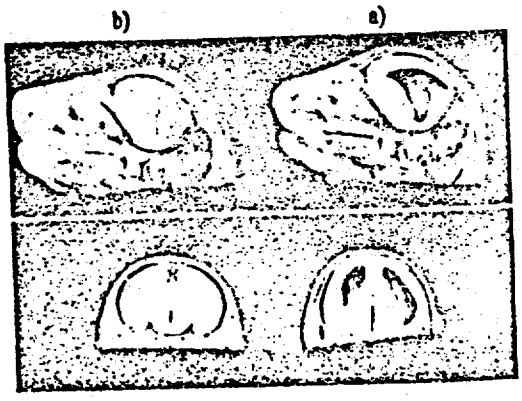


Fig. 2. a) Hydrozephalus in Frontal- und Sagittalschnitten [illegible] Rattenfeten der Gruppe II. b) Kopf eines gleichaltrigen Kontrolltieres (Kontrollgruppe IX) in entsprechenden Schnittebenen.

1 N. P. NAPALKOV und V. A. ALEXANDROV, Z. Krebsf. *71*, 32 (1968).
2 J. SANDER und G. BERKLE, Z. Krebsf. *73*, 54 (1969).
3 S. IVANKOVIC und R. PREUSSMANN, Naturwissenschaften *57*, [illegible] (1970).

Missbildungen bei Rattenfeten nach gleichzeitiger Verabreichung von Äthylharnstoff (ÄH) und Nitrit an tragende Muttertiere

Gruppe	Substanzbeimengungen	Zahl der graviden Ratten	Zahl der implant. Embryonen	Zahl der abgestorb. Embryonen	Lebende Feten Total	Lebende Feten Mit Missbildungen	Art der Missbildungen (zum Teil kombiniert)
I	1% Nitrit im Futter 0,5% ÄH im Wasser 200 mg/kg ÄH durch Magensonde	3	33	20 (60,6%)	13	10 (76,9%)	Hydrozephalus 6 Exenzephalie 3 Anophthalmie 3
II	0,3% Nitrit im Futter 0,5% ÄH im Wasser 100 mg/kg ÄH durch Magensonde	9	82	13 (15,8%)	69	21 (30,4%)	Hydrozephalus 21 Anophthalmie 9
III	0,3% Nitrit im Futter 0,5% ÄH im Wasser	8	78	6 (7,7%)	72	3 (4,2%)	Hydrozephalus 2 Anenzephalie 1 Anophthalmie 1 Eventeration der Bauchorgane 1
IV	Kontrolle 0,5% ÄH im Wasser 200 mg/kg ÄH durch Magensonde	9	76	8 (10,5%)	68	0	
V	Kontrolle 0,5% ÄH im Wasser 100 mg/kg ÄH durch Magensonde	7	82	6 (7,3%)	76	0	
VI	Kontrolle 0,5% ÄH im Wasser	8	76	3 (3,9%)	73	0	
VII	Kontrolle 1% Nitrit im Futter	4	30	0	30	0 (6 weitere Muttertiere 1 Tag nach Nitritfütterung verstorben)	
VIII	Kontrolle 0,3% Nitrit im Futter	10	92	5 (5,4%)	87	0	
IX	Kontrolle unbehandelt	15	118	8 (6,8%)	110	0	

Zusatz von 1% Natrium-nitrit zum Futter allein (Gruppe VII) verursacht eine Intoxikation der Muttertiere, die bei 6 von 10 tragenden Ratten tödlich verlief, aber keinen embryotoxischen oder teratogenen Effekt an der Nachkommenschaft der überlebenden Muttertiere erkennen liess. Mit abnehmender Konzentration von Äthylharnstoff und Nitrit im Futter bzw. Trinkwasser verschwindet die embryotoxische Wirkung, denn die Zahl der abgestorbenen Früchte unterscheidet sich nicht signifikant von den Werten der Kontrollgruppen. Die teratogene Aktivität bleibt dagegen noch erkennbar (Gruppen II und III).

Die Resultate zeigen, dass die Menge des endogen entstehenden ÄNH für eine teratogene Wirkung ausreicht, während die Vorstufen allein keine Missbildungen hervorrufen. Es ist möglich, dass auch im menschlichen Organismus eine endogene Synthese derartiger Stoffe aus Nahrungsbestandteilen stattfindet. Damit ergeben sich Ansatzpunkte für eine Verminderung der teratogenen Gefährdung durch Eliminierung von Vorstufen teratogener Substanzen aus der Nahrung von Schwangeren.

Summary. Simultaneous peroral application of ethylurea and sodium nitrite to pregnant Sprague-Dawley rats, on the 9th and 10th days of gestation, causes embryotoxic and/or teratogenic effects. Each substance alone does not afflict the fetuses, whereas the combined addition to the food permits the formation of ethyl nitroso-urea in the digestive tract. The latter readily passes the placenta and develops the teratogenic effect. It is to be considered that teratogenic substances can occur from food constituents in the human organism also.

V. A. Alexandrov und W. Jänisch

Laboratorium für experimentelle Tumorforschung, Onkologisches Institut, Pesotschnaja 2, Leningrad, (USSR) und Pathologisches Institut, Medizinische Akademie, Nordhäuser Strasse 74, DDR-50 Erfurt (DDR).

2. Dezember 1970.

Species Differences in the Influence of Chlorothiazide on Curarization

Potentiation by chlorothiazide and other sulphonamides of D-tubocurarine induced (-) neuromuscular blockade in the rabbit has been reported; displacement of tubocurarine from nonspecific binding sites by the sulphonamides was proposed as the mechanism underlying the observed potentiation[1,2]. However, it was found[3] that chlorothiazide antagonized the curarization produced by D-tubocurarine in the dog and also in the isolated phrenic-diaphragm preparation of the rabbit. These conflicting results prompted the present study of the influence of chlorothiazide on the curarization induced by D-tubocurarine and other paquicurares in different mammalian species. Experiments were also performed to investigate whether interaction of D-tubocurarine

UG OFFICIALS

., others who attended.

in Pennsylvania and New
Officer, Philadelphia Dis-

"her" by David Kronick,

round-table discussion.
of mutual interest.

w Calorie, High Pro-
ing,' by Dr. Harry J.
ssue of the *Quarterly*
per ounce for New
'87." This value should

AGRICULTURE LIBRARY

46

OLUME XXII NUMBER 3

(13)

ASSOCIATION OF FOOD & DRUG OFFICIALS OF THE UNITED STATES

Quarterly Bulletin

OHIO STATE UNIVERSITY
AUG 20 1958
LIBRARY

JULY 1958

Report from the Food and Drug Administration—*John L. Harvey* .. 97
Chemicals and Antibiotics in Foods—*Curtis R. Joiner* 106
Staphylococcic Food Poisoning—*C. J. Gentzkow* 112
Public Health Aspects of Air Pollution—*J. W. E. Harrisson* 116
The Grade A Law in Indiana—*John Schlegel* 127
Frozen Foods Quality: Role of Regulatory Officials—*Lowell D. Oranger* 133
Quarterly Report to the Editor on Topics of Current Interest: Nitrates and Nitrites in Meat Products 136
Sectional Association Report 139
Local Conference Reports 140
Items and Comments 144

Edited and Published by
THE EDITORIAL COMMITTEE
Editorial Office, 2411 N. Charles St., Baltimore 18, Md.
Business Office, Joe F. Lakey, Bureau of Food & Drugs,
Texas State Department of Health, Austin 1, Texas

46 (13)

QUARTERLY REPORT TO THE EDITOR ON TOPICS OF CURRENT INTEREST

NITRATES AND NITRITES IN MEAT PRODUCTS

The use of sodium and potassium nitrate as curing agents for meats has been an established practice for so long that its origin is unknown. The nitrates have no more preservative action than common salt (1), but do have a special function. The function in the curing process is to fix and heat-stabilize the red color in meat. This color fixation takes place through the reduction of nitrate to nitrite by bacterial action and the reaction of the nitrite with hemoglobin to form nitric oxide hemoglobin. Heat will convert the nitric oxide hemoglobin to nitric oxide hemochromogen, which is also a red pigment. Thus, both pigments may be present in cured meats.

Since nitrate must be changed to nitrite by bacterial action before the above reaction can take place, unsatisfactory or insufficient formation of nitrite results in insufficient or irregular color fixation. It was found that a combination of nitrate and nitrite in the curing salt or pickling solution gave the most satisfactory results (1). Combinations of potassium or sodium nitrate and nitrite are permitted as curing ingredients for meat prepared under Federal meat inspection with the limitations of no more than 200 parts per million of nitrite in the finished product (2).

NATURAL OCCURRENCE OF NITRATE IN FOOD

The normal urinary output of nitrate averages 0.5 gram per day primarily derived from vegetables in the diet. It has been shown that the nitrate content of green vegetables varies from 3,636 p.p.m. for spinach to 50 p.p.m. for asparagus. Strained baby foods were found to contain from 0 p.p.m. for squash and tomatoes to 833 p.p.m. for spinach (3). Traces of nitrate are also found in many potable water supplies. There have been no reports that nitrite occurs in plants, although it has been demonstrated that human blood contains 10 micrograms per 100 cc. as an average (0.1 p.p.m.) (4)

CHRONIC TOXICITY OF NITRATE AND NITRITE

Nitrate. Sodium nitrate has been fed to rats at levels up to 10% in the diet for their life-time (5). Other than some depression in growth at levels above 1% of nitrate, no adverse effects were noted in these animals.

Two dogs were fed 2% sodium nitrate in their diet for a period of 105 and 125 days respectively. No adverse effects were noted and no disturbance in the blood was observed in these animals. Gross and microscopic pathological study of the rats showed nothing attributable to the nitrate

except changes of inanition at 10%, with no morphological difference from the controls at 5% or less in the diet. Nothing of significance was observed in the dogs.

Nitrite. Rats were fed a sodium nitrite supplement for a period up to 168 days (6). In a typical experiment one rat received a total of 0.167 gram sodium nitrite in 121 days. This represents about 93 p.p.m. in the daily diet. No effects on growth and organ weights of important organs were noted.

In a similar experiment in cats, one animal received about 4.1 grams total dose of sodium nitrite during a period of 105 days. This represents approximately 390 p.p.m. nitrite in the daily diet. Again, no effects on growth rate and weight of important organs were noted.

No histopathological examination was done on any of the animals fed nitrite.

THE EFFECT OF SODIUM NITRITE ON BLOOD PIGMENTS

The reaction of nitrite on the hemoglobin of meat has already been mentioned. In the body the usual effect of nitrite is on the hemoglobin of the blood. There is some controversy concerning the molar ratios involved in the interaction of nitrite with hemoglobin to produce methemoglobin (7). The ratios are stated to vary from 2 mols of nitrite to 1 mol of hemoglobin to 0.5 to 1. Thus, 1 gram of sodium nitrite could convert from 463 to 1855 grams of hemoglobin to methemoglobin.

The rate of conversion of methemoglobin to hemoglobin is also of great importance. From the evidence presently available this conversion proceeds at a rate approximating 10–15% of the total methemoglobin in the blood per hour. Thus, the body mechanisms are capable of rapidly reducing methemoglobin to the oxygen-carrying hemoglobin. It is of interest to note that the normal blood of man contains about 0.7% methemoglobin.

THE HAZARD OF RESIDUAL NITRITE IN MEAT

An individual consuming 100 grams of corned beef containing 200 p.p.m. residual nitrite would ingest 20 milligrams of nitrite. This represents about 13 p.p.m. in the day's diet. In light of the facts related above, this quantity of nitrite, if all absorbed, could convert approximately 10–40 grams of hemoglobin to methemoglobin representing about 1.4 to 5.7 per cent of the total blood pigment. In view of the fact that a concentration of 40% methemoglobin in the blood is necessary before compensatory mechanisms of the body are brought into play to increase the oxygen supply to the tissues, the methemoglobin contributed by 20 milligrams of ingested sodium nitrite is of no significance.

It must be borne in mind that the most significant feature in estimating

the health hazards of nitrite is the molar ratio between the nitrite utilized and the methemoglobin formed. As already pointed out 1 gram of sodium nitrite could convert from 463 to 1855 grams of hemoglobin to methemoglobin. The blood of the average adult contains about 700 grams of hemoglobin. As stated previously a concentration of about 40 per cent methemoglobin in the blood brings about compensatory mechanisms to maintain the oxygen supply to the tissues. A concentration of 80–85 per cent is considered lethal (7). Because of the small molecular weight of sodium nitrite, 69, in relation to the large molecular weight of hemoglobin, 64,000, only a small margin of safety exists between the amount of nitrite that is safe and that which may be dangerous. The margin of safety is even more reduced when the smaller blood volume and the corresponding smaller quantity of hemoglobin in children is taken into account. This has been emphasized in the recent cases of nitrite poisoning in children who consumed weiners and bologna containing nitrite greatly in excess of the 200 p.p.m. permitted by Federal regulations (8). The application of nitrite to other foods is not to be encouraged.

REFERENCES

1. Kerr, R. H., Marsh, C. T. N., Schroeder, W. F., and Boyer, E. A., The use of sodium nitrite in the curing of meat. J. Agri. Research, *33*, 541, 1926.
2. Regulations Governing the Meat Inspection of the United States Department of Agriculture, June 1952.
3. Wilson, J. K., Nitrate in foods and its relation to health. Agronomy J., *41*, 20, 1949.
4. Rath, M., and Krantz, Jr., J. C., Blood-nitrite content of man and other species. J. Pharmacol. and Exptl. Therap., *76*, 27, 1942.
5. Fitzhugh, O. G., and Nelson, A. A., Unpublished data, Division of Pharmacology, Food and Drug Administration.
6. Tarr, H. L. A., and Carter, N. M., The effect of sodium nitrite on experimental animals. J. Fish. Res. Bd. Can., *6*, 63, 1942.
7. Bodansky, O., Methemoglobinemia and methemoglobin-producing compounds. Pharmacol. Reviews, *3*, 144, 1951.
8. Orgeron, J. D., et al., Methemoglobin from eating meat with high nitrite content. Public Health Reports, *72*, 189, 1957.

A. J. L.

of the medications despite the continuance of a usual diet. During the period of placebo administration, the average concentration of serum cholesterol was 341 mg. per 100 ml. (range 277 to 522). The average changes for the group, expressed as per cent decrease from 341, were as follows: niacin, 24 per cent; safflower oil, 6 per cent; and sitosterol, 13 per cent. There was little correlation between the response of a given patient to one agent or another. The average weight of the patients varied slightly as follows: placebo, 146 pounds; niacin, 144 pounds; safflower oil, 150 pounds; and sitosterol, 147 pounds. Safflower oil added approximately 540 calories to the daily diet.

Combined therapy of nine patients with both sitosterol and niacin (average dose 4.3 g. per day) resulted in a mean decrease in cholesterol level of 25 per cent when compared with the level during placebo administration (346 mg. per 100 ml.). On the other hand, administration of 4.3 g. of niacin alone per day to these patients caused only a 17 per cent decrease in cholesterol level, and sitosterol alone a 12 per cent decrease.

Side reactions which accompanied administration of niacin were flushing, itching and gastrointestinal distress. Occasional abnormalities were found in the results of the cephalin-cholesterol flocculation test and in the concentration of alkaline phosphatase and glutamic-oxalacetic transaminase in the serum. There was no evidence of clinical hepatic disease. There was also some impairment of the carbohydrate tolerance test. All of these abnormalities were reversed as soon as niacin was discontinued. Neither sitosterol nor safflower oil caused any discomfort other than unpalatability and a tendency to cause loose stools.

The authors discussed possible mechanisms whereby these agents might reduce the concentration of cholesterol in the serum. Presumably sitosterol inhibits absorption of cholesterol from the gut. Unsaturated oils are most effective if substituted for saturated fats, but, aside from the factor of essential fatty acids, the reason is obscure. Niacin is effective but its amide is not, even though it also is an active vitamin. The cutaneous flushing and itching seem to be inseparable from the cholesterol lowering effect. Explanations for this have ranged from an oxidative enzymatic process (J. M. Merrill, *Circulation Res.* **6**, *482* (*1958*)) to enhancement of the metabolic rate (R. Altschul and A. Hoffer, *Arch. Biochem. Biophys.* **73**, *420* (*1958*)). Niacin induces urinary excretion of nicotinuric acid but niacinamide does not. Whatever the mechanism, it must be a pharmacologic one since the necessary dose is so great.

This report describes an experimental method whereby hypercholesterolemia can be reduced by 25 per cent without dietary change or undue risk. This must be considered an investigational form of therapy, and should not be adopted as a routine measure until more experience has been gained. The possibility of late toxic effects needs further study. Finally, it will require critical evaluation to determine whether artificial lowering of the concentrations of cholesterol in the serum is actually of any value to the patient.

NITRATES OR NITRITES AND ABORTION IN CATTLE

Nitrates or nitrites in the diets of pregnant dairy cattle produced non-infectious abortion similar to that observed in cattle on unimproved marshland pasture. Sodium nitrite was more toxic than potassium nitrate.

The problem of non-infectious abortion in dairy cattle has concerned veterinarians and dairymen for many years. In fact, one form of this type of abortion has been reported

among cattle on unimproved marshland pasture in Wisconsin for the past 50 years. Early work from the Wisconsin Agricultural Experiment Station indicated that nitrates or nitrites present in rather high levels in certain species of weeds were probably responsible for this abortion syndrome.

J. Simon *et al.* (*J. Am. Vet. Med. Assn.* **135,** *311* (*1959*)) have recently reported on experiments in which they attempted to correlate the quantity of nitrate or nitrite in the feed with the development of an abortion in pregnant dairy heifers. Earlier observations of oat-hay poisoning (W. B. Bradley, H. F. Eppson and O. A. Beath, *Wyoming Agric. Exper. Sta. Bull.* **241** (*1940*)) indicated that the lethal dose of potassium nitrate for cattle was 25 g. per 100 pounds of body weight. E. I. Whitehead and A. L. Moxon reported (*South Dakota Agric. Exper. Sta. Bull.* **424,** *1* (*1952*)) that excessive nitrate in the diet would result in abortion.

Simon *et al.* postulated that a continuous intake of nitrate or nitrite in sufficient quantity would produce a methemoglobinemia and that the resulting anoxemia in the fetal calves would cause death and subsequent abortion. They set up experiments in which pregnant heifers were given varying levels of potassium nitrate or sodium nitrite at regular intervals in an effort to ascertain whether the resulting syndrome of abortion was identical with that occurring on the marshland pastures.

Ten grams of sodium nitrite given twice daily for three weeks produced methemoglobin levels as high as 12 per cent of the hemoglobin after one week. At the end of two weeks this had risen to 18 per cent, but did not result in abortion. One cow produced a normal calf shortly after the nitrite was discontinued, but the fetal membranes showed lesions similar to those reported for marshland abortions. Interestingly enough, 40 g. of potassium nitrate fed twice daily for three weeks gave no signs of illness or abortion and no measurable methemoglobin in the blood.

In a more critical experiment, Simon *et al.* fed potassium nitrate by capsule twice daily at levels ranging from 10 to 20 g. per 100 pounds of live weight. Those animals given 20 g. per 100 pounds of live weight died following the second dose, having received a total of 140 g. of potassium nitrate. All of the animals receiving the intermediate level, a total of 100 g. of potassium nitrate per day, aborted and the lesions of the fetal membranes resembled those seen in the naturally occurring abortions on marshland pasture. Those animals receiving 10 g. per 100 pounds of live weight did not abort, although they did develop high levels of methemoglobin in their blood.

These very interesting studies emphasize that animals on poor grass hay or pasture may be far more susceptible to nitrate or nitrite poisoning than animals on diets high in carbohydrate concentrates. Certainly this work shows conclusively that nitrates or nitrites will cause severe methemoglobinemia and that one of the results in pregnant cattle may be abortion associated with anoxemia.

It is also evident from a consideration of this work that the methemoglobin levels of blood do not consistently reflect the level of nitrate or nitrite in the diet. Furthermore there was a wide variation between different animals on the same nitrate level, suggesting that methemoglobin in the blood is a poor measure of nitrate toxicity. Apparently individual variation, diet composition, sampling technique, time from blood sampling to analysis and method of analysis all influence methemoglobin values.

Simon *et al.* suggest that some better technique of measuring the effect of nitrate and nitrite is required and propose the analysis of nitrate in the blood as a method for determining the reaction of cattle to nitrate in the feed.

While the work of these authors has established the close relationship between nitrate or nitrite in non-infectious abor-

in dairy cattle, the limited number of animals, the individual variation and the wide spread in nitrate levels used prevented the determination of levels at which this syndrome will develop. More particularly, they have not indicated the levels at which nitrite, a more toxic ingredient than nitrate, will result in abortion nor what is the likely relationship between nitrite and nitrate under naturally occurring pasture conditions. These questions are of particular importance to dairymen and cattlemen in areas where high nitrate levels in pasture forage are a problem.

BLOOD LOSS AND PLASMA CHOLESTEROL LEVELS

The removal of blood samples from rats may be responsible for increases in plasma cholesterol levels.

In many projects using small laboratory animals, the effect of diet on plasma cholesterol levels is studied by taking samples of blood at frequent intervals.

In the course of such a project, I. Coleman and J. M. R. Beveridge (*Nature* 184, *1041 (1959)*) found that some of the increases observed in the cholesterol levels with a high-fat diet in one rat experiment were not reproduced when the experiment was repeated. The only difference in the conditions of the two experiments that might provide an explanation for this was in the frequency of the blood samples and their size.

In the first experiment, samples corresponding to 2 per cent of the blood volume of the rats were taken on alternate days for the first eight days, and then at weekly intervals for a month. In the second experiment lasting two weeks, samples corresponding to 0.5–1.0 per cent of the blood volumes were taken on the first and last day only, and there was no change in cholesterol level in the rats receiving the high-fat diet.

Two further trials were then set up to measure the response of rats to two experimental diets under different degrees of bleeding stress. In one diet, just under 60 per cent of the calories were calculated to come from corn oil, while the second diet was essentially fat-free. Each trial lasted for six days and the rats were bled on alternate days.

In the first of the two trials, using twenty animals for each diet, the initial level of plasma cholesterol was approximately 72 mg. per 100 ml. Six days later after three blood samplings (each of which were equivalent to 1.0–1.5 per cent of blood volume), the values for the high-fat diet had increased significantly by 31 per cent while those for the fat-free diet had increased by only 10 per cent (a nonsignificant difference). In the second trial, in which 15 animals were allotted to each diet, the mean initial level of plasma cholesterol was 67 mg. per 100 ml. The conditions were the same as for the first trial except that the samplings corresponded to 2.5–4.0 per cent of blood volume on each occasion. After six days, the values on the high-fat diet had increased by 122 per cent, and on the fat-free diet by 59 per cent.

With respect to experimental design, it was unfortunate that the size of blood sample was not used as a variable within a test. Nevertheless the results do strongly suggest that the taking of ordinary blood samples can itself be a factor influencing plasma cholesterol levels in experiments with rats. Work has been reported previously by J. J. Spitzer (*J. Lab. Clin. Med.* 46, *461 (1955)*) confirming the older observations that hemorrhage could result in lipemia in rabbits, but showing that daily blood losses of the order of 10 to 15 per cent of blood volume were needed in order to observe the phenomenon. The suggestion from the present work is that very much

Fd Cosmet. Toxicol. Vol. 2, pp. 61–81. Pergamon Press 1964. Printed in Great Britain

55

ARTICLES OF GENERAL INTEREST

UP-TO-DATE WITH NITROSAMINES

We have previously abstracted publications in the field of nitrosamines (NA) and related compounds such as N-methyl-N-nitrosourethane (*Cited in F.C.T.* 1963, 1, 156, 157 & 253). With several research units at work, and new discoveries coming thick and fast, this is potentially one of the epoch-making developments in the history of cancer research. We owe it to our readers therefore to summarize the present position.

A suitable starting-point is the comprehensive review by Heath & Magee (*Brit. J. industr. Med.* 1962, 19, 276) of the toxic properties of the whole class of compounds RR'N·NO, where R and R' are alkyl groups, or hydroxyalkyl or one may be an aryl group; the amino N may be part of a morpholine or piperidine ring; the NO group may be replaced by -CHO to give the corresponding dialkylformamides. In fact the formamides do not appear to be carcinogens and may be dismissed from further consideration.

The work of Schoental (*Nature, Lond.* 1961, 192, 670) on N-methyl-N-nitrosourethane and the demonstration that a daily dose of 4 mg/kg to rats produces carcinoma of the forestomach (Druckrey *et al. Naturwissenschaften* 1961, 48, 165) has been followed by studies on the effects of intravenous injection of this material into rats. The toxicity by this route is high (LD_{50}=5 mg/kg) and repeated doses of 1 mg/kg induced lung carcinomas in all 18 surviving animals. Tumours of the kidney also occurred in 2 instances (Druckrey *et al. Naturwissenschaften* 1962, 49, 451; Druckrey & Preussmann, *Nature, Lond.* 1962, 195, 1111).

In the rat the outstanding characteristic of NA is that they are 'one-shot' carcinogens: a single exposure can ultimately lead to tumour production, but not necessarily in the organs damaged by the acute action of the toxic agent. Thus while the liver is the main site of necrosis, and the lungs may be affected by haemorrhagic lesions, tumours not only develop in the liver, but also in the kidney, bladder or oesophagus, depending on the compound, the dose rate and the strain of rat used.

Magee & Barnes (*J. Path. Bact.* 1962, 84, 19) have reported on the production of kidney tumours in rats after short intensive feeding of dimethyl-NA. Very low levels (5 ppm) were without effect whereas 10–50 ppm given for long periods (20 ppm for 102 weeks; 50 ppm for 42 weeks) resulted in a high incidence of liver tumours without the development of kidney tumours. Feeding at 200 and 100 ppm for periods exceeding 1–4 weeks resulted in death from liver necrosis but if dimethyl-NA was withdrawn within this time the majority of rats made a rapid recovery, with healing of the damaged liver and subsequent production of kidney tumours. The optimum intake scheme for the production of kidney tumours without hepatic tumours was 200 ppm for 1 week or 100 ppm for 4 weeks. Even 1 dose of dimethyl-NA (30 mg/kg) resulted in a 20% incidence of kidney tumours many months after, even though there is evidence that most or even all of the compound is metabolized or excreted within 24 hr.

It is not only mice and rats that are affected. In the golden hamster dimethyl-NA acts with clockwork precision to produce tumours of the trachea and lungs (Dontenwill & Mohr, *Klin. Wschr.* 1961, 39, 493). Diethyl-NA brings about liver tumours with great regularity in

guinea-pigs, usually regarded as a somewhat resistant species (Druckrey & Steinhoff, *Naturwissenschaften* 1962, 49, 497). There is a suggestion also of ready transference across the placenta and secretion into milk.

These results are the outcome of the special qualities of NA, compounds that combine chemical stability with extraordinary solubility in many and various solvents (what a boon to the experimentalist!). It is only in the course of metabolism that their toxic potentialities are unmasked, beginning with dealkylation to a monoalkyl-NA. This is an unstable intermediate with three possible routes of decomposition—and all of them are potential winners in the carcinogen stakes. For example, in the case of dimethyl-NA, which yields monomethyl-NA, the first route leads to methylamine and nitrous acid (Fig. 1). As a carcinogen nitrous acid is an outsider, but it does just qualify for consideration by virtue of its powerful mutagenic effect on tobacco mosaic virus (Fraenkel-Conrat, *Biochim. biophys. Acta* 1961, 49, 169). The second decomposition reaction involves the production of diazomethane and the third a methyl carbonium ion, CH_3^+. Diazoalkanes are potent carcinogens, probably functioning by decomposition to carbonium ions which act as alkylating agents. How metabolic alkylation leads to cancer is the crucial question. The formation of methylated nucleic acid, and in particular of 7-methylguanine, has been demonstrated (*Cited in F.C.T.* 1963, 1, 156) but this event is not necessarily the vital link.

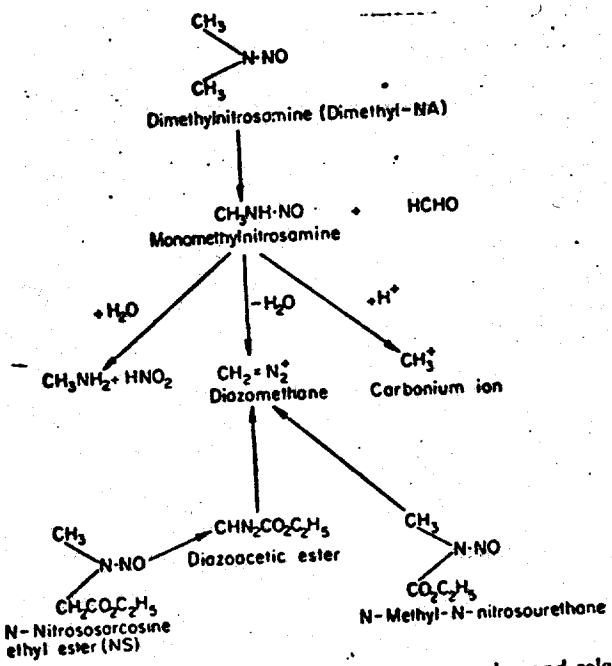


FIG. 1. Possible carcinogenic intermediates of dimethylnitrosamine and related compounds.

In continuation of work reported earlier (*Nature, Lond.* 1961, 192, 42), it has been found that the toxic effect of alkyl-NA can be altered by the administration of some SH compounds (Mizrahi & Emmelot, *Biochem. Pharmacol.* 1963, 12, 55). Subcutaneous injection of excess cysteine to rats leads to a decrease of activity of some of the liver microsomal enzymes which catalyse the transformation of NA. The inhibition of amino acid incorporation into liver microsomes *in vitro* is also prevented or reduced following injection of cysteamine. The

degree of this effect is dependent on the route of administration, the dose of cysteamine and on the nature of the alkyl groups of NA. It is of interest to note that an attempt to combine the distinctive organotropic actions of diethyl-NA and X-irradiation failed to show an additive effect (Schmähl *et al. Naturwissenschaften* 1963, 50, 308).

Oehlert & Hartje (*Naturwissenschaften* 1963, 50, 358) have attempted to follow the development of carcinoma in the parenchymal cells of the liver by injecting into rats [^{3}H] cytidine at various times after the start of diethyl-NA feeding. They conclude that the neoplastic transformation is a progressive change in chromosomal deoxyribonucleic acid consequent upon the incorporation of atypical ribonucleotides. This process can account for several of the observed quantitative aspects of NA carcinogenesis, but even without having the complete answer to this conundrum, it is instructive to consider the relationship between chemical structure, carcinogenic activity and site of tumour development. Symmetrical dialkyl-NA have been synthesized up to the di-*n*-amyl compound (Druckrey *et al. Naturwissenschaften* 1961, 48, 134). Although activity falls off with increasing chain length, it is invariably liver cancer that results on oral administration. Lower doses also induce carcinoma of the oesophagus while some compounds such as di-*n*-butyl-NA similarly affect the bladder (Druckrey *et al. Naturwissenschaften* 1962, 49, 19). Unsymmetrical derivatives bring about cancer of the oesophagus with amazing regularity; so do dimethyldinitrosoethylenediamine, N-nitrososarcosine (NS) and its ethyl ester (Druckrey *et al. Naturwissenschaften* 1963, 50, 100). The exception is ethyl-*tert*-butyl-NA, where steric factors may operate to prevent a carcinogenic action.

With the study of NS and its ester the topic enters a new phase since we are dealing here with a nitrosated amino acid. Druckrey and his colleagues (*Naturwissenschaften* 1963, 50, 99) have drawn attention to the distinction between the 'transport form' and the 'active form' of a carcinogenic NA. By assuming that the transport form is not carcinogenic, and that it is converted to the active carcinogen at a particular site in the body, one can perhaps account for the unusual, not to say peculiar, distribution of the tumours observed with different compounds. Thus metabolic demethylation of the ethyl ester of NS would yield diazoacetic ester (Fig. 1). This compound was tested by administration of 5 mg/kg daily in the drinking-water of rats, yielding tumours of the forestomach and, in a high proportion of rats, also tumours of the base of the tongue. When the daily dose was 50–100 mg/kg daily there was a much more rapid development of tumours, exclusively in the oesophagus, in the same way as with NS and the unsymmetrical dialkyl-NA referred to above.

Thus it is postulated that the toxic potentialities of these compounds are unmasked on the spot in the course of metabolism, beginning with dealkylation to a monoalkyl-NA. The site at which this reaction occurs is doubtless determined by local factors. In the case of NS, Druckrey *et al.* suggest that the acidity of the forestomach liberates alkylating carbonium ions. Similarly diazoacetic ester acts locally to produce tumours of the tongue but once absorbed it reacts so rapidly as to be inactivated without further effect. In the case of NS, since tumours of the oesophagus are readily produced to the same extent by intravenous injection, it follows that the active form of the carcinogen must be produced within the tissue and not in the lumen—the position of the tumours rules out the possibility of oesophageal reflux from the stomach. Liver, lungs and bladder all present opportunities for biochemical explanations of specific locations for tumour production.

Two points of special importance need to be emphasized. Firstly, the experimental induction of carcinoma of the oesophagus has brought about a type of tumour histologically similar to that which occurs in man. The overall picture of tumour distribution in response

to NA strikes one as more immediately relevant to the so-called 'spontaneous' cancers of man. Secondly the daily doses necessary to produce tumours are relatively low; methylbenzyl-NA, 1 mg/kg; ethylvinyl-NA, 1·3 mg/kg; dimethyldinitrosoethylenediamine, 2 mg/kg; ethyl-*n*-butyl-NA, 5 mg/kg and ethyl*iso*propyl-NA, 10 mg/kg. It would be surprising indeed if these findings did not prove to have some immediate application to our environment.

CARCINOGENIC LACTONES

Because of the then known mutagenic activity possessed by β-propiolactone (I) Walpole *et al.* (*Brit. J. Pharmacol.* 1954, 9, 306) tested this compound along with a variety of alkyl ethylenimine derivatives for carcinogenic activity. Repeated twice-weekly subcutaneous injections into rats of 2 mg I/100 g body weight for 12·5 weeks produced malignant tumours in weeks 28–55. The weak activity of I can be accentuated by simultaneous administration of a cocarcinogen or promoter such as croton oil (Salaman, in *Ciba Foundation Symposium on Carcinogenesis*, edited by Wolstenholme & O'Connor, Churchill, London, 1958, p.70). In contrast to the aromatic polycyclic carcinogens, lactones are widespread in nature and are present as constituents in plants, micro-organisms and animal tissues. It is therefore not only of great interest but also of considerable practical importance that lactones should be tested for carcinogenicity.

This task has been undertaken by Dickens & Jones (*Cited in F.C.T.* 1963, 1, 158) who examined a series of fourteen 4- and 5-membered ring lactones. Work along these lines was later extended to a further 7 lactones and preliminary results obtained were reported by Dickens & Jones (*Cited in F.C.T.* 1963, 1, 158), together with the suggested structural requirements for lactone carcinogenicity. High chemical reactivity towards nucleophilic substances e.g. -SH groups, intact cyclic ringed structure and αβ-unsaturation or presence of external double bond *para* to the lactone ring or preferably both, appear to be closely linked with carcinogenic activity.

The extended investigation, more details of which have since become available (Dickens & Jones, *Brit. J. Cancer* 1963, 17, 100), involved repeated subcutaneous injections into 2-month-old rats of 0·1–2 mg of each of the following substances: ββ-dimethyltrimethylene oxide (II), maleic anhydride (III), sodium maleate (IV), β-hydroxypropionic acid (V), parasorbic acid (VI), penicillic acid (VII) and sodium penicillin G (VIII). They were administered in aqueous or oily vehicles twice weekly for periods ranging from 32–61 weeks. Control animals were subjected to repeated subcutaneous injections of 0·5 ml arachis oil over a period of 61 weeks. Following dosage with the test compounds, tumours some of which were more highly malignant than others developed at the site of injection—nearly all tumours survived and grew well after transplantation. Tumours which were remote from the injection site were not considered to be associated with the action of the test substance. Newcomers to the circle of carcinogenic lactones II, III and VI were now firmly established. The carcinogenic activity of VII and VIII and the lack of effect of IV, V and the oil itself were confirmed.

This experiment was terminated after 106 weeks (Dickens & Jones, *A.R. Brit. Emp. Cancer Camp.* 1962, No. 40, p.158) and the following final incidence of tumours/rats was observed: oil alone, 0/14; 2 mg aqueous VII, 4/5; 2 mg aqueous VIII, 5/12; 0·2 mg VI, 4/6; 2 mg VI, 4/5; 1 mg aqueous V, 0/4; 1 mg III, 2/3; 1 mg aqueous IV, 0/3 and 1 mg II, 2/4. So far

Iowa State University Veterinarian
Vol 28 (1966)

57

An Experimentally Induced Case of Acute Nitrate Toxicity*

Nitrate toxicity in ruminants has caused a great deal of interest in the past few years, and many articles have appeared in various journals dealing with this problem.[2-4] However, various investigators have arrived at so many different conclusions that it is difficult to know just when nitrate poisoning is or is not a problem.[3] It has been recognized for many years that high levels of nitrate in the feed of ruminants can result in symptoms of nitrate poisoning. Nitrate itself is believed to be only slightly toxic, with poisoning being associated with reduction of nitrate in the rumen to nitrite and other nitrogenous substances.[9] A general review of the problem indicates that two types of toxicity occur, acute and chronic. This experiment was undertaken to gain information about the acute syndrome in cattle fed a high level of nitrate.

MATERIALS AND METHODS

An eight month old Holstein-Angus heifer weighing approximately 455 pounds was used as the experimental subject. Prior to confinement, the animal was given a complete physical examination with special reference given to any abnormalities that might have interfered with the experimental results. No abnormalities were noted. The animal was confined to a clean pen on December 13, 1965, and all feed withheld for twelve hours.

The following morning the animal was given a known quantity of Sudax hay, the nitrate content of which varied from one to seven percent, depending on where the sample for analysis was taken. The consumption of hay was measured and the amount of nitrate per pound of body weight determined. Blood samples were taken prior to feeding and throughout the duration of the experiment to determine the blood methemoglobin and serum nitrate and nitrite levels. One sample was to be taken six hours after feeding with additional samples to be taken at twelve hour intervals until signs appeared, at which time samples were to be taken as often as seemed appropriate. Samples for methemoglobin determination were to be tested within one hour of being obtained. On post mortem examination samples of ingesta were to be collected for nitrate determination. However, during the course of the experiment, for economic reasons, it was decided to attempt to save the animal's life using the recommended antidote, methylene blue, given at the rate of 10 mg/kg body weight. Sudax was to be fed for forty-eight hours, and if no symptoms were noted the animal would be drenched with potassium nitrate at the rate of 725 mg/kg body weight.[8] At the end of forty-eight hours no symptoms were evident and the calculated lethal dose of potassium nitrate was administered via a stomach tube. At this time thirty pounds of Sudax had been consumed.

RESULTS

After feeding the high nitrate Sudax hay for forty-eight hours, no symptoms were visible. Five hours and fifteen minutes post drenching with the potassium nitrate, an increase in respiratory rate and degree of salvitation were noted. Respiratory difficulty rapidly developed into a dyspnea, accompanied by a muscular weakening beginning in the rear quarters and progressing forward until complete collapse occurred. A blood sample at this time revealed a very chocolate-brown

* This reported prepared by Group 10 of Toxicology 451. Wylie, Freitager, Wynia, Froelich, Gavin, Hinricks, and Orlando.

Table I

Sample	Date	Time	Methb as % of Total Hb	Serum NO^3 ug/ml	Serum NO_2 ug/ml	Comments
1	12/13	11:30 AM	0.5%	0.2	—	Control
2	12/14	8:30 AM	0.7%	0.3	—	Control
3	12/15	12:30 PM	4.6%	2.8	—	Sudax-24 hrs.
4	12/16	8:30 AM	8.1%	3.1	—	Sudax-48 hrs.
5	12/16	9:30 AM	3.3%	43.3	—	KNO_3-1 hr.
6	12/16	10:15 AM	3.0%	52.0	—	KNO_3-2 hrs.
7	12/16	10:30 AM	1.4%	60.0	trace	KNO_3-2 hrs.
8	12/16	1:30 PM	—	59.0	—	KNO_3-5 hrs.
9	12/16	1:30 PM	1.0%	59.3	0.1 (Plasma	KNO_3-5 hrs.
None	12/16	1:45 PM	—	—	—	Signs appear

colored blood. The mucous membranes revealed extreme cyanosis. At this point the animal was given 200 cc of a .4% methylene blue solution intravenously (800 mg total or 4 mg/kg body weight)*. Remission of clinical signs was rapid, and recovery appeared to be complete within two hours of administration of the antidote.

Laboratory reports indicated that blood samples taken during the course of the experiment became progressively more brown as determined by visual examination. Chemical results (table I) revealed an inconsistent methemoglobin level, a constantly rising serum nitrate level, and a trace of nitrite in the plasma.

As is evident from able I, a definite increase in the serum nitrate level occurred while the animal was being fed Sudax hay (sample 3, 4), although no clinical signs were evident. Following oral administration of potassium nitrate, serum nitrate levels rose rapidly (samples 5–9) with the appearance of signs coinciding with a serum nitrate level above 60 ug/ml. The erratic methemoglobin levels could not be correlated with the appearance of clinical signs. Nitrite levels show a slight rise with the appearance of symptoms (samples 7, 9).

DISCUSSION

Chemical results revealed that poisoning from feeding Sudax hay alone may have occurred had the hay been fed a longer period. With the serum nitrate level rising from a base of .57% to 8.1% in the forty-eight hours on hay alone, another forty-eight to seventy-two hours may have raised the nitrate level to the point of acute toxicity. This would be an interesting point to pursue in further experiments. The lack of detectable serum nitrite until symptoms were about to appear is explained by an understanding of the pathogenesis of nitrate toxicity.

When nitrate is consumed by the ruminant, the majority of the anion is oxidized to nitrite in the rumen by the rumen microorganisms. The unchanged nitrate and nitrite ion are absorbed into the blood stream where the nitrate ion accumulates while the nitrie ion combines with hemoglobin oxidizing it to methemoglobin. That is, the ferrous portion of the hemoglobin is oxidized to the ferric state while the nitrite ion is reduced.[1, 6, 7, 8] Symptoms appear because methomoglobin is not able to carry oxygen and the animal dies of suffocation. The delay of approximately five hours is due to the time necessary for conversion of nitrate to nitrite, absorption of the nitrite, and subsequent conversion of hemoglobin to methemoglobin. In addition, signs are not exhibited until 30–40% of the hemoglobin is oxidized to methemoglobin, and death does not occur until 90% of the hemoglobin is methemoglobin.[7] In this experiment, however, the methemoglobin level was low (1.0%) near the time signs appeared. Because the determination of methemoglobin was not conducted within the recommended one hour interval of being obtained, little significance can be placed on this variable in table I.

* Radeleff suggests methylene blue be given at the rate of 10 mg/Kg body weight.[5] Blood and Henderson suggest a dosage of .5–1 mg/lb of body weight for this condition.[1]

With regard to the antidote, methylene blue is the chemical of choice. Although it acts as both an oxidizing and a reducing agent in an isolated chemical reaction, it always acts to reduce ferric (methemoglobin) to ferrous (hemoglobin) iron when used as an antidote for nitrate toxicity, providing the dosage is not too high. (Three different dosage levels were mentioned in this report.)

Prevention of nitrate toxicity when high nitrate feeds are being fed can be enhanced by feeding a high energy feed along with the nitrate feed, and by reducing the amount of nitrate forage fed. Perhaps the best method of preventing acute nitrate toxicity is to feed a forage with less than 1.5% nitrate on a dry weight basis, and not over 0.5% nitrate in the total ration.[5]

Nitrate toxicity may be suspected on the basis of clinical signs. It may be confirmed by laboratory anlaysis of nitrate levels in the feed, water, ingesta, serum and the methemoglobin level in the blood. It must be differentiated from cyanide poisoning, anaphylaxis, chlorate poisoning, and pulmonary edema and emphysema. Color of the blood can be used to differentiate cyanide; anaphylaxis may have a history of some kind of foreign substance administration; chemical analysis will determine chlorate levels; and post mortem lesions will reveal pulmonary edema and/or emphysema. Chronic nitrate toxicity is very hard to diagnose, and can only be accomplished by elimination of other possible causes.[1]

SUMMARY

This experiment was conducted to gain information about the acute syndrome of nitrate toxicity. An eight month old, 455-pound Holstein-Angus heifer was fed a high nitrate hay (Sudax, 1–7% nitrate) for forty-eight hours, and then given 150 grams of potassium nitrate orally. Acute symptoms appeared five hours later and progressed rapidly toward a fatal termination. However, methylene blue (800 mg) was administered intravenously with rapid recovery resulting.

Laboratory results revealed a progressive brown coloration of the blood, an elevated serum nitrate level, a trace of serum nitrite, and an inconsistent methemoglobin level.

The pathogenesis of the disease and action of the antidote, as well as prevention of nitrate toxicity and differential diagnosis were discussed.

REFERENCES

1. Blood, D. C. and J. A. Henderson. *Veterinary Medicine*, 2nd edition. The Williams and Wilkins Company, Baltimore: 1963.
2. *Extension Veterinary Medical Letter*. Iowa State University Extension Service. No. 39. August, 1963.
3. Ibid., No. 58. March, 1965.
4. Kingsbury, John M. *Poisonous Plants of the United States*. Prentice-Hall, Inc., Englewood Cliffs, New Jersey.
5. "Nitrate Poisoning in Livestock." California Agricultural Experiment Station Extension Service. Circular 506. December, 1961.
6. "Nitrate Problem." Iowa State University Extension Service. Special Report No. 34. August, 1963.
7. Prewitt, R. D. and C. P. Merilan. *Journal of Dairy Science*, 41:807, 1958.
8. Radeleff, R. D. *Veterinary Toxicology*. Lea and Febriger, Philadelphia, 1964.
9. Sinclair, K. B. and D. I. H. Jones. "The Effect of Nitrate on Blood Composition and Reproduction in the Ewe." British Veterinary Journal. 120:78, 1964.

zyme derangement in the experimental animals, although if present, it appeared only to affect succinate and possibly pyruvate metabolism, not all links in the respiratory chain as the authors have suggested. If enzyme insufficiency is part of the atherosclerotic process, it will have to be demonstrated with better preparations than crude tissue homogenates.

The question of associated impairment of carbohydrate metabolism is of sufficient interest to warrant study of tissues from animals with naturally occurring atherosclerosis. The present data were not obtained from naturally diseased animals, but from those which had been fed high amounts of cholesterol. Other investigations have shown that there are many non-atherosclerotic effects of feeding cholesterol to rabbits that could complicate the evaluation of causes of disturbed oxygen uptake (*see Nutrition Reviews* **20**, *55*, (1962)). These include fatty liver, loss of adipose tissue, increased intake and excretion of water, increased excretion of ions, anemia, jaundice, and enlarged spleen—most of which are not considered normal concomitants of spontaneous atherosclerosis.

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

Prolonged feeding of nitrite reduced storage when vitamin A alcohol or carotene was administered, but had little effect when vitamin A aldehyde was fed. This seems to indicate that the decreased storage is a direct effect of nitrite oxidation on the vitamin or provitamin.

Nitrates in feeds have been suggested as causes of vitamin A deficiency in cattle under feed lot conditions. Nitrates in the diet are converted to nitrites by gastrointestinal microorganisms. This has been observed concurrently with the appearance of methemoglobin in the blood. Although well controlled experiments have shown that nitrate has little if any effect on vitamin A nutrition in cattle (G. S. Smith, E. E. Hatfield, W. M. Durdle, and A. L. Neumann, *J. Animal Sci.* **21**, *1013* (*1962*); K. L. Davison *et al.*, *J. Dairy Sci.* **47**, *1065* (*1964*)), a number of questions regarding nitrate and nitrite remain unanswered for other species.

Several investigators have reported that carotene and vitamin A can be destroyed *in vitro* by nitrite, but not by nitrate (O. E. Olson, D. L. Nelson, and R. J. Emerick, *J. Agr. Food Chem.* **11**, *140* (*1963*); D. L. Pugh, G. B. Garner, R. A. Bloomfield, and M. E. Muhrer, *J. Animal Sci.* **21**, *1009* (*1962*); Pugh and Garner, *J. Agr. Food Chem.* **11**, *528* (*1963*); B. S. Reddy and J. W. Thomas, *J. Animal Sci.* **21**, *1016* (*1962*); W. K. Roberts and J. L. Sell, *J. Animal Sci.* **22**, *1081* (*1963*); Davison and J. Seo, *J. Dairy Sci.* **46**, *862* (*1963*)). This *in vitro* reaction is dependent on pH, and certain compounds related to carotene are less severely attacked than beta-carotene itself. J. Bruggemann and J. Tiews (*Z. f. Vitaminforsch.* **34**, *233* (*1964*)) have suggested that nitrate interferes with absorption of vitamin A and prevents its accumulation in the liver of chickens.

W. E. J. Phillips, (*Canad. J. Biochem.* **44**, *1* (*1966*)) has conducted investigations attempting to answer the controversial question of whether nitrite acts on vitamin A metabolism before or after the vitamin is absorbed from the diet. Sprague-Dawley female rats were started at 21 days of age on a semipurified diet, free of vitamin A, and supplemented with a stabilized form of the vitamin to provide 180 μg. vitamin A alcohol per kilogram daily. This quantity of vitamin A is adequate for growth but not liver storage.

In the first of two experiments the basal diet was supplemented with 1 per cent potassium nitrite and 6 mg. carotene per rat per day. During a 20-day period of feeding, total liver vitamin A and its concentration increased with time in the absence, but not in the presence, of nitrite. When 3 mg. vitamin A alcohol per rat per day were fed, the concentration of vitamin A and the total stored in the liver increased with time in control animals, but, again, no such increase was observed when nitrite was fed simultaneously.

A third series of rats was fed the basal diet with or without 1 per cent nitrite. After 30 days of feeding, vitamin A alcohol or equivalent amounts of aldehyde were injected intraperitoneally. One per cent nitrite reduced body growth by 33 and 25 per cent respectively, and there were proportional decreases in liver mass. The feeding of nitrite had led to a reduction in the total amount of vitamin A in the liver at 24 hours after injection, although the concentration per unit weight of liver remained about the same.

The rate of disappearance of vitamin A from the liver between days 32 and 52 of feeding was followed in a group of animals given vitamin A acetate orally. Feeding 1 per cent potassium nitrite did not alter the rate of decrease of liver vitamin A during this interval. In the fifth experiment vitamin A alcohol, vitamin A aldehyde, or beta-carotene was administered in a single oral dose by stomach tube, instead of intraperitoneal injection, to animals fed the basal diet alone or with 1 per cent potassium nitrite for 30 days. Overall body growth and liver weight were depressed in the animals fed nitrite.

Contrary to the results with intraperitoneal injection of vitamin A alcohol or aldehyde, which increased the concentration of vitamin A in the liver tissue, no significant increase was noted when vitamin A alcohol, vitamin A aldehyde, or carotene was administered in this manner. Instead, the total amount of vitamin A stored in the liver was significantly reduced following administration of vitamin A alcohol or carotene to the animals fed nitrite, but there was no reduction in the rats given vitamin A aldehyde.

It is readily apparent from these published experiments that feeding 1 per cent potassium nitrite concomitantly with either carotene or vitamin A decreased the amount of vitamin A in the liver. This, according to Phillips, did not result from the liver's inability to store vitamin A. Evidence supporting this statement is provided by the increased liver concentrations of the vitamin found after intraperitoneal injection when nitrite was fed, and is in accord with observations of Emerick and Olson (*J. Nutrition* 78, 73 (*1962*)).

The data show that the rate of decrease of liver vitamin A was unaffected by feeding nitrite over a 20-day period. The author concludes that the lower levels of liver vitamin A that occur after feeding nitrite are not a direct effect of nitrite on the organ or on hormonal mechanisms which release vitamin A from it. Rather, he believes that prolonged feeding of nitrite reduces the storage of vitamin A only when carotene or vitamin A alcohol is administered, but has little effect on the aldehyde. Since vitamin A aldehyde is an intermediate in carotene metabolism and is unaffected, it is probable that absorption and transport mechanisms are also not affected.

If nitrite influences enzymatic processes which convert carotene to vitamin A, then the decrease in liver storage of vitamin A would be much greater when carotene was given rather than vitamin A itself. Since storage would reflect effects of nitrite on vitamin A per se, and on the conversion of carotene to vitamin A, the like degree to which nitrite affected storage of the vitamin from carotene and from vitamin A is believed to demonstrate that conversion was not directly influenced. The experimental data support Phillips' conclusion that, in

the rat, reduction of liver storage of vitamin A from carotene or the preformed vitamin is the direct effect of oxidation by nitrite on the vitamin or provitamin. It does not involve interference with absorption, enzymatic processes associated with conversion of carotene, or liver function.

The author has pointed out the importance of certain dietary factors, including methods and duration of storage, that may affect dietary vitamin A concentration. When nitrites are present, alteration of the microflora by nitrite, with secondary destruction of the vitamin or provitamin in the gastrointestinal tract, is a possibility which Phillips did not mention specifically. Data are not presented showing the effect of nitrite on food intake.

DIETARY CHOLESTEROL AND REGULATION OF LIVER CHOLESTEROL SYNTHESIS

Acetate-C^{14} incorporation into mevalonic acid was only 3 to 4 per cent of normal in cell-free liver preparations from rats fed cholesterol. Cholesterol feeding had no effect; however, on acetate-C^{14} incorporation into hydroxymethylglutaric acid, the immediate precursor of mevalonic acid. Thus, feedback inhibition of cholesterol biosynthesis by dietary cholesterol seems to occur primarily at the point of synthesis of mevalonate from hydroxymethylglutarate.

The early balance experiments of R. Schoenheimer and F. Breusch (*J. Biol. Chem.* 103, *439* (*1933*)) suggested that the concentration of cholesterol in animals was regulated by a homeostatic mechanism. Evidence for a possible mechanism was first shown by R. G. Gould (*Am. J. Med.* 11, *209* (*1951*)), who found that hepatic cholesterol synthesis was reduced by feeding cholesterol. These results were confirmed by several other investigators (*see Nutrition Reviews* 23, *187* (*1965*)). This type of depression in synthesis is called negative feedback control. Feedback control may be defined as a process in which formation of the product is regulated directly or indirectly by the product itself.

Considerable study has been given to defining the exact steps in cholesterol synthesis that are inhibited by cholesterol. Gould and G. Popjak (*Biochem. J.* 66, *51* (*1957*)) showed that inhibition occurred before formation of mevalonic acid in the synthetic pathway. M. D. Siperstein and V. M. Fagan (*J. Clin. Invest.* 37, *1185* (*1958*)) obtained evidence that the inhibition was localized more specifically at the conversion of beta-hydroxy, beta-methylglutaric acid (hydroxymethylglutaric acid) to mevalonate. This is the fourth of 26 reactions in the biosynthesis of cholesterol from acetate. These conclusions were based on the fact that cholesterol feeding had no effect on conversion of mevalonate-C^{14} to cholesterol. However, a more direct effect of cholesterol on mevalonate synthesis was not demonstrated.

Studies of mevalonate synthesis from hydroxymethylglutarate have been hindered by problems in the separation and isolation of mevalonate from hydroxymethylglutarate. Recently, however, Siperstein, Fagan, and J. M. Dietschy, (*J. Biol Chem.* 241, *597* (*1966*) were able to separate mevalonate and hydroxymethylglutarate by gas-liquid chromatography, and detailed studies of mevalonate synthesis were then possible (Siperstein and Fagan, *J. Biol. Chem.* 241, *602* (*1966*)).

The first experiments tested mevalonate synthesis from acetate-C^{14} in liver slices. Approximately 19 mμmoles of acetate-C^{14} were converted to cholesterol when unlabeled mevalonate was not added to the medium. When unlabeled mevalonate was added to trap mevalonate-C^{14}, acetate-C^{14}

THE LANCET

LONDON 18 MAY 1968

Nitrites, Nitrosamines, and Cancer

FOR centuries meat has been preserved with salt, though the origin of the practice is unknown. At first, it seems, the meat was rubbed with pulverised rock salt and then stored in contact with similar material for three to four weeks. It was later observed that when the preserved meat was cooked there were red patches intermingled with the usual brown colour of cooked fresh meat. These red patches were caused by impurities in the rock salt (which proved to be metallic nitrates) that had reacted with the meat pigments. These observations led to the deliberate use of nitrate (saltpetre) to produce a more uniform colour, and fixed formulations of salt and nitrate came into general use. Subsequently, large amounts of nitrite were found in meat treated in this way, and it became clear that the red colour was due to a chemical reaction between the nitrite and the meat pigments. The nitrate had been reduced to nitrite by microorganisms in the meat. This being established, it was logical to substitute nitrite for nitrate in the original salt mixture, and nitrites became widely used, especially for curing pork. The chemistry of the curing process is complex and is based on reactions between sodium or potassium nitrite and myoglobin and hæmoglobin with the formation of nitrosomyoglobin and nitrosohæmoglobin, which are the red pigments.[1] Nitrites are used as food additives in many countries and they have an especially valuable preservative action against *Clostridia*.[2] In Britain their use is permitted in cured and pickled meat products and in some cheeses.[3]

The acute and subacute toxic effects of nitrites (vasodilatation, lowering of blood-pressure, and formation of methæmoglobin) are well known. Other toxic actions include reduction of vitamin-A stores in the liver and disturbance of thyroid function. Babies are particularly sensitive to nitrites because fœtal hæmoglobin is more readily oxidised to methæmoglobin than is adult hæmoglobin. This sensitivity has led to fatal methæmoglobinæmia in babies who had been given well-water containing nitrates,[4] which can be reduced to nitrites by the microbial flora of the gut. Nitrates are present in several vegetables, and samples of spinach used as baby foods may contain considerable quantities. Some of this nitrate may be reduced to nitrite during preparation of the baby's feed, and this has sometimes led to methæmoglobinæmia.[5,6] These aspects of nitrite toxicity have been taken into account in regulating the use of nitrites in food, but the situation has become more complicated since the discovery of the remarkable toxic, carcinogenic, and other biological actions of the nitrosamines, because, under certain conditions, these compounds may be formed by interaction between nitrites and secondary or tertiary amines.

Since the hepatotoxic[7] and carcinogenic[8] actions of dimethylnitrosamine were described by BARNES and MAGEE, much work on this and other nitroso compounds has been published, and the subject has been reviewed by DRUCKREY et al.[9] and by MAGEE and BARNES.[10] The nitroso carcinogens are extremely potent and are active in a wide range of species.[11] They have induced tumours in virtually all organs of the rat, and they have the remarkable capacity to induce cancer after only one dose. Some nitroso compounds are teratogenic and have also induced tumours in the progeny when given to pregnant rats. Several of the nitroso carcinogens are very powerful mutagens. Clearly, then, the possible occurrence of these biologically highly active compounds in food or other environmental situations is a matter of the gravest concern.

The unsuspected formation of a carcinogenic nitrosamine was dramatically demonstrated in Norway by KOPPANG and his colleagues,[12,13] who investigated an outbreak of severe liver disease in ruminants in 1961 and 1962. The incidence of the disease was found to be related to the feeding of meal made from herring preserved with sodium nitrite, and this observation suggested that a nitrosamine had been formed. A similar liver lesion could be induced in sheep by the administration of dimethylnitrosamine and this species was apparently more sensitive to the acute effects of dimethylnitrosamine than others previously tested. Mink were similarly affected. Samples of the herring meal with and without known toxic properties were analysed for dimethylnitrosamine and diethylnitrosamine. Six samples from toxic batches after prolonged storage at 2–4°C were found to contain dimethylnitrosamine in concentrations ranging from 30 to 100 p.p.m. Dimethylnitrosamine was not detected in six non-toxic batches of meal. It is highly significant that dimethylnitrosamine could be formed at such levels in this meal, since the feeding of 5 p.p.m. or even 2 p.p.m. of dimethylnitrosamine in the diet is sufficient to induce liver tumours in the rat.[14] Of 68 rats fed 5 p.p.m. dimethylnitrosamine throughout their life-span, 5 (7%) had liver tumours. It seems highly probable that a smaller incidence of tumours would be induced at even

1. Halliday, D. *Process Biochem.* 1967, 2, 32.
2. Spencer, R. *F. Aff.* 1966, 41, 39.
3. Preservatives in Food Regulations. S.I. 1532. H.M. Stationery Office, 1962.
5. Simon, C. *Lancet*, 1966, i, 872.
6. *Br. med. J.* 1966, i, 250.
7. Barnes, J. M., Magee, P. N. *Br. J. ind. Med.* 1954, 11, 167.
8. Magee, P. N., Barnes, J. M. *Br. J. Cancer*, 1956, 10, 11.
9. Druckrey, H., Preussmann, R., Ivankovic, S., Schmahl, D. *Z. Krebsforsch.* 1967, 69, 103.
10. Magee, P. N., Barnes, J. M. *Adv. Cancer Res.* 1967, 10, 163.
11. Schmahl, D., Osswald, H. *Experientia*, 1967, 23, 497.
12. Ender, F., Havre, G., Helgebostad, A., Koppang, N., Madsen, R., Ceh, L. *Naturwissenschaften*. 1964, 51, 637.
13. Sakshaug, J., Sognen, E., Hansen, M. A., Koppang, N. *Nature, Lond.*

lower dietary levels of nitrosamine when clinical evidence of toxicity would probably be absent. If man is sensitive to nitrosamine carcinogenesis—and there seems no reason to assume that he is not—the implications of the above experiments are obvious.

Nitrosamines may be found in other environmental situations. DRUCKREY and PREUSSMANN[15] suggested that they might be present in cigarette smoke, and they have in fact been detected there in minute amounts.[16] The presence of trace amounts of nitrosamine in white flour has been claimed,[17] confirmed,[18] and denied.[19] The quantities of nitrosamine thus reported are extremely small and their biological significance is impossible to assess in the absence of much more experimental work on dose-response relationships at much lower doses than have been studied so far. The formation of N-nitroso compounds by nitrosation of amines might happen in the human stomach as well as in stored food, and SANDER[20] has shown that nitrosamines can be formed in vitro from secondary amines and nitrite when mixed together in human gastric juice. Chronic feeding of nitrite to rats, however, even when diethylamine was given at the same time, did not induce tumours.[21] Apparently, therefore, carcinogenic nitrosamines could be formed by interaction of nitrites with amines present in food, and hazardous levels might be attained in foods of high amine content. Any possible risk to human health arising from the addition of nitrites to food must, however, be balanced against their value as preservatives, especially their action against *Clostridia*.

The diabetogenic antibiotic, streptozotocin, isolated from *Streptomyces achromogenes*, is a nitrosamide and induces kidney tumours in the rat.[22] This compound seems to be the first known naturally occurring nitroso carcinogen, but a related series of natural carcinogens found in cycad plants has been well investigated since their carcinogenic action was reported by LAQUEUR and his colleagues in 1963.[23 24] These plants are widely distributed throughout the tropics and subtropics. The active carcinogenic components are glucosides of methylazoxymethanol, and their biological and biochemical actions seem to be virtually identical with those of the nitroso compounds. A hepatotoxic azoxy compound of this general type, elaiomycin, has been isolated from a strain of *Streptomyces*.[25] Like the fungi that produce aflatoxin, *Streptomyces* are widely distributed organisms, and the possibility that they too may contaminate human food must not be dismissed.

15. Druckrey, H., Preussmann, R. *Naturwissenschaften*, 1962, 49, 498.
16. Neurath, G. *Experientia*, 1967, 23, 400.
17. Marquardt, P., Hedler, L. *Arzneimittel-Forsch.* 1966, 16, 778.
18. Kroller, E. *Dt. LebensmittRdsch.* 1967, 63, 33.
19. Thewlis, B. H. *Fd Cosmet. Toxic.* 1967, 5, 333.
20. Sander, J. *Arch. Hyg. Bakt.* 1967, 151, 22.
21. Druckrey, H., Steinhoff, D., Beuthner, H., Schneider, H., Klärner, P. *Arzneimittel-Forsch.* 1963, 13, 320.
22. Avison, R. N., Feudale, E. L. *Nature, Lond.* 1967, 214, 1254.
23. Laqueur, G. L., Mickelsen, O., Whiting, M. G., Kurland, L. T. *J. natn. Cancer Inst.* 1963, 31, 919.
24. *Lancet*, 1965, i, 593.
25. Ehrlich, J., Anderson, L. E., Coffey, G. L., Feldman, W. H., Fisher, M. W., Hillegas, A. B., Karlson, A. G., Knudsen, M. P., Eston, J. K., Youmans, A. S., Youmans, G. P. *Antibiotics Chemother.* 1954, 4, 338.

Vesico-ureteric Reflux

WHEN urine enters the bladder it normally stays there until it is passed through the urethra at the next act of micturition. It is prevented from re-entering the ureters, even when intravesical pressure is raised by contraction of the detrusor muscle during micturition, by a valvular mechanism at the ureterovesical junction which is controlled by the muscle-fibres of the intramural ureter and the trigone of the bladder.[1-4] Peristaltic activity in the lower end of the ureter may also contribute towards this mechanism by maintaining an intraureteric pressure which is slightly higher than the intravesical pressure.[5-7] If the mechanism is deficient, urine re-enters the ureters from the bladder and sometimes flows into the renal pelvis and calyces. Such a situation is known as vesico-ureteric reflux. Since it is commonest in children, it is of special interest to pædiatricians and pædiatric urologists. There is general agreement that vesico-ureteric reflux seldom arises in normal subjects.[8-14] Usually, the condition is discovered during the investigation of children with urinary-tract infections, and it is present in about 35% of such cases seen at hospital.[15-20] It is revealed by a cystogram which, as much as an intravenous pyelogram, must be part of the routine investigation of every child shown to have a urinary infection. Reflux is occasionally seen in children with uninfected urinary tracts who are investigated because of conditions such as enuresis. Such children should, however, be regarded as candidates for attacks of urinary infection and be observed carefully.

The cause of reflux is in doubt. The ureterovesical junction may be congenitally incompetent or it may become incompetent as a result of damage produced by infection or by obstruction to the bladder-outflow tract.[21] This last possibility seems the least likely, since, although in children reflux may accompany some mechanical obstructive urethral lesions, such as posterior urethral valves, there is evidence that even in these cases reflux is due to a coincident congenital abnormality of the ureterovesical junction, rather than to the secondary effects of the obstruction,[1] and doubt is increasing about the existence of non-mechanical

1. Stephens, F. D., Lenaghan, D. *J. Urol.* 1962, 87, 669.
2. Johnston, J. H. *Ann. R. Coll. Surg.* 1962, 30, 324.
3. Tanagho, E. A., Pugh, R. C. B. *Br. J. Urol.* 1963, 35, 151.
4. Tanagho, E. A., Hutch, J. A., Myers, F. H., Rambo, O. N., Jr. *J. Urol.* 1965, 93, 165.
5. Scott, J. E. S., De Luca, F. G. *Br. J. Urol.* 1960, 32, 216.
6. Scott, J. E. S., De Luca, F. G. *ibid.* p. 320.
7. Melick, W. F., Brodeur, A. E., Karillas, D. N., Naryka, J. J. *J. Urol.* 1962, 88, 38.
8. Campbell, M. F. *Am. J. Dis. Child.* 1930, 39, 386.
9. Gibson, H. M. *J. Urol.* 1949, 62, 40.
10. Iannoconne, G., Panzironi, P. E. *Acta radiol.* 1955, 44, 451.
11. Jones, B. W., Headstream, J. W. *J. Urol.* 1958, 80, 114.
12. Leadbetter, G. W., Duxbury, J. H., Dreyfuss, J. R. *ibid.* 1960, 84, 69.
13. Politano, V. A. *J. Am. med. Ass.* 1960, 172, 1252.
14. Lich, R., Jr., Howerton, L. W., Jr., Goode, L. S., Davis, L. A. *J. Urol.* 1964, 92, 436.
15. Ericsson, N. O. *Acta pædiat., Stockh.* 1960, 49, 196.
16. Rosenheim, M. L. *Br. med. J.* 1963, ii, 1433.
17. Smellie, J. M., Hodson, C. J., Edwards, D., Normand, I. C. S. *ibid.* 1964, ii, 1222.
18. Normand, I. C. S., Smellie, J. M. *ibid.* 1965, i, 1023.
19. Hodson, C. J., Wilson, S. *ibid.* 1965, ii, 191.
20. Stansfeld, J. M. *ibid.* 1966, i, 631.
21. Stewart, C. M. *J. Urol.* 1961, 85, 119.

63 (26)

Fd Cosmet. Toxicol. Vol. 6, pp. 519–532. Pergamon Press 1968. Printed in Great Britain

ARTICLES OF GENERAL INTEREST

ON THE TRAIL OF NITROSAMINES

The hazard of carcinogens in food has repeatedly claimed our attention in this Journal and a fresh look at the problem is prompted by a recent Leading Article (*Lancet* 1968, i, 1071), which examines the possibility of the presence of nitrosamines in our food.

Nitrosamines form a group of versatile and potent carcinogens. Of a large number of nitroso-analogues that have been synthesized, very few appear to be incapable of inducing tumours in the rat. The target organ varies with the compound, but tumours have been produced in practically all the major tissues of this species (*Lancet, loc.cit.*; *Cited in F.C.T.* 1965, 3, 498). In addition, members of this chemical group have been shown to be carcinogenic to the hamster (Herrold, *J. Path. Bact.* 1966, 92, 35) and the new-born mouse (*Cited in F.C.T.* 1965, 3, 499). Even the guinea-pig, generally found resistant to other liver carcinogens, is quite susceptible to the alkylnitrosamines (*ibid.* 1964, 2, 61). The most disturbing features of these compounds are that very low concentrations in the diet (2 ppm in the case of dimethylnitrosamine) have been shown to be tumorigenic to rats (Terracini *et al.*, *Br. J. Cancer* 1967, 21, 559) and that a single dose of only a few milligrams also suffices to produce tumours (*Cited in F.C.T.* 1964, 2, 61).

Concern about the presence of nitrosamines in food resulted from an investigation into outbreaks of liver disease among ruminants in Norway in 1961 and 1962 (*ibid.* 1965, 3, 525). This condition was found to be related to the feeding of fish meal preserved with sodium nitrite, and further studies revealed the presence of nitrosamines, which were shown to be formed by the interaction of nitrite with secondary and tertiary amines.

Nitrites are used as preservatives in cheeses and in a variety of meats and meat products, in which they are particularly valuable as a defence against Clostridia. They are also found in some brands of flour and other foods. In the UK, their use is permitted only in certain cheeses and in cured and pickled meat products, in which they may also have some apparently beneficial effect on the flavour. The known presence of nitrites indicated the advisability of looking for nitrosamines in food intended for human consumption. The occurrence of nitrosamines in flour was first reported in Germany (*ibid.* 1967, 5, 112) and their concentration has been shown to increase when the samples are heated at baking temperatures (170°C) for 0·5 hr. Early tests on British flour failed to reveal any nitrosamines or more than traces of nitrates and nitrites (Thewlis, *Fd Cosmet. Toxicol.* 1967, 5, 333) but subsequently, Hedler & Marquardt (*Fd Cosmet. Toxicol.* 1968, 6, 341), using the same analytical method as they used for German flours, have demonstrated the presence of nitrosamines also in samples of British flour. Nitrogen dioxide is no longer used as a bleaching agent in flour, either in the UK or Germany, and it has been suggested that the presence of nitrite in flour might be due to contamination from motor-exhaust gases (Buré, *Annls Falsif. Expert. chim.* 1962, 638, 52). However, Hedler & Marquardt (*loc. cit.*) have also found nitrosamines in the stalk, leaves and grain of wheat, and suggest that the nitrogen content of the fertilizer used may be an important factor in this finding. These workers have also demonstrated the

presence of nitrosamines in milk and cheese, but so far their methods do not permit any estimation of the quantities of nitrosamines involved.

Recent work indicates that nitrosamines present in meat and meat products probably constitute a greater threat to health than those in flour. Investigating the presence of nitrosamines in Norwegian bacon and smoked sausage and in smoked ham from Iceland, Professor Fr. Ender (1967, personal communication) found up to 2·4, 6·5 and 5·7 μg/kg respectively. It would be of great interest to know whether nitrosamines are present in British meat products.

To quote the *Lancet* Leader (*loc.cit.*): "... the possible occurrence of these biologically highly active compounds in food or other environmental situations is a matter of the gravest concern." Such concern must be reflected in a growing determination to solve the outstanding questions relevant to this problem. Extensive analysis of foodstuffs for nitrosamines would not only give an indication of how widespread these compounds are in the diet, but might also provide some clues to their origins and to the extent to which their presence is affected by processing, particularly by high-temperature cooking procedures such as grilling or roasting, and by storage at low temperatures. Another big question-mark hangs over man's sensitivity to the carcinogenic effects of nitrosamines. Although there is no reason to suppose that he is not sensitive, the evidence from industrial exposure is equivocal, since the extensive hepatic damage reported (Barnes & Magee, *Br. J. ind. Med.* 1954, **11**, 167) did not appear to be associated with cancer. Perhaps intensive study of the susceptibility of primates to nitrosamine carcinogenesis and of nitrosamine metabolism in these species would assist in solving this problem.

Some investigations have been carried out, others are in progress and it is expected that more will be started in the near future. It may be that comprehensive analytical and toxicological investigations will show that our concern is in fact misplaced and that nitrosamines do not constitute a significant dietary hazard for man, but this problem should be regarded as of the highest priority by government and industry.

SYNERGISM IN CARCINOGENESIS

The appearance of a further paper in a series concerned with the possible complementary action of orally-administered carcinogens has prompted another look at the possible enhancement of the tumorigenic activity of one material by a second carcinogen fed simultaneously. We have reported some of this work before (*Cited in F.C.T.* 1966, **4**, 196 & 247) but the moment seems opportune to collate some of the relevant information and review the situation.

While a great deal of attention has been paid to the effect of applying mixtures of carcinogens to the skin (Poel, *National Cancer Institute Monograph* No. 10, 1963, p. 611) and of administering them subcutaneously (Steiner & Falk, *Cancer Res.* 1951, **11**, 56), comparatively few studies have been concerned with the combined effects of orally-administered carcinogens. MacDonald *et al.* (*ibid.* 1952, **12**, 50) found that the simultaneous feeding of two hepatocarcinogens, either 2-acetylaminofluorene and 3′-methyl-4-dimethylaminoazobenzene or 4-dimethylaminoazobenzene and 4′-methyl-4-dimethylaminobenzene, produced an incidence of liver tumours considerably higher than the sum of the tumours obtained by the separate feeding of the same amounts of each compound of the pair. However, simultaneous administration of 2-acetylaminofluorene or 4-dimethylaminoazobenzene with non-

Volume 9 Number 3 June 1971

PHARMACY LIBRARY
AUG 20 1971
C.S.U. LIBRARIES

Toxicology

An International Journal published for the British Industrial Biological Research Association

CONTENTS

Page

RESEARCH SECTION

Short-term toxicity of Orange G in rats (*I. F. Gaunt, M. Wright, P. Grasso and S. D. Gangolli*) 329

Short-term toxicity of Yellow 2G in rats (*I. F. Gaunt, F. M. B. Carpanini, Ida S. Kiss and P. Grasso*) 343

Short-term toxicity of isobornyl acetate in rats (*I. F. Gaunt, Consuelo E. Agrelo, J. Colley, A. B. G. Lansdown and P. Grasso*) 355

Bioassay for antioxidants based on protection of isolated rat liver mitochondria against the photodynamic toxicity of benzo[a]pyrene (*S. S. Epstein, Inna B. Saporoschetz, C. Katsioules and Y. Bishop*) 367

The wholesomeness of irradiated mushrooms (*M. J. van Logten, Engelina M. den Tonkelaar G. van Steenis, R. Kroes and G. J. van Esch*) 379

Intolerance to cottonseed oil in rats fed a low protein diet (*E. M. Boyd and C. J. Krijnen*) 389

Teratogenic studies on 2,4,5-trichlorophenoxyacetic acid in the rat and rabbit (*J. L. Emerson, D. J. Thompson, R. J. Strebing, C. G. Gerbig and V. B. Robinson*) 395

Study of the teratogenicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the rat (*G. L. Sparschu, F. L. Dunn and V. K. Rowe*) 405

Reduction of pesticide residues in human adipose tissue with diphenylhydantoin (*J. E. Davies, W. F. Edmundson, A. Maceo, G. L. Irvin, III, Janet Cassady and Ana Barquet*) 413

REVIEW SECTION

REVIEWS OF RECENT PUBLICATIONS 425

BOOK REVIEWS 431

INFORMATION SECTION

LEGISLATION 435

ARTICLES OF GENERAL INTEREST 439

TOXICOLOGY: ABSTRACTS AND COMMENTS 445

FORTHCOMING PAPERS 461

RA1211
A1F6
V.9(3)

daily, whereas smokers of 15 or more cigarettes/day consumed 53·4 g and the consumption of sucrose generally reflected the number of cigarettes smoked. Elwood *et al.* (*ibid* 1970, i, 1014) found no statistically significant difference between the sucrose intake of women in a Welsh mining valley who suffered from angina pectoris and the intake of those who did not. Among the men, those with electrocardiographic evidence of ischaemic heart disease took on average less sucrose than the others; but it was again found, in both sexes, that those who took most sucrose were also those who smoked most cigarettes. Elwood *et al.* (*loc. cit.*) therefore support the suggestion that there is an association between the consumption of sucrose and of tobacco which might give the appearance of an association between sucrose intake and ischaemic heart disease. However, Yudkin (*ibid* 1970, i, 1111) refuses to recognize any logic in the argument that because a high sugar consumption and heavy smoking both go with a tendency to develop ischaemic heart disease, and because heavy smoking tends to be accompanied by heavy sugar consumption, smoking, but not a high sugar intake, is a cause of the disease. The basis for such a conclusion certainly seems, at present, too confusing to be convincing.

Effect of sucrose on drug absorption

Leaving the highly unsatisfactory evidence that sucrose may or may not be a factor in atherosclerotic heart disease, we may look into a rather different aspect of its effects. Kato *et al.* (*Jap. J. Pharmac.* 1969, 19, 331) have reported that when rabbits were given an intragastric dose of aminopyrine in water, the serum concentration of the drug rose more rapidly, and to nearly twice the peak value, than when the dose was given in a syrup composed (in g/litre) of sucrose (400), sorbitol (50), sodium cyclamate (4) and saccharin (0·5). The same phenomenon occurred when dipyrone (sulpyrine) was given, the concentration of its serum metabolite, *N*-methyl-4-aminophenazone, being higher and rising more quickly with an aqueous solution than a syrup. The explanation was found after the gastric emptying rates of 50% sucrose and 1% carboxymethylcellulose (CMC) were measured in rats, using phenol red indicator to demonstrate the entry of the solutions into the small intestine. CMC emptied more rapidly from the stomach than did sucrose. Moreover, pentobarbitone narcosis and strychnine convulsions were retarded when the drugs were given in a sucrose syrup, compared with the rate of reaction when they were given in CMC. The high osmotic pressure of the sucrose solution was probably an important factor in the rate of gastric emptying.

Here we have a significant piece of evidence showing that the action of drugs can be modified by the concurrent taking of sucrose. Whether this factor can be brought to bear on the controversy over the possible atherosclerotic potential of sugar remains to be seen.

NUCLEIC-ACID ALKYLATION IN RELATION TO NITROSAMINE CARCINOGENESIS AND MUTAGENESIS

Dimethylnitrosamine and several homologous compounds have been shown, *in vivo*, to alkylate nucleic acids in organs susceptible to their tumorigenic action. Such an alkylation was thought to be brought about by some intermediate product of metabolism (Magee, *N.Z. med. J.* 1968, 67, 59). Druckrey *et al.* (*Z. Krebsforsch.* 1967, 69, 103) postulated that those nitrosamines capable of being converted by oxidative dealkylation into a monoalkylnitrosamine and thence into a carbonium ion were carcinogenic, while those incapable

of such conversion, such as *tert*-butylmethylnitrosamine, were not. These studies suggested a correlation between alkylation of nucleic acids and tumour production, a hypothesis which received further support from the finding that the cyclic carcinogenic nitrosamines, nitrosopyrrolidine, nitrosopiperidine and nitrosomorpholine, alkylated hepatic RNA (*Cited in F.C.T.* 1968, **6**, 651). Hopes of establishing a direct causal relationship between *in vivo* alkylation of nucleic acids and carcinogenesis soon began to fade however (*ibid* 1970, **8**, 79), and a further study on cyclic nitrosamines has speeded this trend.

The four carcinogenic nitrosamines selected for this study (Lijinsky & Ross, *J. natn. Cancer Inst.* 1969, **42**, 1095) were nitrosoazetidine (which causes tumours in the liver and lung), nitrosohexamethyleneimine (tumours in the oesophagus and liver), nitrosomethylcyclohexylamine (causing oesophageal but not liver tumours) and nitrosomethylaniline (another oesophageal carcinogen). The compounds were labelled with tritium and in each case male rats were given a single dose of the compound below the LD_{50}. DNA and RNA were isolated from the liver and hydrolysed with acid, the products being separated by ion-exchange chromatography and the fractions assayed for radioactivity. Appropriate fractions were then subjected to paper chromatography and mass spectrometry. Evidence of RNA alkylation was found in the livers of rats treated with nitrosoazetidine, but no alkylated RNA was recovered from the livers of rats treated with nitrosohexamethyleneimine, also a hepatocarcinogen. On the other hand, nitrosomethylcyclohexylamine, though not a liver carcinogen, did give rise to an alkylated base in liver RNA, and this base was identified as 7-methylguanine by mass spectrometry. No alkylated base was detected in the liver RNA of rats treated with nitrosomethylaniline, and no alkylation of DNA was detected with any compound. As far as the cyclic *N*-nitrosamines were concerned, therefore, no correlation between *in vivo* alkylation of nucleic acids and carcinogenesis could be established.

This unsatisfactory development led to a search for other types of reaction with DNA and RNA. The powerful carcinogen, ethylnitrosourea, failed to ethylate tissue RNA but readily produced *O*-alkyldeoxyguanosine from DNA (Loveless, *Nature, Lond.* 1969, **223**, 206). It was found that O-6-alkylation of deoxyguanosine correlated better with mutagenesis than did 7(N)-alkylation. Thus both methyl and ethyl methanesulphonates alkylate guanine at the 7(N)-position but only ethyl methanesulphonate is mutagenic to T-even phages (Brookes & Lawley, *Biochem. J.* 1963, **89**, 138; Loveless, *Proc. R. Soc. B.* 1959, **150**, 497). It seems, therefore, that despite the disappointing results with 7(N)-alkylation of nucleic acids it may yet prove possible to relate carcinogenicity to nucleic-acid reaction by taking into account the extent of O-alkylation (Loveless, 1969, *loc. cit.*).

Yet another field of interaction between nitrosamines and nucleic acids was opened by the studies of Wunderlich *et al.* (*Biochem. J.* 1970, **118**, 99). Using ^{14}C-labelled material, these authors demonstrated that alkylation of mitochondrial DNA by methylnitrosourea was 3–7 times that of nuclear DNA *in vitro*. Furthermore alkylation of embryonic mitochondrial DNA *in vitro* was about twice that of adult liver-mitochondrial DNA. The preferential alkylation of mitochondrial DNA has also been demonstrated *in vivo* and may be attributable to the freedom of mitochondrial DNA from histones, its much higher rate of synthesis compared with nuclear DNA and/or the probable accumulation of the lipophilic methylnitrosourea in the mitochondria and particularly in the membranes, which are rich in lipid. The significance of this reaction in the carcinogenic process is not clear. Alkylating agents induce mutations in phages and monocellular organisms and may perhaps induce mitochondrial mutations. If mitochondrial mutations do occur, the effect of the mutants on the behaviour of the cell becomes an important consideration. It is known that

mutated mitochondria in Neurospora can determine the phenotype of a cell, and if there is any possibility that mitochondrial mutations might have a similar influence on mammalian cells, the interaction of alkylating agents with mitochondrial DNA needs to be taken into account in the carcinogenic process.

ous feed grain and wheat crops under the warehouse stored reseal loan program for the 1973–74 storage period, see F.R. Doc. 72-18879, Agricultural Stabilization and Conservation Service, *supra*.

[7 CFR Part 1464]

FIRE-CURED, DARK AIR-CURED, AND VIRGINIA SUN-CURED TOBACCO

Notice of Advance Grade Rates for Price Support on 1972 Crop

Correction

In F.R. Doc. 72-17535 appearing at page 21956 of the issue for Tuesday, October 17, 1972, in § 1464.17 *1972 crop—Virginia Fire-cured tobacco, Type 21—advance schedule*, the first figure in the left-hand column, now reading "A2F," should read "A1F."

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE

Food and Drug Administration

[21 CFR Part 121]

FOOD ADDITIVES

Proposed Revocation of Use of Morpholine

An order published in the FEDERAL REGISTER of June 30, 1962 (27 F.R. 6232), provided for morpholine to be added to boiler water in the preparation of steam that will contact food. Subsequent orders were published (1) in the FEDERAL REGISTER of July 20, 1962 (28 F.R. 6878), to provide for the use of morpholine and its salts of fatty acids derived from animal or vegetable oils, as corrosion inhibitors for steel or tinplate intended for food-contact use and (2) in the FEDERAL REGISTER of November 30, 1962 (28 F.R. 11799), to provide for the use of morpholine as the salt(s) of one or more of the fatty acids meeting the requirements of § 121.1070, as a component of protective coatings applied to fruits and vegetables.

Since the establishment of the regulations cited above, questions have been raised about the safety of morpholine. It has been shown that under acid conditions, it is possible for this substance at significant levels to combine with nitrite, forming nitrosomorpholine that may be a health hazard. In view of this information, the above listed uses of morpholine and its salts, can no longer be regarded as having been shown to be safe as required by section 409 of the Federal Food, Drug, and Cosmetic Act.

Therefore, pursuant to provisions of the Federal Food, Drug, and Cosmetic Act (secs. 409, 701(a), 72 Stat. 1785–1788, as amended, 52 Stat. 1055; 21 U.S.C. 348, 371(a)) and under authority delegated to him (21 CFR 2.120), the Commissioner of Food and Drugs proposes to amend Part 121, as follows:

1. In § 121.1088 *Boiler water additives* by deleting the item "morpholine" from the substances listed in paragraph (d).
2. By revoking § 121.1105 *Morpholine*.
3. In § 121.2551 *Corrosion inhibitors used for steel or tinplate* by deleting the item, "morpholine and its salts of fatty acids derived from animal or vegetable oils" from the "List of substances" in paragraph (b)(1).

Interested persons may, within 60 days after publication hereof in the FEDERAL REGISTER, file with the hearing Clerk, Department of Health, Education, and Welfare, Room 6-88, 5600 Fishers Lane, Rockville, Md. 20852, written comments (preferably in quintuplicate) regarding this proposal. Comments may be accompanied by a memorandum or brief in support thereof. Received comments may be seen in the above office during working hours, Monday through Friday.

Dated: November 1, 1972.

SAM D. FINE,
Associate Commissioner for Compliance.

[FR Doc.72-18906 Filed 11-2-72;8:53 am]

[21 CFR Part 121]

FOOD ADDITIVES

Use of Sodium Nitrite, Sodium Nitrate, Potassium Nitrite, and Potassium Nitrate

Recent information indicates that under some conditions nitrites may react with secondary and tertiary amines, which occur naturally in many foods, to form nitrosamines in food and perhaps that nitrites may also react with such amines in man's gastrointestinal tract to form nitrosamines. Certain nitrosamines have been found to be carcinogenic in test animals, and are, therefore, potentially hazardous to human health.

Nitrite is normally present in human saliva. Nitrates occur naturally in many foods, including vegetables, meat, poultry, fish, and water and may be reduced to nitrite by harmless microorganisms present in such food and the human intestinal tract.

Nitrites and nitrates have long been used in the curing of meat and poultry, such use predating the Food Additives Amendment of 1958. Nitrites prevent red meat from turning brown and are responsible for the familiar red color of cured meats. The unique organoleptic qualities of cured meats are often the result of the use of nitrites. At the present time the use of nitrites and nitrates (insofar as nitrates are converted to nitrites) is essential in the production of certain staple foods in maintaining the characteristics of color, taste, and texture which the consumer through long exposure has come to associate with and demand of such foods. For example, without nitrite bacon is salt pork, frankfurters are bratwurst and ham is salty roast pork.

The appreciation of the critical importance of nitrites in retarding growth and toxin development of *Clostridium botulinum* has grown over the last 40 years. *C. botulinum* is a microorganism that is ordinarily quite harmless and which is naturally present in the soil, in the sea, and in the food supply. *C. botulinum* becomes a source of concern only when it grows in food because it then produces a toxin which, when ingested, causes botulism, an acute food poisoning marked by a high mortality rate. Nitrites are essential to inhibit the growth of *C. botulinum* in canned ham, bacon, and in some processed meat, poultry, and fish products.

There have been no outbreaks of botulism attributable to commercially prepared foods which have been treated with nitrites or nitrates. On the other hand, there have been a number of fatalities due to botulism associated with consumption of commercially prepared foods prior to use of nitrite as a preservative. There is need, therefore, to consider with extreme care any changes in regulations governing the use of these substances. From a public health standpoint, the choice to be made is between the risk of the possibility of a chronic illness (cancer) and a very real and more immediate hazard (botulism).

Regulations promulgated by the Food and Drug Administration permit the use of nitrites and nitrates in certain foods as follows:

An order published in the FEDERAL REGISTER of September 23, 1961 (26 F.R. 8972), permitted sodium nitrite to be used as a preservative and color fixative in canned pet food containing meat or fish (21 CFR 121.223).

An order published in the FEDERAL REGISTER of September 23, 1961 (26 F.R. 8973), permitted sodium nitrate and sodium nitrite, respectively, to be used as preservatives and color fixatives in smoked cured salmon. The regulations were amended by orders published in the FEDERAL REGISTER of (1) March 3, 1962 (27 F.R. 2090), to permit the additives to be used in meat-curing preparations for the home curing of meat and meat products, (2) July 31, 1963 (28 F.R. 7776), to permit the use of the additives in smoked cured shad, and (3) November 5, 1964 (29 F.R. 14985), to permit the use of the additives in smoked cured sablefish. The order published in the FEDERAL REGISTER of September 23, 1961 (26 F.R. 8973), also permitted sodium nitrite to be used as a color fixative in smoked cured tuna fish products (21 CFR 121.1063, 121.1064).

An order published in the FEDERAL REGISTER of July 26, 1963 (28 F.R. 7594), permitted potassium nitrate to be used as a curing agent in the processing of cod roe (21 CFR 121.1132).

An order published in the FEDERAL REGISTER of August 26, 1969 (34 F.R. 13659), permitted sodium nitrite to be used in combination with salt (NaCl) to aid in inhibiting the outgrowth and toxin formation from *Clostridium botulinum* type E in the processing of smoked chub in accordance with prescribed conditions of manufacturing and storage of the finished product. The regulation was

amended by an order published in the FEDERAL REGISTER of March 25, 1970 (35 F.R. 5034), to revise the cooling parameters after cooking (21 CFR 121.1230).

Nitrite and nitrate also have prior sanction for use as curing agents in meat and meat and poultry products. These uses were first sanctioned under the Meat Inspection Act of 1907 and the Federal Food and Drug Act of 1906 and are currently recognized under regulations of the U.S. Department of Agriculture (9 CFR 318.7, 381.147).

The Commissioner of Food and Drugs concludes that, in view of the possible health hazard of nitrosamines, the use of nitrites and nitrates in food should be limited to only those uses which are essential in inhibiting the growth of *C. botulinum* and for obtaining the essential characteristics of cured meats at this time. Thus he proposes to amend the Food Additive Regulations to delete the following non-essential uses:

1. Sodium nitrite in canned pet food containing meat or fish. These products are fully retorted and require no further preservation.

2. Sodium nitrate in smoked cured sablefish, salmon, and shad. Sodium nitrite (together with the use of salt, cooking, and refrigeration) is sufficient to control *C. botulinum*, and there is no need for the sodium nitrate as well. Although the available information indicates that there is no need for the use of sodium nitrate in smoked cured salmon, Nova Scotia salmon which is lightly salted (NaCl) may be a possible exception and the Food and Drug Administration will give careful consideration to any data or other information regarding such use that interested persons may wish to submit to the Hearing Clerk.

3. Sodium nitrite in smoked tuna fish products. The only function of the additive is to fix the color of the fish.

4. Potassium nitrate in cod roe. The additive is used as a curing agent. There are no data indicating its usefulness in inhibiting *C. botulinum*. It appears that this inhibition is accomplished by the use of a high percentage of sodium chloride in the cure.

In the FEDERAL REGISTER of August 12, 1972 (37 F.R. 1640), the Commissioner proposed to expand Subpart E under Part 121, within which would be established regulations governing all prior sanctioned direct and indirect food ingredients known to the Commissioner. As set forth below, the Commissioner proposes that two new sections, § 121.2007 *Sodium or potassium nitrite* and § 121.2008 *Sodium or potassium nitrate* be added to Subpart E of Part 121 as proposed at 37 F.R. 1640. The limitations proposed are the prior sanctions known to the Commissioner. Documented evidence of additional or different prior sanctions for these ingredients may be submitted in comments.

Therefore, pursuant to provisions of the Federal Food, Drug, and Cosmetic Act (sec. 409, 701, 72 Stat. 1785–1788, as amended, 52 Stat. 1055; 21 U.S.C. 348, 371) and under authority delegated to him (21 CFR 2.120), the Commissioner proposes to amend Part 121 as follows:

§ 121.223 [Revoked]

1. By revoking § 121.223 *Sodium nitrite.*

§ 121.1063 [Amended]

2. Paragraph (a) of § 121.1063 *Sodium nitrate* is amended by deleting subparagraph (1), relating to the use of sodium nitrate in smoked cured sablefish, salmon, and shad, and by deleting the words "and color fixative" from subparagraph (2).

§ 121.1064 [Amended]

3. Paragraph (a) of § 121.1064 *Sodium nitrite* is amended by deleting subparagraph (1), relating to the use of sodium nitrite in smoked tuna fish products, and by deleting the references that relate to the use of sodium nitrate in smoked cured sablefish, salmon, and shad in subparagraph (2), and by deleting the words "and color fixative" from subparagraphs (2) and (3).

§ 121.1132 [Revoked]

4. By revoking § 121.1132 *Potassium nitrate.*

5. By adding §§ 121.2007 and 121.2008 to Subpart E as follows:

§ 121.2007 Sodium or potassium nitrite.

Sodium and potassium nitrite are subject to prior sanctions for use as curing agents, with or without sodium or potassium nitrate, in meat and meat products and in poultry products in the following amounts: 2 pounds to 100 gallons of pickle at 10 percent pump level; 1 ounce to 100 pounds of meat (dry cure) or poultry product (dry cure); ¼ ounce to 100 pounds of chopped meat and/or chopped meat by product or chopped poultry meat. Sodium and potassium nitrite used in food shall comply with the specifications contained in the Food Chemicals Codex. The use of nitrites, nitrates, or any combination thereof shall not result in more than 200 parts per million of nitrite in the finished product.

§ 121.2008 Sodium or potassium nitrate.

Sodium and potassium nitrate are subject to prior sanctions for use as sources of nitrite in the production of cured meat and meat products and cured poultry products in the following amounts: 7 pounds to 100 gallons of pickle; 3½ ounces to 100 pounds of meat (dry cure) or poultry product (dry cure); 2¾ ounces to 100 pounds of chopped meat or chopped poultry meat. Sodium and potassium nitrate used in food shall comply with the specifications contained in the Food Chemicals Codex. The use of nitrites, or nitrates, or any combination thereof shall not result in more than 200 parts per million of nitrite in the finished product.

Interested persons may, within 60 days after publication hereof in the FEDERAL REGISTER, file with the Hearing Clerk, Department of Health, Education, and Welfare, Room 6-88, 5600 Fishers Lane, Rockville, MD 20852, written comments (preferably in quintuplicate) regarding this proposal. Comments may be accompanied by a memorandum or brief in support thereof. Received comments may be seen in the above office during working hours, Monday through Friday.

Dated: November 1, 1972.

SAM D. FINE,
Associate Commissioner for Compliance.

[FR Doc.72-18905 Filed 11-2-72;8:53 am]

DEPARTMENT OF TRANSPORTATION

Coast Guard

[46 CFR Part 12]

[CGD 72-204P]

CERTIFICATION OF SEAMEN

Proposed Firefighter Endorsement

The Coast Guard is considering amending its rules for the certification of seaman to add a "firefighter" endorsement.

Interested persons are invited to submit written comments, suggestions, or objections regarding the proposed rule to the U.S. Coast Guard (GCMC), 400 Seventh Street SW., Washington, DC 20590. The comments should refer to this notice number CGD 72-204P and should give the reasons upon which any recommended changes are based, any specific wording recommended, and the name, address, and organization, if any, of the commenter.

All written comments received on or before December 8, 1972, will be considered before final action is taken on this proposal. The proposed rule may be changed in the light of comments received.

Copies of all written comments received will be available for examination in Room 8234, Department of Transportation, Nassif Building, 400 Seventh Street SW., Washington, DC both before and after the closing date for receipt of written comments.

The endorsement as "firefighter" is being proposed to recognize those seamen who have attended a formal training course and to identify those seamen who have had special training in firefighting techniques. This proposal conforms to the Intergovernmental Maritime Consultative Organization resolution (A. 124(V)) dated October 25, 1967, which recommends that each member State should aim at training all its seafarers in fire prevention and firefighting to an extent appropriate to their function on board ship.

Following a disaster in the Adriatic Sea, the Greek Government has announced that after April 15, 1972, no Greek seaman will be allowed to embark in any ship flying the Greek flag unless he has taken a special course in firefighting. Although at the present time the United States does not intend to require

This will be the first plant anywhere using large oxygen-blast generators. The gas made in such a generator will contain more hydrogen and tar compounds but no nitrogen. Oxidation of CO will be carried out by the conventional Haber-Bosch process, as in Berezniki. The scheme of purification of the gas is unique in that, after removal of sulfur compounds from the gas by activated charcoal and washing CO_2 out with large volumes of [illegible] (as in Berezniki), the gas is sent to fractionating columns similar to the Linde-Bronn columns used for coke-oven gases. Here, by cooling the gas to very low temperatures by boiling ammonia and liquid nitrogen, fractions of acetylene, ethane, methane, and finally CO are condensed from it. For more complete removal of CO, the gas is washed in the fractionating column by liquid nitrogen, thus reducing its CO content to about 0.5 per cent. The nitrogen will be separated from air in another plant and mixed with hydrogen just before synthesis. The oxygen by-product from the nitrogen plant will be used in the blast of gas generators.

The contact apparatus here will be similar to that in Berezniki, but to prevent quick poisoning of the catalyst in all four columns when operating on comparatively impure gas, one contact mass is made to serve as a precatalyst, following practice in plants using Claude's process. All gas passes first through a precatalyst column and is then circulated through three other catalysts where the reaction is completed.

The next plant will be built in Chirchiki, near Tashkent, with a capacity of about 150,000 tons of ammonia per year, to supply fertilizers to the cotton fields in this section of the country. Electric current, supplied by a 200,000-horsepower hydroelectric plant built on the rapids of river Chirchiki, will be used for the electrolysis of water to supply hydrogen, and nitrogen will be made either by burning hydrogen or by the liquefaction of air. The synthesis will be similar to that of other Soviet plants and the Haber-Bosch process will be used.

Two other nitrogen plants will be built in the Magnitogorsk and Kuznetz metallurgical plants. Here the source of hydrogen will be coke-oven gas. The Nitrogen Research Institute in Moscow is working on methods for the complete and economical conversion of methane in coke-oven gas into hydrogen. If no satisfactory method is found in the near future, two of the above-mentioned plants will use the liquefaction process for extraction of hydrogen which will resemble the existing installation in Gorlovka.

Mexico

G. G. Colin, 3a. Guerrero 74-Mexico, D. F.

Public Works Planned as Aid to Unemployment

In order to relieve conditions among the unemployed, approximately $7,000,000 (pesos) are to be spent in improvements in Mexico City. The work will include construction of efficient drainage systems, homes for workers, street and road improvements, and insuring a good potable water supply. A large number of men will be employed. Many Mexican laborers who have left the United States in the past two years will be given preference.

New Oil Reserve Law Gives State Wide Power

In accordance with the provisions of the new oil reserve law, the present limits of national oil reserve zones will be extended 50 kilometers. With regard to rights acquired before the law came into effect, the new extensive zone falls within the jurisdiction of the state for the national oil reserve.

At present there are confirmatory concessions covering an area of 6,226,063 hectares and ordinary concessions covering 2,490,902 hectares; 4,194,110 hectares pending concession and other areas make a total of over 20,000,000 hectares, which far exceed the actual demand. According to the present law, confirmatory concessions are not subject to immediate compulsory exploitation, and may be considered as reserves out of state control.

The new law gives the state economic control over this source of public wealth; insures the nation's supply in emergencies; and permits price regulation of oil and its derivatives in the Mexican market. This law does not affect acquired interests, but only free land.

Office of Exterior Commerce Is Created

Mexico cannot escape the universal movement to defend her exterior interests by giving careful attention to her exterior commerce. To solve these problems the Division of Exterior Commerce was created in January, 1933, in the Department of National Economy. This division is studying the production and exploitation of various Mexican articles that may be or are being exported to foreign countries, and compiling statistics on quantities, prices, terms, transportation, freights, export duties, etc. A directory of foreign buyers is being prepared for the information of exporters. The problem of credit has also been given special attention. From the comparison of statistics on imports and exports, the division will suggest the propriety of establishing adequate commercial treaties with other countries.

Its publicity campaign includes a very efficient information service to exporters and importers, as well as information concerning the demand for Mexican products as transmitted by our consuls and commercial attachées in foreign countries.

June 6, 1933

72

Fatal Poisoning with Sodium Nitrite

H. B. Arbuckle and O. J. Thies, Jr., Davidson College, Davidson, N. C.

Sodium Nitrite is handled by many textile supply firms, and is distributed without poison labels to cotton mills and dye plants. We have in our laboratory stock room containers from three different manufacturing chemists bearing sodium nitrite labels, but without a poison mark of any kind.

Search of the chemical literature reveals no reported case of fatal poisoning with sodium nitrite in the United States. Two deaths in Bavaria, and four in Algeria, were attributed to sodium nitrite.

Sodium nitrite is not included in the long list of poisonous substances that the North Carolina Code specifies must be sold under poison label. Since this substance is used in large quantities in the dye rooms of many mills, we consider it wise to place on record a striking case of fatal poisoning that has occurred in North Carolina.

On October 28, 1932, we were called to investigate the death of the two-year-old son of L. D. Ray, a worker in a cotton mill in Huntersville, Mecklenburg County, N. C.

When we arrived on the scene, the child was dead. His brother directed us to the junk pile near the mill, and stated that the child picked up a small quantity of a white substance, which we saw on the junk pile, put it in his mouth, and at once ran crying toward his home, which was about 300 feet distant. Before he reached there, he fell to the ground vomiting. Members of his family hurried to him, and found him pale and weak. On picking him up his limbs hung limp, he was desperately weak, and still vomiting. He was carried directly to his home and the mill physician summoned at once. So rapid was the action of the poison that the child died before the physician arrived. His mother stated that not more than 15 minutes had elapsed since she had picked him up.

With the aid of the physician, a portion of the stomach contents was removed. The white substance obtained from the junk pile on analysis in the laboratory proved to be sodium nitrite. Examination of the stomach contents showed that they were alkaline and contained a considerable quantity of sodium nitrite. The solid particles of the stomach contents showed a distinct chocolate brown color. Stomach contents are normally acid, but the hydrolysis of a substance like sodium nitrite would cause the alkalinity. This confirms the detection of a large amount of sodium nitrite in the stomach.

To verify our opinion that sodium nitrite is a violent poison, and was the cause of this child's death, we administered 65 milligrams (1 grain) in a capsule to a cat weighing 2.6 pounds. In 5 minutes the cat began violent vomiting. Four minutes later it lost control of muscles, sprawled on the floor, and began screaming. The screaming slowly died down and in 6 minutes more, 15 minutes after the dose was given, the cat was dead. This certainly shows the extremely poisonous nature of sodium nitrite.

Chemical literature records the two following cases of fatal nitrite poisoning:

H. Molitoris reports two cases of fatal sodium nitrite poisoning occurring within a few months in the same factory, one on October 4, 1910, and one on April 13, 1911. These were workmen in a factory where sodium nitrite was manufactured in Innsbruck, Austria.

L. Musso in 1925 reports four cases of death from sodium nitrite poisoning in a drug store in Algeria, by taking sodium nitrite from a bottle labeled "Sodium Tartrate."

The facts presented in this paper show that the use of sodium nitrite as a meat-curing agent might be attended with great danger.

Bibliography

Czaplewski, *Vierteljahrsschr. gericht. Med.*, [3] 43, 356.

Hesse, Erich, *Arch. exptl. Path. Pharmakol.*, 126, 209–21 (1927).

Heubner, W., and Meier, Rolf, *Nachr. Ges. Wiss. Göttingen. Math. physik. Klasse*, 1925, 73–7.

LaFranca, S., *Arch. fisiol.*, 8, 14–16; *Zentr. Biochem. Biophys.*, 12, 524.

Mladoveanu, M. C., *Compt. rend. soc. biol.*, 99, 606–10 (1928).

Molitoris, H., *Vierteljahrsschr. gericht. Med.*, [3] 43, 2nd Suppl., 289–97 (1912).

Musso, L., *J. pharm. chim.*, [8] 4, 345–60 (1926).

Walsh, T. C., *Food Manuf.*, 7, 49–51 (1932).

ABSTRACTS OF PAPERS

162nd National Meeting

American Chemical Society

Washington, D.C. September 12 - 17, 1971

Creative Printing, Inc.
Hyattsville, Md.

GC-mass spectrometry. Identifications were based on I_R values and mass spectra. A total of 29 sulfur chemicals were identified in the HVP-L-cysteine·HCL-D-xylose system, and 21 in the cysteine·HCL-D-xylose system of which 15 were not present in the former model system. The synthesis of 12 sulfur chemicals identified in the model systems will be described.

39. WATER-SOLUBLE FLAVOR PRECURSORS OF BEEF. PEPTIDE-BOUND NUCLEOTIDES IN BEEF MUSCLE. A. F. Mabrouk, Food Chemistry Division, Food Laboratory, US Army Natick Laboratories, Natick, Mass. 01760.

A method which is of general application to animal and plant tissues is described for preparing purified peptide-bound nucleotides. The isolation of individual peptidyl nucleotides from 50% ethanolic extracts of beef has been accomplished using ion exchange chromatography. These compounds have been characterized with respect to mononucleotides composition as well as amino acids content. Structural studies of individual compounds revealed that they are nucleotide-5-phosphoanhydride peptide. The possible relation of beef peptidyl-nucleotides to beef flavor will be discussed.

634

40. THE FATE OF NITRITE IN MEAT. J. B. Fox, Jr., R. A. Nicholas and W. L. Sulzbacher, Eastern Marketing and Nutrition Research Division, Agricultural Research Service, U. S. Department of Agriculture, 600 East Mermaid Lane, Philadelphia, Pennsylvania 19118.

The loss of nitrite during processing of meat products is greater than can be accounted for by reaction with meat pigments and reductants alone. Nitrite disappearance in meat slurries and extracts, and in model systems was followed by the AOAC Greiss reagent analysis and by the availability of nitrite for nitric oxide myoglobin formation dithionite reduction. The rate and amount of nitrite loss was increased by the presence of oxygen and/or added reductants. Although a reduction-oxidation cycle is thus implied, reaction products of such a cycle could not be found in any significant amounts. A spectrally distinct compound was formed from ascorbate and nitrite, concomitant with loss of nitrite accounted for by the formation of nitric oxide myoglobin. The formation of reaction products with ascorbate and other reductants, with or without air, is concluded to be the principal pathway for nitrite loss during meat processing.

WEDNESDAY AFTERNOON - SECTION A - GENERAL - S. Kazeniac, Presiding

41. REACTION OF NITRITE WITH CREATINE AND CREATININE. Michael C. Archer, Stephen D. Clark, Joan E. Thilly, and Steven R. Tannenbaum, Dept. of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Mass. 02139.

73

Treatment of creatine in acid solution with excess sodium nitrite yielded N-nitrososarcosine (N-nitroso-N-methylglycine). This material, obtained as a yellow oil and purified by elution from a silica gel column with diethyl ether, was identical to authentic N-nitrososarcosine. A possible mechanism for this reaction involves deamination of creatine to yield N-carbamyl-N-methylglycine as the first step. This can then react with more nitrous acid to yield N-carboxyl-N-methylglycine which decarboxylates to give N-methylglycine (sarcosine). A final nitrosation step yields N-nitrososarcosine Nitrosation of creatinine has been examined previously by Schmidt (Arch. Pharm., 250, 330, 1912), but the products have never been characterized unambiguously. Treatment of creatinine in acid solution with excess sodium nitrite at room temperature gave a peach coloured precipitate. Recrystallization from water yielded a white solid which gave a negative Liebermann test for a nitroso group, and the compound was identified as creatinine-5-oxime. If the same reaction was carried out at 0°C, however, a pink precipitate was formed which on recrystallization from water gave white crystals. The compound gave a negative Liebermann test and was identified as 1-methylhydantoin-5-oxime. The significance of these reactions will be considered with respect to the environmental problem of carcinogenic N-nitroso compounds.

42. CHROMIUM IN SELECTED FOODS. Edward W. Toepfer, Walter Mertz, Edward E. Roginski, Marilyn M. Plansky, Human Nutrition Research Division, Agricultural Research Center, Beltsville, Maryland 20705.

The relative biological activity for chromium glucose tolerance factor, GTF, was determined in a number of common food substances, and in extracts of these materials. Activities of acid hydrolysates were compared to activities of alcohol extracts.

Chromium was chemical
analytical procedure
color stability. The
recoveries, and confi
relative bioassay acti
available or useful ch

43. INTERACTIONS OF
L.B. Chen and
chusetts Institute

Reactions betwe
no acids and prote
lysine in smoked f
into phenolic, neu
fractions caused a
ground pork prepar
identified in wood
casein, a variety
significant loss i
nolic and carbonyl
compared. Interac
teins may play an
nutritional and fu

44. CHANGES IN BOV
D. Harrison, D
Kansas State Unive

The effects
fresh bovine semit
conditions were st
filters was used t
of light in air, c
Spectral reflecta
ascertain changes
nitrogen atmosphe
metmyoglobin), whe
greatest damage.
muscle color than
acrylamide gel el
disruption of the
of myoglobin, oxy

45. PRESSURE FREE
IN DRYING TISS
General Foods Cor

Freeze drying has
duce shrivelling
tion. We have fo
pressure prior to
rehydration of ma
dried products ca
of foods. Only c
but certain other
Carbon dioxide, r
made to correlate
this process. Th
dried product obt
unshrivelled stru
The gas which has
into the tissues
meanwhile preven
changes could be
often yields a m
more rapidly than

also possible with the use of nystatin counterselection and by substituting a nonfermentable carbon source (glycerol) for glucose, along with a few modifications as follows. (i) Cells were grown for 48 hours on yeast extract and peptone (YEP) plus 5 percent glucose before EMS treatment in order to reduce the number of mitochondria per cell. These glucose-repressed conditions result in cytological changes similar to those seen with anaerobic growth, namely, a reduction from 15 or 20 mitochondria in a respiring cell to two or three mitochondria per repressed cell. (ii) The cells were returned to YEP plus 5 percent glucose after treatment with ethylmethanesulfonate to enhance the segregation of a single mitochondrial population of a sensitive mutant. (iii) After nitrogen starvation, the cells were grown on petite medium plus 1 mg of neomycin per milliliter before addition of nystatin. (iv) Finally the cells were grown on petite medium for 24 hours after the nystatin was washed away. One of the ten mutants isolated is unable to grow on petite medium containing 800 μg of neomycin per milliliter, but grows well on petite medium without neomycin and on YEPD plus 3 mg of neomycin per milliliter. This indicates that some mitochondrial function is impaired by the addition of neomycin. We have been unable to analyze this mutant genetically because the ascospores do not germinate.

Strain AA-89, which was selected for streptomycin sensitivity by suppression with low concentrations of streptomycin and which is also cold-sensitive (allelic with streptomycin sensitivity), has an altered ribosomal profile (Fig. 1) when grown at the nonpermissive temperature (15°C); thus it shows the cold-sensitive phenotype defective in subunit assembly similar to those reported for bacteria (*17*). The cytoplasmic ribosomes isolated from this mutant when grown at the permissive temperature (30°C) are sensitive to neomycin and streptomycin in an in vitro protein-synthesizing system (*13*). Our results indicate that AA-89 is a ribosomal mutant in the sense of Apirion (*18*), and we conclude that the streptomycin and neomycin sensitivity of the strain results from this alteration in ribosome structure.

FRANK T. BAYLISS*
ROBERT T. VINOPAL†
Department of Bacteriology, University of California, Davis 95616

References and Notes

1. M. Nomura, *Bacteriol. Rev.* 34, 228 (1970); B. Weisblum and J. Davies, *ibid.* 32, 493 (1968); D. Schlessinger and D. Apirion, *Annu. Rev. Microbiol.* 23, 387 (1969).
2. J. F. Speyer, P. Lengyel, C. Basilio, *Proc. Nat. Acad. Sci. U.S.* 48, 648 (1962).
3. E. C. Cox, J. R. White, J. G. Flaks, *ibid.* 51, 703 (1964); J. E. Davies, *ibid.*, p. 659; F. N. Chang and B. Weisblum, *Biochemistry* 8, 836 (1966).
4. L. Breckenridge and L. Gorini, *Genetics* 65, 9 (1970); L. Gorini, *Cold Spring Harbor Symp. Quant. Biol.* 34, 101 (1969).
5. L. Gorini, J. L. Jacoby, L. Breckenridge, *Cold Spring Harbor Symp. Quant. Biol.* 31, 657 (1966).
6. H. J. Whitfield, Jr., R. G. Martin, B. N. Ames, *J. Mol. Biol.* 21, 335 (1966).
7. L. Gorini and E. Kataja, *Biochem. Biophys. Res. Commun.* 18, 656 (1965).
8. H. Morimoto and H. O. Halvorson, *Proc. Nat. Acad. Sci. U.S.* 68, 324 (1971); A. J. Lamb, G. D. Clark-Walker, A. W. Linnane, *Biochim. Biophys. Acta* 161, 415 (1968).
9. D. Y. Thomas and D. Wilkie, *Genet. Res.* 11, 33 (1968).
10. D. Cooper, D. V. Banthorpe, D. Wilkie, *J. Mol. Biol.* 26, 347 (1967).
11. Streptomycin sulfate and neomycin sulfate were used.
12. G. D. Clark-Walker and A. W. Linnane, *J. Cell Biol.* 34, 1 (1967).
13. F. T. Bayliss and J. L. Ingraham, in preparation.
14. L. A. Shevchenko and I. I. Filippovich, *Dokl. Akad. Nauk SSSR* 161, 1461 (1965); L. A. Shevchenko and A. I. Novichkova, *Mikrobiologiya* 34, 656 (1965); L. A. Shevchenko, V. I. Biryuzova, M. N. Meisel, *ibid.* 35, 85 (1966).
15. S. R. Snow, *Nature* 211, 206 (1966).
16. J. Lederberg, *J. Bacteriol.* 61, 549 (1951); P. F. Sparling, J. Modolell, Y. Takeda, B. D. Davis, *J. Mol. Biol.* 37, 407 (1968).
17. P. C. Tai, D. P. Kessler, J. L. Ingraham, *J. Bacteriol.* 97, 1298 (1969); C. Guthrie, H. Nashimoto, M. Nomura, *Proc. Nat. Acad. Sci. U.S.* 63, 384 (1969).
18. D. Apirion, *J. Mol. Biol.* 30, 255 (1967).
19. N. R. Eaton, *J. Bacteriol.* 83, 1359 (1962).
20. G. A. Bray, *Anal. Biochem.* 1, 279 (1960).
21. We thank Dr. Richard Snow for his advice on genetic experiments and the use of his micromanipulator. We also thank John Ingraham, in whose laboratory this work was done, for providing encouragement, useful discussion, and a critical review of the manuscript. This work was supported by PHS grant AI 05526 and NIH predoctoral traineeships under grant GM 1041.
* Present address: Department of Biology, Edinboro State College, Edinboro, Pa. 16412.
† Present address: Department of Bacteriology and Immunology, Harvard Medical School, Boston, Mass. 02115.

28 July 1971

74

Environmental Nitroso Compounds: Reaction of Nitrite with Creatine and Creatinine

Abstract. *Creatine reacts with nitrite under acid conditions to produce first sarcosine and then N-nitrososarcosine, which is a weak carcinogen in the rat. Creatinine reacts with acidified nitrite to produce either creatinine-5-oxime or 1-methylhydantoin-5-oxime, depending on reaction conditions. The toxicity and environmental significance of these compounds is not yet known.*

The carcinogenicity of large numbers of nitrosamines has been demonstrated in various tissues of many animal species (*1*). It is possible that nitrosamines will form in the environment whenever nitrite and secondary or tertiary amines occur together. Certain foodstuffs have already been shown to contain simple alkyl nitrosamines (*2*), and reaction of nitrite and various secondary amines in human gastric juice at *p*H 1.5 in vitro also yielded nitrosamines (*3*). These compounds might represent a health hazard (*4*), and their possible relevance to the etiology of human cancer has been reviewed (*1, 5, 6*).

Nitrates and nitrites are frequently used as preservatives and for color fixation in foods. Extensive use of nitrates as fertilizers can lead to high accumulated levels in water supplies and plant tissue. Microorganisms, particularly the coliform group or *Clostridia*, reduce nitrate to nitrite; thus nitrite can accumulate during the storage of fodder crops and during the storage and processing of vegetables and food to which nitrate has been added.

Creatine, present in muscular tissue of many vertebrates, is a normal constituent of meat. A large portion of creatine in muscle is combined with phosphoric acid as phosphocreatine, but this compound is readily hydrolyzed to creatine and creatinine in acid solution (*7*). Creatinine, the end product of creatine metabolism, is found together with creatine in muscle tissues, milk, and blood. Other significant sources of creatinine include grain seeds and other vegetable matter, certain fish, and crab meat extract (*8*).

In view of the abundance of nitrite, creatine, and creatinine in our environment, and because these compounds are frequently ingested simultaneously in a normal diet, we thought that it was important to investigate their mutual reactivity.

We first demonstrated the formation of *N*-nitrososarcosine by reaction of creatine with nitrite: *N*-nitrososarcosine has been shown to induce cancer of the esophagus in the rat (*6*). To a stirred solution of 5.2 g of creatine (Nutritional Biochemicals) dissolved in 15 ml

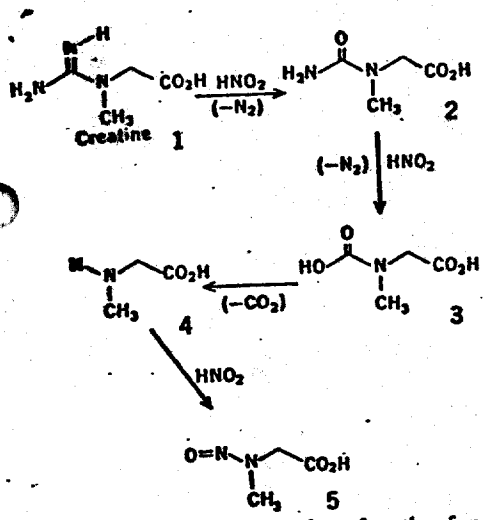


Fig. 1. Proposed mechanism for the formation of *N*-nitrososarcosine (5) by reaction of creatine and nitrite.

of 25 percent HCl, 5.3 g of $NaNO_2$ in 20 ml of distilled water was added dropwise. After standing at 25°C for 2½ hours, the reaction mixture was saturated with NaCl and extracted four times with 10-ml portions of diethyl ether. The extracts were combined and then dried over anhydrous magnesium sulfate. Removal of the solvent under reduced pressure yielded 1.1 g of a yellow oil. The oil was purified by elution from a silica gel column with diethyl ether, and yielded 1.07 g of *N*-nitrososarcosine that was characterized by its identity to authentic material (*9*) synthesized directly from sarcosine.

A possible mechanism for this reaction (Fig. 1) involves reaction of creatine (1) with 1 mole of nitrous acid to yield *N*-carbamyl-*N*-methylglycine (2) as the first step. This product can then react with more nitrous acid to yield *N*-carboxyl-*N*-methylglycine (3), which decarboxylates to give *N*-methylglycine (sarcosine, 4). A final nitrosation yields *N*-nitrososarcosine (5).

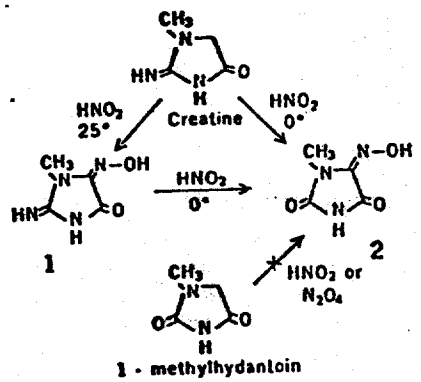


Fig. 2. Formation of creatinine-1-oxime (1) and 1-methylhydantoin-5-oxime (2) by reaction of creatinine and nitrite.

Nitrosation of creatinine has been examined by Schmidt (*10*), but the products have never been characterized unambiguously.

To a stirred solution of 6.1 g of creatinine (Nutritional Biochemicals) dissolved in 25 ml of 10 percent HCl we added dropwise, over a period of 10 minutes, 26.3 g of $NaNO_2$ in 30 ml distilled water. The solution became hot and turned green and then orange, and a yellow solid precipitated. Stirring was continued for 1 hour; the solid was removed by filtration and washed with water. Recrystallization of the solid from hot water, after treatment with animal charcoal yielded 2.2 g of a white solid, m.p. 255°C (with decomposition), which gave negative results in a Liebermann test for a nitroso group. The compound was identified by analysis and spectroscopic methods (*11*) as creatinine-5-oxime (1; Fig. 2).

When the reaction described above was carried out at 0°C, the solution turned orange; a pink solid precipitated which, after 1 hour, was collected, washed, and dissolved in hot water. The hot water solution was treated with animal charcoal, filtered, and subsequently yielded 1.75 g of white crystals melting at 181°C. This compound also gave a negative Liebermann test and was identified by analysis and spectroscopic methods (*12*) as 1-methylhydantoin-5-oxime (2; Fig. 2).

Creatinine-5-oxime was readily converted to 1-methylhydantoin-5-oxime. One gram of the crude yellow creatinine-5-oxime was suspended in 10 ml of 10 percent HCl and cooled to 0°C. Over a period of 5 minutes, 4 g of $NaNO_2$ in 10 ml of distilled water was added to the stirred suspension. Stirring was continued for 3 hours at 0°C, and the slurry gradually turned pink. Recrystallization of the pink solid from water after treatment with animal charcoal gave 0.35 g of 1-methylhydantoin-5-oxime.

An attempted synthesis of 1-methylhydantoin-5-oxime from the parent 1-methylhydantoin (Aldrich Chemical Co., Inc., Milwaukee, Wis.) by reaction with excess sodium nitrite in acid solution failed to yield any product at either 0° or 60°C. Direct reaction with nitrogen tetroxide in acetic acid (*13*) also yielded no product.

These reactions are summarized in Fig. 2. Understanding of the mechanisms of the reactions remains obscure, although the imino group in creatinine does seem implicated in its reactivity. The toxicities of creatinine-5-oxime and 1-methylhydantoin-5-oxime are as yet unknown.

We have demonstrated in vitro the formation of a known weak carcinogen for the rat, *N*-nitrososarcosine, and two oximes of undetermined toxicity, from precursors in abundant supply in man's environment. Since the relative proportion of creatine and creatinine in any system will be a function of temperature and *p*H (*7, 14*), the amounts of various end products formed by reaction with nitrite will vary from system to system. It remains to be determined whether these reactions actually take place in foods or in the mammalian stomach, and their significance in the incidence of human cancer must be evaluated.

MICHAEL C. ARCHER
STEPHEN D. CLARK
JOAN E. THILLY
STEVEN R. TANNENBAUM

Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge 02139

References and Notes

1. P. N. Magee and J. M. Barnes, *Advan. Cancer Res.* 10, 163 (1967).
2. Staff writer, *Food Cosmetics Toxicol.* 6, 519 (1968); T. Fazio, J. W. Damico, J. W. Howard, R. H. White, J. O. Watts, *J. Agr. Food Chem.* 19, 250 (1971).
3. J. Sander, *Arch. Hyg. Bakteriol.* 151, 22 (1967).
4. Staff writer, *Lancet*, 1968-I, 1071 (1968).
5. W. Lijinsky and S. S. Epstein, *Nature* 225, 21 (1970).
6. H. Druckrey, R. Preussmann, S. Ivankovic, D. Schmähl, *Z. Krebsforsch.* 69, 103 (1967).
7. C. H. Fiske and Y. Subbarrow, *J. Biol. Chem.* 81, 629 (1929); H. Borsook and J. W. Dubnoff, *ibid.* 168, 493 (1947).
8. Editors, *The Merck Index* (Merck & Co., Rahway, N.J., ed. 8, 1968), p. 291.
9. D. L. Hammick and D. J. Voaden, *J. Chem. Soc.* 1961, 3303 (1961); F. H. C. Stewart, *Aust. J. Chem.* 22, 2451 (1969); W. Lijinsky, L. Keefer, J. Loo, *Tetrahedron* 26, 5123 (1970).
10. E. Schmidt, *Arch. Pharmakol. Exp. Therap.* 250, 330 (1912).
11. Data for creatinine-5-oxime are as follows. Calculated (percent) for $C_4H_6N_4O_2$: C, 33.80; H, 4.23; N, 39.44. Values (percent) found were: C, 33.55; H, 4.35; N, 39.40. The mass spectrum [*m/e* (relative intensity)] showed 142 [parent mass ion, (20)], 126 (13), 125 (29), 112 (10), 72 (33), 69 (20), 57 (17), 56 (31), 55 (44), 54 (16), 53 (20), 44 (38), 43 (88), 42 (100 percent), 41 (47), 40 (15), 39 (15). [The mass spectrum had a rather high peak at 143 (4.5), which was shown, by high resolution mass spectrometry, to be $C_4H_7N_4O_2$ and corresponds to M+H]. The infrared data (cm^{-1}) revealed 3320 (s); 3115 (s); 2500 (m, broad); 1720 (m); 1655 (s); 1585 (s); 1535 (w); 1495 (w); 1420 (m); 1340 (s); 1240 (m); 1210 (s); 1110 (s); 1075 (m); 1015 (s); 985 (m); 885 (m), where s is strong, m is medium, and w is weak. Nuclear magnetic resonance (in D_2O/NaOD) showed τ 6.5 (singlet); (in trifluoroacetic

acid) τ 6.5 (singlet). Ultraviolet absorption in 0.1N HCl showed peaks (λ_{max}) at 231 nm (ϵ = 10,760); and at 284 nm (ϵ = 9,650). In 0.1N NaOH, λ_{max} was 269 nm (ϵ = 10,150) and at 309 nm (ϵ = 12,800).

12. The data for 1-methylhydantoin-5-oxime are as follows. Calculated (percent) for $C_4H_5N_3O_3$: C, 33.57; H, 3.50; N, 29.37. Values (percent) found were C, 33.30; H, 3.54; N, 29.36. The mass spectrum [*m/e* (relative intensity)] indicated 143 [parent mass ion (23)], 127 (11); 126 (50); 83 (15); 72 6(4); 70 (44); 57 (11); 56 (47); 55 (32); 54 (23); 53 (20); 43 (27); 42 (100); 41 (12); 30 (18). The infrared data (cm^{-1}) showed 3260 (s, broad); 1780 (m); 1730 (s); 1655 (s); 1440 (s); 1240 (w); 1130 (m); 1070 (m); 1000 (s) NMR (in D_2O/NaOD) showed τ 6.55 (singlet); (in trifluoroacetic acid) τ 6.9 (singlet). Ultraviolet absorption in 0.1N HCl showed λ_{max} at 225 nm (ϵ = 6300); 283 nm (ϵ = 5750). In 0.1N NaOH, λ_{max} was 259 nm (ϵ = 9000); 311 nm (ϵ = 8850).

13. E. H. White, *J. Amer. Chem. Soc.* 77, 6008 (1955).

14. G. Edgar and H. E. Shirer, *ibid.* 47, 1179 (1925).

15. This work was supported by NIEHS grant 1-PO1-ES00597, and the high-resolution mass spectrometry was supported by NIH research grant RR00317 from the Division of Research Facilities and Resources.

23 August 1971

Isolation and Characterization of Larvicidal Principle of Garlic

Abstract. *The larvicidal principles of garlic,* Allium sativum *L., have been isolated and identified as diallyl disulfide and diallyl trisulfide. Both natural and synthetic samples of these larvicides are fatal at 5 parts per million to* Culex pipiens quinquefasciatus *Say.*

Active search for effective plant agents that will destroy mosquitoes has been prompted by the controversy concerning the general harmfulness of DDT and by the development by insect pests of resistance to various other chemical insecticides. At one time DDT was considered a panacea for eliminating the mosquito problem. Although medicinal and antibacterial properties of garlic (*Allium sativum* L., N.O. Liliaceae) have been extensively studied (*1*), only recently has the larvicidal property of its oil, for at least four species of mosquitoes in *Culex* and *Aedes* genera (*2*), been demonstrated. Greenstock (*3*) has shown that garlic oil could destroy aphids, cabbage-white butterfly caterpillars, and Colorado beetle larvae. We now report the isolation, characterization, and testing of the active principle in garlic responsible for the mosquito control. We used *Culex pipiens quinquefasciatus* Say (the same as *C.p. fatigans* Wiedemann) as test organism.

The medicinal properties of garlic and related plants have prompted study of their chemical composition (*4*). Several *S*-substituted cysteines and cysteine sulfoxides, partly in the free form and partly as γ-glutamyl peptides have been isolated from various species of *Allium*. However, it has been proved that the physiologically active compounds are formed through the enzymatic reactions and spontaneous decomposition of parent compounds. The alkyl sulfides, cysteine sulfoxides, and thiols that have been reported to be present are produced by the degradations of the precursors (*1*).

In our work the crude garlic oil was obtained by steam distillation of homogenized garlic cloves. The oil was purified on a silica gel column and was eluted with solvents of increasing polarity. The fractions obtained from the column were tested for larvicidal activity as described (*2*). The fractions eluting with light petroleum had pronounced larvicidal activity. The active fraction contained sulfur. The infrared spectrum (1640, 990, and 910 cm^{-1}), nuclear magnetic resonance (NMR) spectrum (3.5 δ, 2H; 5.25 δ, 2H; 5.98 δ, 1H), and mass (*m/e*, 41) spectrum show the presence of allyl ($CH_2{=}CH{-}CH_2{-}$) group. Color reactions and absence of lowfield signal in the NMR spectrum indicated the absence a thiol group. Since there are no sulfur-oxygen absorptions (*5*) in the infrared spectrum, sulfur should be present as sulfide linkage only. Gas-liquid chromatography (GLC) (*6*) indicated the presence of several components. Two major components could be separated by preparative GLC and were subjected to mass spectroscopy. The more volatile component was identified as diallyl disulfide (*m/e*, 146) while the other fraction corresponded to diallyl trisulfide (*m/e*, 178). A trace amount of dialyl tetrasulfide (*m/e*, 210) was also indicated. The above conclusions were confirmed by comparison with synthetic preparations. Diallyl disulfide was prepared as was described by Carson and Wong (*7*). A mixture of diallyl disulfide and diallyl trisulfide was obtained by the interaction of sodium polysulfides and allyl bromide. Diallyl trisulfide could be separated from the mixture by preparative GLC (*6*). The presence of diallyl disulfide and diallyl trisulfide in the natural sample was confirmed by the infrared and mass spectra and GLC comparisons with the synthetic samples.

The larvicidal action of the natural samples has been compared to several synthetic samples as shown in Table 1. The relative effectiveness of diallyl disulfide and diallyl trisulfide alone or in mixture even at a concentration of 5 ppm, as against the ineffectiveness of the related compounds diallyl sulfide and dipropyl disulfide and dipropyl trisulfide at 200 ppm is noteworthy. We have also observed that antagonistic properties of diallyl di- and trisulfides against several pests of economic and medical importance such as potato tuber moth, red cotton bug, red palm weevil, houseflies, and mosquitoes. The nontoxic nature of garlic to higher animals has been established on the basis that it has been used for edible pur-

Table 1. Toxicity tests of active fraction of garlic oil and synthetic samples to late third-instar larvae of laboratory-reared *Culex pipiens quinquefasciatus* Say.

Compounds used	Mean percentage of mortality at indicated treatment concentrations (ppm)*							
	1	3	5	7	10	50	100	200
Natural sample†	3	64	100	100	100			
Synthetic mixture of diallyl di- and trisulfides	4	76	100	100	100			
Diallyl disulfide	4	70	100	100	100			
Diallyl trisulfide	0	49	92	100	100			
Diallyl sulfide	0	0	0	0	0	0	0	16
Dipropyl disulfide and dipropyl trisulfide	0	0	0	0	0	0	0	0

* Each mean based on five replications; 50 larvae per replicate; mortality scored after 24 hours.
† The ratio of diallyl di- and trisulfide varies with the variety of garlic used.

76

Comparative Study of the Carcinogenic Activity of Nitrosamines [1, 2]

MARY F. ARGUS [3] *and* CORNELIA HOCH-LIGETI,[4]
Cancer Research Laboratory, Department of Pharmaceutical Chemistry, J. Hillis Miller Health Center, University of Florida, Gainesville, Florida; Laboratory Service, Veterans Administration Center, Martinsburg, West Virginia; and Department of Pathology, George Washington University School of Medicine, Washington, D.C.

SUMMARY

The carcinogenic activity of dimethyl-, diethyl-, and diphenylnitrosamine and dimethylhydrazine was studied in the rat. The high carcinogenic activity of dimethylnitrosamine and diethylnitrosamine was confirmed; the localization and morphology of the tumors differed from those previously described. Diphenylnitrosamine and dimethylhydrazine had no carcinogenic activity. Dimethylnitrosamine produced tumors with a variety of histological characteristics in the liver, lung, and kidney. Primary malignant tumors arose in 1, 2, or all 3 of these organs of the same rat. In the lung, squamous-cell carcinomas were found either as the only pulmonary tumor or in association with bronchiolar or alveolar adenocarcinoma. Diethylnitrosamine induced hepatocellular carcinomas in all rats, with metastases into the lung in many instances. One primary kidney tumor was found.—J. Nat. Cancer Inst. 27: 695–709, 1961.

IN THE course of investigating the causes of liver damage in two industrial workers, Barnes and Magee in 1954 demonstrated that dimethylnitrosamine (DMN) provokes liver injury in rats (*1*). When this substance was fed for many months, tumors were produced in the liver (*2*); later, tumors in the kidney and in the lung were also observed (*3*). It was reported that primary liver and kidney tumors did not occur together in the same rat. A similar impression could be gained from the work of Zak *et al.* (*4*) in which the production of kidney and lung tumors in rats by DMN was reported, but in the liver only "hyperplastic and cystic nodules could be observed in a relatively high incidence."

While experiments on the effect of structural changes in the nitrosamine molecule on the carcinogenic and metabolic activities of these substances were in progress, a note was published (*5*) on the production of cirrhosis

[1] Received for publication March 31, 1961.
[2] This work was supported by grant CY-4939 from the National Institutes of Health, Public Health Service, and a grant-in-aid from the Greater New Orleans Cancer Association and the Cancer Society of Greater Baton Rouge.
[3] *Present address:* Departments of Medicine and Biochemistry, Tulane University Medical School, USPHS Hospital Research Laboratory, 210 State St., New Orleans 18, La.
[4] The authors wish to thank Mrs. Judith F. Kane for her expert assistance, and Dr. Joseph C. Arcos for helpful discussion.

600915—61——21

and cancer of the liver in rats by diethylnitrosamine (DEN) fed in drinking water. Hemorrhages in the lungs, thymus, para-aortic lymph nodes, and the gastric mucosa were also observed.

In the present paper the comparative carcinogenicity and tissue specificity of DMN, DEN, diphenylnitrosamine (DPhN), and dimethylhydrazine (DMH) are reported.

MATERIALS AND METHODS

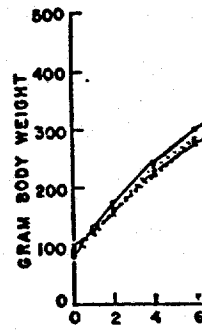
The nitrosamines and dimethylhydrazine were purchased from the Eastman-Kodak Company. Equimolar amounts of DMN (400 μg), DEN (550 μg), DPhN (1070 μg), and DMH (325 μg) were given daily 5 times a week in 1 ml water by stomach tube, except DPhN which was suspended in 1 ml of 1 percent aqueous methylcellulose. It was shown in previous experiments that methylcellulose is not carcinogenic and does not interfere with the carcinogenic activity of another compound (*6*). Solutions or suspensions were prepared fresh daily just before administration.

Male Wistar rats supplied by Carworth Farms were used, 25 per experimental group. They weighed on the average 92 g at the beginning of the experiment and were maintained on Purina laboratory chow and water *ad libitum*. The experiment with DMN was terminated between 35 and 48 weeks, that with DEN between 22 and 35 weeks, and that with DPhN and DMH in the 53d week; the feeding of the two latter substances was discontinued after 45 weeks. Animals that were killed or died on or after the 22d week and all those killed at the end of the experiments were autopsied, and portions of liver, spleen, kidneys, lung, and any other organs with macroscopic changes were fixed in Mossman's fixative. Hematoxylin-eosin stain was used routinely; mucicarmine, periodic acid-Schiff, or aldehyde fuchsin stains were employed when indicated.

RESULTS

Text-figure 1 shows that the average weight of the rats receiving DMN or DEN was slightly above that of the rats on DPhN or DMH until 30 weeks. After that time the average weights in the groups receiving DMN or DEN declined slowly. Some individual animals lost considerable weight. The slight increase in the average weight of rats on DMN after the 36th week may have been caused by ascites or an increase in the size of the tumor.

The duration of the experiment, the amount of nitrosamine derivatives and DMH given, and the tumor incidence are shown in table 1. Twenty-two of 25 rats, receiving DMN lived longer than 31 weeks. In 1 animal, found dead in the 34th week, a tumor was seen in the liver on gross examination, but no microscopic investigation was performed. The histopathological findings in 21 rats are summarized in table 2. All rats



TEXT-FIGURE 1.—
Rats fed dimet
Rats fed dieth
Rats fed diphe
Rats fed dimet
Each point rep

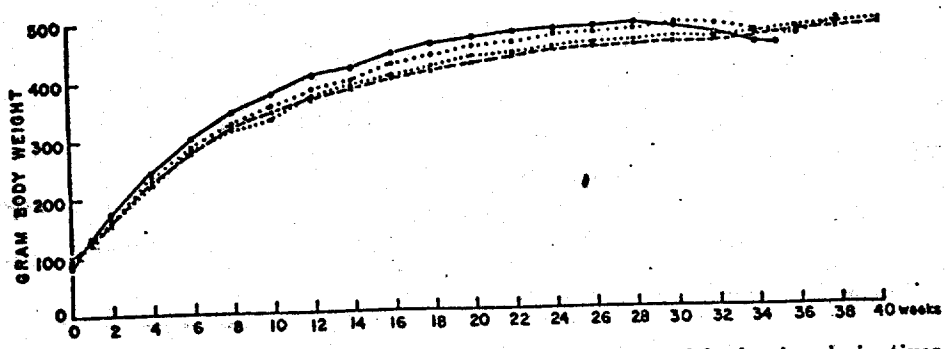
TABLE 1.—Carci

$(CH_3)_2N{\cdot}NO$, D
$(C_2H_5)_2N{\cdot}NO$, D
$(C_6H_5)_2N{\cdot}NO$, D
$(CH_3)_2N{\cdot}NH_2$, 1

*In 1 rat a tumor w
†Compound given

TABLE 2

Single primary
Liver
Kidney
Lung
Two primaries
Liver and kidne
Liver and lung
Lung and kidne
Three primaries
Liver, kidney, l



TEXT-FIGURE 1.—Growth curves of rats fed nitrosamine and hydrazine derivatives.

Rats fed dimethylnitrosamine ●.....●
Rats fed diethylnitrosamine O———O
Rats fed diphenylnitrosamine X-------X
Rats fed dimethylhydrazine ▲.....▲
Each point represents the average weight of 22 to 25 rats.

TABLE 1.—Carcinogenic activity of nitrosamine derivatives and dimethylhydrazine to rats

	Duration of experiment (weeks)	Total amount per rat (mg)	Rats with tumor/ rats in experiment
$(CH_3)_2N{\cdot}NO$, DMN	31–48	62–96	22/22*
$(C_2H_5)_2N{\cdot}NO$, DEN	22–35	61–96	25/25
$(C_6H_5)_2N{\cdot}NO$, DPhN	53†	244	0/25
$(CH_3)_2N{\cdot}NH_2$, DMH	53†	74	0/25

*In 1 rat a tumor was seen in the liver but not examined microscopically.
†Compound given for 45 weeks.

TABLE 2.—Location and histological type of tumors produced by DMN

		Predominant type of tumor							
		Liver			Kidney			Lung	
	Number of rats	Liver cell	Bile duct	Sarcoma	Adenocarcinoma	Solid carcinoma	Sarcoma	Squamous cell	Adenocarcinoma
Single primary	8								
Liver	3	2		1					
Kidney	1						1		
Lung	4							2	2
Two primaries	7								
Liver and kidney	2	1	1		1	1			
Liver and lung	2		2					1	1
Lung and kidney	3				2	1		1	2
Three primaries	6								
Liver, kidney, lung	6	4	2		4	2			6

developed tumors, which were localized in the liver, kidney, or lung. Besides the primary tumors, metastatic tumors from the liver were found in the lungs. The diagnosis of multiple primary tumors was based on the usually accepted criteria: marked differences in the morphology of the tumors and demonstrations of specific metabolic products in the cells or the transformation of normal to neoplastic epithelium. No organs apart from the liver, lung, or kidney were involved in the neoplastic process. In 6 rats primary tumors were in all 3 locations, in 7 rats 2 primary sites were observed, and in 8 rats tumors in a single location were found.

The organs involved were grossly much enlarged and had multiple, pale, elevated nodules. In the liver the nodules reached several cm in diameter; in the lungs and kidneys they rarely exceeded 1 cm. Occasionally the tumors in all three locations appeared cystic with a dark-yellow or slightly hemorrhagic content. On histological investigation the tumors of the liver consisted either of long, 3 to 5 cells thick, finger-like strands of large, cytoplasm-rich cells, which produced the pattern of hepatocellular carcinomas (fig. 1) or of glandular arrangements of cuboidal or columnar cells showing secretion and often containing bile pigment (fig. 2). Both types of tumor were occasionally in the liver of the same animal. Invasion of the vessels by the tumor could be observed in some instances. In 1 rat the tumor consisted of long, spindle-shaped cells with abundant intercellular substance and was diagnosed as a fibrosarcoma (figs. 3 and 4). Sarcomatous elements were found in the stroma of another tumor with predominantly cholangiocarcinomatous features. No cirrhosis was found, but multiple foci of necrosis were present. Cells with hyperchromatic or bizarre nuclei and many mitoses were present in the non-neoplastic areas.

The tumors in the kidneys were mostly papillary adenocarcinomas, single or, more often, multiple, and measured grossly from 0.5 to 1.0 cm in diameter. The tumors seemingly originated from tubules and, in some instances, appeared to lie in cysts or dilated tubules (fig. 5). Solid tumors, formed by large, cytoplasm-rich cells, resembling clear cell carcinomas, were also observed (fig. 7). In one kidney, spindle-shaped cells with elongated nuclei and small round cells with sparse cytoplasm formed the bulk of the tumor. Interspersed epithelial structures resembling newly formed glomeruli and small tubules were found. From the histological similarity the lesion was designated as Wilms' tumor (fig. 9).

Of particular interest are the tumors that developed in the lung. Four squamous-cell carcinomas were found: in 2 animals as the only tumor in the body, and in the 2 others, simultaneously with primary liver or kidney tumors (figs. 10 and 11). The squamous-cell carcinomas were about 1.5 to 2 cm in diameter. Squamous metaplasia of the bronchial epithelium was demonstrated, and in 2 animals there was evidence of direct connection between the metaplastic epithelium and the carcinoma. In 1 animal both an adenocarcinoma and a squamous-cell carcinoma were present (fig. 8). Also, in 1 rat areas of squamous-cell carcinoma

r, kidney, or lung.
the liver were found
umors was based on
the morphology of
products in the cells
helium. No organs
d in the neoplastic
ocations, in 7 rats 2
in a single location

d had multiple, pale,
veral cm in diameter;
. Occasionally the
n a dark-yellow or
ation the tumors of
nger-like strands of
ern of hepatocellular
nboidal or columnar
ent (fig. 2). Both
same animal. Inva-
ome instances. In 1
ells with abundant
rcoma (figs. 3 and 4).
another tumor with
cirrhosis was found,
with hyperchromatic
the non-neoplastic

ry adenocarcinomas,
rom 0.5 to 1.0 cm in
bules and, in some
fig. 5). Solid tumors,
ar cell carcinomas,
e-shaped cells with
ytoplasm formed the
s resembling newly
oin the histological
r (fig. 9).
l in the lung. Four
as the only tumor
ith primary liver or
ll carcinomas were
sia of the bronchial
here was evidence of
and the carcinoma.
ous-cell carcinoma
amous-cell carcinoma

were found in a predominantly adenomatous tumor. Adenocarcinoma occurred in 11 animals. This tumor was nearly always of multicentric origin. Most of the tumors were only a few millimeters large and were located in the periphery of the lung. They consisted of papillary or tubular arrangements of large, mostly columnar or cuboidal neoplastic cells (fig. 12). The tumors occurred in or were accompanied by areas of severe inflammation. Lesions—considered metastatic—were in 4 animals (figs. 4 and 6).

The tumors produced when DEN was fed were much more uniform than those produced by DMN. Five rats killed between the 22d and 35th weeks and 20 rats killed the 35th week all had liver tumors. On gross examination the left underlobe seemed most extensively involved. Cirrhosis was not found. Each tumor had the histological character of a hepatocellular carcinoma. Nine showed invasion of the small vessels of the lung and had multiple metastases in the parenchyma (figs. 13, 14, 15, and 16). In 1 animal a primary tubular carcinoma was in one kidney. Primary lung tumors were not seen. The hemorrhagic changes described by Schmähl *et al.* (*5*) were not observed.

DPhN and DMH were fed for 45 weeks and the rats killed in the 53d week. There were no tumors in any of these rats. The livers were normal grossly and microscopically; in many kidneys the tubules contained albuminous precipitation. Most lungs had peribronchial lymphocytic infiltration, as is very common in older rats; in some, squamous metaplasia of the bronchial epithelium was present, particularly in areas of bronchiectasis.

DISCUSSION

Although in the present experiment a striking difference was found in the location and morphology of the tumors produced by either DMN or DEN, the difference might have been caused in part by experimental conditions. The rats on DMN were kept alive longer than those on DEN. One kidney tumor was present in a rat fed DEN; therefore, in all likelihood more such tumors could be expected in a more prolonged experiment. No primary lung tumor was produced with DEN in this experiment, but it is possible that such tumors might have occurred under different conditions. The observation of Magee and Barnes (*3*) that liver and kidney tumors do not occur together in the same animal has not been confirmed in the present experiment.

The localization of the tumors produced by ingestion of DMN points to organs in which the chemical is either metabolized or excreted. Using C^{14}-labeled DMN, Dutton and Heath (*7*) showed in mice and rats that the main radioactive product is expired CO_2. In rats about 40 percent of the injected C^{14} is recovered by that route in 8 hours, 7 percent appears in the urine, and the remainder is fairly evenly distributed in the tissues, at the end of the experiment. The authors conclude that, because of the rapidity with which DMN is metabolized, the biologically active sub-

stance is a metabolite and not the DMN itself. Studies *in vitro* showed that DMN is rapidly metabolized by liver slices or homogenates, but little or not at all by other organs (*8*). Comparable studies on the metabolism of DEN are not known.

The production of squamous-cell carcinoma in the lung of rats by an *ingested* carcinogen is observed here for the first time. Lung tumors similar in morphological character and variety to those in the present experiment were produced by intratracheal inoculation of the polyoma virus to the Syrian golden hamster (*9*).

The type of relationship that has been formulated between electron density and the carcinogenic activity of the polycyclic hydrocarbons [reviewed *e.g.*, in (*10*)] may also hold for the nitrosamines, where carcinogenic activity is lost when the *N*-methyl or *N*-ethyl substituents are replaced by *N*-phenyl groups. Although the influence of steric hindrance, because of the relatively greater size of the phenyl groups, may not be excluded, circumstantial evidence suggests that these groups may actually function as electron traps redistributing the high electron density of the nitroso group. In fact, in both active compounds, DMN and DEN, the aminonitrogen has no basic properties in aqueous solutions, which indicates that there is a displacement of the free electron doublet of the nitrogen toward the nitroso group. The reactivity of the nitroso group, because of its high electron density, might then be instrumental in establishing the carcinogenic activity of the molecule following two possible mechanisms: (*a*) by altering vital metabolic control sites (*11*) or endoplasmic morphology (*12*), such as the nonmetabolized DMN or DEN, by virtue of their potent protein-denaturing ability (*13*); this denaturing ability has been shown to be in relation with the hydrogen-bonding ability of the nitroso group, which in turn is related to electron density. (*b*) by facilitating intramolecular cyclization of the *N*-monomethyl compound, an intermediate of metabolic breakdown (*14*), to diazomethane, which was shown to have carcinogenic activity (*15*); a similar mechanism may exist by analogy with DEN. It is also possible that situations (*a*) and (*b*) are not mutually exclusive, but are concurrently operative in living tissues.

The observations that DMN is toxic to the human liver and that nitrosamine derivatives are carcinogenic in experimental animals emphasize the danger of occupational and possibly nutritional exposure to this class of substances. Nitrosamine derivatives are still used industrially, particularly in Europe, in the vulcanization of rubber (*16*) and in the preparation of textile fibers (*17*).

REFERENCES

(*1*) Barnes, J. M., and Magee, P. N.: Some toxic properties of dimethylnitrosamine. Brit. J. Indust. Med. 11: 167–174, 1954.

(*2*) Magee, P. N., and Barnes, J. M.: The production of malignant primary hepatic tumours in the rat by feeding dimethylnitrosamine. Brit. J. Cancer 10: 114–122, 1956.

(3) ———: The experimental production of tumors in the rat. Acta Unio internat. contra cancrum 15: 187–190, 1959.

(4) ZAK, F. G., HOLZNER, J. H., SINGER, E. J., and POPPER, H.: Renal and pulmonary tumors in rats fed dimethylnitrosamine. Cancer Res. 20: 96–100, 1960.

(5) SCHMÄHL, D., PREUSSMAN, R., and HAMPERL, H.: Leberkrebs erzeugende Wirkung von Diäthylnitrosamin nach oraler Gabe bei Ratten. Naturwissenschaften 47: 1–2, 1960.

(6) ARGUS, M. F., and RAY, F. E.: Possible relation between carcinogenicity and ease of hydrolysis *in vitro* of derivatives of 2-aminofluorene. Nature, London 184: 2018–2019, 1959.

(7) DUTTON, A. H., and HEATH, D. F.: Dimethylation of dimethylnitrosamine in rats and mice. Nature, London 178: 644, 1956.

(8) MAGEE, P. N., and VANDEKAR, M.: Toxic liver injury. The metabolism of dimethylnitrosamine *in vitro*. Biochem. J. 70: 600–605, 1958.

(9) RABSON, A. S., BRANIGAN, W. J., and LEGALLAIS, F. Y.: Lung tumors produced by intratracheal inoculation of polyoma virus in Syrian hamsters. J. Nat. Cancer Inst. 25: 937–965, 1960.

(10) PULLMAN, A., and PULLMAN, B.: Electronic structure and carcinogenic activity of aromatic molecules. New developments. Advances Cancer Res. 3: 117–169, 1955.

(11) ARCOS, J. C., GOSCH, H. H., and ZICKAFOOSE, D.: Structural alterations in cell particles during chemical carcinogenesis. III. Selective action of hepatic carcinogens other than 3′-methyl-4-dimethylaminoazobenzene on different types of mitochondrial swelling. Effect of stimulated liver growth. J. Biophys. & Biochem. Cytol. In press.

(12) EMMELOT, P., and BENEDETTI, E. L.: Changes in the fine structure of rat liver cells brought about by dimethylnitrosamine. J. Biophys. & Biochem. Cytol. 7: 393–396, 1960.

(13) ARGUS, M. F., LEUTZE, C. J., and KANE, J. F.: Nitrosamines and other carcinogens as agents of protein denaturation. Experientia. In press.

(14) HEATH, D. F., and DUTTON, A. H.: The detection of metabolic products from dimethylnitrosamine in rats and mice. Biochem. J. 70: 619–626, 1958.

(15) SCHOENTAL, R.: Carcinogenic action of diazomethoxane and of nitroso-*N*-methyl urethane. Nature, London 188: 420–421, 1960.

(16) SCHULTHEISS, E.: Berufsdermatosen, Gummi und Ekzem, Editio Cantor, Aulendorf i. Wurtt., 1959, vol. 3, p. 213.

(17) HUEPER, W. C.: Personal communication.

PLATE 85

FIGURE 1.—Hepatocellular carcinoma, rat fed DMN for 48 weeks. *Note* long strands of large cytoplasm-rich cells. Hematoxylin and eosin. × 150

FIGURE 2.—Cholangiocarcinoma, rat fed DMN for 37 weeks. Neoplastic cells are arranged in glandular pattern. Hematoxylin and eosin. × 150

FIGURE 3.—Fibrosarcoma in liver of rat fed DMN for 46 weeks. Spindle-shaped cells with elongated nuclei. Hematoxylin and eosin. × 150

FIGURE 4.—Same rat as figure 3. Lung metastasis resembling primary tumor. Hematoxylin and eosin. × 150

eeks. *Note* long
× 150

eoplastic cells are

s. Spindle-shaped

tumor. Hema-

600915—61——22

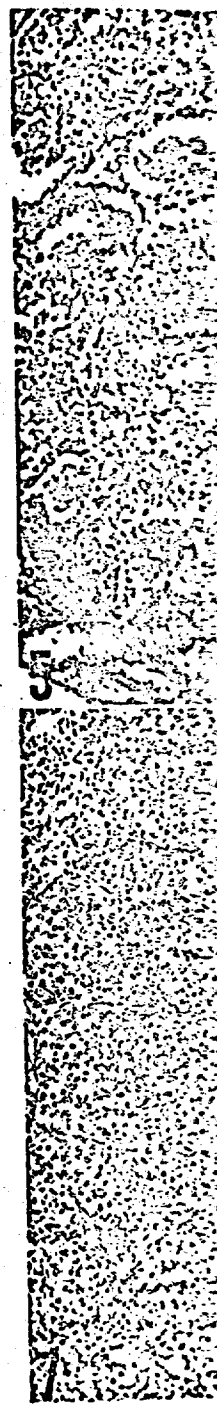
PLATE 86

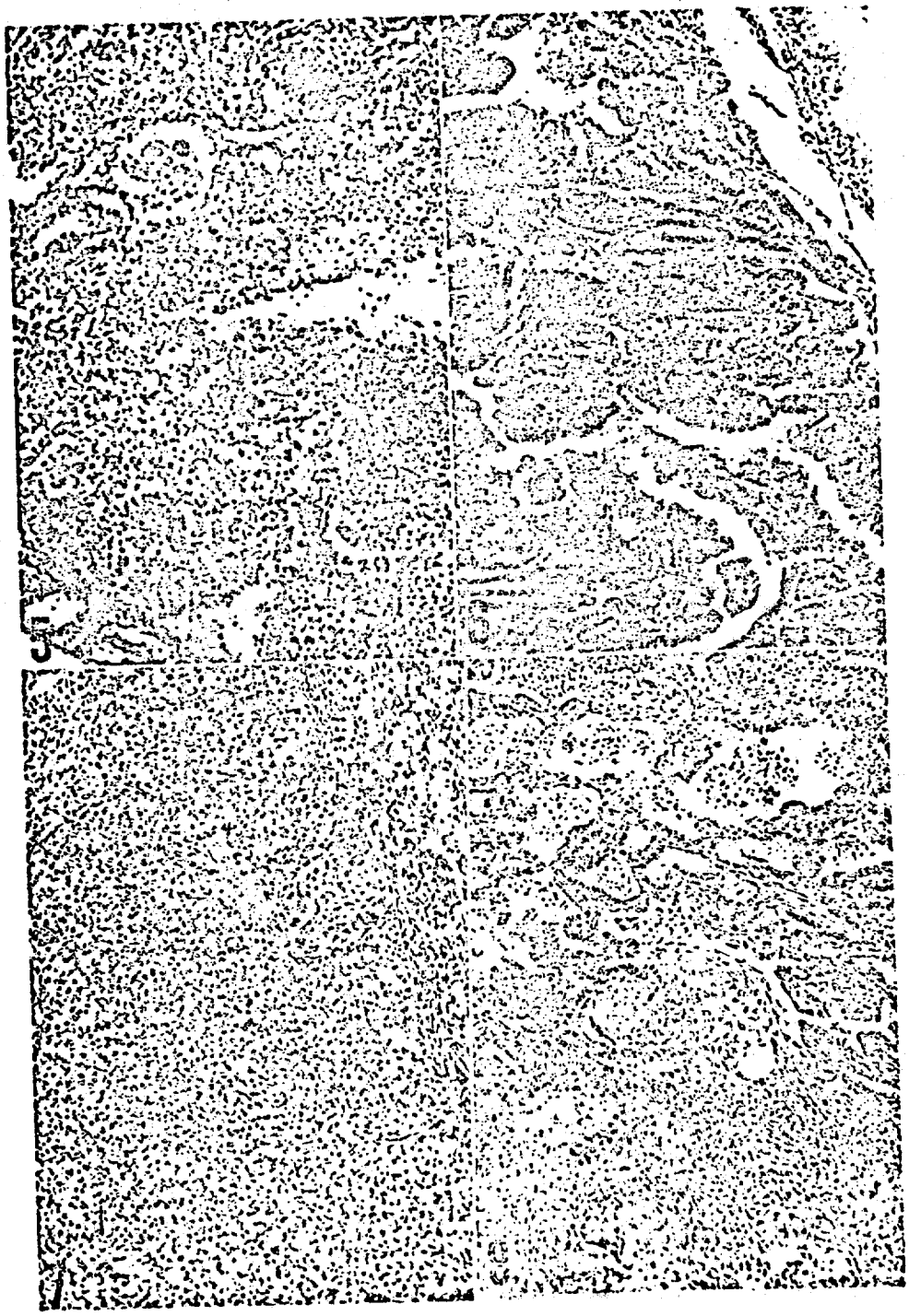
FIGURE 5.—Papillary tumor in kidney of rat fed DMN for 48 weeks. Hematoxylin and eosin. × 150

FIGURE 6.—Same rat as figure 5. Papillary tumor in lung, possibly metastatic. Mucin secretion could not be demonstrated by special stains. Hematoxylin and eosin. × 150

FIGURE 7.—Solid tumor in kidney of rat fed DMN for 31 weeks. Hematoxylin and eosin. × 150

FIGURE 8.—Same rat as figure 7. Lesion in lung shows both adenocarcinoma and squamous-cell carcinoma. Hematoxylin and eosin. × 150





ARGUS AND HOCH-LIGETI

eks. Hematoxylin

ossibly metastatic.
s. Hematoxylin and

Hematoxylin and

lenocarcinoma and

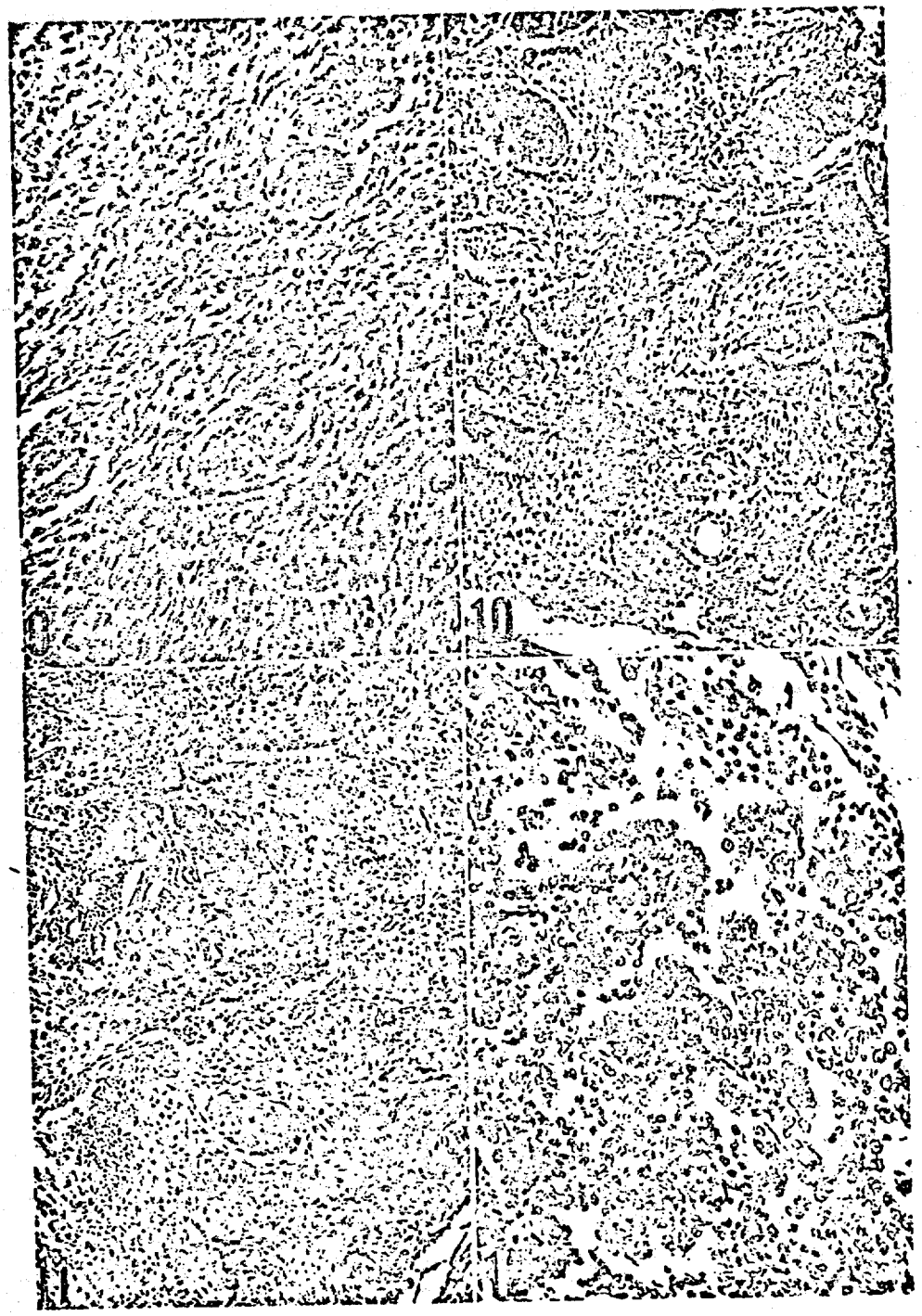
PLATE 87

FIGURE 9.—Tumor in kidney of rat fed DMN for 42 weeks. Elongated sarcomatous cells with indication of newly formed glomeruli. Hematoxylin and eosin. × 300

FIGURE 10.—Squamous-cell carcinoma in lung of rat fed DMN for 37 weeks. No other tumor found in this rat. Hematoxylin and eosin. × 150

FIGURE 11.—Squamous-cell carcinoma in lung of rat fed DMN for 48 weeks. This rat had tumors also in liver and kidney. Hematoxylin and eosin. × 150

FIGURE 12.—Epithelial tumor with alveolar structure in lung of rat fed DMN for 48 weeks. Hematoxylin and eosin. × 300



Elongated sarcomatous
lin and eosin. × 300

MN for 37 weeks. No
150

MN for 48 weeks. This
eo × 150

of rat fed DMN for 48

600915—61——23

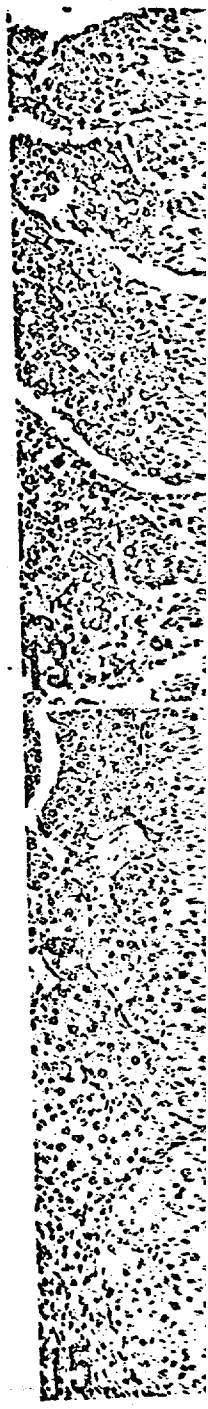
PLATE 88

FIGURE 13.—Hepatocellular carcinoma in the liver of rat fed DEN for 35 weeks. Long strands of neoplastic tissue, with invasion of veni. Hematoxylin and eosin. × 150

FIGURE 14.—Same rat as figure 13. Thrombi of liver-cell carcinoma in pulmonary artery. Hematoxylin and eosin. × 150

FIGURE 15.—Hepatocellular carcinoma in the liver of a rat fed DEN for 35 weeks. Long finger-like arrangement of neoplastic cells. Hematoxylin and eosin. × 150

FIGURE 16.—Same rat as figure 15. Tumor-cell thrombi in the pulmonary vessels with small metastatic nodule. Hematoxylin and eosin. × 150



[CANCER RESEARCH 31, 1201–1205, September 1971]

77

Acute Synergistic Toxicity and Hepatic Necrosis following Oral Administration of Sodium Nitrite and Secondary Amines to Mice[1]

Shogo Asahina, Marvin A. Friedman, Elsie Arnold, Gail N. Millar, Myra Mishkin, Yvonne Bishop, and Samuel S. Epstein

[Laboratories of Environmental Toxicology and Carcinogenesis [S. A., M. A. F., E. A., G. N. M., M. M., S. S. E.] and Laboratories of Biostatistics [Y. B.], Children's Cancer Research Foundation, Inc.; Department of Pathology, Harvard Medical School [S. S. E.]; and Department of Biostatistics, Harvard School of Public Health [Y. B.], Boston, Massachusetts 02115

SUMMARY

Combined p.o. administration of sodium nitrite and dimethylamine or methylbenzylamine to male mice produced acute synergistic toxicity, as evidenced by relative weight loss, mortality, and liver necrosis. Similar results were obtained when p.o. dosage of dimethylamine was followed by single administration of sodium nitrite at intervals up to 3 hr; toxicity was, however, markedly reduced when sodium nitrite was administered prior to dimethylamine. The incidence of mortality and liver necrosis was dependent on the time interval separating successive administrations of sodium nitrite and dimethylamine.

INTRODUCTION

Nitrosamines are a major candidate class of carcinogens likely to be causally related to human cancer (12) Nitrosamines act systemically and produce cancer in a wide variety of organs of many species at the ppm dietary level (5, 13, 14); some nitrosamines are also mutagenic and teratogenic (12). Although practical methods for detection lack specificity and sensitivity, nitrosamines have been found in tobacco smoke, grains, and alcoholic beverages in concentrations of <5 ppm (15, 16, 18). Higher concentrations of nitrosamines, particularly dimethylnitrosamine, have been found in nitrite-preserved fish meals which were highly toxic to ruminants (7, 19); dimethylnitrosamine is formed during cooking of canned or smoked fish with nitrite (26). However, even more widespread and of possibly greater significance than nitrosamines *per se* are their precursors, nitrites and secondary amines (12). Nitrite and methylbenzylamine or morpholine are carcinogenic when fed simultaneously to rats (22, 24); however, these secondary amines are not naturally occurring.

Secondary amines, particularly dimethylamine, are found in fish, fish meal, fish products, cereals, tea, flavoring agents, crude liquors, tobacco, and tobacco smoke (12); various drugs (piperazine, thiocarbasone, tolbutamide, tryparsamide, and streptomycin) are also secondary amines. Inorganic nitrite is produced from nitrate, in a wide variety of sources (plants, vegetables, and water) by nitroreductases present in many saprophytic and parasitic bacteria. The average human daily intake of nitrite has been estimated as 22 μmoles, equivalent to 1.5 mg of $NaNO_2$ (20); levels in excess of this have produced acute methemoglobinemia in infants (12).

Nitrosamines are formed chemically from the interaction of nitrites or oxides of nitrogen and secondary amines in acidic conditions (17). Such reactions also occur *in vitro* at nearly neutral pH in the presence of enteric bacteria (21), *in vitro* in the gastric juice of various species including man (20), and *in vivo* in the stomach of laboratory animals and man (23, 25). Nitrosopiperidine is formed *in vivo* in the small intestine of the rat from piperidine and sodium nitrite (1, 2).

Combined p.o. administration of sodium nitrite and dimethylamine or methylbenzylamine produced inhibition of mouse liver protein and nRNA synthesis (10) and alkylation of guanine in liver RNA;[2] these effects are characteristic of those produced by the corresponding nitrosamines, dimethylnitrosamine and methylbenzylnitrosamine. This communication extends these studies and reports acute synergistic toxicity (weight loss, mortality, and hepatic necrosis) following p.o. administration of single doses of sodium nitrite and dimethylamine or methylbenzylamine.

MATERIALS AND METHODS

Solutions of dimethylamine hydrochloride, methylbenzylamine hydrochloride, and sodium nitrite were freshly prepared in distilled water and administered by gavage to groups of adult male HaM/ICR mice (Charles River Breeding Laboratory, Wilmington, Mass.) maintained on Purina chow and tap water *ad libitum* and housed 5 per mesh-bottom hanging cage.

The dose-response effects of dimethylamine and sodium nitrite were tested by their combined or immediately successive administration by gavage; combined dose-response effects of methylbenzylamine and sodium nitrite were tested by successive gavage of separate solutions. Sodium nitrite was administered at doses of 100 and 150 mg/kg; dimethylamine

[1] Supported by NIH Grants C-6516 and FR-05526 and National Air Pollution Control Administration Contract CPA 70-17.

Received November 17, 1970; accepted April 28, 1971.

[2] M. A. Friedman, G. N. Millar, and S. S. Epstein, Acute Inhibition of Liver Protein and Nuclear RNA Synthesis and Methylation of Guanine following Oral Administration of Sodium Nitrite and Dimethylamine to Mice, unpublished data.

Table 1

Acute synergistic toxicity due to simultaneous p.o. administration of sodium nitrite and dimethylamine hydrochloride to mice

Dose (mg/kg)			Mortality by 3 days (%)		Body weight change (g)				Liver/body weight ratio (× 10^{-2}) for all mice		Mice with gross liver necrosis (%)			
					All mice		Surviving mice				All mice		Surviving mice	
Sodium nitrite	Dimethylamine hydrochloride	Total no. of mice (replicates)	Mean	S.D.[a]	Mean	S.D.[b]	Mean	S.D.[b]	Mean	S.D.[b]	Extensive	Localized	Extensive	Localized
0	0	70 (5)	0		+1.7	1.4	+1.7	1.4	8.5	0.8	0	0	0	0
0	500	40 (3)	0		+1.7	1.6	+1.7	1.6	8.5	0.8	0	0	0	0
0	1000	40 (3)	0		+2.1	1.9	+2.1	1.9	8.5	0.9	0	0	0	0
0	2000	40 (3)	0		+1.5	1.9	+1.5	1.9	8.4	1.0	0	0	0	0
0	2500	40 (3)	0		+0.1	1.9	+0.1	1.9	8.2	0.8	0	0	0	0
100	0	30 (2)	0		+1.3	1.3	+1.3	1.3	9.0[c]	0.7	0	0	0	0
100	500	30 (2)	0		+1.2	1.5	+1.2	1.5	8.7	0.7	0	0	0	0
100	1000	30 (2)	0		+1.0	2.3	+1.0	2.3	9.1[d]	1.2	7	50	7	50
100	2000	30 (2)	5	5	+1.3	1.5	+1.4	1.6	8.6	0.9	53	40	50	43
100	2500	30 (2)	18	10	+0.5	2.0	+0.6	2.1	8.6	1.3	57	20	54	25
150	0	70 (5)	0		+1.1	1.8	+1.1	1.8	8.3	1.0	0	0	0	0
150	500	70 (5)	0		+0.9	1.6	+0.9	1.6	7.9[c]	0.9	10	47	10	47
150	1000	70 (5)	19	17	−0.1	1.8	0.0	1.8	7.3[c]	1.0	51	40	42	49
150	2000	70 (5)	70	18	−0.2	2.1	+0.2	2.4	7.7[c]	0.9	77	9	67	24
150	2500	30 (2)	77	3	−0.7	1.3	−1.5	1.3	7.5[c]	0.8	87	7	86	14

[a] Variability between replicates.
[b] Variability between mice, all replicates combined.
[c] A 5% significance level for proportion of points falling on one side of regression line based on controls.
[d] A 1% significance level for proportion of points falling on one side of regression line based on controls.

Table 2

Acute synergistic toxicity due to simultaneous p.o. administration of sodium nitrite and methylbenzylamine hydrochloride to mice

Dose (mg/kg)			
Sodium nitrite	Methylbenzylamine hydrochloride	No. of mice tested	Mortality by 3 days (%)
0	0	10	0
0	800	10	0
0	1600	10	0
100	0	10	0
100	800	10	10
100	1200	10	40
100	1600	10	90

was administered at doses of 500, 1000, 2000, and 2500 mg/kg (Table 1); and methylbenzylamine was administered at doses of 800, 1200, and 1600 mg/kg (Table 2). Mice given water, dimethylamine, methylbenzylamine, and sodium nitrite alone served as concurrent controls. Mice were tested in groups of 10 to 15; tests with dimethylamine were more extensive and were replicated on 2 to 5 occasions.

The effects of sequential administration of dimethylamine (2500 mg/kg) and sodium nitrite (150 mg/kg) were determined as follows. First, sodium nitrite was administered and followed by a single dose of dimethylamine at subsequent intervals of 0, 15, 30, 60, or 120 min; second, dimethylamine was administered and followed by sodium nitrite at intervals of 0, 15, 30, 120, or 180 min (Table 3). Tests based on a minimum of 10 mice at each test dose level were replicated on 2 to 6 occasions.

Mice were observed and weighed daily up to 3 days; survivors were killed with ether. Postmortem examination was carried out on all animals, and the liver and both kidneys were weighed. Livers from 10 to 20% of mice per dose group were fixed in Tellyesniczky's solution for 24 hr; tissues were stained with hematoxylin and eosin.

RESULTS

No mortality occurred following administration of sodium nitrite or either secondary amine alone. Single doses of sodium nitrite produced temporary inertia; weight gain was reduced at both doses of sodium nitrite and at the highest dose of dimethylamine alone (Table 1). Combined administration of sodium nitrite and dimethylamine produced acute toxicity, as evidenced by progressive inertia, anorexia, ascites, relative weight loss, and mortality; relative weight loss and mortality were both clearly dose dependent (Table 1). Mortality was similar irrespective of whether dimethylamine and sodium nitrite were administered singly in combined solution or successively in separate solutions. Dose-dependent mortality was also seen following combined administration of sodium nitrite and methylbenzylamine (Table 2). In general, the onset of mortality was more rapid when sodium nitrite was administered in combination with methylbenzylamine rather than with dimethylamine. The median lethal dose of dimethylamine, in combination with 150 mg/kg sodium nitrite, was 1600 mg/kg; the median lethal dose of

Table 3

Synergistic toxicity in mice due to p.o. administration of dimethylamine hydrochloride and sodium nitrite at timed intervals

Dose (mg/kg)		Interval between doses (min)	Total no. of mice (replicates)	Mortality by 3 days (%)		Liver/body weight ($\times 10^{-2}$) for all mice		Overall no. of mice with liver necrosis		Mice with liver necrosis (%)	
Dimethylamine hydrochloride	Sodium nitrite			Mean	S.D.[a]	Mean	S.D.[b]	Extensive	Localized	Mean	S.D.[a]
0	0	0	40 (4)	0	0	7.7	0.8	0	0	0	0
0	150	0	15 (3)	0	0	8.0	0.8	0	0	0	0
2500	0	0	15 (3)	0	0	8.0	0.9	0	0	0	0
No interval between dimethylamine and nitrite											
2500	150	0	80 (6)	43	22	7.3[c]	0.8	59	13	90	9
Nitrite before dimethylamine											
2500	150	15	20 (2)	15	7	7.4[d]	0.8	1	7	40	10
2500	150	30	20 (2)	5	7	7.6	0.6	0	7	35	7
2500	150	60	20 (2)	0	0	7.6	0.6	0	1	5	7
2500	150	120	20 (2)	0	0	7.7	0.8	0	4	20	14
Dimethylamine before nitrite											
2500	150	15	65 (6)	32	13	7.3[c]	0.9	34	19	81	17
2500	150	30	50 (5)	30	20	7.1[c]	0.9	21	12	66	25
2500	150	60	80 (6)	26	24	7.4[c]	0.8	26	28	68	23
2500	150	120	50 (5)	20	16	7.3[d]	0.8	13	14	54	21
2500	150	180	39 (4)	13	14	7.2[c]	0.8	14	8	56	13

[a] Variability between replicates.
[b] Variability between mice, all replicates combined.
[c] A 1% significance level for proportion of points falling on one side of regression line based on controls.
[d] A 5% significance level for proportion of points falling on one side of regression line based on controls.

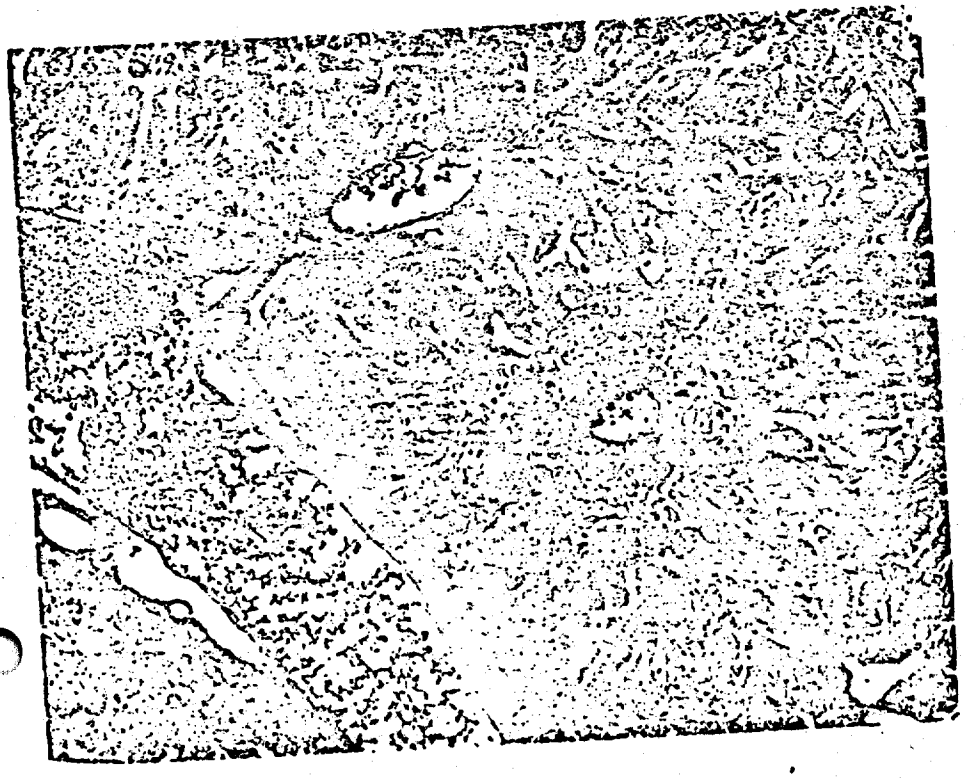


Fig. 1. Centrilobular necrosis and congestion in portal veins in a mouse which died 3 days after p.o. administration of 2000 mg/kg dimethylamine in combination with 150 mg/kg sodium nitrite. × 100.

methylbenzylamine, in combination with 100 mg/kg sodium nitrite, was 1300 mg/kg.

At autopsy, no significant abnormalities were seen in mice treated with sodium nitrite, dimethylamine, or methylbenzylamine alone. However, ascites and congestion of the kidney and spleen were generally seen in surviving mice and in those dying within 3 days of administration of sodium nitrite and dimethylamine; such changes were minimal in combination groups involving sodium nitrite and methylbenzylamine. The ascitic fluid was generally clear and transparent, although occasionally it was hemorrhagic. Males dying within 24 hr after treatment had distended and food-free stomachs which, in some cases, revealed submucosal hemorrhages; no free blood was found in the gastrointestinal tract. The ratio of kidney to body weight was comparable in all dimethylamine test and control groups; the ratio of liver to body weight was, however, reduced in mice simultaneously treated with high doses of dimethylamine and sodium nitrite (Table 1).

Livers in animals from high-dosage combined treatment groups were generally swollen and mottled and, in some cases, hemorrhagic, friable, and necrotic. Macroscopically, the hepatic lesions were either extensive, involving 1 or several lobes, or localized, involving only a discrete area in 1 or more lobes. No necrotic liver lesions or hemorrhage was seen in any mice treated with dimethylamine, methylbenzylamine, or sodium nitrite alone.

Microscopic examination of livers of mice treated with sodium nitrite and dimethylamine revealed widespread centrilobular and midzonal parenchymal liver necrosis, sinusoidal and portal congestion, and hemorrhage. In some cases, necrosis was extensive and diffuse and included all but immediately periportal areas (Fig. 1); the incidence and

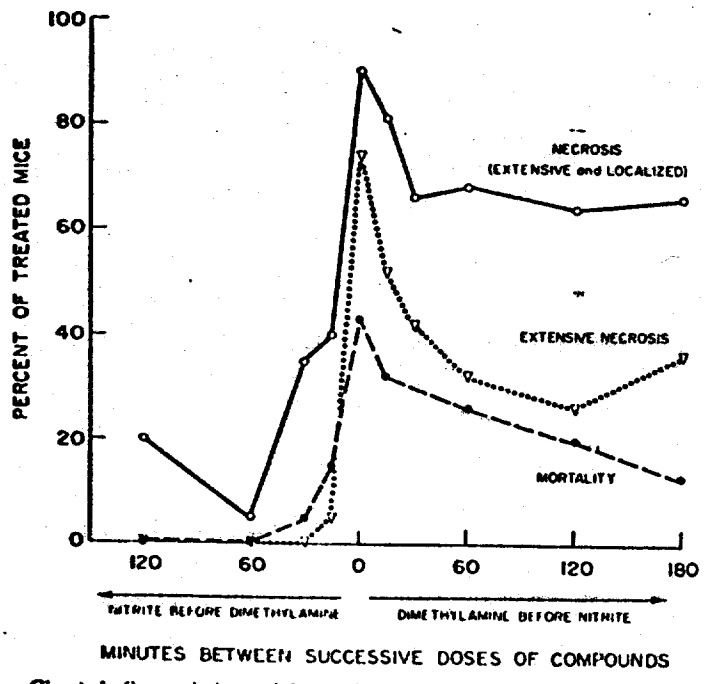


Chart 1. Synergistic toxicity and hepatic necrosis in mice following successive p.o. administration of dimethylamine and sodium nitrite at varied intervals.

severity of liver necrosis were clearly dose related (Table 1). Hepatic changes were similar in mice receiving high doses of methylbenzylamine and sodium nitrite, except that the necrosis was largely periportal. Necrosis was reduced and accompanied by leukocytic infiltration and minimal parenchymal regeneration in surviving mice of all combined treatment groups. No proliferative endothelial changes were observed in central or portal veins nor in the sinusoids; no epithelial changes were noted in bile ducts.

In studies on sequential administration of dimethylamine and sodium nitrite, mortality and hepatic necrosis were highest following simultaneous administration of dimethylamine and sodium nitrite (Chart 1; Table 3). Mortality and hepatic necrosis were reduced in groups receiving sodium nitrite 15 or 30 min before dimethylamine; no deaths occurred when this interval exceeded 30 min. Mortality and extensive hepatic necrosis occurred in all groups receiving dimethylamine before sodium nitrite; the mean percentage of mortality decreased with increasing intervals between doses.

DISCUSSION

The acute synergistic toxicity induced by combined administration of high doses of sodium nitrite and dimethylamine or sodium nitrite and methylbenzylamine is consistent with *in vivo* biosynthesis of nitrosamines from amine and nitrite precursors. Liver lesions in mice treated simultaneously with dimethylamine and sodium nitrite or methylbenzylamine and sodium nitrite were morphologically identical with those in mice receiving the corresponding nitrosamine, dimethylnitrosamine or methylbenzylnitrosamine, respectively (3, 4, 11). Similar liver changes were induced even when sodium nitrite and dimethylamine were administered on closely separate occasions. Toxicity, mortality, and necrosis were reduced with increasing time intervals between successive administrations; such time dependency was particularly marked when sodium nitrite was given before dimethylamine. Sodium nitrite is absorbed very rapidly from the stomach of the mouse; 70 to 100% of administered nitrite is absorbed within 30 min (9, 24). However, marked mortality and extensive necrosis were observed when sodium nitrite was given as long as 3 hr after dimethylamine, suggesting that dimethylamine is absorbed relatively slowly and is thus available over prolonged periods for reaction with subsequently ingested nitrates and nitrites to yield endogenous nitrosamines. Similarly, there was no inhibition of mouse liver nRNA synthesis when sodium nitrite was administered 30 min prior to dimethylamine; however, there was significant inhibition when dimethylamine was administered 1 hr before sodium nitrite.[2]

Zonal hepatic necrosis may be induced by shock (6). However, no significant necrotic lesions in these experiments were observed in animals receiving sodium nitrite or high doses of secondary amines alone, although these produced temporary inertia in some mice; similar results were seen following parenteral administration (S. Asahina and S. S. Epstein, unpublished data). Synergistic toxicity (mortality and hepatic necrosis) resulting from combined dosage with sodium

nitrite and either of 2 secondary amines, in the present experiments, is consistent with previous data on synergistic inhibition of mouse liver protein and nRNA synthesis by sodium nitrite and dimethylamine or methylbenzylamine (10). The use of such toxicological and biochemical markers as indirect indices of *in vivo* biosynthesis of dimethylnitrosamine, rather than its chemical identification, is necessary due to the rapid absorption and metabolism of this particular nitrosamine (14); however, 7-methylguanine has been isolated from liver RNA following combined administration of dimethylamine and sodium nitrite.[2] These studies further extend data on *in vivo* biosynthesis of nitrosamines (1, 2, 23–25) and are of particular practical relevance in view of the widespread dietary occurrence of dimethylamine (12). Synergistic carcinogenicity has been demonstrated following combined feeding of rats with sodium nitrite and morpholine or methylbenzylamine (22), but neither of these amines occur naturally.

The relevance of these findings to *in vivo* biosynthesis of nitrosamines in man, particularly at normal dietary levels of nitrites and secondary amines, requires further consideration. Critical attention should be directed to the use of secondary amines as food-flavoring agents as well as their use as drugs, to the drinking of water with elevated nitrate levels, and to the propriety of continued use of nitrates and nitrites as food additives in meat and fish at presently permissible concentrations of 500 and 200 ppm, respectively (8).

ACKNOWLEDGMENTS

We thank Dr. W. Lijinsky, Dr. S. Preussman, and Dr. B. S. Alam for their comments.

REFERENCES

1. Alam, B. S., Saporoschetz, I. B., and Epstein, S. S. Formation of *N*-Nitrosopiperidine from Piperidine and Sodium Nitrite in the Stomach and the Isolated Intestinal Loop of the Rat. Nature, in press.
2. Alam, B. S., Saporoschetz, I. B., and Epstein, S. S. The Synthesis of Nitrosopiperidine from Nitrate and Piperidine in the Stomach and Small Intestine and in Isolated Gastric Contents of Rats. Nature, in press.
3. Bailie, M. J., and Christie, G. S. The Acute Toxic Action of Dimethylnitrosamine on Liver Cells. Biochem. J., *72:* 473–479 1959.
4. Barnes, J. M., and Magee, P. N. Some Toxic Properties of Dimethylnitrosamine. Brit. J. Ind. Med., *11:* 167–174, 1954.
5. Druckrey, H., Preussman, R., Ivankovic, S., and Schmähl, D. Organotrope Carcinogene Wirkungen bei 65 verschiedenen *N*-Nitroso-Verbindungen an BD-Ratten. Z. Krebsforsch., *69:* 103–201, 1967.
6. Ellenberg, M., and Osserman, K. The Role of Shock in the Production of Central Liver Cell Necrosis. Am. J. Med., *11:* 170–178, 1951.
7. Ender, F., Havre, G., Helgebostad, A., Koppang, N., Madsen, R., and Ceh, L. Nitrosamines Strike in Fish Meal. Naturwissenschaften, *51:* 637–638, 1964.
8. Federal Register, Title 21, Ch. 1, No. 121.1063 and No. 121.1064. 1968.
9. Friedman, M. A., Greene, E. J., and Epstein, S. S. Rapid Gastric Absorption of Sodium Nitrite in Mice. J. Pharm. Sci., in press.
10. Friedman, M. A., Millar, G., Sengupta, M., and Epstein S. S. Inhibition of Mouse Liver Protein and Nuclear RNA Synthesis following Combined Oral Treatment with Sodium Nitrite and Dimethylamine or Methylbenzylamine. Experientia, in press.
11. Jacobsen, K. H., Wheelwright, H. J., Clem, J. M., and Shannon, R. N. Studies of the Toxicology of *N*-Nitrosodimethylamine Vapor. A. M. A. Arch. Ind. Health, *12:* 617–622, 1955.
12. Lijinsky, W., and Epstein, S. S. Nitrosamines as Environmental Carcinogens. Nature, *225:* 21–23, 1970.
13. Magee, P. N., and Barnes, J. M. The Production of Malignant Primary Hepatic Tumors in the Rat by Feeding Dimethylnitrosamine. Brit. J. Cancer., *10:* 114–122, 1956.
14. Magee, P. N., and Barnes, J. M. Carcinogenic Nitroso Compounds. Advan. Cancer Res., *10:* 163–246, 1967.
15. Marquardt, P., and Hedler, L. Uber das Vorkommen von Nitrosaminen in Weizenmehl. Arzneimittel-Forsch., *6:* 778–779, 1966.
16. McGlashan, N. D., Walters, C. L., and McLean, A. E. M. Nitrosamines in African Alcoholic Spirits and Esophageal Cancer. Lancet, *2:* 1017, 1968.
17. Mirvish, S. Kinetics of Dimethylamine Nitrosation in Relation to Nitrosamine Carcinogenesis. J. Natl. Cancer. Inst., *44:* 633–639. 1970.
18. Neurath, G. Zur Frage des Vorkommens von *N*-Nitrosoverbindungen im Tabakrauch. Experientia, *23:* 400–404, 1967.
19. Sakshauge, V. J., Sögnen, E., Hansen, M. A., and Koppang, N. Dimethylnitrosamine; Its Hepatotoxic Effect in Sheep and Its Occurence in Toxic Batches of Herring Meal. Nature, *206:* 1261–1262, 1965.
20. Sander, J. Kann Nitrit in der menschlichen Nahrung Ursache einer Krebsentstehung durch Nitrosaminbildung sein? Arch. Hyg. Bakteriol., *151:* 22–28, 1967.
21. Sander, J. Nitrosamine Synthesis durch Bakterien. Z. Physiol. Chem., *349:* 429–432, 1968.
22. Sander, J., and Burkle, G. Induktion maligner Tumoren bei Ratten durch gleichzeitige Verfütterung von Nitrit und sekundären Aminen. Z. Krebsforsch., *73:* 54–66, 1969.
23. Sander, J., and Seif, F. Bakterielle Reduktion von Nitrat im Magen des Menschen als Ursache einer Nitrosamine-bildung. Arzneimittel-Forsch. *19:* 1091–1093, 1969.
24. Sander, V., Schweinsberg, F., and Menz, H. P. Untersuchungen uber die Entstehung cancerogener Nitrosamine im Magen. Z. Physiol. Chem., *349:* 1691–1697, 1968.
25. Sen, N. P., Smith, D. C., and Schwinghamer, L. Formation of *N*-Nitrosamines from Secondary Amines and Nitrite in Human and Animal Gastric Juice. Food Cosmet. Toxicol., *7:* 301–307. 1969.
26. Sen, N. P., Smith, D. C., Schwinghamer, L., and Howsam, B. Formation of Nitrosamines in Nitrite-treated Fish. Can. Inst. Food Technol. J., *3:* 66–69, 1970.

(82)

J. Fd Technol. (1972) 7, 111–124.

The Perigo effect in pork

J. ASHWORTH AND R. SPENCER*

Summary

Perigo, Whiting & Bashford (1967) showed that nitrite heated in a laboratory medium was more inhibitory to clostridia than was explicable by the nitrite level itself. They suggested that this effect may be involved in the safety of canned cured meats.

A suitable experimental procedure has been developed to enable the inhibitory effect of nitrite heated in meat to be investigated. Using this procedure a Perigo type effect has been shown with minced pork. The roles of physical conditions, and of chemical additives, in the formation of this inhibition, have been examined.

In most of the work described in this report *Clostridium sporogenes* NCTC 8594 was used as the test organism. Spores of this organism, and also vegetative and sporing cultures of *Cl. botulinum* (types A, B and E), together with some other strains of *Cl. sporogenes*, have also been tested in the meat/nitrite system.

Finally, the work described in this report is considered in the light of the still unexplained stability and safety of canned cured meat products which have only received a mild heat process.

Introduction

The stability and safety of canned cured meats which have received a relatively mild heat process—much less than that necessary for the stability and safety of canned uncured meats—are well established. Although the detailed mechanism of the stability and safety of these products is not yet known, it results from a number of factors, of which the presence of sodium nitrite in the cured meat is one. The literature on the antimicrobial effect of nitrite, with particular regard to clostridia and cured foods, has been extensively reviewed by Spencer (1969).

A new facet of the antimicrobial effect of nitrite was revealed by the investigations of Perigo *et al.* (1967). These workers used a model system of a complex bacteriological

Authors' address: British Food Manufacturing Industries Research Association, Randalls Road, Leatherhead, Surrey.

* Present address: J. Sainsbury Ltd., Stamford House, Stamford Street, London, S.E.1.

medium in place of meat and investigated the ability of sodium nitrite to inhibit the growth of *Clostridium sporogenes* at various pH values when a sterile solution of nitrite was added to the heat sterilized medium, and also when the nitrite was heated in the medium. They confirmed that the effect of unheated sodium nitrite was pH dependent and that 200–400 ppm sodium nitrite was necessary to inhibit the growth of *Cl. sporogenes* at pH values around neutrality. Additionally, they showed that as little as 3–5 ppm sodium nitrite heated in the medium for 20 min at 105–115°C, could inhibit growth, and that this inhibition was largely independent of the pH value of the medium.

Perigo *et al.* (1967) postulated that when nitrite was sufficiently heated in the basic medium, it was involved in a reaction resulting in the production of some unknown inhibitory substance. They suggested that such an inhibitor might play an important role in the stability of certain sublethally processed cured meats.

Perigo & Roberts (1968) examined the activity of sodium nitrite heated in the complex medium of Perigo *et al.* (1967), subsequently referred to as Perigo medium, against a range of clostridia including *Cl. botulinum* of various types, and obtained results similar to those obtained previously using *Cl. sporogenes*.

Johnston, Pivnick & Samson (1969) considered four possible roles for nitrite in the stability of cooked cured meats:

The enhanced destruction of spores by heat.

An increased rate of germination of spores during the heat process followed by death of the germinated spores.

The prevention of germination of spores that survived the heat process.

The production of an inhibitory substance from the reaction of nitrite with some component of the meat during the heat process.

Johnston *et al.* (1969) concluded that a Perigo inhibitor was probably not produced during the heat treatment of meat with nitrite and hence was of little relevance in explaining the role of nitrite in the safety of canned cured meats.

Labbe & Duncan (1970) while confirming the Perigo effect in media using *Cl. welchii*, were of the opinion that sodium nitrite does not combine with some component of heated cured meats to give an inhibitor. They suggested that sodium chloride and sodium nitrite, combined with heat and a low pH, alone maintain safety and stability in canned cured meats.

To summarize, a highly potent inhibitor of the growth of several species of clostridia is produced when sodium nitrite is heated in certain complex media. The nature of this inhibitor is unknown, and although there is some evidence that it is not of importance in the stability and safety of cooked meats, this evidence is far from conclusive.

The aim of the work described was to determine whether a Perigo effect could be

shown in meat, and whether such an effect could be involved in the safety of cooked, cured meats.

Preliminary work was concerned with establishing experimental procedures with meat. Using fresh pork, a bioassay, based on that used in media, has been developed. As in previous work, *Clostridium sporogenes* NCTC 8594 (NCIB 8053; PA 3679) was the main test organism.

In contrast to the findings of Johnston *et al.* (1969) a Perigo type effect has been shown in minced pork. Johnston *et al.* (1969) used sodium nitrite at levels up to 200 ppm in a 50% m/v slurry of meat in a bacteriological medium. With slightly higher levels of sodium nitrite introduced into whole minced pork, a situation similar to that described by Perigo *et al.* (1967) in their laboratory medium occurs, with nitrite heated in the system being more inhibitory than unheated nitrite. The Perigo effect in pork has been demonstrated with different muscle types and using *Cl. botulinum* types A, B and E as well as the normal test organism, and other strains of *Cl. sporogenes*.

Materials and methods

The standard meat bioassay

This was a modification of the original Perigo bioassay. The meat was minced twice through a 5 mm screen, and then filled, in 25 g amounts as compressed plugs, into 1 oz Universal bottles with serum caps. Half these bottles (tightly closed), were given the standard heat treatment of 115°C for 20 min in a domestic pressure cooker.

After heat treatment, these samples were divided into groups of at least five bottles. One group was taken as the controls; the other groups were inoculated aseptically via the caps with not more than 2 ml of a Millipore (HA membrane) sterilized sodium nitrite (AnalaR) solution, to give the desired nitrite levels (see Table 1).

The remaining unheated bottles were also divided into groups of at least five bottles. Each one of these groups was then inoculated with not more than 2·5 ml of the sodium nitrite solution, directly into the meat plug, to give the desired concentrations. After closure, these bottles were given the standard heat treatment.

All bottles were stored in the dark at room temperature for 18 hr to ensure even distribution of nitrite throughout the plug. At the end of this period a nitrite determination was carried out on the meat plug at each nitrite level, added either before or after heating.

All the remaining bottles were aseptically inoculated with *Cl. sporogenes*. An 18 hr culture of *Cl. sporogenes* in Robertson's cooked meat broth was diluted with an equal volume of uninoculated cooked meat broth, and 0·1 ml of this dilution inoculated directly through the serum cap, with a hypodermic syringe, into the meat plug. All inoculated samples were then incubated at 37°C, and examined daily for growth during the first week and subsequently at weekly intervals up to 1 year.

TABLE 1. The Perigo effect in pork

Initial sodium nitrite concentration (ppm meat)	Residual sodium nitrite at time of challenge (ppm meat)	Growth out of 4 replicates				
		Incubation period at 37°C				
		1 day	3 days	5 days	10 days	40 days
$NaNO_2$ added before heating						
150	32	2/4	4/4	4/4	4/4	4/4
200	36	1/4	4/4	4/4	4/4	4/4
300	66	0/4	2/4	2/4	2/4	2/4
400	108	0/4	0/4	0/4	0/4	0/4
500	112	0/4	0/4	0/4	0/4	0/4
600	129	0/4	0/4	0/4	0/4	0/4
$NaNO_2$ added after heating						
75	52	4/4	4/4	4/4	4/4	4/4
100	68	2/4	4/4	4/4	4/4	4/4
150	114	0/4	1/4	2/4	3/4	3/4
200	142	0/4	0/4	0/4	0/4	0/4
300	219	0/4	0/4	0/4	0/4	0/4
Controls		4/4				

Considerable gas production, haziness of the supernatant, and an increase in colour of the meat were used as indications of microbial growth.

The nitrite assay

When meat is heated with sodium nitrite up to 90% of the nitrite may be lost; further nitrite loss occurs during storage. Nordin, Rubin & Barnett (1966, unpublished data) reported half-lives of nitrite in stored meat of 200 hr at 20°C, and 80 hr at 37°C.

Because of these losses, a sensitive, and rapid, nitrite assay was essential to determine the residual nitrite contents of the meat/sodium nitrite systems at the time of challenge with the test organism. A modification of the method of Schall & Hatcher (1968) was adopted for use with meat.

Four grams of the minced meat were placed with 5 ml of an ammonia/hydrochloric acid buffer (pH 9·6) and approximately 100 ml of distilled water in a 250 ml flask, and heated for 60 min on a boiling water bath.

After this period soluble protein was precipitated by addition of a saturated solution of potassium aluminium sulphate, using bromocresol purple as indicator. At the grey end point (pH 5·8–6·4) precipitation of soluble proteins was complete. The solution

was filtered; 10 ml of the filtrate were placed in a 50 ml volumetric flask and about 10 ml of water added. One ml of a 5% m/v sulphanilamide solution in diluted hydrochloric acid was added and the solutions were mixed well. One ml of a 0·1% m/v aqueous N-1-naphthyl-ethylene-diamine dihydrochloride (NED) solution was added, the flask contents were made up to volume, allowed to stand at room temperature for 30 min, and the extinctions read at 540 nm.

Bacterial cultures

Normally, an 18 hr cooked meat broth culture of *Cl. sporogenes* NCTC 8594 was used to challenge the meat/nitrite systems. Spore cultures of this organism were prepared by the method described by the National Canners Association Research Laboratories (1968). Other clostridial strains were also used from time to time.

These experimental procedures were used as the basis of the work described in this paper; these conveniently fall into four categories:

(a) The demonstration of a Perigo effect in pork. Lean leg pork was used in the standard meat bioassay, with various levels of nitrite added before or after the heat treatment.

(b) The influence of additives on the Perigo effect in pork. Various additives were introduced into the meat bioassay system, and their effect evaluated.

(c) The influence of the heat treatment on the Perigo effect in pork. In addition to the standard heat treatment, the effect of other heat treatments upon inhibitor production was examined. Inhibitor production was also assessed, using the standard meat bioassay, after the meat had received a preliminary heat treatment.

(d) Other clostridial cultures. In addition to the standard test organism, i.e. vegetative *Cl. sporogenes* NCTC 8594, spores of this, and vegetative and sporing cultures of other clostridia, have also been evaluated in the meat test system.

Results

(a) *The demonstration of a Perigo effect in pork*

The results of one investigation using 150–600 ppm sodium nitrite added to pork before heating, and 75–300 ppm sodium nitrite added to pork after heating are shown in Table 1.

When the quantal growth response (from Table 1) was plotted against the residual nitrite level (Fig. 1) a considerable difference was seen between the effects of pre- and post-heat nitrite treatments. As a convenient method of quantifying the nitrite required for inhibition, the ED_{50} values were derived for this, and all subsequent work. (The ED_{50} value is defined as that level of residual nitrite which would bring about inhibition in 50% of the samples, based on the quantal response in sets of bottles at each nitrite concentration). From the graph the ED_{50} values after 3 days incubation were:

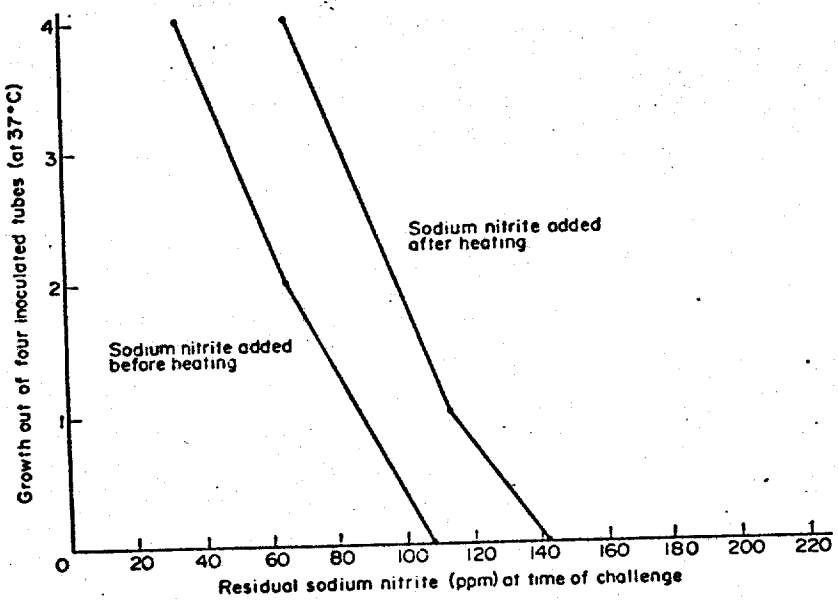


FIG. 1. Quantal growth response of *Cl. sporogenes* in minced pork with added sodium nitrite.

pre-heat nitrite – 66 ppm residual nitrite
post-heat nitrite – 99 ppm residual nitrite.

This shows that by adding the sodium nitrite before heating enhanced inhibition is seen, as might be expected from the work of Perigo *et al.* (1967).

This work has been repeated many times on both shoulder and leg pork, and comparable results obtained. A large difference in quantal response has been observed between different muscles of either the same or different types.

No correlation has been found between the ED_{50} sodium nitrite levels and the loss of nitrite during the heating process, in any of our investigations. Furthermore, no correlation was found between the ED_{50} values of sodium nitrite added either before or after heating, with the pH of the meat used (in the range pH 5·7–6·3)—see Table 2.

(b) *The influence of additives on the Perigo effect in pork*

Sodium thioglycollate is an ingredient of the medium used by Perigo *et al.* (1967) in their original work. It has been shown that this, or certain other reducing agents are necessary for the production of a Perigo effect in this medium (unpublished findings). Therefore the effect of the addition of reducing agents on the standard meat bioassay has been examined. Protein hydrolysates (also components of the original medium of Perigo *et al.*, 1967) and amino acids have been added to the standard meat bioassay, and their effects assessed.

TABLE 2. Comparison of pH values with the inhibitory nitrite levels in a minced pork/nitrite system

pH	ED_{50} values of $NaNO_2$ in meat (ppm)	
	$NaNO_2$ added before heating	$NaNO_2$ added after heating
5·7	46	62
5·8	130	175
5·9	76	170
6·0	28	no data
6·1	57	79
6·2	116	190
6·3	51	103

The results obtained using 0·1% m/v additions of reducing agents to this system are given in Table 3. Because cysteine and sodium ascorbate gave enhanced inhibition more detailed experiments were carried out. These confirmed that sodium ascorbate and cysteine (free base) produced an increase in inhibition, thioglycollate gave a slight increase, but with SFS and SFB there was a marked decrease in the inhibitory activity of the system.

In addition to cysteine, the effect of 0·1% m/v methionine, an amino acid mixture (detailed below), 1% m/v acid hydrolyzed casein (AHC), and 1% m/v Lab Lemco/Yeast extract (2 : 1 mixture) were all evaluated independently in the standard meat bioassay. The results given in Table 4 indicate that none of these additives markedly affected the production of inhibition. These results were confirmed in repeat

TABLE 3. The effect of various reducing agents on the ED_{50} values of sodium nitrite added to meat before heating

Additive (0·1% m/v)	ED_{50} values of $NaNO_2$ in meat (ppm)	
	$NaNO_2$ alone	$NaNO_2$ plus additive
Sodium ascorbate	57	14
Sodium thioglycollate	80	52
Sodium formaldehyde sulphoxylate (SFS)	80	> 130
Sodium formaldehyde bisulphite (SFB)	80	> 150
Cysteine (free base)	57	12

TABLE 4. The effect of the addition of amino acids and protein hydrolysates on the Perigo effect in meat

Additive	ED_{50} values of $NaNO_2$ in meat (ppm)	
	$NaNO_2$ alone—added before heating	$NaNO_2$ plus additive —added before heating
Methionine (0·1%)	47	>43
Amino acid mixture (0·1%)	57	48
AHC (1·0%)	57	54
Lab Lemco/Yeast extract (1·0%)	57	63

experiments, where it was also shown, that in combination with reducing agents, an actual loss in inhibitor production may occur.

The amino acid mixture contained:

L Lysine HCl	470 mg	L Cystine	10 mg
L Leucine	210 mg	DL Tryptophan	100 mg
L Tyrosine	4 mg	DL Serine	300 mg
L Glutamic acid	83 mg	L Histidine HCl	420 mg
DL-β-Phenyl-α-alanine	280 mg	DL Alanine	450 mg

During experiments with additives, the Eh of each system, and that of the control (meat alone plus nitrite) were measured using gold electrodes. The range examined was −50 to −150 mV, and in this range the inhibition produced could not be correlated with the Eh of the system.

(c) *The influence of the heat treatment upon the Perigo effect in pork*

In addition to using a heat treatment of 115°C for 20 min, other processing conditions were also used. The results of these various treatments are shown in Table 5. These results, which were basically confirmed by further experimentation, agree with results we have obtained using liquid media. Excessive heating appears to cause some loss in inhibition in the meat/nitrite system.

The results in Table 6 show that preheating pork at 115°C for 20 min before a standard meat bioassay does not significantly affect inhibitor production.

TABLE 5. The efficacy of different heat treatments in the production of a Perigo effect in pork

°C	Steam pressure (psi)	Time (min)	ED_{50} values as $NaNO_2$ added to the meat before heating	
			Initial*	Residual
108·5	5	30	234	138
115	10	20	300	117
121	15	15	300	139
		30	>500	148

*These figures are equivalent ED_{50} values based on initial nitrite levels.

(d) *Other clostridial cultures*

The results in Table 7 show that spores of *Cl. sporogenes* NCTC 8594, used in place of the normal vegetative culture initially behave in a manner similar to the vegetative culture. Subsequently they grew at much higher nitrite levels than did the vegetative cell inocula. After incubation for 42 days at 37°C, growth of the spores had occurred at all nitrite levels, in all three experiments. A comparable situation had not occurred with the vegetative culture, where a Perigo effect was still apparent.

TABLE 6. The Perigo effect in pork after preheating of the meat

Experiment	ED_{50} values of $NaNO_2$ in meat (ppm)			
	$NaNO_2$ added before heating		$NaNO_2$ added after heating	
A. Single heat treatment				
a	(300)	44	(133)	98
b	(350)	42	(75)	52
c	(250)	36	(75)	42
Mean	(300)	41	(94)	64
B. Double heat treatment				
a	(367)	43	(118)	85
b	(350)	30	(75)	76
c	(367)	31	(92)	59
Mean	(361)	35	(95)	73

The figures in parentheses are the equivalent ED_{50} values based on initial nitrite levels.

TABLE 7. The growth of *Clostridium sporogenes* spores in a heated meat/nitrite system

	Inoculum	ED_{50} values of sodium nitrite in meat (ppm)			
		6 days	17 days	28 days	42 days
$NaNO_2$ added before heating	Vegetative	<30	<30	30	48
		65	65	65	65
		44	44	46	48
	Spores	<30	152	>152	>152
		40	65	96	>129
		<40	65	168	>168
$NaNO_2$ added after heating	Vegetative	<56	<56	72	96
		105	105	105	105
		135	135	135	142
	Spores	<56	120	180	>196
		23	105	195	>235
		96	142	140	>314

Other vegetative cultures of *Cl. sporogenes*, (received from Dr T. A. Roberts, MRI, Bristol) were tested in place of the normal organism; the results in Table 8 show that a Perigo effect ultimately occurred with all the strains tested, i.e.:

1075, received by McClung (Indiana University) in 1952 from N.C.A., Washington, and believed to be an authentic strain of PA 3679.

1077, received by McClung (Indiana University) in 1952 from John Morrell & Co., and believed to be an authentic strain of PA 3679/ S_2.

MRI-13, a putrefactive anaerobe isolated from a processed meat product.

NCTC 532.

In Table 9 are the results obtained with vegetative cultures and also trypticase spore cultures (Schmidt & Nank, 1960) of *Cl. botulinum* types A, B and E used in the standard meat bioassay. These results clearly show that despite minor differences, both vegetative and spore cultures of the *Cl. botulinum* tested behave in a manner similar to the normal test organism. It is feasible, that with longer incubation, the *Cl.*

botulinum spores might eventually grow at all the sodium nitrite concentrations in a similar manner to *Cl. sporogenes* (NCTC 8594) spores.

TABLE 8. Comparison of the growth of various strains of *Clostridium sporogenes* after incubation at 37°C

Strain	Time of incubation at 37°C (days)	ED_{50} values of $NaNO_2$ in meat (ppm)	
		$NaNO_2$ added before heating Residual	$NaNO_2$ added after heating Residual
1075	18	59	108
1077	3	72	88
NCTC 532	3	72	152
MRI-13	3	65	118
NCTC 8594	3	33	88

Discussion

Perigo *et al.* (1967) postulated that the effect which they had observed, i.e. that the inhibitory activity of nitrite heated in a medium was very much increased, may be involved in the safety and stability of canned, cured, meats. The investigations reported here have shown that a similar effect to that observed by Perigo *et al.* (1967) in

TABLE 9. Comparison of the inhibition of vegetative cells and spores of *Cl. botulinum* types A, B, and E with vegetative *Cl. sporogenes*

	ED_{50} values of $NaNO_2$ in meat (ppm) after 5 days at 37°C			
	Vegetative form		Spore form	
	$NaNO_2$ added before heating	$NaNO_2$ added after heating	$NaNO_2$ added before heating	$NaNO_2$ added after heating
Cl. sporogenes NCTC 8594	71	114	no data	no data
Cl. botulinum A NCTC 7272	83	158	64	114
Cl. botulinum B NCTC 7273	71	130	67	156
Cl. botulinum E NCTC 8266	78	89	99	182

a bacteriological medium can be produced, under certain circumstances, in pork. In order to demonstrate a Perigo effect in meat a new experimental procedure, based on the original method used by Perigo *et al.* (1967) in a medium, was developed.

Because of the need to ensure uniform distribution of sodium nitrite in the meat, it was not possible to challenge the meat/nitrite system immediately after the heat treatment and/or the addition of nitrite, but only after overnight storage at 20°C. When sodium nitrite was heated with the meat there was a considerable loss in the assayable nitrite, and even with overnight storage at 20°C some nitrite losses occurred. The inhibition produced, following a loss of nitrite during heating, was compared with that produced when nitrite was aseptically added to the meat, by relating the inhibitory levels of assayable nitrite present at the time of inoculation with the test organism. Furthermore, in order to be able to detect any inhibitory effects of small differences in nitrite concentrations between these two systems, a series of nitrite levels (differing by 25–150 ppm) were used in each experiment, and the ED_{50} residual nitrite levels, based on a quantal response in four tubes, was used as the basis for the estimation of any inhibitory effect. The differences between the ED_{50} values when (i) nitrite was heated in the meat, and (ii) when nitrite was added after a heat treatment, were often small, and varied from experiment to experiment; in every case a Perigo effect was evident, i.e. in meat heated with nitrite greater inhibition was found than was explicable by the residual nitrite levels alone. This effect was consistently reproducible, but its magnitude differed considerably from the findings of Perigo *et al.* (1967) using media. At pH 6 Perigo *et al.* (1967) reported ED_{50} values of 8 and 52 ppm with the nitrite added before and after heating respectively. In pork, the corresponding ED_{50} values obtained were of the order of 60–70 ppm and 90–100 ppm residual nitrite (these represent input levels of about 300 and 150 ppm before and after heat treatment respectively).

When meat was heated with nitrite, the production of an inhibitory effect was relatively independent of the pH value. This is similar to the findings reported in media by Perigo *et al.* (1967). However, with the addition of nitrite to the meat without a subsequent heat treatment, the results differed. Here the inhibitory action of nitrite in medium was found to be influenced considerably by the pH of the medium: in meat no comparable effect was seen.

Johnston *et al.* (1969) used 50% suspensions of comminuted pork luncheon meat, thermally processed in cans. Meat processed without sodium nitrite, and then subsequently reheated in the medium with or without various levels of sodium nitrite up to 200 ppm failed to show a Perigo effect. This is understandable on the basis of the work described here because such input levels of nitrite would not be expected to produce inhibition after heat treatment. Johnston *et al.* (1969) also describe work carried out with slurries prepared from meat heated in cans with various sodium nitrite levels. It is interesting to note that in this case inhibition was found and that this was comparable to the findings reported here, i.e. the inhibition was related to the nitrite concentration

at the time of inoculation, and not necessarily to the level of sodium nitrite actually heated with the meat.

Although attempts were made to increase inhibitor production by the addition of various compounds to the meat/nitrite systems, no appreciable increases in the Perigo effect were observed.

Thus in summary the following points emerge. First, although other workers have failed to demonstrate a Perigo effect in meat, we have shown that such an effect can be produced in a well defined model system, based on that first used by Perigo *et al.* (1967) with a complex laboratory medium. Secondly, as yet a Perigo effect has been shown only in this model meat system. In this system attempts to appreciably magnify the effect, by altering the conditions for formation of inhibition, have failed. Thirdly, from the work reported here no evidence has been obtained which would indicate that a Perigo effect is likely to be involved, under practical conditions, with the safety and stability of canned cured meats. The joint effect of a heat treatment and the Perigo inhibitor on sublethally heated spores (analogous with the joint effect of a heat treatment and either sodium chloride or sodium nitrite, reported by Roberts & Ingram, 1966) was not investigated in this work. This, together with the low incidence of spores in canned cured meats, may on further examination indicate that some role should be ascribed to the Perigo effect in the safety and stability of canned, cured, meats.

Acknowledgments

Much of the work described in this paper would not have been possible without the assistance and advice of many members of the staff of the British Food Manufacturing Industries Research Association. The authors are indebted to all these colleagues, and especially so to Miss L. L. Hargreaves and Mr T. Wright for their invaluable assistance in the execution of much of the experimental work, and to the Director of the Research Association, Dr A. W. Holmes, for permission to publish these findings.

Acknowledgments are also due to Dr T. A. Roberts of the Meat Research Institute (Langford, Bristol) for kindly supplying some of the strains of *Cl. sporogenes* used in these investigations.

References

Johnston, M.A., Pivnick, H. & Samson, J.M. (1969) *Can. Inst. Fd. Technol. J.* 2, (2), 52.

Labbe, R. G. & Duncan, C. L. (1970) *Appl. Microbiol.* 19 (2), 353.

National Canners Association Research Laboratories. (1968) *Laboratory Manual for Food Canners and Processors, Volume I—Microbiology and Processing*, p. 166. Avi Publishing Company Inc., Westport, Connecticut.

PERIGO, J.A. & ROBERTS, T.A. (1968) *J. Fd Technol.* 3, 91.
PERIGO, J.A., WHITING, E. & BASHFORD, T.E. (1967) *J. Fd Technol.* 2, 377.
ROBERTS, T.A. & INGRAM, M. (1966) *J. Fd Technol.* 1, 147.
SCHALL, E.D. & HATCHER, D.W. (1968) *J. Ass. off. analyt. Chem.* 51, 763.
SCHMIDT, C.F. & NANK, W.K. (1960) *Fd. Res.* 25, 321.
SPENCER, R. (1969) *Food Poisoning due to Clostridia: the Factors Affecting the Survival and Growth of Food Poisoning Clostridia in Cured Foods.* B.F.M.I.R.A. Scientific and Technical Survey No. 52. British Food Manufacturing Industries Research Association, Leatherhead, Surrey.

(*Received* 15 *October* 1971)

MEDICAL INTELLIGENCE

BRIEF RECORDING

Sausage Cyanosis — Acquired Methemoglobinemic Nitrite Poisoning*

SURAJ P. BAKSHI, M.D.,† JOHN L. FAHEY, M.D.,‡ AND LAWRENCE E. PIERCE, M.D.§

BUFFALO, NEW YORK

KISZKA (kish-ke), or blood sausage, is a common table food among Polish nationals in western New York. It is composed of fresh pork and a coarse cereal filler to which beef blood, seasonings and both sodium nitrite and nitrate are added before the mixture is stuffed into casing. Five patients with unexplained sudden cyanosis with "cardiovascular collapse" shortly after ingestion of sausage have been admitted to regional hospitals. The purpose of this report is to describe another patient in whom more intensive investigation gave indirect evidence that nitrite poisoning was responsible for methemoglobinemic cyanosis.

CASE REPORT

A 48-year-old factory worker was admitted to the hospital on November 5, 1966, with intense cyanosis. Twenty minutes earlier he complained of increasing nausea, became vertiginous, vomited 3 times, collapsed to the pavement and turned a bluish color. There was no previous history of cardiopulmonary disease. Except for generalized cyanosis, physical examination was unremarkable.

On examination the blood pressure was 100/70, the respirations 24, the pulse regular at a rate of 140 per minute, and the temperature 100.5°F.

The hematocrit was 41 per cent, and the white-cell count 21,200, with 88 per cent neutrophils and 7 per cent band forms. An electrocardiogram showed supraventricular tachycardia. X-ray study of the chest and ^{131}I macroaggregated albumin lung scan were negative. Arterial-blood pH, carbon dioxide tension and total venous carbon dioxide were normal. Arterial oxygen saturation, expressed as the proportion of total heme pigment, was 50 per cent, rising to 80 per cent after 3½ hours (inhalation of 100 per cent oxygen) and to 97 per cent on the following morning, when the patient was comfortable and acyanotic. Methemoglobin determination was 4.1 gm and 4 days later 0.7 gm per 100 ml. A detailed review of the previous days' activities revealed ingestion of approximately 1 pound of kiszka 30 minutes before the symptoms began. The patient had eaten a similar amount on many separate occasions over the past 35 years. A residual portion of the same sausage ring from which he had eaten showed a total nitrite concentration of 0.181 mg per gram of meat (the maximum allowable limit being 0.200 mg per gram of meat). Bacteriologic culture of the sausage grew no pathogens. The patient's erythrocyte DPNH-linked methemoglobin reductase activity by the dichlorophenolindophenol assay of Scott[1] was normal. The blood was then nitrited, and the rate of methemoglobin reduction measured with the use of glucose as substrate. Reduction of methemoglobin was normal both with and without methylene blue. The patient was discharged as recovered after 5 hospital days.

DISCUSSION

In the preparation of kiszka the nitrite-nitrate is used primarily as a curing agent but also acts as a preservative and color stabilizer. Near maximum permissible levels of nitrite are sought to preserve the bloody appearance since color represents a desirable marketing characteristic of the product. Although there was an acceptable nitrite concentration in the residual portion of kiszka examined, the suspicion that the ingested portions contained poorly distributed nitrite clusters sufficient to induce acute nitrite poisoning remained the best explanation. This conclusion is strengthened by the facts that the patient had often previously eaten kiszka without adverse effect, the meat was bacteriologically nonpathogenic, there was a normal in vivo and vitro capacity to reduce methemoglobin, and the symptoms were consistent with those of acute nitrite poisoning.[2,3]

SUMMARY

A previously healthy patient had an acute episode of acquired methemoglobinemia mimicking cardiopulmonary catastrophe that was indirectly shown be due to nitrite poisoning; the nitrite was being used as a curing and preservative agent in a locally manufactured Polish speciality sausage named kiszka

REFERENCES

1. Scott, E. M., and McGraw, J. C. Purification and properties of diphosphopyridine nucleotide diaphorase of human erythrocytes. *J Biol. Chem.* 237:249-252, 1962.
2. Gleason, M. N., Gosselin, R. E., and Hodge, H. C. *Clinical Toxicology of Commercial Products: Acute poisoning (home and farm)* Second edition. 1211 pp. Baltimore: Williams & Wilkins, 1963. Pp 116-118.
3. Roueché, B. *Eleven Blue Men and other Narratives of Medical Detection.* 215 pp. Boston: Little, Brown, 1954.

*From the Department of Hematology, Mercy Hospital (requests for reprints should be addressed to Dr. Pierce at 565 Abbott Road, Buffalo, New York 14220).

†Chief medical resident, Mercy Hospital.

‡Associate in hematology, City of Hope Medical Center, Duarte, California.

§Director of hematology, Mercy Hospital; instructor in medicine, State University of New York at Buffalo.

113 ✓ 20-12

THE

AMERICAN JOURNAL

OF

PHYSIOLOGY

VOLUME LXXXI

BALTIMORE, MD.
1927

64726

113

THE INFLUENCE OF SODIUM NITRITE, PEPTONE AND PILOCARPINE ON THE EXTERNAL SECRETION OF THE PANCREAS

A Correlation of the General and Splanchnic Vascular Changes with the Increased Pancreatic Secretion Rate

O. W. BARLOW

From the Hull Physiological Laboratory of the University of Chicago

Received for publication November 24, 1926

In an earlier publication, in which the vascular and pancreatic secretory changes following the vein administration of standard secretin preparations were correlated (Barlow, 1926) it was observed that a unit dose of a control secretin, containing a small amount of vasodilatins, showed a higher secretagogue efficiency than an equivalent dose of a vasodilatin-free secretin (Weaver et al., 1926) made from a portion of the control extract..

The present study was undertaken in an attempt to clarify the above assumption, i.e., to determine whether vasodilatins per se may actually be additive with pancreatic secretins and whether they may act as pancreatic secretagogues as postulated by Popielski (1912).

Since the vasodilatin principles as represented by certain portions of intestinal mucosal extracts are of relatively unknown character, it was deemed advisable to repeat the work of Popielski (1912) with Witte's peptone and to duplicate the results through the use of a known chemically pure vasodilatin substance, i.e., sodium nitrite. The responses obtained from peptone and sodium nitrite were then compared with two other types of pancreatic secretagogues, i.e., Bayliss and Starling secretin and pilocarpine respectively, with the idea of interpreting the mode of action of the vasodilatins. The usual pancreatic secretory responses obtained with them were then compared with those obtainable after cutting the cervical vagi and after atropine and ergotamine tartrate respectively.

Methods. During the course of study, 36 dogs ranging from 15 to 20 kilograms in weight were used. Eighteen to 24 hours after feeding, the animals were placed under a light ether anesthesia, preliminary to the administration of the general hypnotic. The femoral vein was quickly isolated, and a cannula inserted and connected with a burette for washing in injections. The ether mask was then removed and barbital sodium, in a dosage of 225 mgm. per kilo body weight, slowly injected. The

animals were thus maintained under very constant conditions as to anesthesia, blood pressure and respiration rate during the succeeding 10 to 12 hours of the experiments.

General blood pressure was recorded from the carotid artery by the usual methods. The splanchnic vascular changes were recorded from the kidney volume by means of an egg-shaped oncometer, each inner half of which was fitted with a rubber dam, the graphic record of the volume changes being obtained by air transmission from the oncometer to a tambour.

The pyloric valve was tightly ligated in all except a few experiments. In the latter a loose ligature was placed around the pylorus and the ligature ends led through the abdominal wall by means of a metal tube similar to that frequently used for constricting arteries. In this manner the pylorus could be ligated or freed at will simply by varying the tension on the ligature ends, with the minimal disturbance to the animal. The duodenum was then isolated and a small glass cannula inserted in the duct of Santorini and ligated in place, the pancreatic juice being led to the exterior by means of a short rubber tube attached to the cannula. The duodenum was then replaced in the abdomen and all incisions closed by sutures.

Results. In order to have some standard criteria by which to judge the secretagogue efficiency and the vascular changes produced by the preparations to be tested, a standard dose of Bayliss and Starling (1902) secretin, representing 0.034 gram of the original intestinal mucosa per kilo body weight, was used as a control, the same secretin preparation thus being compared with the respective secretagogues on each dog of a series.

The usual course of an experiment was to obtain a simultaneous graphic record of the blood pressure, kidney volume and pancreatic secretory rate during a ten-minute period. A secretin injection was then made and the observations continued until the blood pressure and secretory rate returned to that immediately preceding the injection. A dose of the vasodilatin was then injected and the observations repeated during the succeeding 40 minutes.

Sodium nitrite. The general and splanchnic vascular changes obtainable after the vein administration of 15 mgm. of sodium nitrite are shown and compared with those obtainable from a standard dose of secretin (fig. 1). The general blood pressure after nitrite shows the typical gradual fall to a maximum at the 5th minute with a gradual progressive recovery thereafter. The splanchnic vascular changes, including the pancreas, show a definite dilatation and in spite of the slight decrease in kidney volume after the 2nd minute, the splanchnic vasodilatation is even more evident when the blood pressure level is observed.

The increased pancreatic secretion following the administration of sodium nitrite shows a latent period and a maximal peak corresponding to

that of the control secretin injection. Likewise the nitrite secretory period corresponds to that observed for secretin in that the secretory values bear an inverse relation to the general blood pressure values. Consequently, since the blood pressure was depressed by sodium nitrite over a period longer than that of the secretin, the secretory values remain above normal correspondingly.

The external pancreatic secretory rate was increased with all dosages of sodium nitrite studied, i.e., 2 to 300 mgm. The secretory efficiency increased with the dosage up to a maximum which was obtained with an optimum dosage of 20 mgm. With larger doses the secretory efficiency decreased from the maximum, inverse in degree to the size of the dose administered.

Gottlieb (1894), in a study of the relations between the pancreatic blood supply and the secretion rate, concluded that secretion seemed dependent on the amount of blood coming to the gland and quotes Heidenhain to the effect that the connection between glandular activity and vessel width was unmistakable. It seems quite probable that the most important factor governing the external pancreatic secretion, following the administration of sodium nitrite is the total blood supply to the gland and this in turn directly dependent on the degree of splanchnic vasodilatation (Burton-Opitz, 1910; Sollmann, 1922) and the general blood pressure level. Low blood pressure in itself does not necessarily favor pancreatic secretion but dilatation of splanchnic vessels certainly favors an optimum secretion. Consequently with the larger nitrite dosages administered, the marked and prolonged fall of blood pressure tended to embarrass the general functions, including the pancreas, irrespective of the marked splanchnic dilatation as shown with different methods by Franck and Hallion (1896).

It is of interest to note in comparison with the nitrite reactions that frequently barium chloride has been observed to increase the pancreatic secretory rate through a vasodilatation of the pancreatic vessels (Edmunds, 1910-11).

The nitrite secretagogue action may be obtained following the injection of sodium nitrite either initially, i.e., the first injection in any dog, or the total secretion obtainable with a certain dosage may be duplicated in sequence over a period of three hours; further, the volume of juice obtained at the beginning of an experiment compares favorably with that obtainable under the same conditions twelve hours later.

The pancreatic responses obtainable, both preceding and following ligation of the pyloric valve, or the intravenous administration of large doses of atropine, or ergotamine tartrate, 1 mgm. per kilo body weight, and repeated at intervals, are of the same order of magnitude. In two out of twenty-nine injections in six different dogs, an optimal dose of sodium nitrite gave a greater secretory response preceding ligation of the pylorus

than was obtained afterwards. This response, obviously due to the expulsion of a small amount of acid from the stomach (Pawlow, 1912; Barlow, 1926) occurred near the end of the nitrite response and must be considered exceptional.

Synergism of pancreatic secretins and vasodilatins. The assumption that the vasodilatin and secretin fractions of standard secretin preparations are, within limits, additive, was tested out on 4 animals. The responses, obtainable in sequence, to a single dose of Bayliss and Starling secretin; 15 mgm. of sodium nitrite; to a dose of Bayliss and Starling secretin plus 15 mgm. of sodium nitrite, and to two doses of Bayliss and Starling secretin, were compared in figure 2.

The marked differences between the pancreatic secretory curve of the combined secretin and nitrite injection, curve *C*, and those of *A*, *B* or *D* might apparently be explained by the greater and more prolonged depression of the general blood pressure. The secretin contained two fractions, i.e., vasodilatin and pancreatic secretin (Weaver et al., 1926). Presumably the combined vasodilatation produced by the secretin vasodilatins and the sodium nitrite resulted in a secretion through purely vascular changes as well as furnishing the optimal pancreatic secretory conditions for the secretin fraction.

Peptone. The definite secretagogue reaction of the pancreas to the vein administration of Witte's peptone and to a lesser degree, Merck's peptone as well, figure 3, confirms the results of Popielski (1912) and Skarżynska (1924).

The general and splanchnic vascular responses to peptone are comparable with those obtained with sodium nitrite, the greatest difference being the degree of the peptone blood pressure depression. As a consequence, the pancreatic secretory response following the administration of Witte's peptone bears an inverse relationship to the blood pressure level during the first 20 minutes only, at which time the secretory rate has returned to normal while the blood pressure is only 80 per cent normal and the splanchnic vasodilatation maximal (Kondo, 1919).

The only explanation offered for these minor differences between the actions of the nitrites and peptones is an apparent difference in dosage and the complex character of the latter which, according to Hanke and Koessler (1920), consists mainly of proteoses, some peptone and various quantities

---

All graphs represent the median data for all dogs of each series.

Figs. 1–4. Blood pressure computed in per cent of level preceding injection.

Kidney volume—a change of ± 1 mm. in the oncometric graphic record was arbitrarily taken as equivalent to ± 1 per cent.

Pancreatic secretory rate—

$$\frac{X - \text{rate in drops per minute at a given period}}{N - \text{normal rate in drops per minute}}$$

to the expul-
2; Barlow,
must be con-

assumption
preparations
responses,
g secretin;
secretin plus
ng secretin,

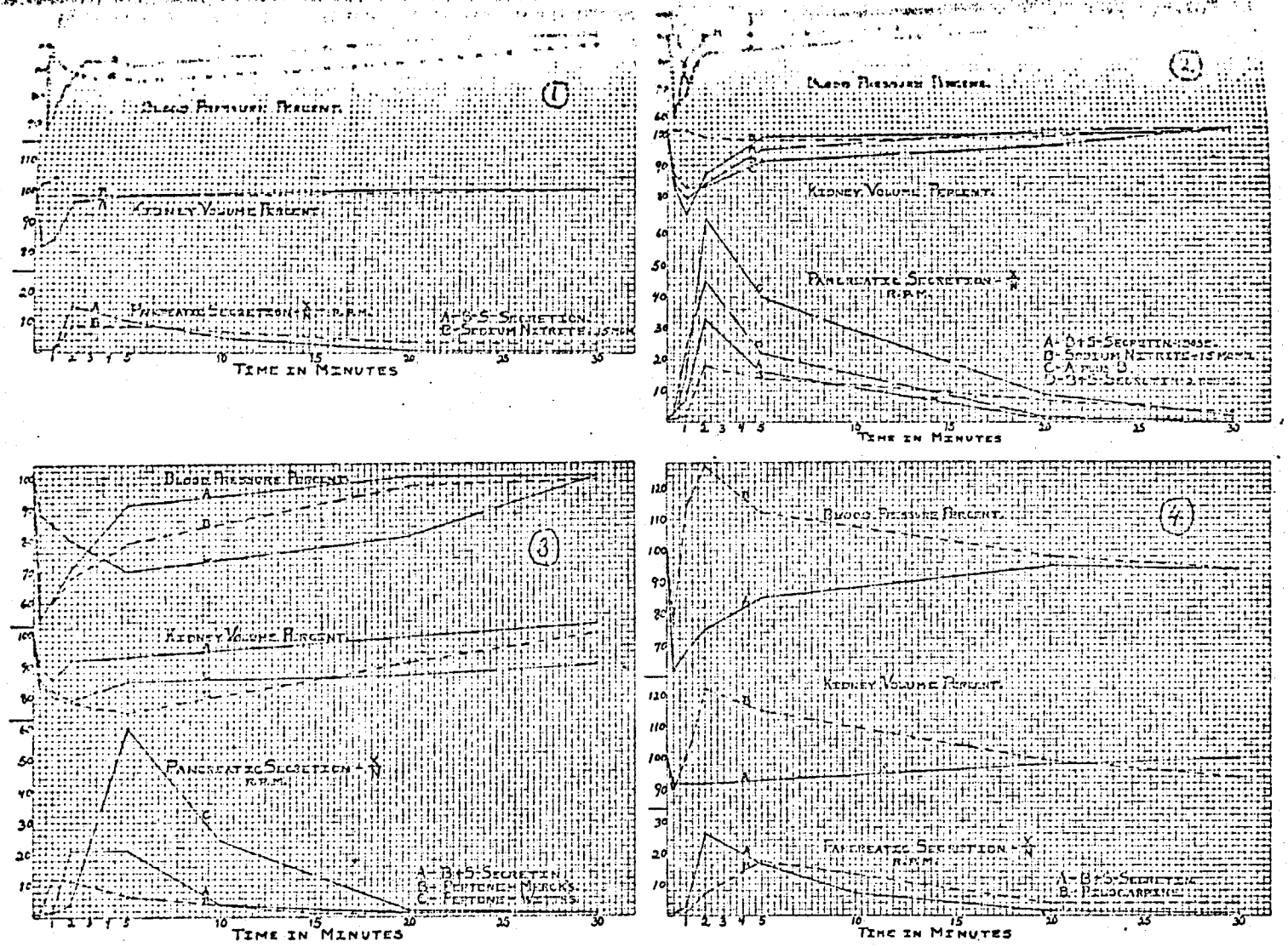
curve of the
A, B or D
ed depres-
wo fractions,
726). Pre-
a vasodila-
rely vascular
conditions

s to
vein
's peptone
karzynska

omparable
, being the
equence, the
of Witte's
during the
returned to
e splanch-

between the
losage and
d Roessler
us quantities

injection.
record was



of histamine. In this connection it may be observed that although the secretory efficiency of Merck's peptone was found to be much greater than similar data presented by Skarzynska (1924), the pancreatic secretory response to Merck's peptone, as observed by the author, compares favorably with similar data by Skarzynska (1924), Parsons (1925) and Gutkowski (1926) for histamine.

The peptone secretagogue efficiency follows a curve similar to that of sodium nitrite, in that the efficiency increases with the degree of vasodilatation up to a maximum obtained with a dosage of 30 cc. of a 5 per cent solution and decreases with higher dosages.

Pilocarpine. The comparison of the vascular and pancreatic secretory responses to secretin and pilocarpine respectively, as shown by figure 4, gives the following contrasts. The general blood pressure and organ volume changes following the administration of pilocarpine, irrespective of the initial cardiac slowing, are directly opposite those obtainable with secretin. The splanchnic organ volume changes after pilocarpine follow the blood pressure almost exactly, while secretin, on the other hand, produces a passive vasodilatation of the splanchnic region similar to the nitrites.

The secretin secretory curve, in rapidly attaining a maximum and gradually decreasing to normal by approximately the 20th minute after administration, differs widely from that obtained with pilocarpine. With the latter, a longer latent period was observed, as previously shown by Bayliss and Starling (1904), the maximal secretion peak was reached later and the augmented secretion continued for 45 to 75 minutes.

By the administration of atropine it was possible to stop the pancreatic secretion induced by pilocarpine at any stage within a period of 30 to 90 seconds. The further administration of pilocarpine, following atropinization, had no demonstrable effect on the blood pressure or the pancreatic flow even though a total dosage of pilocarpine equivalent to 500 times that of the atropine, previously injected, was administered.

SUMMARY. It has been shown that the intravenous administration of several vasodilatins including sodium nitrite, Merck's peptone, Witte's and histamine results in a greater or less external secretion of the pancreas as compared with secretin. This pancreatic stimulation is paralleled by a greater or less fall in blood pressure, depending on the character and dosage of the vasodilatin injected, and an increased splanchnic blood flow and organ volume. The secretagogue response to sodium nitrite is not influenced by ligation of the pylorus, cutting the cervical vagi, or the administration of either atropine or ergotamine in large doses. As a consequence, the mechanism resulting in the stimulated pancreatic flow seems closely associated with the improved blood supply of the pancreas as a result of the general splanchnic vasodilatation.

It may be pointed out that a similar increase in the splanchnic organ

volume as well as pancreatic blood flow was observed following the introduction of dilute HCl into the duodenum (Burton-Opitz, 1920; Barlow, 1926) and likewise resulted from the vein administration of vasodilatin-free secretin. It is possible, therefore, that the splanchnic vascular change observed is one of the important chemical responses resulting from the normal expulsion of acid chyme into the duodenum (Pawlow, 1912), and consequently may be the intermediary or the end point of one of the "causes of the external secretion of the pancreas" (Ivy, 1926). Aside from the fact that a variety of substances, including nitrites, proteoses, cleavage products such as histamine and various intestinal mucosal extracts, produce a comparable vascular response as well as pancreatic secretory responses, a number of questions are raised as to certain physiological responses under conditions of hypotension.

The vascular and pancreatic secretory responses following the administration of pilocarpine have been correlated with similar data from comparable dosages of a standard pancreatic secretin. The splanchnic vascular changes observed paralleled very closely the general blood pressure curve; presumably, the splanchnic vessels were not affected noticeably by pilocarpine since no vascular response of any kind, within limits, was obtainable after atropine.

Pilocarpine stimulated the external pancreatic flow irrespective of ligation of the pyloric valve, as observed by Camas and Gley (1913). Since the vascular and pancreatic secretory responses following the injection of pilocarpine may be entirely inhibited by atropine, it appears that the point of attack of pilocarpine, in producing a copious external secretion of the pancreas, is not on the nerves of the intestine or even through the production of secretin as maintained by Hustin (1920), since the pancreatic secretion produced by secretin or even by placing dilute HCl in the intestine is wholly unaffected by the administration of atropine either by vein or subcutaneous injection (Bayliss and Starling, 1902; Farrell and Ivy, 1926).

CONCLUSIONS

1. It has been shown that vasodilatins per se are both additive and synergistic with standard pancreatic secretin preparations.

2. Peptone, histamine and sodium nitrite increase the external pancreatic secretory rate presumably by improving nutritional conditions, i.e., blood supply to the splanchnic organs, since the secretory rate subsequent to the administration of sodium nitrite was unaffected by cutting the cervical vagi, ligation of the gastric pylorus, or the administration of atropine or ergotamine.

3. The general and splanchnic vascular changes have been correlated with the pancreatic secretory rate following the administration of pilo-

carpine. Further proof of the mechanism of the pilocarpine secretagogue action has been presented.

BIBLIOGRAPHY

Barlow, O. W. 1927. This Journal, (in press).

Bayliss, W. M. and E. H. Starling. 1902. Journ. Physiol., xxix, 31.
1904. Journ. Physiol., xxx, 61.

Burton-Opitz, R. 1920. Wien. med. Wochenschr., xlii, 1.

Camas, L. and E. Gley. 1913. Arch. int. d. Physiol., xiii, 102.

Dale, H. H. and P. P. Laidlaw. 1910. Journ. Physiol., xli, 318.

Edmunds, C. W. 1910–11. Journ. Pharm. Exper. Therap., ii, 559.

Farrell, J. I. and A. C. Ivy. 1926. This Journal, lxxviii, 325.

Franck, F. and L. Hallion. 1896. Compt. rend. d. soc. d. biol., 561.

Gutkowski, B. 1926. Abs. commun. to 12th Int. Physiol. Congress.

Gottlieb, R. 1894. Arch. f. exper. Path. u. Pharm., xxxiii, 265.

Hanke, M. T. and K. K. Koessler. 1920. Journ. Amer. Med. Assoc., lxxv, 1227.

Harnack, E. and H. Myer. 1880. T. Sollmann's Manual of pharmacology, 1922.

Hustin, A. 1920. Travaux d. Lab. d. physiol., 1914–20, xiii, 345.

Ivy, A. C. 1926. Ann. Clin. Med., iv, 798.

Launoy, L. 1905. Arch. int. d. Physiol., iii, 62.

Olivecrona, H. 1921. Journ. Pharm. Exper. Therap., xvii, 143.

Parsons, E. 1925. This Journal, lxxi, 479.

Pawlow. 1912. Work of the digestive glands.

Popielski, L. 1912. Münch. Med. Wochenschr., lix, 534.

Skarzynska, M. 1924. Compt. rend. d. l. soc. d. biol., xc, 1476.

Weaver, M. M., A. B. Luckhardt and F. C. Koch. 1926. Journ. Amer. Med. Assoc., lxxxvii, 640.

Brit. J. industr. Med., 1954, 11, 167.

116

SOME TOXIC PROPERTIES OF DIMETHYLNITROSAMINE

BY

J. M. BARNES and P. N. MAGEE

From the Toxicology Research Unit, Serum Research Institute, Woodmansterne Road, Carshalton Beeches, Surrey

(RECEIVED FOR PUBLICATION MAY 22, 1954)

The appearance of two cases of cirrhosis of the liver among three men working in the research laboratory of a large industrial undertaking led to suspicion becoming attached to dimethylnitrosamine (D.M.N.) as the causative agent. Dimethylnitrosamine had been introduced into the laboratory as a solvent about 10 months before the first case of cirrhosis was discovered. One of the men had died of bronchopneumonia, and cirrhosis of the liver was an incidental finding at necropsy. The second man with a long-standing history of dyspepsia had a laparotomy after five months' exposure to D.M.N. At that time no note of any abnormality of the liver was made. Six months later he was examined for an incisional hernia that had developed in the operation scar. It was then recorded that the liver felt hard and was suspected of being cirrhotic. Liver function tests carried out at this time showed evidence of damage. When repeated three months later after all further exposure to D.M.N. had ceased these tests indicated an improved liver function. The third man has remained in good health but has had no liver function tests carried out on him.

A search of the literature revealed only a single short report of the toxic properties of D.M.N. (Hamilton and Hardy, 1949). Dimethylnitrosamine had been used in an automobile factory and some illness had followed. Experiments on dogs showed it to be capable of producing severe liver injury, but no experimental details are provided. The medical officer of the firm therefore asked if we would examine the toxic properties of D.M.N. towards laboratory animals.

Dimethylnitrosamine, $\begin{matrix}CH_3\\CH_3\end{matrix}\!\!>\!N.NO$, is a mobile yellow liquid with a faint, characteristic odour. It boils at 151° C. and its specific gravity is 1·015 at 20° C. It is miscible in all proportions with water, ethyl alcohol, methylene chloride, or vegetable oil. Under ordinary conditions it is a stable substance, but with zinc dust and acetic acid it can be reduced to dimethylhydrazine $\begin{matrix}CH_3\\CH_3\end{matrix}\!\!>\!N.NH_2$.

Preliminary experiments revealed that D.M.N. readily produced a severe liver injury and this paper gives an account of some of the features of the lesions.

Materials and Methods

The D.M.N. was a sample of pure material specially prepared by the firm who had both made and used the D.M.N.

Male and female albino rats were used in the majority of the experiments. Additional observations were made on mice, guinea-pigs, rabbits, and dogs.

For single oral doses a solution of D.M.N. in water was administered. For injection either an aqueous solution was used or the undiluted D.M.N. was given by a micro-syringe. Percutaneous absorption was studied on rats depilated with barium sulphide. The D.M.N. was applied undiluted and the skin left uncovered. The rats were placed in small individual wire cages for four hours after each dose so that they could not lick themselves.

For the feeding experiments solutions of D.M.N. were made up in arachis oil. These solutions were added at the rate of 10 ml./kg. of powdered rat diet (M.R.C. diet 41).

For histological work animals were killed with coal gas unless already dead. Tissues were fixed in 10% formol-saline and in Helly's fluid. Paraffin sections cut at 7μ were stained by haematoxylin and eosin, by the van Gieson and the Mallory azocarmine methods for connective tissue, Gomori's silver method for reticulin, and by the periodic-acid-Schiff technique (McManus, 1946). Frozen sections of material fixed in formol-saline were stained with Sudan IV. Sections of pancreas, after fixation in Bouin's fluid, were stained by the aldehyde fuchsin and the chromium haematoxylin phloxin methods (Gomori, 1941, 1950).

Results

Acute Poisoning.—In all species examined a single lethal dose of D.M.N. by mouth or by injection

produced extensive liver necrosis but the differences in the reactions of each species merit a separate description.

Rats.—The L.D. 50 by intraperitoneal injection was 26·5 (20·5–34·2) mg./kg. calculating the results by the method of Weil (1952). Although the response of rats to an oral dose varied somewhat in different experiments, the average lethal dose was less than 50 mg./kg. The rats never died in less than two days or more than six days after a single oral dose. They appeared ill, sitting quietly with ruffled coats, but displayed no other sign of poisoning except that abdominal swelling was visible in some animals surviving several days.

At necropsy there was a variable amount of blood-stained fluid in the peritoneal cavity. The volume might exceed 30 ml. and sometimes had a colour and consistency approaching that of pure blood. The omentum and pancreas often had a jelly-like oedema in their interstices, and subcutaneous oedema over the abdominal wall was occasionally seen. Haemorrhage into the lumen of the upper part of the intestine was sometimes observed. The liver was swollen, dark and mottled, and somewhat friable. Apart from a little clear pericardial and pleural exudate in some animals there were no other lesions visible to naked-eye examination. Haemorrhage was confined to the peritoneal cavity and lumen of the gut. In a number of animals where this was pronounced, the possibility that cerebral haemorrhage might have occurred was excluded by direct examination.

A dose of 40 to 50 mg./kg. had a similar effect whether given by mouth, by intravenous, intraperitoneal, or subcutaneous injection. But acute poisoning could not be produced by percutaneous application of undiluted D.M.N. A dose of 100 mg./kg. applied on four successive days had no effect on the general condition of six rats.

Rabbits.—A dose of 15 mg./kg. killed rabbits in less than 24 hours. Those given 10 mg./kg. either died in less than 24 hours or survived at least 10 days. A straw-coloured peritoneal exudate was usually present at death but it was never haemorrhagic. The liver was large, dark, and friable but no other lesions were visible to naked-eye examination.

Mice.—Mice behaved like rabbits. Death always took place within 30 hours and all mice receiving 20 mg./kg. died. Any peritoneal fluid present at death was clear. The livers were swollen, red, and mottled.

Guinea-pigs.—Eight animals receiving doses of 25 to 50 mg./kg. all died, but none in less than four days or more than eight days after the D.M.N. In the majority a haemorrhagic peritoneal exudate was present at death and the liver was congested and mottled.

Dogs.—One dog was given a single oral dose of 50 mg./kg. An hour later it vomited several times. Next morning it was conscious but unable to stand and died quietly 20 hours after receiving the D.M.N. At necropsy the peritoneum contained about 100 ml. of bright red blood and the stomach and gut were full of blood, some obviously recently shed. There were no other lesions or haemorrhages. The liver was not grossly abnormal to naked-eye examination.

A second dog died in less than 24 hours after a single oral dose of 20 mg./kg. Again it vomited repeatedly soon after the D.M.N. Within six hours it was comatose though moved when touched. At necropsy there was no peritoneal haemorrhage but the stomach and upper parts of the gut contained much bright red blood. The liver appeared congested.

A third dog received 10 mg./kg. by mouth on three separate occasions. Eight days elapsed between the first and second doses and four days between the second and third. It vomited about an hour after the first two doses. It was not obviously ill until the seventh day after the last dose, when it was found unconscious in the morning. At necropsy there was about 100 ml. of fluid resembling pure blood in the peritoneal cavity. The stomach and small intestine contained a black fluid and the bowel contained typical melaena. There were no erosions in the stomach. The mesenteric lymph nodes were red. The liver was firm and tense; the cut surface was mottled and had a "nutmeg" appearance. There was no obstruction or gross lesion within the lumen of the hepatic vein. The pleural and pericardial cavities and the lungs were healthy.

Subacute Effects.—Rats that lived for seven days or more after a dose of 20 to 40 mg./kg. frequently appeared ill and ate little. They had a pronounced abdominal swelling due to fluid that could be aspirated. This fluid accumulation masked any loss of weight due to emaciation. The liver was of variable size and often irregular in outline. In some animals it showed evidence of fibrosis.

Two rabbits surviving a dose of 10 mg./kg. appeared ill 10 days later and at necropsy both had some peritoneal exudate and small, symmetrically shrunken livers.

Chronic Poisoning.—Chronic poisoning was produced by giving a diet containing 50, 100, and 200 parts per million (p.p.m.) D.M.N. to groups

of six young rats. The reactions of the groups were as follows :—

50 *p.p.m.*—After 110 days on the diet the mean weight of the group on D.M.N. was significantly lower than that of the control group. No rats died nor was anything abnormal noted at necropsy on those killed at this time.

100 *p.p.m.*—All the rats died after 62 to 95 days on the diet. At 60 days the mean weight of the group was only 65% of that of the controls.

200 *p.p.m.*—All rats died or were killed *in extremis* after 34 to 37 days on the diet. At necropsy all rats in the last two groups showed extreme emaciation and complete absence of body fat. The food intake was unaffected during the first two weeks on these diets indicating that D.M.N. in the concentration used did not reduce the palatability of the diet. Emaciation must have been the result of the toxic effects of D.M.N. Gross peritoneal exudate was not seen in these animals but in some there was evidence of recent haemorrhage into the gut. The retroperitoneal and retrosternal lymph glands were sometimes red. The liver was small and in those dying later there was obvious fibrosis. The pleural surfaces of the lungs often showed a number of discrete brown stains scattered over them.

Histological Observations

Liver.—A detailed account of the changes seen in the rat is given and findings in the other species briefly compared. A single dose of 5 to 10 mg. D.M.N./kg. produced no significant damage, but a dose of 20 mg./kg. or above led to characteristic changes. Within six hours there was a pallor of the centrilobular and midzone regions where the liver cells showed an eosinophilia of the cytoplasm greater than in those cells in the periportal zones. The central veins were moderately congested and the sinusoids dilated. Fat staining showed a deposition of fine sudanophilic droplets in the cells of the centrilobular areas. In the same area glycogen was depleted.

At 18 hours the changes were more conspicuous though still confined to the centrilobular and midzone regions. The parenchymal cells were obviously damaged. The cytoplasm was amorphous and eosinophilic, frequently vacuolated but only rarely to the extent of producing large hydropic cells (Fig. 1). The nuclei were swollen, pale and irregular in outline. The periportal zone and portal tracts were unaffected. The contrast was more striking in sections stained for fat. The periportal areas were free from any deposit of sudanophilic material (Fig. 2). Glycogen was almost completely absent.

By 24 hours the necrosis of the centrilobular and midzone cells was general. The necrotic areas were becoming confluent and haemorrhage into them was frequent. The isolated periportal areas of apparently healthy liver cells were surrounded by bands of congested necrotic tissue. The reticular framework remained intact.

By 48 hours this pseudo-lobular appearance was very marked (Fig. 3) and the necrotic bands were haemorrhagic. The red blood cells showed evidence of degeneration. There was an early invasion of the damaged area by polymorphonuclear cells. The proportion of necrotic to apparently healthy tissue varied considerably from animal to animal but the general picture was the same. By this time active regeneration as witnessed by numerous mitotic figures and binucleate cells had started (Fig. 4). The reticular pattern was preserved but the reticulo-endothelial cells were swollen and deeply stained but not obviously damaged. The portal tracts were normal.

By 72 hours the areas of necrosis had begun to shrink and were separated from the regenerating parenchyma by empty clefts (probably artefacts) (Fig. 5). This gave the lesions a "punched out" appearance. The necrotic areas contained only polymorphs and reticulo-endothelial cells among the debris and a few nuclear remnants from the parenchymal cells. The reticulin framework and the central veins were intact (Figs. 6 and 7).

Sections from animals killed at a later stage were almost certainly from animals that would have ultimately survived. The picture was very variable depending as it must upon the extent of the original injury. There was a progressive shrinkage of the necrotic areas and its replacement by actively growing liver cells from the undamaged areas. At first the regenerated areas showed irregular arrangement of the cells but by 17 days the liver had almost regained its normal appearance with only occasional fine strands of young connective tissue as a reminder of the pre-existing necrosis. In one animal killed 17 days after a dose of 45 mg./kg. there was evidence of incomplete repair with persistent islands of necrosis and more prominent branches of young connective tissue.

The livers of rats dying while being fed diets containing 200 and 100 p.p.m. of D.M.N. showed a similar picture of acute liver injury with considerable variation in degree from animal to animal. The islands of periportal tissue often contained parenchymal cells with unusual nuclear forms but rarely showing mitosis. There was little fatty change and glycogen was always absent. The bands of necrotic tissue might show variation in the proportion

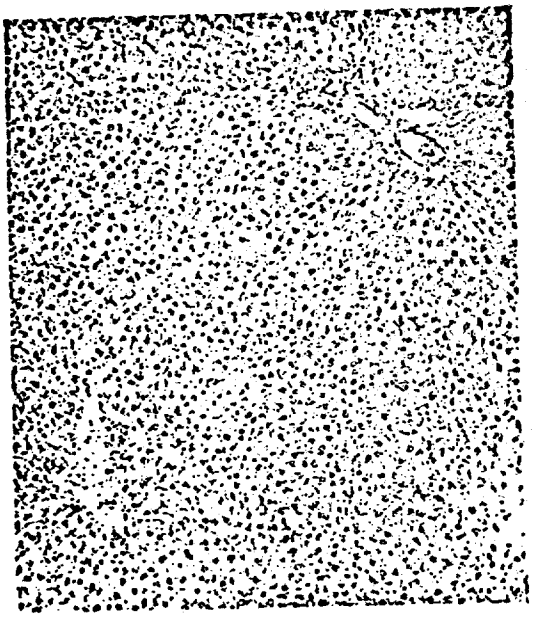


FIG. 1.—Rat liver after ingestion of D.M.N., 50 mg. per kg., for 18 hours. Haematoxylin and eosin. Early centrilobular and midzonal necrosis. × 100.

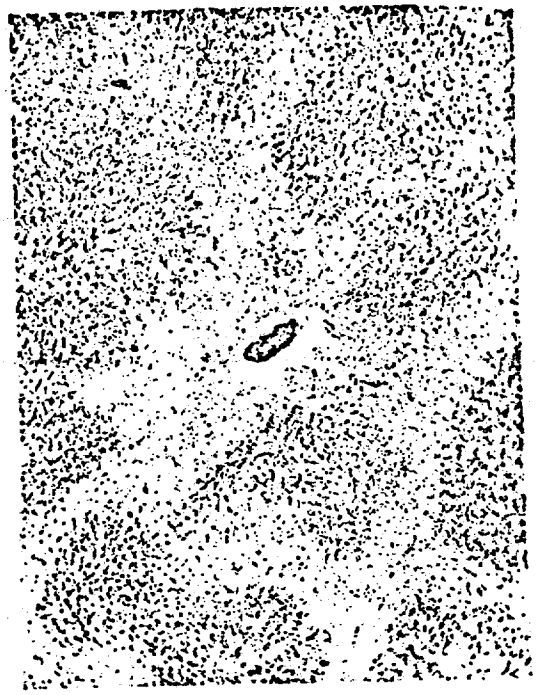


FIG. 2.—Rat liver after ingestion of D.M.N., 50 mg. per kg., for 18 hours. Sudan IV green filter. The pale areas represent surviving periportal parenchyma: the darker areas represent the necrotic zones containing many sudanophil droplets. × 60.

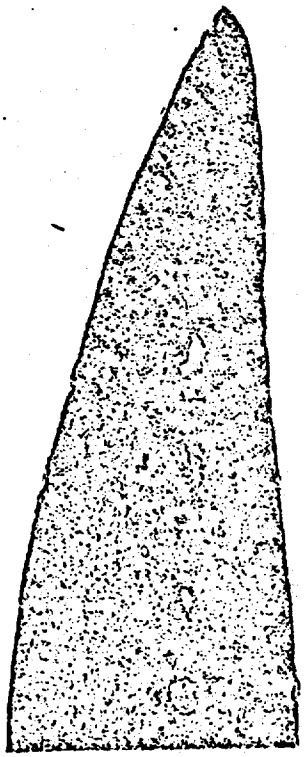


FIG. 3.—Rat liver after ingestion of D.M.N., 30 mg. per kg., for 48 hours. Haematoxylin and eosin. The darker areas of surviving periportal parenchyma are isolated by large confluent zones of necrosis. × 18.

FIG. 4.—Rat liver after ingestion of D.M.N., 30 mg. per kg., for 48 hours. Haematoxylin and eosin. Regenerating parenchymal cells with three mitotic figures. × 500.

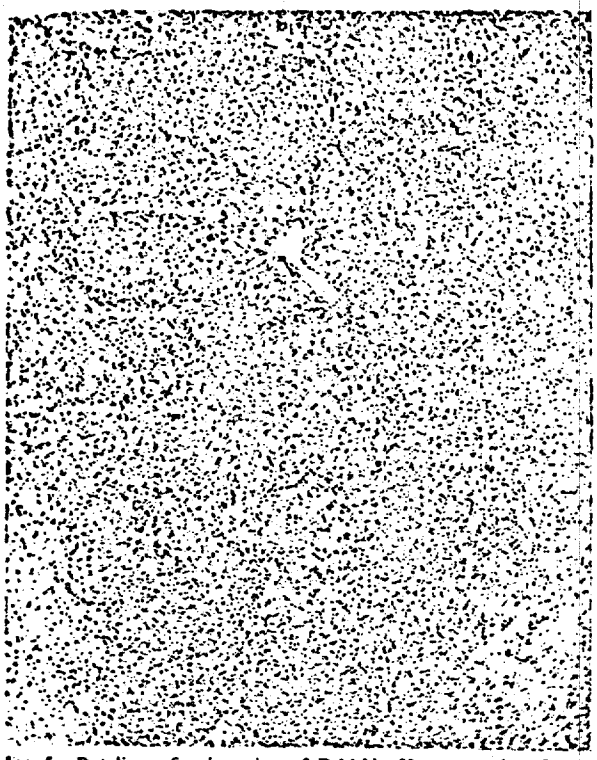


FIG. 5.—Rat liver after ingestion of D.M.N., 50 mg. per kg., for 72 hours. Haematoxylin and eosin. The necrotic zones are abruptly demarcated from the regenerating parenchyma. × 60.

FIG. 6.—Rat liver after ingestion of D.M.N., 50 mg. per kg., for 72 hours. Gomori silver impregnation. The reticular network persists in the necrotic area with no obvious reduction in the distance between the hepatic vein and the portal tract. × 100.

of its different constituents—degenerating hepatic cells, red cells and red-cell debris, macrophages, fibroblasts, and young connective tissue (Fig. 8).

In those surviving longest there was some bile duct proliferation and more fibrous tissue (Fig. 9). There was often a deposit of a yellow pigment staining for iron.

A histological picture similar to that in the rat was seen in the liver of the dog, with typical centrilobular haemorrhagic necrosis extending out into the mid-zones and leaving only narrow rims of surviving parenchyma around the portal tracts. The portal veins showed severe congestion and dilatation and there was frequent haemorrhage into the connective tissue of the portal tracts. The lymphatics of the portal tracts were widely dilated. The hepatic veins were patent. In the rabbits and mice there was less evidence of a zonal injury to the liver lobules and the effect more closely resembled that of a massive necrosis (Himsworth, 1947). In neither species did animals survive more than 30 hours after the administration of D.M.N.

Other Organs.—These showed little change. The lungs were congested and sometimes showed small

FIG. 7.—Rat liver after ingestion of D.M.N., 50 mg. per kg., for 72 hours. Gomori silver impregnation. Same preparation as Fig. 6 to show intact central vein, also persisting reticular strands in the necrotic tissue. × 400.

FIG. 8.—Rat liver after ingestion of D.M.N., 100 p.p.m., for nine weeks. Mallory's azocarmine. Irregular formation of fibrous tissue. × 100.

FIG. 9.—Rat liver after ingestion of D.M.N., 100 p.p.m., for nine weeks. Mallory's azocarmine. Same preparation as Fig. 8 showing fibrous tissue and bile duct proliferation. × 100.

haemorrhagic areas. The kidneys were normal. The spleen had a reduced red cell content and a more prominent reticulo-endothelial component. The retrosternal lymph glands contained amorphous eosinophilic debris, degenerating erythrocytes and macrophages filled with yellow-brown pigment. In the dog there was no evidence of congestion in the gastro-intestinal tract and the mucous membrane was well preserved.

Discussion

Dimethylnitrosamine appears to act primarily as a liver poison producing severe liver necrosis in rats, mice, rabbits, guinea-pigs, and dogs. The species examined seem to be more or less equally susceptible to D.M.N. and the general picture of poisoning is consistent with that following a severe liver injury. Some features of the condition merit further discussion.

The haemorrhagic peritoneal exudate and the bleeding into the lumen of the gut are striking features of poisoning in rats, dogs, and guinea-pigs. It has not been described in animals with liver injury following carbon tetrachloride, beryllium, phosphorus, or diphtheria toxin, for example. However, poisoning by senecio (ragwort) can produce liver damage and internal haemorrhage in horses and cattle and the condition has been produced experimentally in rats (Davidson, 1935). Although the loss of blood may be a contributory cause of death after D.M.N. in those species in which it occurs, death is just as rapid in those species in which there is no haemorrhage. Dimethylnitrosamine produces no histological evidence of widespread damage to any important organ other than the liver. The congestion and other signs of injury in the lungs were probably terminal.

Like carbon tetrachloride, D.M.N. produces a centrilobular necrosis and complete recovery is possible after the extensive damage produced by a single near-lethal dose. Repeated doses, as in the feeding experiment, may lead to fibrosis. However, the lesions due to D.M.N. differ from those due to carbon tetrachloride in that they are haemorrhagic and show a sharp transition from the damaged to undamaged liver tissue. In the central part of the lobule the hepatic cells appear totally destroyed while at the periphery they appear normal. The swollen hydropic or otherwise apparently injured liver cells are rarely seen after D.M.N.

In some respects the distribution of the lesions gives the liver the nutmeg appearance associated with passive venous congestion. In dogs a centrilobular necrosis that resembles the lesions of chloroform poisoning has been described after temporary

ligation of the hepatic vein (Simonds and Callaway, 1932). In cats and monkeys similar lesions have been described after experimentally produced chronic venous congestion (Bolton, 1914–15). Permanent partial occlusion of the hepatic vein in dogs leads to death within 72 hours if the occlusion is too severe (Zimmerman and Hillsman, 1930). Two of three dogs so dying had a serosanguineous peritoneal exudate and pools of blood were present throughout the greater part of the liver lobules, but no detailed histological descriptions were given.

In rats poisoned with senecio (Davidson, 1935) the lesions are very similar to those produced by D.M.N. and there was an accompanying oedema of the parietal peritoneum. Histologically in both cases there was a sharp line of demarcation between the healthy and necrotic tissue. In senecio poisoning, however, the central veins of the liver lobules were frequently seen to have been ruptured and there was endothelial proliferation of the hepatic and pulmonary veins. Such changes were not seen in rats poisoned with D.M.N.

The cause of the haemorrhages into both the injured liver and the peritoneal cavity and lumen of the gut is not apparent. The lungs may be the seat of small haemorrhages but otherwise there seems to be no general tendency to haemorrhage outside the area draining into the portal veins.

A striking feature of the liver lesion in rats poisoned with D.M.N. is the sharp line of demarcation between the totally destroyed parenchyma and apparently uninjured liver cells. This may indicate a short-lived action of a severe, rapidly acting poison. The fact that D.M.N. is freely soluble in water means that it would probably be excreted rapidly if not quickly metabolized and broken down in the body. In the rabbit death takes place rapidly even after a dose of D.M.N. that is just lethal. In this species, few if any liver cells seem to be spared.

The way in which D.M.N. damages the liver cannot yet be surmised. If it is a rapidly acting poison it might be assumed that after oral administration the gut, portal vessels, and liver are directly damaged by the passage of D.M.N. during its absorption. The fact that similar lesions can follow the intravenous administration of D.M.N. suggests that these tissues possess some unusual susceptibility to the toxic action of D.M.N. The tendency to haemorrhage in certain species may be the result of some impairment of blood clotting-mechanisms or to action on blood vessels. If the latter is the case the peculiar distribution of the lesions might be the result of an accentuation of venous back pressure in the portal system as a sequel to the injurious action of D.M.N. on the liver. In the dog the lesions in the liver are very similar to those described in dogs killed during anaphylactic shock (Dean and Webb, 1924). On the other hand a careful examination of the intestinal mucosa in the dogs poisoned by D.M.N. failed to reveal any of the intestinal lesions said to accompany anaphylaxis in this species (Manwaring, Beattie, and McBride, 1923).

Turning to the possible chemical mechanisms whereby D.M.N. exerts its toxic action, there is again no evidence as to how this is brought about. It is conceivable that D.M.N. might be reduced, by circulating or intracellular sulphydryl groups, for example, to dimethylhydrazine. Hydrazine itself produces liver necrosis but spares other tissues (Wells, 1908).

However, preliminary observations in this laboratory by W. N. Aldridge have shown that rabbits given dimethylhydrazine die in convulsions 24–48 hours after a dose of 100 mg./kg. without histological evidence of any liver damage. Bodansky (1924) examined a number of substituted hydrazines, not however including dimethylhydrazine, and found that not all of them damaged the liver.

There remain therefore three possibilities to consider in further work on D.M.N. Dimethylnitrosamine itself may be toxic and react with some important cell constituent. It may be metabolized to produce a toxic breakdown product. Or its metabolism and breakdown may require some additional factor which, when withdrawn for this purpose, leaves the cells of the liver irreparably injured.

The experimental observations indicate that D.M.N. can produce severe liver injury in a variety of experimental animals. This makes it difficult to exclude a direct causal connexion between the handling of D.M.N. and the development of cirrhosis of the liver in two out of the three men who did so.

As a solvent D.M.N. is obviously toxic, and, from the limited experience of its use, it is clearly a dangerous one to handle despite its low volatility and consequent comparative freedom from hazards from its vapour. The absence of a strong smell or other irritant properties means that contamination of the skin and clothes may have passed unnoticed. Though it does not readily penetrate the skin of rats, it may more easily be absorbed if clothing is soaked and applied to the skin. Nevertheless there are no observations which suggest that such was the route by which the two men absorbed D.M.N. in doses sufficient to produce cirrhosis of the liver. If one assumes that man is no less sensitive than the rat, then a daily dose of 260 mg. for a man weighing 70 kg. would be the equivalent dose to that depressing

the growth of the rats on 50 p.p.m. in their diet. Twice such a daily dose would obviously be very dangerous. With an air intake of 5 litres per min. for seven hours a day a very dangerous level in the atmosphere would be 250 mg./cu.m. A safe atmospheric concentration would clearly have to be very much less, certainly not more than 25 mg./cu.m.

Summary

Dimethylnitrosamine (D.M.N.) is a potentially useful solvent on which little toxicity data have yet been presented.

Prompted by the occurrence of two cases of liver injury in men handling D.M.N., in the laboratory of a large firm, a study of its action on experimental animals was undertaken.

In rats, rabbits, mice, guinea-pigs, and dogs D.M.N. in doses of 20 to 40 mg./kg. produces a severe liver necrosis ending fatally. Other organs are not damaged.

The lesions have some features in common with those produced by carbon tetrachloride but also some distinctive features.

In dogs, rats, and guinea-pigs a striking feature is haemorrhage into the gut and peritoneal cavity.

The features of intoxication are briefly discussed.

We wish to thank the medical officer of the firm concerned for his help and interest and for arranging for supplies of pure material. We are also very grateful to Professor G. R. Cameron, F.R.S., for his advice and help on the histological material. R. F. Legge provided valuable technical assistance.

References

Bodansky, M. (1924). *J. biol. Chem.*, 58, 799.
Bolton, C. (1914–15). *J. Path. Bact.*, 19, 258.
Davidson, J. (1935). *Ibid.*, 40, 285.
Dean, H. R., and Webb, R. A. (1924). *Ibid.*, 27, 51.
Gomori, G. (1941). *Amer. J. Path.*, 17, 395.
—— (1950). *Amer. J. clin. Path.*, 20, 665.
Hamilton, A., and Hardy, H. L. (1949). *Industrial Toxicology*, 2nd ed. Hoeber, New York.
Himsworth, H. P. (1947). *Lectures on the Liver and its Diseases*, p. 7. Blackwell, Oxford.
McManus, J. F. A. (1946). *Nature, Lond.*, 158, 202.
Manwaring, W. H., Beattie, A. C., and McBride, R. W. (1923). *J. Amer. med. Ass.*, 80, 1437.
Simonds, J. P., and Callaway, J. W. (1932). *Amer. J. Path.*, 8, 159.
Weil, C. S. (1952). *Biometrics*, 8, 249.
Wells, H. G. (1908). *J. exp. Med.*, 10, 457.
Zimmerman, H. M., and Hillsman, J. A. (1930). *Arch. Path., Chicago*, 9, 1154.

nente boje vinskog crvenila, već sivo-plavi i čokoladno-smeđi tonovi karakteristični za prisustvo methemoglobina. Ta boja podsjeća na plavo-smeđu boju čovjeka pod ultravioletnim svijetlom. Methemoglobinemička cijanoza je tamnija, pa ponekad daje gotovo crni izgled. Zbog toga se i lakše primjećuje, naročito iz udaljenosti. Najintenzivnija je na istaknutim dijelovima lica i na jeziku, zatim na rukama, naročito na noktima (17).

Naši su bolesnici svi odreda više ili manje pokazivali karakteristike upravo takve cijanoze, pa se već na temelju njihova izgleda kod dolaska u bolnicu posumnjalo na methemoglobinemiju. Spektrofotometrijskom analizom krvi nađeni procenat methemoglobina (6–48%) potpuno je odgovarao stupnju kliničke manifestacije – cijanoze. Promptno stvaranje methemoglobina, karakteristično za djelovanje nitrita, očitovalo se veoma brzo i kod naših bolesnika. Svi su oni »pomodrili« u roku od 10–20 minuta nakon ingestije otrova. Ostali simptomi otrovanja kod naših bolesnika mogu se smatrati posljedicom anoksije i pada krvnog tlaka.

Methemoglobinemije uzrokovane nitritima su reverzibilne i ne oštećuju crvena krvna tjelešca, jer methemoglobinemija sama po sebi ne uzrokuje hemolizu (18). Zato ni kod naših bolesnika nije bilo promjena u krvnoj slici, pa ni kod naknadnih kontrolnih pregleda. Ni kod jednog bolesnika nisu nađena Heinzova tjelešca, koja su indikator toksičkog oštećenja eritrocita i obično popratna pojava kod methemoglobinemija uzrokovanih nekim otrovima, koji indirektno oksidiraju hemoglobin (aromatski amino spojevi i nitro-spojevi).

Redukciju methemoglobina mogu izazvati agensi, koji reduciraju (askorbinska kiselina i glutation) ili se može izazvati katalizom normalnog enzimatskog mehanizma redukcije. Zbog toga je za kliničko liječenje methemoglobinemija shodno davanje visokih doza askorbinske kiseline (1 g ili više intravenozno ili peroralno), ma da askorbinska kiselina djeluje sporije od djelovanja normalnog staničnog mehanizma za redukciju. Mnogo brži je učinak metilenskog modrila. Metilensko modrilo nema redukcionog djelovanja, već djeluje katalitički, t. j. ubrzava djelovanje staničnog mehanizma za redukciju. Zato je kod toksičkih methemoglobinemija, osobito kad se razina methemoglobina približava opasnoj vrijednosti (60% i više), indicirano davanje metilenskog modrila. Daje se obično 1 mg boje na kg tjelesne težine kod odraslih, a 2 mg kod djece. Ako cijanoza ne iščezne u roku od jednog sata, može se davanje ponoviti, i to 2 mg na kg tjelesne težine. Metilensko modrilo se može dati i peroralno u dozi od 3 do 5 mg na kg tjelesne težine. Ipak, prema Bodanskome (19), većina toksičkih methemoglobinemija, kod kojih razina methemoglobina ne prelazi 40%, ne treba specifičnog liječenja, već samo pomno promatranje bolesnika i, zbog anoksije, ograničenje njegove fizičke aktivnosti. Inhalacija kisika i davanje infuzije glukoze nemaju fiziološkog opravdanja (19).

Među našim bolesnicima samo je kod P. J.-a bilo potrebno, zbog visoke razine methemoglobina (48%), dati metilensko modrilo. Međutim je upravo taj bolesnik odbio svako liječenje. Kod ostalih bolesnika davana je askorbinska kiselina (1 g) intravenozno.

Liječenje otrovanja nitritima je inače simptomatsko i odnosi se na popratne pojave poznate kod terapeutskog davanja nitrita.

Kako smo već spomenuli, alimentarna otrovanja nitritima iz mesnih proizvoda uglavnom se događaju zabunom, pa je njihovo sprečavanje svedeno na samo održavanje propisa kod pripremanja smjese za konzerviranje. Budući da su naši bolesnici radnici iz mesne industrije, koji su se otrovali na istom radnom mjestu u tri navrata s duljim vremenskim razmacima, to možemo smatrati, da su prilike na radnom mjestu bar donekle učestvovale kao vanjski faktor kod nastanka ovih nesretnih slučajeva.

Literatura

1. *Goodman, L., Gilman, A.:* The Pharmacological Basis of Therapeutics. Macmillan, New York, 1941.
2. *Fertman, M., Doan, C.:* Blood. 3 (1948) 349.
3. *Finch, C.:* New Engl. J. M., *239* (1948) 470.
4. *Naider, S., Venkantrao, P.:* Calcuta M. J., *42* (1945) 79.
5. *Comley, H.:* J. A. M. A., *129* (1945) 112.
6. *Rosenau M. J.:* Preventive Medicine and Hygiene. Appleton – Century – Crofts Inc., New York, 1951.
7. *Bosch, H., Rosenfield, A., Huston, R., Shipman, H., Woodward, F.:* J. Am. Water Works Assn., *42* (1950) 101.
8. *Bushoff:* Tierärztl. Umschau. 2 (1947) 32. cit. prema Chem. Abstr. *42* (1948) 8983.
9. *Barilari, Benedetto:* Semana Méd., 2 (1945) 228. cit. prema Chem. Abstr., *41* (1947) 222.
10. *Schmidt, H., Stich, W., Kluge, F.:* Dtsch. Med. Wschr. (1949) 961.
11. *Schulze, W., Scheibe, E.:* Z. Ges. Inn. Med., 3 (1948) 580. cit. prema Moeschlin, S., Klinik und Therapie der Vergiftungen. Schwalbe, Bern, 1952.
12. *Büch, O.:* Slg. Vergift. fälle, *14* (1952) 53.
13. *Savić, I.:* Klanice i tehnologija mesa. Naučna knjiga, Beograd 1952.
14. *Jensen, L. B.:* Microbiology of Meats, The Garrard Press, Champaigne, Illinois, 1945.
15. *Mc Cord, C., Harrold, G., Meedk, S.:* J. Ind. Hyg. Toxicol., 23 (1941) 200.
16. *Heilmeyer, L.:* Spectrophetometry in Medicine. Adam Hilger, London, 1943.
17. *Beritić, T.:* Lij. Vjesnik, 72 (1950) 162.
18. *Clark, B., Morissey, R.:* Blood, 6 (1951) 532.
19. *Bodansky, O.:* Pharmacol. Rev., 3 (1951) 144.

Summary

TOXIC METHAEMOGLOBINAEMIA DUE TO THE ACTION OF NITRITES

Seven cases of toxic methaemoglobinaemia due to accidental nitrite poisoning in workers engaged in the preparation of meat and meat products are reported. In all these cases the meat-pickling salt was ingested as a consequence of the accidental water and food contamination. The main sources of nitrites in clinical intoxications are listed and the importance of the meat preserving solution for mass poisoning is emphasized. The general chemistry of the meat curing process is therefore briefly described. The clinical picture and the treatment of toxic methaemoglobinaemia are discussed.

Institute of Industrial Hygiene, Zagreb

Received for publication 10. 10. 1955.

Arh. hig. radu, 6 (1955) 311

PROFESIONALNA ABRAZIJA I ATRICIJA ZUBA

Z. Njemirovskij

Zavod za odontologiju Medicinskog fakulteta u Zagrebu

(Primljeno 10. XII. 1955.)

Iznesena su opažanja autora o profesionalnoj abraziji zuba kod stakloduvača. Pregledano je 77 radnika-duvača stakla, ali nisu nađena velika oštećenja, koja bi se mogla očekivati. Opažanja o profesionalnoj atriciji izvršena su kod radnika u jednoj kotlovnici. Opažena je atricija zuba kod radnika, koji rade s pneumatičnim čekićem. Iznesenu su i podaci o učestalosti karijesa kod jedne i druge grupe radnika.

U evropskoj literaturi opisali su Misch (1), a u američkoj Shour i Sarnat (2) velike konkavne zareze na centralnim gornjim sjekutićima kod duvača stakla. Ti su urezi pravilni i na oba sjekutića jednaki. Pregledavši 77 radnika u jednoj tvornici stakla nisam našao takvih pojava. Ukratko ću opisati nalaze i pokušati da ih rastumačim. U tvornici stakla, gdje sam vršio preglede, postoji samo jedno radilište, gdje radnici rade na stari način puhanjem ugrijanog stakla na staklarsku lulu. Istu lulu primaju četiri radnika za redom. Stara metoda postepeno se napušta i upotrebljava se samo za izradbu specijalnih predmeta i velikih boca. Postepeno se uvode poluautomatski i automatski uredaji zbog jeftinije i brže proizvodnje, a način rada je higijenski.

Pregledi su izvršeni na uobičajeni način. Ukupno je bilo pregledano 77 radnika. Budući da na oštećenja zuba sigurno utječe dob i radni staž, iznosim u tablicama 1 i 2 te podatke.

Iz podataka se vidi, da je pretežni broj radnika srednje dobi i da ih velik broj radi u tvornici duže od 6 godina. Tu činjenicu želim istaknuti, jer je caklina najtvrda supstancija u čovječjem tijelu pa abrazija može nastati samo dugotrajnim djelovanjem nekog mehaničkog faktora. Kod 77 ispitanika našao sam samo 14 zuba s naročito velikim abrazivnim defektima u centralnim gornjim sjekutićima. Očekivane velike defekte nisam našao. Jedino kod trojice radnika zauzimali su defekti jednu petinu labijalne plohe, dok su kod ostalih bili manje izraženi. Kod četiri

LABORATORY INVESTIGATION
Copyright © 1966 by the International Academy of Pathology
Vol. 15, No. 1, Part 1
Printed in U.S.A.

Effect of Dimethylnitrosamine on the Endoplasmic Reticulum of Rat Liver Cells

149

E. L. Benedetti and P. Emmelot

In previous papers[2, 3, 7] we have been able to show that in the early stage after administration of the liver carcinogen dimethylnitrosamine (DMNA) to rats, a swelling of the endoplasmic reticulum and a detachment of ribosomes coincided with the inhibition of amino acid incorporation into the liver ribosomal protein. As regards the latter, evidence has been presented that the polyribosomes of rat liver are the target of a metabolite of DMNA.[4, 8]

RESULTS

With reference to the upper and lower layers (containing fragments of smooth and rough endoplasmic reticulum) and the pellet (containing polyribosomes), as described in the previous paper in this Symposium, by Benedetti, Bont, and Bloemendal, the following differences between liver preparations from DMNA-treated rats (25 mg. of DMNA per 250 gm. of rat/body weight for 4 hours) and normal rats, have been observed.

THE LAYERS CONTAINING "MICROSOMES"

1. The number of (poly)ribosomes attached to the membranes is decreased (Fig. 1).

2. The ribosomal aggregates are orientated irregularly, no spiral or helical configurations being observed.

3. The ribosomes have a spongelike appearance, with areas of decreased stainability by uranyl acetate (Fig. 2).

4. The membrane vesicles are of a nonuniform size; among them so-called liposomes are present (Figs. 1 and 2).

5. A fair number of the membrane vesicles is disrupted.

6. The stainability of the membrane element by uranyl acetate is very irregular (Fig. 2). This is also borne out by negative staining (Fig. 3), which confirms observation 5 and, in addition, reveals membrane subunits of a similar character as found in the membranes, lying free from the latter (Fig. 3).

POLYRIBOSOME PELLET

1. The polyribosomes present in the pellet also show a damaged appearance (Fig. 4).

2. The monomers are broken down into (or at least give the appearance of being made up by) smaller components (Fig. 4).

RIBOSOMAL FRACTIONS ISOLATED BY SUCROSE-DENSITY GRADIENT CENTRIFUGATION

The polyribosome pellets prepared from normal and DMNA-treated liver were used for continuous sucrose-gradient centrifugation as described previously.[8] Aliquots of the various fractions were studied as spread preparations under the electron microscope, following either negative staining with uranyl acetate or shadowing with platinum and carbon while rotating.

The size of the polyribosomes in the various fractions and the preponderant presence of monomers in the 80 S particle fraction was according to expectancy on the basis of the sedimentation profiles.[8] The differences in morphology between the ribosomal units of DMNA-treated and normal preparations were similar to those described under the two preceding headings. In addition, in the DMNA-fractions (around fraction number 20; see reference 8) sedimenting after the heavy polyribosomes and preceding the light material (monomers and dimers), long threads with a thickness of about 80 to 90 Å (in the shadowed preparations) revealing patches of amorphous appearance, were observed

From the Departments of Electron Microscopy and Biochemistry, Antoni van Leeuwenhoek-Huis, the Netherlands Cancer Institute, Amsterdam, The Netherlands.

FIG. 1

(Fig. 5). This
been observed
in contrast to
smaller thickn
both DMNA-c
tions (see pape
Symposium).

Our previous
effect of DMN
ticulum of rat
by the present
somes" and ri
free method fo
elements from
liver.

1. As shown
this Symposium
tween ribosome
lum membranes
normal liver pr
to affect this co

2. The mem
markedly affect
the coarse appe
tern of the m
size and fragm
the membrane
(clusters of)
branes.

3. Gradient
that as a resul
occurs a break
lower units incl
ticle peak;[8] evi
down to smaller
tained. The ribo
livers tend to s
negatively stai
spongelike and
than do those i
of these feature
the monomer, is
ribonuclease, ba
this activity co
threads of 80 t

FIG. 1. A.
section stain
with many
of rat for 4
membrane
variety of

(Fig. 5). This type of strand has never been observed in the control preparations, in contrast to the strands or fibers of smaller thickness, which were present in both DMNA-treated and normal preparations (see paper by Benedetti *et al.*, this Symposium).

DISCUSSION

Our previous results concerning the early effect of DMNA on the endoplasmic reticulum of rat liver cells are substantiated by the present data on isolated "microsomes" and ribosomes, using a detergent-free method for the separation of these elements from normal and DMNA-treated liver.

1. As shown in the preceding paper in this Symposium, a structural contact between ribosomes and endoplasmic reticulum membranes has been demonstrated in normal liver preparations. DMNA appears to affect this contact.

2. The membranes, *per se*, are also markedly affected by DMNA, as shown by the coarse appearance of the globular pattern of the membranes, the nonuniform size and fragmentated state of (some of) the membrane vesicles, and the release of (clusters of) subunits from the membranes.

3. Gradient centrifugation has shown that as a result of DMNA action, there occurs a breakdown of polyribosomes to lower units including a prominent 80 S particle peak;[8] evidence for a further breakdown to smaller products has also been obtained. The ribosome units of the DMNA livers tend to show, both in positively and negatively stained preparations, a more spongelike and fragmented appearance than do those from normal liver. Certain of these features, *i.e.*, the central "pit" in the monomer, is reminiscent of the action of ribonuclease, but biochemical evidence for this activity could not be established.[8] The threads of 80 to 90 Å thickness, only observed in the DMNA preparations, show some morphologic similarity with denatured nucleic acid.[5] Whether they represent some (changed) form of messenger or ribosomal RNA cannot be stated. Neither do the present results allow to conclude that the breakdown of the polyribosomes to smaller aggregates and monomers results from the breakdown of methylated messenger RNA[6, 8] or from the detachment of the affected ribosomes from the messenger strand.

In the maintenance of the polyribosome-membrane complex, one or more of the following factors may operate: (1) the messenger-RNA strand, (2) the nascent polypeptide chain, and (3) membrane constituents. The disruption of this complex may accordingly be due to an effect of the DMNA metabolite on any of these three components. Suggestive evidence for such effects involving all three components is provided, respectively, by the breakdown of the polyribosomes, the inhibition of amino acid incorporation (this being considered the result of the former effect[8]), and the fine-structural alteration of the endoplasmic reticulum membranes, observed after the administration of DMNA. The metabolic conversion of DMNA to diazomethane occurs in the membranes, and diazomethane (or the methylcarbonium ions derived therefrom) may react with the membrane phospholipids and/or the membrane proteins.[4, 7] As emphasized in earlier papers from this laboratory,[3, 7] and since then, corroborated by new data (see this Symposium), the endoplasmic reticulum may be the primary target of the hepatocarcinogens—a view also shared by Pitot and Peraino[9, 11] as a result of his findings with minimal-deviation hepatomas. The significance of this type of interaction of carcinogens for an interpretation of the carcinogenic process alternative to the somatic mutation mechanism, has recently been stressed.[1, 10] Furthermore, the

FIG. 1. *A*, Normal rat liver. Lower layer (interface between 2 M and 1.5 M sucrose). Thin section stained with uranyl acetate. The preparation consists of membrane profiles dotted with many ribosomes. *B*, Rat liver treated with dimethylnitrosamine (25 mg. per 250 gm. of rat for 4 hours). Lower layer prepared as in *A*. The ribosomal aggregates attached to the membranes are scarce (as compared with *A*) and the membrane profiles show a greater variety of size and shape. The *arrows* point to some liposomes.

FIG. 2. Rat liver treated with dimethylnitrosamine. Upper layer: thin section stained with uranyl acetate. The membrane profiles are partially disintegrated: note the irregular stainability of the membrane elements. The ribosomes, either attached to the membranes or free, have a starlike shape (compare with the control preparation in *B*) and show less dense areas apparently not stained by uranyl acetate.

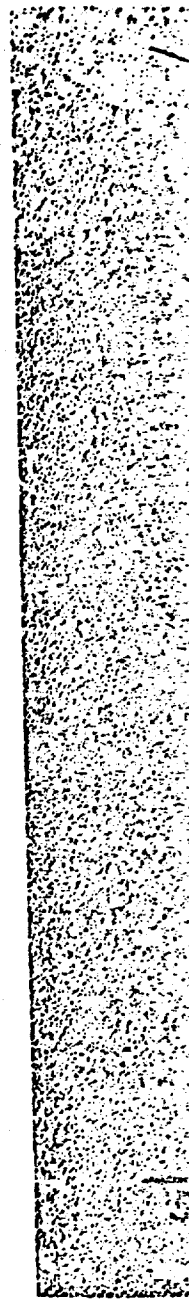


FIG. 3. Rat
negatively stai
suggestive of
sulting from th

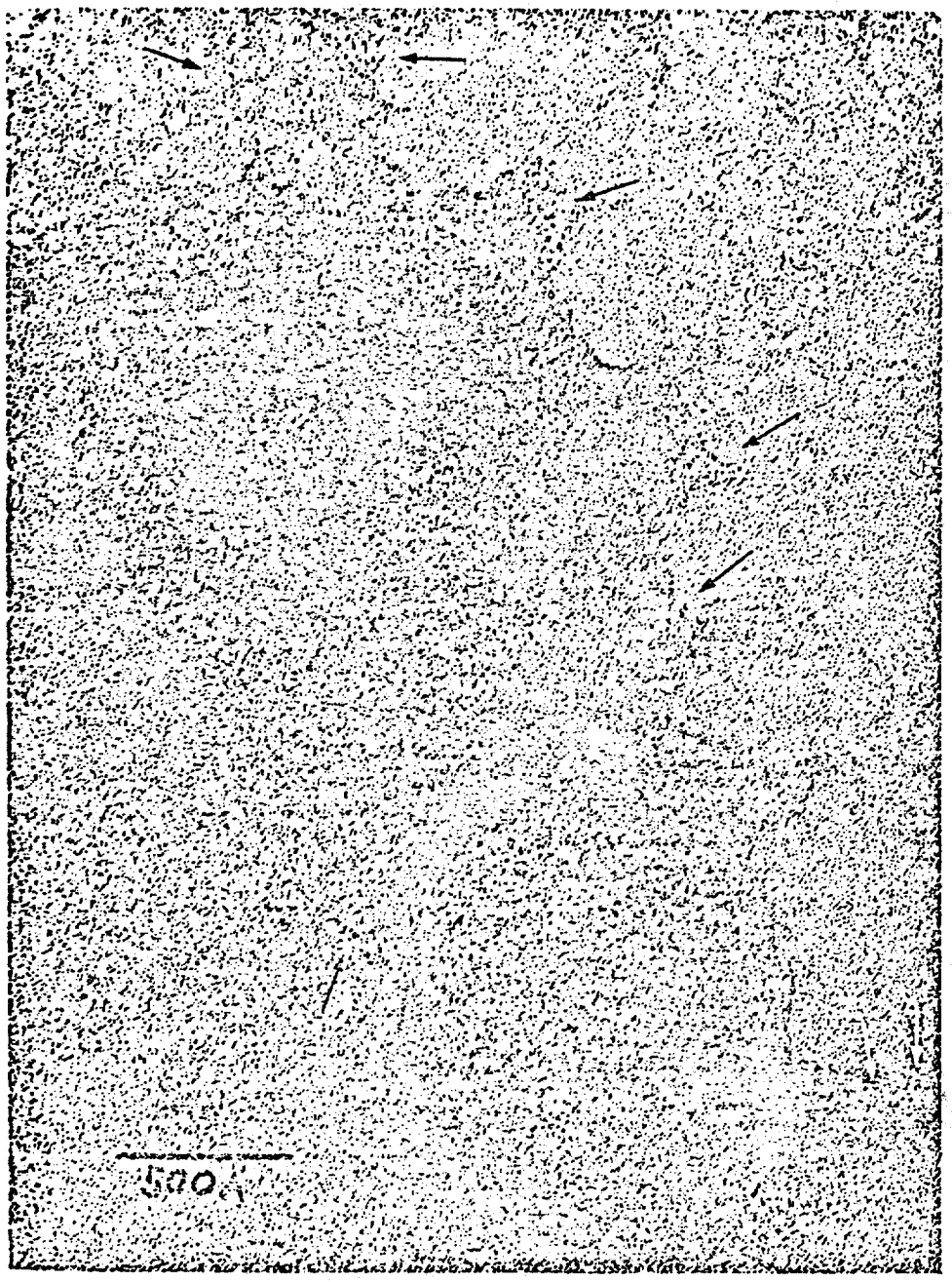


FIG. 3. Rat liver treated with dimethylnitrosamine. Upper layer: "smooth" membranes negatively stained with uranyl acetate. The membranous sheets show a coarse granularity suggestive of a subunit pattern. The *arrows* point to fragments (subunits ?) probably resulting from the disintegration of the membrane.

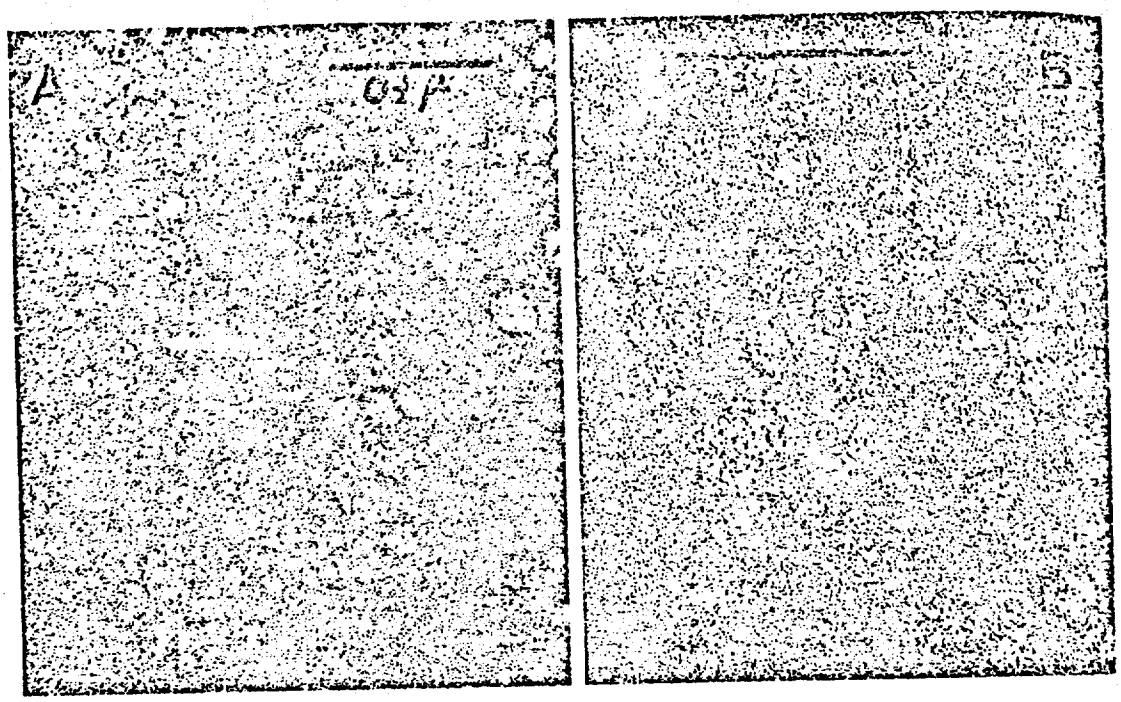


FIG. 4. Rat liver treated with dimethylnitrosamine. Polyribosomal preparation negatively stained with uranyl acetate (1 per cent). The ribosomal aggregates consist of monomers, irregularly shaped, some of them being split in smaller units (*arrows*).

interaction of hepatocarcinogens with endoplasmic reticulum *membranes* may transcend the mere coincidence between the site of metabolism (conversion to a reactive product, *e.g.*, the ultimate carcinogen) and the primary target of these compounds, if it can be shown that the membranes contribute in some way to the macromolecular specificity and/or are involved in regulatory devices of the liver cell.

Acknowledgments. This paper is dedicated by the authors to the memory of their beloved friend and colleague Dr. Isaac Jacques Mizrahi, who lost his life in a tragic car accident on January 7, 1966. Jacques Mizrahi has made an essential contribution to the study of the biochemical effects of carcinogenic nitrosamines. The present electron microscopic observations, which would have been impossible without his continuous stimulating support, corroborate the results of his work, lastly published in *Biochemical and Biophysical Research Communications 21:* 555, 1965.

The very skilled technical assistance of Miss M. Gerhards and Miss C. van der Voorn is also greatly appreciated.

FIG. 5. Rat liver treated with dimethylnitrosamine. Ribosomes from the middle fractions of the continuous sucrose gradient (see text). The spread preparation has been shadowed with carbon-platinum while rotating. The ribosomal aggregates consist of monomers partially disintegrated in small units and held together by strands of various lengths and thicknesses. In *B* a long thick strand, apparently not connected with the ribosomes, is shown. In *C* the *arrows* point to fragmented strands showing patches of amorphous appearance.

...ron negatively
... of monomers,

...st his life in a
January 7, 1966.
...ade an essential
... of the biochemi-
...e nitrosamines. The
...pic observations,
...mpossible without
...ing support, cor-
... work, lastly pub-
... *i Biophysical Re-*
... 555, 1965.
...ical assistance of
... Miss C. van der
...ecreciated.

...mole fractions
...seen shadowed
... monomers par-
...n lengths and
...mes, is shown.
...ne appearance.

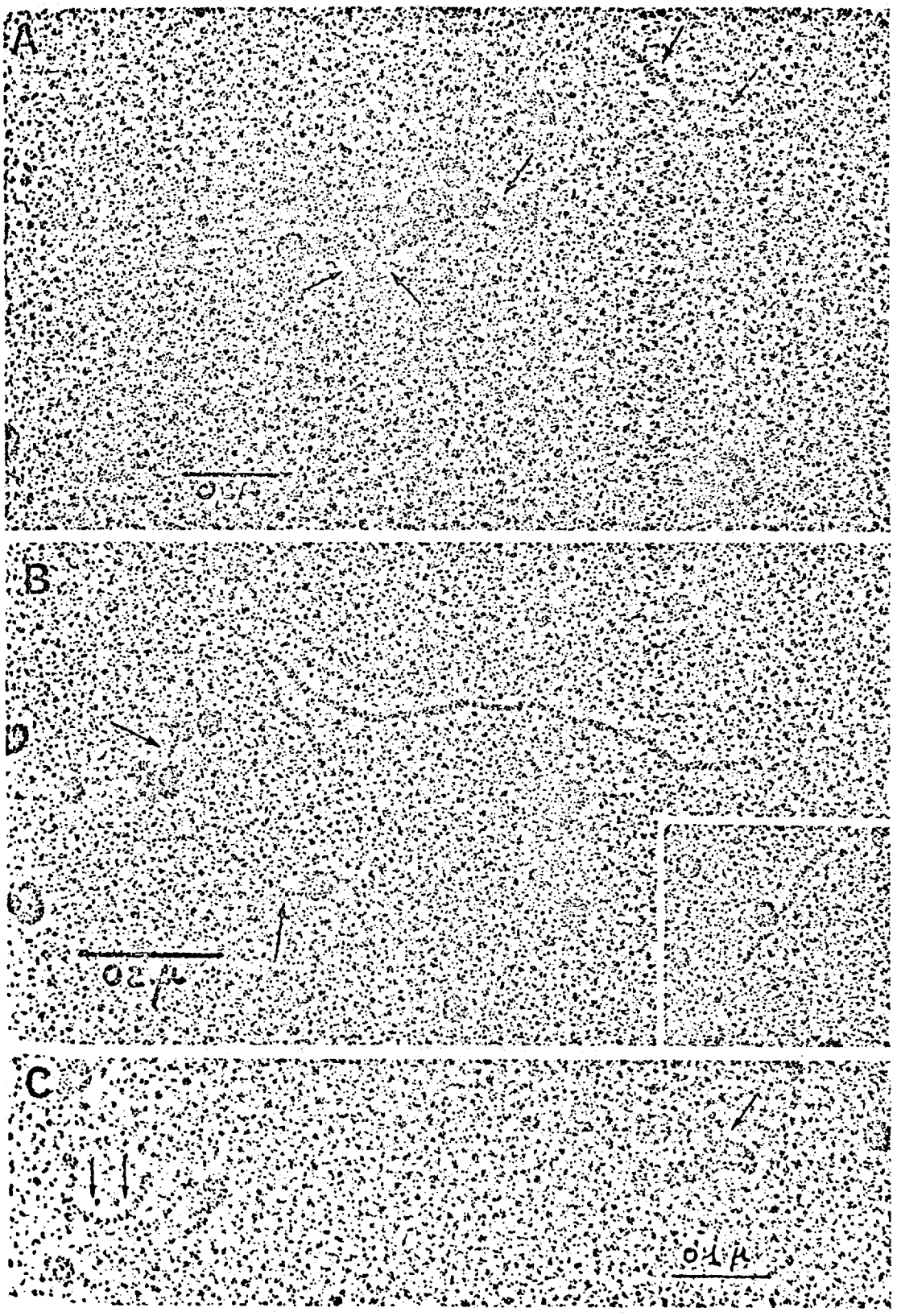


FIG. 5

REFERENCES

1. Emmelot, P. Introduction. In *Cellular Control Mechanisms and Cancer*, edited by Emmelot, P., and Mühlbock, O., p. 1. Amsterdam, Elsevier, 1964.
2. Emmelot, P., and Benedetti, E. L. Changes in the fine structure of rat-liver cells brought about by dimethylnitrosamine. *J. Biophys. Biochem. Cytol. 7:* 393, 1960.
3. Emmelot, P., and Benedetti, E. L. Some observations on the effect of liver carcinogens on the fine structure and function of the endoplasmic reticulum of rat-liver cells. In *Protein Biosynthesis*, edited by Harris, R. J. C., p. 99. London, Academic Press, Inc., 1961.
4. Emmelot, P., Mizrahi, I. J., and Krick, E. Prevention by cysteamine of the inhibitory effect of carcinogenic *N*-nitrosodialkylamines on the incorporation of amino acids in rat liver. *Nature 193:* 1158, 1962.
5. Hall, C. E., and Litt, M. Morphological features of DNA macromolecules as seen with the electron microscope. *J. Biophys. Biochem. Cytol. 4:* 1, 1958.
6. Krick, E., and Emmelot, P. Methylation and breakdown of microsomal and soluble ribonucleic acid from rat liver by diazomethane. *Biochemistry 2:* 733, 1963.
7. Mizrahi, I. J., and Emmelot, P. The effect of cysteine on the metabolic changes produced by two carcinogenic *N*-nitrosodialkylamines in rat liver. *Cancer Res. 22:* 339, 1962.
8. Mizrahi, I. J., and Emmelot, P. On the mode of action by which the carcinogen dimethylnitrosamine inhibits protein synthesis in the liver. *Biochim. Biophys. Acta 91:* 362, 1964.
9. Pitot, H. C. Altered template stability: the molecular mask of malignancy? *Perspect. Biol. Med. 8:* 50, 1964.
10. Pitot, H. C., and Heidelberger, C. Metabolic regulatory circuits and carcinogenesis. *Cancer Res. 23:* 1694, 1963.
11. Pitot, H. C., and Peraino, C. Endoplasmic reticulum and hepatic carcinogenesis. *Biochemical Clinics, Vol. 3, The Liver*, p. 139 New York, The Reuben H. Donnelley Corporation, 1964.

G 1673-1
Croatian Trsl.
DKD

163

TOXIC METHEMOGLOBINEMIA CASES CAUSED BY NITRITES

(Toksičke methemoglobinemije zbog djelovanija nitrita)

Authors: T. Beritić and F. Valić

Source: Arhiv za Higijenu Rada i Toksikologiju (Yugoslavia)
Vol. 6, 1955, P. 303-310.

Methemoglobin (hemiglobin, ferrihemoglobin) is a product of oxidation of hemoglobin, the divalent Fe of which has become trivalent by the oxidative elimination of one electron. The resulting pathological blood pigment is physiologically inert, but is useless for the transportation of O. Obvious pathological conditions, in which a part of the hemoglobin is converted into methemoglobin, are called methemoglobinemias. Even in the blood of healthy people, quite small amounts of methemoglobin are present. A greater accumulation is prevented by the rapid reduction of methemoglobin, which is constantly performed by a special enzymatic mechanism in the red blood cell. On the other hand, through a congenital defect, this mechanism may be absent or may be suppressed (congenital methemoglobinemia). Through the action of some poisons or of their metabolites, which directly or indirectly oxidize the hemoglobin, this mechanism becomes inadequate (toxic methemoglobinemia). Among the poisons, which oxidize hemoglobin directly, the best known are the nitrites.

In pharmacological and toxicological literature, one usually includes among the nitrites not only different inorganic and organic nitrites, but also the nitrates. According to Goodman and Gilman (Reference 1), this is possible because the biological action of nitrite and of nitrate is mainly due to the effect of the released nitrite ion (NO_2^-). The organic nitrite and the inorganic nitrite easily release this ion, irrespective of whether the carrier is the organic radical or the metal. In the body the organic nitrates release the nitrate ion and if these nitrates reach the gastrointestinal tract, they can be reduced into nitrite under the action of the bacterial flora. Thus, in this way, the nitrates act as nitrites.

Nitrite poisonings are medicamentous, suicidal or alimentary. Bismuth subnitrate, ammonium and K nitrate are medicines still prescribed nowadays. Cases of poisoning by Bi subnitrate are known, especially in children, sometimes with fatal termination (Reference 1). A mortal poisoning by erythrol tetranitrate is described (Ref. 2). Female patients with ulcerative lesions of the intestines easily acquire methemoglobinemia after nitrate ingestion (Reference 3). Rather frequent cases of suicidal poisoning by Na and K nitrite are recorded only in India (Reference 4), where the sale of these chemicals is free, and where home weavers make a considerable use of them for texture dying.

Alimentary nitrite poisonings usually derive from two sources, that is well-water and meat, specifically meat products which have been treated with inorganic nitrites and nitrates. Hardly ten years ago, toxic cases of methemoglobinemia in suckling babies (Reference 5), who were received milk food artificially, were elucidated. Poisoning became manifest, since in each case the food was prepared with the help of well-water with a relatively high nitrate content. Since only the babies became sick, the mechanism of these cases of toxic methemoglobinemia is interpreted as follows: the acidity of the gastric juice in suckling babies is relatively high (pH about 4.9); in the intestines it renders possible the growth of bacteria, which reduce the nitrates, that is develop nitrites. These nitrites become resorbed into the blood circulation and develop toxic methemoglobinemia. Especially babies less than two months old are sensitive to this process. Interesting is the so far unexplained specific geographical distribution of this baby disease (Reference 6). In the United States, only in the State of Minnesota, there were in three years (1947 - 1949) 139 cases (14 mortal) of methemoglobinemia in suckling babies (Ref. 7). In well-water, a nitrate content exceeding 20 p.p.m. (expressed as N) is dangerous. Among food poisonings one must include sad cases in which nitrite were used by mistake instead of common salt (Reference 4). The most frequent cases of food poisoning, however, are caused by wrongly processes meat or meat products containing to much nitrite and nitrate added as chemical preservatives during preparation. Such poisonings assume the from of real epidemies. Bushoff (Reference 8) in 1947 described mass poisonings by some broth which contained brine (pickles ?), and Barilari and Benedetto (Reference 9) mentioned similar cases caused by sausages. Schmidt and others (Reference 10) described 51 cases of poisoning by blood puddings, and Schulze and Scheibe (Reference 11) 79 cases, and in 1952

Büch (Reference 12) reports a mass poisoning by Na nitrite through the use of sausage broth; up to 146 persons became sick at the same time. Usually the responsibility for such cases rests with the meat production industry which, in recent time, made an increasing use of nitrite and nitrate to preserve meat. Since there is now a growing demand for preserved meat products in which nitrite and nitrate are a necessary addition, it is mainly from these products that these chemicals derive here. For this reason a knowledge of the main principles of meat curing and of the manufacture of chemical preservatives is important.

Meat preservation is the basic task of the meat industry, and curing without damaging the organoleptic properties of the products is the main problem. Any preserving method involves at least a partial loss of the nutritive value of the meat, yet curing is necessary as the only way to keep meat over a long period of time from deteriorating.

In most cases, common salt is used in preserving meat (salting), or a mixture of common salt, nitrate, nitrite and possibly some additives such as sugar, rum and some spices (pickling) (Reference 13) are applied to the same purpose. Salting serves to preserve parts not predominantly muscular.. Pickling (brine) is used for muscular parts because of the unfavorable effect of salt on hemoglobin and myoglobin, resulting in an alteration of the color of the meat.

The meat preserving mechanism is not yet fully elucidated and to be sure involves a complex combination of physical, chemical, fermentative and bacterial processes. It is mainly through its bacteriostatic action that common salt exerts its preservative effects. The bactericidal action at the relatively low concentrations involved here is insignificant. Furthermore, salts destroys the animal parasites also. It also exerts the unwelcome effect of altering the color of meat. Na chloride decomposes the hemoglobin, or the myoglobin, so that meat loses it natural color. In order to prevent this, nitrate or nitrite is added to meat and imparts to it the pleasant color of cured meat. This color persists even after cooking. The process involved is as follows: The denitrifying bacteria cause the reduction of the nitrates to nitrites. In the acid medium, the nitrites pass into nitric acid (? u dušičastu kiselinu ?) and this acid passes into NO. This oxide combines with the hemoglobin, or the myoglobin, forming the markedly red nitrohemoglobin (NOHb)

or nitrosomyoglobin. Cooking causes these compounds to pass into the intensely red nitrosohemocromogens, which are thermostable. Since Na chloride does not destroy nitrosohemoglobin or nitrosohemocromogens, the meat keeps its red color even after cooking. This action of nitrate and nitrite on the blood pigment can be shown diagramatically as follows (Reference 14) (See Diagram Page 305):

Formerly in curing, one used nitrates nearly exclusively, in addition with common salt. In recent time, nitrites or a nitrite and nitrate combination, have been utilized more and more. The nitrates must first be reduced into nitrites. The time required for this reduction is saved by the direct use of nitrite. Thus the pickling period is shortened by half, and in some cases by two thirds. Since the degree of nitrate reduction depends on the amount of bacteria present, which cause denitrification, it is never possible to accurately calculate the amount of nitrate required. The direct use of nitrite precludes this difficulty, and in this way there is never an excess of nitrite in the finished products. Since, also, less nitrite than nitrate is required, nitrite is used more and more in the curing process, for economic reasons. The nitrites strengthen the preserving action of salt, since to some extent they are bacteriostatic (they prevent the growth of putrefaction bacteria, especially the anaerobes).

The nitrite action depends on the amount used. If nitrite is in excess, the red nitroso-compounds fail to develop; Fe becomes oxidized and brown or green pigments are found. These are compounds which contain Fe in the trivalent form (methomoglobin to holeglobin). Hence, in preparing curing mixtures, special attention must be given to the nitrite concentration. The pickling mixture should be prepared only by authorized enterprises, and not by the the meat product plants themselves.

Such is the amount of nitrite used in pickling, that the finished products should never contain more than 25 mg of nitrite for 100 grams of meat. Thus nitrite cannot cause poisoning, since the highest amounts of correctly processed meat that a man may consume cannot introduce toxic quantities into the organism. Only a mistake during brine preparation can cause poisoning through finished products.

In industry, poisoning through nitrites is nearly unknown. On the other hand, in the meat processing industry, there can occur sad cases of poisoning through food contact with nitrite and nitrate, especially under unfavorable sanitary conditions (drinking-water from pipes, places of food consumption far from the food storage). In one meat processing plant, more cases of poisoning of this type occurred in recent time. They should be described at this point.

N. M., 19 years old, workwoman in a meat plant. On the day of her hospitalization, friends invited her to have some drink which had the odor and taste of rum. About 15 minutes later, she became sick, things around appeared as through a fog, she became blue and lost consciousness. On arriving at the hospital, she was pale and cyanotic, she complained of a strong headache in the neck region. RR: 90/60. After a gastric lavage, the ingestion of an analeptic and of a high dose of vitamin C, her state subjectively improved. Methemoglobin in her blood amounted to 23% of the total hemoglobin. The gastric content showed the presence of nitrate and nitrite. The day after, the patient was out of danger; cyanosis had completely disappeared.

M.L., 27 years old, workman in the same plant. On the day of his hospitalization, at about 10:00 AM he drank about 4 dcl of water from a container used in keeping potable water at his working place. He did know that at the time this water had been mixed with brine. Half an hour after, he felt very hot in his head whereas his heart was pulsating strongly; then he had a strong headache in the neck region, his sight became blurred, he suffocated and had accesses of coughing. At this time his fellow-workers warned him that he had become very blue especially at his lips. On his arrival at the hospital, he was in a state of excitement and fright; he was very cyanotic, especially at his lips and tongue. There is tremor of the hands, and later on of the entire body. RR: 100/65. After a gastric lavage, and the ingestion of an analeptic, the pressure decreases progressively, the patient feels better. Methomoglovin in the blood amounted to 38%. Nitrite is present in the stomach content. High doses of ascorbic acid is used as treatment.

The day after, the patient feels quite well. Cyanosis has disappeared. When he leaves the hospital, his pressure is 115/75.

M. I., 26 years old, drank water, on the same day and about at the same time, after breakfast, from the same container as his fellow-workers. About 10 minutes later, he felt very hot but did not vomit. His face and hands turned blue; then he had a strong headache in the back region and in the temples. Upon arrival at the hospital, he exhibited considerable cyanosis, especially at his lips. He was upset and restless. RR: 95/60, other findings were normal. After a gastric lavage, the ingestion of an analeptic and of high doses of vitamin C, his state improved substantially, the cyanosis gradually decreased; the day after, the patient feels well again. Methemoglobin in the blood amounted to 26% of the entire hemoglobin. Nitrites were found in the stomach content.

P.V., 24 years old, drank far less water than his fellow-workers. Beside a slight headache, there were no subjective hindrances. He comes with the others to the hospital, but only for a check. He barely shows some cyanosis, which corresponds to the detection of methemoglobin in the blood (6%). After receiving vitamin C and at the end of a few hours, he is discharged.

P.S., 27 years old, after drinking some water, feels quite sick and vomits; he feels a strong pain in the back of his head and at the temples; he breathes with difficulty and feels pressure on the heart. His lips, then his hands, especially his nails, turn blue. He arrives at the hospital in a state of restlessness and marked cyanosis. RR: 100/65. Methemoglobin 23%; all the other laboratory findings are normal. The nitrite reaction in the gastric content is positive. Ascorbic acid and an analeptic are administered. He state improves. After 24 hours, cyanosis has completely disappeared.

P.J., 46 years old, does not know how much water he drank, but he drank from the same container as the others on the same morning. Himself he acknowledge, and the others explain, that he ingested a fair amount of brandy (rakija) before coming to work and immediately before he drank water. After drinking the water, he felt sick immediately and vomited considerably; he felt much pressure on the heart and breathed with difficulty. Upon arriving at the hospital, considerable

cyanosis, especially at the lips, tongue and face, the face showing telangiectasia. Strong tachycardia, 114 per minute. RR: 130/60. Methemoglobin in the blood 48% of the entire hemoglobin. Alcohol in the blood (Nicloux method) 1.74 per thousand. The patient refuses the gastric lavage and any other treatment. On his own request, he leaves the hospital a few hours after his arrival. The following day, the patient is checked; there is no cyanosis, and he feels subjectively very well.

V.J., 49 years old, used by mistake Na nitrite instead of salt, for his breakfast food. Soon after eating, he felt an insipid taste in his mouth, yet he continued working until he felt sick, and then vomited. At this time, fellow-workers warned him that his face had turned blue. Half an hour later, he felt dizzy and very weak on his feet. He was cyanotic and pale, upon arriving at the hospital. No blood was sampled for methemoglobin; the other findings were normal. An analeptic and vitamin C were administered, and the patient improved considerably. The day after the cyanosis disappeared completely.

In all the patients, in whom methemoglobin was determined, blood was sampled immediately after hospitalization and before any therapeutic intervention. For the quantitative determination of methemoglobin, the Heilmeyer differentially spectrophotometric method was used (Reference 16). Measurements were taken with the help of the Beckman spectrum photometer, model DU. The extinctions of the blood solutions were measured with proper lengths of 560, 576 and 590 millimicra in an aperture width of 0.1 mm. The methomoglobin concentration was calculated on the basis of the characteristic quotients of extinction $\frac{E\ 576}{E\ 560}$ and $\frac{E\ 576}{E\ 590}$. For photometering purpose, one used blood solutions in 0.4% ammonia (1:100). The measurements were performed in ("kivetama ?) of a layer thickness of 0.5 cm.

DISCUSSION:

The main clinical symptom of methemoglobinemia is cyanosis. Methemoglobinic cyanosis exhibits a color of its own, different from the well known color of usual cyanoses, that is of the cyanoses involving the flow of an increased amount of reduced hemoglobin in the capillary blood. In methemoglobinic cyanosis what predominates are not the purple color component of a vinous red, but the the grey-blue and chocolate-brown shades characterizing the presence of methemoglobin. This color reminds one of the blue-brown color of man under UV illumination.

Methemoglobinic cyanosis is darker, and sometimes has almost a black appearance. That is why it is more easily noticed, especially from afar. It is most intense on the prominent parts of the face and on the tongue, then on the hands, especially on the nails (Reference 17).

Our patients, without exception, exhibited more or less characteristic features of this cyanosis, on the basis of which, already at the time of hospitalization, methemoglobinemia was suspected. The perventage of methemoglobin (6 - 48%), found by spectrophotometric blood analysis, corresponded entirely to the degree of clinical cyanosis manifestation. A prompt formation of methemoglobin, characteristic of the nitrite action, was detected quite rapidly in our patients. All became blue 10 to 20 minutes after ingesting the poison. The other symptoms of poisoning can be regarded as sequels of anoxia and of the drop of blood pressure.

Methemoglobinemias caused by nitrites are reversible and do not damage the red blood corpuscles, since methemoglobinemia by itself does not cause hemolysis (Reference 18). Hence, in our patients, there was no change in the blood picture, or in the subsequent control examinations. Heinz bodies were found not even in one patient. They indicate a toxic damage of the erythrocites and are a concomitant phenomenon of methemoglobinemias caused by poisons which indirectly oxidize the hemoglobin (aromatic amino-compounds and nitro-compounds).

Methemoglobin reduction can be caused by reducing agents (ascorbic acid and glutathione), or can be induced by the catalysis of a normal enzymatic reduction mechanism. Hence, for the clinical treatment of methemoglobinemia, it is convenient to administer high doses of ascorbic acid (1 g or more intravenously or orally), although this acid act more slowly than the action of the normal cellular mechanism on the reduction. The effect of methylene blue is very rapid. It does not have any reducing action, but acts catalytically, that is it accelerates the action of the cellular mechanism on the reduction. Hence, in toxic methemoglobinemias, especially when the methemoglobin level approaches a dangerous value (60% and more), it is proper to administer methylene blue. Usually 1 mg of the color for 1 kg of body weight is given to adults, and 2 mg to children. If the cyanosis does not disappear within one hour, one can renew the administration, that is 2 mg per kg of body weight. Methylene blue can be given by mouth in doses from 3 to 5 mg per kg of body weight.

According to Bodansky (Reference 19), however, most toxic cases of methemoglobinemia, in which the level of methemoglobin does not exceed 40%, do not need a specific treatment, but only a careful observation of the patient because of anoxia, and of a limitation of his physical activity. Oxygen inhalation and the ingestion of glucose infusions are not physiologically justified (Reference 19).

Only P.J., among our patients, had to receive methylene blue because of the high methemoglobin level (48%). On the other hand, it is precisely this patient who refuse every treatment. The other patients received ascorbic acid (1 g) intravenously.

Otherwise, the treatment of poisoning caused by nitrites is symptomatic and is referred to the concomitant phenomena known in therapeutic nitrite administration.

As was already mentioned, food poisoning through nitrites from meat products is mainly caused by mistake, and can be prevented only by adhering to the regulations regarding the preparation of preserving mixtures. Since our patients are meat plant workers which were poisoned at the same working place on three occasions at a rather long time interval, it can be said that the conditions there participated at least to some extent as an external factor in the development of these unfortunate cases.-

O P A Ž A N J A

Observations *Замечания*

Arh. hig. rada. 6 (1955) 303

163

TOKSIČKE METHEMOGLOBINEMIJE ZBOG DJELOVANJA NITRITA

T. BERITIĆ i F. VALIĆ

Institut za medicinska istraživanja Jugoslavenske akademije znanosti i umjetnosti, Zagreb

(Primljeno 10. X. 1955.)

Opisane su methemoglobinemije prouzrokovane nitritima s naročitim obzirom na kemijske konzervanse u industriji mesnih proizvoda, koji su glavni izvor alimentarnih otrovanja nitritima. Iznesen je kazuistički prikaz nekoliko slučajeva otrovanja radnika u mesnoj industriji, koji su zabunom uzeli toksičke doze nitrita.

Methemoglobin (hemiglobin, ferihemoglobin) je oksidacioni produkt hemoglobina, kojega je dvovaljano željezo oksidativnim izbijanjem jednog elektrona postalo trovaljano. Novonastali patološki krvni pigment je fiziološki inertan, ali za transport kisika neupotrebljiv. Manifestna patološka stanja, kod kojih je dio hemoglobina pretvoren u methemoglobin, nazivaju se methemoglobinemijama. Sasvim neznatne količine methemoglobina prisutne su i u krvi zdravih ljudi: veće nakupljanje spriječeno je brzom redukcijom methemoglobina, koju stalno vrši poseban enzimatski mehanizam u crvenoj krvnoj stanici. Međutim, urođenom griješkom taj mehanizam može biti odsutan ili zatajiti (»kongenitalna methemoglobinemija«), a djelovanjem nekih otrova ili njihovih metabolita, koji direktno ili indirektno oksidiraju hemoglobin, taj mehanizam postaje nedovoljan (»toksičke methemoglobinemije«). Među otrovima, koji direktno oksidiraju hemoglobin, najpoznatiji su nitriti.

U farmakološkoj i toksikološkoj literaturi obično se među »nitrite« ubrajaju ne samo razni anorganski i organski nitriti već i nitrati. To je prema Goodmanu i Gilmanu (1) dopušteno zato, što se biološko djelovanje i nitrita i nitrata može uglavnom svesti na učinak oslobođenog iona nitrita (NO_2^-). I organski i anorganski nitriti lako otpuštaju taj ion, bez obzira na to, da li je nosilac organski radikal ili metal. Što se tiče nitrata, i organski nitrati u tijelu otpuštaju nitrit-ion, a anorganski se, ako dospiju u gastrointestinalni trakt, mogu djelovanjem bakterijske flore reducirati u nitrite. I nitrati, dakle, na taj način djeluju kao nitriti.

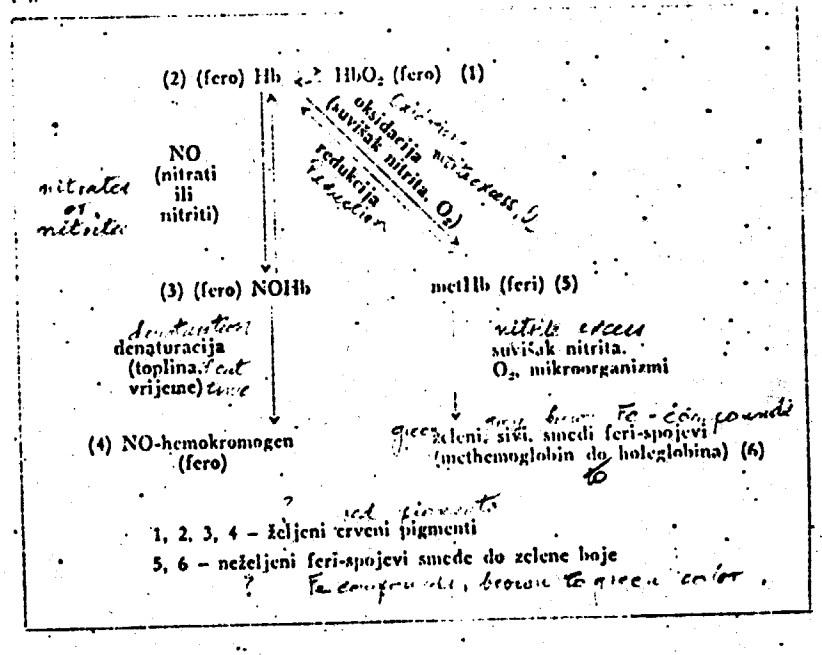
Otrovanja nitritima su medikamentozna, suicidalna ili alimentarna. Bizmutov subnitrat, amonijev i kalijev nitrat su lijekovi, koji se još i danas u medicini propisuju. Poznata su otrovanja bizmutovim subnitratom, naročito kod djece, katkad i sa smrtnim svršetkom (1). Opisano je i letalno otrovanje eritrol tetranitratom (2). Bolesnici s ulceratívnim lezijama crijeva naročito lako dobiju methemoglobinemiju nakon uzimanja nitrata (3). Česta suicidalna otrovanja natrijevim i kalijevim nitritom zabilježena su jedino u Indiji (4), gdje su te kemikalije u slobodnoj prodaji, jer ih tkalci u kućnoj radinosti mnogo upotrebljavaju kod bojadisanja tkanina.

Alimentarna otrovanja nitritima obično potječu iz dva izvora: to su bunarska voda i meso odnosno mesne prerađevine prethodno tretirane anorganskim nitritima i nitratima. Tek su prije deset godina razjašnjene toksičke methemoglobinemije kod dojenčadi (5), koja je bila na umjetnoj mliječnoj ishrani. Istraživanja su, naime, pokazala, da je kod pripremanja hrane u svim slučajevima bila upotrebljena bunarska voda s relativno visokim sadržajem nitrata. Budući da je samo dojenčad obolijevala, mehanizam tih toksičkih methemoglobinemija protumačen je ovako: aciditet želučanog soka kod dojenčadi fiziološki je relativno visok (pH oko 4,9), što u crijevu omogućava rast bakterija, koje reduciraju nitrate, t. j. stvaraju nitrite, koji se resorbiraju u krvni optok i izazivaju toksičku methemoglobinemiju. Osjetljiva su naročito dojenčad ispod dva mjeseca starosti. Zanimljiva je i desad još nerazjašnjena naročita geografska raspodjela ovog oboljenja dojenčadi (6). U SAD je samo u državi Minnesota u tri godine (1947–1949) bilo 139 slučajeva (14 smrtnih!) methemoglobinemija kod dojenčadi (7). Opasni sadržaj nitrata u bunarskoj vodi je koncentracija iznad 20 p. p. m. (izraženo kao dušik, N). Među alimentarna otrovanja treba ubrojiti i nesretne slučajeve, kod kojih je zabunom umjesto kuhinjske soli upotrebljen nitrit (4). Ali najčešća su alimentarna otrovanja neispravno konzerviranim mesom ili mesnim prerađevinama uzrokovana previsokim sadržajem nitrita i nitrata, koji se kao kemijski konzervansi dodavaju kod pripreme mesnih prerađevina. Takva se otrovanja pojavljuju u obliku pravih epidemija. Bushoff (8) je godine 1947. opisao masovna otrovanja juhom, koja je sadržavala salamuru, a Barilari i Benedetto (9) su javili o istim otrovanjima uzrokovanim kobasicama. Schmidt i dr. (10) opisali su 51 slučaj otrovanja krvavicama, Schulze i Scheibe (11) 79 slučajeva, dok je 1952. godine Büch (12) saopćio o masovnom otrovanju natrijevim nitritom uživanjem juhe od kobasica, pri čemu je najednom oboljelo čak 146 osoba. Odgovornost za ta otrovanja obično leži na industriji mesnih proizvoda, koja u posljednje vrijeme sve više upotrebljava nitrite i nitrate za konzerviranje mesa. Budući da suvremena prehrana pučanstva sve više zahtijeva konzervirane mesne produkte, kod kojih je gotovo neophodna upotreba nitrita i nitrata, to su za nas ti prehrambeni artikli praktički i najvažniji izvor nitrita i nitrata. Zbog toga je važno poznavati glavne principe konzerviranja mesnih proizvoda i djelovanja kemijskih konzervansa.

Osnovna zadaća industrije mesnih proizvoda je konzerviranje mesa. Glavni je problem kod toga konzervirati na takav način, da se pritom ne kvare organoleptička svojstva produkta. Kod svih se načina konzerviranja barem jednim dijelom gubi nešto od hranjive vrijednosti mesa, ali je konzerviranje ipak neophodno, jer se jedino na taj način meso može sačuvati od kvarenja dulje vrijeme.

Najčešće se meso konzervira samo kuhinjskom soli (soljenje) ili smjesom kuhinjske soli, nitrata, nitrita i eventualno još nekih dodataka kao što su šećer, rum i neki začini (salamurenje) (13). Soljenjem se konzerviraju dijelovi, koji nisu pretežno ispunjeni mišićjem, dok se mišićni dijelovi uvijek konzerviraju salamurom zbog nepovoljnog utjecaja soli na hemoglobin i mioglobin, što dovodi do promjene boje mesa.

Mehanizam procesa konzerviranja mesa nije još sasvim razjašnjen. To je svakako kompliciran skup fizikalnih, kemijskih, fermentativnih i bakterijskih procesa. Kuhinjska sol pretežno konzervira svojim bakteriostatičkim djelovanjem. Baktericidno djelovanje pri relativno niskim koncentracijama, o kojima se ovdje radi, je neznatno. Osim toga sol ubija i životinjske parasite. Ali sol djeluje nepovoljno na meso, i to na boju mesa. Natrijev klorid razgrađuje, naime, hemoglobin, odnosno mioglobin, pa na taj način meso gubi svoju prirodnu crvenu boju. Da bi se to spriječilo, dodaje se u smjesu za konzerviranje nitrata ili nitrita. Njihovim se djelovanjem dobiva ugodna crvena boja salamurenog mesa, koja se ne gubi ni kuhanjem. Proces, koji se pritom zbiva, je ovaj. Nitrati se utjecajem bakterija, koje denitrificiraju, reduciraju u nitrite; oni u kiselom mediju prelaze u dušičastu kiselinu, a dušičasta kiselina u dušični oksidul (NO). Dušični se oksidul spaja s hemoglobinom, odnosno mioglobinom u izrazito crveni nitrohemoglobin (NOHb), odnosno nitrozomioglobin. Ti spojevi kuhanjem prelaze u živo crvene nitrozohemokromogene, koji su termostabilni. Kako natrijev klorid ne razara ni nitrozohemoglobin ni nitrozohemokromogene meso zadržava crvenu boju i nakon kuhanja. To se djelovanje nitrata i nitrita na krvni pigment može shematski prikazati ovako (14):



Nekad su se za salamurenje uz kuhinjsku sol upotrebljavali gotovo isključivo nitrati, ali se u novije vrijeme sve više upotrebljavaju nitriti ili kombinacija nitrita i nitrata. To je jasno s obzirom na to, što se i nitrati moraju najprije reducirati u nitrite, pa se direktnom upotrebom nitrita uštedi vrijeme potrebno za redukciju. Na taj se način vrijeme salamurenja skraćuje na polovicu, a u nekim slučajevima i na trećinu. S obzirom na to, da stupanj redukcije nitrata zavisi od količine prisutnih bakterija, koje denitrificiraju, nikad se ne može točno izračunati potrebna količina nitrata. Kod upotrebe nitrita ta teškoća otpada, pa na taj način nikad ne dolazi do suviška nitrita u gotovim produktima. Kako je, osim toga, potrebna količina nitrita manja od količine nitrata, to i iz ekonomskih razloga sve više prevladava upotreba nitrita u smjesi za salamurenje. Nitriti pojačavaju i konzervirajuće djelovanje soli, jer donekle djeluju i bakteriostatički (sprečavaju rast bakterija gnjileži, osobito anaerobnih).

Djelovanje nitrita zavisi od upotrebljene količine. Uz suvišak nitrita ne stvaraju se crveni nitrozo spojevi, nego dolazi do oksidacije željeza, pa se javljaju smeđi, pa i zeleni pigmenti. To su spojevi, koji sadržavaju željezo u trovaljanoj formi (methemoglobin do holeglobina). Zato se pri pripravljanju smjese za salamurenje mora obratiti naročita pažnja koncentraciji nitrita. Smjesu za salamurenje trebalo bi da pripravljaju samo za to ovlaštena poduzeća, a ne same tvornice mesnih prerađevina.

Količina nitrita pri salamurenju je takva, da u gotovim produktima nikad nema više od 25 mg nitrita na 100 g mesa. Uz taj sadržaj nitrita ne može doći do otrovanja, jer se ni najvećim količinama ispravno obrađenog mesa, što ih čovjek može konzumirati, ne mogu u organizam još unijeti toksične količine. Do otrovanja gotovim produktima može doći jedino zabunom pri pripravljanju salamure.

Otrovanja nitritima u industriji gotovo su nepoznata. Međutim, u industriji mesnih proizvoda može doći do nesretnih slučajeva otrovanja zbog alimentarnog kontakta s nitritom i nitratom naročito kod nedovoljnih sanitarnih uređaja (vodovodna voda za piće, blagovaonice udaljene od pohranjenih sirovina). U jednom našem poduzeću industrije mesnih proizvoda pojavilo se u posljednje vrijeme više takvih slučajeva otrovanja, pa mislimo, da ih je vrijedno ukratko prikazati.

N. M., 19 godina, radnica u mesnoj industriji. Na dan primitka u bolnicu ponudili su je drugovi kod posla nekom tekućinom, koja je imala miris i tek po rumu. Oko 15 minuta nakon toga pozlilo joj je, zamaglilo joj se pred očima, pomodrila je i izgubila je svijest. Kod dolaska u bolnicu blijeda je i cijanotična, tuži se na jaku glavobolju u zatiljku. RR: 90/60. Nakon ispiranja želuca, davanja analeptika i visokih doza vitamina C stanje se subjektivno poboljšava. Methemoglobin u krvi iznosio je 23% od ukupne količine hemoglobina. U želučanom sadržaju dokazano je prisustvo nitrata i nitrita. Bolesnica je sutradan bez teškoća, a cijanoza je potpuno nestala.

M. L., 27 godina, radnik u istom poduzeću. Na dan primitka u bolnicu popio je oko 12 sati ujutro približno 4 dcl vode iz posude u kojoj su na radnom mjestu držali vodu za piće, neznajući da je u njoj zabunom primiješana salamura. Pol sata nakon toga osjetio je jaku vrućinu u glavi i jako lupanje srca, a zatim vrlo jaku glavobolju u zatiljku, omaglicu, gušenje i podražajni kašalj. U isto su ga vrijeme drugovi upozorili, da je jako pomodrio, osobito na usnama. Kod dolaska u bolnicu je uzbuđen i zaplašen, vrlo jako cijanotičan, naročito na usnama i na jeziku. Pojavljuje se tremor ruku, a kasnije i cijelog tijela. RR: 100/65. Nakon ispiranja želuca i davanja analeptika tlak se postepeno diže, bolesnik se osjeća bolje. Methemoglobin u krvi iznosio je 38%. U sadržaju želuca dokazano je prisustvo nitrita. Liječen je visokim dozama askorbinske kiseline. Sutradan se osjeća potpuno zdrav, cijanoze nestaje. Tlak je kod otpusta iz bolnice 115/75.

M. L., 26 godina, pio je tog istog dana i otprilike u isto vrijeme, nakon doručka, vodu iz iste posude kao i njegovi drugovi. Nakon 10 minuta osjetio je vrućinu, ali nije povračao. Pomodrilo mu je lice i ruke, a zatim je dobio jaku glavobolju u za-

tiljku i sljepočicama. Kod prijema u bolnicu: jaka cijanoza, osobito na usnama. Zaplašen je i nemiran. RR: 95/60, ostali nalazi uredni. Nakon ispiranja želuca, davanja analeptika i visokih doza vitamina C stanje se bitno poboljšava, cijanoze postepeno nestaje, i bolesnik se sutradan osjeća zdrav. Methemoglobin u krvi 26% od sveukupne količine hemoglobina. U želučanom sadržaju proba na nitrite pozitivna.

P. V., 24 godine, popio je mnogo manju količinu vode nego njegovi drugovi. Osim lagane glavobolje nema subjektivnih smetnja, a u bolnicu dolazi zajedno s ostalima jedino radi kontrole. Kod njega je cijanoza jedva primjetljiva, što odgovara i nalazu methemoglobina u krvi (6%). Dobiva C vitamin i nakon nekoliko sati otpušta se s odjela.

P. S., 27 godina, nakon pića vode tuži se na mučninu i povraća, a zatim osjeća jaku bol u zatiljku i sljepočicama, teško disanje i pritisak na srce. Pomodrile su mu usne, a zatim ruke, naročito nokti. Kod dolaska u bolnicu je zaplašen i pokazuje izrazitu cijanozu. RR: 100/65. Methemoglobin 23%, svi ostali laboratorijski nalazi uredni. Reakcija na nitrite u želučanom sadržaju pozitivna. Davanjem askorbinske kiseline i analeptika stanje se popravlja, cijanoza je nakon 24 sata potpuno nestala.

P. J., 46 godina, ne zna, koliko je vode popio, ali je pio iz iste posude kao i ostali tog jutra. Po vlastitom priznanju i po izjavama drugova, popio je dosta rakije prije dolaska na posao, a i neposredno prije, nego što je pio vodu. Kad je popio vode, osjetio je odmah mučninu i mnogo je povraćao, imao je jaki pritisak na srce i teško je disao. Kod primitka u bolnicu vrlo jaka cijanoza, naročito izražena na usnama, jeziku i na licu, koje inače pokazuje teleangicktazije. Jaka tahikardija od 144 u minuti, RR: 130/60. Methemoglobin u krvi 48% od sveukupne količine hemoglobina. Alkohol u krvi (po metodi Nicloux) 1,74 pro mile. Bolesnik odbija ispiranje želuca kao i svako drugo liječenje. Na vlastiti zahtjev napušta bolnicu nekoliko sati nakon primitka. Sutradan, kod kontrolnog pregleda, nema cijanoze i subjektivno se vrlo dobro osjeća.

V. J., 49 godina, jednom je prilikom u istom poduzeću zabunom namjesto soli uzeo natrijev nitrit, da zasoli jelo kod doručka. Ubrzo nakon jela osjetio je bljutavost u ustima, ali je unatoč tome nastavio raditi, sve dok se nije pojavila mučnina, a zatim i povraćanje. U isto vrijeme su ga drugi upozorili, da je modar u licu. Pol sata nakon toga dobio je vrtoglavicu i osjećao se vrlo slab na nogama. Kod dolaska u bolnicu cijanotičan je i blijed. Krv na methemoglobin nije uzeta, a ostali nalazi su uredni. Davanjem analeptika i C vitamina stanje se bitno popravlja. Sutradan cijanoza potpuno iščezava.

Kod svih bolesnika, kod kojih je bio određivan methemoglobin, krv je uzeta odmah po dolasku u bolnicu, a prije svakog terapeutskog zahvata. Za kvantitativno određivanje methemoglobina bila je upotrebljavana Heilmeyerova diferencijalno spektrofotometrijska metoda (16). Mjerenja su bila izvršena pomoću Beckmanova spektralnog fotometra model DU. Ekstinkcije krvnih otopina bile su mjerene kod valnih duljina od 560, 576 i 590 milimikrona uz širinu pukotine od 0,1 mm. Koncentracija methemoglobina bila je izračunavana iz karakterističnih kvocijenata ekstinkcija $\frac{E\ 576}{E\ 560}$ i $\frac{E\ 576}{E\ 590}$.

Za fotometriranje su bile pripravljane otopine krvi u 0,4% amonijaku, razređenja 1:100. Mjerenja su vršena u kivetama debljine sloja od 0,5 cm.

DISKUSIJA

Prominentni klinički simptom methemoglobinemije je cijanoza. Methemoglobinemička cijanoza je boja svoje vrste, koja nije identična s dobro poznatom bojom običnih cijanoza, t. j. onih cijanoza, kod kojih u kapilarnoj krvi protječu povećane količine *reduciranog* hemoglobina. U methemoglobinemičkoj cijanozi ne prevladavaju ljubičaste kompo-

Biochemical Journal Vol 22 (1928) (163)

XVIII. THE REDUCTION OF NITRATES IN ANIMAL TISSUES.

By FREDERICK BERNHEIM
AND MALCOLM DIXON (*1851 Exhibition Senior Student*).
From the Biochemical Laboratory, Cambridge.

(*Received January 2nd, 1928.*)

It has long been known that many animal tissues contain systems capable of reducing nitrates to nitrites. Stepanow [1902] investigated the presence of nitrite in the tissues of rabbits and dogs. By means of the Griess-Ilosvay reagent he found nitrite in the white matter of the brain, the lungs, bronchi, parotids, small intestine, medullary substance of the kidney, adrenals, testicles, and lymph glands. He found it absent from the grey matter of the brain, the liver, stomach, spleen, cortex of the kidney, muscle and blood. He showed, moreover, that certain tissues which normally contained no nitrite were able to form it when ground with nitrates; and also that if the animal had been fed on food free from nitrates no nitrite was found in any of the tissues.

More recently Lipschitz [1921] has shown that the dehydrases of frog's muscle (studied by Thunberg [1920] with the methylene blue technique) were able to reduce aromatic nitro-compounds, *e.g.* *m*-dinitrobenzene, and that the nitro-compound was simply equivalent to methylene blue as a hydrogen acceptor in these systems. Bach [1911] found that nitrate could replace methylene blue in the aldehyde oxidase system, which rapidly reduced it to nitrite; and Dixon and Thurlow [1924] showed that the same was true of the xanthine oxidase system.

In view of these facts it was thought interesting to investigate the reduction of nitrates by animal tissues, in order to determine which of the known systems could use nitrate as a hydrogen acceptor.

Muscle tissue was first investigated. Preliminary experiments showed that the ability of muscle to reduce added nitrates is by no means general, and seems to be confined to a comparatively small number of species. We have found that the muscles of ox, sheep, pig, rabbit, dog and chicken are quite unable to reduce nitrates, and positive results were only obtained with rat and guinea-pig muscle.

We found, on the other hand, that the livers of all these animals reduce nitrates very readily, and the liver appears to be the main seat of the phenomenon. Rat's liver reduces five to ten times as much nitrate in a given time per unit weight as rat's muscle.

The results of these preliminary experiments led us to choose rat's muscle and ox liver as convenient tissues for the investigation of the nature of the systems responsible for nitrate-reduction, and the results obtained with these two tissues are given in the two following sections of this paper.

THE REDUCTION OF NITRATE BY RAT'S MUSCLE.

The first problem was to see whether any of the known dehydrase systems present in muscle could be responsible for the reduction. Experiments were carried out anaerobically in Thunberg vacuum-tubes as follows. Muscle from the hind legs of a freshly killed rat was finely minced and washed by shaking four times successively with 100 cc. distilled water. The washed muscle was squeezed as dry as possible, and portions of 0·3 g. were weighed out into the Thunberg tubes. 2 cc. phosphate buffer (p_H 7·5) were added to each tube, together with solutions of the appropriate hydrogen donator and acceptor. The hydrogen donators were in all cases made up in 1 % solution in water and carefully neutralised, and 1 cc. of the solution was used. With methylene blue as the hydrogen acceptor the presence in the washed muscle of systems which could oxidise the following substances was readily demonstrated: succinic, formic, citric, acetic, lactic, fumaric, maleic, malic, and glutamic acids. It was found that the washed muscle by itself reduced methylene blue slowly, even after very thorough washing, so that in all cases simultaneous experiments with and without the hydrogen donator were carried out and the rates of reduction compared. The rate of the spontaneous reduction was however, in general, small in comparison with that induced by the hydrogen donator.

The experiments with nitrate were carried out anaerobically in a similar way. 1 cc. of 5 % sodium nitrate (a considerable excess) was added to each tube instead of the methylene blue, the tubes were incubated for 2 hours at 37°, and the nitrite formed during this period was estimated roughly colorimetrically after the addition of 2 cc. of Griess-Ilosvay reagent. As before, the tubes were in all cases compared with control tubes to which no hydrogen donator had been added.

The results of these experiments showed quite definitely that the reduction of nitrate to nitrite, which still occurred with the washed muscle, could not be referred to the action of any of the dehydrase systems mentioned above, since these were all totally unable to reduce nitrate. The amount of nitrite produced by the muscle itself was not increased in the slightest by the addition of any of the hydrogen donators mentioned, and the depth of colour given by the Griess-Ilosvay reagent was always exactly the same as in the control tubes with no added donator. We had previously observed that a solution of succinoxidase (prepared from muscle by Ohlsson's [1921] method) was unable to reduce nitrates in presence of succinate. In addition to the substances mentioned, the following possible hydrogen donators were tried with the washed muscle, also with negative results: acetaldehyde, pyruvic acid,

glucose, acetone, diethyl ketone, creatinine, lecithin, cholesterol, ammonia, and various extracts of liver. The latter were prepared by extracting liver with 2 % sodium carbonate and destroying their own power of reducing nitrate by acidification and subsequent reneutralisation or by boiling (see below) and were added to the muscle in order to see whether any of the thermo- or acid-stable hydrogen donators present in the liver would increase the nitrate-reduction. Boiled and acidified muscle broths were also added to the active muscle, but no greater reduction occurred.

Gscheidlen [1874] stated that muscle which had been exercised reduced more nitrate than unexercised muscle, but we have not found this. The gastrocnemii of the rat were dissected out, and one was placed in Ringer-Locke solution and made to contract by stimulation with induction shocks until too fatigued to respond. The exercised and the control muscles were then chopped finely, a portion of each was thoroughly washed with distilled water, and 0·3 g. of each portion incubated with 2 cc. buffer + 1 cc. 5 % sodium nitrate in Thunberg tubes as before. The results were as follows:

	Nitrite reaction
Exercised muscle	+ +
Washed exercised muscle	+ +
Unexercised muscle	+ +
Washed unexercised muscle	+ +

It was impossible to detect the slightest difference in the strength of the nitrite reaction between any of the tubes, so that it appears that the nitrate-reducing system has no direct connection with the products of muscular activity.

The significance of the results described above is discussed later.

Properties of the nitrate-reducing system of rat's muscle.

The system is not readily washed out with water: after 20 washings there is considerable activity left. Nor does previous treatment with ether to break up the cell-membranes cause it to come more readily into solution.

The system can however be extracted from muscle by $M/15$ disodium hydrogen phosphate solution. Rat's muscle was chopped, washed with water, ground with the alkaline phosphate solution, and filtered. The cloudy filtrate was precipitated by saturation with ammonium sulphate and again filtered. The precipitate contained the system unimpaired, and the filtrate, when added to muscle, did not increase the reduction.

Treating the muscle with acetone does not affect the reduction. Alcohol treatment decreases it. Standing at room temperature in the air gradually destroys the reducing power, probably because the system becomes oxidised.

If the incubation with nitrate is not carried out anaerobically, but in open tubes, the amount of nitrite formed is greatly diminished. This may be due to the system using oxygen in preference to nitrate, so that the two hydrogen acceptors compete; or it may be due to a coupled oxidation of the nitrite formed in the sense of Thurlow [1925].

The system is very sensitive to acid. If brought to p_H 5 and back to neutrality the reduction is completely abolished. It is, however, more stable to alkali, and can be brought to p_H 10 and back without affecting the reduction. The actual reduction process, however, is limited to a very narrow range of p_H, and occurs only between p_H 6·5 and p_H 8, with a maximum velocity at about 7·4.

The system is destroyed by heating to 70°, but survives exposure to 60° for 5 minutes.

These properties make it extremely probable that the system is enzymic in nature, but so far we have not been able to obtain separately catalyst and substrate.

Whether the reduction goes further than the nitrite stage has not been determined.

The system is specific for inorganic nitrates, and *m*-dinitrobenzene is not reduced. This gives us the apparently paradoxical result that while this system can reduce nitrate but not nitrobenzene, the muscle dehydrases can reduce nitrobenzene but not nitrate.

Particularly interesting is the effect of cyanide on the system. The presence of $M/500$ KCN completely prevents the reduction of nitrate by the muscle, so that even after several hours no nitrite can be detected by the very sensitive Griess-Ilosvay reagent. The system is therefore cyanide-sensitive in Warburg's sense in the same way as those systems which have been shown to depend on the action of small amounts of catalytic metals. We have no data to show whether this is the case in this system or not; but it is clear that the fact of a tissue respiration process being cyanide-sensitive is not in itself sufficient reason for assuming that the effect of the cyanide is due to the paralysis of an oxygen activator, as is often done, since the cyanide may inhibit reactions in which molecular oxygen is not concerned, as here.

The reduction of nitrate by liver.

As in the case of muscle, it was found that washing with water did not remove the nitrate-reducing power of the tissue. Washed liver was therefore used in most of the experiments. The following procedure was found to give the most satisfactory results. Half a pound of ox liver was finely minced and shaken in a flask with an equal volume of ether in order to break down the cell-membranes and render the water-washing more effective. It was then placed in a two-litre flask and washed 15 to 20 times with water, until no more haemoglobin was washed out. During this process about half the liver was washed away. The final preparation consists of small light-brown granular masses. It can be kept indefinitely in the ice chest, reduces nitrate readily, and is very convenient to use.

On investigation it was found that the reduction of nitrate by the liver was due to two distinct factors: (*a*) a system apparently identical with the muscle system described above, and (*b*) the aldehyde (and xanthine) oxidase

of liver, which is probably identical with that of milk. This latter enzyme was shown to be capable of reducing nitrate (in the presence of its substrate) by Bach [1911] and by Dixon and Thurlow [1924]. The xanthine oxidase is absent from muscle [Morgan, 1926].

It is possible to separate the two factors. On extracting the washed liver by grinding with 2 % sodium carbonate solution, filtering through muslin, and neutralising the liquid, it is found that both the factors have passed into solution. In order to obtain the xanthine oxidase free from the other factor charcoal is added, the suspension shaken for a few moments, and the charcoal filtered off with suction. A clear light-brown solution of the oxidase is obtained. The separation depends upon the fact that the nitrate-reducing factor (a) is readily adsorbed by charcoal, whereas the oxidase is not [Dixon and Kodama, 1926]. To obtain the former, the sodium carbonate extract is brought to p_H 10, and on reneutralising it is found that the oxidase has been completely destroyed, leaving the other factor intact.

The distinction between the nitrate-reducing system and the oxidase can also be shown by means of cyanide. The nitrate-reduction by the washed liver alone is completely inhibited, while in the presence of aldehyde that part of the reduction which is due to the oxidase is unaffected. The following is a typical experiment.

Contents of tube	Nitrite reaction
0·3 g. washed liver + 2 cc. buffer + 1 cc. $NaNO_3$	+ +
" + " + " + 1 cc. 1 % aldehyde	+ + + +
" + " + " + 0·5 cc. $M/30$ KCN	−
" + " + " + " + 1 cc. aldehyde	+ +

On adding to the liver the possible hydrogen donators previously tested with muscle it was found that all gave entirely negative results with nitrate, with the exception of aldehyde and pyruvic acid. These two substances, and also hypoxanthine, reduced nitrate freely. The increase of reduction in presence of aldehyde and hypoxanthine is clearly due to the action of the oxidase, and was to be expected if the oxidase is the same as that in milk. Pyruvic acid, however, is quite unable to reduce nitrate, either with the oxidase alone or with the nitrate-reducing system alone; it only reduces with the liver. This reaction is in all probability to be attributed to the presence of a decarboxylase in the liver. This would convert the pyruvic acid into acetaldehyde, which would then act as a substrate for the oxidase, and so bring about the reduction. Gottschalk [1924] obtained acetaldehyde on incubating pyruvic acid with liver.

The addition of boiled or acid-treated liver broth to the active preparation of liver produced, as in the case of muscle, no increase in the nitrate-reduction.

The other properties of the nitrate-reducing system of liver are similar to those of the system in muscle. Treatment with acetone does not affect the reduction, but alcohol partially destroys it. It is thermolabile, and is destroyed by heating to 70°, but not by heating to 60° for 5 minutes. It is

acid-sensitive, and is completely destroyed at p_H 5; but, on the alkaline side, p_H 10 leaves it quite unaffected. It is cyanide-sensitive, as mentioned above. The similarity in properties strongly suggests that the nitrate-reducing systems in muscle and liver are identical.

Attempts have also been made to separate catalyst and substrate in the liver system by precipitation and other methods, but without success.

The washed liver contains very little glutathione, to judge from the nitroprusside reaction, but it will very readily reduce oxidised glutathione. A preparation which gives an almost negative nitroprusside test will, after having been incubated anaerobically for a short period with oxidised glutathione, give a very strong reaction. This fact may be of importance in the study of the glutathione system. This reduction is unaffected by boiling the liver, whereas of course the nitrate-reduction is completely destroyed, so that the two reductions cannot be due to the same factors.

The thoroughly washed liver is still able to reduce methylene blue, but we are as yet unable to say whether part or all of this reducing power is due to the nitrate-reducing system or not. The liver also reduces the series of dyes used by Mansfield Clark as r_H indicators. The results obtained with these dyes, however, are somewhat unexpected and difficult to explain, though perfectly definite. They are as follows. The dyes were freshly made up in aqueous solution in concentrations equivalent to the 1/5000 methylene blue solution used. (The names and r_H ranges of the dyes have been previously given in this *Journal* [Dixon, 1926, p. 714], and they are here referred to simply by their series-numbers. They are numbered in ascending r_H order, so that No. 9 is theoretically the easiest and No. 1 the most difficult to reduce. No. 3 *a* is methylene blue.) The reductions were carried out in Thunberg tubes as before, and 0·3 g. of thoroughly washed liver + 2 cc. buffer + 1 cc. of the dye were used in each tube. The table gives the reduction-times (in minutes) with and without the addition of 1 cc. 1 % acetaldehyde.

Dye No.	Reduction-time		Dye No.	Reduction-time	
	Alone	+aldehyde		Alone	+aldehyde
1	120	70	5	45	50
2	420	100	6	17	35
3	480	100	7	20	38
3 *a*	60	40	8	25	54
4	45	50	9	8	25

It will be seen that while the first four dyes behave normally in being reduced more rapidly in the presence of aldehyde than in its absence, reduction of the last six dyes is actually *slowed* by the aldehyde. The cause of this is not clear, but it is possible that there is some active reducing system in the liver whose potential is such that it can reduce dyes 4 to 9, but cannot reduce dyes 1 to 3 *a*. If now this system is inhibited by the presence of aldehyde, results similar to those found will be produced. For, in the case of the last six dyes, where the reduction may be mainly due to this system, the inhibition produced by the action of the aldehyde on the system may

obscure the acceleration produced by the action of the aldehyde oxidase. With the first four dyes, on the other hand, the system plays no part, and consequently the action of the oxidase shows up. This is merely a suggestion, but it is not easy at the moment to see any other explanation.

Discussion.

Though the significance of some of the facts given in this paper is not yet clear, a number of interesting points are brought out by the work.

It appears fairly certain that the nitrate-reducing system in muscle, and the corresponding system in liver, cannot be identical with any of the hitherto described reducing enzymes, and it seems that we must attribute the reduction to a new enzyme system. As to its significance in connection with tissue respiration processes we can of course say little as yet.

As we have pointed out above, the system is very active in the livers of all the animal species examined; but the fact that it is absent from the muscles of all but two of the species affords another example of the curious and apparently haphazard distribution of some of the systems concerned in biological oxidations (compare the xanthine oxidase [Morgan, 1926]).

The fact that the Thunberg tissue dehydrases are unable to reduce nitrate is a fact of considerable importance in considering the ability of nitrate to act as a direct hydrogen acceptor. Dixon and Thurlow, working on the milk oxidase, believed that nitrate was simply equivalent to methylene blue as a hydrogen acceptor, and it is possible that this is the case in the system with which they were dealing. It is clear, however, that the two substances are by no means always equivalent.

We have not sufficient data as yet to decide whether the failure of nitrate to replace methylene blue in the Thunberg dehydrase systems is due to an insufficiently high reducing power in these enzyme systems, to a difference in the accessibility of the two substances to the active enzyme surface, or to the lack of a nitrate-activating catalyst of the type studied by Quastel, Stephenson and Whetham [1925]. Each of these possibilities would seem to provide an adequate explanation.

The first possibility involves the assumption that methylene blue is more easily reduced than nitrate, and that the dehydrases, with the exception of the aldehyde and xanthine oxidase, have reducing powers greater than that necessary to reduce methylene blue but less than that necessary to reduce nitrate. The xanthine oxidase seems to be a particularly strongly reducing system compared with the other dehydrases. Its reducing power must be much higher than that of the succinoxidase for instance, for while the latter system comes to an equilibrium with methylene blue considerably before the dye is completely reduced [Thunberg, 1925], the xanthine oxidase reduces methylene blue completely (*i.e.* more than 99·9999 %), as shown by Clark [1927]. Although a number of systems are known which will reduce methylene

blue but not nitrate, we are not aware of any[1] which will reduce nitrate but not methylene blue.

The possibility that the difference in behaviour is due to a difference in accessibility must be borne in mind; but, although it may explain the facts in the systems under consideration, it cannot account for the difference in behaviour with homogeneous systems (*e.g.* cysteine, referred to below).

In connection with the third possibility it should be pointed out that, while it seems probable that the muscle dehydrases may be able to reduce nitrate only when the latter is activated by a catalyst, it seems quite possible nevertheless that nitrate may be directly reduced without activation in such systems as the xanthine oxidase. Quastel, Stephenson and Whetham used as a test of nitrate-activation the ability of the activated nitrate to oxidise reduced methylene blue, which nitrate by itself is unable to do. We have found that using this test no nitrate-activation can be detected in the xanthine oxidase system: in one limb of a branched vacuum-tube was placed sodium nitrate solution, and in the other limb xanthine oxidase solution in phosphate buffer together with methylene blue and an amount of hypoxanthine just sufficient to reduce the latter; after careful evacuation of the tube and allowing to stand until the methylene blue was reduced, the nitrate solution was immediately run into this solution by tilting the tube. Not the slightest trace of blue colour appeared. If then the oxidation of leuco-methylene blue is a reliable indication of the presence of activated nitrate it would seem that the xanthine oxidase system does not contain a nitrate-activating catalyst and that the oxidase is able to reduce nitrate directly. If the nitrate is added initially with the methylene blue the reduction of the latter is slowed, presumably because the nitrate competes with the methylene blue as a hydrogen acceptor.

If it is assumed that nitrate invariably requires activation before it can be reduced in the tissues it is clear that the catalyst responsible must be associated in a very special way with particular oxidase systems in the cell. For example, the succinic oxidase in, say, a liver extract cannot reduce nitrate, while *in the same solution* the aldehyde oxidase reduces nitrate freely, so that the activator, if present, must be in intimate association with the latter system in such a way that its action is not available for other systems. The same applies also to the nitrate-reducing system in muscle.

Nitrates differ from methylene blue in giving no oxidation-potential at a platinum electrode, in this respect resembling the aromatic nitro-compounds. The latter are nevertheless easily reduced, both *in vitro*, for example by —SH compounds (cysteine, glutathione, etc.), and also by the dehydrases. Nitrates are not reduced by cysteine, unless a nitrate-activating catalyst is present (Quastel, Stephenson and Whetham). They are, however, reduced *in vitro* by such reducing systems as zinc dust suspended in *neutral* solution—a system which has reducing properties which very closely parallel those of the xanthine

[1] One of us (F. B.) will deal with the aldehyde oxidase of the potato in a paper to be published shortly.

oxidase system. For instance, in addition to the reduction of the usual dyes, etc. they both take up molecular oxygen, forming hydrogen peroxide, which is subsequently reduced to water; the oxygen uptake is not prevented by cyanide; they both reduce nitrates and aromatic nitro-compounds; and they both fail to reduce the —SS— group[1]. Other points of similarity might be mentioned. The Thunberg dehydrases, on the other hand, would seem rather to correspond in reducing properties with —SH compounds. They fail to reduce nitrates in the absence of a special catalyst; the oxidation of the —SH group by molecular oxygen is abolished by small amounts of cyanide [Warburg and Sakuma, 1923; Harrison, 1924], which is true also of, at any rate, the oxidation of succinic acid by the succinoxidase [Fleisch, 1924]; and both —SH compounds and the succinoxidase system reduce oxygen some hundreds of times faster than methylene blue, which is not the case with the xanthine oxidase.

We would suggest provisionally that, while the majority of the dehydrases can reduce nitrate only in the presence of a special catalyst, certain of them are able to reduce nitrate directly in the absence of such a catalyst.

Summary.

1. While the livers of all the animals studied reduce nitrate freely, nitrate-reduction in muscle is confined to the rat and the guinea-pig. A study of nitrate-reduction in rat's muscle and ox liver has been made.

2. The reduction of nitrate by muscle cannot be referred to any of the known reducing systems present. The dehydrases of muscle are incapable of reducing nitrate. No hydrogen donator has been found which will increase the reduction of nitrate by washed muscle.

3. The properties of the nitrate-reducing system are described. It appears to be enzymic in nature.

4. The nitrate-reduction is completely abolished by the addition of $M/500$ cyanide.

5. In liver the nitrate-reduction is due to two factors: (*a*) a system having the same properties as that in muscle, and (*b*) the aldehyde (xanthine) oxidase, probably identical with that in milk. These two factors can be separated. No hydrogen donator, other than the substrates of the oxidase, has been found which will increase the reduction of nitrate by liver.

6. These facts are discussed in relation to theories of biological oxidation processes.

We wish to thank Sir F. G. Hopkins for his interest and advice.

[1] Zinc dust in acid solution of course reduces the —SS— group.

REFERENCES.

Bach (1911). *Biochem. Z.* **33**, 282.
Clark (1927). *U.S. Pub. Health Rep. Supplement*, No. **66**.
Dixon (1926). *Biochem. J.* **20**, 703.
Dixon and Kodama (1926). *Biochem. J.* **20**, 1104.
Dixon and Thurlow (1924). *Biochem. J.* **18**, 989.
Fleisch (1924). *Biochem. J.* **18**, 294.
Gottschalk (1924). *Biochem. Z.* **156**, 582.
Gscheidlen (1874). *Pflüger's Arch.* **8**, 506.
Harrison (1924). *Biochem. J.* **18**, 1009.
Lipschitz (1921). *Pflüger's Arch.* **191**, 1.
Morgan (1926). *Biochem. J.* **20**, 1282.
Ohlsson (1921). *Skand. Arch. Physiol.* **41**, 77.
Quastel, Stephenson and Whetham (1925). *Biochem. J.* **19**, 304.
Stepanow (1902). *Arch. exp. Path. Pharm.* **47**, 411.
Thunberg (1920). *Skand. Arch. Physiol.* **40**, 1.
—— (1925). *Skand. Arch. Physiol.* **46**, 339.
Thurlow (1925). *Biochem. J.* **19**, 175.
Warburg and Sakuma (1923). *Pflüger's Arch.* **200**, 203.

The results were presented at the 15th annual meeting of the American Electroencephalographic Society, Atlantic City, 9 June 1961. The drugs used were kindly supplied by Ciba Pharmaceutical Company, Montreal; Hoffman LaRoche Company, Montreal; Lilly Research Laboratories, Indianapolis; and Parke, Davis Company, Montreal.

3. J. A. Wada and F. Bauck, *Electroencephalog. and Clin. Neurophysiol.* 13, 299 (1961).
4. J. A. Wada and L. R. Cornelius, *A.M.A. Arch. Neurol.* 3, 425 (1960).
5. S. Udenfriend, H. Weissbach, D. F. Bogdanski, *Ann. N.Y. Acad. Sci.* 66, 602 (1957).
6. B. B. Brodie, S. Spector, P. A. Shore, *ibid.* 80, 609 (1959).
7. B. B. Brodie and P. A. Shore, *ibid.* 66, 631 (1957).
8. F. Sulser and B. B. Brodie, *Science* 131, 1440 (1960).
9. B. B. Brodie, S. Spector, R. Kunzman, P. A. Shore, *Naturwissenschaften* 45, 243 (1958).
10. E. G. Anderson, D. E. Hutcheon, D. D. Bonnycastle, paper presented before the American Society of Pharmacology and Experimental Therapeutics, 1958; cited by S. Garattini, R. Kato, L. Valzelli, *Psychiat. et Neurol.* 140, 190 (1960).
11. J. A. Wada, paper presented at the Seminar on the Physiology and Biochemistry of Disturbed Brain Function, Vancouver, 15–16 June 1961.
12. M. H. Aprison, paper presented at the 16th annual convention of the Society of Biological Psychiatry, Atlantic City, 10 June 1961.

17 July 1961

194

Effect of Dietary Nitrate on Thyroid Function

Abstract. Experimental results indicate that 0.31 and 0.92 percent dietary nitrate, when consumed by rats and sheep respectively, can affect the normal iodine metabolism of the thyroid gland. The dietary level of iodine appears to be highly important when nitrate is present in the diet.

It has been shown by Wyngaarden *et al.* (*1*) that several monovalent anions, including nitrate, when injected into the animal, can interfere with normal iodine metabolism of the thyroid gland. The effects of nitrate in natural feeds has concerned animal scientists in recent years (*2*). This report attempts to assess the effect of dietary nitrate on the normal functioning of the thyroid gland.

The method of Premachandra and Turner (*3*) was used to determine the goitrogenic effect of nitrate both qualitatively and quantitatively. Adult female rats weighing 200 to 300 g were kept under conditions of uniform temperature of 75°F. The animals were fed a finely ground corn-soybean oil meal diet calculated to contain 135 ppm iodine, for 1 week. Each rat was then injected with 3 μc of carrier-free I^{131}. A 24-hour external thyroid count was made on ether-anesthetized animals placed on a lead plate with the thyroid gland over a scintillation probe. Measurements were made with a scintillation counter connected to a rate meter. Nitrate was added to the ration as KNO_3 at concentrations of 0.5, 1.0, and 2.5 percent.

Table 1. Effect of dietary nitrate on serum total I^{131}, serum-protein-bound I^{131}, and blood NO_3 of sheep.

Activity (count/min per 100 ml serum)		Av. blood NO_3 (mg/100 ml)
Av. total serum I^{131}	Av. PBI^{131}	
Control (five sheep)		
93,170	83,510*	0.80
Treated with 1.5 percent KNO_3 (six sheep)		
59,580	53,950*	1.93

* Significantly different ($p < .01$).

The results, shown in Fig. 1, indicate that dietary KNO_3 at a level of 0.5 percent adversely affected the iodine uptake of the thyroid gland of the rat. This level of nitrate is not uncommon in some hays and ensilages (*4*). The higher levels of nitrate are more commonly found in hay, pasture, and ensilage that are grown either under conditions of unbalanced fertility or the onset of drought or other adverse conditions for plant growth.

Since the above-mentioned feeds are normally given to ruminants, sheep were fed a ration containing 1.5 percent KNO_3. This ration was composed of 58 percent corn cobs, 24 percent corn, 8 percent soybean oil meal, 7 percent molasses, 2 percent minerals, and 1 percent urea. The concentration of serum I^{131} fixed as protein-bound iodine 6 days after 100 μc I^{131} had been injected was used as the criterion (*5*) for normal thyroid function. Methemoglobin was determined and no difference was found between the control and experimental groups. The results in Table 1 indicate that some nitrate passed through the rumen unchanged and that this nitrate interfered with normal thyroid function.

Muhrer *et al.* (*2*) found symptoms of vitamin A deficiency in cattle that consumed nitrate. Garner *et al.* (*6*) showed increased depletion of vitamin A in rats fed nitrate. The function of the thyroid in the conversion of carotene to vitamin A is still debated. However, Johnson and Bauman (*7*) showed that a functioning thyroid gland is necessary for this conversion. In view of these facts, it is conceivable that the vitamin A deficiency is an indirect result of abnormal thyroid function induced by the nitrate.

From a practical view, it is fortunate that the interference of certain monovalent anions with normal iodine metabolism of the thyroid gland can be reversed by increasing the iodine concentration of the diet (*1*). This may help explain the extreme variability in nitrate effect reported by investigators located in different geographic areas. The dietary level of iodine is apparently important for evaluating a feed suspected of containing nitrate.

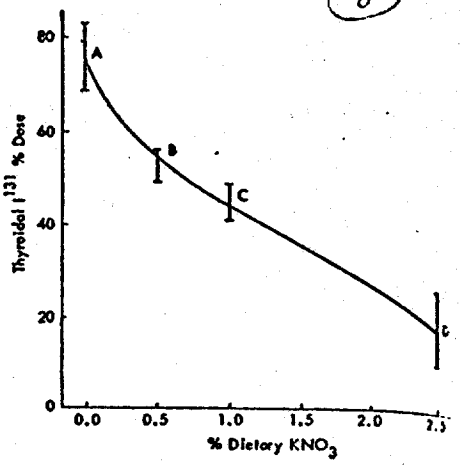


Fig. 1. The effect of dietary KNO_3 on thyroidal uptake of I^{131}. Vertical bars represent standard error of the mean. There were eight, six, six, and four rats in treatments *A*, *B*, *C*, and *D*, respectively. All treatments were significantly different from control *A*: *B*, $p < .05$; *C*, $p < .01$; *D*, $p < .01$.

This research indicates that dietary nitrate can adversely affect the normal functioning of the thyroid gland and suggests that some of the symptoms encountered in the field may be a result of impaired thyroid function of animals. On the basis of this work, we strongly recommend that animal rations suspected of containing nitrates be supplemented with adequate amounts of iodine and vitamin A (*8*).

RICHARD A. BLOOMFIELD,
CLIFFORD W. WELSCH,
GEORGE B. GARNER, MERLE E. MUHRER
Department of Agricultural Chemistry, University of Missouri, Columbia

References and Notes

1. J. B. Wyngaarden and B. Wright, *Endocrinology* 50, 537 (1952).
2. M. E. Muhrer *et al.*, *J. Animal Sci.* 14, 1251 (1955).
3. B. N. Premachandra and C. W. Turner, *ibid.* 19, 1181 (1960).
4. University of Missouri, unpublished data; E. I. Whitehead and A. L. Moxon, *S. Dakota Expt. Sta. Bull.* 424 (1952).
5. I. L. Chaikoff, A. Taurog, W. O. Reinhardt, *Endocrinology* 40, 47 (1947).
6. G. B. Garner, B. L. O'Dell, P. Rader, M. E. Muhrer, *J. Animal Sci.* 17, 1213 (1958).
7. R. M. Johnson and C. A. Bauman, *J. Biol. Chem.* 171, 513 (1947).
8. We are indebted to Dr. C. W. Turner, Dr. W. G. Pipes, and Mr. A. J. Grossie for the use of equipment and materials. This research was supported in part by a grant from Feed Service Corporation, Crete, Neb. This report has been approved by the director of the Missouri Agricultural Experiment Station as journal series paper No. 2298.

2 June 1961

Proceedings of the Society for Experimental Biology and Medicine

195

INCLUDING THE FOLLOWING SECTIONS

Cleveland, O.
District of Columbia
Illinois
Iowa
Maryland
Michigan
Minnesota
Missouri
New York
Northwest
Ohio Valley
Pacific Coast
Rocky Mountain
Southern
Southern California
Southeastern
Southwestern
Western New York
Wisconsin

October-December, 1962 (Inclusive)

VOLUME 111

New York

1. Kuhns, W. J., *J. Exp. Med.*, 1954, v99, 577.
2. Brattsen, L., Colldahl, H., Laurell, A., *Acta Allerg. Kbh.*, 1955, v8, 1.
3. Porter, R. R., *Int. Arch. Allerg. & Appl. Immunol.*, 1957, v11, 61.
4. Stanworth, D., *Acta Allerg. Kbh.*, 1960, 15 (Suppl. VII), 48.
5. Kabat, E. A., personal communication.
6. Sherman W., *Proc. 3rd Int. Cong. Allergology*, Paris,, 1958, p155.
7. Schon, A., Augustin, R., in *Mechanisms of Antibody Formation*, Academic Press, New York, 1959, p79, 94.
8. Kuhns, W. J., *Fed. Proc.*, 1953, v12, 450.
9. Fraser, D. T., *Tr. Roy. Soc. Canada*, 1931, v25, 175.
10. Kuhns, W. J., Pappenheimer, A. M., Jr., *J. Exp. Med.*, 1952, v95, 375.
11. Finger, I., Kabat, E., *ibid.*, 1958, v108, 453.
12. Kuhns, W. J., Pappenheimer, A. M., Jr., *ibid.*, 1952, v95, 363.
13. Cohn, E., Strong, L., Hughes, W., Mulford, D., Ashworth, J., Melin, M., Taylor, H., *J. Am. Chem. Soc.*, 1946, v64, 459.
14. Deutsch, H., Alberty, R., Gosting, L., *J. Biol. Chem.*, 1946, v165, 21.
15. Kunkel, H., Zone Electrophoresis in *Methods of Biochemical Analysis*, Interscience, New York, 1954, v1, p141.
16. Folin, O., Ciocalteau, V., *J. Biol. Chem.*, 1927, v73, 627.
17. Lowry, O., Rosebrough, N., Farr, L., Rosedall, R., *ibid.*, 1951, v193, 265.
18. Kuhns, W. J., *Am. J. Med.*, 1956, v20, 251.
19. ———, *J. Immunol.*, in press.
20. Heremans, J., Vaerman, J., *Nature*, 1962, v191, 1091.
21. Coca, A., Walzer, M., Thommen A., *Asthma and Hay Fever*, Charles C Thomas, Springfield, Ill. 1931.

Received July 2, 1962. P.S.E.B.M., 1962, v111

195

Thyroid Compensation Under the Influence of Dietary Nitrate.* (27769)

R. A. BLOOMFIELD, C. W. WELSCH, G. B. GARNER AND M. E. MUHRER
(Introduced by B. L. O'Dell)
Department of Agricultural Chemistry, University of Missouri, Columbia

The ability of thiocyanate, perchlorate, nitrate and other anions to interfere with normal thyroid function has been the basis of numerous investigations(1,2). Of these ions, nitrate is unique in that it is found in many plant species(3) and in well water(4,5) commonly consumed by domestic animals and man. Recent studies(6) have shown that dietary nitrate interferes with the normal metabolism of injected I^{131}. These studies were conducted on rats and sheep fed nitrate for less than one week. Under normal conditions rats fed KNO_3 show a slight depression in rate of gain; however, when cold stress was applied, the rate of gain was significantly decreased.† This would indicate that rats can compensate for a nitrate load under the usual environmental conditions but not under cold stress. This investigation was undertaken to determine whether or not the thyroid compensates for the nitrate effect on iodine metabolism.

Materials and methods. Sixteen 150 g male rats, originally of the Wistar strain, were divided into 2 groups of 8 each. The animals were maintained on a basal diet of the following % composition: ground yellow corn 58.5, soybean oil meal 35.0, calcium carbonate 1.5, dicalcium phosphate 1.5, sodium chloride 0.2, and Vit. B mix 0.8 (diet calculated to contain 0.83% K and 0.17% Cl). The animals were fasted 24 hours then fed the basal diet supplemented with 2.5% KNO_3 or 1.8% KCl plus 0.7% cerelose. Six hours after the diets were offered, the animals were injected intraperitoneally with 400 μg tapazole per 100 g body weight. One hour later

* Contribution from the Missouri Agri. Exp. Station, Journal Series No. 2451. Supported in part by a research grant from Feed Service Corp., Crete, Nebraska. The authors are indebted to Drs. C. W. Turner and W. G. Pipes for the use of equipment and materials.

† Unpublished data.

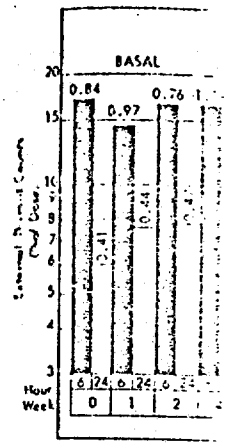


FIG. 1. Compensati-
coming 2.5% dietar
higher than a, p <
than a or b, P <.01,
sent stand

the rats were injec
3 μc I^{131}. Subs
counts(7) were ma
supplemented ratio
5.5 weeks and aver
day in each group.
I^{131}, and the subse
nal counts were re
without 24-hour fa
that iodide "trapp
measured without
binding. Since ta
main as inorganic i
iodide flushing act
toid could be made
The last injectio
without tapazole s
be measured. The
guillotine 36 hou
Weights of the thy
as well as values fo
blood KNO_3(9) w
Results. The re
Six-hour external
while the differenc

TA

Dietary supplement
KCl
KNO_3

* Significan

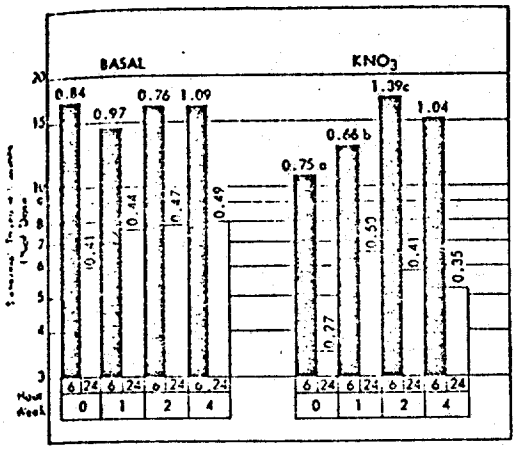


FIG. 1. Compensation of the rat thyroid in overcoming 2.5% dietary KNO_3. b is significantly higher than a, $p < .01$. c is significantly higher than a or b, $P < .01$. Values at top of bars represent standard error of mean.

the rats were injected intraperitoneally with 3 μc I^{131}. Subsequently external thyroid counts(7) were made at 6 and 24 hours. The supplemented rations were fed to the rats for 5.5 weeks and average intake was 14.3 g per day in each group. Injection of tapazole and I^{131}, and the subsequent 6- and 24-hour external counts were repeated at 1, 2, and 4 weeks without 24-hour fast. Tapazole was used so that iodide "trapping" mechanism could be measured without interference from protein binding. Since tapazole caused I^{131} to remain as inorganic iodide, measurements of the iodide flushing action of KNO_3 on the thyroid could be made.

The last injection of I^{131} at 5.5 weeks was without tapazole so that serum PBI^{131} could be measured. The animals were sacrificed by guillotine 36 hours after I^{131} injections. Weights of the thyroids, adrenals, and livers as well as values for serum PBI^{131}(8) and the blood KNO_3(9) were determined.

Results. The results are shown in Fig. 1. Six-hour external counts represent uptake while the difference in 6- and 24-hour counts show the degree of flushing. Rats fed KNO_3 showed a lower uptake of I^{131} at 0 week and lost the majority of this I^{131} within 18 hours. By the end of the second week KNO_3-fed rats had a greater uptake than the KCl group. The greater uptake was not reflected in the ability to overcome the flushing action of KNO_3 as the 24-hour count was still lower than in the control animals. The fourth week showed a slight decrease in uptake with an even greater decrease in the ability to hold the I^{131}.

Table I shows organ weights and serum PBI^{131} in the absence of tapazole. Thyroid weights were significantly higher in the KNO_3-fed animals but there was no difference in adrenal weights. The PBI^{131} was higher in the KNO_3 group. There was no significant difference in weight gains during the 5.5-week period.

Discussion. Mitchell and O'Rourke(10) have recently shown that an increase in thyrotropin (TSH) will overcome the thyroid inhibiting effect of blood thiocyanate. An increased TSH in the KNO_3-fed animals would possibly explain the increased size of the thyroid gland and the progressive increase in uptake of I^{131} over the 4-week period.

A possible explanation for the higher serum PBI^{131} in the KNO_3-fed group after 5.5 weeks is that dietary KNO_3 decreased the inorganic iodine pool in the animal. This concept is supported by the flushing of the thyroidal I^{131} under the influence of KNO_3. Fitting(11) has reported that the circulating inorganic I^{127} is apparently the major determining factor in quantitative fixation of injected I^{131}.

The data presented here show the progressive thyroidal compensation of the rat subjected to excess dietary nitrate under normal environment. The validity of using PBI^{131} as an index of thyroid function in rats that consume perchlorate, thiocyanate or nitrate

TABLE I. Organ Weights and PBI^{131} after 5.5 Weeks on KNO_3 Diet.

Dietary supplement	Thyroid wt, mg/100 g B.W.	Adrenal wt, mg/100 g B.W.	36-hr PBI^{131}, cts/min/ml	Blood KNO_3, mg/100 ml
KCl	8.0 ± .69	11.9 ± .70	3684 ± 534	.3
KNO_3	11.5 ± .48*	12.6 ± .46	7332 ± 665*	24.3

* Significantly different, $P < .01$, from control.

for several weeks is certainly open to question.

Summary. Rats maintained in a normal environment showed a decreased thyroidal I^{131} uptake after 7 hours on a nitrate supplemented diet but were able to overcome this effect after two weeks. Initially the nitrate flushed I^{131} from the thyroid gland; however, this effect was overcome in one week. After 5 weeks, in the absence of tapazole, the nitrate-fed rats fixed more I^{131} as PBI^{131} than did the controls.

1. Wyngaarden, J. B., Wright, B., *Endocrinology*, 1952, v50, 537.

2. Wyngaarden, J. B., Stanbury, J. B., Rapp, B., *ibid.*, 1953, v52, 568.

3. Whitehead, E. I., Moxon, A. L., *S. Dakota Exp Sta. Bull.*, 1952, 424.

4. Schmidt, E. L., *Public Health Rep.*, 1956, v71, 497.

5. Siemens, H., Mallet, C., *Canad. J. Public Health*, 1950, v41, 201.

6. Bloomfield, R. A., Welsch, C. W., Garner, G. B., Muhrer, M. E., *Science*, 1961, v134, 1690.

7. Premachandra, B. N., Turner, C. W., *J. Animal Sci.*, 1960, v19, 1181.

8. Chaikoff, T. L., Taurog, A., Reinhardt, W. O., *Endocrinology*, 1947, v40, 47.

9. Garner, G. B., Baumstark, J. S., Muhrer, M. E., Pfander, W. H., *Anal. Chem.*, 1956, v28, 1589.

10. Mitchell, M. L., O'Rourke, M. E., *J. Clin. Endocrinol. Metab.*, 1960, v20, 47.

11. Fitting, W., *ibid.*, 1960, v20, 569.

Received July 2, 1962. P.S.E.B.M., 1962, v111

Effect of Thyroxine on Maturation of the Testes and Prostate Gland of the Rat. (27770)

GEORGE B. TALBERT (Introduced by James B. Hamilton)
State University of New York, Downstate Medical Center, Brooklyn, N. Y.

Injection of thyroxine beginning at birth has been shown to accelerate such maturational processes as opening of the eyelids, hair growth, epiphyseal plate closure and tooth eruption in rats(1,2,3). Attempts to advance differentiation of the gametogenic cells of the testis or to induce earlier and more rapid growth of the male accessory reproductive glands by thyroid hormone administration have uniformly failed(4,5,6). In these latter studies, however, hormone administration was not begun until the animals were 2 to 3 weeks of age. Since spermatogenesis is well under way at this time(7,8) it seemed possible that the thyroid hormone had been administered at too late an age to significantly accelerate this process. For this reason in the present study thyroxine was administered from birth at which time the earliest Type A spermatogonia are not yet recognizable(8).

Methods and materials. Forty-seven rats from Wistar stock were raised in a room maintained at 76°F and 50% relative humidity. All animals used in this study were obtained from litters which had been reduced to 7 at birth and contained at least 3 males. Day-old males from each litter were divided into 3 groups. The animals in 2 of these groups were injected subcutaneously daily from birth to 36 days of age with DL thyroxine* in alkaline saline. The control group received alkaline saline only.

The thyroxine solutions were prepared at 2 concentrations: 6 μg/0.05 ml ("A" dose) and 2 μg/0.05 ml ("B" dose). Fresh solutions were prepared at 7-day intervals. The daily dose given to the animals in each experimental group was maintained at 6 μg and 2 μg until the rats were 10 days of age. At 11, 16, 21 and 28 days of age the 2 doses were increased so that the "A" dose was maintained at about 9 times the normal physiological level and the "B" dose at 3 times this level. Six micrograms per 100 g of body weight was considered normal.

Necropsy was performed on the day after the last injection. The testes and ventral prostate gland were weighed and the testes

* Thyroxine Crystals Squibb, E. R. Squibb and Sons, New York.

It is suggested, however, that prophylactic measures against enterotoxaemia would provide the greatest benefit.

Acknowledgments

The writers wish to acknowledge the help given by the Hawke's Bay Veterinary Club members who donated the sheep used in this experiment, and also the many members of the staff at Wallaceville who contributed to this work.

REFERENCES

Bennetts, H. W. (1932): *Bull. Coun. sci. industr. Res. Aust.*, No. 57.

Habermann, R. T., and Carlson, F. N. (1946): *Vet. Med.*, *41*: 306.

McBarron, E. J. (1952): cited by Seddon, H. R. (1953): *Serv. Publ. Dep. Hlth. Aust. vet. Hyg.*, No. 9.

McCulloch E. C., and McCoy, J. E. (1941): *J. Amer. vet. med. Ass.*, *99*: 496.

Seddon, H. R. (1931): *Ann. trop. Med. & Parasit.*, *25*: 431.

224

CLINICAL COMMUNICATION

NITRATE POISONING CAUSED BY TURNIPS AND REDROOT

D. T. Brakenridge, *Pukekohe*

Introduction

Variegated thistle, mintweed, sorghum, mangolds, and oaten hay have been shown to cause nitrate poisoning in animals under certain conditions. To our knowledge, this is the first observation in New Zealand on nitrate poisoning of cattle by turnip tops and redroot (*Amaranthus retroflexus*). Connor (1951) states that redroot contains appreciable quantities of nitrate in the stems and branches and could possibly lead to poisoning.

History of Outbreak

The drought conditions during the summer of 1955 created many feeding problems in this area. This farmer was forced into feeding out limited quantities of immature soft turnips in order to sustain his stock. The crop was planted on 11th December, 1954, and was thus only two months old when feeding was commenced on 6th February. Good top growth had occurred despite the dry conditions, but the crop was contaminated by the weed Red Root. The turnips and redroot were pulled and fed out each morning in an estimated quantity of 25 lb. per cow. It was estimated that the proportion of redroot to turnip being fed out was 1 part to 8. At 4 p.m. on the fourth day of feeding, one cow was found dead and two others were sick.

Clinical Observations

The two affected cows were depressed, standing immobile with their heads low and forced against a fence. There was some muscle trembling and, when forced to move, the animals staggered. Respiration seemed little affected, heart rate was rapid, temperature was 100.5° F, and the vaginal mucous membrane was greyish-purple and cyanosed.

At the first visit, no diagnosis was made nor treatment given. However, two hours later the animals were re-examined. The jugular vein of the dead cow was opened to reveal chocolate-brown-coloured blood. The two affected animals were then bled, and the blood was found to be distinctly brown although not so intense as in the dead animal. Nitrate poisoning was then strongly suspected and the two cows were treated with an intravenous injection of 1 g. methylene blue as a 1 per cent. solution. Both animals survived, and, when seen the following day, had venous blood of normal colour. Full recovery followed in about 4 days.

Diagnosis

Unfortunately, blood was not forwarded to a diagnostic laboratory for demonstration of methaemoglobinaemia. However, four days later, a sample of 50 lb. of turnips and 50 lb. of redroot was forwarded to Ruakura Diagnostic Centre for nitrate estimation with the following results:

Turnip tops only: approximately 8 per cent. nitrate as KNO_3 on a dry-matter basis.

Redroot: 6 per cent. nitrate on the same basis.

About two weeks later, a further sample of turnips was analysed with similar results.

Ruakura Diagnostic Centre carried out dosing trials and were successful in proving that both the redroot and turnip tops produced a fatal methaemoglobinaemia.*

Management and Environment

Manurial Programme and Planting History

April 10, 1954	10 cwt lime per acre
April 20	6½ cwt super potash mixture (proportion of 10–1) per acre
May 20	Paddock ploughed and worked
June 8	Potatoes planted 2½ cwt blood per acre 2½ cwt potash per acre 5 cwt super per acre 7½ cwt bone fertiliser per acre
November 22	Potatoes dug
December 7	Ploughed and worked
December 11	Sowed Imperial Green Globe soft turnips with 5 cwt bone fertiliser.

Prevailing Weather Conditions

The summer was hot and dry. Grass and earth temperatures prior to and at the time of the outbreak were as follows:

February	Grass Temp. °F	Earth Temp. 8 inches °F
2	52·8	71·0
3	52·5	72·4
4	51·8	72·9
5	56·8	72·3
6	61·8	73·2
7	59·8	72·1
8	47·6	70·2
9	51·2	70·6

*To be reported in a subsequent paper.

The grass temperature dropped to 47.6° F on the day before the mortality, this being the minimum for the month.

Overcast thundery weather occurred on the day of the outbreak and some rain fell.

Soil Sample

A research soil sample was taken from the paddock and examined by the Soil Fertility Station, Hamilton. The available molybdenum level was extremely high at 0.96 p.p.m., and the nitrifying capacity was found to be somewhat high.

Summary

An outbreak in adult cows of nitrate poisoning associated with feeding turnip tops and redroot (*Amaranthus retroflexus*) is described.

One cow died, whilst two which were affected recovered after treatment by intravenous injection of 1 per cent. methylene blue.

Clinical diagnosis was made on the distinct brown colour of the jugular blood.

The turnip tops contained about 8 per cent. and the redroot about 6 per cent. nitrate.

A subsequent dosing trial proved the toxicity of both plants by the formation of methaemoglobin.

Acknowledgment

My thanks are due to the Rukuhia Soil Research Station and the Ruakura Diagnostic Centre for their assistance.

REFERENCE

Connor, H. E. (1951): Dept. Sci. & Indust. Research. Bull. No. 99.

258

Semiquantitative Analysis of Low Levels of Volatile Nitrosamines by Gas Chromatography–Mass Spectrometry

A method is described for the analysis of volatile nitrosamines in food using a high-resolution mass spectrometer coupled to a gas chromatograph. An ion, characteristic of each nitrosamine, is chosen and the gas chromatograph effluent is monitored by the spectrometer (at a resolution of 10,000) for any compound whose mass spectrum contains the characteristic ion. The amount of a nitrosamine is estimated from a knowledge of the sensitivity of the mass spectrometer to that nitrosamine. Recoveries of a range of nitrosamines, added to food, have been measured at 10, 2, and 1 ppb.

Recently we described a method (Telling *et al.*, 1971) for identifying volatile nitrosamines using gas chromatography and monitoring the chromatograph effluent for compounds whose high-resolution mass spectrum contained an ion at mass 30 of composition NO^+. It was possible to detect 0.2 μg of an individual nitrosamine in this way. Any compound giving a response was subsequently identified by recording low-resolution mass spectra. However, measurement of recoveries from samples to which nitrosamines had been added showed that as much as 0.5 μg of a nitrosamine could be required to give a sufficiently intense low-resolution mass spectrum for positive identification because of interferences from coextracted materials. As the amount used in the determination was $^1/_{10}$th of the total extract, 0.5 μg of a nitrosamine corresponded to 20 ppb of the original sample (250 g), assuming 100% recovery. It was found that the practical limits ranged from 25 to 65 ppb (0.125 to 1.625 μg injected on the column) depending on the nitrosamine. Although this technique could be used as a screening test, it could not be used for the quantitative measurement of nitrosamine content.

We also pointed out that it was possible to identify certain specific nitrosamines at a level of 10 ppb (0.25 μg injected on the column) by monitoring the gas chromatograph effluent for the molecular ion or any other characteristic ion of the nitrosamine. We now wish to describe the application of the latter technique to the semiquantitative analysis of nitrosamines and report developments which enable us to identify nitrosamines at a level of 1 ppb (0.025 μg injected on the column).

EXPERIMENTAL

Increased sensitivity has been achieved by a modification to the mass spectrometer. The value of the grid resistor of the electrometer stage of the signal amplifier has been increased by a factor of 10, resulting in a tenfold increase in signal strength for the same number of ions at the collector. The signal-to-noise ratio is also improved by this factor since the noise is not appreciably increased at the low bandwidth (20 Hz) used when monitoring a single mass at high resolution. In practice, sample losses on the gas chromatograph and in the gas chromatograph-mass spectrometer interface prevent the full increase being realized at the lower limit. It is now found that the detection limit for the NO^+ ion monitoring technique is 5 to 10 ppb (0.125 to 0.25 μg injected on the column).

Greater sensitivity can be achieved by monitoring for a characteristic ion which is more abundant than the NO^+ ion. The molecular ion is the obvious choice for some of the lower molecular weight nitrosamines. For others with weak molecular ions, *e.g.*, less than 10% relative abundance, an intense fragment ion can be selected if its elemental composition is known. The mass spectrometer is adjusted to detect ions of the same elemental composition as the characteristic ion. This is readily achieved on our mass spectrometer (AEI MS 902) using the peak matching facilities and perfluorotri-*n*-butylamine as the mass calibrant. The resolution of the spectrometer should be at least 10,000 so that most of the possible interfering ions from other compounds are separated from the characteristic ion. A compound eluting from the gas chromatograph is identified as a nitrosamine if it has the correct retention time and possesses the characteristic ion in its mass spectrum. The amount of nitrosamine in the sample can be calculated from a knowledge of the sensitivity of the mass spectrometer for that nitrosamine. Several nitrosamines can be monitored in a single run, provided that the time between the elution of successive nitrosamines from the column is sufficient to allow readjustment of the spectrometer.

The apparatus and extraction procedure have been previously described (Telling *et al.*, 1971).

RESULTS AND DISCUSSION

The compounds chosen for study were *N*-nitrosodimethylamine (DMN), *N*-nitrosodiethylamine (DEN), *N*-nitrosodiisobutylamine (Di-BN), *N*-nitrosodibutylamine (Dn-BN), and *N*-nitrosopyrrolidine (NNPyrr), since they cover a wide range of volatility. The molecular ion of DMN, DEN, and NNPyrr was chosen as the characteristic ion for these compounds; *m/e* 84 was chosen for Di-BN and Dn-BN, as the molecular ion is of low abundance in both spectra. The composition of *m/e* 84 was established as $C_5H_{10}N$ by high-resolution mass spectrometry.

The mass spectrometer sensitivity for each compound was determined by injecting known amounts of the nitrosamine on the column and measuring the height of the characteristic ion peak as it was repetitively scanned and displayed on the mass spectrometer oscilloscope. A standard solution containing all five nitrosamines was used and it was possible to measure the response of each compound during the course of a single determination.

The response of the mass spectrometer should be linearly dependent on the amount of nitrosamine injected on the gas chromatograph, and this was confirmed for all five nitrosamines. Various amounts of nitrosamine in the range 0.010 to 0.5 μg were injected and the mass spectrometer response at each characteristic mass was measured, in turn, as the compounds eluted from the column. The sensitivity of the mass spectrometer for each nitrosamine was calculated from these data. The minimum amount of nitrosamine which can be accurately measured is of the order of 0.025 μg injected

Table I. Percentage Recovery of Nitrosamines Added to a 250-g Sample of Luncheon Meat at the 10, 2, and 1 ppb Levels

Level, ppb, of nitrosamine	10 % Recovery			2 % Recovery			1 % Recovery		
	Max	Min	Mean[a]	Max	Min	Mean[a]	Max	Min	Mean[a]
DMN	94	55	75	88	35	56	Interference could not be measured		
DEN	93	65	82	132	61	102	125	41	96
Di-BN	100	52	78	92	51	72	133	60	98
Dn-BN	73	47	58	80	51	69	106	53	77
NNPyrr	52	29	41	56	27	42	101	30	69

[a] The mean value of at least six determinations.

on the column. This corresponds to 1 ppb of the original sample after allowing for the quantities used in the method. However, these values vary on a day-to-day basis and frequent calibration of the mass spectrometer is necessary. Recalibration is essential if the mass spectrometer conditions are changed, *e.g.*, to record low-resolution spectra between nitrosamine determinations. A single measurement of the five nitrosamines used in this study takes 1 hr. Thus it is possible, in a day, to carry out several such measurements to calibrate the instrument and still analyze two or three samples. The time required to analyze a sample for each of the five nitrosamines is governed by the chromatographic conditions, which were chosen so that the nitrosamines are adequately separated from each other. The analysis of an extract for one or two nitrosamines only can be accomplished in a much shorter time by altering the chromatographic conditions.

The overall procedure was checked by adding all five nitrosamines to a typical food substrate, luncheon meat, and measuring the amount recovered. Percentage recovery values of nitrosamines added to samples at 10, 2, and 1 ppb are given in Table I. There is a wide spread in the data, especially at the lowest level. The low precision of the quantification by gas chromatography–mass spectrometry and variations in recovery in the extraction procedure are mainly responsible for the spread in the data. The recovery values which are greater than 100% arise through measurement errors and not from nitrosamines originally present in the sample, since no nitrosamines were detected in the original samples. The lack of precision of the measurements means that only semiquantitative analyses can be carried out in the 1–5 ppb range. However, the technique still offers a means of qualitative analysis at these levels.

SAFETY NOTE

Nitrosamines are highly carcinogenic compounds and all experimental work should be done in a well ventilated area. Safety gloves should be worn whenever nitrosamines are being handled.

LITERATURE CITED

Telling, G. M., Bryce, T. A., Althorpe, J., J. Agr. Food Chem. 19, 937 (1971).

Thomas A. Bryce*
Geoffrey M. Telling

Unilever Research Laboratory
Colworth House
Sharnbrook, Bedford, U.K.

Received for review November 26, 1971. Accepted February 7, 1972.

Factor Responsible for Varietal Differences in Aflatoxin Production in Maize

Varietal differences in aflatoxin production in food grains is of considerable practical significance. The present study on seven hybrid varieties of maize indicated marked differences in aflatoxin production. Opaque-2 produced markedly low toxin, while Deccan hybrid produced maximal amounts. The possible reasons for the differences in toxin production have been investigated. Different extracts of the two varieties of maize (Opaque-2 and Deccan hybrid) have been incorporated into a standard synthetic medium and their ability to support toxin production has been examined. A 5% NaCl extract of Opaque-2 could result in almost total inhibition of the toxin production. Further fractionation of the NaCl extract revealed that the inhibitory factor is a protein of low molecular weight. The concentration of this protein is markedly higher in Opaque-2 than Deccan hybrid variety.

Aflatoxin contamination of food grains is now recognized to be a potential health hazard, in view of the known hepatotoxic and carcinogenic effects of the toxin (Goldblatt, 1969). The production of aflatoxin by toxigenic strains of *Aspergillus flavus* L. on different food grains is known to vary widely (Diener and Davis, 1969; Hesseltine *et al.*, 1966; Kriz, 1970). Maximal production of the toxin has been demonstrated on natural substrates such as rice, while meager production is obtained on soya beans (Hesseltine *et al.*, 1966). Varietal differences in aflatoxin production have been observed in peanuts (Rao and Tulpule, 1967; Doupnik, 1969) and in sorghum (Anandam, 1970). Such wide variation in toxin production in different varieties of the same crop could lead to the identification of varieties which

J. Food Sci. 37(1), 81-85 (1972)

ROBERT L. BUCHANAN and MYRON SOLBERG
Dept. of Food Science, College of Agriculture
& Environmental Science, Rutgers, The State University, New Brunswick, NJ 08903

INTERACTION OF SODIUM NITRATE, OXYGEN AND pH ON GROWTH OF Staphylococcus aureus

INTRODUCTION

MEAT CURING utilizing sodium nitrite is an ancient practice which persists to the present. The role of the nitrite may have originated primarily as a color fixative, but considerable evidence has developed supporting a preservative function as well.

Sodium nitrite was inhibitory for a wide variety of bacteria in fish muscle or bacterial medium, provided the initial pH was acidic (Tarr, 1942). In the same study, Tarr reported that *Staphylococcus aureus* was inhibited by 0.02% $NaNO_2$ if the initial pH was less than or equal to 6. Tarr (1944) demonstrated that 1.0% $NaNO_2$ was bactericidal to *S. aureus* and other facultative anaerobes at an initial pH of 5.6, but was not bactericidal at pH 7.2. Castellani and Niven (1955), using *S. aureus*, reported that the amount of sodium nitrite needed to produce a no-growth response decreased as the initial pH of the bacterial medium was decreased. Various researchers (Castellani and Niven, 1955; Shank et al., 1962; Duncan and Foster, 1968c) have suggested that the pH dependency of nitrite-induced bacterial inhibition reflects the conversion of nitrite to nitrous acid.

Castellani and Niven (1955) reported that aerobically cultured *S. aureus* were able to grow in the presence of significantly higher concentrations of $NaNO_2$ than were cultures grown in an anaerobic environment.

Numerous investigators (Tarr, 1942; Barr and Cameron, 1942; Roberts and Gram, 1966; Pivnick et al., 1967; Duncan and Foster, 1968a; Mol and Timmers, 1970), using meat products and bacterial media, demonstrated that nitrite at commercial levels ($\leq$ 200 ppm $NaNO_2$) was inhibitory for spores and vegetative cells of various species of the spore-forming genera, *Clostridium* and *Bacillus*. Using phase-contrast microscopy, Gould (1965) Duncan and Foster (1968b) determined that commercial levels of sodium nitrite inhibited the outgrowth of spores of various *Bacillus* strains and *Clostridium botulinum* (PA 3679), respectively, but did not prevent germination. Duncan and Foster (1968c) reported that sodium nitrite induced germination of *C. sporogenes* (PA 3679).

ticipate in the formation of carcinogenic nitrosamines exists (Lijinsky and Epstein, 1970), it is necessary that a complete understanding of the function of nitrite in food be obtained. Only with this knowledge can safe substitutions or lower usage levels be established without the creation of new potential hazards.

Qualitative data have been accumulated to demonstrate that pH and oxygen pressure influence the bacteriostatic action of sodium nitrite toward *S. aureus*. Objectives of this study were to: quantitatively examine the effects and interactions of sodium nitrite concentration, initial pH and oxygen pressure on growth of *S. aureus*; determine the effect of nitrite upon the adjustment and the exponential phases of growth and examine the change in $NaNO_2$ concentration as a function of growth.

MATERIALS & METHODS

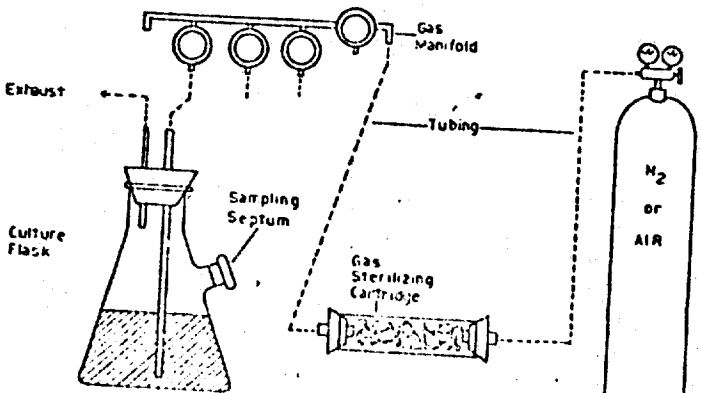
S. AUREUS, ATCC 9664, a food-poisoning strain, was used throughout the study. Stock cultures of the microorganisms were maintained on slants of Brain-Heart Infusion Agar (BHI Agar, Difco), stored at 5 ± 2°C and transferred every 90 days.

Starter cultures were prepared by inoculation of 250-ml Erlenmeyer flasks containing 50 ml Brain-Heart Infusion (BHI, Difco), followed by incubation at 37°C for 24 hr in a shaker water bath (Model 66802, Precision Scientific Co., Chicago, Ill.) at 170 oscillations/minute. This procedure was repeated for a second 24-hr period with an inoculum of 1.0 ml from the first BHI flask.

Growth curves were obtained using a modification of the controlled-atmosphere culture method of Parekh and Solberg (1970). Figure 1 presents a schematic of the cultivation system. The culture flasks used were 300-ml Erlenmeyer-type flasks constructed with a side-arm port (Model 520 without the nephlo tube, Bellco Glass Inc., Vineland, N.J.), fitted with a rubber septum. The gas was sterilized by passage through a sterile cotton cartridge and the gas flow distributed to the individual flasks via a gas manifold. Gas exchange in the culture flasks was obtained by two sterile glass tubes fitted in a two-hole, No. 6 rubber stopper seated in the mouth of the culture flasks. Exhausted gases were passed to a central container for collection of any media which might have foamed out of the flasks. The flow of gas was regulated to minimize foaming and still ensure agitation of the growth medium. All gas exchange and culturing apparatus was autoclaved for 15 min at 15 psi prior to use.

Each flask was filled with 150 ml of BHI at pH 7.3 or adjusted to pH 6.3 with 12 N HCl, prior to autoclaving for 15 min at 15 psi. Reagent-grade $NaNO_2$ was dissolved in 100 ml of sterile, distilled water and filter-sterilized (Filter Type GS-0.22 μ, Millipore Filter Corp., Bedford, Mass.). 1 ml of the $NaNO_2$ solution was added to the autoclaved medium with a sterile, plastic syringe (B-D, disposable, 23G needle, 2.5 cc). Culture flasks were incubated in a 37°C water bath (Model 66648, Precision Scientific Co., Chicago, Ill.). The nitrite concentrations used were 0, 200, 500, 1,000 and 2,000 ppm. The partial pressure of oxygen (pO_2) was controlled by constant flushing with compressed N_2 (pO_2 = 0) or pumped air (pO_2 = 160 mm Hg).

The preincubated culture flasks were inoculated to a level of 10^2–10^3 microorgan-



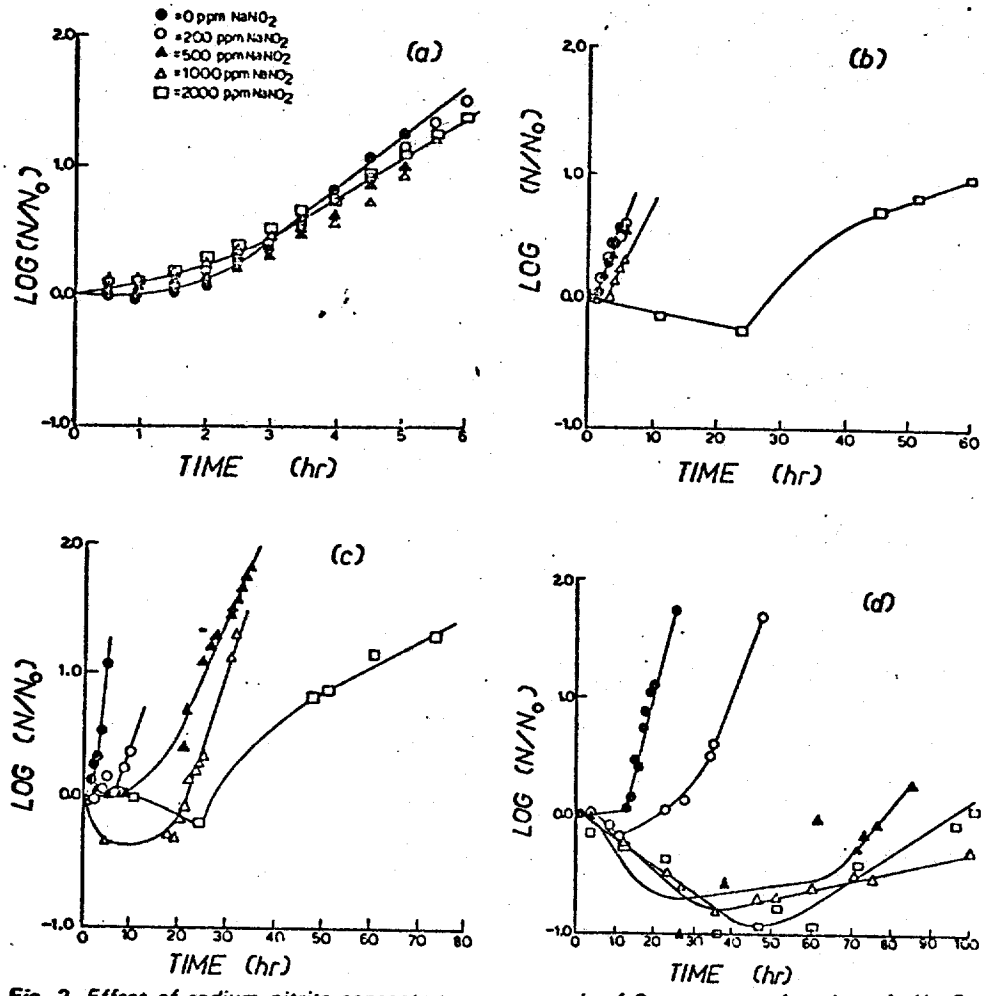


Fig. 2–Effect of sodium nitrite concentration on growth of S. aureus as a function of pH:pO_2 combinations: a) pH 7.3-aerobic, b) 7.3-anaerobic, c) pH 6.3-aerobic and d) pH 6.3-anaerobic.

isms/ml from the starter culture by adding 1 ml of a 10^6 dilution through the septum. Growth was measured by direct count of BHI agar pour plates incubated for 24 hr in a 37°C incubator (Model 1435, Precision Scientific Co., Chicago, Ill.). Sampling was through the septum with a syringe and hypodermic needle.

Adjustment phase duration was defined as the time required for the 0-time inoculum to undergo a twofold increase in number (Solberg and Nickerson, 1963). Exponential growth rates were calculated as the slope of the least-squares regression line of the exponential growth curve, and generation times were calculated from the exponential growth curve slopes.

Determinations of nitrite concentration were performed on duplicate 1.0-ml samples of the culture medium at various times during the adjustment and exponential phases. The concentration of nitrite was analyzed colorimetrically, using a 1-Naphthylamine indicator (Kamm et al., 1965). The colorimetric responses were measured by triplicate readings in a DB-G Spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) at 555 nm, and converted to nitrite concentration by comparison with a standard curve. To determine the degree of nonmicrobial depletion of $NaNO_2$, an uninoculated culture was examined for each of the four pH: oxygen combinations containing 200 ppm $NaNO_2$.

The pH characteristics of the cultures were followed by determining the pH of 1.5-ml samples extracted from the culture flasks at various times during incubation. The pH levels of the samples were determined with an Expandomatic pH Meter (Beckman Instruments, Inc., Fullerton, Calif.) fitted with a combination surface electrode (Model 39182, Beckman Instruments, Inc., Fullerton, Calif.). The first significant pH change was defined as the elapsed time between inoculation of the culture and a pH decrease of 0.3 pH unit.

The adjustment phase duration data were subjected to analysis of covariance and stepwise multiple regression analysis. The exponential growth rate data were subjected to analysis of variance and stepwise multiple regression analysis. The pH data were analyzed by stepwise multiple regression.

RESULTS & DISCUSSION

THE VARIOUS COMBINATIONS of sodium nitrite ($NaNO_2$) concentration: 0, 200, 500, 1,000, 2,000 ppm; initial pH: 6.3 and 7.3 and partial pressure of oxygen: 0 and 160 mm Hg were tested in triplicate to determine their effects on the growth of *S. aureus*.

Figure 2 depicts the growth curves observed under each of the various environmental conditions being studied. Each point is the average of three experiments. It is apparent from Figure 2a that sodium nitrite had no effect even at 2,000 ppm, which is ten times the level permitted in cured meat products, when *S. aureus* was cultivated at pH 7.3 aerobically. At the same pH but under anaerobiosis there was a slightly extended adjustment phase in the presence of 1,000 ppm $NaNO_2$ and a significantly altered growth process when 2,000 ppm of $NaNO_2$ were present, as illustrated in Figure 2b. These nitrite levels are in the range which might be found in curing brines which may be pumped into meat cuts or diffused into meat cuts during submerged storage in a "cover pickle" curing procedure.

The effect of reduced pH is very significant (see Figures 2c and 2d). Under aerobic and anaerobic conditions the adjustment phase is generally extended as the level of $NaNO_2$ increases and the slope of the exponential phase curve is generally reduced.

The complete comparison of the effects of the variables upon the adjustment and the exponential phases of growth of *S. aureus* can be seen more clearly in Figures 3 and 4. These figures present the data in terms of the effect upon growth as a function of increasing $NaNO_2$ concentration. From Figure 3 it can be seen that under anaerobic conditions, pH 6.3 (closed circles) alone had a significant effect upon the adjustment phase duration which was extended from the usual 2 hr to approximately 14 hr. Figure 3 also shows that $NaNO_2$ is an effective inhibitor of *S. aureus* at lower concentrations under anaerobic conditions at either the pH of in vivo muscle (~7.3), or the pH of cooked cured processed meats (~6.3). It is also evident that $NaNO_2$ is inhibitory to *S. aureus* at levels permitted in food products such as cooked cured meats at the pH of 6.3, which is in the pH range for such products.

The inhibitory effects depicted by Figure 3 involve extension of the adjustment phase duration. Figure 4 presents the inhibitory aspects of $NaNO_2$ concentration with respect to the exponential phase of growth expressed as generation time. It is apparent that it took longer for cells of *S. aureus* to multiply anaerobically than aerobically at all levels of $NaNO_2$ concentration. (Compare open circles with open triangles and closed circles with closed triangles.)

Generation time was increased or growth was inhibited by reduced pH in all cases. (Compare open triangles with closed triangles and open circles with closed circles.) The inhibitory effects in both the adjustment and exponential phases of growth are complementary. Termination of adjustment phase in the pH 6.3-anaerobic cultures having $NaNO_2$ concentrations ≥ 500 ppm was unpredictable. Under these conditions, bactericidal

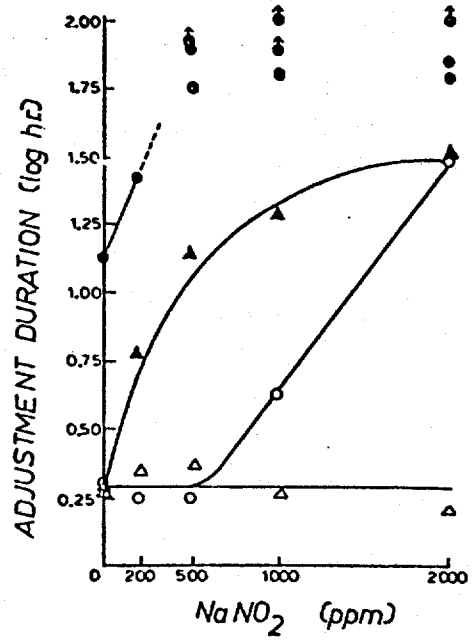


Fig. 3—Average effects of sodium nitrite concentration on duration of the adjustment phase of S. aureus, according to the pH.pO₂ combinations of pH 7.3-aerobic (△), pH 7.3-anaerobic (○), pH 6.3-aerobic (▲) and pH 6.3-anaerobic (●). Dotted line indicates that not all cultures attained exponential growth before termination of the experiment. Such cultures were assigned a value equal to the elapsed time at termination and are identified by an arrow.

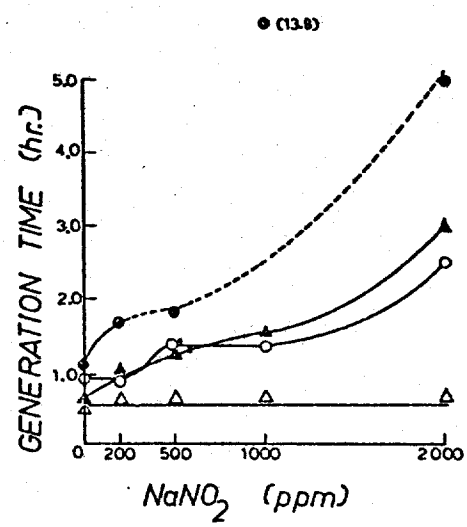


Fig. 4—Average effects of sodium nitrite concentration on generation time of S. aureus according to the pH:pO₂ combinations of pH 7.3-aerobic (△), pH 7.3-anaerobic (○), pH 6.3-aerobic (▲) and pH 6.3-anaerobic (●). The dotted line indicates a postulated response curve, employed since not all cultures of the pH 6.3-anaerobic group attained exponential growth.

properties were observed for some of the cultures. For statistical analysis, those cultures of the pH 6.3-anaerobic group that did not begin exponential growth before termination of an experiment were assigned an adjustment phase duration value equal to the elapsed time at termination. These are indicated by arrows above the points in Figure 3. Stepwise multiple regression analysis indicated that the duration of the adjustment phase was dependent on the three-way interaction of the initial pH, the pO_2 and the square of the $NaNO_2$ concentration.

Cultures of the pH 6.3-anaerobic group that did not achieve exponential growth before termination of an experiment were assigned a value of 0.00 for rate of growth. Statistical analysis indicated that the exponential growth rate was primarily dependent on the pH:pO_2 interaction, but also indicated a significant three-way interaction between the variables as well as significant two-way interactions between the square of the nitrite concentration and the initial pH or pO_2.

This study reconfirms the effect of pH and pO_2 upon nitrite inhibition of *S. aureus* and demonstrates that the effect of nitrite on *S. aureus* is an extension of the adjustment phase, a decrease in the rate of growth and the damage or destruction of some cells. Injury and cell destruction was most apparent at the lower pH level in the presence of nitrite concentrations $\geqslant$ 500 ppm (Fig. 2c and 2d), particularly in the anaerobic cultures (Fig. 2d). These results show that there are bactericidal effects of $NaNO_2$ at concentrations as low as 500 ppm at pH levels as high as 6.3, compared to concentrations of 10,000 ppm at pH 5.6 previously reported bactericidal by Tarr (1944). Results of the present study also indicate that 200 ppm $NaNO_2$ is inhibitory for *S. aureus* at a higher pH level than reported by Tarr (1942). This finding is in closer agreement with Nurmi and Turunen (1970), who reported that various *Micrococcus* and *Staphylococcus* strains were inhibited at pH 6.0 by 200 ppm $NaNO_2$ and did not grow out within 36 hr when the $NaNO_2$ concentration was 1,000 ppm.

Various researchers (Castellani and Niven, 1955; Shank et al., 1962; Duncan and Foster, 1968c) suggested that the pH dependency of nitrite inhibition reflects the conversion of nitrite to nitrous acid. This explanation does not account for the inhibition observed of the anaerobic cultures of *S. aureus* at pH 7.3 in this study or Tarr's (1942) observation that *C. botulinum* was inhibited by 200 ppm $NaNO_2$ at pH 7.05. At both of these pH levels there would be minimal nitrous acid formation. The nitrous acid hypothesis does not take into account the observed inhibition of bacteria by hydroxylamine (Tarr, 1945; Jensen, 1954), which may be involved since it can be formed during reduction of nitrite.

From this study, it might be anticipated that 200 ppm $NaNO_2$ in cured meats would offer significant protection against growth of *S. aureus*, particularly if the meat product is vacuum-packed. However, Bulman and Ayres (1952) and Nordin (1969) reported that the concentration of nitrite in pasteurized cured meats was rapidly depleted during processing and storage. Thus, the protection against *S. aureus* by nitrite would probably be transitory or equivalent to the effect offered by a lower residual level. The exact relationship between the effectiveness of nitrite in bacterial medium as opposed to a meat system awaits elucidation by future research.

To determine the metabolic activity of *S. aureus* for NO^-_2, the concentrations of nitrite recoverable from the bacterial medium of one culture for each variable combination were determined periodically. The method of nitrite analysis (Kamm et al., 1965) had a coefficient of variation equal to 5.78%. While this method of analysis is one of the most sensitive for detecting nitrite, the high coefficient of variation shows the method's precision to be relatively poor, thus limiting this series of experiments to qualitative interpretation. Microbial depletion of nitrite was observed during the exponential growth of most of the aerobic cultures. Anaerobic cultures demonstrated no change in recoverable nitrite. Since there was no nitrite reduction in uninoculated culture flasks, nitrite depletion in aerobic cultures was microbial in nature at both pH levels.

Nason (1962) reported that nitrite was used by facultative anaerobes as an electron acceptor. The nitrite competed with oxygen for electrons derived from the cytochrome system. If nitrite was being used as an electron acceptor by *S. aureus*, it would be anticipated that the maximum utilization would occur during anaerobic growth, and that minimum utilization would occur in aerobic cultures. The observed results of this study were completely contrary to the anticipated effect, indicating that nitrite is either not being employed as an electron acceptor or the ability to reduce nitrite (nitrite reductase system) is somehow blocked during anaerobic growth.

Most bacterial broth cultures will undergo pH changes during some phase of the growth cycle. To determine the pH characteristics of *S. aureus* and how they are affected by sodium nitrite concentra-

tio initial pH and pO_2, the pH of one culture for each test condition was followed through the growth cycle. Most cultures underwent a pH decrease. The aerobic cultures, after the initial pH drop, demonstrated a subsequent rise in pH to a level greater than the initial pH; whereas, the anaerobic cultures remained at the pH level of maximum decrease. An example of the differential response between aerobic and anaerobic cultures is presented in Figure 5. The pattern of the aerobic pH change in this study is in agreement with that observed by McLean et al. (1968), who also reported an initial pH decrease, followed by a pH rise.

This study found that a pH decrease of 0.3 unit occurred at a time which extension of the exponential phase growth curve would describe as a population density of 10^7. This is in agreement with the observation by McLean et al. (1968), who reported that *S. aureus* cultures underwent a pH decrease at the initiation of the negative acceleration phase of growth.

A graphical presentation of the time required for the initial pH to drop 0.3 pH unit as influenced by initial pH, partial pressure of oxygen and $NaNO_2$ concentration appears in Figure 6. Since the points of Figure 6 depict the late exponential or the beginning of the negative acceleration phase of growth, the curves are essentially a summation of those previously presented in Figures 3 and 4. It is evident from Figure 6 that anaerobiosis is an inhibitor in the absence of $NaNO_2$ and that reduced pH becomes significant as $NaNO_2$ concentration increases. Stepwise multiple regression analysis of the time required for initial pH to drop 0.3 unit indicated dependence upon the interaction of nitrite concentration, initial pH and pO_2.

Several attempts have been made to develop a hypothetical mechanism to explain nitrite inhibition. In addition to the proposal for increased conversion of nitrite to nitrous acid as pH is lowered (Shank et al., 1962; Castellani and Niven, 1955), Castellani and Niven also suggested the possibility that the nitrite may be directly causing the inactivation of an essential nutrient or blocking a metabolic reaction.

Castellani and Niven (1955) reported that autoclaving glucose in bacterial media or adding filter-sterilized pyruvate to bacterial media enhanced the bacteriostatic action of nitrite in anaerobic cultures. Addition of sulfhydryl groups neutralized enhancement by the added carbohydrates. Castellani and Niven (1955) suggested that the bacteriostatic action of nitrite might be due to interference with the normal metabolism of hypothetical pyruvate-sulfhydryl com-

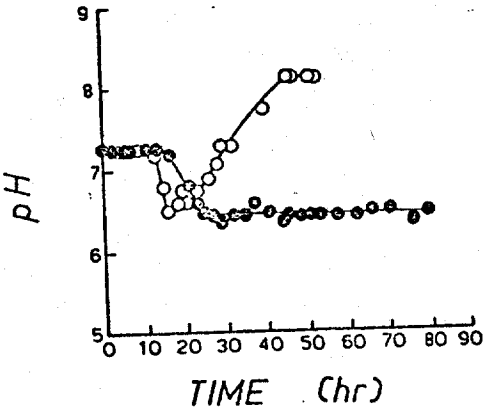


Fig. 5—The pH changes in Brain Heart Infusion cultures of S. aureus grown at 37°C in 200 ppm $NaNO_2$—pH 7.3 anaerobic (•) and 200 ppm $NaNO_2$—pH 7.3 aerobic (o).

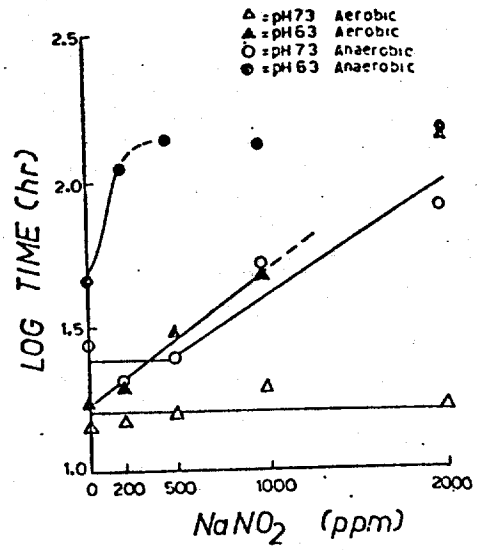


Fig. 6—Effect of sodium nitite concentration on time required to obtain a 0.3-unit pH decrease in S. aureus cultures, according to pH:pO_2 combinations. The dotted lines indicate that a pH decrease did not occur before experiment termination and that the value expressed is the elapsed time at termination.

fered with carbohydrate metabolism, resulting in accumulation of pyruvic acid. In a similar study, Bernheim (1943) reported that nitrite caused pyruvate accumulation in *Vibrio comma*. In a review of carbohydrate metabolism, Baldwin (1963) stated that the normal decarboxylation necessary for the metabolism of pyruvate requires the transfer of the molecule to two successive cofactors, alpha-lipoic acid and coenzyme A, both of which have sulfhydryl groups as their active sites. Baldwin (1963) also reported that arsenite, a compound similar in many ways to the nitrite molecule, blocks this reaction. Testing a large number of enzyme systems, Barron and Singer (1945) reported that the pyruvate-metabolizing enzyme system was the most sensitive to sulfhydryl inhibitors. At this time, we would like to suggest that nitrite may inhibit the growth of *S. aureus* by blocking the sulfhydryl sites of either coenzyme A or alpha-lipoic acid, thus blocking the normal metabolism of pyruvate. Evidence to support this speculation must await results of future studies.

CONCLUSION

NITRITE INHIBITION of *S. aureus* involves extension of the adjustment phase, decrease in growth rate and damage or destruction of some cells. The magnitude of inhibition is dependent on the interaction of sodium nitrite concentration, initial pH and pO_2. *S. aureus* metabolizes nitrite when cultured aerobically, but the [illegible] of anaerobic cul-

aureus cultures demonstrate a pH decrease during the growth cycle. Aerobic cultures, after the initial pH decrease, show a subsequent rise in pH to a level greater than the initial pH, whereas anaerobic cultures remain at the pH level of maximum pH decrease.

If *S. aureus* functions similarly in a cooked cured meat system as in BHI containing nitrite at pH 6.3, and if vacuum packaging of cooked cured meats produces an environment equivalent to BHI continually flushed with nitrogen, this study indicates that the practices of curing with $NaNO_2$ and packaging in vacuum may provide protection against growth of *S. aureus*, both by extending duration of the adjustment phase of growth and by reducing the multiplication rate during the exponential phase of growth.

REFERENCES

Baldwin, E. 1963. Dynamic Aspects of Biochemistry. 4th ed. Cambridge University Press, Cambridge, Eng. pp. 443.

Barron, E.S.G. and Singer, T.P. 1945. Studies on biological oxidations. XIX. Sulfhydryl enzymes in carbohydrate metabolism. J. Biol. Chem. 157: 221.

Bernheim, F. 1943. The significance of the amino groups for oxidation of various compounds by the Cholera vibrio. Arch. Biochem. 2: 125.

Bulman, C. and Ayres, J.C. 1952. Preservative effect of various concentrations of curing salts in comminuted pork. Food Technol. 6: 255.

Castellani, A.G. and Niven, C.F. Jr. 1955. Factors affecting the bacteriostatic action of sodium nitrite. Appl. Microbiol. 3: 154.

Duncan, C.L. and Foster, E.M. 1968a. Role of curing agents in the preservation of shelf [illegible] products. Appl. Micro-

Duncan, C.L. and Foster, E.M. 1968b. Effect of sodium nitrite, sodium chloride, and sodium nitrate on germination and outgrowth of anaerobic spores. Appl. Microbiol. 16: 406.

Duncan, C.L. and Foster, E.M. 1968c. Nitrite-induced germination of putrefactive anaerobe 3679h spores. Appl. Microbiol. 16: 412.

Gould, G.W. 1965. Effect of some food preservatives on the development of bacilli from spores. Intern. Congr. Food Sci. Technol. 2: 247.

Jensen, L.B. 1954. Microbiology of Meats. 3rd ed. Garrard Press, Champaign, Ill. pp. 42.

Kamm, L., McKeown, G.G. and Smith, D.M. 1965. New colorimetric method for the determination of the nitrate and nitrite content of baby foods. J.A.O.A.C. 48: 892.

Lijinsky, W. and Epstein, S.S. 1970. Nitrosamines as environmental carcinogens. Nature 225: 21.

McLean, R.A., Lilly, H.D. and Alford, J.A. 1968. Effect of meat-curing salts and temperature on the production of Staphylococcal Enterotoxin B. J. Bacteriol. 95: 1207.

Mol, J.H.H. and Timmers, C.A. 1970. Assessment of the stability of pasteurized comminuted meat products. J. Appl. Bacteriol. 33: 233.

Nason, A. 1962. Enzymatic pathways of nitrate, nitrite, and hydroxylamine metabolisms. Bacteriol. Rev. 26: 16.

Nord, F.F. and Mull, R.P. 1945. Recent progress in the biochemistry of Fusaria. Advan. Enzymol. 5: 165.

Nordin, H.R. 1969. The depletion of added sodium nitrite in ham. Can. Instit. Food Technol. J. 1: 79.

Nurmi, E.V. and Turunen, S.O. 1970. The effect of nitrite on the growth curve of lactobacilli and micrococci. 16th European Meet. Meat Research Workers, Vol. 1. pp. 413.

Parekh, K.G. and Solberg, M. 1970. Comparative growth of Clostridium perfringens in CO_2 and N_2 atmospheres. J. Food Sci. 35: 156.

Pivnick, H., Rubin, L.J., Barnett, H.W., Nordin, H.R., Ferguson, P.A. and Perrin, C.H. 1967. Effect of $NaNO_2$ and temperature on toxinogenesis by Clostridium botulinum in perishable cooked meats vacuum-packed in air-impermeable plastic pouches. Food Technol. 21: 100.

Roberts, T.A. and Ingram, M. 1966. The effect of sodium chloride, potassium nitrate, and sodium nitrite on the recovery of heated bacterial spores. J. Food Technol. 1: 147.

Shank, J.L., Silliker, J.H. and Harper, R.H. 1962. The effect of nitric oxide on bacteria. Appl. Microbiol. 10: 185.

Solberg, M. and Nickerson, J.T.R. 1963. Growth support potential of irradiated chicken for Escherichia coli and Staphylococcus aureus. Food Technol. 17: 939.

Tarr, H.L.A. 1942. The actions of nitrite on bacteria: Further experiments. J. Fish. Res. Bd. Can. 6: 74.

Tarr, H.L.A. 1944. Actions of nitrates and nitrites on bacteria. J. Fish. Res. Bd. Can. 6: 233.

Tarr, H.L.A. 1945. Bacteriostatic action of hydroxylamine. J. Fish. Res. Bd. Can. 6: 349.

Yesair, J. and Cameron, E.J. 1942. Inhibitive effect of curing agents on anaerobic spores. Canner 94: 89.

Ms received 6/13/71; revised 8/2/71; accepted 8/5/71.

A paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University, The State University of New Jersey, Food Science Department, New Brunswick, New Jersey.

This research was supported in part by a grant from the U.S. Public Health Service Grant No. FD-00124 from the Food and Drug Administration.

The authors gratefully acknowledge the help of Dr. Richard Trout for his guidance in the statistical analysis of the data and assistance with laboratory analyses by Mr. Chris Schmid and Mr. Roger Keefe.

Without Documentation," the following guidelines are offered to assist in diagnosis and to aid in staying out of court.

1) Keep records—list what has been done, and see that nothing has been neglected. In all cases, attempt to have a diagnosis confirmed by a laboratory. Always conduct a necropsy examination if the occasion arises. Keep carbon copies of all letters.

2) Avoid making statements that may be admissions—not even to a third party. "Respect your clients and trust no one."

3) Be sensitive to your client. Allow your ego to call in a consultant, even if it is to firm up your judgment and diagnosis.

4) Refrain from optimistic prognoses. Be guarded!

5) Never experiment. Know what you are doing, at least to the client's knowledge!

6) Know the statute of limitations and its significance.

7) Use the best equipment and drugs.

8) Always have a firm understanding of fees.

9) Do not advertise that you have liability insurance. Make no statements about it without benefit of legal advice. In all cases have adequate liability insurance.

The best motto is: "I try never to do anything that I can't immediately justify on the basis of helping the patient." Further, consult with others when you don't know!

261

Diagnosis of Feed-Related Toxicoses

William B. Buck, D.V.M.

SUMMARY

Some of the more common feed-related toxicoses in cattle include inorganic arsenic, urea, organic insecticides, nitrate, cyanide, and imbalance of minerals such as copper and molybdenum. Problems resulting from moldy feeds have not been elucidated but are undoubtedly of major significance. The philosophy behind feeding ruminants no-roughage diets is criticized and placed in a category of a toxicologic problem.

The diagnoses of feed-related toxicoses can be made by obtaining accurate histories and by making astute observations of clinical signs and postmortem changes. Chemical examination of feeds correlated with blood and tissue analyses can generally be used to confirm or substantiate a diagnosis. Cooperation and communication between the practitioner and the toxicology laboratory is essential if a rapid, proper diagnosis is to be made.

IN RECENT YEARS, drugs, pesticides, nutrient substitutes, and other chemicals have been added to animal feeds in an effort to more efficiently and economically dispense these agents for use in livestock and poultry. Also, many feed companies distribute fertilizers and agricultural chemicals not intended for mixing in feeds.

With the advent of these practices problems have arisen, either due to human error or otherwise, which have resulted in death losses; and beyond that, there has been chemical contamination of the public meat supply in the form of pesticides and drug residues. In addition, there may be toxicants in feeds that are influenced by management procedures, specifically fungal and bacterial toxins, cyanide, and nitrates, which seriously affect the value and safety of animal feeds. Other problems encountered have resulted from imbalance in animal feeds of micronutrients and trace minerals such as copper, molybdenum, iron, selenium, arsenic, and manganese.

The accurate diagnosis of feed-related toxicoses, like many other diseases, is made by utilizing information obtained from 5 types of evidence. These have been discussed by others[1,4,6,7] and are: circumstantial, symptomatic, pathologic, experimental, and chemical. In cases of suspect toxicosis, it is imperative that a thorough history be obtained, that astute observations be made, and that intelligent questions be asked. The veterinarian should apply the professional skills that only he possesses in determining the signs of illness and performing a thorough necropsy examination. More often than not, feed-related toxicoses result in litigations, making it all the more imperative that the case be handled in a thorough and professional manner. A complete history should be recorded including such things as number of animals in the herd, number affected, number dead, the course of events in hours or days, the breed, age, sex, type of management and feeding program, history of past illnesses, and other pertinent information regarding possible sources of exposure to toxicants. In recording the clinical signs, one should pay close attention to details, noticing particularly the course of events and progression of clinical signs. Likewise, if a necropsy examination is conducted, it should include examination of all organ systems including the brain and cranium. Chemical evidence is often indispensable in diagnosing toxicologic problems. Precautions should be taken to assure that specimens are obtained and transported to a toxicology-chemistry laboratory by an unbiased party and that contamination, either intentional or otherwise, is not allowed to occur. The toxicology laboratory should be notified if the case involves potential litigation, thus enabling the laboratory to handle the caese in a manner that will be acceptable in court. All information obtained during the investigation should be relayed to the laboratory.

In this report, certain feed-related toxicoses will be discussed, including those resulting from inorganic and organic arsenicals, copper and molybdenum imbalances, cyanide, nitrate, and urea.

Inorganic Arsenic

Man and all lower animals are highly susceptible to inorganic arsenic, but poisoning is most frequently encountered in the bovine and feline species, resulting from contamination of the food supply in some way. Inorganic arsenicals such as sodium arsenite and arsenic trioxide are frequently used as herbicides; other sources of inorganic arsenic include pesticides for use against ants, snails, and rodents, insulation materials such as vermiculite and others[a] containing arsenic, and lumber treated with arsenical preservatives. Animals will frequently seek out and eat materials such as insulation and rodent baits, or dirt and foliage that are contaminated with inorganic arsenic.

From the Toxicology Section, Veterinary Diagnostic Laboratory, Iowa State University, Ames, Iowa 50010.

[a] Celotex, the Celotex Corp., Tampa, Fla.

Episodes of poisoning by inorganic arsenic are usually explosive with high morbidity and high mortality over a short period, usually no longer than 2 to 3 days. Clinical signs include extreme weakness, trembling, violent colic, vomition (in dogs, cats, pigs, and perhaps even cattle), diarrhea, fast feeble pulse, prostration, normal body temperature, dehydration, intense thirst and coma followed rapidly by death.

Lesions found at necropsy are usually associated with the gastrointestinal tract, ranging from mild inflammation to severe hemorrhages and necrosis of the mucosa; edema of the submucosa and muscularis mucosa; and fluid contents in the large bowel and rumen. If death occurs early in the course of the disease, fluid contents in the gastrointestinal tract may be the only noticeable change from normal.

Inorganic arsenic poisoning in cattle is second only to lead poisoning among all toxicoses detected in our laboratory. Whenever an episode of illness occurs that is characterized by rapid onset and gastroenteritis, with only minor signs of central nervous system involvement resulting in weakness, prostration, and rapid death, inorganic arsenic poisoning should be considered.

A finding of 10 to 15 p.p.m. elemental arsenic (on a wet-weight basis) in the liver or kidneys is sufficient to substantiate a diagnosis of arsenic poisoning provided clinical signs, necropsy findings, and history are compatible. Inasmuch as arsenic is eliminated rapidly via the kidneys, the renal cortex may contain greater amounts than the medulla and the kidneys may contain more arsenic than the liver. Also, the urine may contain several p.p.m. arsenic, indicating rapid excretion.

Diseases frequently confused with arsenic poisoning include hypomagnesemia (grassy tetany), organophosphorus insecticide poisoning, bovine viral diarrhea-mucosal disease complex, urea poisoning, and poisoning from plants containing nitrates, cyanide, oxalates, or alkaloids.

Organic Arsenic

Poisoning by organic arsenicals rarely occurs in cattle and other ruminants but is commonly encountered in swine and poultry as a result of excessive levels of phenylarsonic acids or their salts in the feed.[3]

In contrast to inorganic arsenical poisoning, mortality is very low, although morbidity may be very high. In swine and poultry, clinical signs appear after 3 to 10 days of exposure to high concentrations in the feed (1,000 p.p.m. arsanilic acid or 250 p.p.m. 3-nitro-4-hydroxyphenylarsonic acid) and within 3 to 6 weeks on lower concentrations (250 p.p.m. arsanilic acid and 100 p.p.m. "3-nitro").

Cattle are apparently not very susceptible to organic arsenicals in the feed. Data from our laboratory indicate that 500 p.p.m. of sodium arsanilate in the feed of a 400-lb. (181.4-kg.) calf for six weeks failed to cause any obvious detrimental effects. When the content was increased to 1,000 p.p.m., the animal refused to eat the feed.

Clinical signs of organic arsenical toxicity in swine and poultry include incoordination, inability to control body and limb movements, and ataxia, resulting from peripheral nerve demyelination. Data from our laboratory indicate that calves given sodium arsanilate orally at a dosage of 100 mg./kg. developed clinical signs similar to those noticed with inorganic arsenic poisoning; namely, gastroenteritis, weakness, depression, diarrhea, coma, and death.

Although there is limited knowledge concerning the toxicity of organic arsenicals in cattle, it appears that the accidental incorporation of phenylarsonic acids in cattle feeds would not result in an acute arsenic poisoning syndrome as one might expect but rather would cause the animal to refuse to eat the feed if a sufficient amount was present. On the other hand, if a cow or calf consumed a large dose of one of the phenylarsonic acids, enough to produce acute toxicity, the clinical signs would likely be similar to those of inorganic arsenic poisoning

Urea

Urea is used as a protein substitute for ruminants. Cattle and sheep are most susceptible to overdosage and, therefore, most frequently affected. Problems usually arise because of (1) inadequate mixing of urea in the feed, (2) allowing animals to eat too much initially or failing to allow them to become accustomed to urea in the diet, (3) feeding highly concentrated urea-containing protein supplements without mixing them with grain concentrate, or (4) feeding urea to animals on a high-roughage diet.

Urea is broken down in the rumen by microorganisms releasing ammonia, which is the potentially toxic substance. Ordinarily rumen microorganisms utilize the ammonia for protein synthesis. However, if the rumen microflora are not accustomed to synthesizing protein from ammonia, a buildup of ammonia in the rumen may occur. When the concentration in the rumen reaches 80 mg./100 ml., toxicity is produced. The toxicity of a single dose of urea depends on whether the animal is accustomed to eating urea, fullness of the rumen, and amount of carbohydrates in the feed. Animals on low carbohydrate diets, marginal nutritional status, and unaccustomed to urea may develop clinical signs of poisoning after consuming as little as 0.3 Gm. urea/kg. body weight; 1.0 Gm. urea/kg. body weight may be lethal under certain conditions. If an animal is accustomed to consuming urea and is on a high plane of nutrition, as much as 1 to 2 Gm./kg. body weight may not cause toxicity.

To calculate urea concentration in a feed product, the nonprotein nitrogen protein equivalent should be divided by the factor 2.62. This is because 1.0 lb. of urea is equivalent to 2.62 lb. of nonprotein nitrogen protein. Thus, if a label indicates 64% protein from urea (or "nonprotein nitrogen source"), the concentration of urea is 64/2.62 = 24.5% urea. The clinical course of urea toxicity is usually rapid and acute, from 10 minutes to as much as 4 hours after consuming feed containing urea. Clinical signs indicating abdominal pain include frothy salivation, grinding of the teeth, and kicking at the abdomen. There is usually polyuria, muscle tremors, incoordination, weakness, forced rapid breathing, bloat, violent struggling and bellowing, and terminal tetanic spasms. There is usually a marked jugular pulse. At necropsy examination, there is usually no characteristic lesion. Pulmonary edema, congestion, and hemorrhages are commonly found.

Diagnosis of urea poisoning is based on the history of acute illness following consumption of feeds containing urea. It is usually very important that the feed be analyzed for urea content and calculations be made to determine the possibility of poisoning under the conditions of consumption. If the clinical signs and necropsy findings are compatible with urea poisoning, the diagnosis may be confirmed by analysis for ammonia in the tissues. Tissue and rumen samples for ammonia determination should be frozen immediately. Rumen contents, serum, and urine are most suitable. In most cases of urea poisoning, the rumen-ammonia content will be greater than 100 mg./100 ml. and may go as high as 1,000 to 2,000 mg./100 ml. Serum ammonia-nitrogen concentrations of 2 to 4 mg./100 ml., or greater, are usually found.

Diseases that may be confused with urea toxicity include acute encephalitic diseases such as thromboembolic encephalitis and polioencephalomalacia; grain engorgement; acute nitrate toxicity; cyanide poisoning; organophosphate and chlorinated hydrocarbon insecticide poisoning; and enterotoxemia.

Nitrates and Related Problems

The nitrate ion is both a product and a reactant in the chain of animal and plant interrelationships. Atmospheric nitrogen (N) utilized by nitrogen-fixing bacteria is converted to nitrate (NO_3). The nitrate is used by plants to form plant protein. Vegetable protein is either utilized by animals to form animal protein or is returned by decay to the soil. Ani-

mal metabolic wastes (urea and ammonia) are then converted to nitrites or ammonia, or both, and thence to nitrogen to begin the cycle again. As long as proper balance in this cycle is maintained, problems are not encountered.

Nitrate or nitrite toxicity are related most commonly to plant or water sources. Fertilizers (e.g., ammonium nitrate or potassium nitrate) are sources of high nitrate to either plants or animals. Nitrates may be present in varying amounts in water, plants, animal wastes, and chemicals. Nitrates and nitrites are water soluble. Thus, any nitrate added to soil may be leached away, moving with ground water into the water table. Any source, such as decaying organic matter, nitrogen fertilizers, animal wastes, silage juices, and soil high in nitrogen-fixing bacteria may be a source of contamination to wells or reservoirs. Contamination of water supplies from surface drainage is the usual source of nitrates in water.

Abnormal accumulation of nitrate in plants is influenced by a number of factors. Some of the most important include (1) species of plant, (2) content and form of nitrogen in the soil (soils high in nitrate or ammonia supply nitrate more readily to plants), (3) soil conditions favoring nitrate uptake such as adequate or high moisture, acidity, and aeration or low molybdenum, sulfur, phosphorus, and temperature of the soil [below 55.0 F. (12.8 C.)], (4) drought conditions, (5) decreased sunlight, and (6) application of certain herbicides favoring increased growth rate and nitrate accumulation in early stages.

Nitrates accumulate in vegetative tissue, not in fruits or grain. Accumulation is usually greatest in the stalk and less in the leaves.

When silage is made from high-nitrate forages, aerobic fermentation in the silo may result in reduction of nitrates to oxides of nitrogen. These compounds are usually pungent, yellowish brown gases which are heavier than air and may travel down a silo chute or duct, collecting in low places or tight buildings in sufficient concentration to kill livestock and human beings.

Nitrate itself is not particularly toxic However, nitrite (NO_2^-), the reduced form of nitrate, is readily absorbed and is quite toxic. Ruminants and herbivores can readily reduce nitrate to nitrite and toxicity may occur. The nitrite ion readily oxidizes hemoglobin to methemoglobin resulting in tissue anoxia and poorly oxygenated blood.

Both acute and chronic poisoning may occur. Acute poisoning results when forage nitrates exceed 0.5% nitrate (dry weight basis) or 500 p.p.m. nitrate in water. The effects of nitrate in feed and water are additive, and both sources must be considered when evaluating a nitrate problem.

Acute nitrate poisoning may occur within a few hours after ingestion of feed or water containing high amounts of nitrate. On the other hand, signs of acute toxicity may occur only after an animal has consumed nitrate-containing forages for several days. Clinical signs become apparent when methemoglobin concentrations reach 30 to 40%, and death ensues at about 80 to 90% methemoglobin. This may be greatly influenced by the stress and exertion to which an animal is subjected. The most characteristic and likely clinical signs of nitrate poisoning are referrable to anoxia: dyspnea, cyanotic mucous membranes, rapid weak pulse, incoordination, and collapse. The blood is typically brown or "chocolate" colored. Muscle tremors, weakness, ataxia, and low tolerance to exercise are often observed. Animals may appear relatively normal until forced to move about.

Subacute or chronic nitrate poisoning has been described as causing various problems. Chief among these are poor growth rate, abortion, infertility, vitamin A deficiency, goiter, and increased susceptibility to infection. Although such problems have been associated in the field with elevated nitrate contents in forages and water, experimental evidence to substantiate many of these claims is lacking

The diagnosis of acute nitrate poisoning is based on compatible history, clini-

TABLE 1—Comparison of Clinical Effects of Common Toxicants That May Be Confused with Nitrates (Nitrites)

	Nitrate (nitrite)	Sodium chlorate	Silo gases (nitrous dioxide, nitric oxide)	Cyanide	Carbon dioxide	Carbon monoxide
Blood color	Brown	Brown	Slight brown	Cherry red	Dark	Bright red
Mechanism	Methemoglobin	Methemoglobin	Slight methemoglobin	Anticytochrome oxidase	Displaces oxygen	Carbon monoxide hemoglobin (stable)
Treatment	Methylene blue	Methylene blue	Methylene blue Ca gluconate	Nitrite-thiosulfate	Oxygen fresh air	Fresh air oxygen + 5% CO_2 thionine solution
Physical characteristic	.....	.....	About same density as air or heavier	.....	Heavier than air	Lighter than air

cal signs, and necropsy findings, plus chemical examination of water, forages, and rumen contents, as well as the methemoglobin content in the blood. In determining whole blood methemoglobin content, the sample should be analyzed within 1 hour; or if this is not possible, addition of an equal volume of water will hemolyze the erythrocytes and allow analysis to be performed at a later date. Nitrate and nitrite concentrations in the rumen or stomach contents, forage, and water should be considered in light of the methemoglobin concentrations.

A comparison of clinical effects of common toxicants that may be confused with nitrates (nitrites) is given (Table 1). Other disease conditions that may be confused with nitrate poisoning include sodium chlorate poisoning (this herbicide causes the same signs and lesions), cyanide poisoning (cyanide produces characteristic bright red-colored blood), urea poisoning, organic insecticide poisoning, and acute encephalitic diseases.

Cyanide

Cyanide, hydrocyanic acid, or prussic acid are all terms relating to the same toxicant, which is one of the most rapidly acting poisons available to mammals. It is present as inorganic salts, as a naturally occurring glycoside in certain plants, or as free hydrocyanic acid. Cyanides are used in industry for metal cleaning, electroplating, and chemical synthesis. Preparations containing cyanide are used as rodent or vermin killers and for legal execution of criminals in some states. Hydrocyanic acid is sometimes used as a fumigant.

A number of common plants may accumulate large quantities of cyanogenetic glycoside, which hydrolyzes to form free cyanic acid. Of chief agricultural importance are the sorghums, sudan grasses, and corn. Other species with characteristic high cyanide potential are flax, lima beans, cherry, apple, peach, and apricot seeds and leaves.

Under natural conditions, the hydrolysis of cyanogenetic glycosides in plants is inhibited. On wilting, frosting, or stunting of the plant, free cyanic acid may be released. Ingestion of the glycoside by ruminants with subsequent action of rumen microflora may release cyanic acid from the glycoside. This release is also accomplished by the acid medium of the monogastric stomach. Salts of cyanide (e.g. potassium cyanide) also hydrolyze readily to form free cyanide. Fertilizers (e.g. calcium cyanamide) may contain a cyanogenetic principle. Plants of the mustard family (Cruciferae) may contain thiocyanates that inhibit the production of thyroid hormone resulting in hyperplastic goiter and symptoms of hypothyroidism.

The cyanide ion reacts readily with the ferric (trivalent) iron of cytochrome oxidase. This reaction is more stable than the cytochrome oxidase prosthetic groups. This results in iron being maintained in the trivalent state; electron transport stops, and the chain of cellular respiration is brought to a halt. Thus, cyanide

causes cellular hypoxia. At the same time, hemoglobin cannot release its oxygen to the electron transport system. The clinical appearance then is of bright red (oxygenated) blood which cannot be utilized by the cells.

Cyanide is a potent rapid-acting poison. Animals will commonly be found dead, inasmuch as clinical signs last only a few minutes after ingestion. Clinical effects, when seen, occur in rapid succession. Initially, there is excitement and generalized muscle tremor. Pronounced polypnea and dyspnea follow. There may be salivation, lacrimation, and voiding of feces and urine. The animal goes down, gasps for breath, and may have clonic convulsions due to anoxia. The pupils are dilated, and mucous membranes are bright. The characteristic blood color is a bright cherry red. At necropsy examination, the only consistent changes are those relating to the oxygenation of the blood. Mucous membranes are pink and appear well oxygenated. The blood itself is a bright red color and often clots slowly or not at all.

A diagnosis of cyanide poisoning should be considered whenever animals consuming cyanogenetic plants or cyanide salts are affected acutely and have signs of hypoxia and bright-colored blood or mucous membranes. Chemical confirmation of cyanide poisoning can be accomplished either directly or indirectly. Proper preservation of samples for analysis is imperative. Forages, blood, rumen contents, liver, and muscle tissue may be submitted for laboratory diagnosis. All samples should be quick-frozen as soon as possible and maintained in a frozen state until analysis. This prevents further hydrolysis of the glycoside to free cyanic acid and its subsequent loss in the gaseous form. Levels of cyanic acid in plant material in excess of 20 mg./100 Gm. (200 p.p.m.) are significant. A simple test for cyanide in plant material is available.[1] Even low concentrations of cyanide found in tissues are diagnostically significant.

Other conditions that may be confused initially with cyanide poisoning are nitrate poisoning, pulmonary adenomatosis or emphysema, urea poisoning, and acute organophosphate insecticide poisoning.

Organic Pesticides

Contamination of animal feeds by organic insecticides has been discussed elsewhere.[2] Contamination may occur as a result of misidentification of an insecticide, or of adding it to a grain mixture in place of minerals or salt. Three classes of organic insecticides comprise the major portion of such problems. They include the chlorinated hydrocarbons such as aldrin, dieldrin, chlordane, toxaphene, heptachlor, and DDT; the organophosphate compounds characterized by parathion, diazinon, and thimet; and carbamates characterized by carbaryl and pyrolan.

When cattle are exposed to insecticides via the feed, it is usually in massive doses and results in high morbidity and mortality. The course is usually rapid, and the clinical signs are acute. The chlorinated hydrocarbon insecticides cause hyperirritability and intermittent convulsive seizures interrupted by cerebral depression. The organophosphate and carbamate insecticides are cholinesterase inhibitors and produce a cholinergic effect characterized by excessive salivation, dyspnea, and stiffness of the body musculature. Other clinical signs include hyperactivity of the gastrointestinal tract, constricted pupils, cerebral depression, and in some cases, twitching of the muscles of the face, eyelids, tongue, and ultimately the general musculature. This hyperactivity is followed by weakness and paralysis of the skeletal muscles.

In diagnosing poisoning by the organic insecticides, it is imperative to obtain a good clinical history, notice clinical signs and necropsy findings, and finally, chemically analyze the feed, thus confirming the opportunity of exposure. The significance of chemical analyses of the tissues may be limited. In the case of the chlorinated hydrocarbon insecticides, fat, liver, kidneys, and serum should be analyzed. Interpretation of the results should be made with caution as animals may contain several p.p.m. of most of

the chlorinated hydrocarbon insecticides allowing chronic exposure without noticeable detrimental effects. In the case of organophosphate and carbamate insecticides, determination of the whole blood cholinesterase activity may aid in making a diagnosis. Usually tissue analyses are of little value because most of the organophosphate and carbamate compounds are rapidly metabolized and may be difficult to identify.

Copper-Molybdenum Imbalance

The problem of copper-molybdenum imbalance pits the feed manufacturer against the Food and Drug Administration, with the farmer in between. It is a man-made problem. Copper is recognized as a safe and necessary ingredient for animal feeds. Molybdenum is not recognized as safe and necessary, and, by law, cannot be incorporated in animal feeds. Yet, years of clinical veterinary experience and numerous scientific reports unquestionably indicate that a balanced ratio between copper and molybdenum is necessary for most livestock, especially sheep and, to a lesser extent, cattle. The fact is that copper-molybdenum-sulfate ion metabolism and excretion are closely interrelated. If sheep, for instance, are fed a diet containing a normal concentration of copper (8 to 11 p.p.m.) but with no molybdenum, copper toxicity may result. Therefore, when a vitamin-mineral preparation containing copper but no molybdenum is added to the ration, the copper concentration of the ration may be elevated to as much as 25 to 35 p.p.m.; and since the natural molybdenum concentration in feed is usually low (1 to 2 p.p.m.), copper poisoning may occur. On the other hand, excessive molybdenum in the presence of low or even normal concentrations of copper may result in molybdenum poisoning.

Presently, mineral-vitamin preparations are being combined in complete feeds for sheep and, to a lesser extent, for cattle.[1,5] We have encountered at least 20 episodes of copper poisoning in sheep in Iowa this year. Reports from other states and Canada indicate that this problem is widespread. Usually expensive lambs for show purposes or ram lambs on test are involved. We have seen the problem in the Iowa State University sheep flock for several years. Often ram lambs valued from $500 to 1,000 are lost. Usually only about 5% of a flock are affected, however. Clinical signs and necropsy findings are classical of copper poisoning: rapid onset, weakness, anorexia for 12 to 24 hours, hemoglobinuria, icterus, and severe hemolytic anemia. Mortality is very high and occurs within 2 or 3 days.

The Bureau of Veterinary Medicine, FDA, is aware of this problem, but the obstacles preventing its correction go beyond them to other bureaus in the Food and Drug Administration. Because scientific reports indicate that the addition to feeds of excessive molybdenum without copper in balance will cause molybdenum poisoning, it has been concluded that the addition of molybdenum is not safe and necessary. The fact is ignored that if molybdenum had been added in natural balance with copper (1 part molybdenum to 6 to 10 parts copper) toxicity would not have resulted. Also ignored is the fact that if copper were added to the ration without molybdenum or sulfate being present, copper poisoning would result. There are numerous documented studies confirming this phenomenon. Thus, used alone, copper should not be considered as safe and necessary. However, in balanced combination with molybdenum, both are safe and necessary.

Failure to recognize molybdenum as safe and necessary is based partly on the fact that molybdenum is accumulated in tissues and eliminated in milk when given in excessive amounts in the absence of copper, thus creating a potential public health hazard. Apparently ignored, however, is the fact that under present regulations, animals consuming a ration containing copper but no molybdenum may have copper concentrations of 2,000 p.p.m. or greater in the liver, which could also be hazardous. This problem could be solved by adding molybdenum and

copper to animal feeds at a ratio of about 1 part molybdenum to 6 to 10 parts copper. Another solution might be the addition of copper to animal feeds only in the form of copper sulfate. Because the sulfate ion facilitates excretion of copper, copper in combination with sulfate is less toxic.

Diagnosis of copper poisoning may be confirmed using chemical analysis of the tissues and blood of affected animals correlated with copper and molybdenum concentrations in the ration, clinical signs, and necropsy findings. Normal copper concentration in whole blood ranges from 0.7 to 1.3 p.p.m. Concentrations above this level are often associated with copper poisoning. Liver concentrations associated with copper poisoning are usually greater than 150 p.p.m. on a wet-weight basis, whereas kidney concentrations are usually greater than 15.0 p.p.m. Complete sheep feed containing 25.0 p.p.m. or greater copper with no added molybdenum may produce copper poisoning. Because cattle are less susceptible to this poisoning than sheep, somewhat greater concentrations of copper may be necessary to produce poisoning in this species.

Mycotoxins

Toxic chemical compounds produced by fungi are called mycotoxins. Moldy feeds have been fed since the beginning of a feeding period, usually with no resulting problems. Several disease conditions, however, have been observed, which might be caused by mycotoxins, e.g., polioencephalomalacia in cattle, hemorrhages in swine, gastrointestinal disturbances in all animals, and estrogenic effects in swine and cattle. We know that toxins can frequently be isolated from moldy feeds that are extremely toxic to laboratory animals; in fact, some mycotoxins are the most toxic compounds known. The problem with mycotoxins, I believe, is one in which we have just begun to scratch the surface of knowledge. Many questions are unanswered. Do mycotoxins, in fact, cause disease problems in livestock? Are they as toxic to livestock as they are to laboratory animals and human beings? What management practices will reduce the production of mycotoxins by molds? Do all molds produce toxins? If not, which ones do and under what environmental conditions? Are there mycotoxins yet to be identified?

No-Roughage Diets

A truly toxicologic problem that usually is not considered as such is the use of no-roughage rations for beef cattle. We hear the predictions of a population explosion in the world; and unless major catastrophe intervenes, these predictions will undoubtedly come true. Swine and poultry compete with man for cereal grains and high-protein crops. Therefore, as the human population increases, swine and poultry numbers will decrease. The only animals that will survive the competition with man for food will be the ruminants. This is because they have stomachs that have the ability to convert cellulose and other forms of roughage, which are not suitable for human consumption, to protein, which is suitable for human consumption. Yet, today we are directing much of our research effort in an attempt to convert the cow and other ruminants into a hog—an animal that eats mostly cereal grains. Shouldn't we seriously consider directing our research and developmental efforts toward the long-range goal of making the ruminant more efficient in the conversion of unusable nitrogenous materials and cellulose into protein for human food? A statement was recently made by the director of an animal research station in England. "We will do no more work on the dairy cow since she is out because of milk substitutes." Such a statement is serious, and I believe that we should support and join in with individuals in animal research who are looking for better ways of feeding unusable materials to the ruminant for the production of human food. And besides, many of our feedlot problems stem from the practice of trying to convert the beef cow into a hog.

References

1. Buck, W. B.: I
and Their Interp
(Dec. 15, 1969): 1
2. Buck, W. B.:
Poisonings in Cattl
1970): 1434–1443.
3. Buck, W. B.:
countered with M
of Drugs in Anim
of Sciences, Pub.

Congenita
Defects i
Diarrhea-
in Pregn

Robert F. Kah
Alexander de

Conger
varying fr
natural o
infection
In fiel
collected
with natu
Two catt
The d
calves pr
for deter

From the New
University, Ithac
professor of Epid
of Veterinary
associate profess

The work des
General Research
AI09137-05 from
Infectious Disea

Food Technology Vol 6 255-259 (1952)

Preservative Effect of Various Concentrations of Curing Salts in Comminuted Pork[a,b]

CORNELIUS BULMAN[c] AND JOHN C. AYRES
Department of Bacteriology, Iowa State College, Ames, Iowa

(Manuscript received April 18, 1952)

The preservative effect of sodium chloride, sodium nitrate, and sodium nitrite in comminuted pork trimmings has been investigated. The pork was inoculated with known amounts of P. A. 3679 spores and incubated at 98.6° F. (37° C.) after which the preservative effect of the curing salts was determined by bacteriological and chemical analyses as well as by the appearance of the meat. The minimum amounts of the curing salts necessary both singly and in combination for the preservation of the meat are reported and discussed.

Processing schedules used by various manufacturers to assure satisfactory shelf life of canned meat products differ widely. However, regardless of the processing times followed in sterilizing pork luncheon meat products in 12-ounce oblong or 40-ounce round tins, no concrete evidence has ever been received from the field that any of these meats were understerilized *(16)*. A similar item merchandized in 3- and in 6-pound rectangular cans generally is considered to be understerilized since the heat believed necessary to destroy spores would cause excessive rendering and purging of the meat and result in an unpalatable product having low drained weight. While the perishable product does not have the pronounced canned or "overcooked" flavor associated with the item receiving a sterilizing treatment, the necessity of refrigeration restricts the use of luncheon meat packed in the larger containers.

Most spoilage of canned meat is considered to be caused by the group of bacteria known as putrefactive anaerobes. These organisms have heat resistant spores which can germinate and decompose meat under the anaerobic conditions provided within the sealed container. Generally, non-acid food, such as meat, is given a heat process calculated to destroy spores of *Clostridium botulinum*, an extremely toxigenic putrefactive anaerobe. Before a process of lower sterilizing value can be used, these and other viable putrefactive anaerobic spores must either be absent or be inhibited to a point where they will not produce toxin or cause spoilage. Earlier results from this laboratory *(2)* and elsewhere *(8)* have shown that fresh and cured pork trimmings contain light loads of anaerobic spores. Since these organisms have been found to persist in the product, it seemed important that the tolerance of such forms of life for the several chemical agents which comprise curing salt mixtures should be determined.

While the preservative value of curing salts has been recognized for some time, many conflicting reports regarding the concentrations which proved effective against putrefactive anaerobes are to be found in the literature. Some of the causes of these discrepancies were explained by Tanner and Evans *(17, 18, 19)* who observed that species and strains varied in susceptibility to the same salt and that similar organisms tolerated various levels of the same salt in different substrates. These workers also pointed out that another possible reason for contradictory results was the failure of many investigators to control salt concentrations by chemical analyses. The hydrogen ion concentration of the medium used to test the salt was shown to have an important influence upon the results by Tarr *(20)* who found that sodium nitrite was a more effective preservative in acid media. Lack of agreement of results might be due also to use of different spore loads in the curing salt tests. Jensen and Hess *(9)* and Stumbo, Gross, and Vinton *(15)* have shown that, as the number of spores added is increased, the curing agents are less effective in preventing spoilage.

There appears to be agreement among investigators on the value of sodium chloride in checking putrefactive anaerobic spoilage of canned meat, but there is difference of opinion regarding the preservative value of sodium nitrate and sodium nitrite. Ordinarily, commercial mixed cures contain relatively small amounts of sodium nitrate and sodium nitrite as compared with the amount of sodium chloride used. From a study made by Moulton and Lewis *(11)* they conclude that $NaNO_3$ and $NaNO_2$ exerted a specific inhibitory effect greater than that of an equal amount of NaCl. Yesair and Cameron *(21)* also concluded from their studies that nitrate and nitrite had a specific effect. On the other hand, Evans and Tanner *(3)* reported that putrefactive anaerobes would tolerate concentrations of a commercial mixture of NaCl, $NaNO_3$, and $NaNO_2$ up to the level where NaCl was effective when used alone. Gross, Vinton, and Martin *(6)* pointed out that the amount of $NaNO_3$ used in canned cured meat is too small to be of preservative value and that $NaNO_2$, while an effective preservative, is destroyed during the heat process.

It has been suggested by Jensen and Hess *(9)* that, in the presence of curing salts, putrefactive anaerobic spores may be destroyed at lower temperatures than in their absence. However, investigations by Yesair and Cameron *(21)* and by Stumbo, Gross, and Vinton *(15)* showed that the heat resistance values for putrefactive anaerobic spores were the same in cured as in uncured meat.

[a] Journal Paper No. J-2076 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 1017. Presented at the Twelfth Annual Meeting of the IFT, Grand Rapids, Michigan, June 9, 1952.

[b] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned Number 374 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

[c] Present address: Goodyear Tire and Rubber Company, Akron, Ohio.

The present study was undertaken to determine levels of the 3 curing salts which would be tolerated by putrefactive anaerobes in comminuted pork trimmings. Also, the effectiveness of a commercial curing salt mixture was investigated.

EXPERIMENTAL

Pork samples were obtained after they had been collected by a member of the laboratory staff of a commercial meat packing plant.[4] In securing samples, aseptic precautions were taken in transferring portions of the meat from each of the various trucks in the assembly room to a sterile metal container. Since the samples consisted of lean trimmings from the belly, jowl, backbone, loin end, lean butt, neckbone lean, blade meat, ham trims, and picnic trims and were selected from various loads, they represented meat from a considerable number of hogs. The composition of the pork trimmings was recorded and consisted of about 75% lean and 25% trimmable fat. The percent fat content was not determined by extraction but was that amount that could be trimmed by knife using ordinary care. Gross *(5)* indicated that at a level of 75 to 25 the trimmable fat and extracted fat values are approximately the same. After filling a 6-pound luncheon meat can with known quantities of each of the different types of trimmings in proper proportion, the can was hermetically sealed and immediately placed on a refrigerating coil at —25° F. (—31.7° C.). The contents were maintained in the frozen state during transit and until just prior to sampling in the college laboratory.

In order to prepare the meat for use, the can of frozen pork trimmings were placed in running tap water [60-65° F. (15.6-17.7° C.)] for ½ hour and, after opening the cans observing aseptic precautions, chunks of the still partially frozen meat were ground in a sterile meat grinder equipped with a 3/16 inch grinding plate. Following grinding the can contents were blended in a KitchenAid Model K4-B mixing bowl for 5 minutes at speed 10.

A standard spore suspension was made by transferring an inoculum of *Clostridium* species, No. 3679 National Canners Assoc. (P.A. 3679) to agar slants of Difco Linden thioglycollate medium modified by the addition of 1.5% agar and 0.1% soluble starch. The slants were incubated in the presence of purified "Seaford" nitrogen gas in accordance with the method described by Ogilvy, Burke, and Ayres *(12)* at 98.6° F. (37° C.) for 10 days. At that time a microscopic examination was made to determine purity and the presence of spores. The growth was washed from the slants with sterile physiological saline into a sterile container where it was heated at 176° F. (80° C.) for 20 minutes to destroy the vegetative cells. The spore suspension was held at 33° F. (0.6° C.) and, from time to time, counts were made using the "Most Probable Number" (M.P.N.) method.

Chemically pure curing salts were used, and in each instance a spore count was made to ascertain the degree of contamination.

The pork had a pH range of pH 5.8 ± 0.2. Addition of curing salts did not alter the pH of the fresh pork to any appreciable extent.

The preservative value of each of the salts was estimated in a preliminary experiment and, after the levels of the agent that would prevent germination completely had been determined, a retest was made using from 3 to 4 salt concentrations within that immediate range. Unsalted pork was used to check the effect of the added salt while uninoculated pork was tested to determine the extent of contamination of the meat. Each of from 5 to 7 replicate tubes were filled with approximately 15 g. of meat containing the appropriate level of salt and number of spores. A sterile calking gun fitted with an attachment for delivering the meat paste to the bottom of the test tube was used; care was taken to avoid entrapping excessive amounts of air. The tubes were held overnight at 33° F. (0.6° C.) and then heated in a water bath to an internal temperature of 176° F. (80° C.) for 20 minutes. After heating, one tube from each set was placed aside for the initial spore count and salt analysis; the remaining tubes were sealed immediately by the addition of a layer of sterile paraffin and then were incubated at 98.6° F. (37° C.) and examined at intervals of 1, 2, 4, 7, 14, and 28 days for visual signs of spoilage as indicated by color changes, gas production, and digestion of meat. At the end of 28 days one sample was withdrawn from each set for a spore count and salt analysis. In addition, this opened tube was judged for spoilage by odor and texture of the meat.

Spore counts were made by weighing 10 g. of meat, placing the meat in a Waring blendor and adding 250 ml. of physiological saline. After meat and saline were blended for 10 minutes, 5 replicate 10 ml., 1 ml., and 0.1 ml. quantities of the mixture were each added to tubes containing Difco Linden thioglycollate medium which had been modified by the addition of 0.1% soluble starch. These tubes then were heated at 176° F. (80° C.) for 20 minutes, sealed with a layer of sterile paraffin, and incubated for 10 days at 98.6° F. (37° C.). At the end of the incubation period positive tubes were determined by color change, meat digestion, cloudiness, and putrefactive odor; a microscopic examination was made of material from suspected and spoiled tubes. From the number of positive tubes in each dilution the most probable spore count was obtained by referring to M. P. N. tables.

Chloride was determined by precipitation with a known amount of standard $AgNO_3$ and titration of the excess Ag^+ with standard KSCN. The organic matter in the meat sample was destroyed by ashing in an oven or by wet oxidation with HNO_3 and $KMnO_4$. Results using the wet oxidation method proved to be both faster and more reproducible and, after the initial experiments, this latter procedure was used exclusively. A modification of the Schlössing-Wagner, or $FeCl_2$, method, recommended by Blake and Lewis *(1)* was used to determine nitrate. Sodium nitrite was determined by the method given by Morpeth *(10)* in which the nitrite ion is reacted with sulfanilic acid and α-naphthylamine to produce a red colored compound the intensity of which is an index of the nitrite present. Color intensity was measured by means of an Evelyn photoelectric colorimeter.

RESULTS

Sodium chloride. Various levels of sodium chloride were tested in attempts to determine the approximate minimum concentration of that salt required to prevent spoilage of inoculated and uninoculated pork trimmings. The amount of sodium chloride required in the meat containing no added spores varied from 2.0 to 4.3% between experiments. It was found that when the unsalted controls spoiled quickly, higher salt levels were necessary to keep the meat.

When a load of 175 spores of *Clostridium* spp. (P.A. 3679) per g. was added to pork, sodium chloride at a level of 4% by weight extended the storage life of the meat an additional 3 weeks beyond that of the raw unsalted meat or of pork containing only 2% added sodium chloride (Table 1). None of the inoculated samples containing 6% or 8% NaCl spoiled within the

TABLE 1

Spoilage of inoculated* pork containing different levels of NaCl (5 tubes)

Sodium chloride		Percent spoilage with incubation times at 98.6° F. (37° C.) of		
Added	Found by analysis	1 week	4 weeks	10 months
%	%			
0	0.1	100	....	....
2.0	2.1	100	....	....
4.0	4.1	0	100	....
6.0	6.1	0	0	0
8.0	8.2	0	0	0

* Analyzed to contain 175 P.A. 3679 spores per g.

[4] The authors are indebted to John Morrell and Company whose bacteriologists carefully collected and packed the lots of meat used in this study.

10-month holding period. In another trial, sodium chloride at a level of 5% was shown to delay the onset of spoilage by at least 6 months. Using these findings to establish the maximum and minimum limits of a bacteriostatic range, an attempt was made to determine more accurately the minimum inhibitory level. Concentrations of 3.5, 4.0, and 4.5% NaCl by weight were added to pork trimmings; also, samples containing no added salt and others containing neither salt nor organisms were used as controls. Each of two separate lots of pork were inoculated with 50 spores per g. of P.A. 3679. Results are shown in Table 2.

From these results it may be observed that the critical concentration of sodium chloride appears to be in the neighborhood of 4.0%. Although the minimum level of salt resulting in preservation was not always the same, undoubtedly the lack of reproducibility was due in part to the variability of spores normally present in the fresh pork as well as to those added. The number of spores present in the meat before inoculation was indicated by the fact that from 33 to 100% of the uninoculated tubes also immediately underwent spoilage anaerobically. A difference in susceptibility to sodium chloride among the various anaerobic spores normally present in meat would be expected.

Of some interest is the observation that, at a load of 175 P.A. 3679 spores per g., 4.1% NaCl did not prevent spoilage for 4 weeks (Table 1) but that when a smaller number of spores, 50 P.A. 3679 spores per g., were used (Table 2), lower levels of the agent were sufficient to preserve the meat.

Length of the incubation period also figured in the evaluation of the preservative properties of sodium chloride. As shown in the second trial in Table 2, none of the tubes containing 3.7% NaCl had spoiled during a 9-week incubation period but, at 16 weeks, all had spoiled.

A possible explanation for growth after long periods of incubation was offered by Halvorson *(7)* who suggested that hydroperoxides are likely to be present in media containing unsaturated fatty acids, that these peroxides can be decomposed by various types of enzymes, and that there may be a small amount of such enzyme activity in spores. Also, Halvorson postulates that, if there are a small number of spores, the peroxides may remain stable for relatively long periods of time so that upon standing they gradually disappear to a point where the spores can germinate. An interesting corollary to the above hypothesis would be that sodium chloride delays spore germination by interfering with enzyme elaboration.

Sodium nitrate. Since Tanner and Evans *(17)* indicated that the concentration of the curing agent (rather than the specific effect of the salt used) was the critical factor, the range, 3.5 to 4.5% sodium nitrate was selected for a preliminary trial. However, after a 1-week incubation period, signs of spoilage were evident in all tubes at every nitrate level. Typical anaerobic spoilage appeared first as a reddening of the meat. This was followed by darkening, foul odor, digestion and, with but few exceptions, gas production. Another type of spoilage in meat containing nitrate and arbitrarily considered as atypical, was characterized by change in color to gray-green, much gas production, no foul odor, and no extensive digestion. The pigment of the latter probably was similar to the oxidation pigments described by Greenwood, Urbain, Jensen, and Lewis *(4)* and caused by action of excess nitrite, oxygen, and microorganisms upon meat pigments. All spoilage of this type occurred within the first week of storage and did not increase upon further incubation (Table 3).

TABLE 3

Typical and atypical spoilage of inoculated[h] pork containing different levels of $NaNO_3$ (6 tubes)

Type spoilage	Sodium nitrate		Percent spoilage with incubation times in weeks at 98.6° F. (37° C.) of			
	Added	Found by analysis	1	2	4	12
	%	%				
Atypical	0	0	0	0	0	0
	3.5	3.5	17	17	17	17
	4.0	4.1	33	33	33	33
	4.5	4.7	33	33	33	33
Typical	0	0	100	....	....	....
	3.5	3.5	0	0	17	83
	4.0	4.1	0	0	0	0
	4.5	4.7	0	0	0	0

[h] Approximately 50 P.A. 3679 spores per g.

Some inhibition of anaerobic spoilage was noted in meat containing 3.5% $NaNO_3$ but, as was the case wherein NaCl was used at this level, the meat spoiled upon prolonged storage. Similarly, 4.1 and 4.7% $NaNO_3$ preserved meat for at least 12 weeks.

Since atypical spoilage appeared to be caused by an organism other than a putrefactive anaerobe, an

TABLE 2

Spoilage of inoculated[f] pork containing different levels of NaCl

Trial	Sodium chloride		No. of tubes	Percent spoilage with incubation times at 98.6° F. (37° C.) of				
	Added	Found by analysis		1 week	2 weeks	4 weeks	9 weeks	16 weeks
	%	%						
1	0	0.1	6	100				
	3.5	3.6	6	0	0	0	100	....
	4.0	4.0	6	0	0	0	0	....
	4.5	4.6	6	0	0	0	0	....
[f] Uninoculated pork	0	0.1	6	33	67	67[g]	100	....
2	0	0.1	5	100				
	3.5	3.7	5	0	0	0	0	100
	4.0	4.4	5	0	0	20	20	40
	4.5	4.6	5	0	0	0	0	0
[f] Uninoculated pork	0	0.1	5	100	....	....	....	....

[f] Approximately 50 P.A. 3679 spores per g.
[g] 4 tubes spoiled; other 2 doubtful.

aerobic as well as an anaerobic spore count was made after the meat had incubated for 4 weeks. Atypically spoiled meat contained large numbers of spores capable of growing aerobically.

Also, nitrite analyses indicated approximately 2000 p.p.m. of $NaNO_2$ in atypically spoiled meat and none in sound or in anaerobically spoiled meat. These results indicate that atypical spoilage was due to an aerobic or facultative spore former which reduced nitrate to nitrite. These organisms were able to tolerate 10% $NaNO_3$. Quastel and Stephenson *(13)* have shown that nitrate can take the place of oxygen in the metabolism of some organisms.

In order to avoid the presence of spore forming aerobes, sterile cooked pork was used and a similar experiment was performed. Results corresponded to those of the first nitrate experiment if the atypical spoilage in the earlier trial is ignored (Table 4).

TABLE 4
Spoilage of inoculated [1] cooked pork containing different levels of $NaNO_3$ (5 tubes)

Sodium nitrate		Percent spoilage with incubation time in weeks at 98.6° F. (37° C.) of			
Added	Found by analysis	1	2	4	10
%	%				
0	0	100	....	....	....
2.9	3.1	100	....	....	....
3.4	3.8	0	100	....	....
3.9	4.1	0	0	0	0

[1] Approximately 50 P.A. 3679 spores per g.

Sodium nitrite. Preliminary experiments wherein widely different concentrations of sodium nitrite were used indicated preservation of pork at much lower levels than those which were effective with sodium chloride or sodium nitrate. Also, at high nitrite levels, rapid spoilage of the atypical kind observed in the first nitrate experiment occurred. A preservative effect was indicated at levels of from 0.04 to 0.08% $NaNO_2$ (Table 5) and longer storage life was observed for meat preserved with the higher nitrite concentrations. As shown by comparing the amount of nitrite added with that found by analysis, some destruction took place during the heat process. According to Greenwood, Urbain, Jensen, and Lewis *(4)*, nitrite destruction probably is caused by reaction with meat protein and is accelerated by elevated temperature. Chemical analyses made after 4 and 16-week storage periods indicate a continuing loss. Spoiled meat indicated absence of residual $NaNO_2$ and one sample that was still sound had only 0.0004% nitrite. Other workers, notably Ruyle and Tanner *(14)* have reported complete disappearance of nitrite in canned meats after several months of storage.

There was an appreciable reduction in the anaerobic spore count using the M.P.N. method, in preserved meat containing nitrite after it had incubated for 28 days (Table 6). However, since companion tubes to

TABLE 6
Anaerobic spore counts of meat containing different levels of $NaNO_2$

Sodium nitrite added by wt.	Spores per g. pork	
	At start of incubation	After 4 weeks incubation
%		
0.04	47	2
0.05	71	1
0.06	56	<1
0.07	38	1
0.08	115	1

those having a low spore count after the 4 week incubation period eventually spoiled, spore inhibition was not complete. The question might be raised whether growth of spoilage organisms may be due not to the development of tolerance for nitrite but rather to a gradual lowering of nitrite concentration until a non-toxic level is reached.

Mixed curing salts. Inoculated as well as uninoculated pork was used to test combinations of curing agents. The salts, sodium chloride, sodium nitrate, and sodium nitrite, were tested singly and in combination in concentrations used commercially (Table 7). In uninoculated meat, nitrite in combination with sodium chloride or nitrate appeared to extend storage life of the pork (Table 7A). From results of experiments in which inoculated meat was stored (Table 7B) it is noted that none of the salts or salt mixtures preserved pork upon prolonged incubation. The combination of all three of the salts was found to be the most effective but even this mixture could be tolerated by anaerobes when the meat was incubated at 98.6° F. (37° C.) for 19 weeks. In these experiments all inoculated control tubes spoiled within the first 24 hours of incubation. When used singly or with $NaNO_3$, neither NaCl or $NaNO_2$ had much preservative effect. On the other hand, the combination of chloride and nitrite had inhibitory action. In the experiment wherein $NaNO_2$ was introduced alone at the maximum tolerance permitted in meat products, spoilage was delayed to some extent and 3.5% NaCl when used alone also slightly prolonged storage life. When used by itself at the 0.125% level, $NaNO_3$ had no preservative effect.

TABLE 5
Spoilage of inoculated [1] pork containing different concentrations of $NaNO_2$

No. of tubes	Sodium nitrite				Percent spoilage with incubation time in weeks at 98.6° F. (37° C.) of			
	Added	Found by analysis after incubation time in weeks of						
		0	4	16	1	2	4	16
	%	%	%	%				
5	0	0	0	....	100	....	....	....
6	0.04	0.037 [b]	0.0046	0	33	66	66	100
5	0.06	0.056	0.0058	0	20	20	20	100
5	0.08	0.073	0.0080	0.0004	0	0	0	20

[1] Approximately 50 P.A. 3679 spores per g.
[b] Percent $NaNO_2$ in sound samples of inoculated pork.

TABLE 7

Spoilage of pork containing NaCl, $NaNO_3$ and/or $NaNO_2$ at levels used in commerce

Trial	Spores per g. pork	No. of tubes	Sodium chloride		Sodium nitrate		Sodium nitrite		Percent spoilage with incubation times in weeks at 98.6° F. (37° C.) of					
			Added	Found by analysis	Added	Found by analysis	Added	Found by analysis	1	2	4	12	16	19
A Uninoculated meat			%	%	%	%	%	%						
1	<1	5	0	0.1	0	0	0	0	20	20	20	20	20	20
		5	3.5	3.6	0.125	0.13	0.015	0.0083	0	0	0	0	0	0
2	<1	6	0	0.1	0	0	0	0	17	17	17	17	17	----
		6	3.5	3.6	0	0	0.017	0.0095	0	0	0	0	0	----
3	32	5	0	0.1	0	0	0	0	100	----	----	----	----	----
		5	3.5	3.6	0.125	0.13	0	0	100	----	----	----	----	----
4	52	5	0	0.1	0	0	0	0	80	100	----	----	----	----
		5	0	0.1	0.125	0.13	0.017	0.012	40	40	40	40	40	40
B Inoculated[1] meat														
1		5	3.5	3.6	0.125	0.13	0.015	0.0083	0	0	0	0	20	100
2		5	3.5	3.4	0	0	0.017	0.012	0	0	0	100	----	----
		5	0	0.1	0	0	0.017	0.012	0	80	100	----	----	----
		5	3.5	3.5	0	0	0	0	0	100	----	----	----	----
3		4	3.5	3.7	0.125	0.13	0	0	50	100	----	----	----	----
4		4	0	0.1	0.125	0.12	0.017	0.011	100	----	----	----	----	----
5		5	0	0.1	0.125	0.12	0	0	100	----	----	----	----	----
Control		5	0	0.1	0	0	0	0	100	----	----	----	----	----

[1] Approximately 50 P.A. 3679 spores per g. added.

SUMMARY

An investigation was made of the effective preservative levels of the curing agents sodium chloride, sodium nitrate, and sodium nitrite in comminuted pork trimmings. The minimum concentration of each salt that would preserve pork inoculated with known loads of P.A. 3679 spores (approximately 50 per g.) was determined after incubation at 98.6° F. (37° C.) by bacteriological and chemical analyses as well as by appearance of the meat. Tubes of meat were heated to kill vegetative cells after which the pork trimmings were layered with melted paraffin to insure anaerobiosis. Anaerobic spore counts and curing salt analyses were made of representative samples at the time that the meat was placed in the incubator and again at the time of spoilage or at the end of 28 days (or more) of incubation. Varying degrees of bacteriostasis were shown by levels of 3.5 to 4.4% NaCl while no spoilage was observed at concentrations exceeding 4.4%. The anaerobic spores had about the same tolerance for $NaNO_3$ as for NaCl. Initial levels of $NaNO_2$ of 0.037% or more delayed meat spoilage. A gradual decrease in nitrite was noted during incubation and was accompanied by increased spoilage. Meat containing as little as 0.0004% nitrite apparently was sound while spoiled meat indicated absence of nitrite upon analysis. Inoculated meat containing the three curing agents tested individually or in combinations at levels permitted in commercial mixed cure showed that either $NaNO_2$ or NaCl delayed spoilage for from 1 to 4 weeks but that $NaNO_3$ had no inhibitory effect. The mixture of all three salts prevented spoilage of inoculated meat for 12 but not for 19 weeks.

LITERATURE CITED

1. Blake, J. C., and Lewis, W. L. Recommended procedure for determining nitrate in lean meat. Am. Meat Inst., *Dept. Sci. Research* (1930).
2. Burke, M. V., Steinkraus, K. H., and Ayres, J. C. Methods for determining the incidence of putrefactive anaerobic spores in meat products. *Food Technol.*, 4, 21 (1950).
3. Evans, F. L., and Tanner, F. W. The effect of meat curing solutions on anaerobic bacteria. IV. The effect of mixed curing solutions. *Zentr. Bakt. Parasitenk.*, Abt. II 91, 135 (1934).
4. Greenwood, D. A., Urbain, W. M., Jensen, L. B., and Lewis, W. L. The heme pigments of cured meat. IV. Role of sugars in color of cured meats. *Food Research*, 5, 625 (1940).
5. Gross, C. E. Information on lean to fat ratios in pork luncheon meat. (Private communication. 1950).
6. Gross, C. E., Vinton, C., and Martin, S. Bacteriological studies relating to thermal processing of canned meats. IV. Viability of spores of a putrefactive anaerobic bacterium in canned meat after prolonged incubation. *Food Research*, 11, 399 (1940).
7. Halvorson, H. O. Factors affecting bacterial spore germination. Am. Meat Inst., *2nd Research Conf. Proc.*, 51 (1950).
8. Harriman, L. A., DelGiudice, V. J., Shinn, B. M., and Hansen, R. The incidence of putrefactive anaerobes in pork. Paper delivered to the Society of Illinois Bacteriologists' fall meeting. Peoria, Ill. Oct. 15, 1948. (Abstract of paper with meeting notice. 1948).
9. Jensen, L. B., and Hess, W. R. A study of the effects of sodium nitrate on bacteria in meat. *Canner*, 92 (12), 82 (1941).
10. Morpeth, J. C. The determination of nitrite in brines and cured meats. *J. Soc. Chem. Ind., (London)*, 24, 370 (1946).
11. Moulton, C. R., and Lewis, W. L. *Meat Through the Microscope.* 2nd Ed. 1940, Am. Meat Inst., The Univ. of Chicago, Chicago, Ill.
12. Ogilvy, W. S., Burke, M. V., and Ayres, J. C. A practical method for controlling atmospheres for use in food research. *Food Research*, 15, 343 (1950).
13. Quastel, J. H., and Stephenson, M. Experiments on "strict" anaerobes. I. The relationship of *B. sporogenes* to oxygen. *Biochem. J.*, 20, 1125 (1926).
14. Ruyle, E. H., and Tanner, F. W. The microbiology of certain canned meat products. *Zentr. Bakt. Parasitenk.*, Abt. II. 92, 436 (1935).
15. Stumbo, C. R., Gross, C. E., and Vinton, C. Bacteriological studies relating to thermal processing of canned meat. II. Influence of meat-curing agents upon thermal resistance of spores of a putrefactive anaerobic bacterium in meat. *Food Research*, 10, 283 (1945).
16. Subsistence Research and Development Laboratory, Quartermaster Food and Container Institute for the Armed Forces. Meat Products. Operation Studies No. 1, 11, 97 (1948).
17. Tanner, F. W., and Evans, F. L. Effect of meat curing solutions on anaerobic bacteria. I. Sodium chloride. *Zentr. Bakt. Parasitenk.* Abt. II, 89, 48 (1933).
18. Tanner, F. W., and Evans, F. L. Effect of meat curing solutions on anaerobic bacteria. II. Sodium nitrate. *Zentr. Bakt. Parasitenk.* Abt. II, 89, 48 (1933).
19. Tanner, F. W., and Evans, F. L. Effect of meat curing solutions on anaerobic bacteria. III. Sodium nitrite. *Zentr. Bakt. Parasitenk.* Abt. II, 91, 1 (1934).
20. Tarr, H. L. A. The bacteriostatic action of nitrites. *Nature*, 147, 417 (1941).
21. Yesair, J., and Cameron, E. J. Inhibitive effect of curing agents on anaerobic spores. *Canner*, 94 (13), 89 (1942).

Nature, Vol 183 (1959) pp. 163-165

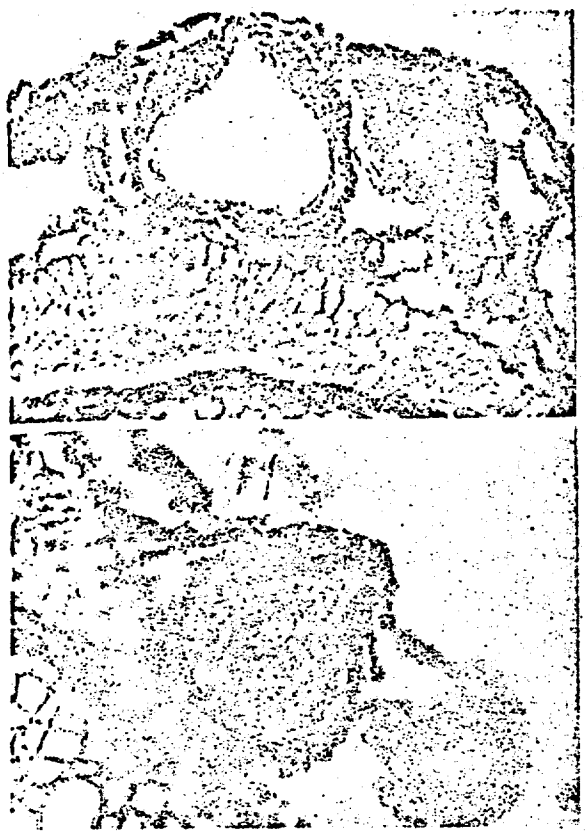


Fig. 1. Top, perithecia of *M. pinodes* treated with 0·75 per cent pentachlorophenol; bottom, normal (untreated) perithecium of *M. pinodes*

The spore counts given in Table 1 suggested that this concentration of pentachlorophenol had a powerful fungicidal effect on mature perithecia of *M. pinodes*, but that the method of application had not given complete coverage.

The second series of laboratory tests was designed to determine the effect of a range of concentrations of pentachlorophenol on 10-gm. samples which were momentarily immersed in the emulsion in order to ensure complete coverage. Spore discharge was assessed in the same way, beginning on the second day after treatment and continuing thereafter at weekly intervals for five weeks. Spore counts are given in Table 2, and these indicate a highly efficient kill of perithecia with all concentrations of pentachlorophenol down to 0·2 per cent.

After each of the first four discharge tests, the samples were allowed to dry in air. After the fifth discharge, all the samples were showered with ascospores of *M. pinodes* and incubated moist in closed containers at room temperature to determine whether recolonization by the fungus could occur at this stage. Straws untreated with pentachlorophenol but steam-sterilized were also subjected to a similar inoculation and incubation.

A very heavy mycelial growth was present on the steam-sterilized straws after incubation for five days; that on the unsterilized untreated controls was less conspicuous, possibly due to partial inhibition by strong bacterial growth. A few isolated colonies were noted on all the samples treated with pentachlorophenol except those which had received the 2 per cent treatment.

In order to examine histologically the effect of pentachlorophenol on the perithecia, representative straws from 0·75 per cent pentachlorophenol and from control samples were embedded in paraffin wax and sectioned by microtome at 10μ. The chemical completely destroyed the contents of the perithecia, which appeared either empty or with a few disorganized remains in the asci, as shown in Fig. 1 (top). Sections of normal untreated perithecia are shown in Fig. 1 (bottom).

Preliminary tests have shown that dinitro-orthocresol (0·2 per cent) and dinitro-secondary butylphenol (0·2 per cent) may be expected to give similar results.

Field experiments with certain of these chemicals are proceeding; but observations to date indicate that the technical difficulties of obtaining a sufficiently complete spray coverage of the pea stubble will present a serious obstacle to successful application of the method for field control of the pathogen.

[1] Hutton, K. E., *Agric. Gaz. N.S.W.*, 68, 535 (1957).
[2] Corke, A. T. K., Rep. Agric. Hort. Res. Sta. Bristol, 1953, 154 (1954).
[3] Byrde, R. J. W., Rep. Agric. Hort. Res. Sta. Bristol, 1949, 81 (1950).
[4] Byrde, R. J. W., Crowdy, S. H., and Roach, F. A., *Ann. App. Biol.*, 39, 531 (1952).
[5] Hirst, J. M., *Ann. App. Biol.*, 39, 257 (1952).

268

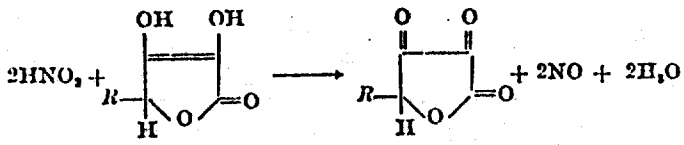
OXIDATION OF ASCORBIC ACID AND SIMILAR REDUCTONES BY NITROUS ACID

By Dr. C. A. BUNTON
Department of Chemistry, University College, London, W.C.1

AND

Prof. H. DAHN and Dr. LOTTE LOEWE
Organisch-chemische Anstalt der Universität, Basel

THE reaction between ascorbic acid and sodium nitrite or nitrous acid was first observed by Karrer and Bendas[1]. This reaction is general for reductones (that is to say, enediols stabilized by conjugation to a carbonyl or similar group)[2,3]; it has the characteristics of a typical oxidation-reduction process, and gives quantitative formation of nitric oxide and dehydro reductone. For example:

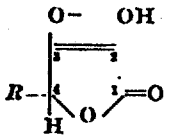


(Ia), Ascorbic acid (R = $CH_2OH.CHOH$—)
(Ib), Phenyl hydroxytetronic acid[3] (R = C_6H_5—)

(IIa), (R = CH_2OH—$CHOH$—)
(IIb), (R = C_6H_5—)

The optimum conditions for these reactions (in aqueous solutions at $pH < 5$) are similar to those in which nitrosation at nitrogen and oxygen occurs[4,5], and we sought and have found a parallel between the oxidation of reductones, and the diazotization of aromatic amines[4], and the exchange of oxygen atoms between water and nitrous acid[5].

The compounds (I*a*) and (I*b*) have characteristic absorptions[6] which are more intense than those of the dehydro reductones (II*a*) and (II*b*) and of nitrous acid, and the reaction can be followed by spectrophotometric methods. The 3-hydroxyl group of (I*a*) and (I*b*) is acidic ($pK_a \sim 4$), while that on C–2 is enolic ($pK_a \sim 12$)[7]. Thus at $pH < 5$ the acid (I) and its mono-anion (III) co-exist.



(III)

The kinetics of the oxidation of ascorbic acid (and to a limited extent of phenyl hydroxytetronic acid (I*b*)) have been examined in detail at 0° C. in aqueous dioxan. A few runs were made in water, but in some conditions the reaction in water is too fast for convenient measurement. In all cases the concentration of the reductone ($0{\cdot}1$–$0{\cdot}2 \times 10^{-3}$ *M*) was much less than that of the nitrous acid ($0{\cdot}8$–$8{\cdot}0 \times 10^{-3}$ *M*).

With sodium nitrite in excess over nitrous acid (pH 3–4 in water) we observe the rate-law for reactions in both water and aqueous dioxan:

$$v = k_2\,[HNO_2]^2$$

The kinetic form of an individual run is of zeroth-order in reductone and the rate is almost independent of the nature and concentration of the reductone:

(I*a*), $10^2\,k_2 = 1{\cdot}7$ (mol.$^{-1}$ l.sec.$^{-1}$)
(I*b*), $10^2\,k_2 = 1{\cdot}5$ (mol.$^{-1}$ l.sec.$^{-1}$)
(0° C.; dioxan : water :: 40 : 60 v/v)

Following the interpretation given to such a kinetic form in the diazotization of the aniline[4], we conclude that the step *determining the rate* of the reaction is the formation of dinitrogen trioxide.

Reaction path A:

$$2HNO_2 \rightleftharpoons H_2NO_2^+ + NO_2^- \quad (1)$$

$$H_2NO_2^+ + NO_2^- \rightleftharpoons N_2O_3 + H_2O \quad (2)$$

$$N_2O_3 + \text{reductone} \rightarrow \text{intermediate (IV)} \quad (3)$$

$$(IV) \xrightarrow[\text{(fast)}]{\text{nitrous acid}} (II) \quad (4)$$

In this acidity region most of the reductone is present as a mono-anion (III): we assume that the dinitrogen trioxide reacts rapidly with this mono-anion (step 3), to give an intermediate (IV), which then reacts rapidly with a further nitrous acid species to give the final products. (The probable nature of the intermediate is discussed below.) This kinetic scheme requires that the rate of reaction of the reductone in water should be close to that of the zeroth-order diazotization of aniline[4], and the oxygen exchange of nitrous acid via dinitrogen trioxide[5]; this is so within the limits of experimental uncertainty.

$k_2 = 0{\cdot}49$ (mol.$^{-1}$ l.sec.$^{-1}$) (oxidation of (I*a*))
$= 0{\cdot}85$ (mol.$^{-1}$ l.sec.$^{-1}$) (diazotization)[4]
$= 0{\cdot}53$ (mol.$^{-1}$ l.sec.$^{-1}$) (oxygen exchange)[5]
(0° C.; water)

At the other extreme of our acidity-range (with $[HClO_4] \gg [HNO_2]$) we observe a different kinetic form:

$$v = k_3^{OH}\,[H^+]\,[HNO_2]\,[\text{ascorbic acid}];$$
$$k_3^{OH} = 7{\cdot}5 \text{ (mol.}^{-2}\text{ l.}^2\text{ sec.}^{-1})$$
(0° C.; dioxan : water :: 40 : 60 v/v)

(So far, this region has been explored only for the oxidation of ascorbic acid in aqueous dioxan.) With an excess of perchloric acid (about $0{\cdot}1$–$1{\cdot}0$ *M*) the concentration of ascorbate ion is very low, and we consider the mechanism to be a slow reaction between the nitrous acidium ion (V) and ascorbic acid.

Reaction path B:

$$H^+ + HNO_2 \rightarrow H_2NO_2^+ \text{ (V)}$$
$$\text{(V)} + \text{ascorbic acid} \rightarrow \text{intermediate (IV)} + H^+$$

followed by a rapid reaction of the intermediate (IV). This process has its analogy with N- and O-nitrosations, for example, the diazotization of aromatic amines in acid[4], and the acid catalysed hydrolysis of alkyl nitrites[8].

In the acidity-range between those which show mechanisms *A* and *B* there is a region in which the kinetics are of mixed order. Part of the reaction proceeds by mechanism *B*, but there is also a rate term which is second order in nitrous acid and first order in stoichiometric reductone. A similar kinetic form has been observed for reactions between nitrous acid and both aliphatic and aromatic amines[4,9]. It arises because in this region dinitrogen trioxide is in facile equilibrium with nitrous acid (steps 1 and 2 of mechanism *A*), but the proportion of the highly reactive reductonate ion is now very low ($< 0{\cdot}1$ per cent), and so the reaction with dinitrogen trioxide (step 3 of path *A*) is slow. A detailed examination of the effect of changes in acidity and nitrous acid concentration upon the reaction-rate between nitrous and ascorbic acids shows that in the region $[H^+] = 10^{-4}$–10^{-3} *M*, dinitrogen trioxide reacts with both ascorbic acid and ascorbate ion, and it is possible to estimate the relative reactivities towards dinitrogen trioxide:

$$v = [HNO_2]^2\,\{k_3^0\,[\text{ascorbic acid}] + k_3^-\,[\text{ascorbate}]\}$$

$k_3^0 = 73$ (mol.$^{-2}$ l.^{2} sec.$^{-1}$); $k_3^- = 1{\cdot}15 \times 10^5$ (mol.$^{-2}$ l.^{2} sec.$^{-1}$) (0° C., dioxan : water 40 : 60 v/v).

Thus we have reactions of the powerfully electrophilic nitrous acidium ion with the undissociated reductone, and of the weakly electrophilic dinitrogen trioxide with both the undissociated reductone and its more reactive anion.

Chloride and bromide ions catalyse the oxidation of ascorbic acid by nitrous acid by forming nitrosyl halides, which are themselves powerful nitrosating agents[4].

$$H_2NO_2^+ + X^- \rightleftharpoons NOX + H_2O$$

$$NOX + \begin{Bmatrix}\text{ascorbic acid}\\ \text{ascorbate ion}\end{Bmatrix} \rightarrow \text{intermediate (IV)} + \begin{Bmatrix}HX\\ X^-\end{Bmatrix}$$

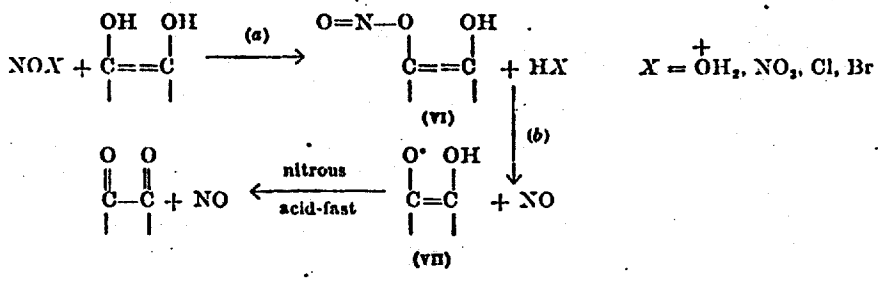
$$(X = Cl, Br)$$

From our kinetic results we can calculate specific rate-constants for the processes, and estimate the relative reactivities of various species towards dinitrogen trioxide and the nitrous acidium ion. In making these calculations we have ignored effects due to activity changes; these should not be large because the ionic strengths of the solutions are low, and approximately constant. Some of the numerical values are uncertain, because they are derived from small differences between large quantities.

We can formulate the reaction between a nitrosating agent and a reductone in various ways. Perhaps the simplest is a preliminary electrophilic attack upon the reductone (*a*), with formation of a nitrite (VI), which then decomposes (*b*).

It is, of course, possible that steps (*a*) and (*b*) may be synchronous, and the process would then be considered as a transfer of a single electron to the nitrosating agent. In either event we conclude that the first step of reaction between a nitrosating agent and a reductone, forming the semiquinone (VII) (which may be identical with the above-mentioned 'intermediate' (IV)), is followed by a rapid reaction between (VII) and nitrous acid, or some species in equilibrium with it.

It seems to us that there is, therefore, a very close similarity between this oxidation – reduction reaction and the well-characterized heterolytic nitrosations by electrophilic reagents.



This work forms part of a research programme on reductones, sponsored by the Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung, to which we express our thanks. A full account of the present work will be given in *Helvetica Chimica Acta*.

[1] Karrer, P., and Bendas, H., *Helv. Chim. Acta*, 17, 743 (1934).
[2] Dahn, H., Fischer, R., and Loewe, Lotte, *Helv. Chim. Acta*, 39, 1774 (1956).
[3] Dahn, H., and Lawendel, J. S., *Helv. Chim. Acta*, 37, 1318 (1954).
[4] Hughes, E. D., Ingold, C. K., and Ridd, J. H., *J. Chem. Soc.*, 58 (1958), and subsequent papers.
[5] Bunton, C. A., Llewellyn, D. R., and Stedman, G., *Nature*, 175, 83 (1955).
[6] Lawendel, J. S., *Nature*, 180, 434 (1957). Dahn, H., and Hauth, H., *Helv. Chim. Acta*, 40, 2249 (1957).
[7] Kunzler, W., and Daniels, T., *J. Amer. Chem. Soc.*, 57, 1929 (1935).
[8] Allen, A. D., *J. Chem. Soc.*, 1963 (1954).
[9] Taylor, T. W. J., *J. Chem. Soc.*, 1099, 1397 (1928).

A TWO-DIMENSIONAL PAPER CHROMATOGRAPHIC METHOD COMBINING ION-EXCHANGE AND PARTITION TECHNIQUES

By C. S. KNIGHT

Research Laboratories, W. and R. Balston, Ltd., Springfield Mill, Maidstone, Kent

ALTHOUGH the two-dimensional paper chromatographic method has been successfully applied to separations involving a wide range of substances, improvements and modifications are continually being proposed to make this technique even more discriminating.

Separations can often be improved by increasing the complexity of one or more of the conditions under which a chromatogram is developed, as, for example, in the use of three- or four-component solvent mixtures. On the other hand, the use of simple conditions to increase the reproducibility is often insufficient to effect an adequate separation. Thus, some approach between the two extremes has to be adopted. The present method, however, offers a means of increasing reproducibility in addition to separability.

This new approach to paper chromatography comes as a result of the development of a range of Whatman ion-exchange celluloses in these Laboratories. These new materials have been prepared by introducing acidic or basic groups into the cellulose molecule such that, under favourable conditions, ion-exchange involving these groups will be the dominating factor in a chromatographic separation[1]. Where, however, it is possible to suppress the activity of these groups such that ion-exchange is no longer the dominating factor, any chromatographic separations under such conditions will be largely as a result of a partition-type mechanism similar to that on unmodified cellulose. On the one material, in paper form, therefore, it is possible to combine ion-exchange and partition techniques in the form of a two-dimensional chromatogram, and this very simply is the basis of the method described below.

The ion-exchange materials investigated contained medium or weak strength functional groups such that where necessary their ion-exchange properties can be suppressed to varying extents by addition of acid or alkali to a developing organic solvent. Details of some separations investigated are as follows.

(1) Phosphorylated cellulose, containing dihydrogen phosphate groups, was used in the form of its mono-ammonium salt. A mixture of copper, manganese, cobalt, cadmium, iron, bismuth, zinc and mercury was applied as a spot near one corner of a

THE ANALYST

PROCEEDINGS OF THE SOCIETY FOR ANALYTICAL CHEMISTRY

DEATHS

We record with regret the deaths of

Edgar Percy Campbell
Albert Edward Fletcher
Harold Wright Hodgson
Reginald Arthur McNicol.

The Toxicology of Nitrates and Nitrites with Particular Reference to the Potability of Water Supplies

A Review*

By E. H. W. J. BURDEN†

(Firthorpe, South Ascot, Berks.)

Introduction

There have been many reports in recent years that high concentrations of nitrates in drinking supplies have caused poisoning and death among infants and also among animals. A review has been made of the evidence available in order to suggest suitable limits for this constituent in drinking water supplies.

The toxicology of nitrates and nitrites

Mode of action—

After ingestion, nitrates are converted to nitrites by bacterial reduction. According to Cornblath and Hartmann,[1] this reduction occurs in the lower intestine of the adult. However, in very young babies, which have a physiological gastric achlorhydria, reduction occurs in the stomach and duodenum from which the nitrites are more readily absorbed into the blood stream. After absorption, nitrites convert oxyhaemoglobin into methaemoglobin and thus interfere with the oxygen transport in the blood. This effect may be cumulative, since the methaemoglobin is reduced only at a slow rate.

* Reprints of this paper will be available shortly. For details, please see p. 492.
† *Formerly* The Government Analyst, Ministry of Health, Khartoum, Sudan.

SYMPTOMS OF POISONING—

Luff[2] describes the symptoms of acute potassium nitrate poisoning. They occur in the order: (*a*) pain in the epigastrium; (*b*) purging, often with blood; (*c*) convulsions of the face muscles; (*d*) weak irregular pulse; (*e*) difficult respiration; (*f*) cold extremities followed by collapse. Cyanosis is usually observed and the blood becomes chocolate coloured.

ANTIDOTE—

Intravenous injection of methylene blue results in a rapid reduction of methaemoglobin to haemoglobin, and the patient makes a dramatic recovery.[3]

LETHAL DOSE: HUMAN—

Nitrates—Smith and Simpson[4] consider the normal fatal dose of potassium nitrate to be 15 to 30 g for an adult, although they quote a report by Peterson, Haines and Webster[5] of the death of an adult after taking 8 g. Luff[2] records the death of an adult after taking 3·5 g of potassium nitrate, although Witthaus[6] reports a recovery after taking 125 g. Comly[7] quotes the observation of Eusterman and Keith[8] that certain adults are extremely allergic to nitrates. These authors record that one adult suffered from extreme cyanosis after taking 7 g of ammonium nitrate daily for 8 successive days. Wood[3] also states that nitrates are a cumulative poison. On the other hand, Amberg[9] reports that a 13-year-old boy (110 lb) with glomerulonephritis took 6 g of potassium nitrate daily for a year without injury. Windmueller[10] states that an adult who took 4 teaspoonsful daily of an equal mixture of sulphur and potassium nitrate for 26 days died 17 days later.

Nitrites—Smith and Simpson[4] consider that the normal fatal dose of sodium nitrite is about 10 g. Manicatide[11] records the death of a 3-month-old child after taking 0·35 g of sodium nitrite, but the recovery of a 2-month-old child after taking 0·4 g. Tankard[12] reports the death of an adult who probably took about 7 g of sodium nitrite. Scholes[13] records the accidental death of three adults, two of them within an hour, after eating a meal seasoned with sodium nitrite instead of sodium chloride. Several other deaths from nitrite poisoning have been recorded.[14,15,16,17,18] However, Kobert, quoted by Autenrieth,[19] mentions the case of a patient who took "several doses" of 0·5 g of sodium nitrite at hourly intervals "without dangerous symptoms," although the symptoms included nausea, diarrhoea, cyanosis and increased diuresis.

Naidu and Venkatrao[20] consider 2·0 g of sodium nitrite to be a fatal dose for a 10-stone man after examining results obtained on dogs. This is equivalent to 32 mg per kg.

FATAL DOSE: ANIMALS—

Crawford[21] reports a case of sodium nitrate poisoning in cattle; he considers that ½ lb of sodium nitrate is a fatal dose. For an animal weighing 500 kg this is equivalent to 450 mg per kg. Bradley, Eppson and Beath[22,23] have shown that "oat hay poisoning" in cattle is caused by abnormally high concentrations of nitrate in the affected hay. They have also found that 1 g of potassium nitrate per kg is a lethal dose for cattle. Death is caused by methaemoglobinaemia, 70 to 80 per cent. of the haemoglobin being converted to methaemoglobin in a few hours. Sheep are similarly affected. Sapiro, Hoflund, Clark and Quin[24] have demonstrated that nitrate is reduced to nitrite by the rumen contents of cattle.

Lewis[25] has shown that, in the rumen of sheep, nitrate is reduced successively to nitrite and ammonia. With large doses of nitrate, the conversion of nitrite to ammonia is limited and the nitrite concentration in the rumen increases rapidly. After giving large doses of nitrate, the methaemoglobin in the blood is found to reach a maximum about 7 hours later. Lewis finds that the doses of nitrate and nitrite required to give 60 per cent. conversion of haemoglobin to methaemoglobin are—

Sodium nitrate (added to rumen)	420 mg per kg
Sodium nitrite (added to rumen)	170 mg per kg
Sodium nitrite (intravenously)	34 mg per kg

Oltmann and Crandall[26] find that the lethal dose of sodium nitrite for rabbits is 80 to 90 mg per kg. Winks, Sutherland and Salisbury[27] report that the lethal dose of sodium nitrite for pigs is 90 mg per kg, and that doses of 50 to 80 mg per kg give toxic symptoms.

The lethal doses of sodium nitrite, calculated as nitrogen, quoted above for adults and various animals can be summarised as shown below—

(1)	For 10-stone adult	33 mg per kg[4]
(2)	For 10-stone adult	22 mg per kg[12]
(3)	Rabbits	16 to 18 mg per kg[26]
(4)	Pigs	18 mg per kg[27]
(5)	Sheep	34 mg per kg[25]
(6)	Dogs	6·4 mg per kg[20]

A summary of the reported lethal doses of nitrate, calculated as nitrogen, is shown below—

(1)	For 10-stone adult	34 to 68 mg per kg[4]
(2)	For 10-stone adult	18 mg per kg[5]
(3)	Cattle	75 mg per kg[21]
(4)	Cattle	140 mg per kg[23]
(5)	Sheep	70 mg per kg[25]

NITRITES AND NITRATES IN WATER SUPPLIES—

In most natural waters, nitrate is the final product of the aerobic decomposition of nitrogenous organic matter. Its presence in a water supply is usually interpreted as an indication of contamination by organic matter, although this may have happened in the remote past and may not necessarily be serious. Nitrite indicates less complete oxidation and may show more recent and possibly dangerous contamination. Traces of nitrate are present in most natural waters, and it is only rarely that the concentration rises to a dangerous level. The recorded cases of poisoning caused by high concentrations in water supplies come under two headings—

(*a*) infantile cyanosis and
(*b*) animal poisoning.

(*a*) INFANTILE CYANOSIS—

Comly[7] cites from personal experience a number of cases of infantile cyanosis where symptoms were observed after ingestion of feeds made from waters containing 64 to 140 p.p.m. of nitrate-nitrogen. He quotes a number of other cases including one fatality.

Taylor[28] records a large number of cases of infantile cyanosis caused by nitrates in feed water.[29,30,31,32,33,34] Betke and Kleihauer[35] also refer to a case of infantile cyanosis occurring under similar circumstances. The reasons that make artificially fed infants particularly susceptible appear to be—

(*i*) the normal gastric achlorhydria of infants, already referred to,[1] and
(*ii*) the high relative fluid intake of an infant compared with that of an adult.[3]

In England, a baby under 15 lb requires about 2½ fluid oz. of water per pound body weight per diem.[3] In the tropics, this figure is much higher and may rise to about 10 fluid oz. per pound body weight per diem.[36] In comparison, a normal adult in England requires about 1·8 pints of liquid a day.[37] For a 10-stone man, this is equivalent to ¼ fluid oz. per pound body weight per diem. In the tropics, the requirement would be about 1⅓ fluid oz. per pound body weight per diem.

Waring[38] suggests that waters used for preparing infant feeds should not contain more than 20 p.p.m. of nitrate-nitrogen.

Ewing and Mayon-White[33] concur with the suggestion. The Committee of the Division of Medical Science of the United States National Research Council[39] regard any well water containing more than 10 p.p.m. of nitrate-nitrogen as unfit for consumption by infants. Wood[3] regards supplies containing less than 20 p.p.m. of nitrate-nitrogen as satisfactory for infant feeding, those containing 20 to 39 p.p.m. as undesirable, those containing 40 to 70 p.p.m. as unsafe and those containing more than 80 p.p.m. as dangerous. Any apparent discrepancy between these authors' findings may be explained by possible differences in climate.

(*b*) ANIMAL POISONING—

Cattle—Fincher[40] reports the death of 4 cattle after drinking from a supply containing 1000 p.p.m. of nitrate-nitrogen. Successive Sudan Government Analysts have recorded

deaths of cattle and camels after drinking from water supplies containing a high nitrate concentration.[41,42,43,44,45,46] In the majority of cases, the nitrate-nitrogen concentration was found to lie in the range 70 to 150 p.p.m., although three supplies containing as much as 870 p.p.m. have been recorded.[45,46] In a recent case, a supply found to contain 320 p.p.m. was fatal to cattle in about 1½ hours after watering.[45] Unfortunately, as far as the records show, the bodies of poisoned animals have not been examined by veterinary inspectors because the supplies have come from wells in remote areas, and any evidence has been lost before a post-mortem examination could be made. On the basis of available evidence I introduced a tentative limit of 50 p.p.m. of nitrate-nitrogen, which has been applied in recent years in Khartoum when making recommendations on the suitability of a particular supply. However, Ibrahim has pointed out[45] that the nitrate concentration of a water supply may fluctuate within a wide range, and he re-examines regularly any supply with a nitrate-nitrogen concentration in the range 30 to 50 p.p.m. Willimott[47] has also had experience of cattle poisoning in Cyprus caused by well waters containing high concentrations of nitrate.

Pigs—Winks, Sutherland and Salisbury[27] record heavy mortality among pigs fed on a soup made by cooking beef and offal in a well water containing 290 to 495 p.p.m. of nitrate-nitrogen and less than 1·5 p.p.m. of nitrite-nitrogen. The soup itself contained 80 to 285 p.p.m. of nitrite-nitrogen.

Fish—Sanborn[48] states that water containing 2000 p.p.m. of sodium nitrate (330 p.p.m. of nitrate-nitrogen) is fatal to fish, whereas a concentration of 1000 p.p.m. of sodium nitrate (165 p.p.m. of nitrate-nitrogen) is without apparent effect.

Discussion and conclusions

In deducing a suitable limit for nitrates in water to be consumed by infants, adults and animals, the following assumptions have been made—

(*i*) that the nitrate is completely reduced to, and absorbed as, nitrite;
(*ii*) that the lethal dose of nitrite-nitrogen for men and cattle is about 20 mg per kg;
(*iii*) that one fifth of the lethal dose, *i.e.*, 4 mg per kg, is the maximum daily amount of nitrite-nitrogen that can be tolerated without giving rise to toxic symptoms.

When computing limits for cattle in tropical climates it should be remembered that in many places cattle are watered only once or twice a day, and that, as a consequence, large volumes are taken at a time.

On the above basis the limits shown in Table I for the permissible concentrations of nitrate-nitrogen in drinking water for infants, adults and cattle in England and in the tropics have been calculated.

Table I

Permissible limits for nitrate-nitrogen in drinking water

Consumer	Assumed weight, kg	Region	Maximum permissible dose of nitrate-nitrogen, mg	Approximate daily water intake, litres	Permissible limit of nitrate-nitrogen in water, p.p.m.
Infant ..	3	England	12	0·5	24
Infant ..	3	Tropics	12	2	6
Adult ..	60	England	240	1	240
Adult ..	60	Tropics	240	5·3	45
Cow ..	500	Tropics	2000	45	45

These figures can only be approximate, but they give general support to the limits already suggested. They emphasise the variations caused by differences in climate. They also show how very susceptible an infant artificially reared in the tropics can be to relatively small amounts of nitrates in drinking water. In areas where the water supplies have a high nitrate concentration special arrangements must be made for supplying infants with low-nitrate water. This is already being done in Norfolk and East Suffolk.[3]

It may also be concluded that, since the lethal doses of nitrate-nitrogen and nitrite-nitrogen for adults are not very different, most of the nitrate consumed is reduced to nitrite and absorbed into the blood stream. Thus it would appear that the significant factor that makes infants susceptible to nitrates is the high relative fluid intake and not the gastric achlorhydria.

References

1. Cornblath, M., and Hartmann, A. F., *J. Pediat.*, 1948, **33**, 421.
2. Luff, A. P., "Text Book of Forensic Medicine and Toxicology," Longmans, Green and Co., London, 1895, Volume I, p. 147.
3. Wood, E. C., personal communication.
4. Smith, S., and Simpson, K., "Taylor's Principles and Practice of Medical Jurisprudence," Eleventh Edition, J. & A. Churchill, London, 1957, Volume II, p. 292.
5. Peterson, F., Haines, W. S., and Webster, R. W., "Legal Medicine and Toxicology," Second Edition, Saunders, Philadelphia.
6. Witthaus, R. A., "Manual of Toxicology," Second Edition, Ballière, Tindall and Cox, London, 1911, p. 305.
7. Comly, H. H., *J. Amer. Med. Ass.*, 1945, **129**, 112.
8. Eusterman, G. B., and Keith, N. M., *M. Clin. North America*, 1929, **12**, 1489.
9. Amberg, S., *Proc. Mayo Clinic*, 1935, 739.
10. Windmueller, E., *J. Amer. Med. Ass.*, 1921, **77**, 858; *Chem. Abstr.*, 1922, **16**, 756.
11. Manicatide, —., *Bull. Soc. Ped., Paris*, 1932, 379; *Chem. Abstr.*, 1933, **27**, 5824.
12. Tankard, A. R., *Analyst*, 1937, **62**, 734.
13. Scholes, A., *Ibid.*, 1936, **61**, 685.
14. Newcomb, C., *Ibid.*, 1936, **61**, 614.
15. ——, *Ibid.*, 1937, **62**, 740.
16. Manley, C. H., *Ibid.*, 1945, **70**, 50.
17. Padberg, L. R., and Martin, T., *J. Amer. Med. Ass.*, 1939, **113**, 1733; *Chem. Abstr.*, 1940, **34**, 7004.
18. Becker, E., *Dtsch. Med. Rdsch.*, 1949, **3**, 900; *Chem. Abstr.*, 1952, **46**, 11442.
19. Autenrieth, W., "Laboratory Manual for the Detection of Poisons and Powerful Drugs," Sixth Edition, J. & A. Churchill, London, 1928, p. 321.
20. Naidu, S. R., and Venkatrao, P., *Calcutta Med. J.*, 1945, **42**, 79; *Chem. Abstr.*, 1947, **41**, 2487.
21. Crawford, M., *Trop. Agric. (Ceylon)*, 1941, **96**, 359; *Chem. Abstr.*, 1942, **36**, 1097.
22. Bradley, W. B., Eppson, H. F., and Beath, O. A., *J. Amer. Vet. Ass.*, 1939, **94**, 541.
23. ——, ——, ——, *Ibid.*, 1940, **96**, 41.
24. Sapiro, M. L., Hoflund, S., Clark, R., and Quin, J. I., *Onderstepoort J. Vet. Sci.*, 1949, **22**, 357.
25. Lewis, D., *Biochem. J.*, 1951, **48**, 175.
26. Oltmann, T. V., and Crandall, L. A., *J. Pharmacol.*, 1931, **41**, 121; *Chem. Abstr.*, 1931, **25**, 3089.
27. Winks, W. R., Sutherland, A. K., and Salisbury, R. M., *Queensland J. Agric. Sci.*, 1950, **7**, 1; *Chem. Abstr.*, 1951, **45**, 3507.
28. Taylor, E. W., "The Examination of Waters and Water Supplies (Thresh, Beale and Suckling)," Seventh Edition, J. & A. Churchill, London, 1958, p. 548.
29. Walton, G., *Amer. J. Public Health*, 1951, **41**, 986.
30. Ferrant, M., *J. Pediat.*, 1946, **29**, 585.
31. Homolka, J., *abstracted in Bull. Hyg.*, 1949, **24**, 676.
32. Campbell, W. A. B., *Brit. Med. J.*, 1952, **ii**, 371.
33. Ewins, M. C., and Mayon-White, R. M., *Lancet*, 1951, **260**, 931.
34. Fawns, H. T., and Aldridge, A. G. V., *Brit. Med. J.*, 1954, **ii**, 575.
35. Betke, K., and Kleihauer, E., *Dtsch. Med. Woch.*, 1957, **82**, 1127; *abstracted in Bull. Hyg.*, 1957, **32**, 979.
36. Burden, E. H. W. J., personal observation.
37. Mottram, V. H., and Graham, G., "Hutchison's Food and the Principles of Dietetics," Eleventh Editon, Edward Arnold (Publishers) Ltd., London, 1956, p. 399.
38. Waring, F. H., *J. Amer. Wat. Wks Ass.*, 1949, **41**, 147.
39. Maxcy, K. F., *Wat. Pollution Abstracts*, 1951, **24**, 97.
40. Fincher, M. G., *Cornell Veterinarian*, 1936, **26**, 271; *Chem. Abstr.*, 1936, **30**, 8389.
41. Henry, A. J., Annual Report of the Government Analyst, Khartoum, Sudan, 1945, 5.
42. Grindley, D. N., *Ibid.*, 1951/52, 6.
43. ——, *Ibid.*, 1952/53, 6.
44. Burden, E. H. W. J., *Ibid.*, 1956/57, 9.
45. Ibrahim, A. H., *Ibid.*, 1957/58, 8.
46. ——, *Ibid.*, 1957/58, 8.
47. Willimott, S. G., personal communication.
48. Sanborn, N. H., *Canner*, 1945, **101**, 5 and 13; *Chem. Abstr.*, 1945, **39**, 4697.

Received *January 16th*, 1961

J. Fd Technol. (1971) 6, 433–438.

274

Some studies on the photolytic decomposition stage in the estimation of N-nitrosamines

D. THORBURN BURNS AND GERALDINE V. ALLISTON*

Summary

A study of the photolytic decomposition of certain N-nitrosamines has shown that these compounds degrade at different rates in the various solution media employed. The decomposition follows first order kinetics under most conditions and reaction half lives have been measured. Linear correlations were observed between half lifetimes determined with different sources for each media. A suggestion that the criteria of half lives be used to optimize analytical conditions in the quantitative estimation of nitrosamines is discussed.

Introduction

In recent years the toxic and carcinogenic effects of N-nitrosamines have been reported (Magee & Barnes, 1956; Druckrey, Preussmann & Schmal, 1963). It has been established that these compounds may be produced as the result of chemical and bacterial action on secondary amines in the presence of nitrates (Sander, 1968), and hence may present a hazard with foods containing amines and nitrates, such as vegetables. It was also shown that the synthesis may be accomplished in acidic media without bacterial intervention. The risk of nitrosamine contamination has resulted in projects to investigate methods for their specific sensitive and quantitative determination in samples of interest.

Good separation of selected nitrosamines has been obtained by thin layer chromatography (Preussmann *et al.*, 1964; Serfontein & Hurter, 1966; Neurath, Pirman & Dunger, 1964) and by gas chromatography (Foreman, Palframan & Walker, 1970), but they were unable to include all of the isomeric N-nitrosamines which could theoretically be met. The most sensitive colorimetric reaction is that described by Daiber & Preussmann (1964), in which the NO group is cleaved by U.V. light and the nitrite formed identified by the Griess reagent. The reaction can be carried out on silica gel thin-layer plates and also

Authors' Address: Department of Chemistry, University of Technology, Loughborough, Leics.
* Present address, Laboratory of the Government Chemist, Cornwall House, Stamford Street, London, S.E.1.

in alkaline aqueous solution. Nitrosamines reduce polarographically at between −0·8 to 1·0 volt relative to a saturated calomel electrode, half wave potentials being pH dependent. Polarographic methods, though sensitive to less than 1 ppm (English, 1951; Smales & Wilson, 1948) are not specific since compounds such as unsaturated aldehydes and ketones which are likely to be present in foods also reduce in this region (Heath & Jarvis, 1955; Lyderson & Nagy, 1967).

An improved method has recently been described which involves the differential polarography of sample solutions before and after photolysis (Walters, Johnson & Ray, 1970). The nitrosamine concentration in the presence of light stable contaminants is estimated by difference.

Little is known about the factors which influence the rate of photolytic decomposition of nitrosamines and photolysis times ranging from minutes (Sander, 1968; Mohler & Mayrhofer, 1968) to several hours (Ballweg & Schmahl, 1967; Walters *et al.*, 1970; Chow, 1967; Sander, 1967) have been recommended. The present study was undertaken to obtain data on the conditions that affect the rates of photolysis of nitrosamines in order to be able to specify more precisely analytical procedures, prior to the final polarographic of spectrophotometric measurement. The effect of pH was examined because several reports suggest that the photolytic decomposition requires strongly acidic conditions (Chow, 1964, 1967; Burgess & Lavanish, 1964; Chow & Lee, 1967) in contrast with the work of Sander (1967) and Daiber & Preussmann (1964) who studied the production of nitrite under alkaline conditions. Since pH and also buffer composition affect the polarographic behaviour of nitrosamines photolyses were carried out in the presence of various supporting electrolytes. The separation and clean-up of nitrosamines from foods, tobacco and certain medicants, prior to their determination, may require solvent extraction (Möhler & Mayrhofer, 1968) so that the variation in the photolysis rates in certain common solvents was also examined.

Experimental photolysis procedures

(1) Irradiation was carried out in stoppered 10 mm silica spectrophotometric cells under a 15 watt Ultraviolet lamp (B.T.L.) designed for chromatographic use, fitted with a Chance OX7 filter to absorb visible radiation and transmit the 254 nm line. The cell, its optical faces at 90° to the radiation beam, was placed at the bottom of the lamp housing 12 cm from the filter. Subsequent experiments at 2, 4·5 and 7 cm showed less than 10% variation in effect with alteration in position. The source is of comparable size and close to the cell so that the inverse square law does not apply. The solution spectra were recorded after various time intervals using a Unicam SP.800 spectrophotometer with the appropriate solvent in the reference cell.

(2) Further investigations were carried out by irradiating 100 ml nitrosamine solutions in a 1000 ml Hanovia Photochemical reactor. The sample solution was placed in the bottom of the outer flask, the removal of small sample aliquots does not significantly

alter the sample–source geometry and hence the irradiation conditions. The medium pressure mercury arc emits predominantly at 254, 265, 297, 313 and 366 nm. The region round the tube is purged with nitrogen and water circulated in a jacket between the gas purge space and the irradiation container. Aliquots of the solution were withdrawn at 10-min intervals until spectra failed to show any further change.

To avoid photolytic decomposition by daylight (Sander, 1967), solution flasks were protected with aluminium foil. The U.V. sources were carefully shielded to prevent irradiation of workers.

Solutions containing 10 ppm of each nitrosamine were used, these had suitable absorbances for following the photolysis reactions.

Results

The absorption maxima of the nitrosamines studied, dimethyl nitrosamine (DMN), dibutyl nitrosamine (DBN) and N nitrosopiperidine (NNP), were found to be in the range 225–242 nm, the exact position varying only slightly with solvent. On irradiation less absorbing products formed, having maxima in the range 204–228 nm, 10–20 nm below the maximum absorption of the parent materials. The band at about 230 nm is known to obey Beer's law (Möhler & Mayrhofer, 1968). Plots of log $(A_t - A_{t\infty})$, for each nitrosamine, at the peak wavelength, against photolysis time gave linear relationships indicating first order kinetics. Here A_t is the absorbance at time t. In some cases a short initial period of lower order kinetics was observed. The data (Table 1) for dimethylnitrosamine in 0·2 M hydrochloric acid showed the most curvature of any studied. The reaction half life times shown in Table 2 were evaluated from the linear, first order, portions of plots. The reaction half life times observed with the low and high intensity sources are given in Tables 2(a) and 2(b) respectively. Plots of half lifetimes determined with one source against those determined with the other source show reasonably linear correlations for each medium examined.

The possible effect of concentration on half lives was examined using 20, 5 and 2·5 ppm NNP at pH 4 in 5, 20 and 40 mm cells with the B.T.L. lamp. The half lives were 1·5, 1·2 and 1·35 hr respectively, i.e. constant within the normal coefficient of variation of results of 15%.

Discussion and conclusions

The rate of photolysis of the nitrosamines studied followed first order kinetics. The reaction halflives are characteristic of each nitrosamine but varied with the solution and irradiation conditions; values observed ranged from ½ to 7½ hr with the low intensity source and from ¼ to 1 hr with the photochemical reactor. Decomposition was faster at low pH and in methanol, ethanol and in dichloromethane except that dimethylnitrosamine appeared more stable in methanol. The results show that the effect of

Table 1. Decomposition of dimethylnitrosamine in a Hanovia photochemical reactor

Solution	0·2 *M* hydrochloric acid		pH 4 (universal buffer)		
Time (min.)	Absorbance	log $(A_t - A_{t\infty})$	Time (min.)	Absorbance	log $(A_t - A_{t\infty})$
0	1·01	$\bar{1}$·87	0	1·04	$\bar{1}$·91
10	0·92	0·81	10	0·94	0·85
20	0·77	0·70	20	0·82	0·77
30	0·63	0·56	30	0·75	0·72
40	0·52	0·40	40	0·69	0·66
50	0·43	0·20	50	0·64	0·61
60	0·36	0·04	60	0·58	0·54
100	0·27	—	100	0·43	0·30
110	0·27	—	240	0·23	—

Table 2. Reaction half lifetimes for the photolytic decomposition of N nitrosamines

Solvent	DMN	DBN	NNP
(a) B.T.L. lamp (times in hr)			
pH 4 (universal buffer)*	2·2	0·7	1·2
pH 9·2 (universal buffer)*	3·5	1·5	1·4
0·2 M HCl	4·9	2·7	2·1
0·1 M HCl†	5·5	3·1	2·0
0·5 M Na_2SO_4, 0·5 M $NaHSO_4$‡	7·3	3·6	3·4
Ethanol	1·3	1·5	1·3
Methanol	6·3	1·6	2·2
Hexane	3·7	2·8	3·4
Dichloromethane	2·3	1·3	1·5
(b) Hanovia Photochemical Reactor (times in min)			
0·2 M HCl	25	16	15
pH 4 (universal buffer)	49	17	23
pH 9·2 (universal buffer)	64	28	25

* Johnson & Lindsey (1939).
† Smales & Wilson (1948).
‡ Lyderson & Nagy (1967).

acidity are less significant than previously suggested, and that nitrosamines are not stable (Chow, 1964) nor are decomposition reactions hindered in organic solvents (Mohler & Mayrhofer, 1968). Comparison of life times determined in 0·2 M and 0·1 M hydrochloric acid (pH about 0·7 and 1·0 respectively) with results obtained in buffered solutions at pH 4 and 9·2, showing a depression of the effect of pH, indicates that salt effects may be important.

As stated previously, half lives were determined from linear plots of log $(A_t - A_{t\infty})$ against time. In some cases the first few points were not linear, due to an inner filter effect, shown as a tendency to zero order kinetics which results when significant fractions of incident radiation are absorbed by solute molecules. Chow (1967) previously observed zero order kinetics in the photolysis of concentrated solutions of nitrosamines. At the 10 ppm levels examined linearity was always achieved in less than one half life.

The linear correlations between half lifetimes in various media, determined using different sources, are useful in that they should allow estimation of reaction times with a particular source for a series of nitrosamines, from half lives measured by other workers. The relationship can be established, for ease of measurement, using the slowest photolysed member of the series. The variation in slope of the interrelation graphs between media is probably due to a lack of uniformity in spectral characteristics of the U.V. sources.

Walters *et al.* (1970) irradiate for fixed periods, the disappearance of a polarographic wave in 2 hr in acid conditions and the formation of nitrite under alkaline conditions in 15 min being used to detect nitrosamines. The use of fixed irradiation times to complete reactions is not satisfactory. The term 'complete reaction', particularly in slow reactions, is not in itself precise since concentrations decrease asymptotically to the time axis for all but zero order kinetics. Once the systems under consideration follow first order kinetics, use of half lives to specify periods of irradiation for a required percentage of reaction to occur, is suggested. A known decomposition of over 99%, a good approximation to complete reaction, would occur in seven half lives.

The extraction and isolation stages from various products such as fish (Lyderson & Nagy, 1967), animal tissue (Heath & Jarvis, 1955), meat and cheese (Mohler & Mayrhofer, 1968) have been considered by previous workers and also lipid effects noted (Walters *et al.*, 1970).

Acknowledgments

The authors are grateful to Mr J. K. Foreman and Mr J. F. Palframan of the Laboratory of the Government Chemist for bringing the problem to their attention, and assistance with materials and also to Dr A. N. Strachan for helpful discussion.

References

Ballweg, H. & Schmahl, J. (1967) *Naturwissenschaften*, **54**, 116.
Burgess, E.M. & Lavanish, J.M. (1964) *Tetrahedron Lett.*, 1221.
Chow, Y.L. (1964) *Tetrahedron Lett.* 2333.
Chow, Y.L. (1967) *Can. J. Chem.* **45**, 53.
Chow, Y.L. & Lee, A.C.H. (1967) *Can. J. Chem.* **45**, 311.
Daiber, D. & Preussmann R. (1964) *Z. analyt. Chem.* **206**, 344.
Druckrey, H., Preussmann, R. & Schmal, D. (1963) *Acta Un. int. Contr. Cancer* **19**, 510.
English, F.L. (1951) *Analyt. Chem.* **23**, 344.
Foreman, J.K., Palframan, J.F. & Walker, E.A. (1970) *Nature Lond.* **225**, 554.
Heath, D.F. & Jarvis, J.A.E. (1955) *Analyst* **80**, 613.
Johnson, W.C. & Lindsey, A.J. (1939) *Analyst* **64**, 490.
Lyderson, D.L. & Nagy, K. (1967) *Z. analyt. Chem.* **230**, 277.
Magee, P.N. & Barnes, J.M. (1956) *Br. J. Cancer* **10**, 114.
Möhler, K. & Mayrhofer, O.L. (1968) *Z. Lebensm-Untersuch* **15**, 313.
Neurath, G., Pirmann, B. & Dunger, M. (1964) *Chem. Ber.* **97**, 1631.
Preussmann, R., Neurath, G., Lorentizen, G.W., Daiber, D. & Hengy, H. (1964) *Z. analyt. Chem.* **202**, 198.
Sander, J. (1967) *Z. physiol. Chem.* **348**, 853.
Sander, J. (1968) *Z. physiol. Chem.* **349**, 429.
Serfontein, W.J. & Hurter, P. (1966) *Nature* **209**, 1239.
Smales, A.A. & Wilson, H.N. (1948) *J. Soc. Chem. Ind.* **67**, 210.
Walters, C.L., Johnson, E.M. & Ray, N. (1970) *Analyst* **95**, 485.

Reprinted from APPLIED MICROBIOLOGY
Vol. 3, No. 3, May 1955
Printed in U.S.A.

(320)

Factors Affecting the Bacteriostatic Action of Sodium Nitrite[1]

A. G. CASTELLANI[2] AND C. F. NIVEN, JR.

Division of Bacteriology, American Meat Institute Foundation, and the Department of Microbiology, University of Chicago, Chicago, Illinois

Received for publication November 29, 1954

Sodium nitrite is known to be responsible for the production of the desirable heat-stable pink color in cured meats. It has also been used as an inhibitor of bacterial spoilage of fish (Tarr, 1941a, b). However, in cured meats it is not known to have any practical preservative action relative to those organisms not inhibited by the high sodium chloride content. Many varieties of staphylococci and various lactic acid bacteria grow quite readily on cured meats, but it has been observed in this laboratory that staphylococci rarely, if ever, are found in large numbers in the interior of cured meat items. In view of this observation, it was decided to investigate the effect of various other factors on the inhibition of staphylococci by sodium nitrite.

MATERIALS AND METHODS

Analytical. Nitrite added to bacteriological media was determined colorimetrically using the Griess reagent (A. O. A. C., 1945). Deproteinization was found to be unnecessary, and the color density was determined in a Coleman spectrophotometer, at a wavelength of 522 mμ.

Sulfhydryl groups were determined by the method of Shinowara (1935, 1935–36) using the phospho-18-tungstic acid reagent. Cysteine was determined directly; the cystine in the same sample was reduced to cysteine and the concentration of cystine was then calculated by difference. Color density was determined spectrophotometrically at 750 mμ.

The pH was determined using a glass electrode Beckman Model H pH meter.

Bacteriological. For most of the experiments a complex medium was employed which contained 1.0 per cent tryptone, 1.0 per cent yeast extract, 0.5 per cent K_2HPO_4 and 1.0 per cent glucose. The medium was prepared double strength, sterilized by autoclaving at 121 C for 20 minutes, sterile solutions of the variable components added, and made up to single strength with sterile distilled water. In a number of experiments, as indicated, the glucose was omitted from the basal medium, sterilized separately, and added aseptically. The sodium nitrite was sterilized by Seitz filtration of a 10 per cent stock solution.

The cultures used for most of this work were strains 161 and 196 of *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*). These were typical coagulase-positive strains (Evans and Niven, 1950) and were isolated from outbreaks of food poisoning by Dr. G. M. Dack of the University of Chicago.

The inoculum consisted of one drop of a 10^{-2} dilution of a 24-hour broth culture.

For aerobic growth, 18-mm culture tubes containing 10 ml of medium were placed in a nearly horizontal position in the incubator to give a large surface area. For anaerobic growth the medium was covered with a vaspar seal. In all of the experiments reported here maximum incubation was for 48 hours at 30 C since the nitrite may disappear at a rapid rate, even in the uninoculated media under certain conditions.

RESULTS

Effect of pH. Tarr (1941a, b) reported that the pH of the medium greatly influences the bacteriostatic power of nitrite. Preliminary experiments, typical of that presented in table 1, confirmed Tarr's observations. In these experiments, *S. aureus*, strain 196, was cultured aerobically in the complex medium at varying pH levels.

Using a pKa value of 3.4 for nitrous acid the minimum bacteriostatic concentration of this hypothetical undissociated acid was calculated for the various pH levels employed. It will be noted that the concentration of nitrous acid for bacteriostasis remains relatively constant throughout the pH range tested, thus relating the bacteriostatic action of nitrite to the acid. In other words, as the pH of the medium is lowered by one unit the bacteriostatic effect of added nitrite is increased approximately 10-fold.

In these experiments in which the organism was cultured aerobically, the glucose was autoclaved in the double strength medium and the nitrite added aseptically. When sterile glucose was added aseptically to the medium after autoclaving there was no significant change in the results.

Anaerobic tolerance and the effect of manner of glucose sterilization. When similar studies were conducted anaerobically it was noted that sterilization of glucose

[1] Journal Paper No. 105, American Meat Institute Foundation.

[2] From Ph.D. thesis. Present address: American Institute of Baking, 400 East Ontario Street, Chicago, Illinois.

TABLE 1. *Effect of pH on the aerobic nitrite tolerance of Staphylococcus aureus, strain 196, when glucose is autoclaved in a complex medium*

pH*	Nitrite Concentration		Calculated Undissociated HNO_2†	
	Growth‡	No growth	Growth	No growth
	ppm	*ppm*	*ppm*	*ppm*
6.91	3,500	4,000	1.12	1.28
6.32	1,800	2,000	1.37	1.52
6.03	600	700	1.38	1.61
5.80	300	400	1.20	1.60
5.68	250	400	1.32	2.12
5.45	140	180	1.25	1.50
5.20	80	150	1.12	2.10
5.05	40	80	0.92	1.84

* The pH values were determined on duplicate tubes of medium at the time of inoculation.

† The amount of undissociated nitrous acid was calculated from the dissociation curve employing a pKa of 3.4.

‡ Growth was determined after 48 hours at 30 C.

TABLE 2. *Effect of the manner of adding glucose on the nitrite tolerance of Staphylococcus aureus, strain 196, at pH 6.55 in a complex medium*

Incubation Conditions	Nitrite Concentration			
	Glucose sterilized in the medium		Glucose added aseptically	
	Growth	No growth	Growth	No growth
	ppm	*ppm*	*ppm*	*ppm*
Aerobic	1,800	3,000	1,600	1,800
Anaerobic	20	40	800	1,000

in the medium greatly enhanced the bacteriostatic effect of nitrite. Calculations of the amount of undissociated nitrous acid again indicated that this was the substance associated with bacteriostasis anaerobically, as it was aerobically.

Results presented in table 2 demonstrate that when the inhibitory level of nitrite was determined at a constant pH, glucose autoclaved in the medium had no effect on the aerobic tolerance, but decreased the anaerobic tolerance about 30-fold. It should also be noted that when glucose was added aseptically the aerobic tolerance was about twice the anaerobic tolerance.

When sucrose was used rather than glucose, autoclaving in the medium had no effect on the anaerobic tolerance and all results were equivalent to those obtained when glucose was added aseptically. Also, when a fermentable carbohydrate was added, the anaerobic nitrite tolerance was of the same order of magnitude as that found when glucose was added aseptically.

Nitrite tolerance of other microorganisms. Three additional strains of coagulase-positive staphylococci were tested and gave results similar to those with strain 196. As indicated in table 3, a number of other bacterial species were tested for their tolerance to nitrite. Considerable variation was noted among these bacteria. In general, aerobic tolerance to nitrite was higher than anaerobic tolerance among these microorganisms, but for the majority of the strains tested the differences were not as marked as with *S. aureus*.

The *Lactobacillus*, *Pediococcus*, *Streptococcus lactis*, enterococcus, and gram negative species tested were relatively tolerant to nitrite. On the other hand, the *Streptococcus pyogenes* strain tested was extremely sensitive. Quite unexpectedly, *Streptococcus salivarius* and *Streptococcus mitis* were relatively sensitive under anaerobic conditions (glucose autoclaved in the medium) but were resistant under aerobic conditions. These two species, in contrast to *S. aureus*, are generally considered to be "indifferent" to oxygen with respect to their growth requirements and do not possess the heme-containing respiratory enzyme systems for obtaining energy aerobically.

Reversal of anaerobic nitrite inhibition. Screening of a number of amino acids and reducing substances revealed that sulfhydryl compounds (sodium thioglycollate, cysteine, or glutathione) would reverse the nitrite inhibition observed when glucose was autoclaved in the medium. Ascorbic acid and other reducing substances that do not contain a sulfhydryl group were ineffective.

Representative data obtained with glutathione for *S. aureus*, strain 196, are presented in table 4. In all experiments the sulfhydryl-containing substances were

TABLE 3. *Minimum inhibitory concentrations of nitrite for a variety of microorganisms at pH 6.6 to 6.7*

Species	Glucose Sterilized in Medium		Glucose Added Aseptically	
	Anaerobic	Aerobic	Anaerobic	Aerobic
	ppm	*ppm*	*ppm*	*ppm*
Streptococcus salivarius (13 strains)	80	4,000	3,000	6,000
Streptococcus mitis	40	4,000	800	2,000
Streptococcus lactis	6,000	10,000	4,000	6,000
Streptococcus liquefaciens (4 strains)	800	6,000	4,000	4,000
Streptococcus faecalis (16 strains)	4,000	6,000	4,000	5,000
Streptococcus pyogenes	2	20		
Lactobacillus casei, strain 7469	4,000	8,000		
Lactobacillus arabinosus, strain 17-5	8,000	25,000		
Pediococcus cerevisiae, strain 8081	8,000	25,000		
Bacillus megatherium	80	4,000		
Escherichia coli	2,000	4,000	4,000	4,000
Aerobacter aerogenes	2,000	4,000		
Proteus vulgaris	400	4,000		
Salmonella typhosa	800	2,000		
Salmonella aertrycke (*typhimurium*)	2,000	4,000		
Shigella flexneri (*paradysenteriae*)	100	2,000		

TABLE 4. *Effect of glutathione on the anaerobic nitrite tolerance of Staphylococcus aureus, strain 196, at pH 6.65 in a complex medium*

	Control Medium		Medium Plus Glutathione (1000 ppm)*	
	Nitrite added	Nitrite recovered†	Nitrite added	Nitrite recovered†
	ppm	*ppm*	*ppm*	*ppm*
Growth	50	27	2,000	1,770
No growth	100	68	4,000	3,450

* The glutathione was sterilized by Seitz filtration and added aseptically. Glucose was autoclaved in the medium.

† These figures represent the residual nitrite concentration in uninoculated tubes of medium after 48 hours incubation at 30 C.

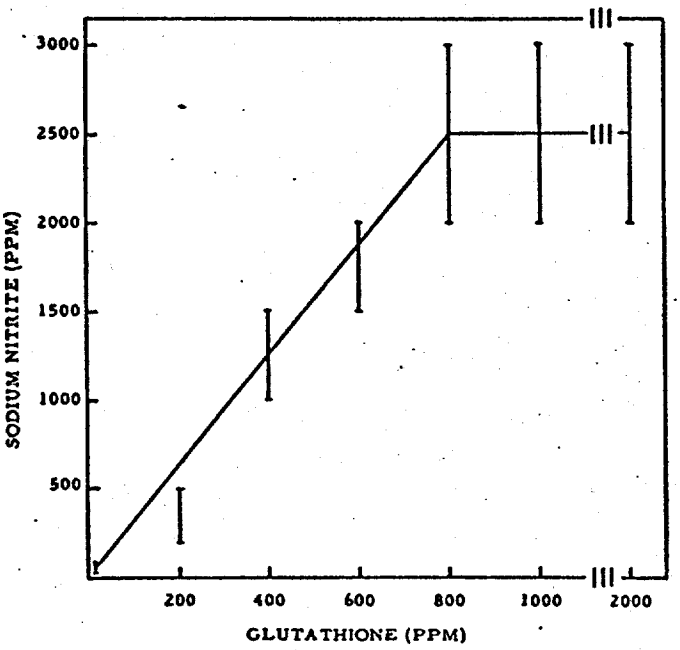


FIG. 1. The quantitative reversal of anaerobic nitrite inhibition by glutathione when glucose is autoclaved in a complex medium; pH 6.68. The vertical lines connect the tested maximum concentration tolerated, with the tested minimum inhibitory concentration for *Staphylococcus aureus*, strain 196.

added aseptically to the medium from a freshly prepared stock solution sterilized by filtration. Recovery values for nitrite indicated that glutathione did not destroy the added nitrite.

As demonstrated in figure 1, the reversal of anaerobic nitrite inhibition was directly proportional to the concentration of added glutathione up to a nitrite concentration of 2500 ppm. At levels of nitrite above this value there was no reversal.

Glutathione appeared to reverse the nitrite inhibition up to a level which approached the nitrite concentration tolerated under aerobic conditions. Glutathione did not affect the aerobic nitrite tolerance of *S. aureus*.

Similar experiments with *Streptococcus salivarius* and *Streptococcus mitis* revealed that the sulfhydryl compounds, especially cysteine, would also reverse the nitrite inhibition observed under anaerobic conditions. The results were not so dramatic, however. Cysteine at a concentration of 1000 ppm increased their anaerobic nitrite tolerance up to 10-fold. Higher concentrations of cysteine failed to increase the nitrite tolerance. These experiments indicate that other nutrilites may be important in overcoming the bacteriostatic effect of nitrite for these streptococci.

Effect of aging the complex medium. Double strength basal medium containing glucose was autoclaved and then aged for 7 days under the following conditions: 1) at room temperature in a slanting position, 2) in an anaerobic desiccator over pyrogallol and sodium carbonate, and 3) in a slanting position at 7 C. The volume was restored with sterile distilled water and nitrite tolerance under anaerobic conditions was determined in the usual manner using *S. aureus*, strain 161. The medium that was aged anaerobically and that aged in the refrigerator gave results comparable to freshly prepared medium. However, the medium that was aged aerobically at room temperature supported growth of the test organism in the presence of about 20 times as much nitrite as did the other media. This was in spite of the fact that the aged medium had a pH of 6.38 compared to a pH of 6.68 for the fresh medium. If the aged medium was steamed just prior to inoculation, the resulting nitrite tolerance was reduced to that found in freshly prepared medium.

Aeration in flasks on a mechanical shaker for 8 hours enhanced the anaerobic nitrite tolerance only 2-fold, indicating that simple saturation with air at room temperature would not explain the effect of aging 7 days at room temperature. Furthermore, the addition of hydrogen peroxide in varying concentrations up to .05 per cent did not enhance the tolerance to nitrite.

Studies using a synthetic medium. A synthetic medium was made up using 19 amino acids, adenine, guanine, uracil, xanthine, mineral salts, thiamine, nicotinic acid, K_2HPO_4, and glucose in amounts similar to those recommended by Gladstone (1937), and Porter and Pelczar (1941). For successful anaerobic growth it was necessary to add pyruvate aseptically at a concentration of 0.5 per cent (Fildes *et al.*, 1936). In this synthetic medium it was found (table 5) that autoclaving glucose with the other constituents decreased the apparent nitrite tolerance about 10-fold. Contrary to the findings in the complex medium, the addition of increased levels of cysteine did not reverse this added inhibition.

Studies with model systems. In an attempt to determine the reason for the lack of a cysteine effect in a synthetic medium, further experiments revealed that the addition of either 0.5 per cent pyruvate or fumarate to the complex medium also tended to nullify the cysteine effect. These results indicated that a chemical reaction takes place between cysteine and pyruvate or fumarate when these substances are mixed together.

TABLE 5. *Effect of cysteine concentration on the anaerobic nitrite tolerance of Staphylococcus aureus, strain 161, in a synthetic medium, pH 6.7*

Cysteine* Added	pH	Nitrite Concentration			
		Glucose sterilized in the medium		Glucose added aseptically	
		Growth	No growth	Growth	No growth
ppm		*ppm*	*ppm*	*ppm*	*ppm*
20	6.70	40	80		
25	6.60			400	800
100	6.60			400	800
400	6.65	40	80		
500	6.55			200	400
1000	6.55	20	40		

* The cysteine was sterilized by Seitz filtration and added aseptically to the sterile medium. The basal medium was devoid of cysteine or cystine and would not support anaerobic growth of the organism.

TABLE 6. *The apparent interaction of pyruvate and cysteine in a phosphate buffer at pH 6.7 under anaerobic conditions**

Time (Hours)	PPM Recovered as:			
	0.5 per cent pyruvate		No pyruvate	
	Cysteine	Cystine	Cysteine	Cystine
0	620	380	620	380
1	37	122	583	304
2	12	95	558	346

* The system contained 0.5 per cent K_2HPO_4 adjusted to pH 6.7 with HCl, 1000 ppm of cysteine, and pyruvate as indicated. Incubation was at 30 C.

Therefore, experiments were designed to demonstrate this chemical reaction in a model system.

Cysteine in a final concentration of 1000 ppm was added to a phosphate buffer, pH 6.7, containing 0.5 per cent pyruvate, and the mixture held at 30 C up to 2 hours under anaerobic conditions. The apparent cysteine-cystine content was determined in this mixture at the time intervals indicated in table 6. Under these conditions it was noted that pyruvate appears to destroy rapidly both cysteine and cystine in the system. The cysteine-cystine destruction was accompanied by a decrease in the pH of about 0.2 units. In the absence of pyruvate, cysteine was relatively stable in the phosphate buffer held under similar conditions.

Similar experiments indicated that fumarate also destroyed cysteine and cystine almost completely within 24 hours when held anaerobically in the phosphate buffer.

The stability of cysteine and nitrite was also determined in the phosphate buffer system without added pyruvate or fumarate. The results are presented in table 7. The results indicate that the nitrite was relatively stable, but the cysteine was partially destroyed in the presence of nitrite. No stoichiometric relationship between nitrite and cysteine could be demonstrated.

TABLE 7. *Stability of cysteine and nitrite in phosphate buffer at pH 6.7 under anaerobic conditions*

Additions	PPM Recovered After 24 Hours		
	Cysteine	Cystine	Nitrite
Cysteine, 1000 ppm*	484	283	—
Cysteine, 1000 ppm*, nitrite, 2000 ppm	186	46	1730
Nitrite, 2000 ppm	—	—	1790

* At zero time there were 620 ppm cysteine and 380 ppm cystine in the system.

Thus experiments in the synthetic medium were not successful in elucidating the exact quantitative relationships between nitrite and sulfhydryl substances as affecting the growth of *S. aureus*. Satisfactory anaerobic growth of the test organisms could not be achieved without the addition of pyruvate. This acid in turn destroyed the effectiveness of the sulfhydryl substances in overcoming the bacteriostatic properties of nitrite.

DISCUSSION

Several explanations have been proposed for the mechanism of nitrite bacteriostasis. The reaction of nitrous acid with alpha-amino groups is the basis for the well known Van Slyke reaction. The bacteriostatic properties of nitrite consequently have been attributed to interference with amino groups of the dehydrogenase systems of *Escherichia coli* (Quastel and Woolridge, 1927).

Philpot and Small (1938a, b) have indicated that nitrous acid can react with monophenols, such as tyrosine, and they attributed the inactivation of pepsin to this reaction. Also to be considered is the possibility that hydroxylamine may be produced from nitrite by a number of organisms (Lindsey and Rhines, 1932) and this substance in turn is responsible in some degree for nitrite bacteriostasis.

A very plausible explanation for the bacteriostatic action of nitrite lies in the ability of the decomposition product, nitric oxide, to react with heme pigments. The production of nitric oxide myoglobin is the basis for meat curing. Ingram (1939) reported that at pH 6 oxygen uptake by *Bacillus cereus* was strongly inhibited by nitrite, thus implying an interference with the cytochrome systems. On the other hand, Tarr (1941a) noted that among two *Achromobacter* strains and two micrococci (whose growth in broth was strongly retarded by nitrite) the aerobic respiration of only one *Achromobacter* strain was inhibited by nitrite at different pH levels. Consequently, he concluded that nitrite does not inhibit bacterial growth by sole virtue of its toxicity toward the aerobic respiratory catalysts.

Further evidence against this theory is given in the present study. Among the collection of bacteria studied, nitrite was strongly inhibitory toward some of the

streptococci even though these bacteria are devoid of the heme-containing respiratory catalysts. Also, nitrite was generally more bacteriostatic under anaerobic conditions where the cytochrome systems would not be important in the metabolism of the microorganisms.

The results presented in this study do not fit any simple theory as to the site of action of nitrite, especially for the staphylococci. Such a theory would have to explain 1) why nitrite is so effective anaerobically in a medium autoclaved with glucose, 2) why aerobic conditions, adding the glucose aseptically, or adding sulfhydryl substances decrease the effectiveness of nitrite, and 3) why aging the test medium containing autoclaved glucose decreases the anaerobic effectiveness of nitrite, and why steaming of this aged medium restores it equivalent to the freshly prepared medium.

Some of the data presented in this study might imply that nitrite inactivates certain bacterial enzyme systems that possess an active sulfhydryl group. Barron and Singer (1945) reported that among a large number of enzyme systems studied the pyruvate-metabolizing enzymes were the most sensitive to sulfhydryl inhibitors. Bernheim (1943), and Nord and Mull (1945) noted that nitrite inactivated the pyruvate-metabolizing enzymes of *Vibrio comma* and *Fusarium*, respectively.

It would seem that the reason for anaerobic nitrite bacteriostasis for *S. aureus* and some of the streptococci in a medium containing autoclaved glucose could best be explained on a nutritional basis. The instability of the sulfhydryl amino acids when autoclaved in a medium containing a reducing sugar is well known. Riesen *et al.* (1947) made the interesting observation that the apparent degree of cystine destruction that occurred in a medium during autoclaving depended upon the test microorganisms employed for assay. In the present study, experiments with *S. aureus*, strain 161, indicated that as much as 90 per cent of the added cysteine content was destroyed when autoclaved with glucose in a synthetic medium for 20 minutes at 121 C. It should be emphasized, however, that in the complex medium employed throughout these studies the medium without added nitrite, even containing glucose during heat sterilization, supported maximum growth for all test microorganisms.

It is therefore logical to postulate that under conditions giving maximum sensitivity to nitrite (glucose autoclaved in the medium), a chemical change occurs, perhaps a condensation or addition, which involves the sulfhydryl constituents and another substance in the medium. The other substance might be a decomposition product of glucose. It would have to be assumed that this product is utilizable by the microorganism anaerobically except in the presence of nitrite. Minimum concentrations of nitrite would interfere with the anaerobic utilization of this complex, either at a physiological level, or else by reacting chemically with it. However, the product would be available under aerobic conditions or else the microorganism would be able to obtain sulfur from other medium constituents in the presence of oxygen.

To account for the reported aging effects it would have to be assumed that the complex is formed only under the influence of heat. Then upon exposure to oxygen at room temperature it would slowly dissociate, again making available sulfhydryl substances for the microorganism even in the presence of nitrite.

Under conditions giving minimum nitrite sensitivity (glucose not autoclaved in the medium), a second vital mechanism, perhaps not involving sulfur nutrition, would be affected by the nitrite to inhibit growth. Also, it might be postulated that those microorganisms which are relatively tolerant to nitrite under all conditions are able to utilize other sources of sulfur in their nutrition, thus eliminating the limited availability of a sulfur source in the presence of small concentrations of nitrite.

Evidence is presented in this study to indicate that a complex is formed between pyruvate (or fumarate) and sulfhydryl substances when added together at room temperature. Evidently, when nitrite is also added, this complex becomes unavailable for some microorganisms, especially *S. aureus*. That addition or condensation products are formed by the interaction of thiols with aldehydes, pyruvic acid or sugars seems to be well documented in the literature (Schubert, 1935, 1936, 1949; Cavallini, 1951). Such reactions between the sulfhydryl group of coenzyme A and substrate are now generally believed to be the basis for the biological activity of this coenzyme.

These studies indicate the need for further research concerning the availability of reaction products between thiols and carbonyl compounds in the nutrition of various microorganisms. Whether such studies will be useful in explaining the apparent inability of *S. aureus* to grow rapidly under anaerobic conditions in cured meats in the presence of nitrite remains to be seen. Further studies along these lines are now being conducted.

Summary

Tolerance toward nitrite varies widely among the different groups of bacteria tested.

A number of factors have been found to drastically affect the bacteriostatic action of sodium nitrite on food-poisoning staphylococci and some related organisms. The pH of the environment influenced the level of nitrite causing inhibition in a manner that tends to confirm the hypothesis that undissociated nitrous acid is the active form.

When glucose was autoclaved in the test medium the anaerobic growth of *Staphylococcus aureus* (*Micro-*

coccus pyogenes var. *aureus*), *Streptococcus salivarius*, and *Streptococcus mitis* was inhibited by much lower levels of nitrite than were required if the glucose was added to the medium after autoclaving. The aerobic tolerance to nitrite by these organisms was not greatly affected by the manner of adding the glucose.

The addition of sulfhydryl compounds such as cysteine or glutathione to a medium that had been autoclaved with glucose restored the nitrite tolerance to the same level as when the glucose was sterilized separately and added aseptically. Aging the medium for 7 days at 25 C after autoclaving also restored the nitrite tolerance to the higher level, but steaming this aged medium completely reversed this aging effect.

An explanation of these findings is offered based upon the assumption that the site of maximum sensitivity in these staphylococci is a system involving an essential sulfhydryl compound.

Evidence is presented to indicate that addition or condensation products are formed between pyruvate (or fumarate) and sulfhydryl substances when added together at room temperature. Nitrite appears to influence the availability of these products in the nutrition of some microorganisms.

REFERENCES

A. O. A. C. 1945 *Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists*, 6th ed. George Banta Publishing Co., Menasha, Wisconsin.

Barron, E. S. G., and Singer, T. P. 1945 Studies on biological oxidations. XIX. Sulfhdryl enzymes in carbohydrate metabolism. J. Biol. Chem., **157**, 221–240.

Bernheim, F. 1943 The significance of the amino groups for the oxidation of various compounds by the *Cholera vibrio* (*V. comma*). Arch. Biochem., **2**, 125–133.

Cavallini, D. 1951 The coupled oxidation of pyruvate with glutathione and cysteine. Biochem. J., **49**, 1–5.

Evans, J. B., and Niven, C. F., Jr. 1950 A comparative study of known food-poisoning staphylococci and related varieties. J. Bacteriol., **59**, 545–550.

Fildes, P., Richardson, G. M., Knight, B. C. J. G., and Gladstone, G. P. 1936 A nutrient mixture suitable for the growth of *Staphylococcus aureus*. Brit. J. Exptl. Path., **17**, 481–484.

Gladstone, G. P. 1937 The nutrition of *Staphylococcus aureus*: Nitrogen requirements. Brit. J. Exptl. Path., **18**, 322–333.

Ingram, M. 1939 The endogenous respiration of *Bacillus cereus*. II. The effect of salts on the rate of absorption of oxygen. J. Bacteriol., **38**, 613–629.

Lindsey, G. A., and Rhines, C. M. 1932 The production of hydroxylamine by the reduction of nitrate and nitrite by various pure cultures of bacteria. J. Bacteriol., **24**, 489–492.

Nord, F. F., and Mull, R. P. 1945 Recent progress in the biochemistry of fusaria. Advances in Enzymol., **5**, 165–205.

Philpot, I. S. L., and Small, P. A. 1938a The action of nitrious acid on *p*-cresol and tyrosine. Biochem. J., **32**, 534–541.

Philpot, I. S. L., and Small, P. A. 1938b The action of nitrous acid on pepsin. Biochem. J., **32**, 542–551.

Porter, J. R., and Pelczar, M. J., Jr. 1941 The nutrition of *Staphylococcus aureus*. The influence of biotin, bios II_B and vitamin H on the growth of several strains. J. Bacteriol., **41**, 173–192.

Quastel, J. H., and Woolridge, W. R. 1927 The effects of chemical and physical changes in environment on resting bacteria. Biochem. J., **21**, 148–168.

Riesen, W. H., Spengler, H. H., Robblee, A. R., Hankes, L. V., and Elvehjem, C. A. 1947 Cysteine and related compounds in the nutrition of lactic acid bacteria. J. Biol. Chem., **171**, 731–748.

Schubert, Maxwell P. 1935 Combination of thiol acids with methylglyoxal. J. Biol. Chem., **111**, 671–678.

Schubert, Maxwell P. 1936 Compounds of thiol acids with aldehydes. J. Biol. Chem., **114**, 341–350.

Schubert, Maxwell P. 1939 The combination of cysteine with sugars. J. Biol. Chem., **130**, 601–603.

Shinohara, K. 1935 The determination of thiol and disulfide compounds with special reference to cysteine and cystine. I. Critical study of the color reaction between phospho-18-tungstic acid reagent and thiol compounds. J. Biol. Chem., **109**, 665–679.

Shinohara, K., and Kirely, E. P. 1935–36 The determination of thiol and disulfide compounds with special reference to cysteine and cystine. VII. Application of the modified phosphotungstic acid method for the determination of cysteine, cystine, and ascorbic acid in urine. J. Biol. Chem., **112**, 709–721.

Tarr, H. L. A. 1941a The action of nitrites on bacteria. J. Fisheries Research Board Can., **5**, 265–275.

Tarr, H. L. A. 1941b Bacteriostatic action of nitrites. Nature, **147**, 417–418.

356

Effect of Cumulative Dose and Dose Rate on Dimethylnitrosamine Oncogenesis in RF Mice [1,2]

N. K. CLAPP *and* R. E. TOYA, Sr., *Carcinogenesis Program, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830*

SUMMARY—Male RF mice were given dimethylnitrosamine (DMN) in drinking water for varied treatment periods, with cumulative doses ranging from 87–243 mg/kg body weight. Incidences of lung adenomas in all treated groups increased over controls by a factor of 2 and reached nearly 100%. Whereas hepatocellular tumors were not apparently affected, incidences of liver hemangiosarcomas increased in higher dose groups and reached a maximum of 96%. The incidences of tumors varied with dose rate as well as with total dose. In two groups receiving the same total dose, hemangiosarcomas appeared only in the group treated at the highest dose rate, which suggested that damage was repaired at the lower dose rate. Since lung tumor incidence was high in DMN-treated mice, metabolism of this chemical into a proximate carcinogen probably occurred in the lung parenchyma as well as the liver. The absence of tumors in the kidney of the RF mouse suggested that the necessary enzyme system was not present. Because liver hemangiosarcomas and hepatocellular tumors, two distinctly different cell types, were induced by DMN and diethylnitrosamine, respectively, the cell type in which metabolism occurs may be specific within an organ.—J Nat Cancer Inst 45: 495–498, 1970.

DIMETHYLNITROSAMINE (DMN) produced tumors in various species of animals (*1*). Efforts were made to evaluate effects of cumulative dose and dose rate in different mouse strains (*2–5*). Magee and Barnes observed a shift in tumor site from liver to kidney in rats by increasing the concentration in food from 50–200 parts per million (*4, 5*). They also observed kidney tumors after intraperitoneal injections of DMN (*4*). Takayama and Oota reported that DMN induced lung, liver, and kidney tumors in ddN, ICR, and C3H mice; however, at high doses ICR mice developed only lung tumors (*2, 3*).

In the present experiments, we determined the relationships between the total dose of DMN and induction of lung and liver tumors in RF mice by varying the length of treatment and the daily dose.

[1] Received February 24, 1970; accepted May 8, 1970.

[2] Research jointly sponsored by the National Cancer Institute and the U.S. Atomic Energy Commission under contract with the Union Carbide Corp.

MATERIALS AND METHODS

Male mice 8–10 weeks old from a noninbred subline of the RF/Un strain were given DMN in the drinking water at a concentration that produced doses of 0.4 (2 groups), 0.91, and 1.8 mg/kg body weight daily for treatment periods of varying lengths, with cumulative doses ranging from 87–243 mg/kg body weight. A total of 241 mice survived the treatment periods; 262 untreated male RF mice were handled similarly and served as controls. Cumulative doses were calculated from the measurement of the volume of water consumed, as previously reported (*6*). The animals were housed 8 per cage and received Purina laboratory chow *ad libitum*.

Mice were necropsied when they died or when they were killed after becoming moribund. Tissues for histologic examination were taken from approximately 70% of the mice, fixed in Zenker-formol solution, embedded in paraffin, and stained with hematoxylin and eosin.

RESULTS

The incidence of primary tumors and their induction times are shown in table 1. The pathogenesis of these tumors in male RF mice, after the administration of DMN, and the criteria for tumor diagnosis were reported in (*1*). The following terms are used to evaluate the results: "incidence," the number of mice with tumors divided by the number of mice alive when the first tumor was diagnosed in each organ; "mean age of death with tumors," the average age at death of all mice with tumors in a particular organ; "minimum induction time," the average age of the first 5 mice having tumors in a particular organ; "mean survival time," the average survival time for the total number of mice in each group.

In all DMN-treated groups, incidences of lung adenomas exceeded control levels by a factor of two, with groups B and E approaching 100%. The highest incidences of liver hemangiosarcomas were in the highest dose groups (D and E). Although their incidence was slightly elevated in group B, liver hemangiosarcomas failed to develop in group C. The incidence of hepatocellular tumors in groups B, D, and E was not affected by DMN. In

TABLE 1.—Incidences of primary tumors and their induction times in DMN-treated male RF mice

Group	Cumulative dose (mg/kg)	Daily dose (mg/kg)	Treatment period (days)	Number of mice	Mean survival time (months)	Lung: Incidence (%)*	Lung: Mean age of death with tumors (months)	Lung: Minimum induction time† (months)	Liver: Incidence (%)*: Hepatocellular tumors	Liver: Incidence (%)*: Hemangiosarcomas	Liver: Mean age of death with all tumors (months)	Liver: Minimum induction time† (months)
A	0	0	0	262	20.5	39 (97/250)	24	12	4 (7/174)	1 (1/120)	24	22
B	87	1.8	49	83	15	99 (82/83)	15	9	0 (0/68)	13 (9/68)	15	14
C	89	0.40	224	17	19	76 (13/17)	20	14	20 (2/10)	0 (0/17)	25	—
D	170	0.43	Life (average = 406)	47	17	85 (40/47)	19	14	2 (1/47)	51 (24/47)	20	16
E	243	0.91	Life (average = 266)	94	12	99 (91/92‡)	12	9	0 (0/93‡)	96 (89/93‡)	12	9

* Number of mice with tumor/number mice alive when first tumor was diagnosed in each organ.
† Average age of first 5 mice with tumors.
‡ Two lungs and one liver were not observed.

378

COMMITTEE ON NUTRITION

INFANT METHEMOGLOBINEMIA

The Role of Dietary Nitrate

ALTHOUGH there have been no documented instances in which naturally occurring nitrates in commercially prepared food have caused infant methemoglobinemia, periodic public statements have suggested that infant foods are implicated as causes of methemoglobinemia because of their nitrate content.[1,2] Nitrate in contaminated well water is known to cause infant methemoglobinemia.[3] Because the intake of naturally occurring nitrates from such foods as spinach and beets could be as high as that from well water, these foods have been suggested as being potentially hazardous to infants. This report will briefly summarize current knowledge of infant methemoglobinemia and examine the possible reasons for the apparent nontoxicity of food nitrates.

METHEMOGLOBINEMIA

Several comprehensive reviews of methemoglobinemia have been published.[4,5] Methemoglobin composes about 1% of the total hemoglobin of the healthy adult. Newborn or premature infants may have two to five times this level.[6] A methemoglobin reductase system normally present in erythrocytes keeps methemoglobin at the 1 to 2% level under normal conditions.[4,7] In the presence of a marked anemia, low methemoglobin concentrations may be fatal. On the other hand, infants and children can tolerate levels from 5 to 8% without manifesting cyanosis. Death from asphyxia may occur when the concentration of methemoglobin exceeds 70% of the total hemoglobin.[5,8]

NITRATES VERSUS NITRITES

Nitrates, *per se*, are toxic only at levels higher than those encountered in foods.[10] The potential hazard of nitrate in water or food is its conversion to nitrite either prior to or after ingestion. The nitrite ion oxidizes ferrous iron in hemoglobin to the ferric state. The resulting compound, methemoglobin, is incapable of binding molecular oxygen.

Conditions for reducing nitrate to nitrite during digestion must exist, or nitrate will be metabolized or excreted without adverse effect. Cornblath and Hartmann[11] have concluded that only infants, the pH of whose gastric juice is higher than 4 and who harbor nitrate-reducing bacteria in the upper gastrointestinal tract, develop sufficient concentrations of nitrite to produce methemoglobinemia. One nonsusceptible infant 10 days of age was fed as much as 100 mg of nitrate per kilogram of body weight per day for an 8-day period without developing any signs of cyanosis. The methemoglobin level increased to only 7.5%.[11]

Jaffe and Heller[4] have reported other factors which may influence the incidence of methemoglobinemia in infancy. Fetal hemoglobin, the concentration of which is relatively high in the early months of life, forms oxyhemoglobin more readily than does adult hemoglobin. By analogy, it may be more susceptible to oxidation to methemoglobin by nitrite. Total circulating hemoglobin also reaches a minimum between the first and second month.[12] Gastric acidity likewise drops to a minimum level at about 10 days of age and then increases gradually.[13] The possibility of a transient deficiency of reduced diphosphopyridine nucleotide (DPNH) methemoglobin reductase has also been suggested.[4]

These factors help to explain the relatively higher incidence of methemoglobinemia in the newborn and young infant; however, because they are variable and only limited feeding tests have been reported, it is not possible to state a specific level of nitrate intake which could be considered safe for all infants.

NITRATES IN FOODS

Extensive reviews[5,11] have failed to identify any infants with methemoglobinemia attributable to the ingestion of plant nitrate

per se. Nitrate poisoning appears to be associated only with ingestion of nitrate in water rather than with naturally occurring nitrate in foods. The data suggest that some protective factors in food may be involved; ascorbic acid and vitamin K, although less effective than methylene blue, have been used in the treatment of methemoglobinemia.[4] These or other naturally occurring reducing agents may prevent the formation or accumulation of methemoglobin in the erythrocytes, or plant nitrates may occur in chemical combinations that are less readily reduced to nitrite in the gastrointestinal tract than is the nitrate ion of well water. The chronicity of water consumption compared to the intermittency of nitrate ingestion from plant food may also be an important factor. As a result of these or other factors, current feeding practices are not likely to supply sufficient plant nitrate to cause methemoglobinemia, even in infants who are susceptible to the disease.

Under some conditions plant nitrates may be converted to nitrite before feeding. If ingested this nitrite may result in serious or fatal methemoglobinemia. Two infants, 2 and 3½ months of age, developed methemoglobinemia shortly after eating spinach prepared at home.[15] The spinach contained only traces of nitrate but as much as 2,180 ppm of nitrite. More recently, 14 German infants, 2 to 10 months of age, developed methemoglobinemia after eating spinach.[16] The spinach involved in these instances also was pureed in the home and stored under questionable conditions; apparently the spinach became toxic during storage because of conversion of nitrate to nitrite.

Schuphan[17] reported that the nitrite content of spinach containing high levels of nitrate increased from 3 to 355 mg of nitrite per 100 gm of dry substance during transportation and storage of the raw leaves. Phillips[18] has confirmed this observation. The conversion of nitrate to nitrite in the raw leaves may be a result of the action of a plant cell enzyme nitrate reductase known to be present in spinach,[19] or it may result from the action of bacterial enzymes. The increased temperature noted during storage and the exclusion of oxygen by tight packing during transportation are thought to be factors which increase the rate of this conversion.

Several investigators[20-22] have found only traces of nitrite in commercially canned infant foods tested under a variety of conditions which might be considered likely to foster conversion of nitrates to nitrites. Phillips[18,23] has demonstrated that nitrite did not accumulate in canned infant foods when opened and sampled for periods of up to 35 days of storage under normal refrigeration. Differences between the canned product and fresh spinach may be attributed to the inactivation of enzymes, elimination of bacteria, and the loss of nitrate during commercial blanching and preparation. In one study as much as 80% of the nitrate originally present in raw leaves was lost during processing.[18] As a precautionary measure, Brown and Smith have recommended that unused portions of spinach be discarded.[24]

The extensive use of baby foods provides additional circumstantial evidence that is worth considering. It is conservatively estimated that more than 350 million jars of canned spinach and beets have been used in the United States and Canada over the last 20 years without causing any proven instances of methemoglobinemia.[25] In view of the dramatic and well recognized symptoms of infant methemoglobinemia, several instances should have been reported by a feeding test of this magnitude if commercially canned spinach and beets were causative factors.

One source of nitrate potentially capable of producing methemoglobinemia in infants has developed in the last few years—nitrate contamination of drinking water from runoff from farms fertilized with nitrates. This is a hazard whose magnitude cannot be properly measured until more systematic surveys of drinking water for nitrate and/or nitrite content have been undertaken in areas where such hazards exists.

SUMMARY

In the United States and Canada, processed infant foods have not been implicated

in methemoglobinemia associated with food or water intake in infants. Although raw spinach and beets have a higher nitrate content than do other infant foods, one or more protective factors may prevent the extrinsic or intrinsic formation of toxic levels of nitrite from these foods as commercially processed for feeding of infants.

Nitrate contamination of drinking water which may occur from run-off from fields fertilized with nitrates, represents a potential hazard.

LLOYD J. FILER, JR., M.D., *Chairman*
CHARLES U. LOWE, M.D., *Chairman, 1964–1969*
LEWIS A. BARNESS, M.D.
RICHARD B. GOLDBLOOM, M.D.
FELIX P. HEALD, M.D., 1963–1969
MALCOLM A. HOLLIDAY, M.D.
ROBERT W. MILLER, M.D.
DONOUGH O'BRIEN, M.D.
GEORGE M. OWEN, M.D., 1963–1969
HOWARD A. PEARSON, M.D.
CHARLES R. SCRIVER, M.D.
WILLIAM B. WEIL, JR., M.D.
O. L. KINE, PH.D., *Consultant*
JOAQUIN CRAVIOTO, M.D., M.P.H. *Consultant*
CHARLES WHITTEN, M.D., *Consultant*

REFERENCES

1. Commoner, B.: The Crisis in the Environment. Paper presented at the National Parks Association. March 18, 1968.
2. Brody, J. E.: Biologist says nitrate fertilizer could be hazard in some food. The New York Times, p. 20, December 28, 1967.
3. Comly, H. H.: Cyanosis in infants caused by nitrates in well water. J.A.M.A., 129:112, 1945.
4. Jaffe, E. R., and Heller, P.: Methemoglobinemia in man. *In* Moore, C. V., and Brown, E. B., ed.: Progress in Hematology, Vol. 4, New York: Grune and Stratton, pp. 48–71, 1964.
5. Walton, G.: Survey of literature relating to infant methemoglobinemia due to nitrate-contaminated water. Amer. J. Pub. Health, 41: 986, 1951.
6. Kravitz, H., Elegant, L. D., Kaiser, E., and Kagan, B. M.: Methemoglobin values in premature and mature infants and children. Amer. J. Dis. Child., 91:1, 1956.
7. Gibson, Q. H.: Reduction of methemoglobin in red blood cells and studies on the cause of idiopathic methemoglobinemia. Biochem. J., 42:13, 1948.
8. Knotek, Z., and Schmidt, P.: Pathogenesis, incidence and possibilities of preventing alimentary nitrate methemoglobinemia in infants. PEDIATRICS, 34:78, 1964.
9. Locket, S.: Clinical Toxicology. London: H. Kimpton, 1957.
10. Stewart, C. P., and Stolman, A.: Toxicology; Mechanisms and Analytical Methods, Vol. 2. New York: Academic Press, p. 787, 1961.
11. Cornblath, M., and Hartmann, A. F.: Methemoglobinemia in young infants. J. Pediat., 33: 421, 1948.
12. Hammond, D., and Murphy, A.: The influence of exogenous iron on formation of hemoglobin in the premature infant. PEDIATRICS, 25: 362, 1960.
13. Miller, R. A.: Observations on gastric acidity during first month of life. Arch. Dis. Child., 16:22, 1941.
14. Simon, C., Kay, H., and Mrowetz, G.: Über den Gehalt an Nitrat, Nitrit und Eisen von Spinat und anderen Gemüsearten und die damit verbundene Gefahr einer Methämoglobinanämie für Säuglinge. Arch. Kinderheilk., 175:42, 1966.
15. Holscher, P. M., and Natzschka, J.: Methämoglobinanämie bei jungen Sauglingen durch nitrithaltigen Spinat. Dtsch. Med. Wschr., 89:1751, 1964.
16. Sionios, A., and Wodsak, W.: Die Spinatvergiftung des Sauglings. Dtsch. Med. Wschr., 90:1856, 1965.
17. Schuphan, W.: Der Nitratgehalt von Spinat (Spinacia oleraces L.) in Beziehung zur Methamoglobinanämie. Z. Ernährungswiss., 5:207, 1965.
18. Phillips, W. E. J.: Changes in the nitrate and nitrite contents of fresh and processed spinach during storage. J. Agr. Food Chem., 16: 88, 1968.
19. Paneque, A., Del Campo, F. F., Ramirez, J. M., and Losada, M.: Flavin nucleotide nitrate reductase from spinach. Biochim. Biophys. Acta, 109:79, 1965.
20. Wilson, J. K.: Nitrate in foods and its relation to health. Agronomy J., 41:20, 1949.
21. Kamm, L., McKeown, G. G., and Smith, D. M.: Food additives; New colorimetric method for the determination of the nitrate and nitrite content of baby foods. J. Ass. Official Agri. Chem., 48:892, 1965.
22. Canada, J. C.: Baby foods as Contributing Factor to Methemoglobinemia. Gerber Products Company Research Report. Unpublished report.

23. Phillips, W. E. J.: Lack of nitrate accumulation in partially consumed jars of baby food. Canad. Inst. Food Technology J., 2:160, 1969.

24. Brown, J. R., and Smith, G. E.: Nitrate accumulation and vegetable crops as influenced by soil fertility practices. Univ. Missouri Agr. Expt. Station Res. Bull. 920, April, 1967.

25. Stewart, R. A.: Gerber Products Company. Private Communication to Lowe, C. U.: Twenty Year Total U.S. Industry Production Data for Strained Spinach and Strained Beets, 1968.

[CANCER RESEARCH 30, 2091-2097, August 1970]

/13

The Effects of 2-Acetylaminofluorene and Nitrite on Free Radicals and Carcinogenesis in Rat Liver[1]

379

Barry Commoner, John C. Woolum, Ben H. Senturia, Jr., and Jessie L. Ternberg

Department of Botany [B. C., J. C. W., B. H. S.], The Center for the Biology of Natural Systems [B. C.], and Department of Surgery [J. L. T.], Washington University, St. Louis, Missouri 63130

SUMMARY

The intensity of the abnormal electron spin resonance signal previously observed in the livers of rats which were fed various chemical carcinogens is found to depend on the intake of the carcinogen, protein, and nitrate or nitrite. Nitrite enhances the intensity of the abnormal electron spin resonance signal and inhibits the carcinogenicity of 2-acetylaminofluorene. The appearance of the signal-bearing complex and the possible mechanism of its anticarcinogenic activity are discussed.

INTRODUCTION

We reported earlier that the livers of rats fed on diets containing 3 chemically unrelated hepatic carcinogens, DAB,[2] thioacetamide, and AAF, develop an abnormal ESR signal in a short time (7 to 21 days) after the onset of the diet (9). The abnormal ESR signal (g value, 2.035) appeared to be associated with a free radical which is quite different from the free radicals which give rise to the ESR signal at g = 2.005 found in normal rat liver. Whereas the normal free radicals arise in the activity of mitochondrial oxidation-reduction enzyme systems (and are, for example, destroyed by boiling), the abnormal signal is little affected by boiling and shows no association with cellular electron transport systems. The abnormal ESR signal disappears within 2 to 3 weeks after its first appearance in the liver, although carcinogen feeding is continued; hepatic tumors are observed after 6 to 9 months. The present paper is concerned with the origin of the abnormal free radical in carcinogen-fed rats and its relationship to AAF carcinogenesis.

MATERIALS AND METHODS

All of the investigations described made use of males of the standard strain of Holtzman rats. In most cases, the rats were received when they weighed about 140 g and were maintained on standard laboratory chow diets for a 1- to 3-week period before being placed on experimental diets. In most experiments, the amounts of experimental diet eaten by the rats previous to sacrifice for ESR studies and the amount of water (or experimental solutions) taken in that period were recorded, as were the initial and final weights of the animals.

For ESR studies, the animals were killed by decapitation: the livers were dissected out and either stored at 0° for up to 3 hr previous to ESR analysis or frozen for 1- to 5-day periods for later analysis. Results are not affected by freezing. Tissue samples (*ca.* 100 mg) were taken from the livers and subjected to ESR analysis according to methods which have been described elsewhere (1). The region of the liver sampled has no effect on the ESR signal observed (see Chart 1). All ESR analyses were made at 15 ± 1° in a spectrometer operating at 9000 MHz with a modulation frequency of 1 MHz. The spectrometer controls were arranged to make a series of 16-sec sweeps over a 400-gauss field encompassing the region in which the ESR signals appeared. The successive runs were averaged with a computer of average transients. The records reported below were obtained as a readout of the summed signal from the computer memory with an X-Y recorder. Generally, a record consisted of the summed results of 15 successive 16-sec sweeps.

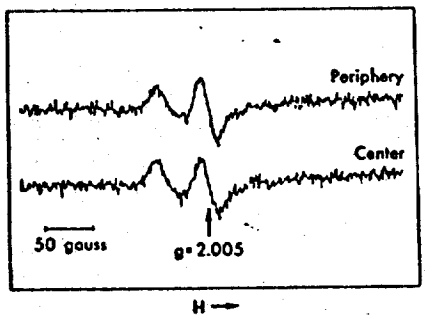


Chart 1. ESR signals from two 100-mg (wet weight) samples taken from the periphery of a lobe of a rat liver and from a central portion of the same lobe. Temperature, 15°; modulation amplitude, 16 gauss; instrumental time constant, 0.01 sec. The recorded signal represents the readout, from a computer of average transients, of the summation of 15 successive signals. *Ordinate*, 1st derivative of the absorption of microwave power.

[1] This investigation was supported by Research Grants 5 RO1 CA-03983 and 5 P10 ES-00139 from the USPHS, HEW.

[2] The abbreviations used are: DAB, *p*-dimethylaminoazobenzene; AAF, 2-acetylaminofluorene; ESR, electron spin resonance.

Received July 8, 1969; accepted April 8, 1970.

Animals retained for pathological studies were sacrificed at appropriate times and subjected to anatomical examination. Abnormal lesions were excised, and standard microscopic examinations were made to determine whether the lesions represented tumors. In addition to certain standard commercial diets, which are specified below, we used a synthetic base diet prepared for us by Nutritional Biochemical Laboratories, Cleveland, Ohio. (See Table 1 for composition.) The diets containing carcinogens were made by adding 0.067% of the carcinogen; in the DAB diet, riboflavin was decreased from 11.2 to 1.67 μg/g. The diet was prepared in the form of approximately 1-g pellets. The required carcinogen (AAF and, in a few instances, DAB) was incorporated into the pellets at Nutritional Biochemical Laboratories. These diets were stored in a cold room until used (for periods up to 6 months).

Table 1

Composition of synthetic diet

Casein	20.11%
Glucose	59.10%
Corn oil	10.05%
Alphacel	4.47%
Salt mix No. 2 USP XIII	4.47%
Cod liver oil	1.12%
DL-Tryptophan	0.447%
Cystine	0.223%
Choline chloride	33.5 μg/g
Niacin	33.5 μg/g
Riboflavin	11.2 μg/g
Thiamin-H	3.35 μg/g
Calcium pantothenate	7.82 μg/g
Inositol	33.5 μg/g
Biotin	0.223 μg/g
Folic acid	0.670 μg/g
Vitamin B_{12}	0.101 μg/g
Pyridoxine-HCl	2.79 μg/g

RESULTS

Carcinogen Feeding Experiments with Rats Maintained on Tap Water. In our initial observations of the abnormal free radical in the livers of carcinogen-fed rats, the animals were provided with ordinary City of St. Louis tap water as drinking water. These initial investigations were followed by a series of comparable experiments with diets containing AAF or DAB extending over a 19-month period. From these experiments, which are summarized in Table 2, it became apparent that rats maintained on an AAF or DAB diet and provided with tap water do not consistently develop the abnormal ESR signal in their livers. Nevertheless, whenever a signal was observed, it was identical with the abnormal ESR signal at g = 2.035 observed in the original experiments (9).

In view of these results, an effort was made to identify the hitherto unappreciated experimental factor that might account for the variability of the response to carcinogen feeding. Fortunately, a valuable clue was provided by the work of Vanin and Nalbandyan (7, 8) (kindly brought to our attention by Dr. L. E. Blumenfeld) who had observed an

Table 2

Results of carcinogen feeding experiments (all animals provided with tap water)

Date of experiment	Carcinogen[a]	No. of animals examined	No. of abnormal ESR signals
December 3, 1965	DAB	20	3
August 31, 1966	DAB	16	0
December 1, 1966	DAB	33	0
December 1, 1966	AAF	33	3
February 7, 1967	AAF	36	4
February 28, 1967	AAF	87	14
March 31, 1967	AAF	52	0
April 23, 1967	AAF	71	1
June 10, 1967	DAB	20	2

[a]In synthetic diet; see Table 1 for diet composition.

ESR signal, apparently identical with the signal which we had found in our initial carcinogen-feeding experiments, in yeast grown on Reader's medium under anaerobic conditions. Given this clue, we conducted a series of experiments, comparable to those reported by Vanin and Nalbandyan (7, 8), which are reported elsewhere (11). These showed that the signal in yeast is related to the presence of nitrate in the medium and that it can be elicited in rat liver by incubation in nitrate or nitrite. Accordingly, it was of interest to study the interaction between AAF and nitrite, both with respect to the induction of the abnormal ESR signal and with respect to the incidence of liver tumors.

The Interaction of AAF and Nitrite in Eliciting the Abnormal Free Radical. Following the discovery of the nitrite effect, in all subsequent feeding experiments rats were maintained on distilled water rather than tap water. A series of experiments was carried out to determine the effects of different levels of nitrite intake on the development of the abnormal ESR signal in the liver. In these experiments, nitrite was added to the distilled drinking water in the form of $NaNO_2$. Measurements were made of the daily fluid intake of the animals, and from these values the actual intake of nitrite was calculated (the amounts are reported in terms of nitrite nitrogen to facilitate comparison with analogous nitrate data). Animals were maintained on diets containing AAF as well as on the basic carcinogen-free diet. In this section, we describe results obtained from feeding experiments with the basic synthetic diet; experiments with other types of diet are described below.

In 1 series of experiments, 2 groups of rats were provided with a graded series of nitrite concentrations in drinking water; 1 group was fed the control synthetic diet, and the other group was fed the AAF synthetic diet. Animals were sacrificed after 10 days on the AAF diet and after 14 days on the control diet, and ESR determinations of liver samples were made in the usual way. (Preliminary experiments showed little change in signal intensity in the livers of rats on the control diet between the 10th and 14th day.) The results are shown in Chart 2. It is evident that nitrite greatly enhances the intensity of the abnormal ESR signal when AAF is included in the diet. The intensity of the signal

reaches a maximum with a daily intake of 2 to 4 mg of nitrite nitrogen. This is evident from Chart 3, which shows the relation between nitrite intake and the intensity of the abnormal ESR signal, expressed in terms of the ratio between the intensity of the abnormal ESR signal and the intensity of the ESR signal normally observed in rat liver at g = 2.005. The maximum-intensity ratio observed in rats fed on the AAF synthetic diet and nitrite is about 8.0. This intensity is significantly greater than that found in the earlier experiments, in which rats fed an AAF synthetic diet were maintained on tap water (maximum intensity ratio, about 2.0).

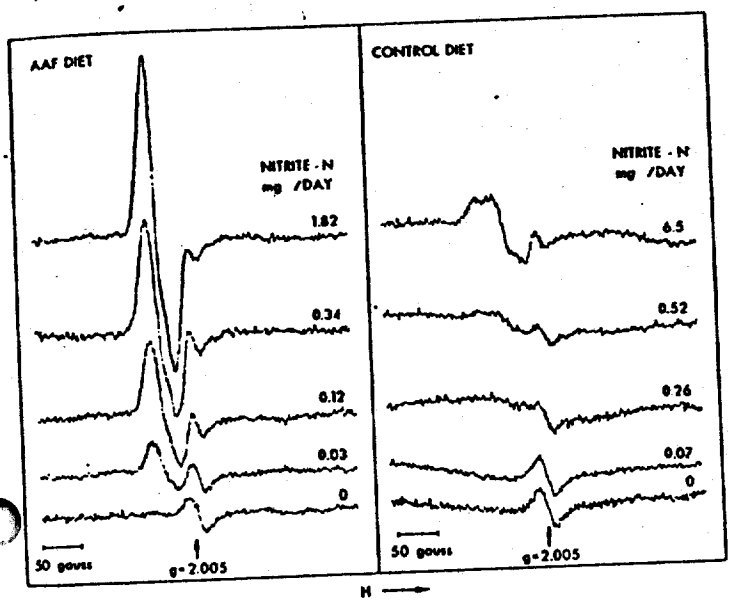


Chart 2. ESR signals from samples of the livers of rats maintained on the AAF synthetic diet and on the control synthetic diet (see Table 1) and provided with varying amounts of nitrite in drinking water. The indicated nitrite intakes were calculated from the known concentration of nitrite in the drinking water and from measurement of the drinking water intake of each animal. The animals on the AAF diet were sacrificed 10 days after the start of the experimental diet, and those on the control diet were sacrificed after 14 days. Instrument conditions as noted in the legend to Chart 1.

Chart 3 also shows that the abnormal ESR signal appears in the livers of rats maintained on the carcinogen-free basic synthetic diet, when the nitrite nitrogen intake is greater than 2 mg/day. Chart 2 shows that the abnormal ESR signals observed in the livers of rats maintained on the carcinogen-free diet (when they are sufficiently intense to provide adequate resolution) are similar with respect to g value and line shape with those observed in the livers of rats fed on the AAF diet. (The signals obtained in most other similar experiments were identical in g value and line shape, whether or not the rats were on the AAF or control diet.) However, it is also evident that in the absence of dietary AAF the maximal intensity ratio of the abnormal signal (about 1.8) is considerably less than it is when AAF is present in the diet. Indeed, when less than 0.5 mg of nitrite nitrogen per day is taken by the rats, the abnormal signal is absent from the liver unless AAF is present in the diet. Thus there is a

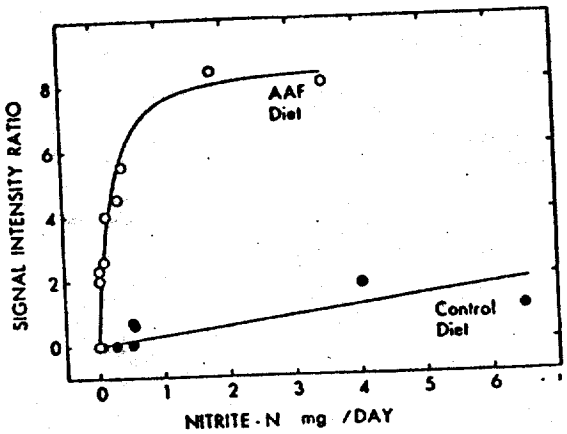


Chart 3. The intensity of the ESR signal at g = 2.035 (relative to the intensity of the normal signal at g = 2.005) in the livers of rats on the AAF synthetic diet and the synthetic control diet, as a function of nitrite intake. Representative signals from this experiment are shown in Chart 2.

synergistic relationship between AAF and nitrite, with respect to the appearance of the abnormal free radicals in the liver. Chart 4 shows that the appearance of the abnormal signal in the livers fed on a synthetic diet containing DAB is also dependent on nitrite.

We have reported elsewhere that nitrate, which may be reduced to nitrite in the gut, depending on physiological conditions and the nature of the intestinal flora, is also capable of inducing the appearance of the abnormal ESR signal in the liver (11). Chart 5 shows that the abnormal signal is absent from the livers of rats fed on the AAF synthetic diet and provided with distilled water, and is present in the livers of animals on the same diet but provided with 1.6 mg/liter of nitrate nitrogen in distilled water (nitrate intake, about 0.18 mg/day).

The Effects of Diet. All of the effects discussed above were based on experiments involving a synthetic diet specially designed to enhance the carcinogenic effect of AAF. However, it appears from the literature on chemical carcinogenesis that the composition of an animal's diet may influence the carcinogenicity of AAF and other organic carcinogens (9). Accordingly, we have investigated the influence of dietary effects on the appearance of the abnormal ESR signal in rat liver.

The importance of the synthetic basic diet in the development of the abnormal ESR signal in AAF-fed rats is evident from the results reported in Chart 6. If AAF is fed in a standard laboratory chow (Purina laboratory chow) to a rat provided with 32 mg/liter of nitrite nitrogen as drinking water, the abnormal signal does not appear, although as expected from the earlier experiments it is observed in the livers of a comparable animal fed on the AAF synthetic diet.

The results of an experiment on the effects of different levels of intake of a standard laboratory chow on the development of the abnormal ESR signal due to the presence of nitrite (*i.e.*, in the absence of a carcinogen) is shown in Chart 7. The nitrite level used in this experiment (120 mg/liter of nitrite nitrogen) is sufficient to induce the abnormal signal in the livers of rats maintained on the

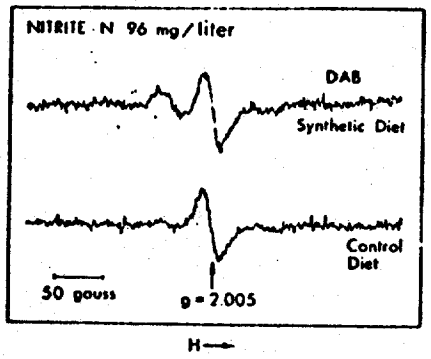


Chart 4. The influence of nitrite on the appearance of the ESR signal at g = 2.035 in the livers of rats maintained on the DAB synthetic diet and the control synthetic diet (see Table 1). Animals were sacrificed 3 days after the start of the experimental diet. Instrument conditions as noted in the legend to Chart 1.

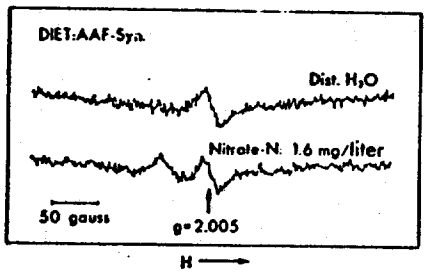


Chart 5. The influence of nitrate on the appearance of the ESR signal at g = 2.035 in the livers of rats maintained on the AAF synthetic diet for 10 days. Instrument conditions as noted in the legend to Chart 1.

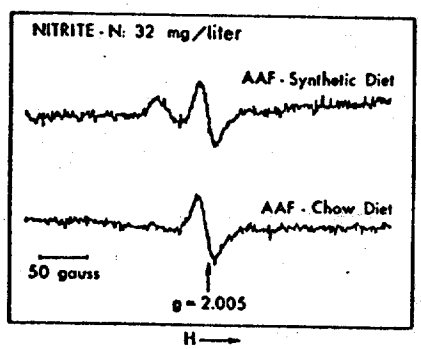


Chart 6. The effects of nitrite and AAF on the appearance of the ESR signal at g = 2.035 in the livers of rats maintained on a synthetic base diet and a laboratory chow base diet. Animals were sacrificed 8 days after the start of the experimental diets. Instrument conditions as noted in the legend to Chart 1.

synthetic carcinogen-free diet (see Charts 1 and 2). However, as shown by Chart 7, if rats are allowed an unlimited laboratory chow diet (about 20 g daily), no abnormal ESR signals are observed in the liver. If the laboratory chow intake is reduced to 15 g/day or less, the abnormal liver signal is observed down to the practical minimum of 2 g/day. Chart 7 also shows that nitrite must be provided if

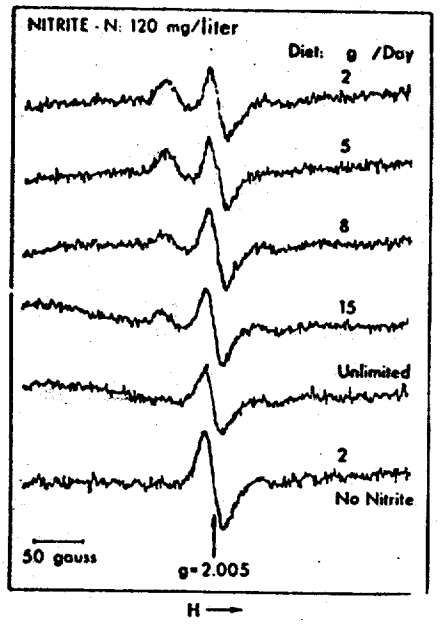


Chart 7. The influence of different intakes of laboratory chow on the appearance of the ESR signal at g = 2.035 in the livers of rats provided with nitrite in their drinking water (except for *bottom curve*, which represents a rat provided with distilled water). Animals were sacrificed 4 days after the start of the dietary regimes. Instrument conditions as noted in the legend to Chart 1.

the effect of the restricted diet is to appear. Chart 8 shows that the abnormal signal due to the restricted diet disappears within 2 days if rats are then allowed an unlimited diet of laboratory chow.

These experiments suggested that the effect of a laboratory chow diet in suppressing the nitrite-induced ESR signal was probably related to some bulk constituent of the diet, such as carbohydrate or protein. We therefore studied the effect of maintaining rats on a simple diet consisting of corn oil, cellulose, and mineral and vitamin mixtures (kindly provided by the Ralston Purina Co., St. Louis, Mo.), to which was added glucose in one case and casein in the other. Distilled water containing 120 mg/liter of nitrite nitrogen was provided in both cases. The results, which are reported in Chart 9, show that the abnormal ESR signal appears in the liver when rats are maintained on the protein-free glucose diet but does not appear when rats are maintained on the glucose-free casein diet.

The foregoing experiments were extended by studying the effects of feeding different amounts of casein, the caloric content of the diet being maintained at a constant level (*ca.* 50 calories/day) by reciprocal additions of glucose to the diet. Sodium nitrite (4.0 mg of nitrite nitrogen/day) was included in the diet, and the rats were provided with distilled water. The results of such an experiment are shown in Chart 10. The general effect is similar to that observed in the case of limited diets of laboratory chow. When 7 to 10 g of casein is provided daily, the abnormal signal is not observed in the liver. With lesser amounts of casein in the diet, the abnormal signal intensity rises to a maximum at 2 g/day. When the diet contains only glucose, the relative

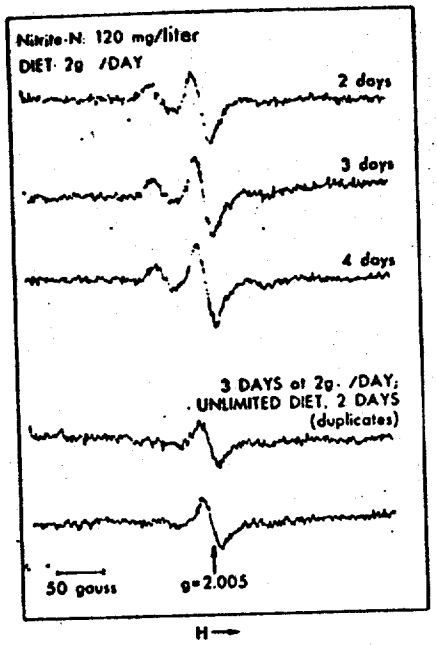


Chart 8. The effect of a return to an unlimited diet of laboratory chow after the g = 2.035 signal has been induced by maintenance of rats on a diet of 2 g/day of laboratory chow. The 3 *upper signals* are from animals maintained on 2 g/day of chow for 2, 3, and 4 days, respectively (with 120 mg/liter of nitrite nitrogen in the drinking water). The 2 *lower curves* are from duplicate animals, which were given an unlimited chow diet after 2 days on the restricted diet. Instrument conditions as noted in the legend to Chart 1.

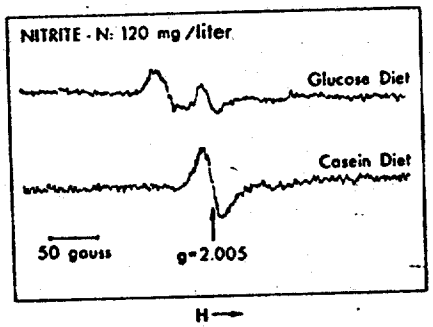


Chart 9. ESR signals from the livers of rats maintained on a glucose diet (casein-free) and a casein diet (glucose-free) for 7 days and provided with nitrite. Both diets contained corn oil, cellulose, and mineral and vitamin mixtures as well. Instrument conditions as noted in the legend to Chart 1.

intensity of the abnormal signal is reduced somewhat; however, food intake is significantly reduced by this diet, so that the intake of nitrite was also reduced.

In another experiment, rats were maintained on the AAF synthetic diet supplemented with casein (1:1 on a weight basis) and provided with various concentrations of nitrite in distilled water. As shown in Chart 11, at this dietary level of casein the synergistic effect of AAF and low levels of nitrite on the intensity of the abnormal ESR signal at g = 2.035 is essentially eliminated (compare with the results shown in Chart 3).

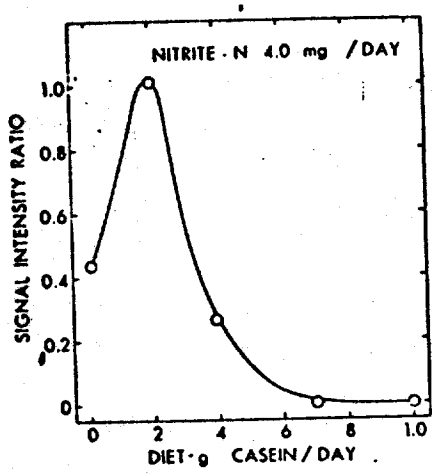


Chart 10. The intensity of the ESR signal at g = 2.035 (relative to the intensity of the normal signal at g = 2.005) in the livers of rats provided with nitrite and maintained on different daily intakes of pure casein. Animals were sacrificed after 7 days on the experimental diet. Instrument conditions as noted in the legend to Chart 1.

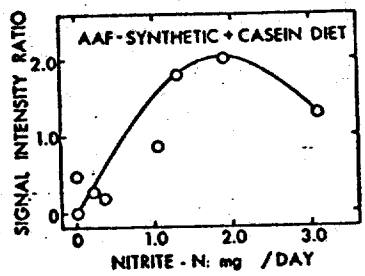


Chart 11. The effect of adding casein (1:1) to the synthetic diet on the intensity of the ESR signal at g = 2.035 (relative to that of the normal signal at g = 2.005), as a function of nitrite intake. (Compare with Chart 3.) Animals were sacrificed 10 days after the start of the experimental diet. Instrument conditions as noted in the legend to Chart 1.

From these studies, it would appear that the presence of a nutritionally sufficient amount of a complete protein in the diet of rats maintained on a level of nitrite which would otherwise induce the appearance of the abnormal signal in the liver completely suppresses this effect. The presence of sufficient dietary protein essentially eliminates the synergistic effect of AAF and nitrite on the intensity of the abnormal signal.

The Interaction of AAF and Nitrite in Carcinogenesis. Given the foregoing results, it was of obvious interest to investigate the effects of various combinations of AAF, nitrite, and synthetic and laboratory chow diets on the incidence of liver tumors. Accordingly, the experiment summarized in Table 3 was carried out. A total population of 240 male Holtzman rats was divided into 6 experimental groups maintained on the diet and drinking water combinations listed in Table 3. Some animals showed external signs of tumors in the head region in the 6th and 7th month after the experiment was started. These were sacrificed and subjected to anatomical and histological examination. The

Table 3

Incidence of tumors on various combinations of diet and nitrite

In addition to the tumors noted, the following were found: 1 tumor of the lower jaw, 3 s.c. sarcomas, 1 tumor of the small intestine (AAF synthetic diet, nitrite); 1 sarcoma of the neck region (AAF synthetic diet, distilled water); 2 tumors of the orbit, 1 lung tumor (AAF-chow diet, nitrite); 2 tumors of the orbit (AAF-chow diet, distilled water).

	Liver tumors			Ear canal tumors		
	AAF[a] synthetic diet	AAF[a] chow diet	Control[a] synthetic diet	AAF synthetic diet	AAF-chow diet	Control synthetic diet
Nitrite[b]	24[c]/38	7/22	0/24	6/29	18/20	1/24
Distilled water	25/26	14/29	0/26	10/19	19/26	0/26

[a]See Table 1 for composition of diet.
[b]Rats were provided with drinking water (distilled) containing 40 mg of nitrite nitrogen/liter.
[c]No. of animals with tumor/total no. of surviving animals in group at 8 months.

remaining animals were sacrificed 8 months after the start of the experiment and similarly examined.

The results, reported in Table 3, revealed several effects on the incidence of tumors. As expected, rats maintained on AAF in synthetic diet and distilled water showed a nearly 100% incidence of liver tumors. The tumor incidence in the group maintained on AAF in a laboratory chow diet and distilled water was significantly less (about 50%). With both of the diets containing AAF, the presence of nitrite in the drinking water significantly reduced the incidence of liver tumors (to approximately 63% in the case of the synthetic diet, and to approximately 32% in the case of the chow diet). No liver tumors were observed in the groups fed on the carcinogen-free synthetic diet, whether provided with nitrite or distilled water. In all of the groups given AAF, the livers which lacked tumors showed histological evidence of cystic changes. Table 3 also shows that AAF induced the appearance of a number of ear canal tumors. Nitrite reduced the incidence of these tumors in animals on the AAF synthetic diet, and somewhat increased their incidence in animals on the AAF-chow diet.

DISCUSSION

The results of the experiments reported above help to elucidate our earlier observation that an abnormal ESR signal at g = 2.035 appears in the livers of rats fed several different chemical carcinogens. To begin with, it is now possible to account for the variability of the results of our earlier experiments, in which rats were maintained on tap water. In recent years, tap water in many areas of the Midwest has been found to contain a significant level of nitrate, which varies with the season and weather conditions. During the period of our earlier experiments, St. Louis tap water contained variable concentrations of nitrate in the range of 0.5 to 1.5 mg/liter of nitrate nitrogen, according to analytical data kindly provided to us by the City of St. Louis Department of Public Utilities (Water Division). As shown in Chart 5, nitrate at the level of 1.6 mg/liter induces the abnormal liver signal in rats fed the AAF synthetic diet. It is likely, therefore, that the positive results obtained in our earlier carcinogen experiments were due to the presence of nitrate in the tap water, and that the variability observed in these experiments resulted from concurrent fluctuations in the tap water nitrate level.

A chief outcome of the experiments reported above is that AAF and nitrite have a synergistic effect on the concentration of the free radical associated with the abnormal ESR signal at g = 2.035 in rat liver. We have previously reported that this signal is due to a free radical complex of ferrous iron, nitric oxide, and a thiol protein (11). It is of interest to consider the relation of this free radical to carcinogenesis in the liver. This free radical appears in the livers of rats provided with 40 mg/liter or more of nitrite nitrogen in their drinking water. Our results (see Table 3) as well as those reported earlier by Druckrey *et al.* (2), show that at such levels nitrite is not carcinogenic in the rat. Similarly, Ross and Henschler (6) find no increase in tumor incidence when golden hamsters are exposed to a mixture of NO_2 and NO. This is evidence, then, that formation of NO-Fe^{++}-thiol protein in the liver is not associated with carcinogenesis. Moreover, as shown above, we find that the intensification of the concentration of this free radical in the liver of AAF-fed rats due to the presence of nitrite in drinking water is accompanied by a significant reduction in the carcinogenicity of AAF. Hence, it would appear not only that the free radical is itself noncarcinogenic but that its enhanced formation in the liver inhibits the carcinogenicity of AAF.

The significant effects of dietary protein on the appearance of the free radical induced by carcinogen-nitrite synergism find a parallel in investigations on dietary influences on carcinogenesis. While the considerable literature on this subject does not permit unequivocal generalizations, certain of the results are worth noting in connection with our own observations. The effects of diet on AAF-induced carcinogenesis have been reviewed by Weisburger and Weisburger (10). They report some evidence of inhibition of AAF-induced carcinogenesis when dietary protein intake is

reduced. This effect parallels our observation of the striking enhancement of the concentration of the NO-FE^{++}-thiol-protein-free radical in the livers of rats treated with AAF and nitrite when protein intake is reduced.

To our knowledge, there are no previous reports of the effects of nitrite and related substances on the incidence of AAF-induced tumors. However, Orzechowski *et al.* (5) report that, while sodium nitrite does not affect the incidence of tumors in mice treated with methylcholanthrene, sodium cobaltinitrite does produce a significant reduction in the incidence of epidermal tumors. Also relevant is the observation reported by Hoffman (4) that exposure to tobacco smoke reduced hepatic damage in rats fed on AAF, for nitrogen oxides occur in tobacco smoke.

Finally, our observation that the protein constituent of the NO-Fe^{++}-protein-free radical contains thiol groups, which on the basis of other studies (11, 12) are involved in the formation of the free radical complex, suggests a relationship to the numerous observations relative to the roles of thiol groups in carcinogenesis. Harington's (3) review of this subject concludes that organic carcinogens tend to interact with critical thiol groups in the cell, thus inhibiting cell division, and that the cells tend to overcompensate for this effect by producing excessive concentrations of thiol-containing compounds. This effect is believed to account for the loss of control of division that is characteristic of tumors. Harington suggests that certain anticarcinogens inhibit the binding of carcinogens to proteins, so that there appears to be competition for cellular thiol groups between the carcinogens and anticarcinogens. This is relevant to our observations, which show that the NO-Fe^{++} complex forms free radicals with 2 protein thiol groups (11). The NO-Fe^{++} complex may therefore bind protein thiol groups so that the formation of this free radical may be regarded as an anticarcinogenic response of the cell.

The foregoing considerations suggest that the NO-Fe^{++}-thiol-protein-free radical which we have observed in the livers of rats treated with AAF and nitrite is associated with a process which inhibits the carcinogenic activity of AAF. This conclusion is supported by observations that those conditions which reduce the incidence of AAF-induced liver tumors in the rat, and in particular the concurrent availability of nitrite and restriction of dietary protein intake, have a positive effect on the appearance of the ESR signal at g = 2.035, which is due to the NO-Fe^{++}-thiol-protein-free radical.

ACKNOWLEDGMENTS

We extend our appreciation and thanks to Mrs. Joyce L. Henry and Mrs. Carol Oborn for their faithful assistance in the laboratory throughout the period of the experiments.

REFERENCES

1. Commoner, B., and Ternberg, J. L. Free Radicals in Surviving Tissues. Proc. Natl. Acad. Sci. U. S., *47:* 1374–1384, 1961.
2. Druckrey, H., von Steinhoff, D., Beuthner, H., Schneider, H., and Klaerner, P. Prufung von Nitrit auf Kronisch toxische Wirkung and Ratten. Arzneimittle-Forsch., *13:* 320–323, 1963.
3. Harington, J. E. The Sulfhydryl Group and Carcinogenesis. Advan. Cancer Res., *10:* 247–309, 1967.
4. Hoffman, H. E. Inhibition of 2-Diacetylaminofluorene Carcinogenesis in the Rat. Dissertation Abstr., *16:* 1332, 1956.
5. Orzechowski, R. F., Gautieri, R. F., and Mann, D. E., Jr. Effect of Sodium Nitrite and *p*-Aminopropiophenone on the Minimal Carcinogenic $Dose_{50}$ of Methylcholanthrene on Mouse Epidermis. J. Pharm. Sci., *54:* 64–66, 1965.
6. Ross, W., and Henschler, D. Fehlen einer cancerogenen Wirkung von Nitrosen Gasen beim Goldhamster. Experientia, *24:* 55, 1968.
7. Vanin, A. F., and Nalbandyan, R. M. Free Radicals of a New Type in Yeast Cells. Biofizika, *10:* 167–168, 1965.
8. Vanin, A. F., and Nalbandyan, R. M. Free Radical States with Localization of an Unpaired Electron on the Sulphur Atom in Yeast Cells. Biofizika, *11:* 178–179, 1966.
9. Vithayathil, A. J., Ternberg, J. L., and Commoner, B. Changes in ESR Signals of Rat Liver during Chemical Carcinogenesis. Nature, *207:* 1246–1249, 1965.
10. Weisburger, E. K., and Weisburger, J. H. Chemistry, Carcinogenicity, and Metabolism of 2-Fluorenamine and Related Compounds. Advan. Cancer Res., *5:* 331–431, 1958.
11. Woolum, J. C., and Commoner, B. Isolation and Identification of a Paramagnetic Complex from the Livers of Carcinogen-treated Rats. Biochim. Biophys. Acta, *201:* 131–140, 1970.
12. Woolum, J. C., Tiezzi, E., and Commoner, B. Electron Spin Resonance of Iron-Nitric Oxide Complexes with Amino Acids, Peptides and Proteins. Biochim. Biophys. Acta, *160:* 311–320, 1968.

Estimation of Steam-Volatile N-Nitrosamines in Foods at the 1 μg/kg Level

SINCE the discovery[1] of the hepatotoxic and carcinogenic properties of N-nitrosodimethylamine (DMN), these properties have been demonstrated in a variety of N-nitroso compounds[2–5]. The effect of feeding mink with herring meal treated with excessive amounts of nitrite is to induce a liver disease[6], and DMN has been isolated as the hepatotoxic factor[7,8]. Various methylamines present in the herring meal, especially dimethylamine and trimethylamine, were found to be responsible for the formation of DMN. Although the concentration of nitrite used in the processing of the fish meal were not typical of normal commercial food preservation practice, it is essential that any potential nitrosamine hazard deriving from the use of nitrites and nitrates in the preserving and processing of foodstuffs should be evaluated.

The principal chemical route to N-nitrosamines is the reaction of secondary amines with nitrous acid in the presence of nitrite ion. Early studies on the kinetics of the reaction[9] have been supported by recent work using radiochemical techniques[10]. Any assessment of a possible hazard due to the occurrence of nitrosamines in the food chain should take into account likely combinations of the necessary precursors and the conditions (for example, concentration, temperature and *pH*) under which such combinations can occur.

Sander *et al.*[11] have shown that it is possible for nitrosamines to be produced *in vivo* from precursors, ingested at relatively high levels. Nitrates are widely distributed throughout nature and are readily reduced to nitrite by bacterial action and they must be considered in any study. There is little information on the distribution of secondary amines in foods, but Lijinsky and Epstein[12] have postulated that they may be produced by pyrolysis of proteins in the cooking of food.

Sensitive and specific analytical methods for the identification and estimation of N-nitroso compounds in food products are essential for the evaluation of any health hazard due to N-nitrosamine formation. Many of the techniques employed in the early studies were of relatively poor sensitivity, and lacked discrimination. Thus the results of analysis of extracts from foodstuffs after clean-up by column chromatography using ion-exchange resins, alumina or charcoal, employing methods such as thin-layer chromatography[13], gas-liquid chromatography[14], polarography[15] or nitrite-release[16], can be ambiguous. Gas-liquid chromatography and mass spectrometry provide more reliable characterization of individual N-nitrosamines. Telling *et al.*[17] reported lower limits of detection varying from 25 μg/kg to 65 μg/kg for a range of volatile nitrosamines by monitoring the common fragment ion NO^+. Gough and Webb[18] reported detection limits of 2 μg/kg by monitoring the appropriate parent ions lowered to 0.2 μg/kg by the use of peak cutting techniques. In all cases reported preparation of a suitable extract gave a concentration factor of about 1,000.

Various methods of separation of volatile N-nitrosamines and clean-up of extracts have been investigated in these laboratories[19]. In this study we have utilized two types of nitrogen selective detector for preliminary examination of food extracts, and where an apparently positive response was obtained the extract was examined further by the combined GLC-mass spectrometry technique of molecular ion monitoring using a high resolution instrument[18]. We describe a method for the detection, identification and estimation of steam-volatile N-nitrosamines in foods down to 1 part in 10^9 (that is, μg/kg). Initial findings in a restricted survey of certain varieties of foods including bacon, fish and cheese are reported. The effects of conventional cooking methods have also been studied and are reported. Compounds studied were the N-nitroso derivatives of dimethylamine (DMN), methylethylamine (MEN), diethylamine (DEN), dipropylamine (DPN), dibutylamine (DBN), pyrrolidine (PYR) and piperidine (PIP).

Varian 1200 and 1740 gas chromatographs were used. The 1200 instrument was fitted with a 5.5 m × 3 mm i.d. stainless steel column packed with a 15% FFAP on 'Chromosorb W' (80–100 mesh). The outlet from the column was connected via a heated transfer line to a 'Coulson Conductivity Detector' operating in the reductive mode for nitrogen. The 1740 instrument was fitted with a 5.5 m × 3 mm i.d. stainless steel column packed with 15% 'Carbowax 20M' on 'Chromosorb W' (80–100 mesh). The modified flame thermionic detector (rubidium sulphate tip) gave the optimum response to standard solutions of N-nitrosamines.

For the mass spectrometry an 'AEI MS902' high resolution instrument was used. The sample was injected into a 'Pye (model R)' gas chromatograph fitted with a 5.5 m FFAP column. The effluent stream from the column was split in a single stage membrane separator, part going by a heated stainless steel transfer line to the mass spectrometer and part into a standard FID detector. Full details of the technique used have been described by Gough and Webb[18].

The sample was minced and 250 g, with 54 g NaCl, was steam distilled; 350 ml. of distillate was collected. 6 ml. of 6 N sulphuric acid and 42 g sodium sulphate (anhydrous) were added to the distillate and the resulting solution was redistilled, 250 ml. being collected. About 0.2 g of solid potassium hydroxide and 50 g sodium chloride were added to this distillate which was then extracted three times with 50 ml. portions of methylene chloride. The organic layers were combined, dried over sodium sulphate, and evaporated in a Danish-Kuderna apparatus at 50° C, until approximately 2.5 ml. of solvent remained. 1 ml. of n-hexane was added and the evaporation was continued until 0.25 ml. remained. The concentrated extract was examined by gas chromatography and the results confirmed where necessary by mass spectrometry.

We initially studied those foods likely to contain the essential precursors for nitrosamine formation; these are cured and processed meats including bacon, ham and salami; cured and

Table 1 Volatile Nitrosamines and Nitrite Levels in Fried Bacon

Description	N-Nitrosamines* μg/kg (uncorrected)				Nitrite content of original sample mg/kg
	DMN	DEN	PYR	PIP	
Back rashers, prepacked	T	ND	A	ND	—
Back rashers, prepacked meat only	T	ND	T	ND	—
Back rashers, prepacked fat	T	ND	T	ND	—
Back rashers, Danish smoked	ND	ND	T	T	—
Collar, Irish	T	ND	ND	ND	—
Middle, Ayrshire, prepacked	T	ND	T	ND	—
Back, smoked, Danish	T	T	ND	ND	50
Back, pale, Danish	A	ND	C	ND	20
Gammon, pale, Danish	A	ND	ND	ND	30
Gammon, smoked, Danish	A	ND	ND	ND	40
Back, pale	A	ND	B	ND	5
Back, smoked	A	ND	A	ND	2
Gammon, smoked	ND	ND	A	ND	30
Gammon, smoked	ND	ND	ND	ND	5
Back, pale	ND	ND	A	ND	10
Back, smoked	A	ND	ND	ND	40
Gammon, pale	ND	ND	ND	ND	10
Gammon, pale	ND	ND	ND	ND	—
Foreback, pale	ND	ND	ND	ND	5
Foreback, pale	ND	ND	A	ND	5
Foreback, pale, Danish	T	ND	ND	ND	20
Forehock, smoked	ND	ND	A	ND	20
Forehock, smoked	ND	ND	ND	ND	60
Forehock, smoked, Danish	ND	ND	A	ND	—

* ND = Not detected. T < 1 μg/kg. A = 1–4 μg/kg. B = 5–9 μg/kg. C = 16–40 μg/kg.

fresh fish; and a range of cheeses, especially those varieties in which the use of nitrate or nitrite is permitted. The results are presented in Tables 1, 2 and 3. The concentrations quoted in the tables were obtained by measurements of the intensities of the molecular ions using the mass spectrometer and at these very low levels are subject to an error of ±50%, and are shown as concentration ranges. In most cases parallel measurements with both GC detectors produced results of a similar order, but for extracts from samples of some cooked foods interference from other nitrogenous compounds occurred which made quantitative measurements of the lower molecular weight species difficult. This was especially true of fried samples. A number of recovery trials have been carried out adding to samples of both the raw and cooked food a mixture of all six volatile N-nitrosamines studied at the 10 μg/kg level. Overall recoveries have been variable but normally fell within the range 70 to 90%. N-nitroso pyrrolidine is exceptional in that very low recoveries were obtained at the initial steam distillation stage and overall recoveries were generally below 30%. No correction factor to allow for losses sustained during the analytical method has been applied to the concentration ranges quoted in the tables.

Table 2 Volatile Nitrosamine and Nitrite Levels in Fish

Description	DMN μg/kg*	Nitrite in original sample mg/kg
Kippers	ND	Tr
Kippers, fried	ND	Tr
Salmon, smoked	ND	—
Haddock, smoked	ND	—
Herring, pickled	ND	—
Cod, salted	A	—
Cod, salted	A	Tr
Cod, fresh	A	Tr
Skate, fresh	ND	Tr
Mackerel, fresh	ND	Tr
Plaice, fresh	ND	Tr
Cod, steak in breadcrumbs, fried	A	Tr
Cod, fillet, fresh	ND	—
Cod, fillet, fried	A	—
Hake, fillet, frozen	ND	—
Hake, fillet, fried	A	—
Cod, steak in batter	ND	Tr
Cod, steak in batter, fried	ND	Tr
Hake in breadcrumbs	ND	Tr
Hake, steak, fresh	A	—
Hake, steak, fried	B	—
Hake, fresh	ND	—
Cod, fresh	ND	—
Cod, fried	A	—
Cod, fresh	ND	—
Cod, fried	A	—
Haddock, fresh	ND	—
Cod, fresh	ND	—
Cod, fried	A	—
Haddock, fresh	ND	—
Haddock, fried	A	—
Haddock, baked	B	—
Cod, fresh	A	—
Cod, fried	B	—
Coley, fresh	ND	—

* ND=Not detected. A=1–4 μg/kg. B=5–9 μg/kg. Tr=2 mg/kg nitrite.

So far only a relatively small number of samples purchased at random has been examined and many of these were of unknown processing history. They may not be typical of the commodities as a whole. Despite these limitations the picture which emerges is that minute quantities of certain N-nitroso compounds which are known to be biologically active at higher levels in animals have been detected in some foods. The available information does not yet permit any definite conclusion concerning the biological effects for man of minute amounts of N-nitrosamines in foods. Meanwhile further analytical information on fish, cheeses, cheese products, meats and meat products is required and additional studies have commenced.

Table 3 Volatile Nitrosamine Levels in Miscellaneous Food Products

Description	Volatile nitrosamines μg/kg* DMN	DEN	Nitrite mg/kg
Spinach, chopped leaf in brine	ND	ND	—
Frying oil (used)	ND	ND	—
Luncheon meat	A	ND	3
Pork, chopped, Danish	A	A	—
Salami, Austrian	ND	ND	2
Salami, Polish	ND	ND	4
Salami, Kosher	ND	ND	380
Salami, German	ND	ND	<2
Salami, Hungarian	A	ND	4
Salami, Italian	ND	ND	<2
Cheese, Cheddar, matured	ND	ND	<2
Cheese, Grinland	A	ND	<2
Cheese, Danbo	ND	ND	—
Cheese, Edam	ND	ND	—
Cheese, Harvant	ND	ND	<2
Cheese, Gouda	ND	ND	<2
Cheese, Danish Blue	A	ND	<2
Cheese, Gouda matured	A	ND	<2
Cheese, St Paulin	A	ND	<2
Cheese, Norwegian Tilsit	A	ND	<2
Cheese, Norwegian goats' milk	A	ND	<2
Cheese, Stilton	ND	ND	—
Bacon, Irish back	ND	ND	—
Ham, smoked, Westphalian	ND	ND	30
Hamburger, minced beef	ND	ND	—

* ND=Not detected. A=1–4 μg/kg.

We thank our colleagues for technical assistance. The work was supported by the Ministry of Agriculture, Fisheries and Food, and the Department of Health and Social Security.

N. T. CROSBY
J. K. FOREMAN
J. F. PALFRAMAN
R. SAWYER

Laboratory of the Government Chemist,
DTI, Cornwall House,
Stamford Street,
London SE1 9NQ

Received February 28, 1972.

1 Magee, P. N., and Barnes, J., *Br. J. Cancer*, 10, 114 (1956).
2 Druckrey, H., Preussmann, R., Ivanokovic, S., and Schmahl, D., *Z. Krebsforsch*, 69, 103 (1967).
3 Weisburger, J. H., Weisburger, E. K., Mantel, N., Hadidian, Z., and Friedrickson, F., *Naturwiss.*, 53, 508 (1966).
4 Schmahl, D., and Osswald, H., *Experientia*, 23, 497 (1967).
5 Magee, P. N., and Barnes, J., *Adv. Cancer Res.*, 10, 163 (1967).
6 Koppang, N., *Proc. IX Nordic Vet. Congr.*, Copenhagen, 777 (1962).
7 Ender, F., Havre, G., Helgebostad, A., Koppang, N., Madsen, R., and Ceh, L., *Naturwiss.*, 51, 637 (1964).
8 Ender, F., and Ceh, L., *Z. Lebensmitt-Untersuch.*, 145, 133 (1971).
9 Taylor, T. W. J., *J. Chem. Soc.*, 2052 (1928).
10 Mirvish, S. S., *J. National Cancer Institute*, 44, 633 (1970).
11 Sander, J., Schweinsberg, F., and Menz, H P., *Hoppe-Seylers' Z. Physical Chem.*, 349, 1961 (1968).
12 Lijinsky, W., and Epstein, S. S., *Nature*, 222, 21 (1970).
13 Preussmann, R., Neurath, G., Wulf-Lorentzen, G., Daiber, D., and Hengy, H., *Z. Analyt. Chem.*, 202, 187 (1964).
14 Foreman, J. K., Palframan, J. F., and Walker, E. A., *Nature*, 225, 554 (1970).
15 Walters, C. L., Johnson, E. M., and Ray, N., *Analyst*, 95, 485 (1970).
16 Daiber, D., and Preussmann, R., *Z. Analyt. Chem.*, 206, 344 (1964).
17 Telling, G. M., Bryce, T. A., and Althorpe, J., *J. Agric. Fd. Chem.*, 19, 937 (1971).
18 Gough, T. A., and Webb, K. S., *J. Chromat.*, 64, 201 (1972).
19 Crosby, N. T., Foreman, J. K., Palframan, J. F., and Sawyer, R., *Symposium on Analysis and Formation of Nitrosamines, Heidelberg, 1971, International Agency for Research in Cancer* (in the press).

Z. Anal. Chem. 206: 344-352 (1964)

416

MAN: Analyst 88, 3 (1963). — [5] COLLINS, P. F., H. DIEHL, and G. F. SMITH: Analy Chemistry 31, 1862 (1959); vgl. diese Z. 176, 389 (1960). — [6] EDMONDS, S. M., a N. BIRNBAUM: J. Amer. chem. Soc. 63, 1471 (1941). — [7] FORRESTER, J. S., a J. L. JONES: Analyt. Chemistry 32, 1443 (1960). — [8] GAHLER, A. R., R. M. HA NER, and R. C. SHUBERT: Analyt. Chemistry 33, 1937 (1961). — [9] JACKWERTH, E., H. SPECKER: diese Z. 168, 340 (1959). — [10] JACKWERTH, E., u. H. SPECKER: die Z. 176, 81 (1960). — [11] KEMULA, W., K. BRAJTER, S. CIEŚLIK u. A. LIPIŃS KOSTROWICKA: Chem. analit. (Warszawa) 5, 229 (1960); vgl. diese Z. 180, 21 (1961). — [12] KHOPKAR, S. M., u. A. K. DE: Anal. chim. Acta (Amsterdam) 22 223 (1960); vgl. diese Z. 178, 298 (1961). — [13] LANGE, B.: Kolorimetrische Analy Weinheim: Verlag Chemie 1956. — [14] MARGERUM, D. W., and C. V. BANK Analyt. Chemistry 26, 200 (1954); vgl. diese Z. 144, 217 (1955). — [15] MELNICK, L. H. FREISER, and H. F. BEEGHLY: Analyt. Chemistry 25, 856 (1953); vgl. diese Z 146, 374 (1955). — [16] MORRIS, R. L.: Analyt. Chemistry 24, 1376 (1952); vgl. die Z. 141, 292 (1954). — [17] MOSS, M. L., and M. G. MELLON: Ind. Engng. Chem anal. Edit. 14, 862 (1942). — [18] MOTOJIMA, K., and H. HASHITANI: Japan Analy 7, 28 (1958); vgl. diese Z. 166, 310 (1959). — [19] NAMIKI, M., Y. KAKITA, a H. GÔTO: Sci. Rep. Res. Inst. Tôhoku Univ., Ser. A, 14, 309 (1962). — [20] PORRET, R. Phil. Mag. 36, 196 (1810). — [21] RIEDEL, K.: diese Z. 159, 110 (1957/58). — [22] SA DELL, E. B.: Colorimetric Determination of Traces of Metals. New York: Inter Publ. 1959. — [23] SPECKER, H., u. W. DOLL: diese Z. 152, 178 (1956). — [24] SPECKE H., u. H. HARTKAMP: diese Z. 140, 353 (1953). — [25] SPECKER, H., u. E. JACKWERTH diese Z. 167, 416 (1959). — [26] SPECKER, H., u. E. JACKWERTH: Naturwissenschaft 46, 446 (1959); vgl. diese Z. 175, 60 (1960). — [27] SPECKER, H., E. JACKWERTH u G. HÖVERMANN: diese Z. 177, 10 (1960). — [28] TESTA, C.: Anal. chim. Acta (Amst dam) 25, 525 (1961). — [29] THOMPSON, J. B.: Ind. Engng. Chem., anal. Edit. 16 646 (1944). — [30] VYDRA, F., and R. PŘIBIL: Talanta (London) 3, 72 (1959); vgl diese Z. 178, 221 (1960). — [31] WAKAMATSU, S.: Japan Analyst 8, 298 (1959); vgl. diese Z. 175, 137 (1960). — [32] WOODS, J. T., and G. MELLON: Ind. Engng. Chem. anal. Edit. 13, 551 (1941). — [33] YOKOSUKA, S.: Japan Analyst 4, 509 (1955); vgl diese Z. 153, 54 (1956).

Dr. E. JACKWERTH, Kontroll-Laboratorium der E. Merck AG, 61 Darmstadt

---

Aus dem Laboratorium Prof. Dr. H. DRUCKREY der Chirurgischen Universitätsklinik Freiburg i. Br. (Direktor: Prof. Dr. H. KRAUSS)

Quantitative colorimetrische Bestimmung organischer N-Nitroso-Verbindungen durch photochemische Spaltung der Nitrosaminbindung

Von

DIETRICH DAIBER und RUDOLF PREUSSMANN

Mit 2 Textabbildungen

(Eingegangen am 1. Juni 1964)

N-Nitroso-Verbindungen sind potente organische Cancerogene, welche im Tierversuch mit hohen Ausbeuten oft selektiv in allen wesentlichen Organen Tumoren erzeugen können[1,3,5-13,17,23,24]. Die erhaltenen

Tumoren gleichen histologisch oft auffäll Pathologie bekannten Befunden. Die Bild ist einfach: N-Nitroso-Verbindungen ent und tertiären Aminen bzw. Alkylamiden nitrosen Gasen. Ihr Vorkommen in der me stand der Diskussion und muß geprüft we

Abgesehen von einigen nicht sehr spez bestanden bislang keine spezifischen Nach Verbindungen. Wir konnten vor kurzem e chromatographischen Trennung von Nit fischen Anfärbung entwickeln[20,21]. Schli Methode zur Reduktion von Nitrosami asym. Hydrazinen und deren Nachweis Derivate bekannt[18].

Quantitativ können einzelne, reine N- polarographisch[15] als auch UV-spektrosk Extinktionskoeffizienten im Maximum b gewiesen werden. Die vorliegende Arbeit b metrische Bestimmungsmethode zur qu wichtigen Stoffklasse.

Die Methode beruht wie der dünnschi weis auf der glatt verlaufenden photoche amin-Bindung[19]. Die im Spektralbereich v N-Nitroso-Gruppe wird unter UV-Bestrahl gespalten. Die entstehende salpetrige S Natriumcarbonat abgefangen und mit d GRIESS-ILOSVAY mit den Kupplungsko α-Naphthylamin nachgewiesen.

Experimenteller

Alle N-Nitroso-Verbindungen, mit Ausnah sulfolans (Fa. Light & Co) sind in unserem Lab Siede- bzw. Schmelzpunkt, molaren Absorptions charakterisiert. Alle Substanzen sind chromatog

Reagens nach Griess-Ilosvay. Gemisch 1:1 a

1. 1%iger Sulfanilsäure in 30%iger Essigsä
2. 0,1%igem α-Naphthylamin in 30%iger E

Zur photometrischen Messung der Extinkti meter PM QII verwendet.

Methode 1: Nachweis von

Modifikation a) in wäßriger Phase.

Reaktionsansatz: 1,0 ml Probe in dest. Wa Wasser, 15 min UV-Bestrahlung, 3,0 ml Griess- verdünnt, 15 min nach Zugabe des Griess-Reag Maximum bei 525 nm.

Tumoren gleichen histologisch oft auffällig den aus der menschlichen Pathologie bekannten Befunden. Die Bildung dieser Verbindungsklasse ist einfach: N-Nitroso-Verbindungen entstehen leicht aus sekundären und tertiären Aminen bzw. Alkylamiden und salpetriger Säure bzw. nitrosen Gasen. Ihr Vorkommen in der menschlichen Umwelt ist Gegenstand der Diskussion und muß geprüft werden[4].

Abgesehen von einigen nicht sehr spezifischen Tüpfelreaktionen[14,16] bestanden bislang keine spezifischen Nachweismethoden für N-Nitroso-Verbindungen. Wir konnten vor kurzem eine Methode zur dünnschicht-chromatographischen Trennung von Nitrosaminen und deren spezifischen Anfärbung entwickeln[20,21]. Schließlich wurde neuerdings eine Methode zur Reduktion von Nitrosaminen zu den entsprechenden asym. Hydrazinen und deren Nachweis als 2-Hydroxy-5-nitrobenzal-Derivate bekannt[18].

Quantitativ können einzelne, reine N-Nitroso-Verbindungen sowohl polarographisch[15] als auch UV-spektroskopisch mit Hilfe des molaren Extinktionskoeffizienten im Maximum bei $\lambda = 230$ nm direkt nachgewiesen werden. Die vorliegende Arbeit beschreibt eine einfache, colorimetrische Bestimmungsmethode zur quantitativen Erfassung dieser wichtigen Stoffklasse.

Die Methode beruht wie der dünnschichtchromatographische Nachweis auf der glatt verlaufenden photochemischen Spaltung der Nitrosamin-Bindung[19]. Die im Spektralbereich von 230 nm stark absorbierende N-Nitroso-Gruppe wird unter UV-Bestrahlung an der N-N-Bindung schnell gespalten. Die entstehende salpetrige Säure wird als $NaNO_2$ durch Natriumcarbonat abgefangen und mit dem bekannten Reagens nach GRIESS-ILOSVAY mit den Kupplungskomponenten Sulfanilsäure und α-Naphthylamin nachgewiesen.

Experimenteller Teil

Alle N-Nitroso-Verbindungen, mit Ausnahme des N-Nitroso-3-methylaminosulfolans (Fa. Light & Co) sind in unserem Laboratorium synthetisiert und durch Siede- bzw. Schmelzpunkt, molaren Absorptionskoeffizienten und Elementaranalyse charakterisiert. Alle Substanzen sind chromatographisch rein.

Reagens nach Griess-Ilosvay. Gemisch 1:1 aus

1. 1%iger Sulfanilsäure in 30%iger Essigsäure
2. 0,1%igem α-Naphthylamin in 30%iger Essigsäure.

Zur photometrischen Messung der Extinktion wurde das Zeiss-Spektralphotometer PM QII verwendet.

Methode 1: Nachweis von Nitrosaminen

Modifikation a) in wäßriger Phase.

Reaktionsansatz: 1,0 ml Probe in dest. Wasser, 0,5 ml 0,5%ige Na_2CO_3 in dest. Wasser, 15 min UV-Bestrahlung, 3,0 ml Griess-Reagens, zu 5,0 ml mit dest. Wasser verdünnt, 15 min nach Zugabe des Griess-Reagenses spektrometrische Messung im Maximum bei 525 nm.

Die Bestrahlung erfolgt in Bechergläsern 30×30 mm; Einstrahlfläche etwa 7 cm²; Schichtdicke 2 mm. Als UV-Lichtquelle dient eine Quecksilberdampf-Quarzlampe der Quarzlampen GmbH, Hanau, Typ Qu 500; Abstand UV-Röhre-Einstrahlfläche: 15 cm.

Modifikation b) in methanolischer Phase.

Reaktionsansatz: 1,0 ml Probe in reinem Methanol, 1,0 ml 0,2% Na_2CO_3 in 80%igem wäßrigem Methanol, 15 min UV-Bestrahlung, 3,0 ml Griess-Reagens, 15 min nach Zugabe des Griess-Reagenses spektrometrische Messung im Maximum bei 525 nm.

Die Bestrahlung erfolgt in Proberöhren 15×100 mm unter Wasserkühlung (etwa 20°C); Einstrahlfläche 1,8 cm²; Schichtdicke 1,3 cm, Abstand UV-Röhre-Einstrahlfläche 17 cm.

Methode 2: Nachweis von Nitrosamiden

Reaktionsansatz. 0,1 ml Probe in reinem Methanol, 1,0 ml 0,067 m Sörensen-Phosphat-Puffer (prim. K-Phosphat, sec. Na-Phosphat) pH 6,5, 15 min UV-Bestrahlung, 3,0 ml Griess-Reagens, zu 5,0 ml mit dest. Wasser verdünnt, 15 min nach Zugabe von Griess-Reagens spektrometrische Messung im Maximum bei 525 nm.

Die Bestrahlung erfolgt in Proberöhren 15×100 mm unter Eiskühlung (4 bis 6°C); Schichtdicke 0,6 cm; Einstrahlfläche 1,8 cm²; Abstand UV-Röhre-Einstrahlfläche 17 cm.

Die Aufstellung der Eichkurven erfolgt durch mindestens 3 Einwaagen im Verhältnis von etwa 1:4:8 Gewichtsteilen. Aus den zugeordneten Probelösungen sind zwei zeitlich unabhängige Doppelbestimmungen gemacht. Die Berechnung der prozentualen Umsetzung erfolgte über eine Eichkurve, die mit jeweils 4 Einwaagen von Natrium- und Kaliumnitrit pro analysi gesichert sind. Diese Eichkurve ist nach Methode 1a) ohne UV-Bestrahlung aufgenommen.

Ergebnisse und Diskussion

Methode 1: Nachweis von Nitrosaminen

Modifikation a). Die angegebene Methode wurde an den in Tab. 1 aufgeführten Verbindungen geprüft und ergab in allen Fällen Eichkurven, die dem Gesetz von Lambert-Beer folgen (Abb. 1). Die Meßpunkte sind mit dem Fehler von durchschnittlich $s_1 = 2{,}17\%$ abs reproduzierbar. Dieser Fehler darf nach dem χ^2-Test als zufällig betrachtet werden[2]. Alle fraglichen Standardabweichungen sind homogen.

Tabelle 1. *Ausbeute an Nitrit bei der photochemischen Spaltung von Nitrosaminen in wäßriger Lösung nach Methode 1a und Fehlerrechnung der Bestimmung*

Nitrosamin	Nitrit-abspaltung in Prozent d. Th.	s_1% abs.	s_2% abs.
N-Nitroso-dimethylamin	100,0	1,78	7,10
N-Nitroso-di-n-propylamin	99,1	1,86	1,67
N-Nitroso-äthyl-tert.-butylamin	95,6	0,87	4,41
N-Nitroso-morpholin	95,6	1,95	1,64
N-Nitroso-4-picolyl-methylamin	98,8	1,60	3,53
N-Nitroso-trimethylhydrazin	101,0	2,69	7,96
N-Nitroso-methyl-allylamin	102,0	1,73	8,07

Die Nitritabspaltung ist du
zu sichern, haben wir eine Reih
einer Doppelbestimmung gepr

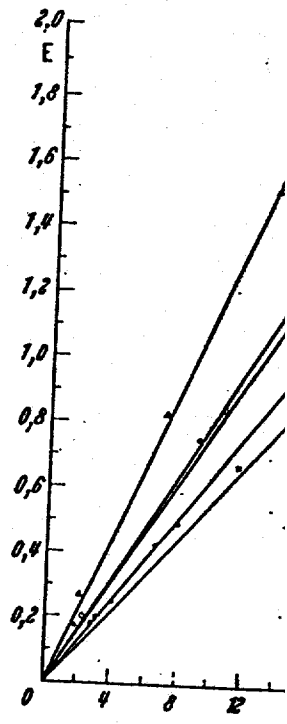


Abb. 1. Eichkurven von N

N-Nitroso-N-methyl-äthylamin, N
Nitroso-N-methyl-butylamin, N-Ni
N'-methylpiperazin und N-Nitroso

Eine Ausnahme hiervon macher
Kern direkt an der N-Nitroso-Gr
verschoben, mit Maxima bei 210 un
nur 70—80% der theoretisch zu
Vertreter seien p-Methylnitrosamin
benzoesäure, N-Nitroso-indolin und
Das Lambert-Beersche Gesetz ist j
klasse voll erfüllt.

Die photochemische Nitrit-Abs
über verschiedene Konzentrationen
nimmt mit steigender Konzentration
Fehler zwischen 0,1 und 0,2 · 10^{-7} M

Die Nitritabspaltung ist durchschnittlich 98%ig. Um diese Aussage zu sichern, haben wir eine Reihe von weiteren Verbindungen mit jeweils einer Doppelbestimmung geprüft. Die Aussage wird erfüllt durch:

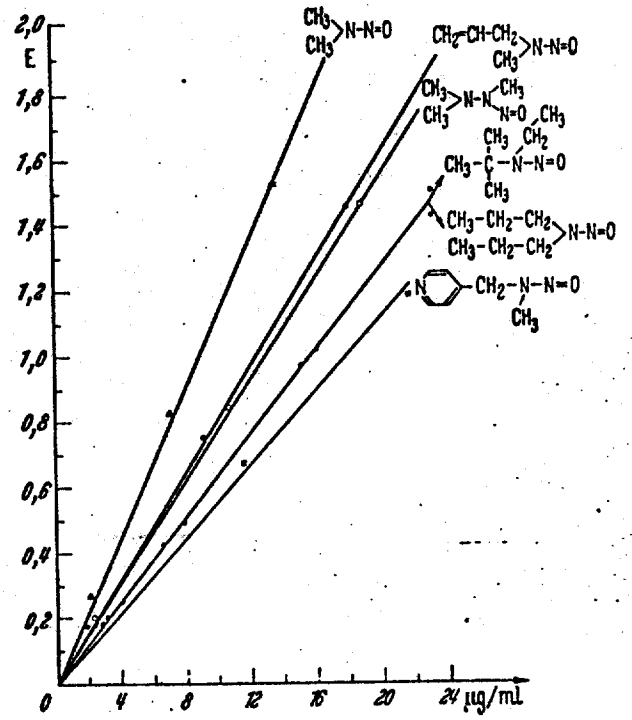


Abb. 1. Eichkurven von Nitrosaminen nach Methode 1a

N-Nitroso-N-methyl-äthylamin, N-Nitroso-N-methyl-propyl-amin, N-Nitroso-N-methyl-butylamin, N-Nitroso-N-methyl-amylamin, N-Nitroso-N'-methylpiperazin und N-Nitroso-N-methyl-β-phenyläthylamin.

Eine Ausnahme hiervon machen Nitrosamine, die einen aromatischen Kern direkt an der N-Nitroso-Gruppe tragen. Ihr UV-Spektrum ist verschoben, mit Maxima bei 210 und 270 nm. Solche Nitrosamine spalten nur 70—80% der theoretisch zu erwartenden Nitritmenge ab. Als Vertreter seien p-Methylnitrosaminobenzaldehyd, p-Methylnitrosaminobenzoesäure, N-Nitroso-indolin und N-Nitroso-N-methyl-anilin genannt. Das Lambert-Beersche Gesetz ist jedoch auch bei dieser Verbindungsklasse voll erfüllt.

Die photochemische Nitrit-Abspaltung typischer Nitrosamine ist über verschiedene Konzentrationen hinweg nicht konstant, sondern nimmt mit steigender Konzentration langsam ab. So ist der systematische Fehler zwischen 0,1 und 0,2 · 10^{-7} Mol/ml Probelösung durchschnittlich

$s_2 = 5\%$ absolut, jedoch sind Werte bis zu 8% möglich. Dieser Fehler ist nach der einfachen Varianz-Analyse nicht mehr zufällig. Es ist zusätzlich ein systematischer Fehler anzunehmen.

Dieser systematische Fehler konnte aufgedeckt werden. Reine Nitritlösungen ändern beim Bestrahlen ihren Nitritgehalt. Die Abnahme ist innerhalb von 15 min etwa 25%. Im Reaktionsansatz wurde eindeutig Nitrat nachgewiesen. Offensichtlich handelt es sich um eine Oxydation des Nitrits zum Nitrat unter Vermittlung von aktiviertem Luftsauerstoff. Folglich sind die bei der Nitritabspaltung aus Nitrosaminen gefundenen Werte Differenzwerte zweier gegenläufiger Reaktionen, nämlich der photochemischen Abspaltung von Nitrit und dessen weitere Reaktionen unter Einfluß des UV-Lichtes. Eine beträchtliche Streuung ist also zu erwarten. Der günstigste Konzentrationsbereich liegt bei etwa 0,6 bis $1{,}0 \cdot 10^{-7}$ Mol/ml Probelösung. Systematische Fehler sind hier am kleinsten. Dieser Sachverhalt ist in Abb. 2 graphisch dargestellt. Die Nitrosamin-Meßpunkte sind um die Nitrit-Eichkurve auf molarer Basis eingeordnet.

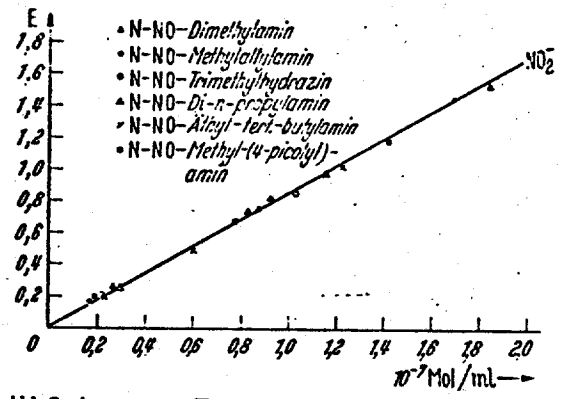


Abb. 2. Ausgezogene Kurve: Nitrit-Eichkurve. Eingetragene Punkte: Nitrosamin-Meßpunkte, berechnet auf NO_2

Die Empfindlichkeit der Reaktion ist gut. $0{,}117 \cdot 10^{-7}$ Mol/ml Nitrosamin ergaben eine Extinktion von 0,100. Bei dem niedermolekularen Dimethylnitrosamin entspricht das 0,085 µg/ml und z.B. bei Methyllaurylnitrosamin 2,7 µg/ml. Bei Einhaltung der angegebenen Bedingungen halten sich die Mängel der Methode in vertretbaren Grenzen. Die Konzentration von Natriumcarbonat ist im angegebenen Bereich optimal. Änderungen nach unten und oben, die den Faktor 2 überschreiten, liefern beträchtliche Fehler. Die Schichtdicke der bestrahlten Lösung hat zwischen 2 und 5 mm keinen Einfluß auf die Reaktion. Die Bestrahlungszeit liefert zwischen 5 und 20 min nahezu konstante Werte; das Maximum der Nitritabspaltung liegt in jedem Fall bei 15 min Bestrahlungsdauer.

Modifikation b). Diese Methode wurde zur Erfassung wasserunlöslicher Nitrosamine entwickelt, d.h. sie ist zum universellen Nachweis von Nitrosaminen geeignet. Durch das Arbeiten in Methanol ist aber die Empfindlichkeit gegenüber Modifikation 1a beträchtlich herabgesetzt.

Wie aus Tab. 2 hervorgeht, ist die
40%ig.

Tabelle 2. *Ausbeute an Nitrit bei der photoche*
methanolischer Lösung nach Methode 1b

Nitrosamin	Nitritabspaltun in Prozent d. Th
N-Nitroso-dimethylamin	37,8
N-Nitroso-methylheptylamin	37,1
N-Nitroso-diphenylamin	33,4
N-Nitroso-dibenzylamin	41,3
N-Nitroso-cyclohexylamin	43,3

Die Reproduzierbarkeit der Meßpu
gut. Der bei Modifikation 1a stark zu
tische Fehler ist hier $s_2 = 1{,}4\%$ abs. un
Zufallsfehlers, was durch die einfache V
gesichert angesehen werden kann. Die
erfüllt. Die Eichkurven folgen dem Ges

$0{,}29 \cdot 10^{-7}$ Mol/ml Probelösung ent
0,100, was einer Nitrosaminkonzentratio
molekularen (N-Nitroso-dibenzylamin)
nol wurde als Lösungsmittel mit der h
keit unter folgenden Lösungsmitteln
Dioxan, Dimethylsulfoxid und Methylä
sinkt in diesen Lösungsmitteln bis unte
tenden Nitritmenge, teilweise bedingt d
tion des Lösungsmittels. Wasserfreie und
von denen wir die gebräuchlichsten gepr
Petroläther, Essigsäureäthylester, Tetra
brauchbar, da ihr Lösungsvermögen f
Gase entweder zu gering ist, oder kein
werden kann. Höhere prozentuale Ant
system bringen nur unwesentliche En
50% H_2O etwa 10%). Die Natriumcarbo
0,10% in 90%igem Methanol einer fast
tionen unter 0,01% bringen Empfindlichk
dauer zeigt bei 15 min optimale Werte.
um ein Verdunsten des Methanols unter
Lampe zu verhindern.

Methode 2: Nachweis vo

In dieser Verbindungsklasse sind ge
zwei Abbaumechanismen möglich. Je na
der eine oder andere Weg beschritten. E

Wie aus Tab. 2 hervorgeht, ist die Nitritabspaltung nur noch etwa 40%ig.

Tabelle 2. *Ausbeute an Nitrit bei der photochemischen Spaltung von Nitrosaminen in methanolischer Lösung nach Methode 1b und Fehlerrechnung der Bestimmung*

Nitrosamin	Nitritabspaltung in Prozent d. Th.	s_1% abs.	s_2% abs.
N-Nitroso-dimethylamin	37,8	1,1	0,6
N-Nitroso-methylheptylamin	37,1	0,8	1,5
N-Nitroso-diphenylamin	33,4	0,8	1,8
N-Nitroso-dibenzylamin	41,3	0,8	0,4
N-Nitroso-cyclohexylamin	43,3	1,3	1,9

Die Reproduzierbarkeit der Meßpunkte ist mit $s_1 = 0{,}9\%$ abs. sehr gut. Der bei Modifikation 1a stark zum Ausdruck kommende systematische Fehler ist hier $s_2 = 1{,}4\%$ abs. und rückt in die Größenordnung des Zufallsfehlers, was durch die einfache Varianzanalyse mit $P = 99\%$ als gesichert angesehen werden kann. Die Nullhypothese ist in jedem Fall erfüllt. Die Eichkurven folgen dem Gesetz nach Lambert-Beer.

$0{,}29 \cdot 10^{-7}$ Mol/ml Probelösung entsprechen einer Extinktion von 0,100, was einer Nitrosaminkonzentration von etwa 2,2 µg/ml bei höhermolekularen (N-Nitroso-dibenzylamin) Nitrosaminen entspricht. Methanol wurde als Lösungsmittel mit der höchst erreichbaren Empfindlichkeit unter folgenden Lösungsmitteln ausgewählt: Aceton, Äthanol, Dioxan, Dimethylsulfoxid und Methyläthylketon. Die Empfindlichkeit sinkt in diesen Lösungsmitteln bis unter 10% der theoretisch zu erwartenden Nitritmenge, teilweise bedingt durch die hohe UV-Eigenabsorption des Lösungsmittels. Wasserfreie und wasserunlösliche Lösungsmittel, von denen wir die gebräuchlichsten geprüft haben (Äther, Chlormethane, Petroläther, Essigsäureäthylester, Tetrahydrofuran, Eisessig) sind unbrauchbar, da ihr Lösungsvermögen für die abgespaltenen nitrosen Gase entweder zu gering ist, oder kein Alkali zu ihrer Bindung gelöst werden kann. Höhere prozentuale Anteile von Wasser im Methanolsystem bringen nur unwesentliche Empfindlichkeitssteigerungen (bei 30% H_2O etwa 10%). Die Natriumcarbonatkonzentration entspricht mit 0,10% in 90%igem Methanol einer fast gesättigten Lösung. Konzentrationen unter 0,01% bringen Empfindlichkeitsverluste. Die Bestrahlungsdauer zeigt bei 15 min optimale Werte. Der Ansatz wird wassergekühlt, um ein Verdunsten des Methanols unter der Wärmeeinwirkung der UV-Lampe zu verhindern.

Methode 2: Nachweis von Nitrosamiden

In dieser Verbindungsklasse sind gemäß ihrer chemischen Struktur zwei Abbaumechanismen möglich. Je nach Reaktionsbedingungen wird der eine oder andere Weg beschritten. Ein Reaktionsweg geht über die

Spaltung der Säureamid-Bindung zum Diazoalkan, katalysierbar durch Hydroxylionen. Der andere Weg ist die Spaltung der N—N=O-Bindung durch UV-Licht.

Es wurde der erste Reaktionstyp zugunsten der Nitritabspaltung durch Senkung des pH und der Reaktionstemperatur zurückgedrängt; dies ist naturgemäß von der spezifischen Reaktivität jeder einzelnen Verbindung abhängig. In früheren Versuchen, in denen die Stabilität dieser Verbindungsklasse gegenüber Alkali untersucht wurde, ergaben sich Unterschiede der Halbwertszeiten von Sekunden bis zu Tagen für verschiedene Nitrosamide. Damit erklären sich zwanglos die stark unterschiedlichen Nitritwerte (Tab. 3). Die Werte schwanken zwischen 30 und 70% der theoretisch zu erwartenden Nitritmenge.

Tabelle 3. *Ausbeute an Nitrit bei der photochemischen Spaltung von Nitrosamiden nach Methode 2 und Fehlerrechnung der Bestimmung*

Nitrosamid	Nitrit-abspaltung in Prozent d. Th.	s_1 % abs.	s_2 % abs.
N-Nitroso-N-methyl-harnstoff	66,0	1,90	3,18
N-Nitroso-N-methyl-urethan	49,6	0,63	1,38
N-Nitroso-N-methyl-p-toluolsulfamid	67,9	1,61	4,50
N-Nitroso-3-methylamino-sulfolan	70,7	4,23	1,70
N,N'-Di-nitroso-N,N'-dimethyloxamid	31,0	0,60	2,00

Die Empfindlichkeit liegt bei 1,8 μg/0,1 ml Probelösung bei niedermolekularen (N-Nitroso-methylharnstoff) und 3,7 μg/0,1 ml bei höhermolekularen Nitrosamiden (N-Nitroso-N-methyl-p-toluolsulfamid). Diese Werte entsprechen einer Extinktion von 0,100. Die Eichkurven folgen mit Ausnahme des N-Nitroso-N-methyl-nitro-guanidins und des N,N'-Dinitroso-N,N'-dimethyl-hydrazodicarbonsäureamids dem Gesetz nach Lambert-Beer. Die Reproduzierbarkeit der Meßpunkte liegt durchschnittlich bei $s_1 = 2{,}27\%$ abs. Dieser Fehler ist nach dem χ^2-Test als zufällig zu beurteilen. Ein systematischer Fehler ist hier nicht nachweisbar. Der Fehler zwischen den Meßwerten aus drei Konzentrationsbereichen liegt mit $s_2 = 2{,}38\%$ abs. in der Größenordnung des Zufallsfehlers. Die Nullhypothese ist mit $P = 99\%$ erfüllt.

Diese Werte sind bei der Zahl möglicher konzentrationsabhängiger Nebenreaktionen bemerkenswert. Trotzdem ist die Reaktion gegenüber Änderungen der Reaktionsbedingungen sehr anfällig. Das Optimum der Nitritabspaltung liegt zwischen pH 7,2 und 6,5. Bei pH 5,0 sind die genannten Werte bereits 20% kleiner. Für eine ausreichende Pufferkapazität ist Sorge zu tragen. Zwischen 0,07 und 0,007 m Sörensen-Puffer besteht kein Unterschied. Die Reaktionstemperatur ist unbedingt einzuhalten. Bei 40°C, einer Temperatur, auf die sich der Reaktionsansatz ohne Kühlung unter Wärmeeinwirkung des UV-Brenners erhitzt

ist eine Nitritabspaltung nicht mehr nachw
der Nitrosamide in den Eichlösungen zu
Methanol hergestellt. Außerdem ist die Me
unlösliche Nitrosamide geeignet. Das Maxi
liegt bei 15 min Bestrahlungsdauer.

Gesamtwertung

Bei Anwendung dieser Methoden wird d
scher N-Nitrosoverbindungen erfaßt. Die unt
liegt durchschnittlich bei etwa 1—2 μg/ml Pr

Im Bedarfsfall kann die untere Nachweis
empfindlicherer Nitrit-Nachweismethoden we
Wir verweisen auf die Arbeiten von SAWICKI
empfindlichen Methode mit Azulen ist die
Werte von etwa 0,1 μg/ml Probe verschiebba

Schließlich sei auf die Möglichkeit aufme
stanzen wie Nitrit und Salpetrigsäureester,
Aktivierung mit Nitritreagentien reagieren
Reaktion von N-Nitrosoverbindungen unters

Zusammenfassung

Es werden Methoden zum quantitativ colo
Nitrosaminen und Nitrosamiden beschrieben.
photochemische Abspaltung von Nitrit zugru
Reagens nachgewiesen wird. Die Empfindlic
2 μg/ml. Die Methoden werden bezüglich der E
duzierbarkeit diskutiert.

Summary

Methods are described for the quantitative
of nitrosamines and nitrosamides. They are b
degradation of these compounds and reaction
Griess reagent. Sensitivity is about 1—2 μg/ml.
with regard to sensitivity and reproducibility.

Die Arbeit wurde durch die Deutsche Forschung

Literatur

[1] BARNES, J. M., and P. N. MAGEE: Brit. J. Ind.
J. Cancer 10, 114 (1956). — [2] DOERFFEL, K: Beurteilun
Ergebnissen. Berlin, Göttingen, Heidelberg: Spring
u. R. PREUSSMANN: Naturwissenschaften 49, 111, 1
u. R. PREUSSMANN: Naturwissenschaften 49, 498
R. PREUSSMANN, G. BLUM u. S. IVANKOVIC: Naturwis
[6] DRUCKREY, H., R. PREUSSMANN u. S. IVANKOVIC:
(1964). — [7] DRUCKREY, H., R. PREUSSMANN u. D. Sc
contra Cancer 19, 510 (1963). — [8] DRUCKREY, H., R.

m Diazoalkan, katalysierbar durch
lie Spaltung der N—N=O-Bindung

-p zugunsten der Nitritabspaltung
aktionstemperatur zurückgedrängt;
ifischen Reaktivität jeder einzelnen
Versuchen, in denen die Stabilität
Alkali untersucht wurde, ergaben
iten von Sekunden bis zu Tagen für
klären sich zwanglos die stark unter-
e Werte schwanken zwischen 30 und
en Nitritmenge.

chemischen Spaltung von Nitrosamiden nach
..echnung der Bestimmung

Nitrit-abspaltung in Prozent d. Th.	s_1 % abs.	s_2 % abs.
66,0	1,90	3,18
49,6	0,63	1,38
67,9	1,61	4,50
70,7	4,23	1,70
31,0	0,60	2,00

1,8 μg/0,1 ml Probelösung bei nieder-
arnstoff) und 3,7 μg/0,1 ml bei höher-
-oso-N-methyl-p-toluolsulfamid). Diese
on von 0,100. Die Eichkurven folgen
-methyl-nitro-guanidins und des N,N'-
dicarbonsäureamids dem Gesetz nach
barkeit der Meßpunkte liegt durch-
bs. Dieser Fehler ist nach dem χ^2-
Ein systematischer Fehler ist hier nicht
n den Meßwerten aus drei Konzentra-
2,38% abs. in der Größenordnung des
ist mit $P = 99\%$ erfüllt.
hl möglicher konzentrationsabhängiger
rt. Trotzdem ist die Reaktion gegenüber
gungen sehr anfällig. Das Optimum der
pH 7,2 und 6,5. Bei pH 5,0 sind di
kleiner. Für eine ausreichende Puffer-
Zwischen 0,07 und 0,007 m Sörensen-
. Die Reaktionstemperatur ist unbeding
Temperatur, auf die sich der Reaktion
ärmeeinwirkung des UV-Brenners erhitzt

ist eine Nitritabspaltung nicht mehr nachweisbar. Um eine Hydrolyse der Nitrosamide in den Eichlösungen zu vermeiden, sind diese mit Methanol hergestellt. Außerdem ist die Methode auf diese Weise für unlösliche Nitrosamide geeignet. Das Maximum der Nitritabspaltung liegt bei 15 min Bestrahlungsdauer.

Gesamtwertung

Bei Anwendung dieser Methoden wird das Gesamtspektrum organischer N-Nitrosoverbindungen erfaßt. Die untere Empfindlichkeitsgrenze liegt durchschnittlich bei etwa 1—2 μg/ml Probe.

Im Bedarfsfall kann die untere Nachweisgrenze durch Einsatz noch empfindlicherer Nitrit-Nachweismethoden wesentlich verschoben werden. Wir verweisen auf die Arbeiten von SAWICKI[22]. Bei Anwendung der sehr empfindlichen Methode mit Azulen ist die untere Nachweisgrenze auf Werte von etwa 0,1 μg/ml Probe verschiebbar.

Schließlich sei auf die Möglichkeit aufmerksam gemacht, daß Substanzen wie Nitrit und Salpetrigsäureester, die ohne photochemische Aktivierung mit Nitritreagentien reagieren, durch diese „direkte" Reaktion von N-Nitrosoverbindungen unterschieden werden können.

Zusammenfassung

Es werden Methoden zum quantitativ colorimetrischen Nachweis von Nitrosaminen und Nitrosamiden beschrieben. Beiden Methoden liegt die photochemische Abspaltung von Nitrit zugrunde, das dann mit Griess-Reagens nachgewiesen wird. Die Empfindlichkeit liegt bei etwa 1 bis 2 μg/ml. Die Methoden werden bezüglich der Empfindlichkeit und Reproduzierbarkeit diskutiert.

Summary

Methods are described for the quantitative colorimetric determination of nitrosamines and nitrosamides. They are based on the photochemical degradation of these compounds and reaction of the nitrite released with Griess reagent. Sensitivity is about 1—2 μg/ml. The methods are discussed with regard to sensitivity and reproducibility.

Die Arbeit wurde durch die Deutsche Forschungsgemeinschaft ermöglicht.

Literatur

[1] BARNES, J. M., and P. N. MAGEE: Brit. J. Ind. Med. 11, 167 (1954). — Brit. J. Cancer 10, 114 (1956). — [2] DOERFFEL, K: Beurteilung von Analysenverfahren- und Ergebnissen. Berlin, Göttingen, Heidelberg: Springer 1962. — [3] DRUCKREY, H., u. R. PREUSSMANN: Naturwissenschaften 49, 111, 166 (1962). — [4] DRUCKREY, H., u. R. PREUSSMANN: Naturwissenschaften 49, 498 (1962). — [5] DRUCKREY, H., R. PREUSSMANN, G. BLUM u. S. IVANKOVIC: Naturwissenschaften 50, 100 (1963). — [6] DRUCKREY, H., R. PREUSSMANN u. S. IVANKOVIC: Naturwissenschaften 51, 144 (1964). — [7] DRUCKREY, H., R. PREUSSMANN u. D. SCHMÄHL: Acta Union Internat. contra Cancer 19, 510 (1963). — [8] DRUCKREY, H., R. PREUSSMANN, D. SCHMÄHL u.

M. MÜLLER: Naturwissenschaften 48, 134 (1961). — [9] DRUCKREY, H., R. PREUSSMANN, D. SCHMÄHL u. M. MÜLLER: Naturwissenschaften 48, 165 (1961). — [10] DRUCKREY, H., R. PREUSSMANN, D. SCHMÄHL u. M. MÜLLER: Naturwissenschaften 49, 19 (1962). — [11] DRUCKREY, H., A. SCHILDBACH, D. SCHMÄHL, R. PREUSSMANN u. S. IVANKOVIC: Arzneimittel-Forsch. (Drug Res.) 13, 841 (1963). — [12] DRUCKREY, H., u. D. STEINHOFF, Naturwissenschaften 49, 487 (1961). — [13] DRUCKREY, H., D. STEINHOFF, R. PREUSSMANN u. S. IVANKOVIC: Z. Krebsforsch. 66, 1 (1964). — [14] FEIGL, F.: Spot Tests. Amsterdam 1960. — [15] HEATH, D. F., and I. A. E. JARVIS: Analyst 80, 613 (1955). — [16] LIEBERMANN, C.: Ber. dtsch. chem. Ges. 7, 247, 287, 806, 1098 (1874). — [17] MAGEE, P. N., and J. M. BARNES: J. Pathol. Bact. 84, 19 (1962). — [18] NEURATH, G.: Chem. Ber. 97, 1631 (1964). — [19] PREUSSMANN, R.: Arzneimittel-Forsch. 14, 769 (1964). — [20] PREUSSMANN, R., D. DAIBER, and H. HENGY: Nature (London) 201, 502 (1964). — [21] PREUSSMANN, R., G. NEURATH, G. WULF-LORENTZEN, D. DAIBER u. H. HENGY: diese Z. 202, 187 (1964). — [22] SAWICKI, E., T. W. STANLEY, J. PFAFF, and A. D'AMICO: Talanta (London) 10, 641 (1963). — [23] SCHOENTHAL, R., and P. N. MAGEE: Brit. J. Cancer 16, 92 (1962). — [24] ZAK, F. G., J. H. HOLZNER, E. J. SINGER, and H. POPPER: Cancer Res. 20, 96 (1960).

Dipl.-Chem. R. PREUSSMANN,
Lab. Prof. DRUCKREY, Chirurg. Univ.-Klinik, 78 Freiburg, Hugstetterstr. 55

Aus dem Institut für Chemische Technologie der Universität Łódź, Polen

Mercurimetrische Bestimmung von Mercaptanen in organischen Lösungsmitteln

Von
MIECZYSŁAW WROŃSKI

(Eingegangen am 13. April 1964)

Die Forderung nach neuen, schnellen und selektiven analytischen Methoden für Schwefelverbindungen in organischen Lösungsmitteln kann in verschiedenen Industriezweigen, vor allem in der Steinkohlenteer- und Erdölindustrie[2], festgestellt werden. Oft wird dabei auf die Bestimmung von Mercaptanen besonderer Wert gelegt. Für diese Zwecke können Quecksilberverbindungen als die besten Reagentien angesehen werden[4].

Aliphatische Mercaptane lassen sich in mit Ammoniak oder Natronlauge alkalisierten, wäßrigen Lösungen sehr genau in Gegenwart von Thiofluorescein als Indicator titrieren, wobei als Reagens o-Hydroxymercuribenzoesäure (HMB) angewendet wird[1,3]. Dieses Prinzip versuchen wir im folgenden zur Bestimmung von aliphatischen und aromatischen Mercaptanen in organischen Lösungsmitteln zu verwenden. Als Lösungsmittel kommen aliphatische und aromatische Kohlenwasserstoffe, die oft Schwefel und Schwefelkohlenstoff enthalten, in Frage.

Experimentel

Titration mit p-Tolylq

Eine Lösung von p-Tolylquecksilber
kann für die Titration von Mercaptane
angewendet werden. In wasserfreien
Ergebnisse ziemlich ungenau aus. Wir
zugefügt, bis beim Rühren eine milchig
die Titration viel genauer durchführen.

Ausführung. Man versetzt 20 ml der Prob
5 ml Äthanol und 0,5 ml 1 n Kalilauge in Wa
gebildete Emulsion nach Zugabe von Thiofl
kleinen Menge KOH) mit 0,001—0,01 n p-T
blaue Farbe verschwindet.

Die Methode wurde an einigen a
Mercaptanen mit gutem Erfolg geprüft
gleich mit dem unten angeführten Ver
hier auf die weitere Diskussion verzicht

Titration mit

Die Titration mit wäßriger Lösung
Zugabe von Äthanol zu den in organi
aliphatischen und aromatischen Mercap
fluorescein durchführen, wobei die Lösu
Ammoniak alkalisiert werden soll. Diese
einfach ausführen, ist aber in Gegenwa
kohlenstoff nicht anwendbar. Schwefelk
aliphatischen Mercaptanen (nicht mit a
Alkalien unter Bildung von mono
$RS^- + CS_2 = RSCS_2^-$. Die Reaktion v
sehr schnell. So werden die Produkte mit
die Ergebnisse fallen deshalb zu niedrig a
mit Mercaptiden, wobei Thiomercaptide
RSS^-, was die falschen Titrationsergeb
Titration ohne Zugabe von Äthanol im
wasserstoff-System ausgeführt wird, so
des Schwefels oder Schwefelkohlenstoffs
bei solchen Titrationen, besonders für aro
nicht scharf aus. Es liegt also der Ge
nach dem Abtitrieren des größten Teil
werden soll.

Ausführung. Man versetzt 15 ml einer Lösu
Lösungsmittel (Toluol) mit 10 ml 1 n wäßrigem
Lösung in verdünntem wäßrigem Ammoniak
stark um und titriert mit der 0,001—0,05 n wäß

Journal of Dairy Science Vol 47 (1964)

429

NITRATE TOXICITY IN DAIRY HEIFERS. I. EFFECTS ON REPRODUCTION, GROWTH, LACTATION, AND VITAMIN A NUTRITION

K. L. DAVISON, WM. HANSEL, L. KROOK, K. McENTEE, AND M. J. WRIGHT

Departments of Animal Husbandry, Veterinary Pathology and Bacteriology, and Agronomy
Cornell University, Ithaca, New York

SUMMARY

Forty-five dairy heifers were fed nitrate at levels of 0, 440, and 660 mg/kg body weight daily beginning three estrous cycles before breeding, or at 40, 150, or 240 days of pregnancy, continuing until they were killed at 30 days after parturition. Conception rate was lower in heifers fed 660 mg nitrate. One abortion occurred in those fed the lower level of nitrate and two abortions and two deaths occurred in those fed the higher level. Growth, length of estrous cycles, length of gestation, birth weight and performance of calves, vitamin A and carotene nutrition, and milk production were similar in all groups. Nitrate feeding increased the nitrate content of milk and meat, but the contamination was so small that it did not appear to constitute a hazard to human health.

The various conditions which may cause nitrate to accumulate in relatively high concentrations in various crops and weeds were reviewed by Wright and Davison (40), who stressed that level of soil nitrogen, drought, intensity of light, and species of plant were major factors influencing nitrate accumulation under practical conditions. Nitrate is found in highest concentration in the plant stem, and in very small quantities, only, in the seed.

The quantity of nitrate required to produce acute toxicity in cattle is still controversial. Estimates of the LD_{50} range from 330 mg/kg body weight daily when given as a drench (3) to 990 mg/kg when fed on hay (7). Bradley et al. (3) arbitrarily set a limit of 0.92% nitrate,[1] or 1.5% KNO_3, as the maximum that could be safely fed in hay, and apparently intended this level to apply when hay was the major, if not the only, dietary constituent. The work of Kretschmer (22) showed that cattle could survive on a diet consisting entirely of forage containing 4.0% nitrate, if they were not overly hungry before given access to the high nitrate forage.

Consumption by cattle of sublethal levels of nitrate, usually meaning 0.5 to 1.0% of the diet, has reportedly caused abortion, lowered milk production, loss in body weight or slower growth, and vitamin A deficiency (4, 11, 15, 23, 30, 31). Many of these reports were based on the detection of nitrate in forages being consumed rather than on controlled experimentation. In preliminary investigations at this station, the feeding of forages containing up to 2.3% nitrate did not cause abortion, lower milk production, or slower growth (7). Investigations were, therefore, intensified. The effects of various levels of nitrate fed for various portions of the reproductive cycle on conception, survival of the embryo and fetus, vitamin A nutrition, growth, and lactation in dairy heifers were studied.

EXPERIMENTAL PROCEDURE

The 20 grade Holstein heifers comprising Replicate 1 (1961-62) were estimated to be 16-20 months old and weighed 600 to 850 lb at the beginning of the experiment. Their reproductive tracts appeared free of abnormalities upon rectal palpation.

During a preliminary 6-wk period the animals were accustomed to the experimental ration. It consisted of first-cutting alfalfa-grass hay, mostly alfalfa, to appetite and 4 lb of concentrate containing 80% ground shelled corn, 18% soybean meal (44% protein), 1% trace mineralized salt,[2] 1% dicalcium phosphate and 2,000 IU vitamin A palmitate per pound. All estrous periods were recorded. Antibody titers were determined in serum for brucellosis and leptospirosis, and in vaginal mucus for vibrio-

Received for publication July 30, 1964.

[1] All quantitative expressions have been converted to nitrate ion, and are on a dry matter basis for feeds.

[2] Guaranteed to contain 96-99% NaCl, 0.25% Mn, 0.1% Fe, 0.05% $SO_4^{=}$, 0.33% Cu, 0.015% Co, 0.008% Zn, and 0.007% I by the processor.

sis; all heifers were free of these diseases.

The 20 heifers comprising Replicate 2 (1962-63) were more uniform in weight and age than those of Replicate 1, being about 16 months old and weighing 550 to 660 lb. They were managed as previously described, except that vitamin A was not added to the concentrate.

After the preliminary period, the animals were randomly assigned to the following experimental groups of ten animals each: (1) control, (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. Groups 2, 3, and 4 were subdivided so that three animals in each group received 440 mg nitrate per kilogram body weight per day and two animals in each group received 660 mg during Replicate 1, and this order was reversed in Replicate 2.

Since heifers in Replicates 1 and 2 appeared to adapt to nitrate feeding by increased erythropoiesis, five Holstein heifers of similar age and weight to those of Replicate 2 were purchased when seven months pregnant and fed nitrate at the 660 mg level beginning at the 240th day of pregnancy. These heifers were sold after 30 days of lactation.

The amounts of nitrate fed were adjusted every 28 days for changes in body weight. Fertilizer grade synthetic sodium nitrate was dissolved in hot water and sprinkled on chopped hay just before each of two equal daily feedings. All animals were permitted to exercise in a small paddock for about 2 hr each morning and evening, at which time they had access to water, trace mineralized salt, and dicalcium phosphate, and were watched for estrus. The heifers were bred artificially, beginning at the fourth observed estrus. Development of the fetus was followed by rectal palpations at 28-day intervals.

Shortly after parturition, all placentae were examined and one calf from each nitrate level within each group was necropsied. The remaining calves, and calves purchased to replace those necropsied, were fed milk from their respective dams at a rate of 4 lb twice daily to 14 days of age, and 5 lb to 30 days when both cow and calf were necropsied. Immediately following parturition, the concentrate was increased at a rate of 1 lb per feeding until the heifers were consuming 18 lb per day.

Daily milk production records were kept. Milk samples were taken at weekly intervals and analyzed for nitrate by the xylenol method of Groweling et al. (14), fat by the Babcock method, total solids with the Watson lactometer and protein by the Orange G dye method previously described by Castillo et al. (5). Percent solids-not-fat was determined by difference.

Plasma from jugular blood samples taken at 4-wk intervals was analyzed for carotene and vitamin A by the method of Kimble (12). Samples of liver tissue were obtained at the start of the experiment and near the 40th and 150th days of pregnancy by the technique of Udall et al. (34) and immediately analyzed for vitamin A by the method of Davies (8).

Samples of meat were collected from the round (semimembranosus and semitendinosus muscles and fascia) and rib (longissimus dorsi muscle and fascia) areas at slaughter, freeze-dried, and analyzed for nitrate by the xylenol method (14).

Samples of the basic feeds were collected biweekly and pooled for a single monthly sample. Nitrate was determined by the phenoldisulfonic acid method of Harper (16), carotene by the AOAC method (1), and crude protein by the Kjeldahl method. Calcium and phosphorus were determined after ashing by flame photometric (13) and colorimetric (2) methods respectively. Total soluble carbohydrate was determined by refluxing 0.5 g samples for 1 hr in 2% sulfuric acid and analyzing the filtrate by the method of Heinze and Murneek (17).

RESULTS

Results of the feed analyses are given in Table 1. Hay fed in Replicate 1 contained much less carotene than that fed in Replicate 2. The hay of Replicate 1 was also lower in soluble carbohydrate and calcium, and higher in crude protein and nitrate. At no time did nitrate in any sample exceed 0.1%.

The productive and reproductive performance of the heifers in Replicates 1 and 2 are summarized in Table 2. Average hay consumption was similar among groups. The amount of hay offered was carefully controlled throughout to attain maximum consumption with minimum refusal, since the hay was the nitrate carrier. Most refusals were less than 5%. One heifer offered 660 mg nitrate/kg. Group 2 consistently left hay when the nitrate was added, despite severe restriction of the quantity offered, and averaged 15% refusals to the end of the trial. Other heifers refused the nitrate-treated hay at first; therefore, the quantity of hay offered was reduced. They adjusted rapidly to it, and in most cases were consuming hay at their original levels within 2 wk.

Data from Groups 3 and 4 were averaged

TABLE 1

Nutrient content of the basic feeds

	Hay		Concentrate	
	1961-1962 Replicate 1	1962-1963 Replicate 2	1961-1962 Replicate 1	1962-1963 Replicate 2
Carotene (μg/g)[a]	5.4	18.2	1.9	1.2
Crude protein (%)	13.5	11.6	16.9	16.2
Nitrate (%)	0.07	<0.01	0.02	<0.01
Calcium (%)	0.80	1.13	0.24	0.24
Phosphorus (%)	0.21	0.19	0.45	0.51
Ash (%)	6.6	6.8	4.1	4.3
Soluble carbohydrate (%)	12.6	15.2		

[a] Carotene analyzed on an as-fed basis, other nutrients determined after drying overnight at 70 C, with a moisture loss of 8 to 10%.

TABLE 2

Productive and reproductive performance of dairy heifers fed various levels of nitrate beginning at various stages of reproduction

	Level of nitrate fed in Replicates 1 and 2, mg/kg			Nitrate beginning 240th day gestation
	0	440	660	
Avg hay consumption (lb/day)	18.1	18.0	17.8	13.5
Avg hay refusal (% of offered)[a]	4.5	4.2	5.2	23.7
Avg estrous cycle length (days)[b]	21.0	20.9	21.1	....
No. of conceptions to first service[b]	25	3	1	....
Avg no. of services per conception[b]	1.3	1.4	2.6	....
No. of abortions	0	1	2	0
No. of deaths	0	0	2	0
Avg daily gain to calving (lb)	1.44	1.51	1.41	1.48
Avg gestation length (days)[c]	278	275	274	278
Avg birth weight of calves (lb)	86	80	77	86
Avg daily gain of calves (lb)	0.67	0.91	0.85	....
Avg milk production (lb/day)	43.6	42.6	40.8	38.9
Avg milk composition				
Nitrate (μg/ml)[d]	5	9	15	9
Total solids (%)	12.5	13.0	12.8	13.2
Fat (%)	3.8	4.0	3.7	4.3
Protein (%)	3.4	3.4	3.6	3.6
Avg nitrate in meat (μg/g)[d]	5	16	21	

[a] Not corrected for moisture gain. Analyses of periodic samples indicated that refusals contained 30 to 40% water.

[b] Values at this stage represent averages of 30, 5, and 5 heifers, respectively. Heifers fed 660 mg nitrate required more services than controls or those fed 440 mg ($P < 0.05$).

[c] Heifers that aborted not included.

[d] Values significantly different from each other, $P < 0.005$; Column 4 not included in comparisons.

with those of the control group for comparing lengths of estrous cycles and conception rates. Nitrate feeding did not alter the length of the estrous cycle. The conception rate of heifers fed 660 mg nitrate was lower than either the controls or those fed 440 mg nitrate, but this difference was probably not due entirely to the nitrate (see discussion).

One heifer fed 440 mg nitrate, Group 3, aborted at 257 days of gestation. Two heifers fed 660 mg nitrate aborted; one at 237 days of gestation in Group 2, and one at 179 days in Group 3. *Pseudomonas aeruginosa* was cultured from the stomach of the 237-day fetus. Abortions were confined to the second replication, and, judging from autolysis of the fetal organs, occurred a few days after fetal death. The exact methemoglobin levels in the dams when fetal death occurred are not known. However, the maximum values recorded were 39% seven days before abortion in the heifer fed 440 mg nitrate, and 84 and 77%, respectively, several months before abortion in those fed 660 mg. These latter heifers had consistently high methemoglobin values.

A heifer fed 440 mg nitrate, Group 3, had a stillborn calf weighing 67 lb at 266 days of gestation. The calf was born during the night with no attendants present. Its lungs showed fetal atalectasis, indicating that respiration had

not occurred. Upon slaughter of the cow 30 days later, a double uterine cervix was discovered. The wall separating the two cervices had been partially torn during calving. It is possible that this condition slowed the birth process, causing death of the calf. The dam was the smallest cow in the experiment, weighing only 800 lb at slaughter. The calf also had a double cervix.

Two heifers in Replicate 2 died, and one heifer in Replicate 1 was near death; all three were receiving the higher level of nitrate. Both deaths occurred during the night; one after 270 days of nitrate feeding, and the other after 249 days of nitrate feeding and ten days postpartum. Both heifers died suddenly, with no previous illnesses. Another heifer collapsed after 162, and again after 181 days of nitrate feeding. She had 93% methemoglobin on the first occasion; no measurement was made on the second. Other signs were a grayish-brown discoloration of the visible mucous membranes, a staggering gait before collapse and during recovery, urination (clear) without attempting to lift the tail, and a weak sway-backed stance when standing. She was fed hay without nitrate both evenings and recovered without treatment. Nitrate feeding was resumed the following morning. She delivered a normal 73 lb calf at 273 days.

Nitrate feeding did not alter the length of gestation in heifers that did not abort. The birth weight of calves from heifers fed nitrate did not differ significantly from controls. Birth weight varied from 65 to 100 lb, with both extremes from heifers fed nitrate. In general, the heifers of Group 4 had lighter calves, probably because all but one of these heifers conceived to the first service and they therefore calved at younger ages and lighter weights. The heifers of Group 2, Replicate 1, had the heaviest calves, averaging 90 lb. The growth rate of calves from nitrate-fed heifers was not significantly greater than control calves.

Milk production was not significantly reduced in the nitrate-fed cows. Average daily milk production ranged from 30 to 53 lb in control heifers, 26 to 57 lb in heifers fed 440 mg nitrate, and 30 to 48 lb in heifers fed 660 mg nitrate. The only change in milk composition associated with treatment was a significant increase in nitrate content. The 15 μg/ml present in milk from those fed 660 mg nitrate does not appear to constitute a health hazard to humans, in view of tolerances of 200 μg nitrite/g in processed meats (35) and 45 μg nitrate/ml in water (36). Apparently, there are no federal regulations specifying safe limits for nitrate in milk.

Nitrate feeding increased the nitrate content of the carcass. The values were the same in samples from the round and rib areas. These increases were so small, however, that they present no health hazard.

The growth curves, shown in Figure 1, were nearly identical for all treatment groups. The

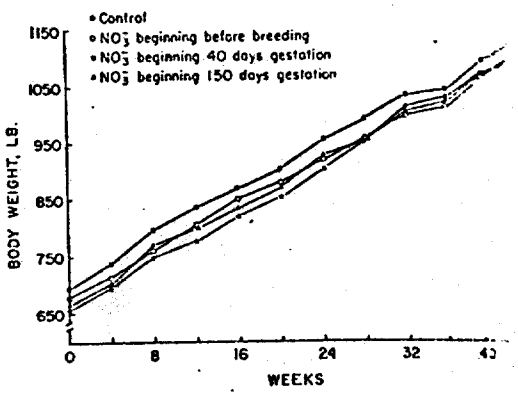


FIG. 1. Growth curves of the heifers of Replicates 1 and 2 as related to grouping.

daily rates of gain, given in Table 2, were similar among nitrate levels and indicate that a near-lethal level of nitrate does not by itself alter growth.

Because of differences between replicates in the carotene and vitamin A content of the feeds, the plasma vitamin A and carotene values of each replicate are presented separately. Plasma vitamin A was moderately low to normal in the heifers of Replicate 1 at the beginning of the experiment (Figure 2) and decreased slowly to parturition, regardless of

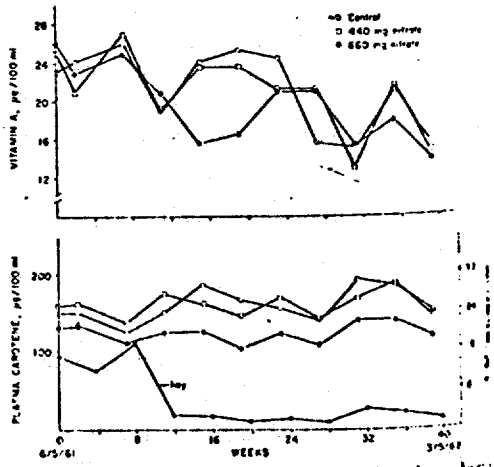


FIG. 2. Vitamin A and carotene in the plasma of the heifers of Replicate 1 as related to level of nitrate and carotene fed.

...atment. Plasma carotene remained static ... animals with higher carotene at the be... remained higher to the end of the ex... The hay was very low in carotene ... the third month to the end of the repli... Some of this hay was fed in the be... of Replicate 2. Plasma vitamin A ... were higher initially in the heifers of ...plicate 2 (Figure 3), but they decreased until

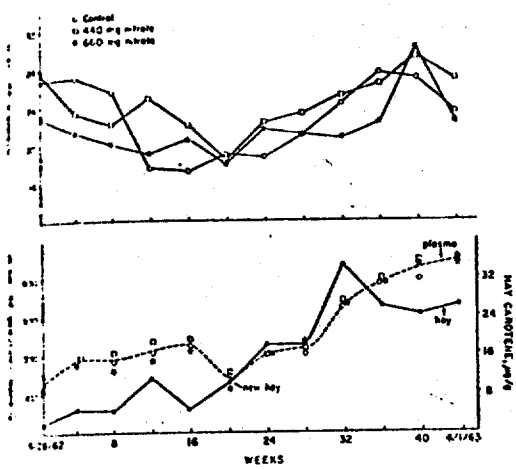


FIG. 3. Vitamin A and carotene in the plasma of the heifers of Replicate 2 as related to level of nitrate and carotene fed.

the new, higher carotene hay was introduced at the 20th week. Plasma carotene and vitamin A then increased to 40 wk, when vitamin A fell as parturition approached, which is a normal occurrence (32).

Liver vitamin A data given in Figure 4 are on a fresh weight basis. Liver storage was somewhat low at the start of both replications and remained low throughout, but this is probably to be expected, because the heifers were

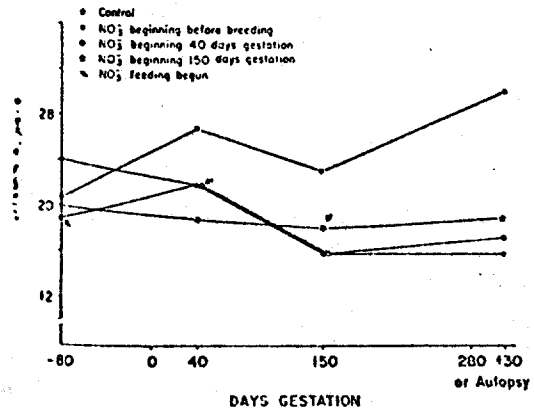


FIG. 4. Vitamin A in the livers of the heifers of Replicates 1 and 2 as related to grouping.

purchased in early spring and kept in drylot thereafter. Although liver vitamin A in the control animals appears to have increased, whereas that of all other animals decreased slightly, the differences were not statistically significant. Liver vitamin A in the animals of Groups 3 and 4 was declining before nitrate feeding was begun at 40 and 150 days of pregnancy, respectively, when that of Group 4 plateaued. One abscessed liver was observed at necropsy, and liver weight did not differ significantly among treatments.

Liver vitamin A had apparently increased over initial levels in all heifers of Replicate 1 when samples were taken at 40 days of pregnancy. This increase was probably an artifact caused by losses of vitamin A in the earlier samples, since they were stored for a short period while the project group was moving into a new laboratory. Vitamin A storage had decreased by 150 days and apparently remained unchanged thereafter.

By the time a few liver samples had been taken at 150 days of pregnancy from the heifers of Replicate 2, it was apparent that liver vitamin A was decreasing in all groups. All heifers were, therefore, biopsied again on December 1. Data are shown in Figure 5. While the data

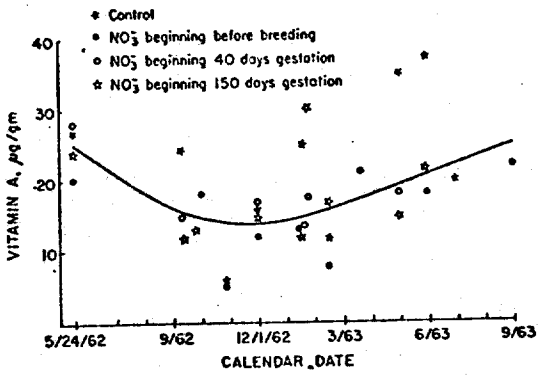


FIG. 5. Variation observed in the vitamin A content of the livers of heifers of Replicate 2, and the changes associated with the level of dietary carotene.

for May 24 and December 1 represent averages of all heifers sampled on the same date, the other points represent single observations, or averages of two to three values, and show variation inherent in the technique as well as the trend in vitamin A storage. The increase in vitamin A after the low period between October and December corresponds with the feeding of the higher carotene hay. Much variation was introduced into the final vitamin A values in Figure 4, because of this period

of increasing vitamin A storage and the wide variation in times that the heifers were necropsied.

Data from the heifers started on nitrate at 240 days gestation are presented in the last column of Table 2. These heifers consumed only about half as much hay as would be expected of 1,200-lb heifers, but the time between the beginning of nitrate feeding and parturition was too short for them to become hungry enough to eat more. Starvation was attempted briefly but was discontinued. Consequently, an average of 510 mg nitrate/kg body weight was consumed daily. One of these heifers did consume 95% of her hay for a 2-wk period; a 42% methemoglobinemia was the only adverse effect. Their milk production and milk composition compare very favorably with those of the previous heifers.

DISCUSSION

The summer of 1961 was very rainy and humid. Hay purchased then was coarse, and although only a small portion was rained on during curing, it had discolored and molded considerably in storage. The summer of 1962 was droughty. Hay purchased that year had a good green color although it was overmature. Some seed pods had formed in the alfalfa despite the apparent fine stems. These differences explain the higher carotene content of hay fed in Replicate 2.

The ration was formulated to meet the nutrient requirements for normal growth of these heifers, as established by the NRC (24). It is well known that additional energy will increase a ruminant's resistance to nitrate (19, 26) and the grain fed herein undoubtedly offered some protection. The nitrate content of the daily rations, including the grain, averaged slightly over 2% during gestation for heifers fed 440 mg nitrate/kg and slightly over 3% for those fed 660 mg. The nitrate content of the total ration decreased somewhat after parturition because of dilution by the additional grain. Hay consumption decreased slightly during lactation.

There was a small but abrupt drop in hay consumption by all heifers in Replicate 2 when feeding of the overmature hay began, but normal consumption was recovered over a period of several days. Hay consumption also dropped abruptly when nitrate feeding began, but in most cases it returned to the original level within a few days. Heifers fed nitrate beginning at 240 days of pregnancy did not regain normal hay consumption.

Consumption of oat hay containing up to 2.3% nitrate was not lower than that of control hay in previous growth and lactation studies with dairy cattle (7), and nitrate additions at similar levels did not reduce feed consumption in growing fattening studies with lambs (6, 12). Nitrate feeding did not reduce lamb weight gains in any of the preceding studies. On the other hand, nitrate additions reduced feed consumption and rate of gain of fattening steers when it was fed with grain or mixed in a ration containing cottonseed hulls (15, 27). Therefore, nitrate probably does not affect growth except in cases where it reduces feed consumption.

There was no indication that nitrate lowered milk production, and perhaps the preceding conclusion on growth applies here. High-nitrate oat hay did not lower milk production in the study of Crawford (7). These observations are in contrast with the conclusions of Muhrer et al. (23), Stewart and Merilan (3) and Case (4). In the report of Case, the reduced milk production observed in cattle feeding high-nitrate silage may have been caused by an energy deficiency per se, since the cows did not have methemoglobinemia and the feeding of molasses improved milk production. All lactation experiments, including this one, could have been improved by prolonging them, and perhaps additional studies are justified on this basis.

The observations by Muhrer et al. (23) and Case (4), that vitamin A deficiency occurred in forages grown during a drought, and that these forages contained more nitrate than normal, suggested that vitamin A deficiency might have been associated with nitrate consumption. Many studies with rats have since shown that nitrate, and particularly nitrite, interferes with conversion of carotene to vitamin A and storage of vitamin A in the liver [See Wright and Davison (40) for review]. The data reported herein indicate that nitrate consumption has very little, if any, effect on vitamin A nutrition of cattle. Most of the differences observed could be explained by the levels of vitamin A and carotene in the feed, and the levels in the plasma and liver initially. Concurrent investigations at other stations with cattle (37) and sheep (6, 12) have supported these observations. Death would probably occur before ruminants could consume enough nitrate to alter their vitamin A metabolism. This does not preclude the possibility of vitamin A deficiency caused by a shortage of vitamin A or carotene in the diet, which may or may not be related to nitrate. Gaseous reduction products from nitrate have been shown under laborat-

...ditions to destroy carotene in silage during ...entation (25), but the extent of this de...ction should be investigated further under ...ied conditions. Nitrate apparently does ...t destroy vitamin A in dry feeds (9).

The extreme variation noted in the vitamin ... contents of the liver was surprising, since ... heifers were fed and managed in an iden...al manner for over 18 months. Animals with ...ater initial reserves tended to maintain their ...pective position, and correction for this ini...al level might be used to lessen variation in ...ture work.

The reproductive problems encountered are ...marized in Table 3. Failure to deposit ...en directly into the uterus probably ex...ains the conception problem with Heifer 287. ...ny heifers not yet consuming nitrate re...ed two inseminations before conception. The reduced fertility of Heifers 257, 263, and ...9, all fed nitrate, could have been caused by ... observations listed. Therefore, the role of ...trate in causing these poor conception rates, ... the problems associated with conception, cannot be assessed.

The embryonic deaths observed in Heifer 310, and possibly 263, were the only ones detected in the 40 heifers. This small number was well within the 16% reported for normal animals by Kidder (20). Heifer 310 probably should not have been bred following estrus on the 49th day, since this probably did not allow sufficient time for the uterus to return to normal.

Many reports have indicated that consumption of high levels of nitrate may cause abortion in cattle (11, 28, 33, 39). In these reports, the abortions were associated with high levels of methemoglobin in the dam, or death of other animals in the same herd. There are enough experimental data to indicate that nitrate consumed at levels less than 2% of the diet, or 440 mg/kg body weight daily, does not cause abortion (7, 10, 27, 29, 38, 39). Abortions reported in animals eating lower levels of nitrate (11, 31), in the absence of other signs such as methemoglobinemia, collapse, or death, may be due to other factors. In the data collected by

TABLE 3

Summary of all the reproductive problems encountered

Cow no.	Level of nitrate	Problem	Observations
		Control	
287	0	5 inseminations for conception.	Cervix very tortuous; catheter not passed beyond third cervical ring until 5th insemination.
316	0	2 inseminations for conception.	No abnormalities observed.
		Nitrate beginning 3 estrous cycles before breeding	
274	440	2 inseminations for conception.	No abnormalities observed.
310	440	Embryonic death between 40 and 49 days. 2 inseminations for 1st conception; 2 for 2nd.	Palpated pregnant 39th day. Estrus 49th day; did not conceive then, returned to estrus and conceived 20 days later.
257	660	3 inseminations for conception. Aborted at 231 days gestation.	Multiple cysts in left oviduct; fluid could not be forced through. Fetus in right uterine horn, side of prior ovulations unknown. *Pseudomonas aeruginosa* isolated from stomach of aborted fetus.
263	660	3 inseminations for conception.	Only 3 days between 1st and 2nd estrus, 39 days between 2nd and 3rd. Ovulation after 1st unknown, ovulated after second. Prior estrous cycles were normal length.
273	660	4 inseminations for conception.	No abnormalities observed.
279	660	2 inseminations for conception. Died after 270 days on nitrate.	Insemination delayed for 24 hr after estrus detected.
		Nitrate beginning 40th day of pregnancy	
237	660	Aborted at 179 days gestation.	No abnormalities observed.
314	660	2 inseminations for conception. Died 10 days after parturition.	No abnormalities observed.
		Nitrate beginning 150th day of pregnancy	
283	440	2 inseminations for conception.	No abnormalities observed.
307	440	Stillborn calf.	Double cervix, septum partially torn.

Hillman et al. (18), approximately 75% of bovine abortions investigated remained unexplained, illustrating the inadequacy of our present knowledge of the causes of abortion.

ACKNOWLEDGMENTS

This study was supported in part by Grant No. GM-07642 of the U. S. Public Health Service, National Institutes of Health. The assistance of M. R. Jainudeen, W. Monson, and W. Wagner, also cooperators in these experiments, was gratefully appreciated.

REFERENCES

(1) Association of Official Agricultural Chemists. Official and Tentative Methods of Analysis. 9th ed. Washington, D. C. 1960.

(2) Barton, C. J. Photometric Analysis of Phosphate Rock. Anal. Chem., 20: 1068. 1948.

(3) Bradley, W. B., Epps on, H. F., and Beath, O. A. Livestock Poisoning by Oat Hay and Other Plants Containing Nitrate. Wyoming Agr. Expt. Sta., Bull. 241. 1940.

(4) Case, A. A. Some Aspects of Nitrate Intoxication in Livestock. J. Am. Vet. Med. Assoc., 130: 323. 1957.

(5) Castillo, L. S., Trimberger, G. W., Henderson, C. R., Herrington, B. L., and Turk, K. L. Comparison of Orange G Dye, Formal Titration, and Kjeldahl Methods for Milk Protein Determinations. J. Dairy Sci., 45: 1079. 1962.

(6) Cline, T. R., Hatfield, E. E., and Garrigus, U. S. Effects of Potassium Nitrate, alpha-Tocopherol, Thyroid Treatments, and Vitamin A on Weight Gain and Liver Storage of Vitamin A in Fattening Lambs. J. Animal Sci., 22: 911. 1963.

(7) Crawford, R. F. Some Effects of Nitrates in Forage on Ruminant Animals. Unpublished Ph.D. thesis, Mann Library, Cornell University, Ithaca, N. Y. 1960.

(8) Davies, A. W. The Colorimetric Determination of Vitamin A by Alkali Digestion Method. Biochem. J., 27: 1770. 1933.

(9) Davison, K. L., and Seo, J. Influence of Nitrate upon Carotene Destruction During in Vitro Fermentation with Rumen Liquor. J. Dairy Sci., 46: 862. 1963.

(10) Epps on, H. F., Glenn, M. W., Ellis, W. W., and Gilbert, C. S. Nitrate in the Diet of Pregnant Ewes. J. Am. Vet. Med. Assoc., 137: 611. 1960.

(11) Garner, G. B. Learn to Live with Nitrate. Missouri Agr. Expt. Sta., Bull. 708. 1958.

(12) Goodrich, R. D., Emerick, R. J., and Embry, L. D. Effect of Sodium Nitrate on the Vitamin A Nutrition of Sheep. J. Animal Sci., 23: 100. 1964.

(13) Greweling, T. Flame Photometry of Calcium and Magnesium in Ashed Plant Materials; Resolution of Anion Interferences with Ethylenediaminetetraacetate. Chem. Analyst, 50: 40. 1961.

(14) Greweling, T., Davison, K. L., and Morris, C. J. Determination of Nitrate Nitrogen in Bovine Blood, Milk, Urine, and Rumen Liquor. J. Agr. Food Chem., 12: 139. 1964.

(15) Hale, W. H., Hubbert, F., Jr., and Taylor, R. E. The Effect of Concentrate Level and Nitrate Addition on Hepatic Vitamin A Stores and Performance of Fattening Steers. J. Animal Sci., 20: 934. 1961. (Abstr.)

(16) Harper, H. J. The Accurate Determination of Nitrates in Soils. Phenoldisulfonic Acid Method. Ind. Eng. Chem., 16: 180. 1944.

(17) Heinze, P. H., and Murneek, A. E. Comparative Accuracy and Efficiency in Determination of Carbohydrates in Plant Material. Missouri Agr. Expt. Sta., Research Bull. 314. 1940.

(18) Hillman, R. B., Gilman, H. L., McEntee, K., and Barber, T. L. Study of Abortion in Domestic Animals. Report of the New York State Veterinary College 1960-61, Cornell University. pp. 69-7[illegible] 1961.

(19) Holtenius, P. Nitrite Poisoning in Sheep with Special Reference to the Detoxication of Nitrite in the Rumen. Acta Agr. Scand 7: 113. 1957.

(20) Kidder, H. E., Block, W. G., Wiltbank, J. N., Ulberg, L. C., and Casida, L. E. Fertilization Rates and Embryonic Death Rates in Cows Bred to Bulls of Different Levels of Fertility. J. Dairy Sci., 37: 691. 1954.

(21) Kimble, M. S. The Photoelectric Determination of Vitamin A and Carotene in Human Blood Plasma. J. Lab. Clin. Med. 24: 1055. 1939.

(22) Kretschmer, A. W., Jr. Nitrate Accumulation in Everglades Forages. Agron. J. 50: 314. 1958.

(23) Muhrer, M. E., Garner, G. B., Pfander, W. H., and O'Dell, B. L. The Effect of Nitrate on Reproduction and Lactation. J. Animal Sci., 15: 1291. 1956. (Abstr.)

(24) National Research Council. Nutrient Requirements of Domestic Animals. 3. Nutrient Requirements of Dairy Cattle. Natl. Acad. Sciences, National Research Council Publ. 464. 1958.

(25) Olson, D. E., Nelson, D. L., and Emerick, R. J. Effect of Nitrate and Some of Its Reduction Products on Carotene Stability. J. Agr. Food Chem., 11: 140. 1963.

(26) Sapiro, M. L., Hoflund, S., Clark, R., and Quin, J. I. Studies on the Alimentary Tract of Merino Sheep in South Africa 16. The Fate of Nitrate in Ruminal Ingesta as Studied in Vitro. Onderstepoort J. Vet. Sci. Animal Ind., 22: 357. 1949.

(27) Setchell, B. P., and Williams, A. J. Plasma Nitrate and Nitrite Concentration

in Chronic and Acute Nitrate Poisoning in Sheep. Australian Vet. J., 38: 58. 1962.

(32) SIMON, J., SUND, J. M., DOUGLAS, F. D., WRIGHT, M. J., AND KOWALCZYK, T. The Effect of Nitrate or Nitrite when Placed in the Rumens of Pregnant Dairy Cattle. J. Am. Vet. Med. Assoc., 135: 311. 1959.

(33) SINCLAIR, K. B., AND JONES, D. I. H. The Effect of Nitrate on Blood Composition and Reproduction in the Ewe. British Vet. J., 120: 78. 1964.

(34) STEWART, G. A., AND MERILAN, C. P. Effect of Potassium Nitrate Intake on Lactating Dairy Cows. Missouri Agr. Expt. Sta., Research Bull. 650. 1958.

(35) SUND, J. M., WRIGHT, M. J., AND SIMON, J. Weeds Containing Nitrates Cause Abortion in Cattle. Agron. J., 49: 278. 1957.

(36) SUTTON, T. S., KAESER, H. E., AND SOLDNER, P. A. Changes in the Level of Vitamin A and Carotene in the Blood Plasma of Dairy Cows Associated with Parturition and Beginning Lactation. J. Dairy Sci., 28: 933. 1945.

(37) THORP, F., JR. Further Observations on Oat Hay Poisoning. J. Am. Vet. Med. Assoc., 92: 159. 1938.

(38) UDALL, R. H., WARNER, R. G., AND SMITH, S. E. A Liver Biopsy Technique for Cattle. Cornell Vet., 42: 25. 1952.

(39) UNITED STATES DEPARTMENT OF AGRICULTURE. Regulations Governing the Meat Inspection of USDA, ARS, Meat Inspection Division. Regulation 18.7 (k). Washington, D. C. 1960.

(40) UNITED STATES PUBLIC HEALTH SERVICE. Drinking Water Standards, 1962. U. S. Public Health Service Publ. 956. Washington, D. C. 1962.

(41) WEICHENTHAL, B. A., EMBRY, L. B., EMERICK, R. J., AND WHETZAL, F. W. Influence of Sodium Nitrate, Vitamin A and Protein Level on Feedlot Performance and Vitamin A Status of Fattening Cattle. J. Animal Sci., 22: 979. 1963.

(42) WINTER, A. J. Studies on Nitrate Metabolism in Cattle. Am. J. Vet. Research, 23: 500. 1962.

(43) WINTER, A. J., AND HOKANSON, J. F. Effect of Long-Term Feeding of Nitrate, Nitrite, or Hydroxylamine on Pregnant Dairy Heifers. Am. J. Vet. Research, 25: 353. 1964.

(44) WRIGHT, M. J., AND DAVISON, K. L. Nitrate Accumulation in Crops and Nitrate Poisoning in Animals. Adv. Agron., 16: 197. 1964.

The Analyst - Vol 84 (1959)

10. Devlin, H. B., and Mattill, H. A., *J. Biol. Chem.*, 1942, 146, 123.
11. Emmerie, A., and Engel, C., *Rec. Trav. Chim. Pays-Bas*, 1939, 58, 283.
12. Kjölhede, K. Th., *Z. Vitaminforsch.*, 1942, 12, 138.
13. Kaunitz, H., and Beaver, J. J., *J. Biol. Chem.*, 1946, 166, 205.
14. Stern, M. H., and Baxter, J. G., *Anal. Chem.*, 1947, 19, 902.
15. Hove, E. L., and Hove, Z., *J. Biol. Chem.*, 1944, 156, 601.
16. Quaife, M. L., *Ibid.*, 1948, 175, 605.
17. Polister, B. H., *Anal. Chem.*, 1954, 26, 407.
18. Eggitt, P. W. R., and Norris, F. W., *J. Sci. Food Agric.*, 1956, 7, 493.
19. Weisler, L., Robeson, C. D., and Baxter, J. G., *Anal. Chem.*, 1947, 19, 906.
20. Green, J., and Marcinkiewicz, S., *Analyst*, 1959, 84, 297.
21. Brown, F., *Biochem. J.*, 1952, 51, 237; 1952, 52, 523.
22. Eggitt, P. W. R., and Ward, L. D., *J. Sci. Food Agric.*, 1953, 4, 176.
23. Green, J., Marcinkiewicz, S., and Watt, P. R., *Ibid.*, 1955, 6, 274.
24. Tosic, J., and Moore, T., *Biochem. J.*, 1945, 39, 498.
25. Green, J., *J. Sci. Food Agric.*, 1958, 9, 801.
26. Datta, S. P., Dent, C. E., and Harris, H., *Biochem. J.*, 1950, 46, xlii.
27. Watt, P. R., and Green, J., *Chem. & Ind.*, in the press.
28. Marcinkiewicz, S., and Green, J., *Analyst*, 1959, 84, 304.
29. Werner, E. A., *Ibid.*, 1933, 58, 335.
30. Hobson-Frohock, A., personal communication.
31. Mitchell, L. C., *J. Ass. Off. Agric. Chem.*, 1957, 40, 999.

A Polarographic Method for Determining Nitrate in Meat and Meat-curing Brines

449

By J. H. DHONT

(Central Institute for Nutrition and Food Research T.N.O., Utrecht, The Netherlands)

A polarographic method for determining nitrate is described; it is based on nitration of phenoldisulphonic acid and subsequent electrolytic reduction of the nitro compound at a dropping-mercury electrode, the diffusion current being proportional to the concentration of nitrate originally present. The effects of nitration time and interfering substances, such as chloride, nitrite and amino acids, the completeness of the nitration reaction and reproducibility have been investigated. The method is suitable for the determination of nitrate in brines contaminated with proteins.

The determination of nitrates in heavily contaminated solutions, such as meat-curing brines, is of special interest in the meat industry, but constitutes a difficult analytical problem.

The usual polarographic procedures involve the catalytic action of polyvalent cations, such as molybdenum[1] or uranyl ion,[2] on the reduction of nitrate ion. Ingram[3] used cerium^{III} ion for the polarographic determination of nitrate in brines. We found that organic compounds present in brines and meat extracts affected the polarographic wave obtained with molybdenum or uranyl ion. An irregular shift of the half-wave potential occurred, together with serious distortion of the reduction wave, and it was difficult or impossible to measure the diffusion current accurately.

Nitration of phenoldisulphonic acid results in the formation of 6-nitrophenol-2:4-disulphonic acid and an extremely small amount of picric acid.[4] This reaction forms the basis of many colorimetric procedures. It was shown that the nitro group of 6-nitrophenol-2:4-disulphonic acid was reducible at a dropping-mercury electrode and gave well defined waves, even in extremely acid solutions and in presence of organic material.

Experimental

Nitration reaction—

The nitration of phenoldisulphonic acid by small amounts of potassium nitrate was carried out in the following way.

A 5·0-ml portion of standard nitrate solution was placed, by pipette, in a small Pyrex dish and evaporated to dryness on a bath of boiling water. To the residue were added 2 ml of phenoldisulphonic acid reagent solution (see "Reagents," p. 374), and the dish was replaced

on the water bath for a few minutes. The contents of the dish were then transferred to a 10- or 25-ml calibrated flask, cooled to room temperature and made up to the mark with water. After the solution had been thoroughly mixed, it was transferred to the electrolysis cell of a polarograph, and, after oxygen had been removed by passing nitrogen through the solution, a polarogram was recorded between 0·0 and −0·7 volt against a mercury-pool anode.

A nitration time of 3 minutes is sufficient at the temperature of the bath of boiling water; longer nitration times, up to 30 minutes, have no effect on the results.

Experiments showed that the nitration product is stable for at least 1 hour. After 6 hours, the diffusion current is too low, as can be seen from the following results—

Time after completion of nitration, hours ..	0·5	6	24
Diffusion current, arbitrary units	56·0	53·0	52·0

The relationship between diffusion current and nitrate concentration is linear, at least between 6·2 and 124 mg of nitrate per litre. The equation of the line was $i = 4{\cdot}85\,c$, where i is the diffusion current in arbitrary units and c is the nitrate concentration in parts per million; the standard deviation of the constant was $\pm 0{\cdot}0455$.

Analytical experience suggests that a constant proportion of the nitrate reacts with phenoldisulphonic acid to form the nitro-derivative. Two methods were used to investigate the completeness of nitration. In one method, the nitro-derivative was formed from known amounts of potassium nitrate and was compared absorptiometrically with known amounts of 6-nitrophenol-2:4-disulphonic acid prepared by King's procedure.[5] In the other method, the nitro-derivative was formed in the same way and its electron uptake per mole was determined during reduction at a dropping-mercury electrode.

The absorptiometric measurements were made at 430 mμ with an Engel absorptiometer manufactured by Kipp, Delft, The Netherlands. The solutions of the nitro-derivative had a pH of 9·7.

The relationship between optical density and concentration was calculated from experiments in which small amounts of potassium nitrate were allowed to react with phenoldisulphonic acid and was found to be $d = 0{\cdot}2295\,c$, where d is the optical density and c is the concentration of nitrate in parts per million; the standard deviation of the constant was $\pm 0{\cdot}00630$.

The equation found for solutions containing known amounts of 6-nitrophenol-2:4-disulphonic acid was $d = 0{\cdot}2271\,c$; the standard deviation of the constant was $\pm 0{\cdot}00536$. These equations show that the values obtained in the two experiments are all on the same straight line. This indicates practically quantitative reaction between phenoldisulphonic acid and nitrate under the conditions described.

Coulometric measurement of the electron uptake confirmed this conclusion. The number of electrons, n, participating in the electrode reaction was determined by a millicoulometric method recently described by Weaver and Whitnack.[6] As the concentration of the compound that is reduced enters into the calculation, the concentration of potassium nitrate used to nitrate the phenoldisulphonic acid can be used instead to control the completeness of the reaction. Three experiments with potassium nitrate solutions between 0·0001 and 0·0040 N gave values of n equal to 5·8, 6·2 and 6·1.

The calculated value of n being 6, the reaction was assumed to be in accordance with the following equation—

$$RNO_2 + 6H^+ + 6e \rightarrow RNH_2 + 2\,H_2O.$$

The pH in these experiments was 0·9 to 1·1, as measured by a hydrogen electrode.

Here, too, quantitative reaction between nitrate and phenoldisulphonic acid can be assumed.

Interfering substances—

Chloride ion commonly interferes in nitration reactions. A series of experiments was carried out with a phenoldisulphonic acid reagent solution consisting of 4 g of phenol, 4 g of water and 100 ml of concentrated sulphuric acid. When this solution was used, it was found that the diffusion current was strongly depressed when chloride ions were present during nitration.

When the amounts of water in the phenoldisulphonic acid reagent and in the residue were decreased, the effect of chloride ion on the nitration reaction also decreased.

The true diffusion current occurred when the residue was heated for a few minutes at 110° to 115° C before the phenoldisulphonic acid reagent solution was added and when, at the same time, 10 g of phosphorus pentoxide were added to the reagent solution. The reaction between hydrochloric and nitric acids does not apparently proceed in absence of water.

The presence of nitrite causes irregular deviations in the polarogram; nitrite must therefore be removed from the sample. This can be accomplished by treatment with urea at a pH of about 3. After 30 minutes, 0·05 per cent. of nitrite in the sample can be completely removed under these conditions.

Added amino acids were found to have no effect. The solutions used in these experiments contained 50 mg each of tyrosine, phenylalanine and glutaminic acid and 47 μg of potassium nitrate per 10 ml.

Although tyrosine can be easily nitrated, no deviations were caused by the presence of the relatively large amount of this compound.

METHOD

APPARATUS—

A photographic recording Heyrovský-type polarograph manufactured by Laboratorni Pristroje, Prague, Czechoslovakia, was used.

REAGENTS—

Phenoldisulphonic acid reagent solution—Dissolve 4 g of crystalline phenol in 100 ml of concentrated sulphuric acid. When dissolution is complete, add 10 g of phosphorus pentoxide. The reagent is ready for use when the phosphorus pentoxide has dissolved; it may contain a slight precipitate, but this is of no importance.

Hydrochloric acid, 19 per cent.—Dilute 1 volume of hydrochloric acid, sp.gr. 1·19, with 1 volume of distilled water.

Lead acetate solution, 20 per cent. w/v, aqueous.

Urea solution, 6 per cent. w/v, aqueous.

Standard nitrate solution—Dissolve 0·200 g of potassium nitrate in water, and dilute to 100 ml in a calibrated flask.

PROCEDURE FOR BRINES—

Place 5 to 10 g of sample in a 250-ml calibrated flask, and add 1 ml of 6 per cent. w/v urea solution and 0·1 ml of 19 per cent. hydrochloric acid. Set aside for 1 hour at room temperature, and then dilute to the mark. Transfer 25 ml of the solution to a 50-ml calibrated flask, add 5 ml of 20 per cent. w/v lead acetate solution, dilute to the mark and filter.

Place 5 to 10 ml of the filtrate in each of two porcelain or borosilicate-glass dishes, and add 0·5 ml of standard nitrate solution to the contents of one dish. Evaporate the solutions to dryness on a bath of boiling water, heat the dishes for 3 to 5 minutes in an oven at 110° to 120° C, and allow to cool in a desiccator.

Add 2 ml of phenoldisulphonic acid reagent solution to each residue when cool, and place the dishes on a bath of boiling water for about 3 minutes. Carefully rinse the contents of each dish into a 10-ml calibrated flask with distilled water, and dilute to the mark when the solution has attained room temperature.

Pour the contents of the flask, including any precipitate that may have formed, into the electrolysis cell of a polarograph, and remove oxygen by passing a stream of nitrogen through the solution. Measure the diffusion current at 0·00 and −0·60 volt against the mercury-pool anode, and correct for the reagent blank value. Calculate the concentration of nitrate in the sample from the increase in diffusion current caused by the addition of the standard nitrate solution.

PROCEDURE FOR MEAT AND MEAT PRODUCTS—

Cut a 10-g sample into small pieces, and place in a 250-ml calibrated flask. Add 5 ml of 6 per cent. w/v urea solution and 0·5 ml of 19 per cent. hydrochloric acid, dilute to the mark with distilled water, and transfer the mixture to a Waring Blendor. Disintegrate the meat, and remove the suspended material by filtration. Place 40 ml of the filtrate in a 50-ml calibrated flask, add 5 ml of 20 per cent. w/v lead acetate solution, dilute to the mark, and filter. Continue as described under "Procedure for Brines," beginning at "Place 5 to 10 ml of the filtrate. . . ."

Discussion of Results

The height of the plateau of the polarographic wave obtained when phenoldisulphonic acid is nitrated in presence of brines and meat is affected by the presence of proteins, salts and fat; these constituents affect the viscosity and surface activity of the solution and may also influence the nitration reaction. However, when a standard addition of nitrate was made, as described, reproducibility was fairly good. Ten determinations on a solution containing 1·00 per cent. of potassium nitrate and 27 per cent. of sodium chloride gave a mean value of 1·006 per cent. of potassium nitrate with a standard deviation of ±0·039 per cent. Eight sets of duplicate determinations on meat extracts containing from 0·36 to 1·43 per cent. of potassium nitrate had a maximum relative error of 6 per cent.

The proposed method was used in a series of recovery experiments on known amounts of potassium nitrate added to 10-g samples of meat; the results were as follows—

Amount of potassium nitrate added, %	0·312	0·780	1·00
Amount of potassium nitrate found, %	0·298	0·770	1·09
Recovery, %	95·5	98·7	109

Table I

Recovery of added potassium nitrate from cover-brine

The sample of brine used contained 0·480 per cent. of potassium nitrate

Amount of potassium nitrate added, %	Total amount of potassium nitrate found, %	Amount of added potassium nitrate recovered, %	Recovery, %
2·22	2·66	2·18	99·0
1·11	1·57	1·09	99·0
	1·59	1·11	100·0
	1·52	1·04	104·0
1·00	1·422	0·942	94·2
	1·476	0·996	99·6

Another series of recovery experiments was carried out on known amounts of potassium nitrate added to a cover-brine containing 0·480 per cent. of potassium nitrate; the results are shown in Table I. A third series of recovery experiments was carried out on known amounts of potassium nitrate added to pump-brine containing 15 to 20 per cent. of sodium chloride; the results were as follows—

Amount of potassium nitrate added, %	1·00	1·00	1·50	0·950
Amount of potassium nitrate found, %	1·00	0·96	1·51	0·973
Recovery, %	100·0	96·0	99·4	102·3

The minimum amount of potassium nitrate that could be determined by the proposed method was 0·015 per cent.; in this instance, however, the relative error was 25 per cent.

I thank Dr. P. Hirsch-Ayalon and Mr. B. Krol for suggestions and advice during this investigation, and Miss J. Jongerden for carrying out many of the experiments.

References

1. Johnson, M. G., and Robinson, R. J., *Anal. Chem.*, 1952, 24, 366.
2. Kolthoff, I. M., Harris, W. E., and Matsuyama, G., *J. Amer. Chem. Soc.*, 1944, 66, 1782.
3. Ingram, M., *J. Soc. Chem. Ind.*, 1941, 60, 126.
4. Chamot, E. M., and Pratt, D. S., *J. Amer. Chem. Soc.*, 1910, 32, 630.
5. King, R., *J. Chem. Soc.*, 1921, 119, 2105.
6. Weaver, R. D., and Whitnack, G. C., *Anal. Chim. Acta*, 1958, 18, 51.

Received *January 12th*, 1959

THE PHYSIOLOGY OF NITRITE POISONING IN SHEEP*

R. H. Diven, R. E. Reed, W. J. Pistor
University of Arizona, Tucson, Ariz.

463

Earlier studies in this laboratory have suggested that sheep, after receiving nitrite, will respond to a subsequent nitrite treatment with lowered methemoglobin (MHb) levels.[1] Davison *et al.*[2] observed an erythropoietic adjustment to continuous nitrate feeding to cattle, and interpreted this as an adaptive response to the reduced oxygen-carrying capacity of the blood. Others[3] have questioned the importance of MHb formation as a lethal factor in nitrite poisoning in sheep. The following study was undertaken to determine the extent of an adaptive response to continuous nitrite administration upon MHb formation and the lethal level of nitrite, and to determine, in part, the significance of MHb formation as the lethal factor in nitrite poisoning.

Two mature ewes were injected intraperitoneally (ip) daily for 35 days with 0.71 mM. KNO_2/kg. body wt. Because of the difference in body weight, one ewe received a total daily injection of 32.9 mM. KNO_2, and the second was administered 23.5 mM.

On day 36 of the study, the two sheep that had received daily injections of nitrite, and a third sheep that had not previously received a nitrite injection, were administered (ip) 0.94 mM. of KNO_2/kg. body wt. On day 37, the two original ewes and a third ewe—not previously injected with nitrite—were treated with 1.06 mM. KNO_2/kg. body wt. Again, on day 38 of the study the two pretreated sheep were matched with a nonpretreated ewe and all three animals were treated with 1.18 mM. KNO_2/kg. body wt.

The nitrite administered to the sheep was prepared immediately before use as a five per cent solution. Jugular blood was collected for analyses at 0, 1, 2, 3, 5, and 7 hours after injection of the nitrite. This sampling procedure was followed for the initial seven days and thereafter on days 8, 11, 14, 21, 28, and 35. The same sampling procedure was followed when the daily nitrite dosage was increased on days 36, 37, and 38 with one exception—the final blood sample from those animals that died was collected when external respiration ceased, but the heart was still beating. All blood samples were analyzed immediately after collection for hemoglobin (Hb) (by the cyanmethemoglobin method), packed-cell volume, MHb[1,5] and plasma nitrite.[6]

The Hb and hematocrit values for the two sheep that received 0.71 mM. KNO_2/kg. body wt. daily for 35 days are reported in TABLE 1. Except for a suggested hemodilution that occurred during the initial 14 days of the study, the values remained relatively constant. The early hemodilution may have resulted from the frequent blood sampling—60 ml./day—or may have been a response to the onset of high environmental temperatures[7] just after the study commenced. These same factors may have prevented the occurrence of a hemoconcentration that would be expected in view of studies with cattle at Cornell.[2,8] In the latter studies, Hb had increased by approximately 28 per cent in five weeks in cattle that were fed 30 gm. NO_3 cwt. each day.

The relationship between the maximum percentage MHb formed each day of sampling and the number of days that the two sheep had received nitrite was analyzed statistically.[9] A test of homogeneity indicated that the observations from

* Arizona Agricultural Experiment Station Technical Paper No. 830.

TABLE 1

HEMOGLOBIN AND HEMATOCRIT VALUES FROM TWO SHEEP TREATED DAILY WITH IP INJECTIONS OF 0.71 MM KNO_2/KG. BODY WT.

Days on experiment	Hemoglobin (gm. %)		Hematocrit (vol. %)	
	Animal #1	Animal #2	Animal #1	Animal #2
1	11.3	10.8	36.0	38.0
2	8.4	9.0	29.0	31.5
3	8.7	9.3	27.5	30.5
4	8.2	9.2	25.0	28.0
5	9.0	9.8	28.5	31.5
6	8.0	8.4	24.5	28.0
8	7.5	8.4	26.0	30.0
11	7.9	8.4	27.0	27.0
14	8.0	9.3	27.5	32.0
21	9.3	9.0	32.0	31.5
28	9.9	9.7	32.0	32.0
35	8.7	8.2	30.5	29.0

the two animals could be combined to form a linear regression of −0.29. The variation due to the regression was significant at the 0.05 probability level. However, the linear correlation coefficient of the pooled MHb values with the number of days on treatment was only 0.42. Therefore, the amount of variation in the maximum MHb values that may be attributed to the passage of time is only 18.08 per cent. Expressing the MHb levels in terms of gms. per cent caused only insignificant changes in the curves that are shown in FIGURE 1.

The time after ip injection of nitrite at which MHb was observed to be at a maximum varied among sampling periods from one to three hours. The relationship between the hour of maximum MHb formation and the number of days that the sheep had been receiving nitrite did not differ from zero.

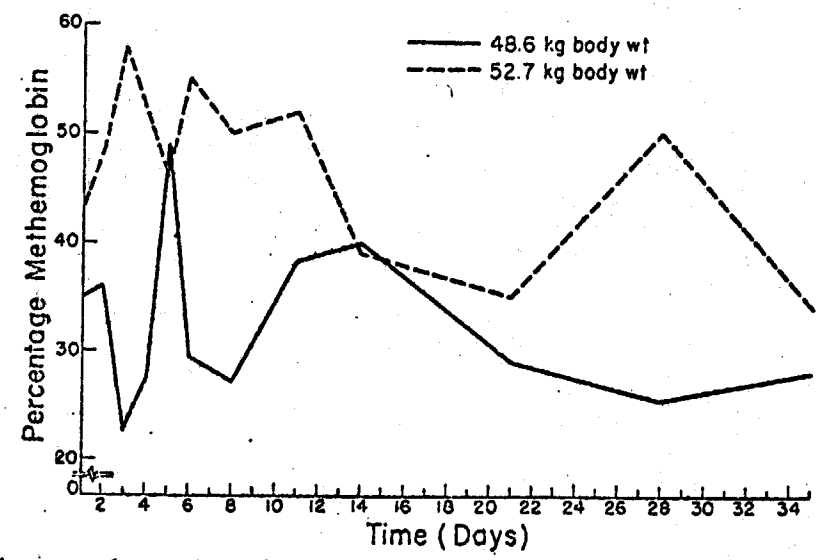


FIGURE 1. Changes in maximum percentage methemoglobin in two sheep that received 0.71 mM. KNO_2/kg. body wt.

Plasma nitrite was consistently higher in the first hourly postinjection sample than in any other subsequent sample. In the larger of the two ewes—treated with a total of 3[illegible] mM. nitrite daily—the first hour nitrite level for all sampling days ranged from 21.8 to 45.0 μmoles per cent. Similarly, the ewe that received 23.5 mM. of total nitrite had first hour plasma nitrite values ranging from 10.9 to 30.2 μmoles per cent. The second hour plasma nitrite values for the respective ewes ranged from 8.3 to 25.7 and from 2.4 to 14.4 μmoles nitrite.

Although the rate of absorption of nitrite from the peritoneal cavity into the circulation may vary, the extreme variation in levels of circulating nitrite seen here may reflect a physiological influence upon the formation of nitrate from the nitrite during oxidation of Hb.[10]

The 0.94 mM. KNO_2/kg. body wt. administered on day 36 to the two pretreated sheep and an additional control sheep had previously been reported as a minimum lethal level for sheep,[1] and was lethal to the nonpretreated sheep in the current study. The animal died at 2 hours and 19 minutes after nitrite injection with a MHb level of 87.3 per cent (FIGURE 2). The two pretreated sheep lived

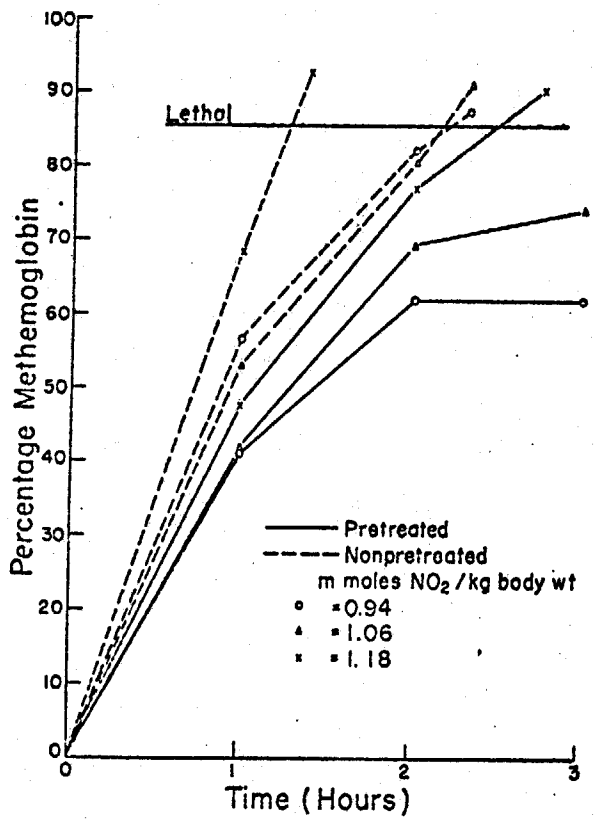


FIGURE 2. Percentage methemoglobin at hourly intervals and at time of death in sheep previously treated for 35 days with nitrite (average of two sheep) and sheep that had not previously received nitrite.

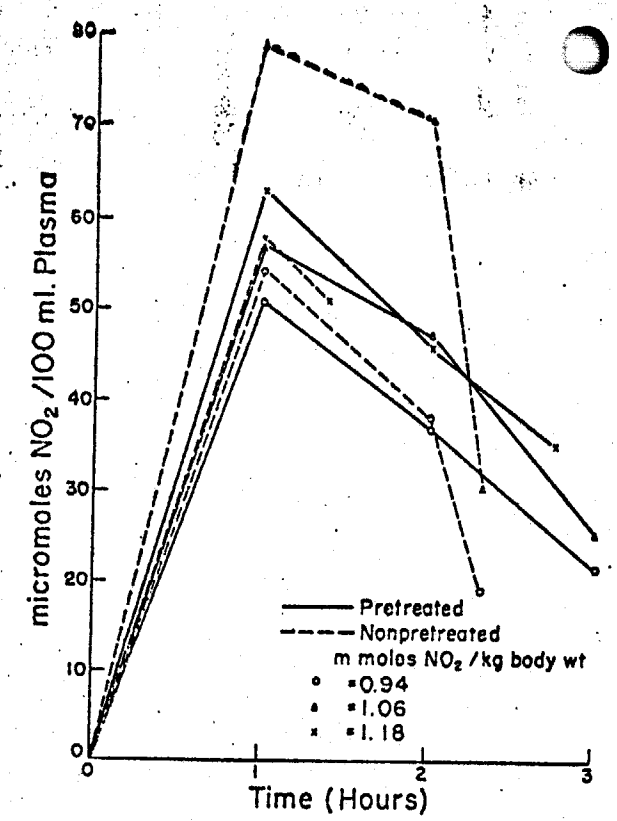


FIGURE 3. μmoles of plasma nitrite at hourly intervals and at time of death in sheep previously treated for 35 days with nitrite (average of two sheep) and sheep that had not previously received nitrite.

and developed maximum percentages MHb of less than 68. The nitrite poisoning symptoms that the pretreated sheep displayed at this and the previous level of nitrite treatment did not differ from the usual signs of sublethal intoxication.[11]

The reason for the effect of previous ip nitrite treatment upon subsequent nitrite lethality is not apparent. Others[1,2] have suggested that there is possibly an altered erythropoietic physiology that could result in an adaptation to nitrite. However, an increased availability of Hb due to a hemoconcentration associated with sustained nitrite treatment[8] did not occur in this study. The two pretreated sheep had fasting Hb values on day 36 of 9.0 and 9.1 gms. per cent, while the nonpretreated sheep's Hb was 11.1 gms. per cent.

As noted above (FIGURE 1), during the initial 35 days of the study, MHb values were higher for the sheep that received the most nitrite although nitrite was given on a body weight basis. When the lethal nitrite was administered, one pretreated sheep that lived and the nonpretreated sheep that died received identical amounts of total nitrite, while the other pretreated sheep that survived received 3.9 mM. more.

It is apparent that the two pretreated sheep had a reduced ability to form

MHb, and that the other suggested toxic actions of nitrite[3] did not cause death. Death in the sheep when MHb was 87.3 per cent was probably due to anoxia. This level of MHb is comparable to results obtained in dogs treated with p-aminopropiophenone,[12] in which death was believed to be due to anoxia resulting from methemoglobinemia.

The relative rates of nitrite disappearance from the plasma (FIGURE 3) is an interesting observation, although its significance is not clear without further study. Nitrite left the circulation of the pretreated sheep at the average rate of 0.26 μmoles/min. between the second and third hour after nitrite injection. Conversely, the rate of plasma nitrite disappearance in the nonpretreated ewe was 1.01 μmoles/min. between the second hour after injection and the time of death.

Similar results were obtained on day 37 of the study when the nitrite dosage was increased to 1.06 mM. KNO_2/kg. body wt. for two pretreated sheep and one nonpretreated sheep. The pretreated sheep survived, although their MHb levels reached as high as 77.0 and 72.6 per cent. The nonpretreated sheep died 2 hours and 20 minutes after nitrite administration with a MHb level of 91.4 per cent. At this level of treatment, the nonpretreated sheep, because of body weight, received less total nitrite than the pretreated sheep. As was the case with the minimum lethal level, 1.06 mM. of injected nitrite resulted in an accelerated rate of plasma nitrite disappearance in the nonpretreated animal (FIGURE 3).

The nonpretreated sheep in this experiment demonstrated the usual signs of fatal nitrite intoxication, but in the two pretreated sheep, response proceded as in the control animal only to the stage of prostration, which lasted several minutes. As the recovery period started, both sheep began reflex licking movements, protruding and withdrawing the tongue rapidly. This progressed to conscious but futile and somewhat desperate efforts to nibble straw while still prone in a lateral position. On assuming sternal recumbency, the sheep began dorsal flexion of the neck, the muzzle being tilted skyward. Soon after this phase, the animals gained their feet, though in a weak and wobbly state. Efforts to walk resulted in stumbling, high-stepping, and stamping. When they bumped into the fence the sheep bit at the wire, or stood and pushed the muzzle through openings. Within an hour and a half, except for weakness and exhaustion, the signs had passed. The similarity of this response to the nervous form of bovine ketosis was striking.

The nitrite treatment was increased to 1.18 mM. KNO_2/kg. body wt. on the last day of the study. The two pretreated sheep and one nonpretreated animal were used in the manner described above for the two days prior to this. One and eighteen-hundreths mM. of nitrite was lethal to all three sheep. MHb levels at the time of death varied from 88.0 to 92.7 per cent in the pretreated ewes, while the control ewe died when MHb reached 92.5 per cent. The only apparent effects of the previous nitrite treatment were the delay in the time of death, and a reduced rate of MHb formation in the pretreated animals (FIGURE 2). The nonpretreated sheep died at 1 hour and 23 minutes after nitrite injection, and the two pretreated ewes died at 2 hours and 34 minutes and 2 hours and 55 minutes after treatment (FIGURE 2). At this, the highest level of nitrite treatment, there was no difference between the pretreated and nonpretreated sheep in the rate of nitrite disappearance from the circulation (FIGURE 3).

Although pretreatment with nitrite produced a condition that increased the lethal levels of nitrite by a factor of 1.25 in sheep, MHb still appears to be the important lethal factor in nitrite poisoning.

References

1. DIVEN, R. H., R. E. REED & W. J. PISTOR. 1963. Maximum methemoglobin produced by nitrite in various animal species. Am. J. Physiol. (in manuscript).
2. DAVIDSON, D. L., WILLIAM HANSEL, M. J. WRIGHT & KENNETH MCENTEE. 1962. Adaptation to high nitrate intake by cattle. Proc. Cornell Nutr. Conf. Feed. Mfr. : 44–51.
3. HOLTENIUS, PAUL. 1957. Nitrite poisoning in sheep, with special reference to the detoxification of nitrite in the rumen. Acta Agr. Scand. 7: 113–163.
4. EVELYN, K. A. & H. T. MALLOY. 1938. Microdetermination of oxyhemoglobin, methemoglobin in a single sample of blood. J. Biol. Chem. 126: 655–662.
5. BODANSKY, OSCAR. 1951. Methemoglobinemia and methemoglobin-producing compounds. Pharmacol. Rev. 3: 144–196.
6. DIVEN, R. H., W. J. PISTOR, R. E. REED, R. J. TRAUTMAN & R. E. WATTS. 1962. The determination of serum or plasma nitrate and nitrite. Am. J. Vet. Res. 23: 497–499.
7. LICHT, SIDNEY. 1958. Therapeutic Heat. Elizabeth Licht. 360 Fountain St., New Haven, Conn.
8. WRIGHT, MADISON, J., W. K. KENNEDY, KENNETH MCENTEE, LENNART P. KROOK, WILLIAM HANSEL, WARREN G. MONSON, KENNETH L. DAVISON & R. F. CRAWOFRD. 1963. Summaries of research on nitrate accumulation and toxicity. Cornell Univ. Conference on Nitrate. New York, N. Y. April 15, 16. : 38–39.
9. OSTLE, BERNARD. 1954. Statistics in Research. Iowa State Coll. Press. Ames, Iowa.
10. LEMBERG, R. & J. N. LEGG. 1949. Hematin Compounds and Bile Pigments. Interscience Publishers, Inc. New York, N. Y.
11. DAVIDSON, W. B., J. L. DOUGHTY & J. L. BOTTOM. 1941. Nitrate poisoning of livestock. Can. J. Comp. Med. Vet. Sci. 5: 303–313.
12. VANDENBELT, J. M., C. PFEIFFER, M. KAISER & M. SIBERT. 1944. Methemoglobinemia after administration of p-aminoacetophenone and p-ammopropiophenone. J. Pharmacol. Exptl. Therapy. 80: 31–38.

G-1673-1
German Trsl.
DKD

490

SCREENING NITRITE FOR CHRONIC TOXICITY IN RATS

(Prüfung von Nitrit auf chronisch toxische Wirkung an Ratten)

Authors: H. Druckrey, D. Steinhoff, H. Beuthner, H. Schneider and P. Klärner
Source: Arzneimittel-Forschung, Vol. 13, April 1963, No. 4, Page 320-323.

1. Underlying Principles:

The extensive utilization of nitrite as food additive for meats, fish and recently for cheese also was accounted for so far only through the investigation of its acute and subacute toxicity. Results of research conducted in the last decase, however, have showed that extremely chronic poisoning effects exist which were described as effects of a second (or as a rule higher) type (Reference 1). They are characterized through higher time constants and thus appear not before a long latent period has elapsed. As far as the primary effects are irreversible, they can lead to permanent changes. Instances in this case are the mutagenic, teratogenic and carcinogenic effects. Hence, in toxicity tests, they must be taken especially into account (References 1, 2). The pertinent Commissions of the German Research Association (Reference 3), of the EUROTOX-Committees (Reference 4) and of the combined FAO/WHO Experts Committees (Reference 5) require the tests to be extend to the entire life duration of the experimental animals, inclusive of the 2nd generation of one animal species at least. In accordance with these directives, Na nitrite orally administered to rats was investigated. This appeared to be especially necessary, since nitrous acid in viruses proved to be intensely mutagenic (Reference 6). Furthermore, the observation of manifold carcinogenic effects in the case of dialkylnitrosamines (Reference 7) had to be taken into account, since these compounds can easily develop from the biologically widely spread organic amines through nitrositation.

The acute poisoning effects of nitrite, which in oral ingestion becomes rapidly resorbed and eliminated in the urine, are involved in the paralysis of vessels (blood pressure drop) and oxidation of the blood pigment to hemiglobin (methomoglobin). The two are typical "concentration effects", hence (according to the

dose involved) appear rapidly and after a short while are completely reversible. The formation of hemiglobin has been very thoroughly performed, since it is practically sole responsible for the lethal result of acute poisoning (Reference 8). In contrast with the vascular effect, the oxidation of the blood pigment must not be attributed to the nitrite anion $NO_2^{\ominus}$, but, as was already surmized (References 8, 9), to the nitrosyl cation $(NO)^{\oplus}$. In accordance with the H-ion concentration, this cation is formed according to the following equation (Ia):

(Ia) P. 320.

Already, however, when nitrite is added to blood in vitro, thus at pH 7.3, it rapidly produces hemiglobine; hence this might suggest a formation of $NO^{\oplus}$ according to Equation (1b):

(1b)

It is at the same time the nitrositing agent proper. It reacts with the hemoglobin Fe through the withdrawal of one electron:

(II)

Through its odd electron number (5 + 6 = 11), the remaining NO possesses a radical nature and thus is capabale of reaction. Through O it can become oxidized to NO_2 and with this it can yield N_2O_3, which in its turn (as nitrosylium nitrite) can form $NO^{\oplus}$ again:

(III)

The same path is possible from the nitrous acid also:

(IV)

On the other hand, one could also assume in the metabolism the direct oxidation of NO to $NO^{\oplus}$ through the donati n of one electron.

Experimentally, it was found that 1 mole of nitrite is able to form from 2 to 5 equivalents of hemiglobin (References 9, 10, 11). In vivo, however, this is not valid, because the hemiglobin formed is reconverted into hemoglobin through reducing substrates or through a specian enzyme even without any damaging of

the erythrocytes (References 12, 13) also in man (Reference 14). Hence, the maximum concentration, actually obtained, is substantially lower. Quantitative tests suggest an a yield of 0.0 (Reference 15) or 0.5 hemoglobin equivalents per mole of nitrite (Reference 16). The reduction depends on the metabolic situation. This reduction is slower in new born children, which still posses fetal hemoglobin (F) and in small children (Reference 17) who thus are especially sensitive to hemiglobin-forming poisons. The same is valid for cats (References 12, 15), whereas reduction in dogs (Ref. 13) and in rats (compare Figure 1) is somewhat as rapid. However, it is always much slower than the formation of hemiglobin through nitrite (compare the definition for the term "poison" in Druckrey, Reference 1).

Figure 1: Kinetics of the hemiglobin formation and reversion after the administration of 100 mg of Na nitrite per kg of body weight with an esophageal sound to BD rats. Curve 1: normal animals, with empty stomach. Curve 2: normal animals, fed. Curve 3: animal chronically treated with nitrite, with empty stomach. Ordinate: Hemiglobin concentration in per cent of the total hemoglobin.

Chronical toxicity tests with nitrite do not exist so far. Only H.L.A. Tarr and M.H. Carter (Reference 18) fed rats with a nitrite containing diet for a prolonged period. The duration, however, was limited to 168 days, and the daily dose of about 1.5 mg/kg was extremely low, so that the assumption adopted regarding the involved harmlessness as to health cannot be regarded as sufficiently justified in this case. Hence it was necessary to conduct investigations which, in accordance with the requirements mentioned at the beginning (References 3, 4 and 5) would extend over the entire life duration and include further following generations. As experimental animals, we selected rats of our BD stocks (Reference 19), because their normal biological data and properties (growth, propagation, life duration as well as diseases and spontaneous tumors) were known to us on the basis of long years of experimentation conducted with many 10,000 of animals. In order to prevent biased results, the collections were constituted of several BD stocks in uniform mixture, also from the sex ratio standpoint.

2. Acute Toxicity:

Of course, the chronical test must start from the acutely lethal dose. The literature data concerning rats exhibit surprizing differences. Whereas, on the one side, a LD_{50} of 10 to 20 mg/kg (subcutaneously) is indicated (Reference 20), on the other hand one finds values of 450 to 2,000 mg/kg (oral), as LD_{100} however (Reference 18). That is why we assumed the complete effective straight lines for the lethal doses of Na nitrite in aqueous solution in different forms of application in our BD rats. Without any exception, they exhibit a parallel trend of high steepness. The standard deflection was determined as $s = \pm 11\%$. We did not find any sex differences.

Table 1: Mean lethal doses of Na nitrite in BD rats as a function of the form of application, of the food intake and of the age.
a. Age b. State c. mean lethal dose d. 1 year e. 3 months f. new born
g. with empty stomach h. after being fed.

The mean lethal doses are grouped in Table 1. It is remarkable that new born rats, in contrast with the conditions in man, are considerably less sensitive than older rats. The LD_{50}, despite subcutaneous administration with 200 mg/kg, is almost double. This might be due to the fact that rats a short time after birth are still relatively less sensitive to the lack of O and only later become necessarily aerobic.

As a criterion of the extent of the resorption and of its rate as compared with that of the effect, we use the comparative determination of the effective doses in oral and in the iv administration. If the evening before, the rats had an empty stomach, the LD_{50} in the iv. administration with 65 mg/kg was only about 20% lower than after administration through esophageal sound (77 mg/kg). Hence, the nitrite resorption, when the stomach is empty, is not only practically complete, but it occurs at a speed that is high as compared as the starting of the effect. On the other hand, in normally fed rats, we found a considerable difference. The LD_{50}, in the case of administration with esophageal sound, amounted to 130 mg/kg as compared with 77 mg/kg in animals maintained with an

empty stomach. After the iv injection, on the contrary, it was 65 mg/kg in the two cases, that is equal, hence independent from the intake of food (Table 1). The difference rests thus only on a delayed resorption.

The course in time of the acute effect of nitrite can be followed easily by determining the hemiglobin concentration. This was done in the Zeiss-spectrophotometer (M4 Qq) at 630 mμ as well as after addition of cyanide at 546 mμ. Then the total hemoglobin was measured as cyanogen hemiglobin (Reference 21). In untreated rats, the hemiglobin concentration ranged between 0 and 3% of the total hemoglobin. The mean value was 0.8%. After a single administration of 100 mg of Na nitrite per kg of body weight with the esophageal sound, we obtained in young rats the concentration courses, rendered by Curve 1 and 2 in Figure 1, as mean values for 6 animals. Remarkable again is the considerable difference between the normally fed rats and the rats maintained with an empty stomach for 16 hours; this difference was already recognizable in the determination of the lethal doses (Table 1). In the two cases it amounts to about 1:1.7, that is, in rats with empty stomachs nitrite is half a time more poisonous. From this group, two animals died after 2 hours. The half value time for the increase of the hemiglobin concentration was 15 to 20 minutes, the one for the decrease was altogether about 220 minutes, hence about 20 times higher. The values agree well with those found by M. Kiese (Reference 13) in dogs. We then tested the reversibility of the toxic total effect. If the dose of 100 mg/kg was repeated after 2 hours, still 2 out 6 rats died. If on the other hand the time difference was 4 hours, all the animal always survived without any troubles.

3. Chronic Tests

a. Nitrite Alone:

In the chronic tests, Na nitrite was diven in drinking water. Since the intake was distributed over the entire day, and the acute effects proved to be rapidly reversible, it was possible to select a high dosage. We gave daily 100 mg/kg which were well tolerated by the animals. The treatment began at the age of about 70 days and was continued basically up to the natural death of the animals. Subsequently, a careful dissection was conducted with determination of the organ weights (wet) of the liver, kidneys and spleen. Suspicious tissues and

tumors were given to Professor H. Hamperl, Bonn, for histological examination. At this point, we thank him for his diagnoses.

In the parents (P) generation, 30 rats of the two sexes from different BD stocks were used. At the beginning the animals did not enjoy drinking the nitrite-containing water. The increase of the body weight was 11% lower than the values of the control animals. After a duration of treatment of 6 months, the animals were coupled for the first time with intentional hybridization. All the 15 couplings were fertile and gave 94 offsprings, hence 6.3 per litter. To this correspond the normal values of our rats. The young animals were normal in every regard. After being weaned from the mother, 30 of them were further treated in the same way as F_1 generation. The weight increase in this case corresponded fully to that of the controls. In 22 couplings, the F_1 rats provided altogether 149 offsprings, hence 6.7 per litter, which again were quite normal. Nevertheless, the tests were pursued with 34 of these animals in the F_2 generation. Hence 3 generations with altogether 94 rats were involved.

Table 2: Survey of chronic toxicity tests in rats with nitrite
a. Treatment, daily b. Number of animals c. Maximum total dose g/kg
d. Average life duration in days e. Tumors f. Day g. Type
h. Mamma fibroadenoma i. 2 sarcoma myel. leukemia sarcoma, lung
j. Myel. leukemia liver cell carcinoma cancer of the skin, head
3 mamma fribroadenomas k. Supra renal (adrenal) adenoma uterus myoma

A survey of the test and of their most important results is given in Table 2. In the evaluation of chronic toxicity tests, we consider the determination of the mean life duration as an essential criterion. This is so not only because a morphological and biochemical examination of all the organs and functions is not possible technically, but because there are also toxic damages which become manifest only through a shortening of the life duration. Moreover, our experiments revealed that only the maximum life duration can be regarded as hereditary characteristic, whereas the amount of average life duration is determined by the surrounding influences. Hence it can exhibit a considerable scattering. For the time being, for untreated rats of our BD stocks, it amounts on the average to 730 days, hence 2 years. In the 3 generations of our tests, on the other hand, we found only 630, 625 and 610 days (Table 2). Hence, no doubt,

there is a shortening of the mean life duration. Yet it is surprisingly low, if one takes into account the extremely high dosage of 100 mg/kg daily, that is the acute LD_{25}. It is rather astonishing that the animals tolerated these doses so long without any recognizable disturbances, although the sensitivity, on the basis of the height of the lethal doses (Table 1), increases considerably with the age, in rats. The maximum life duration was 940, 1065 and 970 days. It corresponds to the values in untreated control rats. The total doses, intaken during this time, of Na nitrite were 75 to 90 g/kg of body weight.

Dissection revealed in practically all the animals pneumonias and pulmonary abscesses, experimentally the most frequent cause of death in rats. The other organ findings corresponded to the age, and revealed no histological changes either (Professor H. Hamperl) to be interpreted in some way as signs of a chronically toxic damage. The tumors observed will be treated together at the end of this article.

After a test duration of about 18 months, the blood of the animals of the 2nd and 3rd generation was investigated. For the erythrocyte number and the hemoglobin concentration, mean values of 6.2 (5.5 to 6.9) million/mm^3 or 16.2 (12.8 to 19) g /100 ml were found. In the case of our rats, these values are quite normal. The white blood count, with otherwise normal figure ratios, exhibited a slight shift to the left; yet this shift could not be considered as pathological. Four hours after renewing the nitrite-containing drinking water, the hemiglobin concentration was detemmined. The values, with considerable scattering, were between practically 0 and 20% of the hemoglobin concentration had a mean value of 7% as compared with 0.8% in the controls (See x in Figure 1) or at 0.9 g/100 ml. Furthermore the kinetics of the hemiglobin formation and the involution in the chronically treated rats were investigated. Ten animals, 16 hours after the last food intake, received a dose of 100 mg/kg of nitrite with the esophageal sound. It was tolerated surprisingly well. The maximum of the hemiglobin concentration was definitely lower than in the untreated control animals which, however, had also an empty stomach (Figure 1, Curve 3). First of all the involution occurred significantly more rapidly with a half time of 3 hours as compared with 4 hours in the controls. A adaptation to the constantly increased formation of hemiglobin must be assumed. No doube, however, in this regard a chronically toxic injury can also be excluded.

b Nitrite and Amine Combination:

Organic amines are easily nitrosited in N through nitrous acid. In the resulting nitrosamines, different carcinogenic effects can be expected (Reference 7). Since amines are present as physiological constituents in food as well as in the body, it appeared to us that this possibility had to be taken into account.

Thirty rats of the F_1 generation from the previously described tests received, from the 30th day of life, 100 mg/kg of Na nitrite daily in drinking water, and at the same time 500 mg/kg of diethyl amine hydrochloride mixed with fresh minced meat. The treaement was tolerated without any disturbance and was pursued until natural death occurred. Propagation was undisturbed and gave quite normally offsprings. From them 37 were taken then as 2nd generation for testing purpose. This time, they were given the amine in the drinking water, and the nitrite in the meat, that is in inverse order as compared as in the previous test. The results of these two tests corresponded in evary way to those of the single administration of nitrite, and thus revealed no basic clues of chronic-toxic injuries.

c Tumors:

The swellings observed in the tests are compared in Table 2. Altoghether 11 tumors developed in the 94 rats treated with nitrite alone. The observed four fibroadenomas of the mamma can be neglected as mild tumors which are very frequent, according to experience. The total number of the malignant swellings then is 7 in 94 animals. Nevertheless, quite different tumors are involved, so that in this connection no causal relationship can be recognized.

It is difficult to evaluate the frequency of "spontaneous cancer". No doubt it is not a constant biological magnitude, but exhibits a remarkably high dispersion. It depends first of all and quite decisively on the age of the animals, and thus can only be evaluated in connection with this age. In accordance with our extensive experiments, the frequency of malignant spontaneous tumors in BD rats is on the average about 2% up to the 700th day of life, and then increases considerably. For later developed tumors, a connection with the treatment can be assumed only if

in its type, location and by the moment of its appearance it reveals a relationship with the nature of the substance tested and its dosage. No indication exists for this. Only 2 of the tumors observed appeared before the 700th day, a myeloic leukemia and a carcinoma of the liver. The two types of swelling are rarely "spontaneous" in the BE rats. If however nitrite had a causal significance, then with the extremely high dosage used, one would have had to expect an accumulation of these types of tumor in the further course of the test. This was not the case. Hence only so-called "spontaneous tumors" must be involved.

In the combination test with diethyl amine, only 3 mild tumores developed, and no real cancer, in 67 rats. Consequently one can exclude with certainty the development of diethyl nitrosamine, which, even with an extremely low dosage of 0.15 mg/kg results in carcinomas of the liver and of the esophagus still after about 610 days (Reference 22). Here it is necessary to make a reservation. The formation of nitrosamines through nitrous acid occurs not before a pH < 4 exists. Our own measurements of the pH in the stomach content of rats showed that this value is not reached even near the pylorus. On the other hand, in man much greater H ion concentrations exist, so that in this case the condintions for the development of $NO^{\oplus}$ from nitrite, and thus also for the nitrositing of amines, are offered as such. One must bear in mind, however, that the highly reactive $NO^{\oplus}$ according to Equation (II) reacts very rapidly and practically quantitatively with Fe II of the hemoglobin or myoglobin, and thus becomes detoxicated. This is why we consider reservations as to the usual and limited use of nitrite in meats in this regard also as unjustified. The situation is different as to fish, and especially as to cheese, because the detoxicating action of hemoglobin or myoglobin does not take place, so that here a development of nitrosamines appears to be possible. This important problem need careful examination. This can be done only in animal tests, because so far no sufficiently sensitive method for the chemical or physical detection of nitrosamines (except in pure solution) exists.

4. Evaluation of the Results:

The present investigations have showed that Na nitrite in the extremely high dosage of 100 mg/kg daily even if administered constantly through the entire life of rats in 3 generations exerts no chronically toxic effects. In 161 test animals and in more than 300 offsptings of the same, there were no indications of a

propagation-injuring, teratogenic or carcinogenic action, and this also if diethyl amine in high dosage was administered at the same time. Thus nitrite exerts only acute poisoning effects, which are rapidly reversible.

As food additive, nitrite is permissible exclusively in mixture with common salt (maximum concentration 0.6 g %). With this reservation, poisoning cases were not observed in man, except in misuses of pure nitrite. The nitrite content in processed food must not exceed 200 mg/kg (calculated as Na nitrite). Thus in the ingestion of 100 g of salt meat a maximum of 20 mg of nitrite would be absorbed. The recommendations of the "Joint FAO/WHO-Expert Committee on Food Additives" (Reference 23) foresee a maximum daily amount of up to 0.4 mg/kg of body weight and of up to 0.8 mg/kg under special conditions. Since in our tests a dose of 100 mg/kg, distributed over the entire, failed, even under constant administration, to provide indications of a chronic-toxic injury, a safety factor of about 200 would be involved.

On the other hand one must bear in mind, however, that nitrite exerts a vasodilator action already pharmacologically in doses of 0.5 to 1 mg/kg of body weight. In this regard, the safety gap is thus quite narrow. Thus a limitation of nitrite in food, for instance through the simultaneous utilization of ascorbic acid, should be welcome.-

English Summary P. 323.

H90

Arzneimittel-Forschung

drug research
MAY 22 1963
LIBRARY
PHARMACY AND BACTERIOLOGY LIBRARY
1955 NEIL AVENUE
MAY 23 1963

4

APRIL 1963
SEITE 245—346
13. JAHRGANG
ARZNEIM.-FORSCH.
DRUG RES.
EDITIO CANTOR KG.
AULENDORF/WÜRTT.

You will recall that we mentioned earlier the disappointing activity of the methyl sulfone group (XIII, b). The ionogenicity concept permitted us to explain that the acidic hydrogens of this compound were responsible for the relatively low activity. The replacement of these hydrogens by fluorines eliminated the acidic hydrogens and also increased the total electron withdrawing effect of the 2-substituent. The effect of this change on biological activity was dramatic.

In the methylsulfide (XIII, f) the hydrogens are not very acidic and hence the compound has good activity. Replacement of hydrogens by fluorines to give SCF_3 analogs resulted in some increase in activity.

Summary

In the broadly investigated phenothiazine series we have developed certain structure-activity correlations, on an empirical basis, which have proved useful in predicting the activity of new ring substituents and sidechains, as well as guiding us to the synthesis of new active compounds. Unfortunately these correlations have so far been found to have limited utility in areas outside the phenothiazines.

Zusammenfassung

Zusammenhänge zwischen Struktur und Wirkung einiger Phenothiazine

Unter den auf breiter Basis untersuchten Phenothiazinen zeigten sich bestimmte Relationen zwischen Struktur und Wirkung bei rein empirischer Beobachtung. Diese Ergebnisse erwiesen sich als brauchbar für die Vorausbestimmung der Wirkung neuer Ringsubstituenten und Seitenketten und waren auch wegweisend für die Synthese neuer wirksamer Verbindungen. Leider scheinen diese Zusammenhänge aber bis jetzt außerhalb des Bereiches der Phenothiazine nur begrenzt übertragbar zu sein.

For the Authors: Dr. Maxwell Gordon, Research and Development Division, Smith, Kline & French Laboratories, Philadelphia 1, Pa. (USA)

Aus dem Laboratorium (Leiter: Professor Dr. H. Druckrey) der Chirurgischen Universitäts-Klinik in Freiburg i. Br. (Direktor: Professor Dr. H. Krauss)

Prüfung von Nitrit auf chronisch toxische Wirkung an Ratten

490

Herrn Professor Dr. h. c. Hans von Euler zum 90. Geburtstag gewidmet

Von H. Druckrey, D. Steinhoff, H. Beuthner, H. Schneider und P. Klärner

1. Grundlagen

Die umfangreiche Verwendung von Nitrit als Lebensmittel-Zusatz für Fleischwaren, Fisch und neuerdings auch Käse war bisher nur durch die Untersuchung seiner akuten und subakuten Toxizität begründet. Forschungsergebnisse des letzten Jahrzehnts haben jedoch gezeigt, daß es auch extrem chronisch verlaufende Giftwirkungen gibt, die als Wirkungen 2. (oder allgemein höherer) Art bezeichnet wurden [1]. Sie sind durch entsprechend größere Zeitkonstanten charakterisiert und treten demgemäß erst nach langer Latenzzeit in Erscheinung. Soweit die Primäreffekte irreversibel sind, können sie zu bleibenden Veränderungen führen. Beispiele dafür sind die mutagene, die teratogene und die carcinogene Wirkung. Sie müssen daher bei Toxizitätsprüfungen besonders berücksichtigt werden [1, 2]. Die einschlägigen Kommissionen der Deutschen Forschungsgemeinschaft [3], des „EUROTOX-Comités" [4] und des gemeinsamen „FAO/WHO-Experten Comités" [5] fordern die Ausdehnung der Versuche über die ganze Lebensdauer der Versuchstiere unter Einschluß der 2. Generation an wenigstens einer Tierart. Nach diesen Richtlinien wurde Natrium-nitrit bei oraler Gabe an Ratten untersucht. Dies erschien besonders notwendig, da sich die salpetrige Säure an Viren als starkes Mutagen erwiesen hat [6]. Ferner war die Beobachtung vielseitiger carcinogener Wirkungen bei Dialkylnitrosaminen [7] zu berücksichtigten, da diese aus den biologisch weit verbreiteten organischen Aminen durch Nitrosierung leicht entstehen können.

Die akuten Giftwirkungen von Nitrit, das bei oraler Gabe schnell resorbiert und im Urin ausgeschieden wird, sind bedingt durch Lähmung von Gefäßen (Blutdrucksenkung) und Oxydation des Blutfarbstoffs zu Haemiglobin (Methaemoglobin). Beides sind typische „Konzentrationswirkungen", treten also (nach Maßgabe der Dosis) schnell ein und sind auch nach kurzer Zeit vollständig reversibel. Besonders gründlich untersucht ist die Bildung von Haemiglobin, weil sie praktisch allein für den tödlichen Ausgang der akuten Vergiftung verantwortlich ist [8]. Die Oxydation des Blutfarbstoffs ist indessen im Gegensatz zur Gefäßwirkung nicht dem Nitrit-Anion $NO_2^{\ominus}$ zuzuschreiben, sondern wie bereits vermutet wurde [8, 9], dem Nitrosyl-Kation $(NO)^{\oplus}$. Dieses wird nach Maßgabe der Wasserstoff-Ionen-Konzentration gemäß Gl. (Ia) gebildet:

$$HNO_2 + H^{\oplus} \rightleftarrows NO^{\oplus} + H_2O \qquad \text{(Ia)}$$

Da Nitrit jedoch schon bei Zugabe zu Blut in vitro, also bei pH 7,5, schnell Haemiglobin erzeugt, wäre auch an eine Bildung von $NO^{\oplus}$ nach Gl. (Ib) zu den-

Es ist zugleich das eigentliche nitrosierende Agens. Mit dem Haemoglobin-Eisen reagiert es durch Entzug eines Elektrons

$$Fe^{++} + NO^{+} \rightarrow Fe^{+++} + NO \qquad \text{(II)}$$

Das verbleibende NO besitzt durch seine ungerade Elektronenzahl (5 + 6 = 11) Radikalnatur und ist entsprechend reaktionsfähig. Es kann durch Sauerstoff zu NO_2 oxydiert werden und mit diesem N_2O_3 liefern, das seinerseits (als Nitrosylium nitrit) wieder $NO^{\oplus}$ zu bilden vermag:

$$NO + NO_2 \rightleftarrows N_2O_3 \rightleftarrows NO^{\oplus} + NO_2^{\ominus} \qquad \text{(III)}$$

Der gleiche Weg ist auch von der salpetrigen Säure her möglich

$$2\,HNO_2 \rightleftarrows N_2O_3 + H_2O \qquad \text{(IV)}$$

Andererseits wäre im Stoffwechsel auch die direkte Oxydation des NO zu $NO^{\oplus}$ durch Abgabe eines Elektrons denkbar.

Experimentell wurde gefunden, daß 1 Mol Nitrit 2 bis 5 Äquivalente Haemiglobin zu bilden vermag [9, 10, 11]. In vivo gilt das jedoch nicht, weil das gebildete Haemiglobin durch reduzierende Substrate bzw. durch ein spezielles Enzym auch ohne Schädigung der Erythrocyten wieder in Haemoglobin zurückverwandelt wird [12, 13], auch beim Menschen [14]. Daher liegt die maximale Konzentration, die tatsächlich erreicht wird, wesentlich niedriger. Auf Grund quantitativer Versuche wird eine Ausbeute von 0,9 [15] bzw. 0,5 Äquivalente Haemiglobin pro Mol Nitrit angegeben [16]. Die Reduktion ist abhängig von der Stoffwechsellage. Sie erfolgt langsamer bei Neugeborenen, die noch fetales Haemoglobin (F) besitzen und bei Kleinkindern [17], die daher gegen Haemiglobin-bildende Gifte besonders empfindlich sind. Das gleiche gilt für Katzen [12, 15], während die Reduktion bei Hunden [13] und bei Ratten (vgl. Abb. 1) in etwa gleicher Weise schneller verläuft. Stets aber erfolgt sie erheblich langsamer, als die Bildung von Haemiglobin durch Nitrit (vgl. die Definition für den Begriff „Gift" bei Druckrey [1].

Chronische Toxizitätsversuche mit Nitrit liegen bisher nicht vor. Lediglich H. L. A. Tarr u. M. H. Carter [18] haben Ratten über längere Zeit mit einer Nitrit-haltigen Diät gefüttert. Die Dauer war aber auf 168 Tage beschränkt, und die tägliche Dosis von etwa 1,5 mg/kg extrem klein, so daß die gefolgerte Annahme der gesundheitlichen Unbedenklichkeit damit nicht als hinreichend begründet gelten kann. Daher waren Untersuchungen erforderlich, die sich gemäß den eingangs erwähnten Forderungen [3, 4, 5] über die ganze Lebensdauer unter Einschluß weiterer Tochtergenerationen erstrecken. Als

Eigenschaften (Wachstum, Fortpflanzung, Lebensdauer sowie Krankheiten und Spontan-Tumoren) aus langjähriger Erfahrung an vielen zehntausend Tieren gut bekannt sind. Zur Vermeidung einseitiger Ergebnisse wurden die Kollektive aus mehreren BD-Stämmen in gleichmäßiger Mischung auch hinsichtlich des Geschlechtsverhältnisses zusammengestellt.

2. Akute Toxizität

Der chronische Versuch muß naturgemäß von der akut letalen Dosis ausgehen. Die in der Literatur vorliegenden Daten an Ratten weisen überraschende Unterschiede auf. Während einerseits eine LD_{50} von 10 bis 20 mg/kg (subcutan) angegeben wird [20], finden sich andererseits Werte von 450 bis 2000 mg/kg (oral), allerdings als LD_{100} [18]. Wir nahmen deshalb die vollständigen Wirkungsgeraden für die letalen Dosen von Natrium-nitrit in wäßriger Lösung bei verschiedenen Applikationsformen an unseren BD-Ratten auf. Sie zeigten ausnahmslos einen parallelen Verlauf von großer Steilheit. Die Standard-Abweichung wurde mit s = ± 11% bestimmt. Geschlechtsunterschiede fanden wir nicht. In der Tab. 1 sind die mittleren letalen Dosen zusammengestellt.

Tab. 1: Mittlere letale Dosen von Natrium-nitrit an BD-Ratten in Abhängigkeit von der Applikationsform, von der Nahrungsaufnahme und vom Lebensalter.

a Alter	b Zustand	c mittlere letale Dosis (mg/kg)		
		oral	intravenös	subcutan
1 Jahr	nüchtern	77	65	
	nach Futter	130	65	
3 Monate	nüchtern	110	105	
neugeboren	—			200

Auffällig ist zunächst, daß neugeborene Ratten im Gegensatz zu den Verhältnissen beim Menschen erheblich weniger empfindlich sind, als ältere. Die LD_{50} beträgt trotz der subcutanen Gabe mit 200 mg/kg fast das Doppelte. Das dürfte daran liegen, daß Ratten kurz nach der Geburt noch relativ wenig empfindlich gegen Sauerstoffmangel sind und erst später obligat aerob werden.

Als Kriterium für das Ausmaß der Resorption und ihrer Geschwindigkeit gegenüber der des Wirkungsablaufs benutzen wir die vergleichende Bestimmung der wirksamen Dosen bei oraler und iv. Gabe. Waren die Ratten am Vorabend nüchtern gesetzt, so lag die LD_{50} bei iv. Gabe mit 65 mg/kg nur etwa 20% niedriger, als nach Gabe durch Schlundsonde (77 mg/kg). Die Resorption des Nitrits erfolgt also bei leerem Magen nicht nur praktisch vollständig sondern auch mit einer Geschwindigkeit, die groß ist gegenüber der des Wirkungseintritts. Dagegen fanden wir bei normal gefütterten Ratten einen erheblichen Unterschied. Die LD_{50} betrug bei Gabe mit der Schlundsonde 130 mg/kg gegenüber 77 mg/kg bei nüchtern gehaltenen Tieren. Nach iv. Injektion war sie dagegen mit 65 mg/kg in beiden Fällen gleich, also unabhängig von der Nahrungsaufnahme (Tab. 1). Der Unterschied beruht danach lediglich auf einer verzögerten Resorption.

Der zeitliche Ablauf der akuten Wirkung des Nitrits läßt sich durch Bestimmung der Haemiglobin-Konzentration leicht verfolgen. Sie geschah im Zeiss-Spektrophotometer (M 4 Q 2) bei 630 mμ sowie nach Zugabe von Cyanid bei 546 mμ. Das Gesamt-Haemoglobin wurde dann als Cyan-Haemiglobin gemessen [21]. Bei unbehandelten Ratten lag die Haemiglobin-Konzentration zwischen 0 und 3% des Gesamt-Haemoglobins. Der Mittelwert betrug 0,8%. Nach einmaliger Gabe von 100 mg Natrium-nitrit pro kg Körpergewicht mit der Schlundsonde erhielten wir an jungen Ratten die in der Abb. 1, Kurve 1 und 2 wiedergegebenen Konzentrationsabläufe als Mittelwerte von je 6 Tieren. Auffällig ist wieder der erhebliche Unterschied zwischen den normal gefütterten und den seit 16 h nüchtern gehaltenen Ratten, der schon bei der Bestimmung der letalen Dosen (Tab. 1) erkennbar war. Er beträgt in beiden Fällen etwa 1 : 1,7, d. h. bei nüchternen Ratten ist Nitrit um mehr als die Hälfte giftiger. Aus dieser Gruppe starben 2 Tiere nach 2 h. Die Halbwertszeit für den Anstieg der Haemiglobin-Konzentration lag bei 15 bis 20 min, die für die Abnahme um (insgesamt) 220 min, war also etwa 10fach größer. Die Werte stehen in guter Übereinstimmung mit denen, die M. Kiese [13] an Hunden gefunden hat. Anschließend prüften wir die Reversibilität der toxischen Gesamtwirkung. Wurde die Dosis von 100 mg/kg nach 2 h wiederholt, starben noch 2 von 6 Ratten. War der Zeitabstand dagegen 4 h, so überlebten stets alle Tiere ohne nennenswerte Störungen.

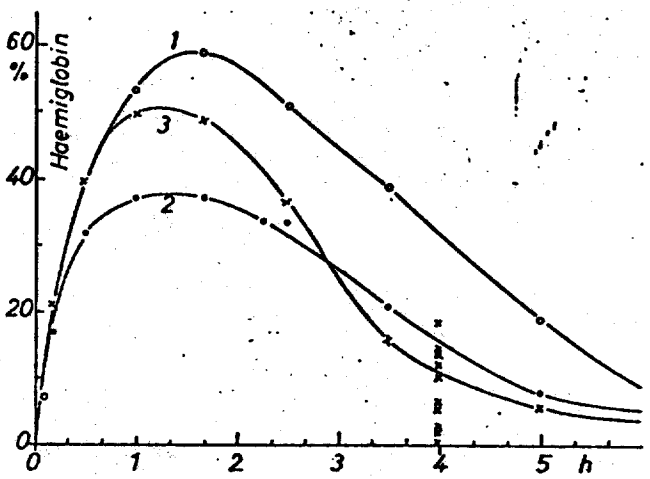


Abb. 1: Kinetik der Haemiglobin-Bildung und Rückbildung nach Gabe von 100 mg Natrium-nitrit pro kg Körpergewicht mit der Schlundsonde an BD-Ratten. Kurve 1: normale Tiere, nüchtern. Kurve 2: normale Tiere, gefüttert. Kurve 3: chronisch mit Nitrit behandelte Tiere, nüchtern. Ordinate: Haemiglobin-Konzentration in Prozent des Gesamt-Haemoglobin.

3. Chronische Versuche

a) Nitrit allein

In den chronischen Versuchen wurde das Natrium-nitrit im Trinkwasser gegeben. Da die Aufnahme dabei nicht zeitlos, sondern über den ganzen Tag verteilt erfolgte, und die akute Wirkung sich als schnell reversibel erwiesen hat, konnte die Dosierung hoch gewählt werden. Wir gaben täglich 100 mg/kg, die von den Tieren gut vertragen wurden. Die Behandlung begann im Alter von etwa 70 Tagen und wurde grundsätzlich bis zum natürlichen Tode der Tiere fortgesetzt. Anschließend erfolgte eine sorgfältige Sektion unter Bestimmung der Organgewichte (feucht) von Leber, Nieren und Milz. Verdächtige Gewebe und Tumoren wurden zur histologischen Untersuchung an Prof. H. Hamperl, Bonn, gegeben, dem wir auch an dieser Stelle für seine Diagnosen danken möchten.

In der Eltern-(P)-Generation wurden 30 Ratten beiderlei Geschlechts aus verschiedenen BD-Stämmen eingesetzt. Anfangs nahmen die Tiere das Nitrit-haltige Trinkwasser nur ungern. Die Zunahme des Körpergewichts lag 11% unter den Werten der Kontrolltiere. Nach einer Behandlungsdauer von 6 Monaten wurden die Tiere zum ersten Mal unter vorsätzlicher Hybridisierung verpaart. Alle 15 Verpaarungen erwiesen sich als fruchtbar und lieferten 94 Nachkommen, also 6,3 pro Wurf. Dem entsprechen die Normalwerte unserer Ratten. Die Jungtiere waren in jeder Hinsicht normal. 30 von ihnen wurden nach Absetzen von der Mutter als F_1-Generation in der gleichen Weise weiterbehandelt. Die Gewichtszunahme entsprach bei ihnen völlig der der Kontrollen. Die F_1-Ratten lieferten in 22 Verpaarungen insgesamt 149 Nachkommen, also 6,7 pro Wurf, die wiederum völlig normal waren. Trotzdem wurden die Versuche an 34 dieser Tiere in der F_2-Generation fortgesetzt. Sie umfassen damit 3 Generationen mit insgesamt 94 Ratten.

Eine Übersicht über die Versuche und ihre wichtigsten Ergebnisse ist in der Tab. 2 gegeben. Bei der Beurteilung chronischer Toxizitäts-Prüfungen sehen wir die Bestimmung der mittleren Lebensdauer als ein wesentliches Kriterium an. Dies nicht nur deshalb, weil eine morphologische und biochemische Untersuchung aller Organe und Funktionen technisch gar nicht durchführbar ist, sondern weil es auch toxische Schädigungen gibt, die

Tab. 2: Übersicht über chronische Toxizitäts-Versuche an Ratten mit Nitrit.

Behandlung, täglich	Generation	Tierzahl	maximale Gesamtdosis g/kg	mittlere Lebenszeit Tage	Tumoren Tag	Tumoren Art
100 mg/kg Na NO_2	P	30	75	630	970	Mamma-Fibroadenom.
	F_1	30	90	625	750	2 Sarkome
					820	myel. Leukaemie
					890	Sarkom, Lunge
	F_2	34	80	610	370	myel. Leukaemie
					470	Leberzell-Carcinom
					720	Hautkrebs, Kopf
					910	3 Mamma-Fibroadenome
Na NO_2 + Diäthylamin 100 mg/kg + 500 mg/kg	F_1	30	67 + 350	630	550	Nebenn.-Adenom
					910	Uterus-Myom
	F_2	37	65 + 300	625	700	Uterus-Myom

sich nur in einer Verkürzung der Lebensdauer manifestieren. Überdies haben unsere Erfahrungen ergeben, daß nur die maximale Lebensdauer als erbliches Merkmal angesehen werden kann, während die Größe der mittleren Lebenszeit von Umwelteinflüssen bestimmt wird. Sie kann daher eine erhebliche Streuung zeigen. Z. Z. beträgt sie bei unbehandelten Ratten unserer BD-Stämme im Durchschnitt 730 Tage, also 2 Jahre. In den 3 Generationen unserer Versuche fanden wir dagegen nur 630, 625 und 610 Tage (Tab. 2). Es liegt also zweifellos eine Verkürzung der mittleren Lebensdauer vor. Sie ist aber bei Berücksichtigung der extrem hohen Dosierung von 100 mg/kg täglich, das ist die akute LD_{25}, überraschend gering. Vielmehr ist es erstaunlich, daß die Tiere diese Dosen so lange ohne erkennbare Störungen vertragen haben, obwohl die Empfindlichkeit, beurteilt nach der Höhe der letalen Dosen (Tab. 1), bei Ratten mit dem Alter erheblich zunimmt. Die maximale Lebensdauer betrug 940, 1065 und 970 Tage. Sie entspricht den Werten bei unbehandelten Kontroll-Ratten. Die in dieser Zeit aufgenommenen Gesamtdosen von Natriumnitrit betrugen 75 bis 90 g/kg Körpergewicht.

Die Sektion ergab bei praktisch allen Tieren Pneumonien und Lungenabszesse, die bei Ratten erfahrungsgemäß die häufigste Todesursache darstellen. Die übrigen Organbefunde entsprachen dem Lebensalter und ließen auch histologisch (Prof. H. Hamperl) keine Veränderungen erkennen, die irgendwie als Zeichen einer chronisch toxischen Schädigung gedeutet werden konnten. Die beobachteten Tumoren sollen am Schluß im Zusammenhang behandelt werden.

Bei den Tieren der 2. und 3. Generation wurde nach einer Versuchsdauer von etwa 18 Monaten das Blut untersucht. Für die Erythrocytenzahl und die Haemoglobin-Konzentration ergaben sich Mittelwerte von 6,2 (5,5 bis 6,9) Millionen/mm³ bzw. 16,2 (12,8 bis 19) g/100 ml. Sie sind bei unseren Ratten völlig normal. Das weiße Blutbild zeigte bei sonst normalen Zahlenverhältnissen eine leichte Linksverschiebung, die jedoch ebenfalls nicht als pathologisch angesprochen werden konnte. 4 h nach Erneuerung des Nitrit-haltigen Trinkwassers wurde die Haemiglobin-Konzentration bestimmt. Die Werte lagen mit beträchtlicher Streuung zwischen praktisch 0 und 20% der Haemoglobin-Konzentration, mit einem Mittelwert von 7% gegenüber 0,8% bei den Kontrollen (s. Kreuze in Abb. 1) bzw. bei 0,9 g/100 ml. Daraufhin wurde die Kinetik der Haemiglobin-Bildung und Rückbildung an den chronisch behandelten Ratten untersucht. 10 Tiere erhielten 16 h nach der letzten Futteraufnahme eine Dosis von 100 mg/kg Nitrit mit der Schlundsonde. Sie wurde überraschend gut vertragen. Das Maximum der Haemiglobin-Konzentration (Abb. 1, Kurve 3) lag deutlich niedriger als bei unbehandelten aber ebenfalls nüchternen Kontrolltieren. Vor allem erfolgte die Rückbildung signifikant schneller mit einer Halbwertszeit von 3 h gegenüber 4 h bei den Kontrollen. Danach ist eine Anpassung an die ständig erhöhte Bildung von Haemiglobin anzunehmen. Sicher aber kann auch in dieser Hinsicht eine chronisch toxische Schädigung ausgeschlossen werden.

b) Kombination von Nitrit und Amin

Organische Amine werden durch salpetrige Säure z. T. leicht am Stickstoff nitrosiert. Bei den entstehenden Nitrosaminen ist mit vielseitigen carcinogenen Wirkungen zu rechnen [7]. Da Amine sowohl in der Nahrung als auch im Körper als physiologische Bestandteile vorkommen, hielten wir eine Berücksichtigung dieser Möglichkeit für notwendig.

30 Ratten der F_1-Generation aus dem vorher beschriebenen Versuch erhielten vom 30. Lebenstage an täglich 100 mg/kg Natrium-nitrit im Trinkwasser und gleichzeitig 500 mg/kg Diäthylamin hydrochlorid mit gehacktem Frischfleisch vermischt. Die Behandlung wurde ohne Störung vertragen und bis zum natürlichen Lebensende fortgesetzt. Die Fortpflanzung war ungestört und lieferte völlig normale Nachkommen. 37 von ihnen wurden dann als 2. Generation in Versuch genommen. Sie erhielten jedoch umgekehrt das Amin im Trinkwasser und das Nitrit im Fleisch. Die Ergebnisse dieser beiden Versuche entsprachen in jeder Weise denen bei alleiniger Gabe von Nitrit, lieferten also keine Anhaltspunkte für chronisch-toxische Schädigungen.

c) Tumoren

Die in den Versuchen beobachteten Geschwülste sind in der Tab. 2 zusammengestellt. Bei den mit Nitrit allein behandelten 94 Ratten entstanden insgesamt 11 Tumoren. Die beobachteten 4 Fibroadenome der Mamma können als gutartige Tumoren, die erfahrungsgemäß spontan am häufigsten vorkommen, außer Betracht bleiben. Die Gesamtzahl der bösartigen Geschwülste ist also 7 bei 94 Tieren. Es handelt sich jedoch um völlig verschiedenartige Tumoren, so daß in dieser Hinsicht kein Kausalzusammenhang erkennbar ist.

Die Häufigkeit von „Spontankrebs" läßt sich schwer beurteilen. Sicher ist es keine konstante biologische Größe sondern weist eine bemerkenswert hohe Streuung auf. Vor allem hängt sie ganz entscheidend vom Alter der Tiere ab und kann daher nur im Zusammenhang mit diesem beurteilt werden. Nach unseren umfangreichen Erfahrungen beträgt die Häufigkeit bösartiger „Spontantumoren" bei BD-Ratten bis zum 700. Lebenstage im Durchschnitt etwa 2% und nimmt dann erheblich zu. Für später entstandene Tumoren kann nur dann ein Zusammenhang mit der Behandlung angenommen werden, wenn sie nach ihrer Art, Lokalisation und dem Zeitpunkt ihres Auftretens eine Beziehung zur Natur der geprüften Substanz und ihrer Dosierung erkennen lassen. Dafür besteht hier kein Anhaltspunkt. Nur 2 der beobachteten Tumoren sind vor dem 700. Tage aufgetreten, eine myeloische Leukaemie und ein Carcinom der Leber. Beide Geschwulstarten sind „spontan" bei BD-Ratten selten. Wenn aber das Nitrit eine ursächliche Bedeutung hätte, so wäre bei der extrem hohen Dosierung eine Häufung dieser Tumorarten im weiteren Verlauf des Versuchs zu erwarten gewesen. Das war nicht der Fall. Es kann sich also nur um sogenannte „Spontantumoren" handeln.

Im Kombinationsversuch mit Diäthylamin sind bei 67 Ratten nur 3 gutartige Tumoren entstanden und kein echter Krebs. Danach kann eine Entstehung von Diäthylnitrosamin, das auch bei extrem geringer Dosierung von 0,15 mg/kg noch nach etwa 610 Tagen zu Carcinomen der Leber und Speiseröhre führt [22], mit Sicherheit ausgeschlossen werden. Hier ist jedoch eine Einschränkung notwendig. Die Bildung von Nitrosaminen durch salpetrige Säure erfolgt erst bei einem pH < 4. Eigene Messungen des pH im Mageninhalt von Ratten ergaben, daß dieser Wert auch in der Nähe des Pylorus nicht erreicht wird. Beim Menschen kommen dagegen weitaus höhere Wasserstoffionen-Konzentrationen vor, so daß hier die Bedingungen für die Entstehung von $NO^{\oplus}$ aus Nitrit und damit auch für die Nitrosierung von

Aminen an sich durchaus gegeben sind. Indessen ist zu berücksichtigen, daß das hoch reaktive $NO^{\oplus}$ nach Gl. (II) mit dem Eisen II des Haemoglobins oder Myoglobins sehr schnell und praktisch quantitativ reagiert und damit entgiftet wird. Deshalb halten wir Bedenken gegen die übliche und begrenzte Verwendung von Nitrit in Fleischwaren auch in dieser Hinsicht für nicht begründet. Anders liegen die Dinge aber für Fisch und vor allem für Käse, weil die entgiftende Wirkung von Haemoglobin oder Myoglobin entfällt, so daß hier eine Entstehung von Nitrosaminen möglich erscheint. Diese wichtige Frage bedarf einer sorgfältigen Prüfung. Sie kann nur in Tierversuchen erfolgen, weil es eine genügend empfindliche Methode für den chemischen oder physikalischen Nachweis von Nitrosaminen — außer in reiner Lösung — bisher nicht gibt.

4. Beurteilung der Ergebnisse

Die vorliegenden Untersuchungen haben gezeigt, daß Natrium-nitrit in der extrem hohen Dosierung von täglich 100 mg/kg auch bei dauernder Gabe über die ganze Lebenszeit von Ratten in 3 Generationen keine chronisch-toxischen Wirkungen hat. Insbesondere ergaben sich an 161 Versuchstieren und an mehr als 500 Nachkommen von ihnen keine Anhaltspunkte für eine Fortpflanzungsschädigende, teratogene oder carcinogene Wirkung, und zwar auch dann nicht, wenn gleichzeitig Diäthylamin in hoher Dosis gegeben wurde. Nitrit hat also nur akute Giftwirkungen, die schnell reversibel sind.

Als Lebensmittelzusatz ist Nitrit ausschließlich in Mischung mit Kochsalz (Höchstgrenze 0,6 g%) zulässig. Vergiftungsfälle am Menschen wurden unter dieser Einschränkung nicht beobachtet, sondern nur bei mißbräuchlicher Verwendung von reinem Nitrit. Der Nitrit-Gehalt im fertigen Lebensmittel darf 200 mg/kg (berechnet als Natrium-nitrit) nicht übersteigen. Bei Verzehr von 100 g gepökeltem Fleisch würden vom Menschen also maximal 20 mg Nitrit aufgenommen werden. Die Empfehlungen des „Joint FAO/WHO-Expert Committee on Food Additives" [23] sehen als Tages-Höchstmenge bis zu 0,4 mg/kg Körpergewicht vor und unter besonderen Bedingungen bis zu 0,8 mg/kg. Da in unseren Versuchen eine Dosis von 100 mg/kg, über den ganzen Tag verteilt gegeben, auch bei dauernder Aufnahme keine Anhaltspunkte für eine chronisch-toxische Schädigung ergeben hat, würde sich ein „Sicherheitsfaktor" von etwa 200 ergeben.

Auf der anderen Seite ist jedoch zu berücksichtigen, daß Nitrit pharmakologisch bereits in Dosen von 0,5 bis 1 mg/kg Körpergewicht als gefäßerweiterndes Mittel wirksam ist. In dieser Hinsicht ist die Sicherheitsspanne also nur gering. Deshalb wäre eine Einsparung von Nitrit in Lebensmitteln, z. B. durch gleichzeitige Verwendung von Ascorbinsäure, zu begrüßen.

Zusammenfassung

1. Natrium-nitrit wird im Hinblick auf seine Verwendung als Lebensmittelzusatz an BD-Ratten toxikologisch untersucht.
2. Die Größe der akut letalen Dosis hängt in starkem Maße vom Lebensalter und bei oraler Gabe von der Nahrungsaufnahme ab, weniger dagegen von der Applikationsart. Die Wirkung ist, beurteilt nach der Haemiglobin-Bildung, in wenigen Stunden vollständig reversibel.
3. Dauerversuche bei oraler Gabe von täglich 100 mg/kg Natrium-nitrit im Trinkwasser über die ganze Lebenszeit an 3 Generationen von Ratten ergaben weder im Blutbild und an den Organen noch an der Fortpflanzung oder den Nachkommen irgendwelche Anhaltspunkte für chronisch-toxische Schädigungen. Auch die Größe der mittleren Lebensdauer war trotz der extrem hohen Dosierung nur in geringem Maße verkürzt.
4. Die zeitlose Gabe von 100 mg/kg Natrium-nitrit führte an den chronisch behandelten Tieren zu einer signifikant geringeren Haemiglobin-Konzentration als bei den Kontrollen. Die Rückbildung erfolgte schneller.
5. Die kombinierte Behandlung mit täglich 100 mg/kg Natrium-nitrit und 500 mg/kg Diäthylamin-hydrochlorid, die wegen der Möglichkeit einer Entstehung von carcinogenen Nitrosaminen an 2 Generationen durchgeführt wurde, verlief ebenfalls ohne erkennbare Schädigungen.
6. Für eine carcinogene oder teratogene Wirkung wurden keine Anhaltspunkte gefunden.
7. Die Verwendung von Nitrit als Lebensmittelzusatz in den gesetzlich vorgeschriebenen Grenzen wird vom toxikologischen Standpunkt als duldbar angesehen.

Die Untersuchungen wurden durch die Deutsche Forschungsgemeinschaft ermöglicht. Die ausführliche Veröffentlichung erfolgt auf Wunsch des „Joint FAO/WHO Expert Commitee on Food Additives".

Summary

Screening of Nitrite for Chronic Toxicity in Rats

1. Sodium nitrite was subjected to toxicity tests in BD-rats in view of its utilisation as food additive.
2. The acute LD depends largely on the age of the animals, and, with oral administration, on their food intake, but less on the route of administration. Judging from hemiglobin formation, the effect is completely reversible after a few hours.
3. Sustained oral administration of 100 mg/kg daily of sodium nitrite in drinking water covering the entire life span of the rats during three generations, did not yield any evidence of chronic toxic impairment of the blood picture or organs, nor were progeny adversely affected. Despite the extremely high dosage, average life expectancy was only moderately reduced.
4. Sustained administration of 100 mg/kg sodium nitrite in the rats subjected to chronic tests led to a significant reduction in hemiglobin concentrations when compared with the controls. Reversal of this effect was more rapid.
5. Combined administration of 100 mg/kg sodium nitrite and 500 mg/kg diethylamine hydrochloride daily was tested on two generations in view of possible formation of carcinogenic nitrosoamines, but did not yield demonstrable results.
6. Evidence of possible carcinogenic or teratogenic effects was not found.
7. The utilisation of nitrite as food additive within legally prescribed limits is considered justified from the toxicity point of view.

Literatur

[1] Druckrey, H., Arzneim.-Forsch. 7, 449 (1957)
[2] Druckrey, H., Münch. med. Wschr. 98, 295, (1956)
[3] Farbstoff-Kommission der Deutschen Forschungsgemeinschaft „Mitteilung 5" (1954) und „Mitteilung 11" (1962)
[4] Berichte der Konferenzen in Bad Godesberg (1954) und Ascona (1957)
[5] World Health Organisation, Technical Report Series, Genf No 144 (1958) und 220 (1961)
[6] Kaudewitz, F., Z. Naturforsch. 14b, 528 (1959)
[7] Druckrey, H., Preussmann, R., Schmähl, D. u. Müller, M., Naturwiss. 48, 134 (1961)
[8] Heubner, W., Methaemoglobin bildende Gifte, Erg. Physiol. 43, 9 (1940)
[9] Meier, R., Arch. exp. Path. Pharmak. 110, 241 (1925)
[10] Greenberg, L. A., Lester, D. and Haggard, H. W., J. Biol. Chem. 151, 665 (1943)
[11] Lehman, A. J., Quart. Bull. Ass. Food Drug Off. 22, 156 (1958)
[12] Jung, F., Arch. exp. Path. Pharmak. 192, 464 (1939)
[13] Kiese, M., Klin. Wschr. 25, 453 (1947)
[14] Moeschlin, S., Klinik und Therapie der Vergiftungen, G. Thieme Verlag, Stuttgart 1952
[15] Issekutz, B. von, Arch. exp. Path. Pharmak. 195, 551 (1939)
[16] Kiese, M. u. Soetbeer, M., Arch. exp. Path. Pharmak. 207, 457 (1949)
[17] Betke, K., Dtsch. med. Wschr. 87, 909 (1962)
[18] Tarr, H. L. A. and Carter, M. H., J. Fish. Res. Board, Can. 6, 63 (1947)
[19] Druckrey, H., Danneberg, P., Dischler, W. u. Steinhoff, D., Arzneim.-Forsch. 12, 911 (1962)
[20] Spector, W. S., Handb. of Toxicology I, W. B. Saunders Co., Philadelphia 1955
[21] Havemann, R., Jung, F. u. von Issekutz, B., Bioch. Zschr. 301, 116 (1939); Fleisch, H., Helv. physiol. pharmacol. Acta 17, 318 (1959)
[22] Druckrey, H., Schmähl, D. u. Schildbach, A., Naturwiss. 49, 217 (1962)
[23] World Health Organis. Techn. Rep. Ser. 1962, 228

Für die Verfl.: Prof. Dr. H. Druckrey, Laboratorium der Chirurgischen Universitätsklinik, 78 Freiburg i. Br., Hugstetterstr. 55

Zeitschrift für Krebsforschung 66, 1—10 (1964)

492

Aus dem Laboratorium H. DRUCKREY der Chirurgischen Universitätsklinik Freiburg i. Br.
(Direktor: Prof. H. KRAUSS)
und dem Pathologischen Institut der Universität Bonn
(Direktor: Prof. H. HAMPERL)

Erzeugung von Krebs durch eine einmalige Dosis von Methylnitroso-Harnstoff und verschiedenen Dialkylnitrosaminen an Ratten*

Von

H. DRUCKREY, D. STEINHOFF, R. PREUSSMANN und S. IVANKOVIC

Mit 6 Textabbildungen

(Eingegangen am 8. Juli 1963)

In quantitativen Versuchen zur Krebserzeugung mit verschiedenen Agentien 4-Dimethylaminoazobenzol (DRUCKREY (1)), DRUCKREY und KÜPFMÜLLER (1), 4-Dimethylaminostilben [DRUCKREY u. Mitarb. (6)], Diäthylnitrosamin [DRUCKREY u. Mitarb. (5)], aromatische Kohlenwasserstoffe (DRUCKREY u. SCHMÄHL) und UV-Licht (BLUM)] bei dauernder Gabe an Ratten wurde zwischen der Höhe der Tagesdosis (d = mg/kg) und der Länge der mittleren Induktionszeit (t = Tage) bis zum Auftreten einer bestimmten Ausbeute an Carcinomen (50% = konst.) im beidseitig log.-Feld eine lineare Abhängigkeit gefunden. Sie entsprach der Formel

$$d\,t^n = \text{konst.} \qquad (1)$$

wobei der Exponent n fast stets zwischen 2 und 3 lag (DRUCKREY u. SCHMÄHL). Das Ergebnis wurde inzwischen von uns an anderen Beispielen reproduziert. Die Gültigkeit der Beziehung nach Gl. (1) erwies sich als so streng, daß die Induktionszeiten nach vorheriger Ermittlung der Größe von n vorausberechnet und mit überraschender Genauigkeit experimentell bestätigt werden konnten (DRUCKREY u. SCHMÄHL). Nach Gleichung (1) wächst die Wirkung bei dauernder Gabe konstanter Dosen des Carcinogens etwa mit dem Quadrat der Expositionszeit an, entspricht also einem beschleunigten Vorgang (BLUM). Dieser neuartige Wirkungstyp wurde zuerst in „Stop"-Versuchen beobachtet [DRUCKREY (2)] und als „Verstärkerwirkung" bezeichnet [DRUCKREY u. KÜPFMÜLLER (2)]. Seine Begründung sehen wir darin, daß die carcinogene Wirkung auf irreversiblen Veränderungen an vermehrungsfähigen Einheiten (Duplikanten, Zellen) beruht. Nach Gabe des carcinogenen Dimethylnitrosamins an Ratten konnten P. N. MAGEE u. E. FARBER (1962) in intakten Nucleinsäuren speziell des betroffenen Organs, nämlich der Leber, eine Methylierung von Guanin nachweisen und aus der RNS das N (7)-Methylguanin isolieren.

Wenn die Carcinogenese tatsächlich gemäß Gl. (1) ein beschleunigter Vorgang ist, dann müßte es möglich sein, sie auch durch einen einzigen „Impuls" genügender Stärke zu „starten". Dafür gibt es bereits experimentelle Beispiele. C. HUGGINS u. Mitarb. (1961) gelang die regelmäßige Erzeugung von Brustkrebs durch eine einmalige Gabe von Methylcholanthren mit der Schlundsonde. Dies ist allerdings nur an jungen Sprague-Dawley-Ratten möglich. An neugeborenen

* Die Arbeiten wurden durch die Deutsche Forschungsgemeinschaft ermöglicht.

Swiss-Mäusen konnten L. Fiore-Donati u. Mitarb. (1961) mit einer einzigen s.c. Injektion von 2 mg Äthylurethan in nur etwa 110 Tagen außer Lungen-Adenomen auch 13/60 maligne Lymphome erzeugen. P. N. Magee u. J. Barnes (1959 u. 1962) beobachteten nach einmaliger Gabe von 30 mg/kg Dimethylnitrosamin mit der Schlundsonde bei 3 von 15 Ratten anaplastische Tumoren der Niere, während die gleiche Substanz täglich gefüttert nur Leberkrebs erzeugt. Schließlich berichtete R. Schoenthal (1960) über ein Carcinom des Vormagens nach einer einzigen Dosis von 50 mg/kg Methyl-nitroso-urethan bei allerdings nur einer Ratte.

Versuche

Wir haben im Rahmen unserer systematischen Untersuchungen mit Nitrosaminen während der letzten Jahre auch orientierende Versuche mit einmaliger Gabe oder kurzdauernder Behandlung bei verschiedenen Applikationsformen (oral, intravenös, subcutan und Inhalation) an Ratten durchgeführt. Dabei erhielten wir eine ganze Anzahl maligner Tumoren, jedoch nicht nur der Nieren sondern auch Carcinome der Leber, Speiseröhre, Ovarien und Nasennebenhöhlen. Die wichtigsten Ergebnisse sind in der Tabelle 1 zusammengestellt. Ihre histologischen Diagnosen verdanken wir Prof. H. Hamperl, Bonn. Danach kann offenbar jede Art von Krebs durch eine einzige Dosis erzeugt werden.

Tabelle 1. *Übersicht über die Erzeugung von Tumoren durch einmalige oder kurzdauernde Behandlung mit verschiedenen Nitrosaminen bei Ratten*

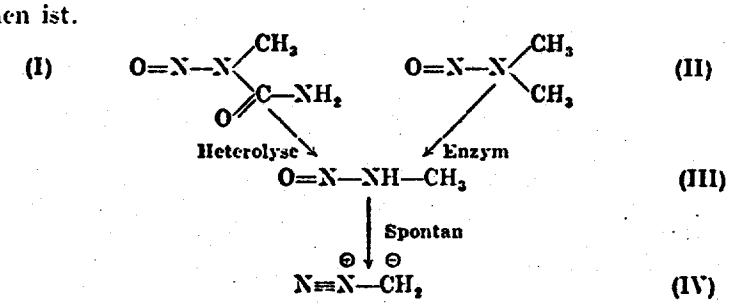
a Substanz	b Dosierung, mg/kg Applikation	c Anzahl der Tumoren bei überlebenden Ratten		d Nach Tagen
Dimethyl-nitrosamin . . .	1 × 37, Inhalation	Ethmoidcarcinom	1/3	300
Diäthyl-nitrosamin	1 × 280, intravenös	Nierenadenom	2/4	310
		Nierencarcinom	2/4	340
		Ovarialcarcinom	1/4	330
	1 × 280, oral	Lebercarcinom	1/3	485
	4 × 25, oral	Nierencarcinom	1/2	490
	4 × 35, oral	Nierenadenom	1/2	500
		Leberadenom	1/2	528
		Oesophaguscarcinom	1/2	520
Methyl-vinyl-nitrosamin .	1 × 44, Inhalation	Nasenhöhlencarcinom	1/1	270
Äthyl-äthanol-nitrosamin .	10 × 200, oral	Nierenadenom	3/4	630
		Nierencarcinom	1/4	695
		Ovarialcarcinom	1/4	645
		Lebercarcinom	1/4	646
Äthyl-butyl-nitrosamin . .	1 × 370, oral	Nierensarkom	1/4	375
Nitrosomorpholin	6 × 30, oral	Nierencarcinom	1/9	540
		Hepatom	1/9	590
		Ovarialcarcinom	2/9	600

Der strenge Beweis für das Vorliegen einer „Verstärkerwirkung" bzw. eines beschleunigten Vorgangs nach Gl. (1) wäre jedoch erst dann gegeben, wenn gezeigt werden kann, daß auch eine sehr kurzlebige Substanz mit einer einzigen Dosis regelmäßig Krebs erzeugt.

Für unsere Versuche wählten wir den N-Methyl-N-nitrosoharnstoff (I), über dessen carcinogene Wirkung wir früher berichtet hatten [Druckrey u. Mitarb. (4)].

Bei täglicher oraler Gabe an Ratten erzeugte er vorwiegend Plattenepithelkarzinome des Vormagens.

Die Substanz wurde durch Umkristallisieren gereinigt ($F = 124°$ C). Sie ist sehr instabil. Schon bei leicht alkalischer Reaktion wird der Acyl-Rest hydrolytisch abgespalten (p_H 8: $t_{50} = 30$min), so daß über das Monomethylnitrosamin (III) das hoch reaktive Diazomethan (IV) entsteht. Die „Wirkformen" sind also die gleichen, die auch aus dem N,N-Dimethylnitrosamin (II) gebildet werden, dessen methylierende Wirkung auf Nucleinsäuren in vivo von P. N. MAGEE und E. FARBER (1962) nachgewiesen wurde. Im Körper erfolgt eine schnell verlaufende Spaltung des Methyl-nitroso-Harnstoffs (vgl. S. P. KRAMER u. Mitarb., 1959), so daß in wenigen Minuten ein sehr weitgehender Abbau anzunehmen ist.



In den vorliegenden Versuchen gaben wir den Methylnitrosoharnstoff (MNH) intravenös in die Schwanzvene von Ratten. Die mittlere letale Dosis betrug 108 mg/kg gegenüber 110 mg/kg bei Gabe mit der Schlundsonde. Die Übereinstimmung deutet daraufhin, daß MNH trotz seiner Instabilität eine für unsere Versuche ausreichende Lebensdauer besitzt. Im Blut wird MNH nur relativ langsam hydrolysiert. Dabei kommt es zur Oxydation des Hämoglobins, ähnlich wie bei Diazoessigester ([DRUCKREY u. Mitarb. (2)].

Das Vergiftungsbild, das nach oraler Gabe des MNH hämorrhagische Schädigungen des Magens und Darms aufwies [DRUCKREY u. Mitarb. (4)], war nach der intravenösen Injektion durch eine schwere Schädigung des Knochenmarks und lymphatischen Gewebes beherrscht. Im Verlauf von 8 Tagen nach der Injektion der LD 50 entwickelte sich eine schwere Leukopenie (mit Werten unter 1000 Zellen) und Anämie, die am den 12. Tag zum Tode führten. Im Kot war reichlich Blut nachweisbar, nicht aber im Urin. Bei der Sektion fanden sich aber ebenfalls Hämorrhagien und Ulcerationen im Magen-Darm-Kanal. Die nicht gestorbenen Ratten erholten sich unter Behandlung mit Penicillin-Streptomycin sowie Eisen schnell.

In den Versuch kamen im ganzen 16 Tiere aus verschiedenen BD-Stämmen. Neun von ihnen hatten eine Dosis von 100 mg MNH pro Kilogramm Körpergewicht injiziert bekommen und die sieben restlichen Ratten 70 mg/kg. Sie wurden unter sorgfältiger Pflege bis zum natürlichen Lebensende beobachtet und dann gründlich seziert und histologisch untersucht. Da beide Gruppen keine irgendwie charakteristischen Unterschiede erkennen ließen, werden sie gemeinsam behandelt.

Am 275. Tage nach der Injektion starben die ersten zwei Ratten aus der Gruppe, die die schwächere Dosierung von 70 mg/kg erhalten hatte. Beide zeigten

mehrere Papillome des Vormagens und eines von ihnen eine etwa 1 g schwere, weiß fleischige Geschwulst der linken Niere, die histologisch als Nierensarkom diagnostiziert wurde. In kurzer Folge starben 14 Ratten und in einem etwas größeren Zeitabstand die letzten zwei. Alle Tiere hatten Tumoren.

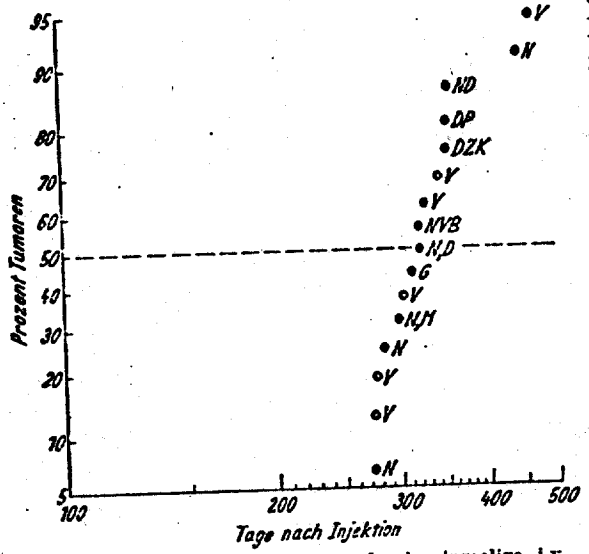


Abb. 1. Erzeugung von Tumoren durch einmalige i.v. Injektion von 70 bzw. 100 mg/kg Methylnitrosoharnstoff an BD-Ratten. ● Carcinom oder Sarkom; *N* Niere; *V* Vormagen; *M* Magen; *Z* Zunge; *K* Kiefer; *D* Darm; *B* Brustdrüse; *G* Gehirn; *P* Plasmocytom; ○ Vormagen-Papillome

Das zeitliche Auftreten der Geschwülste und ihre Verteilung auf die einzelnen Organe wurden in ein Wahrscheinlichkeitsnetz eingetragen (Abb. 1). Für die Latenzzeit bis zum Tode an der Geschwulst ergibt sich ein Mittelwert von 320 ± 40 Tagen nach der einmaligen Injektion. Die Lage der einzelnen Punkte im Wahrscheinlichkeitsnetz entspricht nahezu einer Geraden. Es liegt also praktisch eine Normalverteilung vor. Danach kann der Kausalzusammenhang als gesichert gelten.

Vier Ratten hatten lediglich gutartige Papillome und Hyperkeratosen des Vormagens. Bei den anderen 12 Ratten fanden sich ausnahmslos maligne Tumoren und zwar in den verschiedensten Organen, in sechs Fällen sogar am gleichen Tier. MNH erweist sich damit als ein starkes und bemerkenswert vielseitiges Carcinogen.

Tabelle 2.

Verteilung der Tumoren bei 16 Ratten, die einmalig eine intravenöse Injektion von 70—100 mg/kg Methylnitroso-Harnstoff erhalten hatten. Vier Ratten hatten nur Vormagen-Papillome

Lokalisation	Papillome	Sarkome	Carcinome
Gehirn		1	
Zunge			1
Kiefer			2
Vormagen	10		4
Drüsenmagen		1	
Dünndarm			2
Dickdarm			3
Niere		7	1
Mamma			
Subcutis		1	
Plasmocytom		1	
Summe	10	11	13

Die Verteilung der Tumoren auf die einzelnen Organe ist in der Tabelle 2 noch einmal übersichtlich zusammengestellt. Ähnlich wie in den Versuchen von P. N. Magee und J. M. Barnes (1962) mit Dimethylnitrosamin bei einmaliger Gabe stehen auch hier die malignen *Nieren*-Tumoren im Vordergrund. Sie traten bei 7 Ratten auf, in 2 Fällen beidseitig, sämtlich bei weiblichen Tieren. Ihre Größe lag meist bei 1—3 g, teils waren es aber auch riesige Geschwulstbildungen, die bis zu 50 g wogen. Makroskopisch erschienen sie weißlich, fleischig, oft etwas glasig und mit hämorrhagischen Partien durchsetzt (Abb. 2). Die histologische Diagnose ergab unreife *Sarkome* mit spindelförmigen Zellen und reichlich Mitosen. Bei unseren Fällen traten bei Nitrosomethylharnstoff nur unreife Sarkome auf, gelegentlich auch Mischformen

(Carcinosarkome oder eventuell nach Art der Wilms-Tumoren), jedoch keine rein epithelialen Nierengeschwülste. MAGEE und BARNES dagegen beobachteten nach Gaben von Dimethylnitrosamin sowohl Carcinome wie Sarkome. Die Frage, ob hier wirklich eine selektive Wirkung vorliegt, muß in weiteren Versuchen geklärt werden.

Nicht weniger auffällig ist die Häufigkeit von Tumoren des *Vormagens*. Papillome fanden wir bei 10 von den insgesamt 16 Ratten. Meist lagen sie multipel vor mit 1—2 mm Durchmesser (Abb. 3) und zwar stets vergesellschaftet mit Hyperkeratose. Vier Ratten hatten Carcinome des Vormagens (Abb. 4), die histologisch als infiltrativ wachsende, zum Teil stark verhornende Plattenepithel-Carcinome diagnostiziert wurden. Eine dieser Ratten hatte zusätzlich ein Spindelzell-Sarkom in der Wandung des Drüsenmagens.

Abb. 2. Großer maligner Nierentumor von Sarkom-Typ bei einer weibl. Ratte, 322 Tage nach einmaliger i.v. Injektion von 100 mg/kg Methyl-nitroso-Harnstoff

Tumoren des Vormagens hatten wir bereits nach oraler Gabe von MNH beobachtet [DRUCKREY u. Mitarb. (4)], niemals aber als „Spontangeschwülste". Da

Abb. 3

Abb. 4

Abb. 3. 4494, ♂, NO-CH_3-Harnstoff 1 × 100 mg/kg i.v. Papillom des Vormagens, 355 Tage nach 1 × 100 mg/kg Methyl-nitroso-Harnstoff i.v.

Abb. 4. 3988, ♂, NO-CH_3-Harnstoff. Ausgedehnter Plattenepithelkrebs des Vormagens, 350 Tage nach 1 × 100 mg/kg Methyl-nitroso-Harnstoff

sie nun hier auch nach intravenöser Injektion des Carcinogens mit ähnlicher Ausbeute beobachtet wurden, können die damaligen Befunde nicht auf eine nur lokale und direkte Wirkung allein bezogen werden. Vielmehr ist zumindest auch eine resorptive Wirkung auf das Vormagen-Epithel anzunehmen. Es sei vermerkt, daß bei keinem Tier ein Krebs des Oesophagus beobachtet wurde, während die speziell diesen Krebs erzeugenden (vor allem unsymmetrischen) Dialkylnitrosamine [Druckrey u. Mitarb. (3)] wiederum den Vormagen stets frei ließen. Die carcinogene Wirkung der Nitrosamine weist also eine überraschende Selektivität auf, für die einstweilen keine Begründung gegeben werden kann. Die Befunde zeigen indessen, daß der Vormagen von Ratten nicht ohne weiteres als „verlängerter Oesophagus" angesehen werden kann.

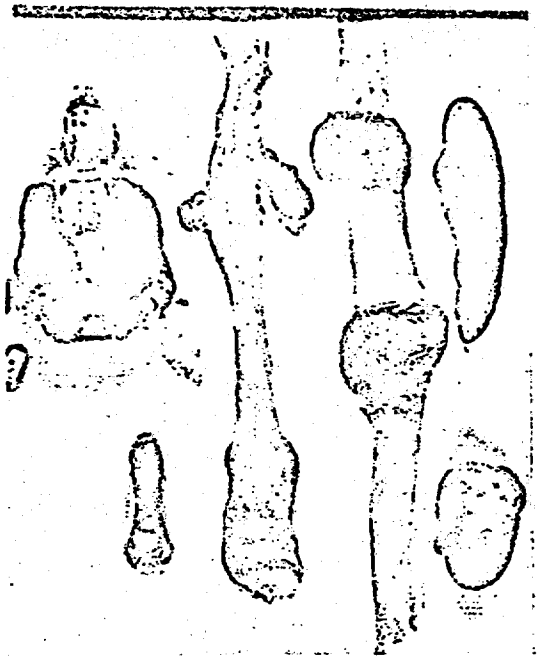


Abb. 5. Nieren-Sarkom, Adenocarcinome des Dünndarms und Rectums, Papillome des Zungengrundes und Plattenepithelkrebs des Oberkiefers, 360 Tage nach 1 × 70 mg/kg Methyl-nitroso-Harnstoff i.v.

Tumoren des Darmkanals fanden sich bei vier Ratten. Stets waren es mehrere, 0,3—1 g schwere Knoten, die bei einem Tier von Jejunum bis zum Rectum verteilt lagen (Abb. 5), bei den anderen entweder nur im Dünndarm oder Dickdarm. Die histologische Diagnose lautete in allen Fällen Adenocarcinom. „Spontan" kommen sie bei unseren Ratten nicht vor. Ihre experimentelle Erzeugung ist auch nur selten gelungen. Auffällig war, daß gerade Darmkrebs niemals allein auftrat sondern stets vergesellschaftet mit Tumoren anderer Organe. Ratte Nr. 1996 hatte außer mehreren Dickdarm-Carcinomen noch Papillome im Vormagen und Sarkome in beiden Nieren, bei Nr. 3991 waren es zusätzlich ein Adenocarcinom der Zunge und ein Plattenepithelkrebs am Unterkiefer. Nr. 4494 hatte außer Carcinomen im Dünndarm wiederum Papillome des Vormagens und ein Plasmocytom im Thoraxraum. Schließlich Ratte Nr. 1991 wies die meisten Geschwülste auf (Abb. 5): Adenocarcinome in allen Darmabschnitten, Papillome im Vormagen und am Zungengrund, ein

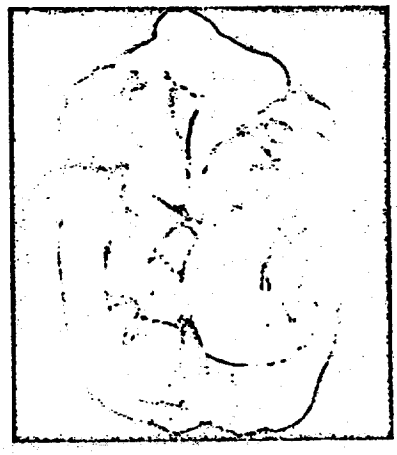


Abb. 6. Hämorrhagisches Sarkom an der Hirnbasis einer Ratte, 312 Tage nach 1 × 160 mg/kg Methyl-nitroso-Harnstoff

Sarkom der linken Niere und ein Plattenepithelcarcinom des Oberkiefers. Carcinome des Kieferbereichs oder der Zunge haben wir auch sonst in Versuchen mit Nitrosaminen beobachtet, so daß die kausale Bedeutung des MNH in diesem Falle nicht zweifelhaft erscheint.

Bei einer Ratte (Nr. 3989, ♂) fand sich ein Tumor im *Gehirn*. Er lag rechts an der Basis, war etwa erbsgroß, hämorrhagisch und deutlich fester als die Gehirnsubstanz (Abb. 6). Die histologische Untersuchung zeigte ein recht buntes Bild. Vorwiegend waren es spindelförmige Zellen mit reichlich Mitosen, daneben aber auch andere Zellen mit aufgetriebenem Cytoplasmaleib und feiner Granulierung. In anderen Bezirken hatten die Zellen einen randständigen Kern und große Vacuolen im Cytoplasma. Die Diagnose lautete: Spindelzellsarkom.

Da die experimentelle Tumorerzeugung im Gehirn durch resorptive Gabe eines Carcinogens bisher nur in einem einzigen Falle, und zwar nach 2,7-Bis-(acetylamino)-fluoren bei dauernder Gabe berichtet wurde (K. C. Snell, H. Stewart u. Mitarb., 1961) hatten wir die Veröffentlichung unserer Beobachtung zunächst zurückgestellt und neue Versuche mit MNH angesetzt. Sie haben inzwischen bereits in zahlreichen Fällen Tumoren nicht nur des Gehirns sondern auch des Rückenmarks ergeben. Darüber wird später berichtet werden (Naturwissensch. im Druck).

Diskussion

Die außerordentlich vielseitige Wirkung des MNH ist in hohem Maße bemerkenswert. Fast erscheint die Substanz als ein ganz unspezifisches Carcinogen, dessen vielseitige Wirkung durch die leichte und schnelle Bildung von Diazomethan verständlich ist. Indessen hat das Methylnitrosourethan, das ebenfalls Diazomethan liefert, bei intravenöser Injektion an unseren Ratten nur Lungenkrebs erzeugt [Druckrey u. Preussmann, Druckrey u. Mitarb. (1)], den wir beim MNH trotz seiner vielseitigen Wirkung überhaupt nicht sahen. Ferner fanden wir nicht ein einziges Carcinom der Leber, Blase oder des Oesophagus, die sich durch bestimmte Nitrosamine sehr sicher erzeugen lassen [Druckrey u. Mitarb. (3)]. Insoweit liegen doch im Positiven und Negativen unverkennbare Organotropien vor, deren Ursachen noch völlig dunkel sind, die aber als pharmakologische Grundprobleme weiter verfolgt werden.

Da MNH die gleiche „Wirkform" liefert, wie Dimethylnitrosamin, nämlich Diazomethan, kann auch seine carcinogene Wirkung auf eine Methylierung von Guanin in Nucleinsäuren und deren irreversible „mutative" Veränderung bezogen werden (Magee u. Farber). Im Gegensatz zum Dimethylnitrosamin ist das MNH aber an sich schon instabil und bedarf keiner enzymatischen Aktivierung, so daß es auf alle Zellen in gleicher Weise wirken müßte. Deshalb schien seine direkte Prüfung auf mutagene Wirkung an Einzellern möglich. Diese Versuche wurden von Prof. H. Marquardt u. Mitarb. in Freiburg durchgeführt. Sie haben z. B. an Saccharomyces cerevisiae eine auffallend starke Mutagenität des MNH und anderer Acyl-Alkyl-nitrosamide nach Maßgabe ihrer Spaltbarkeit ergeben. Der Wirkungsmechanismus der Diazomethan liefernden Nitrosamine und -amide wie des MNH ist also relativ gut überschaubar.

— Die bemerkenswert starke und vielseitige carcinogene Wirkung einer einzigen intravenösen Dosis gerade des besonders instabilen Methyl-nitroso-Harnstoffs

dürfte wohl endgültig beweisen, daß die Carcinogenese gemäß Gl. (1) als beschleunigter Vorgang (Blum), als „Verstärkerwirkung" verstanden werden muß [Druckrey (2), Druckrey u. Schmähl]. Dies wird durch den weiteren Nachweis gestützt, daß auch bei sechs verschiedenen Dialkylnitrosaminen sowohl bei oraler Gabe als auch bei Inhalation nur eine einmalige Dosis zur Krebserzeugung genügt (Tabelle 1). Es wird also durch das Carcinogen ein Prozeß in Gang gesetzt, der, einmal genügend angestoßen, dann schließlich nach eigenen Gesetzen weiterwächst bzw. sich vermehrt oder verstärkt („Verstärkerwirkung"). Dafür lassen sich leicht anschauliche Modelle finden, z. B. in der Biologie: die Befruchtung von Kaltblütereiern und deren Vermehrung, in der Technik: der Start eines Vorgangs durch einen Impuls oder in der Wirtschaft: die Einzahlung eines Kapitels und seine Vermehrung durch Zins und Zinseszins.

Schon bei unseren früheren „Stop"-Versuchen mit dem Leberkrebs erzeugenden 4-Dimethylaminoazobenzol war uns aufgefallen, daß die Leber der Tiere schon wenige Tage nach dem Absetzen der Behandlung sowohl histologisch als auch hinsichtlich ihres Gewebsstoffwechsels oder Riboflavingehaltes völlig normal erschien. Trotzdem entstand nach langer Latenzzeit und dann auffallend plötzlich der charakteristische Leberkrebs. Ganz entsprechende Beobachtungen berichteten P. N. Magee und J. M. Barnes bei der Erzeugung von Nierenkrebs durch eine einmalige Gabe von Dimethylnitrosamin. Auch da erschienen die Nieren lange Zeit völlig unverändert. In unseren Versuchen war eine Untersuchung der verschiedenen Organe in einzelnen Phasen der Latenzzeit nicht möglich, jedoch spricht der Befund, daß bei einseitigem Nierentumor die zweite Niere stets völlig normal war, in gleichem Sinne.

Hier liegen wichtige Probleme, die noch einer sorgfältigen Untersuchung bedürfen. Dennoch zeigen die übereinstimmenden Beobachtungen schon jetzt, daß die Einwirkung des Carcinogens sicher nicht sofort zu fertigen Krebszellen geführt haben kann, die sich dann nur noch zu vermehren hatten. Vielmehr erfolgt in den Minuten oder wenigen Stunden der Einwirkung des MNH mit der irreversiblen Veränderung von Nucleinsäuren offenbar nur eine „Determinierung" der betroffenen Zellen, die dann erst nach langer Latenzzeit spontan zu „fertigen" Krebszellen werden und sich bei Erreichen einer genügenden Anzahl schließlich exponentiell vermehren. Die an vielen Beispielen nachgewiesene spontane Progression der Malignität erscheint damit als die Endphase dieser durch das carcinogene Agens eingeleiteten Vorgänge.

In diese Richtung deutet auch die Beobachtung von *gutartigen* Tumoren in den vorliegenden Versuchen, nämlich von Adenomen der Niere und Leber (Tabelle 1) oder von Papillomen des Vormagens (Tabelle 2), also gerade in jenen Organen, in denen auch die malignen Tumoren entstanden. Wir sehen sie deshalb als echte Vorstadien des Krebs an, zumal wir aus zahlreichen Versuchen mit verschiedenen Carcinogenen bei dauernder Gabe wissen, daß zuerst gutartige Adenome oder Papillome entstehen, die dann bei weiterer Gabe des Carcinogens mit Sicherheit und in kurzer Zeit zu Carcinomen werden. Jedenfalls findet man von einem kritischen Zeitpunkt an praktisch nur noch Carcinome. Auf der anderen Seite gibt es auch bei der Ratte gutartige Tumoren, die erfahrungsgemäß niemals bösartig werden, wie z. B. die bei weiblichen Ratten „spontan" häufig vorkommenden Fibroadenome der Mamma. Diese haben mit Krebs oder Präcancer wahr-

scheinlich überhaupt nichts zu tun, sondern sind eher als äußere Störung der homoeostatischen Kontrolle von Funktion und Wachstum der Brustdrüse zu verstehen.

Warnung!

Methyl-nitroso-Harnstoff und ähnliche Nitrosamide werden in der Chemie zur Herstellung von Diazomethan sehr viel verwendet. Schon unsere früheren Ergebnisse mit der Harnstoff- und Urethan-Verbindung hatten uns veranlaßt, vor der Gefährlichkeit dieser Substanzen eindringlich zu warnen und ihren Ersatz durch weniger gefährliche Produkte zu empfehlen. Die hier mitgeteilte Beobachtung, daß schon eine einzige Dosis solcher Substanzen sogar regelmäßig Krebs erzeugen kann, unterstreicht den Ernst dieser Warnung.

Zusammenfassung

Nach früheren Ergebnissen wächst die carcinogene Wirkung bei täglicher Gabe konstanter Dosen etwa mit dem Quadrat der Induktionszeit an, entspricht also einem beschleunigten Vorgang. Danach müßte Krebs auch durch eine einzige Dosis eines kurzlebigen Carcinogens erzeugt werden können.

16 BD-Ratten erhielten eine einmalige intravenöse Injektion von 70 bzw. 100 mg/kg N-Methyl-N-nitroso-Harnstoff. Nach einer mittleren Latenzzeit von 320 ± 40 Tagen starben alle Tiere mit Tumoren, 12 von ihnen hatten bösartige Geschwülste zum Teil in mehreren Organen, meist in den Nieren, im Vormagen und Darm. Eine Ratte zeigte ein Sarkom des Gehirns.

Ferner wurden nach einmaliger Gabe von Dimethyl-, Methylvinyl-, Diäthyl-, Äthyläthanol- und Äthylbutyl-nitrosamin sowie von N-Nitroso-morpholin in hoher Dosis (oral bzw. Inhalation) bei 15 Ratten Carcinome der Nieren, der Leber, Speiseröhre, der Ovarien und Nasen-Nebenhöhlen beobachtet.

Die mitgeteilten Ergebnisse warnen zu äußerster Vorsicht im Umgang mit Nitrosamiden und anderen Diazoalkane-liefernden Substanzen.

Cancerogenesis in Rats by a Single Dose of Methyl-Nitrosocarbamide and Various Di-Alkyl-Nitrosamines

Summary

As learned from earlier results, the carcinogenic effect of daily administered uniform doses increases with about the square of the induction time: this represents an accelerated process. Accordingly, it should be possible to induce cancer by means of a single dose of a short-living carcinogen.

Sixteen BD rats received a single intravenous injection of 70—100 mgm/kg of N-methyl-N-nitroso-urea. After an average latency period of 320 ± 40 days all animals died with tumors. Twelve of them had malignant tumors, some in several organs with most in the kidneys, forestomac, and intestine. One rat had a sarcoma of the brain. In addition, following single doses of di-methyl-nitrosamine (or the methylvinyl-, di-ethyl-, ethyl ethanol-, and ethylbutyl nitrosamine compounds), and N-nitroso-morpholin in higher doses (oral or inhalation) in 15 rats carcinoma of the kidneys, the liver, esophagus, ovary, and nasal sinuses were observed.

The results reported serve as a warning to use nitrosamides and other diazoalkan-producing substances with the utmost care.

Literatur

BLUM, H. F.: Carcinogenesis by ultraviolet light. Princeton, New Jersey: Princeton Univers. Press 1959.

DRUCKREY, H.: (1) Quantitative Grundlagen der Krebserzeugung. Klin. Wschr. 22, 532 (1943).

— (2) Experimentelle Beiträge zum Mechanismus der cancerogenen Wirkung. Arzneimittel-Forsch. 1, 383 (1951).

—, u. K. KÜPFMÜLLER: (1) Quantitative Analyse der Krebsentstehung. Z. Naturforsch. 3b, 254 (1948).

—, — (2) Dosis und Wirkung. Aulendorf, Wttbg.: Ed. Cantor 1949.

—, u. R. PREUSSMANN: Nitrosomethylurethane a potent carcinogen. Nature (Lond.) 195, 1111 (1962).

— — J. AFKHAM u. G. BLUM: (1) Erzeugung von Lungenkrebs durch Methylnitrosourethan bei intravenöser Gabe an Ratten. Naturwissenschaften 49, 451 (1962).

— — G. BLUM u. S. IVANKOVIC: (2) Carcinogene Wirkung von Diazoessigester und Nitroso-Sarkosinester. Naturwissenschaften 50, 99 (1963).

— — — — u. J. AFKHAM: (3) Erzeugung von Karzinomen der Speiseröhre durch unsymmetrische Nitrosamine. Naturwissenschaften 50, 100 (1963).

— — D. SCHMÄHL u. M. MÜLLER: (4) Erzeugung von Magenkrebs durch Nitrosamide an Ratten. Naturwissenschaften 48, 165 (1961).

— A. SCHILDBACH, D. SCHMÄHL, R. PREUSSMANN u. S. IVANKOVIC: (5) Quantitative Analyse der carcinogenen Wirkung von Diäthylnitrosamin. Arzneimuttel-Forsch. (im Druck 1963).

—, u. D. SCHMÄHL: Quantitative Analyse der experimentellen Krebserzeugung. Naturwissenschaften 49, 217 (1962).

— D. SCHMÄHL u. W. DISCHLER: (6) Dosis-Wirkungs-Beziehungen bei der Krebserzeugung durch 4-Dimethylaminostilben bei Ratten. Z. Krebsforsch. 65, 272 (1963).

FIORE-DONATI, L., L. CHICCO-BIANCHI, G. DE BENEDICTIS e G. MAJORANO: Leukaemogenesis by Urethan in New Born Swiss Mice. Nature (Lond.) 190, 278 (1961).

HUGGINS, C., L. C. GRAND and F. P. BRILLANTES: Mammary Cancer induced by a single feeding of polynuclear hydrocarbons and its suppression. Nature (Lond.) 189, 204 (1961).

KRAMER, S. P., A. M. SELIGMAN, S. D. GABY, R. D. SOLOMON, J. I. MILLER, C. WILLIAMSON and B. WITTEN: The Nature of some N-Nitroso-N-(2-Chloraethyl)-carbamates in animals and man. Cancer (Philadelphia) 12, 446 (1957).

MAGEE, P. N., and J. M. BARNES: (1) The experimental production of tumors in the rat by dimethylnitrosamine. Acta Un. int. Cancr. 15, 187 (1959).

— — (2) Induction of kidney tumors in the rat with dimethylnitrosamine. J. Path. Bact. 84, 19 (1962).

—, and E. FARBER: Toxic liver injury and carcinogenesis. Methylation of rat-liver nucleic acids by Dimethylnitrosamine. Biochem. J. 83, 114 (1962).

MARQUARDT, H., F. K. ZIMMERMANN u. R. SCHWAIER: Naturwiss. 50, 625 (1963).

SCHOENTHAL, R.: Carcinogenic action of diazomethane and of Nitroso-N-methyl-urethane. Nature (Lond.) 188, 420 (1960).

SNELL, K. C., H. L. STEWART, H. P. MORRIS, B. P. WAGNER and F. E. RAY: Intracranial Neurilemmoma and Medulloblastoma induced in rats by the dietary Administration of N,N'-2,7-Fluorenylbisacetamide. Nat. Cancer Inst. Monogr. No 5, 85 (1961).

Prof. Dr. H. DRUCKREY,
Laboratorium der Chirurgischen Universitätsklinik
78 Freiburg i. Br.

Zeitschrift für Krebsforschung 66, 11—28 (1964)

Aus dem Ludwig-Aschoff-Haus, dem Pathologischen Institut der Universität Freiburg i. Br.
(Direktor: Prof. Dr. F. Büchner)

Untersuchungen über die Generationszeit, DNS-Synthesezeit und Mitosedauer von Zellen der hyperplastischen Epidermis und des Plattenepithelcarcinoms der Maus nach Methylcholanthrenpinselung

Von

P. Dörmer, H. Tulinius und W. Oehlert

Mit 13 Textabbildungen

(Eingegangen am 9. Juli 1963)

Das Wachstum einer Zellpopulation mit post- und intermitotischem Zellbestand, und damit die Volumenzunahme des entsprechenden Gewebes, kann theoretisch auf verschiedene Weise geschehen. Entweder verlängert sich die Lebensdauer der sich differenzierenden Zellen bei unveränderter Generationszeit der sich teilenden Zellen, oder es kommt zu einer Verkürzung der Generationszeit der sich teilenden Zellen bei unveränderter Lebensdauer der sich differenzierenden Zellelemente. Letztere Möglichkeit bedeutet eine echte Zunahme der Zellproliferation.

Für die durch Methylcholanthrenpinselung hervorgerufene Wachstumssteigerung der Epidermis über das Stadium der Hyperplasie und des Papilloms bis zum Carcinom sind beide Möglichkeiten in der Literatur diskutiert worden (Setälä u. Mitarb. 1961; Oehlert, Cote u. Büchner 1961). Exakte Aufschlüsse über die Länge der einzelnen Phasen im Generationscyclus einer Zelle und über die Lebensdauer postmitotischer Zellen lassen sich durch Anwendung der autoradiographischen Technik nach Injektion von ^{3}H-Thymidin erhalten.

In vorliegender Arbeit wurde durch autoradiographische Untersuchungen in der hyperplastischen Epidermis der Maus nach Methylcholanthrenpinselung die Lebensdauer der intermitotischen Zellen des Stratum basale und der postmitotischen Zellen des Stratum spinosum bestimmt. In der hyperplastischen Epidermis und im Plattenepithelcarcinom wurde die DNS-Synthese-Phase und die prämitotische Ruhezeit ermittelt. Außerdem versuchten wir, unter Verwendung der erhaltenen Werte die Größe der eigentlichen Wachstumsfraktion im Carcinom zu errechnen. Die Ergebnisse sollten uns Hinweise auf den Entstehungsmechanismus und das Ausmaß der Wachstumsvorgänge im Carcinom erbringen. Außerdem sollte untersucht werden, ob sich neben Veränderungen der Lebensdauer bestimmte Veränderungen im Generationscyclus der einzelnen Zellen fassen lassen, welche für die Carcinomentwicklung an der Epidermis typisch sind.

Versuchsanordnung und Methode

19 ausgewachsene weiße Mäuse vom Stamm Agnes Blum wurden einmal wöchentlich auf der nicht vorbehandelten Rückenhaut mit einer 0,25%igen Lösung von Methylcholanthren in Benzol gepinselt. Nach 90 Tagen, zum Zeitpunkt der ersten Excisionen, fand sich bei allen Tieren eine deutliche Hyperplasie der Epidermis mit fast totalem Haarausfall an den gepinselten Stellen. Auch die zu diesem Zeitpunkt entstandenen Papillome wurden

Seite

BRIZIARELLI, G., Effects of Dosage and Time of Administration of Testosterone Propionate on 7,12-Dimethylbenzanthracene Mammary Carcinogenesis in the Rat . . . 517

DRUCKREY, H., S. IVANKOVIC und B. T. SO, Erzeugung von Carcinomen der Haut bei Ratten durch intravenöse Injektion von Diazoessigester. Mit 1 Textabbildung . . . 523

SCHMÄHL, D., C. THOMAS, W. SATTLER und G. F. SCHEID, Experimentelle Untersuchungen zur Syncarcinogenese. 3. Mitteilung. Versuche zur Krebserzeugung bei Ratten bei gleichzeitiger Gabe von Diäthylnitrosamin und Tetrachlorkohlenstoff bzw. Äthylalkohol; zugleich ein experimenteller Beitrag zur Frage der „Alkoholcirrhose". Mit 2 Textabbildungen . . . 526

SCHMÄHL, D., und C. THOMAS, Dosis-Wirkungs-Beziehungen bei der Erzeugung von Hämangioendotheliomen der Leber bei Mäusen durch Diäthylnitrosamin. Mit 1 Textabbildung . . . 533

MOHR, U., J. ALTHOFF und H. WERRA, Diaplacentare Wirkung des Carcinogens Diäthylnitrosamin beim Goldhamster. Mit 2 Textabbildungen . . . 536

IVANKOVIC, S., H. DRUCKREY und R. PREUSSMANN, Erzeugung von Tumoren im peripheren und zentralen Nervensystem durch Trimethyl-nitroso-harnstoff an Ratten. Mit 5 Textabbildungen . . . 541

BREUER, H., R. KNUPPEN und D. SCHMÄHL, Fehlen einer carcinogenen Wirkung bei einem körpereigenen Steroidepoxyd. Mit 1 Textabbildung . . . 549

ITO, T., T. HOSHINO and K. SAWAUCHI, Further Observations of Urethan-induced Thymic Lymphoma in Mice. With 7 Figures in the Text . . . 552

HÖLZEL, F., P. PFLÜGER, O. BARTH und H. MAASS, Die Hemmung der DNS- und der RNS-Synthese in Ehrlich-Ascitestumorzellen durch Trenimon. Mit 7 Textabbildungen . . . 559

Autorenverzeichnis . . . 574

101-5204 (525)
German Trsl.
DKD

492

CANCER PRODUCTION THROUGH A SINGLE DOSE OF METHYLNITROSO UREA AND DIFFERENT DIALKYLNITROSAMINES IN RATS

(Erzeugung von Krebs durch eine einmalige Dosis von Methylnitroso-Harnstoff und verschiedenen Dialkylnitrosaminen an Ratten)

Authors: H. Druckrey, D. Steinhoff, R. Preussmann and S. Ivankovic

Source: Zeitschrift für Krebsforschung 66, 1 - 10 (1964).

In quantitative tests for cancer production with different agents (4-dimethyl aminoazobenzene (DRUCKREY (1)), DRUCKREY and KÜPFMÜLLER (1), 4-dimethylamino stilbene (DRUCKREY and collaborators (Reference 6), diethylnitrosamine (DRUCKREY and collaborators (Ref. 5)), aromatic hydrocarbons (DRUCKREY and SCHMÄHL) and UV-light (BLUM)), in the case of continuous administration to rats, a linear dependence was found between the height of the daily dose (d = mg/kg) and the length of the average induction time (t = days) until the development of a definite yield of carcinomas (50% = const.) in the bilateral log.- field

$$d\ t^n = \text{const.} \qquad (1)$$

in which case, the exponent n was nearly always between 2 and 3 (DRUCKREY and SCHMÄHL). The result in the meantime was reproduced by us on other instances. The validity of the relation according to Equation (1) proved to be so high that it was possible to calculate in advance the induction periods according to the previous determination of the value of n and to confirm them experimentally with surprising accuracy (DRUCKREY and SCHMÄHL). According to equation (1), the effect of the continuous administration of contant doses of the carcinogenic substance increases somewhat with the square of the exposure period, and thus corresponds to an accelerated process (BLUM). This action of a new type was first observed in "stop" tests (DRUCKREY, Ref. 2) and was called "intensifying effect" (DRUCKREY and KÜPFMÜLLER, Ref. 2). It is founded on the fact, we think, that the carcinogenic action is based on irreversible changes in units that can multiply (duplicants, cells). Following the administration of carcinogenic dimethylnitrosamine to rats, it was possible for P.N. MAGEE and E. FARBER (1962)

to detect,, in intact nucleic acid of the organ in question especially, that is the liver, a methylation of guanine, and to isolate N (7)-methylguanine from the RNS.

If carcinogenesis is actually, according to equation (1), an accelerated process, then it should be possible to start it through a single impulse of sufficient strength. For this, experimental instances already exist. C.HUGGINS and collaborators (1961) obtained the regular production of mammary cancer through a single administration of methylcholanthrene with the help of an esopharyngeal sound. This is possible, however, only with young Sprague-Dawley rats. In newborn Swiss mice, L.FIORE-DONATI and collaborators (1961) were able, by a single s.c. injection of 2 mg ethylurethane, to produce in only about 110 days, not only lung adenomas, but also 13/60 malignant lymphomas. P.N. MAGEE and J. BARNES (1959 and 1962), after a single administration of 30 mg/kg dimethylnitrosamine with the exopharyngeal sound in the case of 3 to 15 rats, observed anaplastic tumors of the kidney, whereas the same substance, daily added to the diet, produces only the cancer of the liver. Finally, R. SCHOENTHAL (1960) reported a carcinoma of the antestomach after a single dose of 50 mg/kg of methyl-nitroso-urethane in one rat only.

Tests:

During recent years, within the scope of our systematic investigations with nitrosamines, we conducted also orientation tests with a single administration or with a short-term treatment and different forms of administration (oral, intravenous, subcutaneous and inhalation) on rats. We thus obtained quite a number of malignant tumors, not only of the kidneys, but also carcinomas of the liver, esophagus, ovaries and secondary nasal cavities. The most important results are grouped in Table 1. Their histological diagnoses are due to Professor H. HAMPERL, Bonn, Accordingly, it is obvious that any type of cancer can be produced by a single dosis.

Table 1: Survey of the production of tumors through a single and short duration treatment with different nitrosamines in rats.

a. Substance b. Dosage, mg/kg of application c. Number of tumors in surviving rats d. After so many days e. Kidney adenoma f. Liver carcinoma g. nasal cavities carcinoma

A strong proof of the presence of an "intensifying action" or of an accelerated process according to equation (1) would not exist before it can be shown that even a very short-lived substance produces cancer regularly with a single dose.

For our tests we selected the N-methyl-N-mitrosourea (I), concerning the carcinogenic effect of which we had reported previously (DRUCKREY and collaborators (Ref. 4)). When administered daily by mouth to rats, it produced mainly pavement epithelium carcinoma od the antestomach (crop).

The substance was purified through recrystallization (F = 124 C). It is a very unstable substance. Already with a slightly alkaline reaction, the acyl residue splits hydrolytically (pH 8: t_{50}=30 minutes), so that, through the monomethyl-nitrosamine (III) the highly reactive diazomethane (IV) develops. Thus the "active forms" are the same as those developed from the N.N-dimethyl nitrosamine (II) for which P.N. MAGEE and E. FARRER (1962) detected the methylating action on nucleic acids in vivo. In the body a rapid splitting of the methyl-nitroso-urea occurs (Compare S.P. KRAMER and collaborators, 1959), so that in a few minutes quite an extensive decomposition must be assumed

(II) P. 3

(III)

(IV)

In the previous tests we administered the methylnitrosourea (MNH) intravenously in the tail vein of rats. The mean lethal dose was 108 mg/kg as compared with 110 mg/kg when administered with esopharyngeal sound. The agreement suggests that MNH, despite its instability, possesses a life sufficient for our tests. In the blood, MNH is hydrolized only relatively slowly. There is oxidation of the hemoglobin, like in the case of diazoacetic ester (DRUCKREY and coll. (2)).

The poisoning picture, which after oral administration of MNH exhibits damages of the stomach and intestine (DRUCKREY and collaborators, Ref. 4), was controlled after the intravenous injection through a severe damage of the bone marrow and

lymphatic tissue. In the course of 8 days after the injection of LD 50, a secere leukopenia (with values below 1000 cells) and anemia developed, which result in death on the 12th day approximately. The feces contained abundant blood, but not the urine. Dissection revealed hemorrhages and ulcerations in the gastrointestinal. The rats that had not dies recovered rapidly through a treatment with penicillin-streptomycin and iron.

The test involved altogether 16 animals from different BD breeds. Nine of them had received through injection a dose of 100 mg MNH per kilogram of body weight and the other seven rats70 mg/kg. They were observed with great care up to their natural death, and then were thoroughly dissected and histologically examined. Since the two groups revealed no characteristic difference so to say, they are treated together.

On the 275th day after the injection, the first two rats of the group which had received the weaker dosage of 70 mg/kg, died. The two rats exhibited several papillomas of the antestomach, and one of them a white-fleshy tumor, about 1 g in weight, of the left kidney, which was diagnozed histologically as a renal sarcoma. In rapid succession, 14 rats died, and at a somewhat larger time interval the last two. All the animals had tumors.

The development in time of the tumors and their distribution in the individual organs were plotted in a probability network (Figure 1).

Figure 1: Production of tumors through a single i.v. injection of 70 or 100 mg/kg of methylnitrosourea in BD rats. . Carcinoma or sarkoma; N kidney; V antestomach; M stomach; Z tongue; K jaws; D intestine; B mammary gland; G brain; P plasmocytoma; o antestomach-papillomas

For the latent period up to the death by the tumor there is a mean value of 320 $\pm$ 40 days after the single injection. The position of the individual dots in the probability net corresponds nearly to a straight line. Thus practically there is a normal distribution. Hence, the causal relation can be considered as obtained.

Four rats had only benign papilloma and hyperkeratoses of the antestomach. In the case of the other 12 rats, there were without exceptions malignant tumors and this in the most different organs, in six cases in the same animals even. MNH thus proves to be a powerful and remarkably versatile carcinogenic compound.

Table 2: Distribution of the tumors in 16 rats, which had received a single intravenous injection of 70-100 mg'kg methylnitrosourea. Four rats had only antestomach papillomas.
a. brain b. tongue c. jaw d. antestomach e. glandular stomach
f. small intestine g. large intestine h. kidney i. mamma j. Subcutis
k. plasmocytoma.

The distribution of the tumors in the individual organs is once more presented as a whole in Table 2. Like in the tests conducted by P.N. MAGEE and J.M. BARNES (1962) with dimethylnitrosamine with a single administration, here also the malignant kidney tumors are prominent. They developed in 7 rats, in two cases bilaterally, totally in female animals. Their size was in most cases about 1 - 3 g; in part it was, however, extremely large tumor formations, which weighed up to 50 grams. Macroscopically they appear to be whitish, fleshy, often somewhat glazed and interspersed with hemorrhagic parts (Figure 2).

Figure 2: Large malignant kidney tumor of the sarcoma type in a female rat, 322 days after a single i.v. injection of 100 mg'kg methyl-nitroso-urea.

The histological diagnosis revealed immature sarcomas with fusiform cells and abundant mitosis. In our cases, with nitrosomethylurea, there were only immature sarcomas, occasionally also mixed forms (carcinosarcoma or possibly of the type of the Wilms tumors), but no purely epithelial kidney tumors. MAGEE and BARNES, on the other hand, observed, after administrations of dimethyl nitrosamine, carcinomas as well as sarcomas. The question whether here a selective action is actually present, must be clarified in further tests.

Figure 3: 4494, ♀, NO-CH_3 urea 1 x 100 mg/kg i.v. Papilloma of the antestomach, 355 days after 1 x 100 mg/kg methyl-nitroso urea i.v.
Figure 4: 3988, ♂, NO-CH_3-urea. Extended pavement epithelium cancer of the antestomach, 350 days after 1 x 100 mg/kg methyl-nitroso-urea.

Not less remarkable is the frequency of tumors of the antestomach. Papillomas were found by us in 10 of the 16 rats. In most cases they were multiple and 1 - 2 mm in diameter (Figure 3) and always accompanied with hyperkeratoses. Four rats (Figure 4) had carcinomas of the antestomach (Figure 4), which were diagnozed as infiltratively growing, partly highly cornifying pavement epithelium carcinomas. One of these rats had also a spindle cell sarcoma in the wall of the glandular stomach.

Tumors of the antestomach had already been observed by us after oral administration of MNH (DRUCKREY and collaborators, Ref. 4), but never as "spontaneous tumors". Since here they were observed even after i.v. injection of the carcinogenic substance with the same yield, the findings made at that time cannot be referred only to a purely local and direct action. One must rather assume at least also a resorptive action on the antestomach epithelium. It must be note that in no animal a cancer of the esophagus was observed, whereas the mainly unsymmetrical dialkylnitrosamines, which produces this cancer especially (DRUCKREY and collaborators, Ref. 3) left again the antestomach free. The carcinogenic action of the nitrosamines exhibits thus a surprising selectivity, for which at present no justification can be provided. The findings show that the antestomach of rats cannot be simply regarded as a "prolonged esophagus".

Figure 5: Kidney sarcoma, adenocarcinomas of the small intestine and of the rectum, papilloma of the base of the tongue and pavement epithelium cancer of the upper jaw, 36^0 days after 1 x 70 mg/kg methyl-nitroso-urea i.v.

Tumors of the intestinal canal were found in four rats. There were always several heavy knots, 0.3 - 1 g in weight, which in one animal were distributed from the jejunum to the rectum (Figure 5) in the other, either only in the small intestine or in the large intestine. The histological diagnosis in each case was adenocarcinoma. They are not "spontaneous" in our rats. Their experimental production succeeded only seldom. It was remarkable that intestinal cancer never developed alone, but always accompanied by tumors of other organs. Rat No. 1995 had also papillomas in the antestomach and sarcomas in the two kidney; in No. 3991 there were also an adenocarcinoma of the tongue and a

pavement epithelium at the lower jaw. No. 4494 had beside carcinomas in the small intestine papillomas of the antestomach and a plasmocytoma in the thorax cavity. Finally rat No. 1991 exhibited most of the tumors (Figure 5): adenocarcinomas in all the section of the intestine, papillomas in the antestomach and at the base of the tongue, a sarcoma of the left kidney and a pavement epithelium carcinoma of the upper jaw. Carcinomas of the maxillary region or of the tongue were observed by us otherwise in tests with nitrosamines, so that the causal importance of MNH in this case does not appear doubtful.

In a rat (No. 3989 ♂) there was a tumor in the brain. It was on the right at the base, it had somewhat the size of a pea, hemorrhagic and definitely more solid than the brain substance (Figure 6).

Figure 6: Hemorrhagic sarcoma at the base of the brain of a rat, 312 days after 1 x 100 mg/kg methyl-nitroso-urea.

The histological investigation revealed a quite diversified picture. Predominantly there were fusiform cells with abundant mitoses, but also other cells with intumescent cytoplasm body and fine granulation. In other regions the cells had a marginal nucleus and large vacuoles in the cytoplasm. The diagnosis was spindle cell carcoma.

Since the experimental tumor production in the brain through resoprtive administration of a carcinogenic substance had been reportet so far only in a single case, and after 2.7-bis-(acetylamino)-fluorene in continuous admistration (K.C. SNELL, H. STEWART and collaborators, 1961) we had at first retarded the publication of our observation and started new tests with MNH. In the meantime they have already given, in a large number of cases, tumors not only of the brain, but also of the spinal marrow (spinal cord). We will report later on in this respect (Naturwissenschaft in the press).

Discussion:

The extraordinary versatile action of MNH is quire remarkable. It almost appears that the substance is quite a nonspecific carcinogenic material, the versatile effect of which is understandable on account of the easy and rapid formation of

diazomethane. In the meantime, methylnitrosourethane, which also gives diazomethane, produced in intravenous injection in our rats only lung cancer (DRUCKREY and PREUSSMANN, DRUCKREY and collaborators, Ref. 1), which we did not observe in the case of MNH despite its many-sided action. Furthermore, we found non one carcinoma of the liver, bladder or esophagus, which can be produced very efficiently through some nitrosamines (DRUCKREY and collaborators, Ref. 3). So far thereare still unmistakable organotropies in the positive and in the negative, the causes of which are still completely unknown, but which are further studied as pharmacological basic problems.

Since MNH provides the same "active form" like dimethylnitrosamine, that is diasomethane, its carcinogenic action can also be referred to a methylation of guanine in nucleic acids and their irreversible "mutative" change. (MAGEE and FARBER). In contrast to dimethylnitrosamine, MNH, however, is already unstable as such and needs no enzymatic activation, so that it should act on all the cells in the same way. Hence its direct examination with respect to a mutagenic action on unicellular organisms seemed to be possible. These tests were conducted at Freiburg by Prof. H. MARQUARDT and collaborators. They gave, for instance in saccharomyces cerevisiae a surprisingly high mutagenicity of MNH and of other acyl-alkyl-nitrosamides according to their splittability. The mechanism of the action of the nitrosamines and amides giving diazomethane as well as of MNH can thus be easily subjected to an overall examination.

The remarkably high and versatile carcinogenic action of a single i.v. dose of the especially unstable methyl-nitroso-urea should no doubt definitively prove that the carcinogenesis according to Equation (I) must be understood to be an accelerated process (BLUM), an "intensification effect" (DRUCKREY, Ref. 2, DRUCKREY and SCHMÄHL). This is supported by the further detection that in the case of six different dialkynitrosamines, administered by mouth or by inhalation, a single dosis suffices to produce cancer (Table 1). Thus the carcinogenic substance starts a process which, once it has received enough impetus, grows further according to its own laws, or increases or intensifies ("intensification effect"). In this respect, models are easily found, for instance in biology: fertilization of eggs of cold-blooded animals and their increase; in engineering: the start of a process through an impulse, or in economics: the deposit of capital and its increase through interests and compound interests.

Already in the case of our previous "stop" tests with liver-cancer-producing 4-dimethylaminoazobenzol, we had been struck by the fact that, after days after the interruption of the treatment, the livers of the animals appeared entirely normal from the histological standpoint as well as to their tissue metabolism or riboflavin content. Nevertheless, after a long latent period the characteristic cancer of the liver suddently develops conspicuously. Quite similar observations were reported by P.N. MAGEE and J.M. BARNES with the production of kidney cancer through a single administration of dimethylnitrosamine. In these cases also, the kidneys appeared for a long time to be quite unchanged. In our tests an investigation of the different organs in individual phases of the latent period was not possible; however, the finding suggests that in the case of a unilateral kidney tumor the second kidney was always fully normal, in the same sense.

We are confronted by important problems still in need of careful investigation. However, the concordant observations show already at this point that the action of the carcinogenic substance kannot have led immediately to complete cancer cells, which then only had to multiply. Rather what happens in the minutes or few hours of the progressive action of MNH with the irreversible change of nucleic acids is obviously only a "determination" of the cells in question, which then, not before a long latent period, become "complete" cancer cells and, when in sufficient number, finally multiply exponentially. The spontaneous progression of the malignancy, detected in many instances, appears thus to be the final phasis of these processes brought about by the carcinogenic agent.

In these direction points also the observation of benign tumors in the present tests, that is adenomas of the kidney and liver (Table 1) or papillomas of the antestomach (Table 2), hence precisely in the organs where the malignant tumors developed. Hence, we regard them as true preliminary stages of cancer, especially since we know, through a large number of tests with different carcinogenic agents and in continuous administration that at first benign adenomas or papillomas develop, which then, with further administration of the carcinogenic substance become definitely and rapidly carcinomas. Anyway, from a critical moment, only carcinomas are practically found. On the other side, there are also in the rats mild tumors which, according to experience, never become malignant, like the fibroadenomas of the mamma, frequently "spontaneous" in female rats.

These adenomas have probably nothing to do with cancer or precancer, but must rather be regarded as external disturbance of the homeostatic control of function and growth of the mammary gland.

Attention!

Methyl-nitroso-urea and similar nitrosamides are abundantly used in chemistry for the preparation of diazomethane. Already our previous results with the urea and urethane compound has induced us to raise a serious warning against the dangerousness of these substances, and to recommend that less hazardous products be substituted for them. The observation recorded here, that already a single dose of substances of this kind can produce cancer even regularly, emphasize the gravity of the warning.

PL. MICROBIOL.
IGHT. 1962. Hy.
conidia from
54:317-319.
VEZINA. 1963.
's compound S
ids 2:93-97.
VEZINA. 1963
ds by spores
2:513-520.
VEZINA. 1965
compound S
by spores of
robiol. 11:351.
SINGH. 1963.
ores of micro
f progesterone
eus. Appl.
SINGH. 1965.
n submerged

APPLIED MICROBIOLOGY, Feb. 1968, p. 401-405
Copyright © 1968 American Society for Microbiology
Vol. 16, No. 2
Printed in U.S.A.

Role of Curing Agents in the Preservation of Shelf-stable Canned Meat Products[1]

CHARLES L. DUNCAN[2] AND E. M. FOSTER
Department of Bacteriology and Food Research Institute, University of Wisconsin, Madison, Wisconsin 53706

Received for publication 29 November 1967

Experiments were conducted to gain a better understanding of the mechanism by which sodium chloride, sodium nitrate, and sodium nitrite supplement the action of heat in preserving canned cured meat products. Heated spores of putrefactive anaerobe 3679h were less tolerant of all three curing agents in the outgrowth medium than were unheated spores. When the curing agents were added to the heating menstruum, but not to the outgrowth medium, sodium chloride and sodium nitrate tended to protect the spores against heat injury, but sodium nitrite did not. When the spores were both heated and cultured in the presence of the curing agents: (i) nitrate and salt increased the apparent heat resistance at low concentrations (0.5 to 1%) but decreased it at concentrations of 2 to 4%; (ii) nitrite was markedly inhibitory, especially at *p*H 6.0. At the normal *p*H of canned luncheon meats (approximately 6.0), nitrite appears to be the chief preservative agent against spoilage by putrefactive anaerobes.

Table-ready, shelf-stable canned luncheon meats represent an important group of food products. They contain the usual curing ingredients (sodium chloride, sodium nitrate, and sodium nitrite) and they are heat-processed after canning. However, the heat treatment is far below that required to effect sterilization, usually amounting to about $F_0 = 0.3$ [equivalent to 0.3 min at 250 F (121 C)]. Nevertheless, these products can be stored for months at room temperature without spoiling. Several billion pounds of luncheon meats have been consumed in the United States without a single known—or even suspected—case of food poisoning.

Numerous workers have commented on the remarkable stability of these products (9, 11). This stability is achieved even though the curing ingredients by themselves are not sufficient to prevent spoilage of the meat, and the relatively mild heat treatment used is not nearly sufficient to destroy all the bacterial spores (5, 6, 12).

A heat treatment which does not necessarily result in complete inactivation of spores may damage them enough to prevent growth and multiplication under the storage or test conditions that prevail after heating (2, 3, 7). This is generally thought to be the case in the preservative system of cured meats.

Recently, Roberts and Ingram (10) reported that spores given increasing heat treatments became progressively more sensitive to the effect of curing agents in the outgrowth medium. The effect of nitrate was roughly equal to that of salt. Nitrite concentrations of the order of 50 ppm had a decisively inhibitory effect on the outgrowth of heated spores.

Surprisingly little is known as to whether the primary effect of the curing salts is exhibited during the actual heat treatment, or later during outgrowth of the bacterial spores. This study was undertaken in an attempt to gain a better understanding of the mechanism by which the curing ingredients supplement the action of heat in effecting preservation.

MATERIALS AND METHODS

Preparation of spore suspensions. Putrefactive anaerobe 3679 strain h (PA 3679h), obtained from Hilmer A. Frank, was used in all experiments. This organism was described by Campbell and Frank (1).

The activated culture technique of Uehara, Fujioka, and Frank (13) was employed to prepare the inoculum for sporulation in the following medium: Trypticase, 6%; glucose, 0.1%; sodium thioglycolate, 0.1%; *p*H 7.0. Cultures were incubated for 1 week at 37 C and for an additional week at 20 C; then the spores were harvested by centrifugation and washed five times at 4C with cold sterile deionized water. They were cleaned with Carbowax 4000 (8) and washed 10 more

[1] Published with the permission of the Director of the Wisconsin Agricultural Experiment Station, Madison.

[2] Present address: Department of Foods and Nutrition, University of Wisconsin, Madison, Wis. 53706.

times as before. The final suspensions were stored at 4 C until used.

Enumeration of spores. For direct counts, the spore suspension was diluted as necessary in 30% glycerol, and refractile spores were counted by phase-contrast microscopy in a Petroff-Hausser counting chamber. For counts of viable spores before and after heating, appropriate dilutions in distilled water were dispensed in Pyrex tubes (16 × 150 mm) containing 12 ml of Difco Liver Veal Agar (LVA). The tubes were capped with about 3 ml of 2% agar containing 0.2% sodium thioglycolate. When used, sodium chloride and sodium nitrate were added to the medium before sterilization. The amounts indicated in the figures are additional to the respective 0.5 and 0.2% present in the formulated LVA. Sodium nitrite was added to the tubes of melted LVA from a filter-sterilized 10% stock solution just before inoculation. The *p*H of the LVA was adjusted to 6.0 or 7.0 with 3 N NaOH or 3 N HCl before sterilization. Colonies were counted after 5 days at 30 C.

Preparation of survivor curves. The basal heating menstruum was 0.067 M phosphate buffer (*p*H 6.0 or 7.0) prepared with Na_2HPO_4 and KH_2PO_4. When sodium chloride or sodium nitrate was added, the final *p*H was adjusted with 3 N NaOH or 3 N HCl before sterilization. Filter-sterilized sodium nitrite was added to the heating menstruum from a 10% stock solution.

Spores were heated at 98 C in a 200-ml three-neck round bottom flask, which was submerged in a bath of high-temperature propylene glycol. The apparatus was similar to the one described by Daoust, Read, and Litsky (4). The flask was fitted with a thermometer and a motor-driven agitator. In preparation for an experiment, 198 ml of the appropriate sterile menstruum was added to the flask and equilibrated at 98 C. Then 2 ml of suspension containing approximately 3.6×10^8 spores was added. Thus, the spores reached the desired temperature almost instantaneously.

Samples were removed at intervals, cooled immediately, and diluted as necessary for preparation of quadruplicate counts in LVA tubes with or without the curing salts. Heating was continued long enough to reduce the viable spore count by at least 99.9%.

A survivor curve was prepared for each experimental variable by plotting per cent survivors against time on semilog paper. The initial value (100% survival) was the count of unheated spores in the particular outgrowth medium being tested (LVA with or without curing salts).

Experimental design. Experiments were planned to allow the separation of effects during heating from those occurring after heating (i.e., effect on outgrowth). All trials were run both at neutrality and at *p*H 6.0, a value near that of canned luncheon meat. Three types of experiments were run.

(i) Spores were heated in phosphate buffer, then cultured in LVA containing various concentrations of the curing salts. This approach should reveal the effect of the salts on outgrowth of spores after heat injury.

(ii) Spores were heated in phosphate buffer containing various concentrations of the curing salts. Then the spores were removed by centrifugation, washed three times in cold deionized water to remove the salts, and cultured in plain LVA. This treatment should reveal the additive effects of the chemicals during heating independent of their effect in the outgrowth medium.

(iii) Spores were heated in phosphate buffer containing various concentrations of the curing salts, then cultured in LVA containing the corresponding chemicals. This treatment should reveal the combined effects of heat and chemicals and most nearly approaches the conditions that exist in practice.

Control survivor curves were prepared for each set of test conditions by omitting the curing salts.

Expression of results. The foregoing plan produced 120 survivor curves. For convenience in comparing the relative resistance of the spores under different conditions of heating, the *D* value of each survivor curve was plotted against concentration of the curing salt. (*D* value is the time in minutes required to kill 90% of the spores at a specified temperature.) Thus, a *D* value higher than the control (survivor curve without the curing salt) shows increased resistance (i.e., protection) of the spores, whereas a *D* value lower than the control indicates decreased resistance or sensitization to the curing agents.

Differences in *D* values of control survivor curves between experiments reflect different batches of spores produced at various times.

RESULTS

Effect of curing agents on outgrowth of heat-injured spores. That heating injures spores in a manner that reduces their ability to grow in the

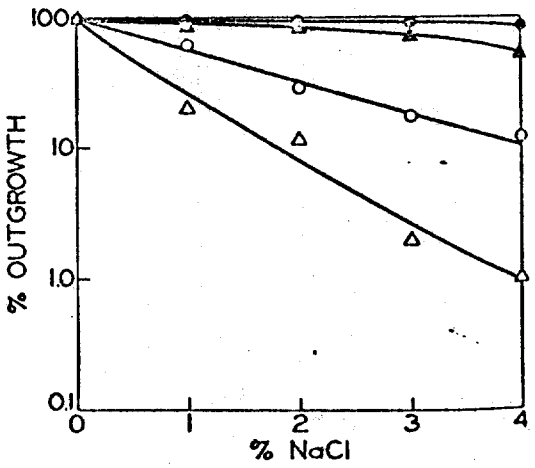


FIG. 1. *Effect of prior heating on outgrowth of PA 3679h spores in LVA containing 0 to 4% NaCl at pH 6.0 and 7.0. Spores heated 10 min at 98 C in 0.067 M phosphate buffer at same pH as outgrowth medium. Colony counts in LVA without NaCl considered 100% outgrowth. Symbols: ●, unheated spores, pH 7.0; ○, heated spores, pH 7.0; ▲, unheated spores, pH 6.0; △, heated spores, pH 6.0.*

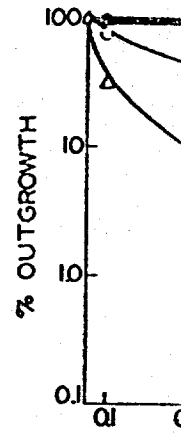


FIG. 2. *Effect*
PA 3679h spores
at pH 6.0 and 7.
bols as in Fig. 1.

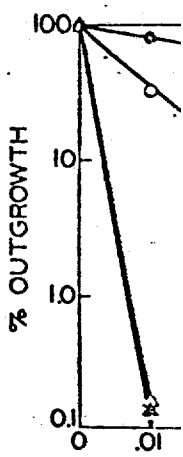


FIG. 3. *Effect*
PA 3679h spores i
at pH 6.0 and 7.0.
as in Fig. 1.

presence of curi
every instance,
min were less tol
unheated spores
*p*H 6.0 than at
inhibitory at the
pletely inhibited
unheated spores
(Fig. 3). The co
had essentially ti

Effect of NaCl
5: (i) heat inc
sodium chloride

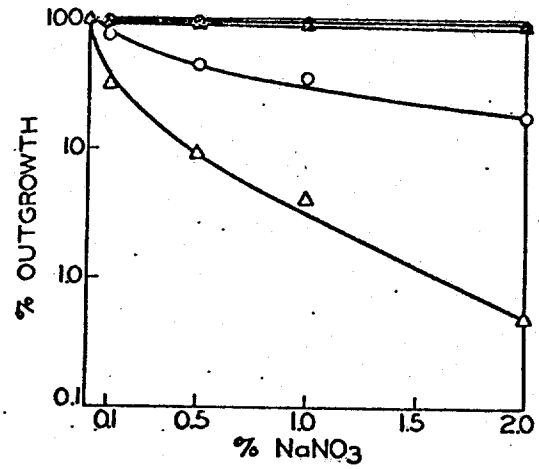


FIG. 2. *Effect of prior heating on outgrowth of PA 3679h spores in LVA containing 0 to 2% $NaNO_3$ at pH 6.0 and 7.0. Experimental conditions and symbols as in Fig. 1.*

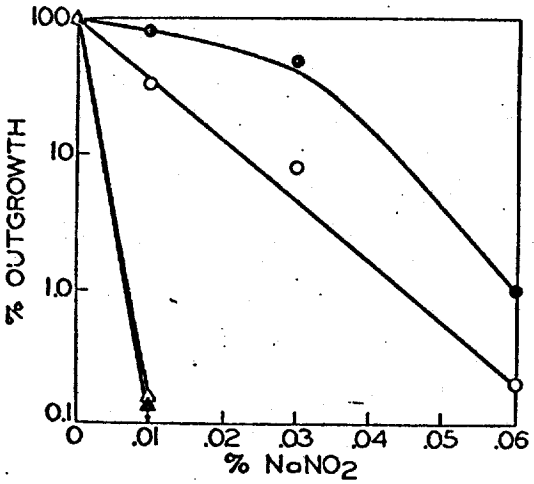


FIG. 3. *Effect of prior heating on outgrowth of PA 3679h spores in LVA containing 0 to 0.06% $NaNO_2$ at pH 6.0 and 7.0. Experimental conditions and symbols as in Fig. 1.*

presence of curing salts is shown in Fig. 1–4. In every instance, spores that survived 98 C for 10 min were less tolerant of the curing salts than were unheated spores. The effect was more marked at *p*H 6.0 than at *p*H 7.0. Nitrite was especially inhibitory at the lower *p*H value. In fact, it completely inhibited outgrowth of both heated and unheated spores at the lowest concentration tested (Fig. 3). The combined curing ingredients (Fig. 4) had essentially the same effect as nitrite alone.

Effect of NaCl. Two effects were observed (Fig. 5): (i) heat increased the sensitivity of spores to sodium chloride in the outgrowth medium, and (ii) sodium chloride tended to protect the spores against heat injury. When the spores were both heated and cultured in the presence of NaCl, the protective effect predominated at the lower concentrations and the sensitizing effect predominated at the higher concentrations. The protective effect was more pronounced at *p*H 6.0 than at *p*H 7.0.

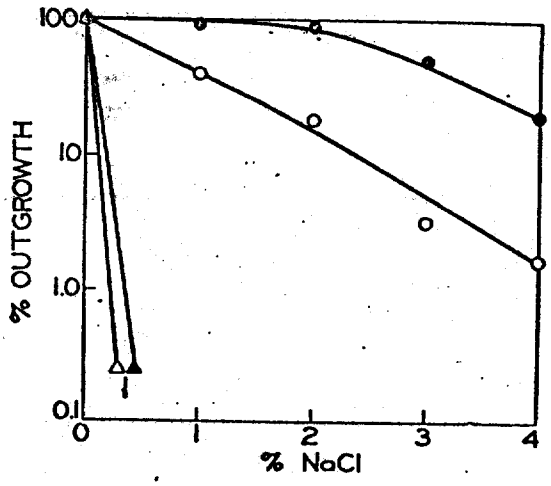


FIG. 4. *Effect of prior heating on outgrowth of PA 3679h spores in LVA containing mixed curing agents (0.1% $NaNO_3$, 0.03% $NaNO_2$, and 0 to 4% NaCl) at pH 6.0 and 7.0. Experimental conditions and symbols as in Fig. 1.*

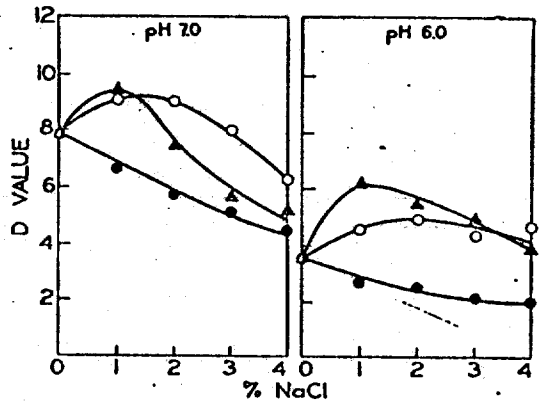


FIG. 5. *Effect of NaCl on D values of PA 3679h spore survivor curves at 98 C. Symbols: ○, spores heated in 0.067 M phosphate buffer, pH 6.0 or 7.0, containing 0 to 4% NaCl, then washed and cultured in LVA at the corresponding pH without NaCl; ●, spores heated in 0.067 M phosphate buffer, pH 6.0 or 7.0, then cultured in LVA at the corresponding pH with 0 to 4% NaCl; ▲, spores heated in 0.067 M phosphate buffer, pH 6.0 or 7.0, containing 0 to 4% NaCl, then cultured in LVA at the corresponding pH and NaCl concentration.*

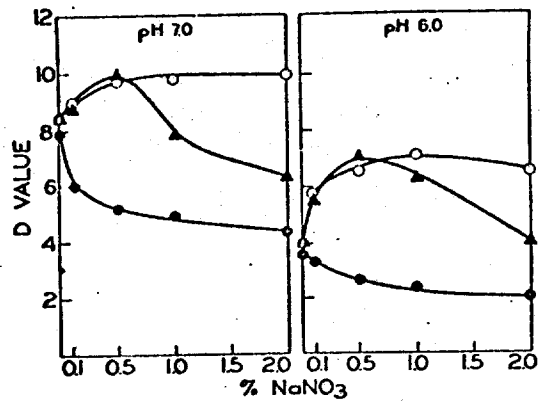


FIG. 6. *Effect of $NaNO_3$ on D values of PA 3679h spore survivor curves at 98 C. Experimental conditions and symbols as in Fig. 5.*

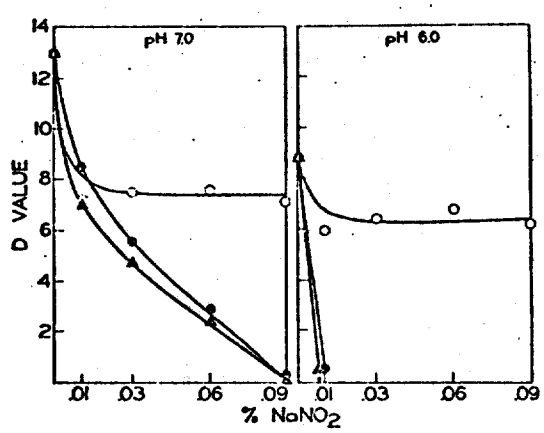


FIG. 7. *Effect of $NaNO_2$ on D values of PA 3679h spore survivor curves at 98 C. Experimental conditions and symbols as in Fig. 5.*

In canned luncheon meats, the effective salt concentration (per cent salt in the aqueous phase) usually is between 4 and 6%; hence, NaCl should play a significant role in preservation.

Effect of $NaNO_3$. Nitrate had essentially the same effect as NaCl (Fig. 6). However, at the concentrations normally used in canned luncheon meats (up to 0.1% based on the weight of the product), it is doubtful that this agent would have a significant preservative effect.

Effect of $NaNO_2$. In contrast to NaCl and $NaNO_3$, nitrite had no detectable protective effect during heating of the spores (Fig. 7). Heating in the presence of as little as 0.01% $NaNO_2$ reduced the ability of the spores to grow in its absence. Even more marked was the inhibitory effect of nitrite in the outgrowth medium, particularly at *p*H 6.0.

Effect of mixed curing salts. The interaction of the curing salts with their combined protective and sensitizing effects is shown in Fig. 8. At *p*H 7.0, the protective effect of NaCl and $NaNO_3$ predominated when the spores were heated with the curing salts and cultured in their absence. However, the spores showed the expected sensitivity when heated in buffer and then cultured in the presence of the curing salts. The inhibitory effect of nitrite overcame the protection afforded by NaCl and $NaNO_3$ when the spores were both heated and cultured with the mixed curing salts.

The importance of nitrite in preservation of cured meats is clearly shown in the results for *p*H 6.0. It had little effect during heating but was decisively inhibitory when present in the outgrowth medium.

DISCUSSION

This work was done prior to publication of and without knowledge of similar experiments being performed in England (10). The results show good agreement. As reported by Roberts and Ingram (10), heat in some way injures or sensitizes bacterial spores, making them less tolerant of meat-curing salts in the outgrowth medium. The result-

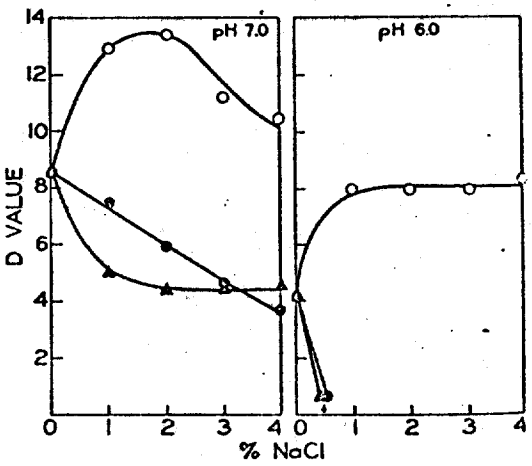


FIG. 8. *Effect of mixed curing salts on D values of PA 3679h spore survivor curves at 98 C. Symbols: ○, spores heated in 0.067 M phosphate buffer, pH 6.0 or 7.0, containing 0.1% $NaNO_3$, 0.03% $NaNO_2$, and 0 to 4% NaCl, then washed and cultured in LVA at the corresponding pH without the curing salts; ●, spores heated in 0.067 M phosphate buffer, pH 6.0 or 7.0, then cultured in LVA at the corresponding pH with 0.1% $NaNO_3$, 0.03% $NaNO_2$, and 0 to 4% NaCl; ▲, spores heated in 0.067 M phosphate buffer, pH 6.0 or 7.0, containing 0.1% $NaNO_3$, 0.03% $NaNO_2$, and 0 to 4% NaCl, then cultured in LVA at the corresponding pH and concentration of mixed curing salts.*

ing inhibitory effect of the curing agents is considerably greater at *p*H 6.0, which is near the usual *p*H of canned cured meat products, than at pH 7.0. No doubt these circumstances contribute to the remarkable stability of canned cured luncheon meats.

The experimental plan of the study reported here allowed the separation of effects during heating from those during outgrowth. At relatively low concentrations, sodium chloride and sodium nitrate actually tended to protect the spores against heat injury, as was shown by Viljoen with sodium chloride over 50 years ago (14). Nitrite, on the other hand, enhanced heat injury.

Examination of the combined effects of heat and the mixed curing salts (Fig. 8) clearly shows the significance of nitrite and *p*H 6.0 in preserving canned cured meat products. Sodium chloride is contributory (Fig. 5), but sodium nitrate probably has no significant effect at the concentrations normally used in practice (Fig. 6).

ACKNOWLEDGMENT

This investigation was supported by a series of annual grants from Oscar Mayer & Co., Madison, Wis.

LITERATURE CITED

1. CAMPBELL, L. L., AND H. A. FRANK. 1956. Nutritional requirements of some putrefactive anaerobic bacteria. J. Bacteriol. 71:267–269.
2. CAMPBELL, L. L., C. M. RICHARDS, AND E. E. SNIFF. 1965. Isolation of strains of *Bacillus stearothermophilus* with altered requirements for spore germination, p. 55–63. *In* L. L Campbell and H. O. Halvorson [ed.], Spores III. American Society for Microbiology, Ann Arbor, Mich.
3. CURRAN, H. R., AND F. R. EVANS. 1937. The importance of enrichments in the cultivation of bacterial spores previously exposed to lethal agencies. J. Bacteriol. 34:179–189.
4. DAOUST, D. R., R. B. READ, JR., AND W. LITSKY. 1961. Thermal inactivation studies on pathogenic bacteria in milk and various milk products. I. *Corynebacterium diphtheriae* ATCC No. 296. J. Dairy Sci. 44:32–40.
5. GOUGH, B. J., AND J. A. ALFORD. 1965. Effect of curing agents on the growth and survival of food-poisoning strains of *Clostridium perfringens*. J. Food Sci. 30:1025–1028.
6. GROSS, C. E., C. VINTON, AND S. MARTIN. 1946. Bacteriological studies relating to thermal processing of canned meats. IV. Viability of spores of a putrefactive anaerobic bacterium in canned meat after prolonged incubation. Food Res. 11:399–404.
7. HACHISUKA, Y. 1964. Studies on amino acids which are required by the sublethally treated spores of *B. subtilis* for colony formation. Japan. J. Bacteriol. 19:162–168.
8. IRIE, R., AND H. UCHIYAMA. 1964. Preparation of a clean sample of bacterial spores. J. Gen. Appl. Microbiol. 10:237–242.
9. RIEMANN, H. 1963. Safe heat processing of canned cured meats with regard to bacterial spores. Food Technol. 17:39–49.
10. ROBERTS, T. A., AND M. INGRAM. 1966. The effect of sodium chloride, potassium nitrate and sodium nitrite on the recovery of heated bacterial spores. J. Food Technol. 1:147–163.
11. SILLIKER, J. H., R. A. GREENBERG, AND W. R. SCHACK. 1958. Effect of individual curing ingredients on the shelf stability of canned comminuted meats. Food Technol. 12:551–554.
11. TANNER, F. W., AND F. L. EVANS. 1934. Effect of meat curing solutions on anaerobic bacteria. Zentr. Bakteriol. Parasitenk. Abt. 1 Orig. 91:1–14.
13. UEHARA, M., R. S. FUJIOKA, AND H. A. FRANK. 1965. Method for obtaining cleaned putrefactive anaerobe 3679 spores. J. Bacteriol. 89:929–930.
14. VILJOEN, J. A. 1926. Heat resistance studies. 2. The protective effect of sodium chloride on bacterial spores heated in pea liquor. J. Infect. Diseases 39:286–290.

APPLIED MICROBIOLOGY, Feb. 1968, p. 406-411
Copyright © 1968 American Society for Microbiology
Vol. 16, No. 2
Printed in U.S.A.

Effect of Sodium Nitrite, Sodium Chloride, and Sodium Nitrate on Germination and Outgrowth of Anaerobic Spores[1]

CHARLES L. DUNCAN[2] AND E. M. FOSTER

Department of Bacteriology and Food Research Institute, University of Wisconsin, Madison, Wisconsin 53706

Received for publication 29 November 1967

The effects of meat-curing agents on germination and outgrowth of putrefactive anaerobe 3679h (PA 3679h) spores were studied in microcultures. Nitrite concentrations up to 0.06% at *p*H 6.0 or between 0.8 and 1% at *p*H 7.0 allowed emergence and elongation of vegetative cells but blocked cell division. The newly emerged cells then lysed. With more than 0.06% nitrite at *p*H 6.0 or more than 0.8 to 1% at *p*H 7.0, the spores lost refractility and swelled, but vegetative cells did not emerge. Even as much as 4% nitrite failed to prevent germination (complete loss of refractility) and swelling of the spores. Sodium chloride concentrations above 6% prevented complete germination (i.e., the spores retained a refractile core). In the presence of 3 to 6% sodium chloride, most of the spores germinated and produced vegetative cells, but cell division was often blocked. Sodium nitrate had no apparent effect on germination and outgrowth at concentrations up to 2%.

Germination and outgrowth of bacterial spores include five sequential steps: (i) germination (becoming nonrefractile, stainable, and heat-sensitive); (ii) swelling of the germinated spore; (iii) emergence of the new vegetative cell; (iv) elongation; and (v) cell division.

Although many substances are known to block the development of spores into actively multiplying vegetative cells, the stage of germination or outgrowth at which a particular substance is effective is known only in a few cases. Gould (5) determined the effects of a number of common food preservatives on growth from spores of six *Bacillus* species, but he failed to include any of the clostridia. Most previous work (2, 6, 7) has dealt with growth of vegetative cells, and Gould's study was the first attempt to define the effects of chemical preservatives on spore germination and outgrowth.

In an earlier report (3), we described the end result, as shown by colony formation, when sodium chloride, sodium nitrate, and sodium nitrite were allowed to act on an anaerobic spore-former during and after heating. The purpose of the work reported here was to define the point in the developmental process at which the curing salts act to prevent outgrowth.

MATERIALS AND METHODS

Spores of putrefactive anaerobe 3679 strain h (PA 3679h) were prepared as previously described (3). Liver Veal Agar (Difco) containing various concentrations of sodium chloride, sodium nitrate, or sodium nitrite was used as the growth medium at *p*H 6.0 or 7.0. Sodium nitrite was added to the base medium from a freshly prepared, filter-sterilized 10% solution immediately before use. The other salts were added before sterilization.

Microcultures were prepared by layering approximately 1 ml of the appropriate agar medium on the surface of a sterile glass slide (25 × 75 mm). The prepared slides were stored in petri dishes to maintain sterility. A loopful of spore suspension was smeared onto the surface of a sterile glass cover slip (24 × 50 mm) and allowed to dry in a petri dish. Then, the cover slip was inverted and carefully pressed onto the hardened agar surface. Air spaces along the sides of the cover slip were filled with additional medium, and the edges of the cover slip were sealed with Vaspar. The slide cultures were incubated at 30 C and examined at intervals up to 6 days with a Zeiss phase-contrast photomicroscope.

RESULTS

The normal development of PA 3679h spores from dormancy to active multiplication is illus-

[1] Published with the permission of the Director of the Wisconsin Agricultural Experiment Station, Madison.

[2] Present address: Department of Foods and Nutrition, University of Wisconsin, Madison, Wis. 53706.

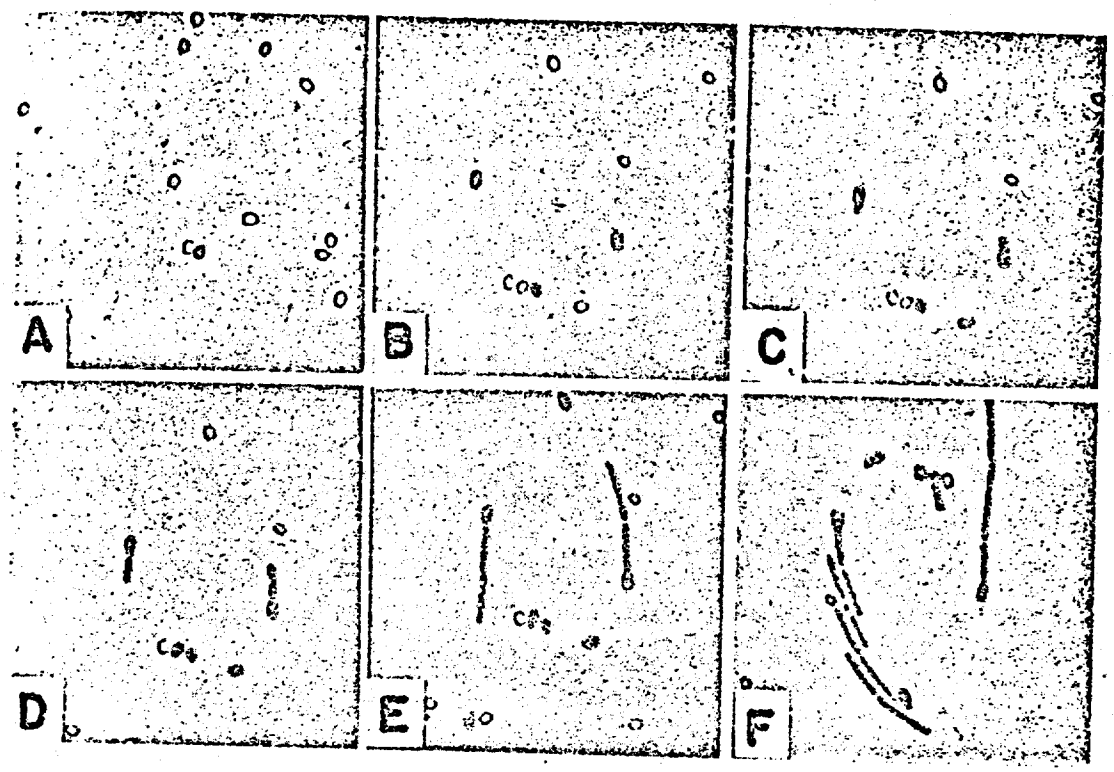


FIG. 1. *Stages in germination and outgrowth of PA 3679h spores in Liver Veal Agar microculture (pH 7.0) at room temperature. (A) At 5 hr, several spores have lost refractility; (B) 7 hr, swelling of spores; (C) 10 hr, emergence of vegetative cell from end of spore; (D) 13 hr, elongation of vegetative cell; (E) 15 hr, cell division; (F) 18 hr, continuing growth. (B) through (E) show the same microscopic field. Original magnification, × 1,070.*

trated in Fig 1. With this organism, emergence follows lysis of one end of the swollen germinated spore. This contrasts with some of the *Bacillus* species, whose spores undergo lysis or rupture of one side of the spore case.

The time intervals shown in Fig. 1 obviously are not representative of every spore observed, because outgrowth was not a synchronous process. The variability in response of individual spores is readily seen.

Effect of sodium nitrite. Preliminary experiments showed that 0.01 and 0.09% sodium nitrite completely inhibited colony formation at *p*H 6.0 and 7.0, respectively. The photographs in Fig. 2 reveal two points of inhibition in the outgrowth process. Even as much as 4% nitrite did not interfere with loss of refractility (Fig. 2A) and swelling (Fig. 2B). However, the process stopped there if the nitrite concentration was as much as 0.06% at *p*H 6.0 or 0.8 to 1% at *p*H 7.0. At lower nitrite levels, the vegetative cells emerged (Fig. 2C) and elongated (Fig. 2D), but cell division did not take place even at levels of 0.01% (*p*H 6.0) or 0.09% (*p*H 7.0). Elongated cells that did not multiply eventually lysed, leaving the empty spore coats (Fig. 2E).

Effect of sodium chloride. With more than 6% sodium chloride, most of the spores underwent only partial germination; that is, they lost part of their refractility but were not completely phase-dark (Fig. 3A and B). This condition persisted for at least 10 days.

At salt concentrations of 1 to 3%, overgrowth of the slide cultures made it impossible to observe inhibition of individual spores. However, concentrations between 3 and 6% were sufficiently inhibitory to prevent complete overgrowth, thereby allowing observation of the effects of salt. The variability in response of individual spores is evident, since no single concentration of salt completely blocked all of the spores at a particular developmental stage. Instead, cells which were inhibited at each of the transitional stages—emergence (Fig. 3C), elongation (Fig. 3D), or cell division (Fig. 3E)—could be found in the same slide culture at salt concentrations between 3 and 6%. Cells that did not develop

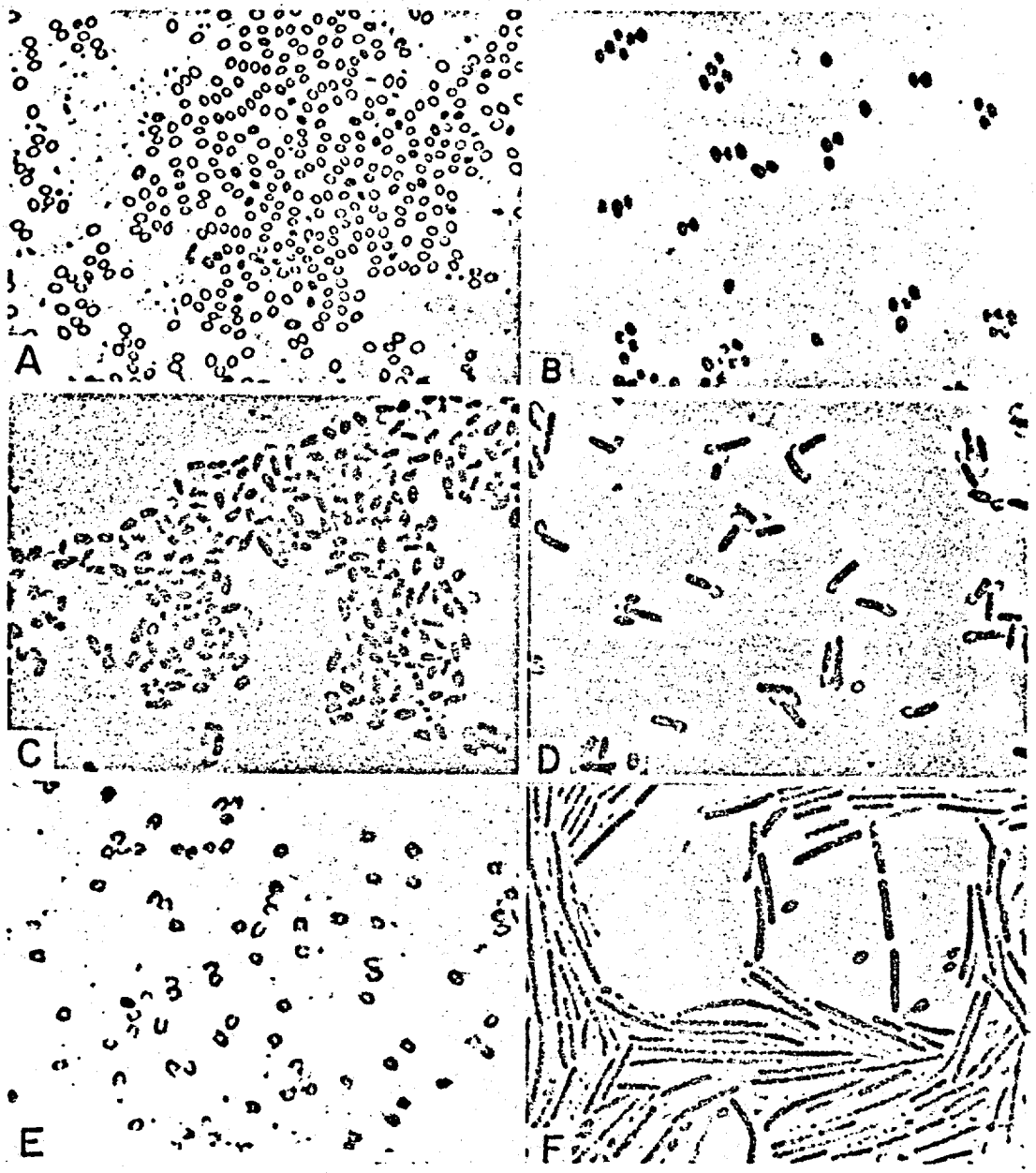


FIG. 2. *Effect of sodium nitrite on germination and outgrowth of PA 3679h spores in microculture at 30 C. Loss of refractility (A) and swelling of the spores (B) took place at all concentrations of nitrite up to 4%. Further development depended on nitrite level and pH. Emergence (C) and elongation (D) occurred at concentrations up to 0.06% at pH 6.0 and 0.8 to 1.0% at pH 7.0, but the process did not go further even with nitrite levels as low as 0.01% (pH 6.0) or 0.09% (pH 7.0). Under these conditions, the newly emerged cells lysed, leaving the spore coats behind (E). The normal pattern of growth without nitrite is shown in (F). Original magnification, × 1,070.*

beyond elongation eventually lysed, leaving the empty spore coats (Fig. 2E).

Effect of sodium nitrate. Overgrowth by vegetative cells at all concentrations of nitrate up to 2% masked any visible effects on individual spores. Since this concentration is at least 20 times that normally employed in commercial practice, no further tests were conducted with sodium nitrate.

A summary of the stages which, in the transformation of a spore into a vegetative cell, are inhibited by various concentrations of sodium

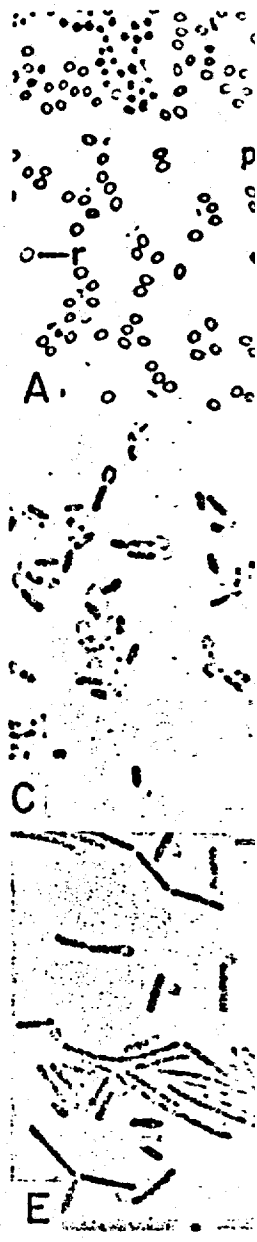


FIG. 3. *Effect of so*
Complete loss of refra
germination (p) are sh
tion of emergence (C
and less sodium chlora
mental stage. The norm
× 1,070.

nitrite and sodium chl
In all cases, the end
particular stage subse
of the newly emerged

FIG. 3. *Effect of sodium chloride on germination and outgrowth of PA 3679h spores in microculture at 30 C. Complete loss of refractility was inhibited by more than 6% sodium chloride (A, B). Various stages of partial germination (p) are shown with completely refractile (r) spores. Variability among spores is reflected in the inhibition of emergence (C), elongation (D), or cell division (E) in some of the population at concentrations of 6% and less sodium chloride. No specific concentration completely blocked all of the spores at a particular developmental stage. The normal pattern of growth without sodium chloride is shown in (F). Original magnification,* × *1,070.*

nitrite and sodium chloride is presented in Fig. 4. In all cases, the end result of inhibition at a particular stage subsequent to swelling was lysis of the newly emerged or elongated cell.

DISCUSSION

With the exception of Gould (5), previous workers have studied the effects of sodium nitrite primarily on vegetative cells. Gould reported

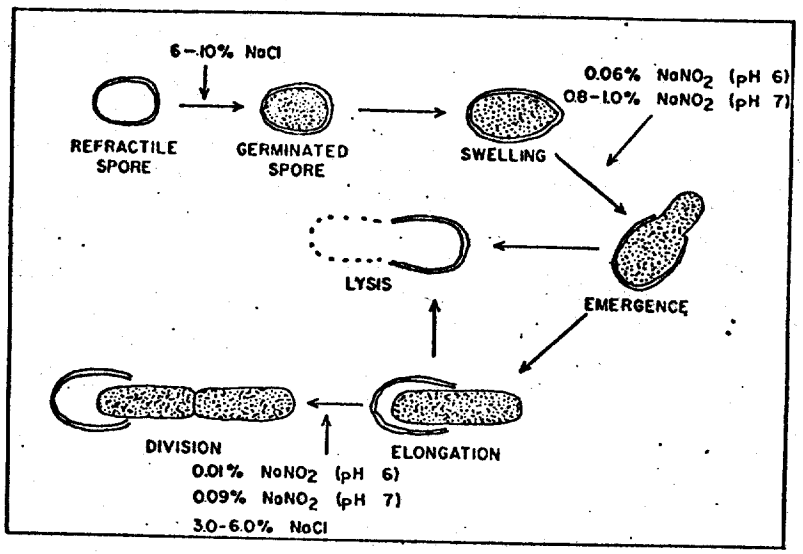


FIG. 4. *Summary of the effects of sodium nitrite and sodium chloride on the germination and outgrowth of PA 3679h spores. The points of inhibition are indicated by arrows perpendicular to those designating the normal process. Inhibition of stages prior to cell division results in lysis of cells.*

that spores of several *Bacillus* species germinated, though at reduced rates, in less than 0.03% sodium nitrite at *p*H 6.0. Development was arrested immediately after germination; i.e., before lysis or rupture of spore coats had occurred. Higher concentrations of nitrite (0.075 to 0.25%) prevented germination altogether at *p*H 6.0. Toxicity was three to five times greater at *p*H 6.0 than at *p*H 7.0.

By contrast, nitrite levels as high as 4% failed to inhibit germination of PA 3679h spores in this study. Furthermore, low concentrations of nitrite allowed emergence and elongation of some of the cells before inhibition occurred.

Gould also reported that up to 8% sodium chloride did not prevent germination of spores of any of the organisms studied. Concentrations ranging from 4 to 7% inhibited outgrowth, whereas concentrations of 10 to 15% progressively reduced and finally prevented germination. These results are comparable to the ones reported here. However, partial germination of PA 3679h spores occurred in 6 to 10% salt.

Partial germination of PA 3679h spores was first reported by Uehara and Frank (Bacteriol. Proc., p. 36, 1965). At hyperoptimal incubation temperatures (above 45 C), germination in the presence of L-alanine was only partial in that most of the spores appeared not to be completely darkened (the core was refractile). Partial germination also was observed when the germination system included D-alanine, a competitive inhibitor of L-alanine-induced germination. These results were interpreted to indicate that L-alanine-induced germination occurred in at least two stages.

Normally, spore germination is considered to be a rapid irreversible process, which is relatively insensitive to the environment (as compared to growth of vegetative cells). The present results confirm those of Uehara and Frank in suggesting that germination of PA 3679h spores may occur in two stages, since the addition of sodium chloride to the medium allowed only partial germination.

The results of these and other studies in our laboratory (3, 4) may help to explain the importance of nitrite in the preservation of canned cured meat products. Nitrite actually stimulates spore germination, especially under acid conditions at elevated temperatures (4). During normal heat processing, therefore, nitrite may induce spores to germinate and thus make them susceptible to thermal inactivation.

Spores that survive the heat treatment may germinate, but outgrowth is blocked by the residual nitrite in the product. At *p*H 6.0, as little as 0.01% nitrite allowed emergence of vegetative cells but prevented cell division. Under these conditions, the vegetative cells lysed.

Canned cured meat products usually contain 4 to 6% brine (i.e., 4 to 6% sodium chloride in the aqueous phase). Although this concentration did not prevent outgrowth of all PA 3679h spores, it did inhibit some of them and its effectiveness should be even greater with heat-injured spores

(3). Therefore, the salt concentrations used in practice may contribute significantly to the preservation of canned cured meat products. As with nitrite, newly emerged vegetative cells whose further development is blocked by 4 to 6% sodium chloride eventually will lyse.

ACKNOWLEDGMENT

This investigation was supported by a series of annual grants from Oscar Mayer & Co., Madison, Wis.

LITERATURE CITED

1. CAMPBELL, L. L., AND H. A. FRANK. 1956. Nutritional requirements of some putrefactive anaerobic bacteria. J. Bacteriol. 71:267–269.
2. CASTELLANI, A. G., AND C. F. NIVEN, JR. 1955. Factors affecting the bacteriostatic action of sodium nitrite. Appl. Microbiol. 3:154–159.
3. DUNCAN, C. L., AND E. M. FOSTER. 1968. Role of curing agents in the preservation of shelf-stable canned meat products. Appl. Microbiol. 16: 401–405.
4. DUNCAN, C. L., AND E. M. FOSTER. 1968. Nitrite-induced germination of putrefactiev anaerobe 3679h spores. Appl. Microbiol. 16:412–416.
5. GOULD, G. W. 1964. Effect of food preservatives on the growth of bacteria from spores, p. 17–24. *In* N. Molin [ed.], Microbial inhibitors in food. Almqvist & Wiksell, Stockholm.
6. INGRAM, M. 1939. The endogenous respiration of Bacillus cereus. II. The effect of salts on the rate of absorption of oxygen. J. Bacteriol. 38:613–629.
7. TARR, H. L. A. 1941. The action of nitrites on bacteria. J. Fisheries Res. Board Can. 5:265–275.

APPLIED MICROBIOLOGY, Feb. 1968, p. 412-416
Copyright © 1968 American Society for Microbiology
Vol. 16, No. 2
Printed in U.S.A.

Nitrite-induced Germination of Putrefactive Anaerobe 3679h Spores[1]

CHARLES L. DUNCAN[2] AND E. M. FOSTER

Department of Bacteriology and Food Research Institute, University of Wisconsin, Madison, Wisconsin 53706

Received for publication 29 November 1967

Sodium nitrite alone has been shown to stimulate germination of PA 3679h spores. The process was accelerated by using increased concentrations of sodium nitrite, a low *p*H, and a high temperature of incubation. At low concentrations of nitrite (0.01 to 0.2%), the delay of 36 to 48 hr occurred before germination commenced at 37 C. However, with 3.45% nitrite at 45 C and *p*H 6.0, most of the spores germinated within 1 hr. At *p*H 7.0, the germination rate decreased markedly, and at *p*H 8.0 it was nil. The greatest acceleration in germination rate occurred near 60 C. Hydroxylamine was completely inhibitory to nitrite-induced germination. Sodium nitrite, in turn, inhibited germination by L-alanine, the degree of inhibition being influenced by nitrite concentration and *p*H.

Several ways have been found to induce germination of bacterial spores. In addition to the "physiological germinants," such as amino acids and carbohydrates, there have also been reports of "ionic germination" (5, 8). Hyatt and Levinson (5) were able to induce germination of *Bacillus megaterium* spores with either KNO_3 or KNO_2, but only if the spores were first subjected to a sublethal heat treatment of 10 min at 60 C. Likewise, Black (Bacteriol. Proc., p. 36, 1964) observed germination of *B. cereus* strain *terminalis* spores in 0.5 to 2.0 M $NaNO_2$ or KNO_2.

Studies of microcultures by Duncan and Foster (3) indicated that sodium nitrite, even in high concentration, would not prevent loss of refractility and swelling of putrefactive anaerobe 3679h (PA 3679h) spores, although very small amounts of the compound were able to inhibit division of newly emerged cells. These observations prompted an investigation of the effect of sodium nitrite on germination of PA 3679h spores.

MATERIALS AND METHODS

Spores of PA 3679h were prepared as described elsewhere (2). Reaction mixtures in new screw-capped tubes (16 × 125 mm) were adjusted to the proper temperature in a thermostatically controlled water bath; then 1.2×10^5 to 1.5×10^5 spores were added per ml. Germination was followed by measuring the decrease in optical density (expressed as change in OD) at 600 mμ with a Bausch & Lomb Spectronic 20 colorimeter. Germination was also validated by phase contrast microscopy and by staining with dilute methylene blue.

All reagents were prepared as concentrated stock solutions in deionized water and diluted as necessary to give a final reaction mixture volume of 6 ml. Fresh solutions of sodium nitrite were prepared before each experiment.

For some experiments, the germination rate of the spores was expressed as the change in OD per minute × 100, as measured over a 10-min period during the first 10 to 25 min of germination.

RESULTS

Increasing the concentration of sodium nitrite from 0.03 to 0.2% dramatically accelerated germination at *p*H 6.0 (Fig. 1). Essentially no germination occurred at *p*H 6.0 in 1 week without nitrite or at *p*H 7.0 with as much as 0.2% nitrite.

The 36-hr lag before germination commenced was eliminated by increasing the nitrite concentration to 0.5 M (3.45%) and the temperature to 45 C. Figure 2 shows the rapid germination at *p*H 6.0 under these conditions in contrast to a much slower rate at *p*H 7.0 and practically none at *p*H 8.0.

Figure 3 shows the effect of temperature on nitrite-induced germination. The greatest acceleration in germination rate occurred in the vicinity of 60 C. These results are in sharp contrast to the 45 C optimum reported by Uehara

[1] Presented in part at the 67th Annual Meeting of the American Society for Microbiology, New York, N.Y., 30 April-4 May 1967. Published with the permission of the Director of the Wisconsin Agricultural Experiment Station, Madison.

[2] Present address: Department of Foods and Nutrition, University of Wisconsin, Madison, Wis. 53706.

Vol. 16, No. 2
Printed in U.S.A.

factive

dison, Wisconsin 53706

PA 3679h
of sodium
entrations of
nation com-
most of the
l markedly,
ate occurred
d germina-
degree of

0⁸ spores were added
wed by measuring the
xpressed as change in
Lomb Spectronic-20
so validated by phase
staining with dilute

s concentrated stock
diluted as necessary
volume of 6 ml. Fresh
prepared before each

mination rate of the
ange in OD per min-
10-min period during
tion.

n of sodium nitrite
tically accelerated
1). Essentially no
) in 1 week without
ch as 0.2% nitrite
mination commenced
the nitrite concen-
he temperature to
id germination at
ns in contrast to a
d practically none

of temperature on
The greatest ac-
occurred in the
s are in sharp con
eported by Uehara

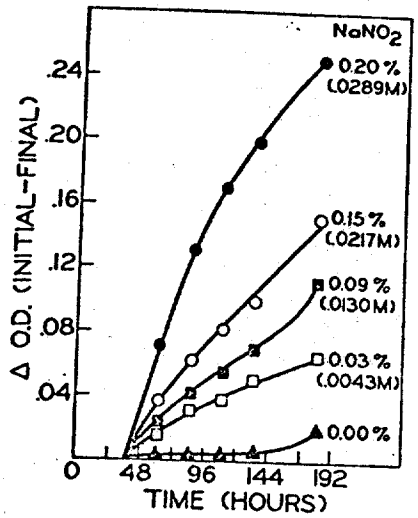


FIG. 1. *Effect of sodium nitrite on germination of PA 3679h spores. Spores were incubated at 37 C in 0.01 M phosphate buffer, pH 6.0, plus added nitrite. No germination was observed at pH 7.0 under the same conditions.*

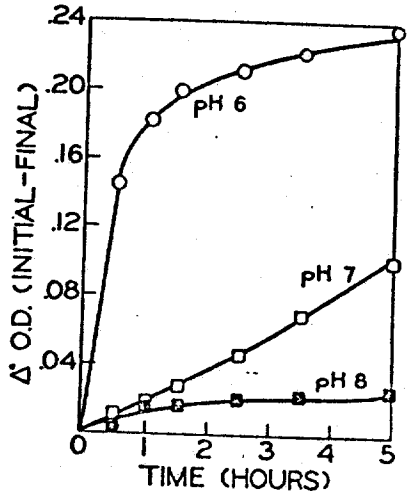


FIG. 2. *Effect of pH on germination of PA 3679h spores by sodium nitrite. Spores were incubated at 45 C in 0.01 M phosphate buffer plus 0.5 M (3.45%) sodium nitrite at the indicated pH values.*

and Frank (10) for the L-alanine germination system with spores of this same organism.

A sublethal "heat shock" is necessary to induce optimal germination in some bacterial spores. To see whether the temperature effect in Fig. 3 was the result of "heat activation," spores and sodium nitrite were heated separately and together, as shown in Table 1. Heating the spores alone (treatment 1), the nitrite alone (treatment 2), or both separately (treatment 3) gave about the same germination rates as no heating (treatment 4). Only when the spores and nitrite were heated together (treatment 5) was the germination rate increased appreciably. Thus, it must be concluded that the effect of increased temperature shown in Fig. 3 was not the result of "heat activation" of the spores or of heat-induced alteration of the nitrite ion, but rather was the result of greater interaction between nitrite and the spores.

Various compounds that have been shown to inhibit germination of bacterial spores were tested for their effect on nitrite-induced germination of PA 3679h (Table 2). Hydroxylamine was markedly inhibitory, as were tris(hydroxymethyl)aminomethane buffer (a known sequestering agent of metal ions), sodium azide, potassium cyanide, and hydrogen peroxide. All other com-

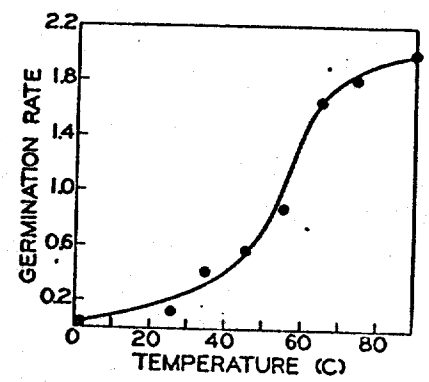


FIG. 3. *Effect of temperature on rate of PA 3679h spore germination by sodium nitrite. Final concentration of sodium nitrite was 0.5 M in 0.01 M phosphate buffer, pH 6.0.*

TABLE 1. *Effect of heating components of germination mixture on the germination rate of PA 3679h spores*[a]

Treatment	Germination rate
1. Spores heated at 65 C for 30 min in 0.01 M phosphate buffer; then removed and suspended in germination mixture at 35 C	0.33
2. Germination mixture heated at 65 C for 30 min; then cooled to 35 C and spores added	0.32
3. Spores and germination mixture heated separately at 65 C for 30 min; then cooled to 35 C and mixed	0.34
4. Unheated spores in unheated germination mixture at 35 C	0.34
5. Spores incubated in germination mixture at 65 C	1.65

[a] Germination mixture: 0.5 M sodium nitrite in 0.01 M phosphate buffer, pH 6.0.

TABLE 2. *Effect of various compounds on the initial rate of sodium nitrite-induced germination of PA 3679h spores*[a]

Test compound	Final concn	Germination rate	Change in germination rate[b]
			%
Control (phosphate buffered, 0.01 M)	—	.56	
Control (Tris-buffered, 0.01 M)	—	.04	−93
Hydroxylamine-HCl	500.0 mM	.00	−100
NaN_3	10.0 mM	.01	−98
KCN	10.0 mM	.17	−70
H_2O_2	2.0%	.23	−59
Glycine	10.0 mM	.56	0
$MnCl_2$	1.0 mM	.83	+48
8-Hydroxyquinoline	1.0 mM	.63	+13
$HgCl_2$	1.0 mM	.62	+11
2,4-Dinitrophenol	5.0 mM	.70	+25
$NaNO_3$	58.8 mM	.59	+5
$NaNO_3$	117.6 mM	.59	+5
NaCl	85.6 mM	.69	+23
NaCl	342.4 mM	.70	+25

[a] Germination mixture: 0.5 M sodium nitrite in 0.01 M phosphate buffer, *p*H 6.0, 45 C.

[b] Based on phosphate-buffered control.

pounds tested either had no effect or were slightly stimulatory. Except with hydroxylamine, the decreased germination rates in Table 2 were eventually overcome, and the final levels of germination were equivalent to those of the phosphate-buffered control. With hydroxylamine, however, there was no germination at all within 10 hr.

"Competitive" inhibition caused by increasing concentrations of hydroxylamine is shown in Fig. 4. This compound is known to inhibit bacterial growth (6). To see whether it prevented germination by reacting with some component of the spore, PA 3679h spores were suspended in 0.5 M hydroxylamine in 0.01 M phosphate buffer, *p*H 6.0, and incubated for 20 hr at 30 C. Then the spores were washed five times with cold deionized water and suspended in 0.5 M sodium nitrite in 0.01 M phosphate buffer, *p*H 6.0. The germination rate at 45 C was approximately the same as that with spores which had not been treated with hydroxylamine. Therefore, it would appear that the mechanism of inhibition by hydroxylamine is not that of a prior combination with some component of the spores.

Uehara and Frank (10) described a germination system for spores of PA 3679h consisting of L-alanine and sodium pyrophosphate. Germination was most rapid at *p*H 8.5 and 45 C; it was practically nil at *p*H 6.5 and below, or at temperatures above 55 C. Figure 5 shows the alkaline *p*H optimum of the L-alanine germination system in contrast to the acidic optimum of the nitrite system (Fig. 2).

Nitrite inhibited germination by L-alanine (Fig. 6). At *p*H 8.0, which is near the optimum

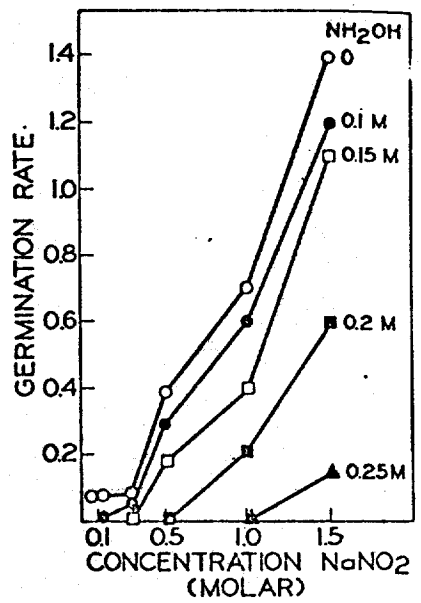


FIG. 4. *Effect of hydroxylamine on germination of PA 3679h spores by sodium nitrite. Spores were incubated at 45 C in 0.01 M phosphate buffer, pH 6.0, with added sodium nitrite and hydroxylamine hydrochloride.*

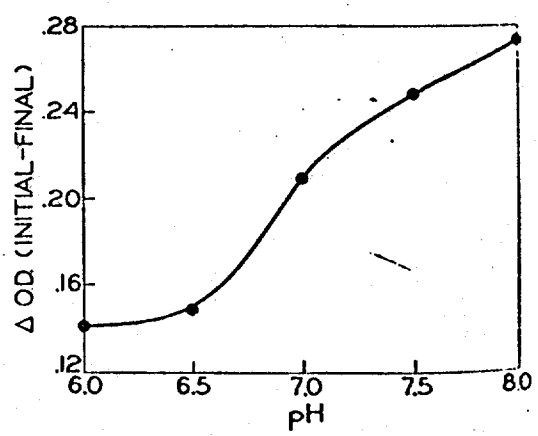


FIG. 5. *Effect of pH on germination of PA 3679h spores by L-alanine and sodium pyrophosphate. The germination mixture contained: L-alanine, 60 mM; sodium pyrophosphate, 35 mM; sodium thioglycolate 8.8 mM; and phosphate buffer, 0.01 M; adjusted to the appropriate pH. The total change in optical density was measured after 9 hr at 45 C.*

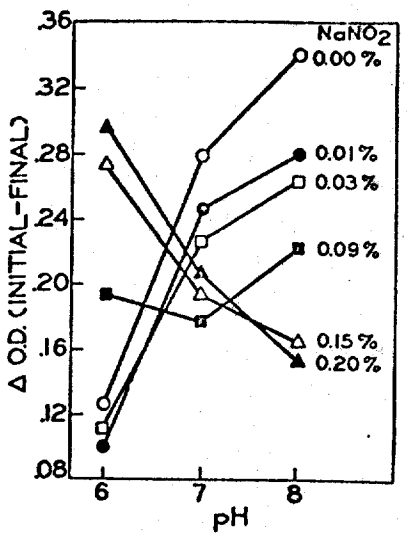


FIG. 6. *Effect of sodium nitrite on* L-*alanine-sodium pyrophosphate induced germination of PA 3679h spores. The germination mixture contained:* L-*alanine, 60 mM; sodium pyrophosphate, 35 mM; sodium thioglycolate, 8.8 mM; and phosphate buffer, 0.01 M; adjusted to pH 6, 7, or 8. Sodium nitrite was added to give the final concentrations shown. The total change in optical density was measured after 36 hr at 45 C. There was no change in optical density of control spore suspensions in 0.01 M phosphate buffer at the respective pH values.*

for the L-alanine system, increasing additions of nitrite decreased germination. However, at *p*H 6.0 which favors nitrite-induced germination, additional nitrite stimulated germination.

Inhibition of the L-alanine system by small amounts of nitrite is contrary to expectation. Fleming and Ordal (4) reported a synergistic effect of L-alanine and inorganic ions on germination of *B. subtilis* spores. They observed negligible germination in L-alanine at low ionic strength, but the rate accelerated as the ionic strength was increased up to about 0.1 M. This was true with a variety of salts, especially monovalent ones. However, with the system reported here, even as little as 0.01% (0.0014 M) sodium nitrite caused inhibition of L-alanine-induced germination.

DISCUSSION

The role of ions in germination of bacterial spores has been given little consideration, and most of the work reported previously has been done with aerobic sporeformers. The results reported here have shown that sodium nitrite is able to induce germination of spores of PA 3679h. This process was affected by the concentration of nitrite, the *p*H, and the temperature of incubation. The relatively low *p*H necessary for optimal activity indicates that undissociated nitrous acid probably is the effective compound.

The lag period of 36 to 48 hr at 37 C before commencement of germination in low concentrations of nitrite could be a reflection of the temperature of incubation. Increasing the temperature from 4 to 90 C resulted in progressively greater rates of germination (Fig. 3), with most rapid acceleration between 50 and 70 C. Black (Bacteriol. Proc., p. 36, 1964) also found nitrite germination of *B. cereus* spores to be accelerated with increasing temperatures above 43 C.

In contrast to our results, Black observed no germination within 60 min; then virtually all of the spores germinated in the next 0.5 hr. Other workers have found a heat shock to be necessary for ionic stimulation of germination, but, in our experience with nitrite, germination commenced immediately and without the necessity of prior heat shock.

It is possible that previous workers' failure to observe germination with ions alone was due to the incubation temperatures employed, usually 30 to 45 C which is well within the growth ranges of the organisms. In the nitrite system reported here, germination was fastest at temperatures far above the growth range of the organism.

Of the substances tested, only hydroxylamine was completely inhibitory to nitrite-induced germination. Results indicated that this inhibition was not caused by a prior reaction of hydroxylamine with some specific component of the spore, since it was effective only when present in conjunction with nitrite. The possibility of a chemical reaction between hydroxylamine hydrochloride (the actual compound used) and sodium nitrite should not be overlooked. It is known (6) that these two compounds may react to yield N_2O, NaCl, and water. However, this reaction is stoichiometric, and yet only 0.25 M hydroxylamine was required to effect complete inhibition of germination by 1.0 M sodium nitrite. It would appear that another reaction, presumably involving some spore structure, does occur. The effect of hydroxylamine may be exerted only after the initial reaction of nitrite with the spore.

The fact that sodium nitrite, even in low concentrations, inhibits germination by L-alanine is not in agreement with reports of a synergistic action between various ions and alanine. The lack of enhancement could be explained by the differences in *p*H optima of the two systems: at *p*H 8.0 nitrite is not an effective germinant, but at *p*H 6.0 it is active. However, this does not explain why very small amounts of nitrite inhibited the L-alanine germination system.

Various suggestions have been put forth to

explain the mechanism by which ions stimulate germination of spores. Alderton and Snell (1) suggested an indirect effect of ions on metabolic activity which might involve a volume change in the spore structure to a point of core hydration. Metabolic activity could begin after hydration.

In view of the variety of ions reported to be effective in germination, any role as an enzyme activator would seem to be nonspecific in nature. Also, the concentration of ions involved in germination is normally higher than that usually considered necessary for enzyme activation.

Rode and Foster (9) suggested that a number of germinants, viz., inorganic and organic ions, chelating agents, surfacants, and hydrogen ions, induce germination by altering the conformational structure of proteins and perhaps other macromolecules responsible for the dormant spore state.

Riemann (7) assumed a spore structure-Ca-dipicolinic acid or Enzyme-Ca-dipicolinic acid complex to be associated with dormancy in spores. He suggested that this complex could be disrupted by the chelation of calcium with agents such as ethylenediaminetetraacetic acid, dipicolinic acid, and amino acids, thus leading to germination.

Fleming and Ordal (4) suggested that Riemann's mechanism for germination could be extended to include germination with both monovalent and multivalent ions, since Fleming (Ph.D. Thesis, Univ. of Illinois, Urbana, 1963) had previously shown that increased ionic strength could favor dissociation of the chelated structure.

Black (Bacteriol. Proc., p. 36, 1964) suggested that germination of *B. cereus* spores by sodium nitrite was effected by reduction, with nitrite serving as an electron donor, and possibly mediated by the activity of spore catalase. If this is correct, it would appear that the action of nitrite is indeed nonspecific, since it also induced germination of PA 3679h spores, which presumably are devoid of catalase.

If nitrite does act by reduction, it could conceivably result in the alteration of the tertiary structure of a spore protein, which in turn may be involved in the calcium-dipicolinic acid complex. The increase in germination rate with increasing temperature and increasing nitrite concentration may be a reflection of such structural alteration.

ACKNOWLEDGMENT

This investigation was supported by a series of annual grants from Oscar Mayer and Co., Madison, Wisc.

LITERATURE CITED

1. ALDERTON, G., AND N. SNELL. 1963. Base exchange and heat resistance in bacterial spores. Biochem. Biophys. Res. Commun. 10:139-143.
2. DUNCAN, C. L., AND E. M. FOSTER. 1968. Role of curing agents in the preservation of shelf stable canned meat products. Appl. Microbiol. 16:401-405.
3. DUNCAN, C. L., AND E. M. FOSTER. 1968. Effect of sodium nitrite, sodium chloride, and sodium nitrate on germination and outgrowth of anaerobic spores. Appl. Microbiol. 16:406-411.
4. FLEMING, H. P., AND Z. J. ORDAL. 1964. Responses of *Bacillus subtilis* spores to ionic environment during sporulation and germination. J. Bacteriol. 88:1529-1537.
5. HYATT, M. T., AND H. S. LEVINSON. 1961. Interaction of heat, glucose, L-alanine and potassium nitrate in spore germination of *Bacillus megaterium*. J. Bacteriol. 81:204-211.
6. MELLOR, J. W. 1928. A comprehensive treatise on inorganic and theoretical chemistry, vol. 8. N. P. Longmans, Green and Co., Ltd., London.
7. RIEMANN, H. 1961. Germination of spores with chelators, p. 24-48. *In* H. O. Halvorson (ed.), Spores II. Burgess Publishing Co., Minneapolis.
8. RODE, L. J., AND J. W. FOSTER. 1962. Ionic germination of spores of *Bacillus megaterium* QMB1551. Arch. Mikrobiol. 43:183-200.
9. RODE, L. J., AND J. W. FOSTER. 1965. Gaseous hydrocarbons and the germination of bacterial spores. Proc. Natl. Acad. Sci. U.S. 53:31-38.
10. UEHARA, M., AND H. A. FRANK. 1965. Factors affecting alanine-induced germination of clostridial spores, p. 38-46. *In* L. L. Campbell and H. O. Halvorson [ed.], Spores III. American Society for Microbiology, Ann Arbor, Mich.

awarded of the Health Research Council of the City of New York.

JACK R. BATTISTO
LEWIS C. CANTOR
FELIX BOREK
ALLAN L. GOLDSTEIN
ESTRELLA CABRERRA

Departments of Microbiology and Immunology,
and Biochemistry,
Albert Einstein College of Medicine,
Yeshiva University,
New York.

Received March 10; revised April 29, 1969.

[1] Kalpaktoglu, P. K., Yunis, E. J., and Good, R. A., *Nature*, 215, 833 (1967).
[2] Haller, J. A., *Transplantation*, 2, 287 (1964).
[3] Kubal, L., and Auerbach, R., *Nature*, 217, 460 (1968).
[4] Moody, J. K., and Reed, N. D., *Nature*, 218, 1056 (1968).
[5] Searle, A. G., *Nature*, 184, 1419 (1959).
[6] Meier, H., and Hoag, W. C., *Naturwissenschaften*, 49, 320 (1962).
[7] Shearer, G. M., Cudkowicz, G., St James Connell, M., and Priore, R. L., *J. Exp. Med.*, 128, 437 (1968).
[8] Dobbs, J., Rivero, I., Sabb, F., and Lee, S. L., *Immunology*, 14, 213 (1968).
[9] Biozzi, G., Stiffel, C., Mouton, D., Bouthillier, Y., and Decreusefond, C., *Immunology*, 14, 7 (1968).
[10] Nussenzweig, R. S., Merryman, C., and Benacerraf, B., *J. Exp. Med.*, 120, 315 (1964).
[11] Jerne, N. K., and Nordin, A. A., *Science*, 14, 405 (1963).
[12] Ovary, Z., Fudenberg, H., and Kunkel, H. G., *J. Exp. Med.*, 112, 953 (1960).
[13] Tata, T., and Ishizaka, K., *J. Immunol.*, 96, 112 (1966).
[14] Osler, A. G., Mulligan, jun., J. J., and Rodriguez, E., *J. Immunol.*, 96, 334 (1966).
[15] Lindquist, K., and Bauer, D. C., *Immunochemistry*, 3, 373 (1966).
[16] Chaperon, E. A., Selner, J. C., and Claman, H. H., *Immunology*, 14, 533 (1968).
[17] Friedman, H., *Proc. Soc. Exp. Biol. and Med.*, 117, 526 (1964).
[18] Adler, F. L., *J. Immunol.*, 95, 26 (1965).
[19] Askonas, B. A., White, R. G., and Wilkinson, P. C., *Immunochemistry*, 2, 329 (1965).
[20] Blinkoff, R. B., *J. Immunol.*, 97, 727 (1966).
[21] Davidsohn, L., Lee, C. L., and Zandrew, F., *Fed. Proc.*, 23, 190 (1934).

503

Carcinogen in a Transkeian Bantu Food Additive

THE high incidence of oesophageal cancer in Bantu people in localized areas of the Transkei, between which there is little or none of the disease, has developed rapidly since the early 1940s. This, and the fact that the women spend most of their lives (and men up to the age of 20) within a mile or so of their homes, presents a rare opportunity for studying the aetiology of the disease. A close association was found between the occurrence of the disease in women and signs of molybdenum deficiency in the leaves of their food plants such as pumpkins, beans and maize[1]. Molybdenum is essential for nitrogen metabolism in plants and a deficiency is known to lead to an accumulation of nitrate[2]. The addition of nitrate to chopped herbage enhances its tendency to form nitrite and nitrous gases[3]. These and secondary amines present in all plants might form nitrosamines, many of which are carcinogenic and several of which have been shown to cause oesophageal cancer selectively in rats[4].

This working hypothesis is being tested by a search for nitrosamines in Bantu food plants. Samples of their staple foods, chiefly maize, pumpkins and beans, and of a wide range of wild plants used by Bantu in various ways, especially when food is scarce, are being gathered from a rectangle 1,200 yards × 600 yards with a population of about 350, in which there have been five deaths (three women and two men).

The first positive result has been obtained with the fruit of a solanaceous bush, the juice of which is used by headboys to curdle milk. The resulting curds are their chief source of sustenance during the day. Because this habit persists to the age of nearly twenty the cumulative total dose of juice must be considerable. Women rarely use this juice. At least three different sorts of fruit are used as the source of the juice.

Solanum incanum fruit (wet weight 4,300 g) was broken up with ethyl alcohol (22 l.) in a high speed macerator, and the mixture was filtered and the filtrate concentrated to a small volume in a vacuum at a temperature below 40° C. This solution was separated on thin-layer plates of 'Kieselgel *G*' using a mixture of carbon tetrachloride and dichloromethane (3 : 2), and the nitrosamine band (R_F 0·2–0·6) was identified by spraying with Preussmann's reagent[5]. The nitrosamine-rich bands were scraped off and extracted with ether. After removal of silica by filtration the extract was dried and the ether was removed in a vacuum, leaving an almost colourless oil (20 mg), which gave a positive Lassaigne's test for nitrogen. When this oil was run on a thin layer of silica gel, as just described, a spot corresponding to dimethylnitrosamine appeared when Preussmann's reagent was sprayed on the plates. When dimethylnitrosamine was added to the oil and the mixture was applied to a plate the same spot was developed, with no sign of separation of any components. In these chromatograms other spots suggested the presence of further nitrosamines. The infrared spectrum of this oil (8 per cent solution in CCl_4) revealed several peaks (3,012 wsh, 2,959 s, 2,933 vs, 2,857 s, 1,466 m, 1,456 msh, 1,287 m, 1,040 w, cm^{-1}; s, strong; m, medium; w, weak; sh, shoulder) coincident with those of dimethylnitrosamine.

The isolation of a nitrosamine-rich fraction from the plant extract was greatly improved by preparative dry column chromatography on alumina in nylon columns[6], with methylene chloride as the developing solvent. In this way 360 g of plant extract, prepared as described, yielded a nitrosamine-rich fraction as an oil (145 mg). Using a Beckman *GC*-4 gas chromatograph equipped with dual flame ionization detectors and a stream splitter (glass columns 1·19 m long with inner diameter 12·5 mm, packed with 15 per cent 'Carbowax 1,000' on 'Gas Chrom *P*80/100' mesh; a column temperature of 100° C; N_2 as carrier gas flowing at a rate of 115 ml./min) the oil (4 per cent in CCl_4) was found to have at least thirteen components with retention times varying from 4·1 to 30 min. A prominent peak at 4·9 min increased in intensity when authentic dimethylnitrosamine was added to the oil solution. Dimethylnitrosamine (4 per cent in CCl_4) alone had a retention time of 4·9 min. When columns were packed with polyethylene glycol succinate the result was the same—the dimethylnitrosamine peak was intensified when authentic material was added.

Gas chromatography was repeated after the solution of the oil in carbon tetrachloride had been neutralized by washing with dilute sulphuric acid, dilute sodium hydroxide solution and water to remove free amines. After drying, the solution gave the same gas chromatographic results.

The fraction with a retention time of 4·9 min (on 'Carbowax') was collected in a "porous plug"[7] of 25 μl. of spectroscopic grade deuterochloroform in a tube immersed in liquid air. After successive injections 1 mg of this fraction had accumulated in the porous plug. After warming to room temperature, the deuterochloroform solution of the fraction was sealed in a micro-ampoule (100 × 1·7 mm inner diameter), which was placed in a nuclear magnetic resonance tube, with a 1·7 cm nylon plug[8] at the bottom. Deuterochloroform containing 2 per cent tetramethylsilane was added to the tube to above the mark of the solution in the micro-ampoule, to serve as an external standard. The nuclear magnetic resonance spectrum (taken in a Perkin-Elmer *R*12 instrument) had several peaks, including two at 6·34 and 7·05 p.p.m. characteristic of the dimethylnitrosamine spectrum taken in the same conditions.

The deuterochloroform solution was then transferred to a micro-infrared cell and the spectrum was taken in a Beckman *IR*-8 instrument. The spectrum had fifteen peaks coincident with those of authentic dimethylnitrosamine taken in deuterochloroform. Among the coincident peaks were those associated with the N to O stretching frequencies at 1,447, 1,321 and 1,290 cm^{-1} and the N to N stretching frequencies at 1,053 cm^{-1}.

These results show that dimethylnitrosamine was present in the sample of *Solanum incanum* examined. Dimethylnitrosamine has since been isolated from several other specimens of this plant. An extensive survey for the presence of nitrosamines in this and several other plants growing in the Transkei is in progress.

We thank Dr H. Parolis for helpful discussions. This work was supported in part by a grant from the Deferred Pay Interest Fund.

L. S. DU PLESSIS
J. R. NUNN

Department of Chemistry,
Rhodes University,
Grahamstown,
South Africa.

W. A. ROACH

Rhodes University,
Bantu Chemical Cancer Research,
Transkei, South Africa.

Received August 27, 1968; revised April 18, 1969.

1 Burrell, R. J. W., Roach, W. A., and Shadwell, A., *J. Nat. Cancer Inst.*, 36, 201 (1966).
2 Hewitt, E. S., and Jones, E. W., *J. Pomology Hort. Sci.*, 23, 254 (1947).
3 [illegible]son, W. H., Burris, R. H., Panesehandra, Sant, P., and Little, H. N., *J. Agric. Food Chem.*, 6, 121 (1958).
4 Druckrey, H., Preussmann, R., Blum, G. V., and Ivankovic, C., *Naturwissenschaften*, 50, 99 (1963).
5 Preussmann, R., Daiber, D., and Hengy, H., *Nature*, 201, 502 (1964).
6 Low, B., and Goodman, M., *Chem. and Ind.*, 2026 (1967).
7 Kais[illegible], R., *Chem. Brit.*, 5, 54 (1969).
8 Provost, L. R., and Jardine, R. V., *J. Chem. Ed.*, 45, 675 (1968).

Absorption of Microwaves by Microorganisms

RECENTLY S. J. W. and Dodds reported that 136 GHz microwaves interfere with the growth of *Escherichia coli* cells[1]. Here we report further investigations of the absorption of 65 to 75 GHz microwaves by cells of *E. coli* B_R as well as isolated protein, RNA and DNA, and its effect on some metabolic processes. Our equipment has been described[1,2]. Cells were grown for 24 h at 37° C in nutrient broth (NB) and either (*a*) washed twice in 0·85 per cent NaCl and deposited as a pellet by centrifugation or (*b*) resuspended after washing to a cell concentration of 1×10^8 cells/ml. in 0·5 per cent glucose + 0·5 per cent casamino-acids + 50 μg/ml. thymine in 0·1 M phosphate buffer, *p*H 6·9 (GCT). To examine the effect of microwaves on cell growth, 0·5 ml. aliquots of cells suspended in GCT were placed in vials 1·5 cm in diameter. Some vials were put on top of microwave horns and others incubated at the same temperature (25° C) out of the radiation field. At various times the vials were removed and the number of cells/ml. determined by dilution and plating on nutrient agar. The effect of microwave absorption on metabolic processes was investigated by resuspending the washed cells to a concentration of 1×10^8 cells/ml. in 1·0 per cent glucose in 0·1 M phosphate buffer, *p*H 6·9. Aliquots of 0·2 ml. of this suspension were placed in the vials and after positioning on the microwave apparatus 0·3 ml. of a solution was added, which contained 0·8 μCi/ml. of one of the following: thymine-2-^{14}C, uracil-2-^{14}C or algal ^{14}C protein hydrolysate plus two of the following: unlabelled thymine, 50 μg/ml.; uracil, 100 μg/ml.; or casamino-acids, 500 μg/ml. After various times the cells from each vial were deposited onto a Millipore filter and washed with 100 ml. of 1 N HCl; the filter was dried and radioactivity counted. The microwave absorption spectra of the cells, H_2O, protein, RNA and DNA were obtained by making films of the material on a thin mica window. The films were dried at 80 per cent relative humidity and covered with a second thin piece of mica. The difference between the microwave absorption of a blank and the film gave the absorption spectrum of the film of cells or biological material.

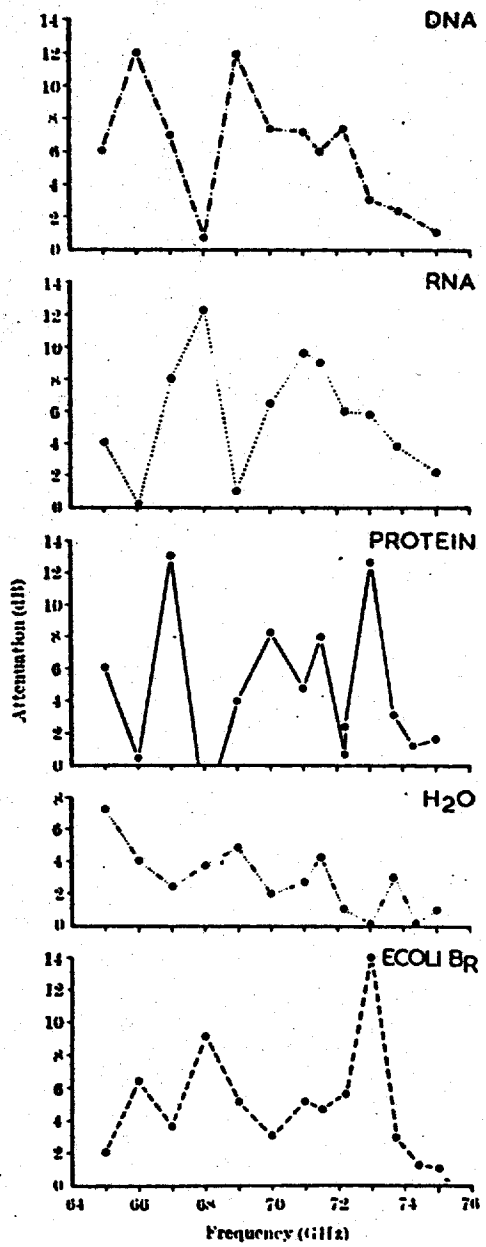


Fig. 1. The 64 to 75 GHz microwave absorption spectra of biological entities.

Fig. 1 shows the absorption of microwaves by cells of *E. coli* B_R as well as by H_2O, protein, RNA and DNA. The water film showed three absorption maxima at 69, 71·5 and 73·7 GHz whereas with the film of cells attenuation peaks occurred at 66, 68, 71 and 73 GHz. Absorption maxima for protein were observed at 67, 70, 71·5 and 73 GHz with 67 and 73 GHz microwaves most strongly

instead. The growth of the chicks receiving about 50% of their phosphorus as phytate P was at least as good as that of chicks receiving inorganic P, but there was some indication of impaired bone calcification.

From balance studies it appeared that about one-fifth of the phytate P was utilisable by the chicks, and the use of radioactive phytin suggested that some of this phosphorus was being incorporated in actively growing tissues.

Acknowledgment

The authors are greatly indebted to Dr. W. Ll. Jenkins for carrying out the determinations of radioactivity.

Dept. of Agricultural Biochemistry
Institute of Rural Science
Penglais
Aberystwyth

Received 30 March, 1960

References

[1] Lowe, J. T., Steenbock, H., & Kreiger, C. M., *Poult. Sci.*, 1939, **18**, 40
[2] Mitchell, H. H., & McClure, F. J., *Nat. Res. Coun., Wash., Bull.*, 1937, **99**, 1
[3] Mellanby, E., *Brit. med. J.*, 1922, ii, 849
[4] Eddy, W. H., Muller, H. R., & Heft, H. L., *Proc. Amer. Soc. biol. Chem., J. biol. Chem.*, 1922, **50**, xix
[5] Common, R. H., *Nature, Lond.*, 1939, **143**, 379
[6] Common, R. H., *J. agric. Sci.*, 1941, **31**, 281
[7] Shohl, A. T., 'Mineral Metabolism', 1939, p. 175 (New York: Rheinhold Corp.)
[8] Rapoport, S., *J. biol. Chem.*, 1940, **135**, 403
[9] Rapoport, S., Leva, E., & Guest, G. M., *J. biol. Chem.*, 1941, **139**, 621
[10] Singsen, E. P., *Storrs agric. Exp. Sta. Bull.*, 1948, No. 260, p. 53
[11] Singsen, E. P., Matterson, L. D., & Kozeff, A., *Poult. Sci.*, 1949, **28**, 781
[12] Neumann, W. F., & Riley, R. F., *J. biol. Chem.*, 1947, **168**, 545
[13] Falkenheim, M., Neumann, W. F., & Hodge, H. C., *J. biol. Chem.*, 1947, **169**, 713
[14] Gillis, M. B., Norris, L. C., & Heuser, C. F., *Poult. Sci.*, 1953, **32**, 977
[15] Keane, K. W., Collins, R. A., & Gillis, M. B., *Fed. Proc.*, 1954, **13**, 463
[16] Ashton, W. M., & Williams, P. C., *J. Sci. Fd Agric.*, 1958, **9**, 505
[17] Boutwell, P. W., *J. Amer. chem. Soc.*, 1917, **39**, 491
[18] Svensson, S. A., *Nature, Lond.*, 1957, **179**, 972
[19] Wilson, P. N., *J. agric. Sci.*, 1954, **45**, 110 (Appendix tables)

516

BACTERIAL METABOLISM OF NITRATE AND NITRITE IN MATURING BACON

By B. P. EDDY, D. P. GATHERUM and A. G. KITCHELL*

Substantial quantities of nitrate can be lost from maturing bacon without any corresponding accumulation of nitrite. This loss of nitrate does not occur if the growth of bacteria on the surface is reduced by swabbing with a solution of formaldehyde. It is suggested that in normal commercial practice this loss of nitrate would take place after the matured sides had left the factory.

* Present address: British Joint Services Mission, Washington, U.S.A.

Introduction

It is generally agreed that the nitrite in normal bacon, necessary for its cured colour, is produced by bacterial reduction of the nitrate (saltpetre) added during curing.[1] It has been plainly shown that this change takes place for the most part during the storage of the bacon after it has been removed from the curing tank, but it has apparently been accepted that reduction of nitrate is the only change involved. Recent work with curing brines,[2, 3] however, makes it clear that nitrite in such systems is frequently destroyed by further reduction, so that much less nitrite may accumulate than might be expected. The investigation here reported was made to see whether this happened in bacon, as well as in curing brines.

Preliminary experiments were carried out in which paired sides of bacon were pumped and tanked by the usual methods employed in the factory. After being tanked, the gammons were cut off and a slice taken from each side immediately and after 7 and 14 days' maturation. The slices were analysed for sodium chloride, potassium nitrate and sodium nitrite. The results suggested that the nitrate content of maturing sides could decrease without any corresponding accumulation of nitrite. These preliminary experiments were considered to be unsatisfactory for two main reasons: first, the impossibility of sampling any side more than once at the same point meant that successive slices had to be taken at increasing distances from the nearest pumping point; second, no attempt had been made to determine differences between the external and internal portions of the slices. Accordingly the experiments described below were carried out in attempts to decide whether the observation concerning nitrate disappearance was real and, if so, whether it was related to the growth of bacteria during maturation.

Experimental

Curing and sampling

Normally the sides were pumped, tanked for 5 days and then stacked in the maturing cellar. The details of each process, however, varied among the several experiments and are therefore given in the appropriate place. The same applies to the details of the sampling techniques used. In the experiments with sides, the samples as removed were divided into two approximate halves so as to give external and internal portions in relation to the skinless surface of the whole side. The relations of the samples to the sides in the two types of experiment concerned are shown diagrammatically in Fig. 1.

Chemical analyses

The samples were minced and extracted by the method of Grau & Mirna.[4] Approximately 15-g. samples were taken: after extraction and cooling, the meat + extract was made up to 200 ml. and the filtered solution used for the analyses. In the case of the surface samples in Expt. 3, which were too small to be extracted in this way, extraction was by three successive treatments with small volumes of boiling water.

Sodium chloride and potassium nitrate were estimated by the methods of Grau & Mirna,[4] and sodium nitrite by the method of Eddy.[5] The results are given as g./100 g. fresh weight for NaCl and as p.p.m. also on a fresh weight basis for nitrate and nitrite.

Bacterial counts

Suspensions of surface tissue, for dilution prior to plating, were prepared by vigorously shaking the material with glass beads in 12·5% NaCl solution. Dilutions were made in a similar solution. Exterior and interior deep samples were homogenised and diluted in 4% NaCl.

The numbers of bacteria present were determined by surface counting (*a*) on nutrient digest agar containing 4% NaCl incubated for 6 days at 25° and (*b*) on pork extract salt agar (20% NaCl), prepared as described by Jespersen & Riemann[6] but with the nitrate omitted, and incubated for 15 days at 25°. Surface counts are given as number/cm.^{2}, deep counts as number/g.

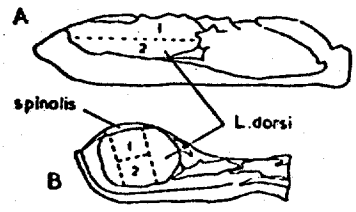


FIG. 1.—*Relation of tissues sampled to the whole side*

A—Expt. 1; B—Expts. 2 and 3
1—exterior samples; 2—interior samples

Results

The experiments described below fall into a series showing progressive refinement of technique and corresponding departure from normal factory practice. In work of this type it is clearly important to adhere as closely as possible to the normal commercial technique; but, in this case, it was found necessary to move from normally cured sides to specially treated portions of back and ultimately to turn to cured fillets (*psoas* muscle) in order to obtain satisfactory results. The basic objective underlying all the experiments was to obtain comparable analyses, week by week, of maturing bacon, for salt, nitrate and nitrite contents with simultaneous bacterial counts.

Expt. 1

Four pairs of sides were chosen, pumped normally, and stacked at the top of the curing tank without being sprinkled with either salt or nitrate. On removal from the tank, two sides were sampled immediately by cutting out a slice about 4 cm. thick from a position 2–3 cm. from the round bone, and the remainder, two at a time and in like manner, after 7, 14 and 21 days of maturation. The scheme of sampling was as follows:

Time of sampling	Sides sampled
After tanking	1L, 4L
,, 7 days' maturation	1R, 2R
,, 14 ,, ,,	2L, 3L
,, 21 ,, ,,	3R, 4R

The *longissimus dorsi* and associated muscle bands were taken for analysis and divided to give exterior (E) and interior (I) samples. These were extracted and analysed for NaCl, KNO_3 and $NaNO_2$. The results are shown in Table I.

Initially there was much more salt in the exterior portion of the slice than in the interior, presumably because the sides had spent only 5 days immersed in brine. During maturation, however, the difference between them narrowed and, after the 14th day, the concentrations remained the same. The nitrate analyses of the exterior samples show a steady fall throughout the period of maturation, while the interior samples show a parallel fall during the second and third weeks, following a rise during the first week. The nitrite contents of the exterior samples rose during the first two weeks of maturation and then fell, whereas those of the interior samples showed little change during the first week followed by rises during the next two. It is, however, at once clear that the changes in nitrite content are of a much lower order of magnitude than those in nitrate content.

Table I

Changes in salt, nitrate and nitrite contents of maturing bacon (Expt. 1)

	External samples			Internal samples		
Out of tank						
Side	1L	4L	Mean	1L	4L	Mean
NaCl, %	5·1	5·4	5·3	1·7	2·3	2·0
KNO_3, p.p.m.	1800	2200	2000	1500	2300	1900
$NaNO_2$, p.p.m.	76	69	73	7	3	5
7 days' maturation						
Side	1R	2R	Mean	1R	2R	Mean
NaCl, %	3·2	5·5	4·4	2·2	3·6	2·9
KNO_3, p.p.m.	1200	2000	1600	1600	3600	2600
$NaNO_2$, p.p.m.	110	180	150	8	15	12
14 days' maturation						
Side	2L	3L	Mean	2L	3L	Mean
NaCl, %	4·4	4·4	4·4	3·9	3·9	3·9
KNO_3, p.p.m.	1000	1200	1100	1900	2100	2000
$NaNO_2$, p.p.m.	260	320	290	76	81	79
21 days' maturation						
Side	3R	4R	Mean	3R	4R	Mean
NaCl, %	4·5	4·2	4·4	4·0	3·6	3·8
KNO_3, p.p.m.	840	510	680	1600	1100	1400
$NaNO_2$, p.p.m.	210	280	250	76	120	100

Expt. 2

The results of the first experiment, particularly the nitrate analyses (Table I), showed considerable differences between duplicate samples and it was thought that these might be reduced by using back bacon, pumped at regular intervals and sampled at points halfway between the needle holes. This technique was used in the second and third experiments.

A pair of sides was chosen and pumped normally, except along the back where they were pumped carefully opposite the heads of the 5th, 7th, 9th, 11th and 13th ribs (see below). No nitrate was sprinkled on the sides prior to covering with tank brine, but otherwise they were treated normally. After removal from the tank, the sides were put on the top of a stack of commercial sides. Samples of 6 cm.^{2} cross-sectional area were cut from the *longissimus dorsi* using a circular cutter (Fig. 1). The samples were divided giving approximately equal exterior (E) and interior (I) portions. The samples were extracted and analysed as already described, with the results given in Table II.

Time of sampling	Left side Rib no.	Right side Rib no.
After tanking	6	12
,, 7 days' maturation	8	10
,, 13 ,, ,,	10	8
,, 20 ,, ,,	12	6

The changes in salt concentration agree with those in the previous experiment in suggesting that internal equilibration is substantially complete in a fortnight, and the equilibrium salt contents are of the same order in both cases. However, while the pattern of change in the internal samples is similar in the two experiments, the changes in the external samples are markedly different. In Expt. 1 there was a fall during the first week followed by 2 weeks with little or no change, whereas in this experiment there was a substantial rise during the first week followed by successive, smaller increases. The overall increase was greater in the internal than in the external sample (approx. 1 : 5 and 1 : 3 respectively) and, since the increases occurred in both samples, it is reasonable to deduce that it was due to diffusion of salt from the pumping sites to the sampling sites.

Table II

Changes in salt, nitrate and nitrite contents of maturing bacon (Expt. 2)

	External samples			Internal samples		
	Left side	Right side	Mean	Left side	Right side	Mean
Out of tank						
NaCl, %	1·17	1·37	1·27	0·82	0·64	0·73
KNO_3, p.p.m.	350	260	310	820	150	490
$NaNO_2$, p.p.m.	6	11	8·5	<1	<1	<1
7 days' maturation						
NaCl, %	2·51	3·61	3·06	1·82	2·21	2·02
KNO_3, p.p.m.	680	1300	990	1900	1700	1800
$NaNO_2$, p.p.m.	28	24	26	4	4	4
13 days' maturation						
NaCl, %	3·51	3·59	3·55	3·49	3·58	3·54
KNO_3, p.p.m.	910	1300	1100	2500	2800	2700
$NaNO_2$, p.p.m.	130	190	160	34	12	23
20 days' maturation						
NaCl, %	3·87	4·31	4·09	3·92	3·58	3·75
KNO_3, p.p.m.	610	1200	910	2000	1200	1600
$NaNO_2$, p.p.m.	88	120	100	61	63	62

The behaviour of the nitrate contents is quite different in the two experiments when judged by the actual nitrate results (cf. Tables I and II). However, the differences largely disappear when the nitrate is considered in terms of the salt present (Fig. 2), a procedure the validity of which depends on the roughly constant ratio of nitrate to salt in the pumping brine. The nitrite contents behaved similarly in both experiments.

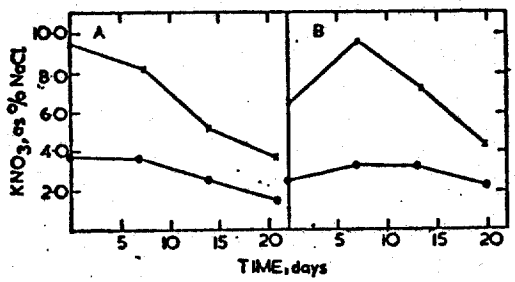


FIG. 2.—*Changes in nitrate contents of maturing sides of bacon*

(Nitrate expressed as % of salt in the same sample. Computed from data in Tables I and II)

A—Expt. 1; B—Expt 2
●——● Exterior samples
×——× Interior samples

Expt. 3

The technique used in Expt. 2 seemed more promising than that in the previous experiment. Accordingly a similar experiment was carried out with the following modifications: (1) bacterial counts were made on the samples; (2) more sides were used, half of them being swabbed each week with formaldehyde solution to reduce bacterial growth; and (3) the external samples were divided into two, a thin surface layer being removed for independent analysis.

Three pairs of sides were selected and pumped as in Expt. 2, beginning opposite the 4th rib. The pockets were stuffed with salt as usual and the sides stacked, unsprinkled, at the top of the curing tank. After being cured, the sides were removed from the tank and the fores and gammons cut off. All six sides were then sampled opposite the 5th rib, the sampling technique throughout the experiment being as follows. An incision was made with a sterile, circular cutter of 4·5 cm. diameter, and the surface layer of flesh (*spinalis* muscle) removed for bacterial counts and nitrite estimations. The exposed surface was seared with a hot iron and then a sample taken right through the *longissimus dorsi* with a sterile cutter of 3·2 cm. diameter. The sample was trimmed free from fat and connective tissue and divided into exterior and interior halves. The sampling hole was plugged with a cork.

Sides 1L, 2R and 3L were swabbed with a mixture of 2 parts of formalin in 100 parts of 25% NaCl immediately and each week thereafter. The treated and untreated sides were then stacked for maturing in separate piles, sides 3L and 3R being placed skin downwards on the floor of the cellar with the other sides, skin upwards, on top. The sides were sampled, as described above, at approximately weekly intervals, according to the schedule in Table III.

The results of the chemical analyses are given in Tables IV and V and the results of the bacterial counts in Fig. 3. In Fig. 4 the nitrate contents are plotted as percentages of the salt in the same sample: this minimises differences due to pumping. The values given are the means of two samples in all cases except the out-of-tank where they are the means of three.

The interpretation of the results of the chemical analyses in Expt. 3 along the lines intended, by using the mean values from pairs of sides differing from week to week, is difficult because of differences in the time-table of changes among the three untreated sides (Table IV). The

Table III

Sampling schedule in Expt. 3

Time of sampling	Untreated sides			Formalin-treated sides		
	1R Rib. no	2L Rib no.	3R Rib no.	1L Rib no.	2R Rib no.	3L Rib no.
After tanking	5	5	5	5	5	5
,, 8 days' maturation	7	7	—	7	7	—
,, 14 ,, ,,	9	—	7	9	—	7
,, 20 ,, ,,	—	9	9	—	9	9
,, 27 ,, ,,	11	11	—	11	11	—
,, 34 ,, ,,	13	—	11	13	—	11
,, 41 ,, ,,	—	13	13	—	13	13

Table IV

Changes in salt, nitrate and nitrite contents of maturing bacon (Expt. 3)

	Surface samples				Exterior samples				Interior samples			
	1R	2L	3R	Mean	1R	2L	3R	Mean	1R	2L	3R	Mean
Out of tank												
NaCl, %	—	—	—	—	1·2	0·83	0·78	0·94	1·9	2·6	1·7	2·1
KNO_3, p.p.m.	—	—	—	—	1400	520	780	900	2400	3000	1900	2400
$NaNO_2$, p.p.m.	150	230	230	200	19	27	4	17	<1	<1	<1	<1
1 week matured												
NaCl, %	—	—	—	—	1·3	2·4	—	1·9	1·2	2·5	—	1·9
KNO_3, p.p.m.	—	—	—	—	300	930	—	620	870	2100	—	1500
$NaNO_2$, p.p.m.	47	91	—	69	29	61	—	45	12	63	—	33
2 weeks matured												
NaCl, %	—	—	—	—	2·0	—	2·1	2·1	1·6	—	2·6	2·1
KNO_3, p.p.m.	—	—	—	—	450	—	700	580	1000	—	1900	1500
$NaNO_2$, p.p.m.	67	—	76	72	22	—	40	31	13	—	46	30
3 weeks matured												
NaCl, %	—	—	—	—	—	4·4	2·8	3·6	—	4·4	2·9	3·7
KNO_3, p.p.m.	—	—	—	—	—	1800	600	1200	—	3300	1700	2500
$NaNO_2$, p.p.m.	—	69	10	40	—	130	39	85	—	150	41	96
4 weeks matured												
NaCl, %	—	—	—	—	3·1	4·5	—	3·8	2·5	6·0	—	4·3
KNO_3, p.p.m.	—	—	—	—	250	2500	—	1400	640	2600	—	1600
$NaNO_2$, p.p.m.	15	12	—	14	43	180	—	110	19	—	270	140
5 weeks matured												
NaCl, %	—	—	—	—	3·5	—	3·7	3·6	3·0	—	4·0	3·5
KNO_3, p.p.m.	—	—	—	—	180	—	370	280	450	—	1100	780
$NaNO_2$, p.p.m.	5	—	6	6	17	—	67	42	18	—	75	47
6 weeks matured												
NaCl, %	—	—	—	—	—	4·9	4·3	4·6	—	4·8	4·2	4·5
KNO_3, p.p.m.	—	—	—	—	—	440	320	380	—	810	810	810
$NaNO_2$, p.p.m.	—	13	13	13	—	89	52	71	—	122	92	110

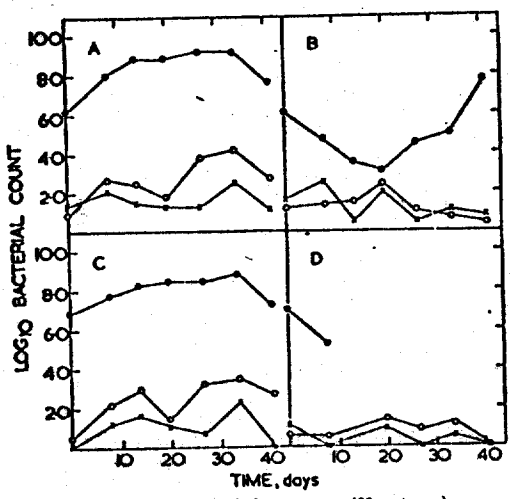


FIG. 3.—*Bacterial counts* (Expt. 3)

A—untreated sides and B—formaldehyde-treated sides, counts on nutrient digest agar + 4% NaCl. C—untreated sides and D—formaldehyde-treated sides, counts on pork extract salt agar (20% NaCl)

●——● Surface samples
○——○ Exterior samples
×——× Interior samples

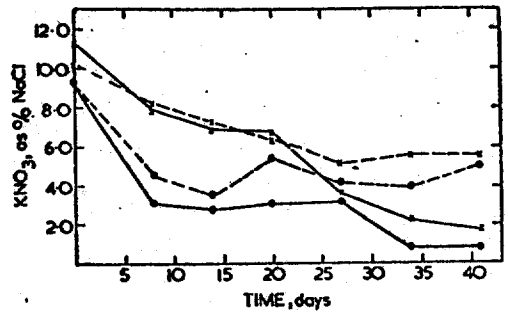


FIG. 4.—*Changes in nitrate contents of maturing sides of bacon, some treated with formaldehyde* (Expt. 3)

(Nitrate expressed as % of salt in the same sample. Computed from data in Tables IV and V)

●——● Exterior samples—untreated
●- - - -● " " —formaldehyde-treated
×——× Interior samples—untreated
×- - - -× " " —formaldehyde-treated

substantial decreases in nitrate content began to occur after the second week of maturation in sides 1 and 3 but not until after the fourth week in side 2. It is, however, clear (when corresponding treated and untreated sides are compared—Fig. 4) that there were considerable

Table V

Changes in salt, nitrate and nitrite contents of maturing bacon swabbed each week with formaldehyde solution (Expt. 3)

	Surface samples				Exterior samples				Interior samples			
	1L	2R	3L	Mean	1L	2R	3L	Mean	1L	2R	3L	Mean
Out of tank												
NaCl, %	—	—	—	—	0·76	0·97	1·3	1·0	4·2	2·3	0·85	2·5
KNO_3, p.p.m.	—	—	—	—	830	830	1000	890	4400	2400	830	2500
$NaNO_2$, p.p.m.	180	200	700	360	10	8	50	23	<1	<1	<1	<1
1 week matured												
NaCl, %	—	—	—	—	2·3	3·8	—	3·1	1·5	3·5	—	2·5
KNO_3, p.p.m.	—	—	—	—	700	2300	—	1500	1100	3200	—	2200
$NaNO_2$, p.p.m.	85	133	—	100	15	59	—	37	2	76	—	39
2 weeks matured												
NaCl, %	—	—	—	—	2·9	—	3·7	3·3	2·3	—	2·9	2·6
KNO_3, p.p.m.	—	—	—	—	880	—	1500	1200	1600	—	2200	1900
$NaNO_2$, p.p.m.	26	—	44	35	20	—	48	34	18	—	39	29
3 weeks matured												
NaCl, %	—	—	—	—	—	4·8	3·8	3·4	—	4·6	4·3	4·5
KNO_3, p.p.m.	—	—	—	—	—	3000	1800	2400	—	3200	2600	2400
$NaNO_2$, p.p.m.	—	98	81	90	—	82	56	69	—	110	130	120
4 weeks matured												
NaCl, %	—	—	—	—	3·1	4·5	—	3·8	2·5	6·0	—	4·3
KNO_3, p.p.m.	—	—	—	—	940	2800	—	1900	1300	3000	—	2200
$NaNO_2$, p.p.m.	72	120	—	96	56	150	—	100	21	110	—	66
5 weeks matured												
NaCl, %	—	—	—	—	3·5	—	3·7	3·6	3·0	—	4·0	3·5
KNO_3, p.p.m.	—	—	—	—	1100	—	2500	1800	1500	—	2900	2200
$NaNO_2$, p.p.m.	76	—	110	94	63	—	95	79	22	—	51	37
6 weeks matured												
NaCl, %	—	—	—	—	—	5·7	4·8	5·3	—	6·2	4·5	5·4
KNO_3, p.p.m.	—	—	—	—	—	2800	2400	2600	—	3200	2600	2400
$NaNO_2$, p.p.m.	—	120	130	130	—	130	110	120	—	180	50	120

decreases in nitrate on prolonged maturation and that these decreases did not occur, at least in the outer half of the muscle, when bacterial growth was reduced by swabbing the surface with formaldehyde solution.

The fate of the nitrite in the surface layers is of some interest, especially the indication (Table V) that values of the order of 700 p.p.m. (roughly the concentration in the tank brine) can be built up during tanking. The indications are that the relatively high surface-nitrite contents at the beginning of maturation are soon lost, possibly by diffusion, and that subsequently, if maturation is prolonged, the nitrite content falls to a very low level (Table IV). This latter fall is due to bacterial action since it does not occur in sides swabbed with formaldehyde solution (Table V).

It is of interest to note that whereas swabbing with formalin caused an initial reduction in the surface flora, after 6 weeks' maturation the numbers (as counted on 4% salt agar) had exceeded the original values in spite of repeated swabbing. The internal flora, however, was kept at a very low level. Since there was no visible evidence of deep penetration of the formaldehyde (discoloration, etc.), it seems possible that during maturation there is normally some migration of bacteria from the surface into the interior. Some support for this speculation is derived from the observation that the exterior samples of *longissimus dorsi* from the untreated sides had higher counts than the interior samples, suggesting a gradient. Further, few of the bacteria present within this muscle were capable of anaerobic growth and thus appear to be aerobes such as are found at the surface, or organisms capable of using nitrate as a hydrogen acceptor.

Expt. 4

At this point it became clear that significant results would only be obtained if the following criteria were satisfied: (1) a larger number of samples should be analysed at each point; (2) the

changes due to diffusion during maturation should be avoided; (3) the starting material should be as homogeneous as possible. Accordingly, an experiment was designed in which the maturing material would consist of pickled fillets (*psoas* muscle) lying in the cavity between two maturing sides of bacon.

Preliminary tests were carried out to determine the degree of fortification of the brine with nitrate, and the duration of immersion in the fortified brine, which would be required to give fillets containing suitable contents of salt and nitrate for a maturation experiment of the type desired.

For the actual experiments, 48 fillets were steeped for 2½ h. with occasional stirring, in factory cover-brine fortified with nitrate, the final composition of the experimental brine being NaCl 23·7%, KNO_3 1·5%, $NaNO_2$ 660 p.p.m. After being brined, the fillets were drained for a few minutes, 8 were set aside for chemical and bacteriological analysis, and the remaining 40 were placed lengthwise in the cavities between four pairs of normally maturing sides of bacon. The lower side of bacon was placed skin downwards, the upper side skin upwards. In the laboratory, a further 16 fillets were pickled similarly, swabbed with formaldehyde/NaCl and hung in a container over a trough of water to provide control material bearing a minimum of bacterial growth. At each sampling, 8 fillets were minced and extracted separately, NaCl, KNO_3 and $NaNO_2$ estimations being made on the extracts. Samples of unextracted mince were also taken for pH determinations and bacterial counts, the latter being made on nutrient agar + 4% NaCl only. The results are shown in Table VI and Fig. 5.

The results of Expt. 4 show clearly that large losses of nitrate can occur during maturation of cured pig muscle without a stoicheiometric accumulation of nitrite: the recovery of nitrate as nitrite was only about one-third. Further, the analyses of the control fillets swabbed with formaldehyde/salt solution indicate that the losses of nitrate occurring in the experimental

Table VI

Mean salt, nitrate and nitrite contents and pH values of cured fillets sampled at weekly intervals during maturation together with the standard deviations (Expt. 4)

(8 fillets taken at each sampling)

	Untreated fillets		Untreated fillets	Formaldehyde-treated fillets
Out of tank		3 weeks matured		
NaCl, %	5·45 ± 0·56	NaCl, %	5·83 ± 0·44	5·85 ± 0·13
KNO_3, p.p.m.	2900 ± 320	KNO_3, p.p.m.	140 ± 84	2600 ± 110
$NaNO_2$, p.p.m.	130 ± 18	$NaNO_2$, p.p.m.	680 ± 200	54 ± 1·1
pH	5·96 ± 0·13	pH	7·07 ± 0·31	5·77 ± 0·03
1 week matured		4 weeks matured		
NaCl, %	6·22 ± 0·57	NaCl, %	5·93 ± 0·61	—
KNO_3, p.p.m.	2900 ± 340	KNO_3, p.p.m.	24 ± 19	—
$NaNO_2$, p.p.m.	110 ± 34	$NaNO_2$, p.p.m.	74 ± 98	—
pH	5·99 ± 0·08	pH	7·80 ± 0·13	—
2 weeks matured		5 weeks matured		
NaCl, %	5·69 ± 0·41	NaCl, %	6·00 ± 0·46	6·42 ± 0·80
KNO_3, p.p.m.	2100 ± 410	KNO_3, p.p.m.	—	2800 ± 320
$NaNO_2$, p.p.m.	310 ± 121	$NaNO_2$, p.p.m.	30 ± 9·7	44 ± 5·3
pH	6·09 ± 0·17	pH	7·76 ± 0·10	5·84 ± 0·05

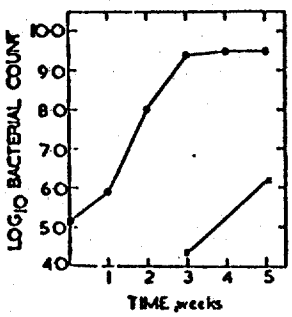


FIG. 5.—*Bacterial counts on nutrient digest agar + 4% NaCl—mean values from 8 fillets*

●——● Untreated fillets
×——× Formaldehyde-treated fillets

fillets are due to the action of bacteria growing on the surface. The organisms growing on the formaldehyde-treated fillets during the later stages of the experiment were mainly yeasts.

Discussion

The fillet experiments provided the most satisfactory evidence of large-scale bacterial metabolism of nitrate and nitrite because of the large quantities involved and the replication of samples, but the curing of fillets under the experimental conditions used does not relate very realistically to the curing of bacon sides. Not only was there no pumping, but the fillets were immersed in pickle for only $2\frac{1}{2}$ h. and were then laid between maturing bacon sides. The surface/volume ratio is high in fillets, hence the effect of surface bacteria would have been greater than on bacon sides. Further, the *psoas* muscle is rather different from the *longissimus dorsi*. Nevertheless the fillet technique is clearly useful within its limitations, and the main points of the investigation have been established. During maturation processes, large losses of nitrate may occur without corresponding accumulations of nitrite, and these losses can be attributed to bacterial activity. It seems likely that, in practice, such losses of nitrate would take place after the sides had left the factory.

The effect in maturing bacon seems to be that the nitrite concentration seldom rises above a few hundred p.p.m., although more material would need to be examined to establish this conclusively. It is natural, from analogy with brines, to attribute the discrepancy between nitrate lost and nitrite found to the ability of the bacteria on the bacon to reduce nitrite, besides nitrate. The two reactions are not necessarily carried out by the same organisms, but it has been indicated previously that about half of the bacteria on bacon may be able to reduce nitrite.[7] It would be interesting to confirm these suggestions by seeking for the reduction products of nitrite: hydroxylamine is known to be produced during the reduction of nitrate and nitrite by some bacteria[8] and to be formed in cures;[9] and nitrous oxide and nitrogen have been shown to be produced from nitrate by a *Bacillus* sp. isolated from a 'blown' can of bacon.[10]

On the other hand, some nitrite may disappear through purely chemical reactions with meat constituents, e.g., haem products and amino-compounds. However, it is known that only about 10 p.p.m. of nitrite are required to combine with the former[1] and that, although nitrite reacts chemically with substances in pork extracts,[11] the amounts so destroyed at the pH values recorded in Expt. 4 ($5{\cdot}96 \pm 0{\cdot}13$ rising to $7{\cdot}80 \pm 0{\cdot}13$) and at the temperature of the curing cellar would account for only a small part of the observed discrepancy (Eddy, unpublished observations).

Low Temperature Research Station
Cambridge

Received 23 November, 1959; amended manuscript 7 April, 1960

References

[1] Brooks, J., Haines, R. B., Moran, T., & Pace, J., *Rep. Fd Invest. Bd Lond.*, 1940, No. 49
[2] Hornsey, H. C., *2nd Int. Symp. Fd Microbiol.*, 1958, p. 303 (London: H.M.S.O.)
[3] Eddy, B. P., & Kitchell, A. G. (in preparation)
[4] Grau, R., & Mirna, A., *Z. anal. Chem.*, 1957, 158, 182
[5] Eddy, B. P., *Leeuwenhoek ned. Tijdschr.*, 1958, 24, 81
[6] Jespersen, N. J. T., & Riemann, H., as reference 2, p. 177
[7] Eddy, B. P., as reference 2, p. 87
[8] Lindsey, G., & Rhines, C., *J. Bact.*, 1932, 24, 489
[9] Jensen, L. B., & Hess, W. R., *Food Manuf.*, 1941, 16, 157
[10] Eddy, B. P., & Ingram, M., *J. appl. Bact.*, 1956, 19, 62
[11] Rose, D., & Peterson, R., *Food Tech., Champaign*, 1953, 7, 369

539

Effect of Nitrate and Nitrite on Vitamin A Storage in the Rat[1]

R. J. EMERICK AND O. E. OLSON
Station Biochemistry Department, South Dakota Agricultural Experiment Station, Brookings, South Dakota

The high nitrate content of some forages has been suggested as a factor responsible for precipitating vitamin A deficiencies in livestock receiving levels of carotene thought to be adequate (Garner, '58). A microbial reduction of nitrate in the rumen with the subsequent accumulation of some of its reduction products, including nitrite, is well known. The feeding of 0.3% of potassium nitrite was reported by O'Dell et al.[2] to cause an increased rate of depletion of vitamin A stores in the liver of rats. Several other workers, among them Muhrer et al.,[3] Smith et al.[4] and Holst et al.,[5] have reported that the feeding of rations having a high nitrate content had an adverse effect upon the vitamin A status of animals.

The experiments reported herein were conducted to determine whether nitrate or its reduction product, nitrite, interferes with the storage of vitamin A in the liver, either from a preformed source or from carotene, under controlled experimental conditions.

EXPERIMENTAL

In each of three trials, Sprague-Dawley female rats were given a low vitamin A diet (table 1) for a period of two to three weeks to deplete liver vitamin A stores. After this time, the rats were divided into 9 groups of 8 rats each for each trial, and were fed the following diets: groups 1, 2 and 3, control (low vitamin A diet, table 1); groups 4, 5 and 6, control plus 3% of sodium nitrate; groups 7, 8 and 9, control plus 0.5% of sodium nitrite. These diets were fed for a period of 6 days in all instances. On the third and fourth days, vitamin A or carotene sources were administered. On the sixth day, the animals were killed and the livers were immediately frozen. Liver carotene or vitamin A, or both, were determined at a later date by the method of Johnson and Baumann ('47a). The vitamin A sources used in these studies were analyzed colorimetrically as described in an industrial bulletin[6] with the exception that the oil solution was analyzed without prior saponification. The procedure used in these experiments for measuring the storage of vitamin A is based on the liver storage test of Guggenheim and Koch as modified by Foy and Morgareidge ('48).

TABLE 1
Vitamin A depletion diet fed to rats

	% of diet
Cornstarch	65
Casein, vitamin-free[1]	18
Brewer's yeast	8
Salt mixture[2]	4
Fortified corn oil[3]	5

[1] "Vitamin Free" Casein, Nutritional Biochemicals Corporation, Cleveland.
[2] Philips and Hart ('35).
[3] Fortified corn oil contained approximately 40 IU vitamin D as irradiated ergosterol and 1 mg DL-α-tocopheryl acetate/gm. This supplied 2 IU vitamin D and 0.05 mg α-tocopheryl acetate/gm of complete diet.

Received for publication March 16, 1962.

[1] Published with the approval of the Director of the South Dakota Agricultural Experiment Station as publication no. 546 of the journal series. The vitamin A sources were kindly supplied by Nopco Chemical Company, Newark, New Jersey.

[2] O'Dell, L. B., Z. Erek, L. Flynn, G. B. Garner and M. E. Muhrer 1960 Effects of nitrate containing rations in producing vitamin A and vitamin E deficiencies in rats. J. Animal Sci., *19:* 1280 (abstract).

[3] Muhrer, M. E., A. A. Case, G. B. Garner and W. H. Pfander 1955 Toxic forage produced in a drought area. Ibid., *14:* 1251 (abstract).

[4] Smith, G. S., A. L. Neumann and E. E. Hatfield 1961 Carotene utilization and vitamin A nutrition as influenced by dietary nitrite and "high nitrate" silage: Laboratory studies. Ibid., *20:* 683 (abstract).

[5] Holst, W. O., L. M. Flynn, G. B. Garner and W. H. Pfander 1961 Dietary nitrite vs. sheep performance. Ibid., *20:* 936 (abstract).

[6] A colormetric assay for vitamin A. Technical Service Bureau Publication VA-84. Nopco Chemical Company, Harrison, New Jersey, 1958.

TABLE 2

Body and liver weights of rats receiving various dietary treatments[1]

Trial	Treatment	Control diet			3% $NaNO_2$ diet			0.5% $NaNO_2$ diet		
		Group no.	Final body wt	Liver wt	Group no.	Final body wt	Liver wt	Group no.	Final body wt	Liver wt
			gm	*gm*		*gm*	*gm*		*gm*	*gm*
1[2]	None	1	120	5.6	4	114	4.5	7	107	4.3
	Vitamin A, oral	2	124	6.0	5	124	5.6	8	115	4.4
	Vitamin A, injected	3	118	5.6	6	122	5.0	9	111	4.3
2[3]	None	1	132	5.8	4	124	5.4	7	116	4.8
	Vitamin A, oral	2	132	7.1	5	122	5.4	8	119	5.3
	Vitamin A, injected	3	135	6.8	6	128	5.5	9	119	5.4
3[4]	None	1	142	6.7	4	135	6.0	7	129	6.2
	Carotene, 1 mg/rat	2	144	7.5	5	138	6.9	8	129	6.4
	Carotene, 1.7 mg/rat	3	144	7.6	6	139	6.9	9	130	6.6

[1] Average body weights for rats prior to receiving the experimental diets were as follows: trial 1, 108; trial 2, 115; trial 3, 128.
[2] Vitamin A source for trial 1 was vitamin A palmitate dissolved in sesame oil. Total dosage was equivalent to 294 μg of vitamin A alcohol/rat.
[3] Vitamin A source for trial 2 was vitamin A palmitate in a carbohydrate base (Capstress, Nopco Chemical Company, Harrison, N. J.) dispersed in water. Total dosage was equivalent to 210 μg of vitamin A alcohol/rat.
[4] Carotene source was pure β-carotene dissolved in corn oil.

In view of the report that nitrate reduces feed consumption and lowers weight gain,[7] the length of time that the rats were fed diets with added nitrate or nitrite was held to a minimum, i.e. 6 days. Vavich and Kemmerer ('50) have shown that although the size of the rat is an important factor in influencing the amount of vitamin A stored (an inverse relationship), the amount of diet consumed has no effect on vitamin A storage, when intake of the vitamin is held constant.

Trial 1. Vitamin A palmitate dissolved in sesame oil (9800 IU/ml) was administered orally from a blunt pointed syringe at the rate of 0.05 ml/rat on each of two consecutive days (total dosage, 980 IU/rat) to groups 2, 5 and 8; and by subcutaneous injection to groups 3, 6 and 9. Groups 1, 4 and 7 remained untreated.

Trial 2. Water-dispersed vitamin A palmitate (3500 IU/ml) was administered orally at the rate of 0.1 ml/rat on each of two consecutive days (total dosage, 700 IU/rat) to groups 2, 5 and 8; and by subcutaneous injection to groups 3, 6 and 9. Groups 1, 4 and 7 remained untreated.

Trial 3. Pure β-carotene was dissolved in corn oil and administered by stomach tube at the rate of 1 mg of β-carotene (total dosage)/rat for groups 2, 5 and 8; and 1.7 mg/rat for groups 3, 6 and 9. In each instance, one-half of the carotene dosage was administered on two consecutive days in 0.5 ml of oil/day. As in the previous two trials, groups 1, 4 and 7 were not treated.

RESULTS AND DISCUSSION

Despite the relatively short experimental period, differences in average final weights (table 2) were apparent with the lower weights generally being associated with nitrate, and especially nitrite feeding. These weight differences were also reflected in the liver weights.

Data pertaining to the liver storage of vitamin A and carotene in the various trials are shown in table 3. In trial 1, oral administration of the oil solution of vitamin A resulted in an increase in liver

[7] Weichenthal, B. A., R. J. Emerick, L. B. Embry and F. W. Whetzal 1961 Influence of nitrate on performance and vitamin A status of fattening cattle. J. Animal Sci., 20: 955 (abstract).

TABLE 3

Average liver vitamin A and carotene levels of rats receiving nitrate or nitrite and given various vitamin A or carotene treatments

Trial	Treatment	Control diet			3% $NaNO_2$ diet			0.5% $NaNO_2$ diet		
		Group no.	Carotene	Vitamin A	Group no.	Carotene	Vitamin A	Group no.	Carotene	Vitamin A

TABLE 3

Average liver vitamin A and carotene levels of rats receiving nitrate or nitrite and given various vitamin A or carotene treatments

Trial	Treatment	Control diet			3% $NaNO_3$ diet			0.5% $NaNO_2$ diet		
		Group no.	Carotene	Vitamin A	Group no.	Carotene	Vitamin A	Group no.	Carotene	Vitamin A
			μg	μg		μg	μg		μg	μg
1[1]	None	1	—	2.6	4	—	1.8	7	—	2.7
	Vitamin A, oral	2	—	49.7	5	—	49.7	8	—	30.9[2]
	Vitamin A, injected	3	—	4.6	6	—	5.8	9	—	18.1[2]
2[3]	None	1	—	1	4	—	1	7	—	1
	Vitamin A, oral	2	—	107.2	5	—	104.0	8	—	86.8[2]
	Vitamin A, injected	3	—	20.8	6	—	17.3	9	—	23.3
3[4]	None	1	4.2	3.0	4	4.1	3.8	7	4.7	3.2
	Carotene, 1 mg/rat	2	7.8	98.1	5	5.7[5]	74.3[5]	8	7.6	64.6[5]
	Carotene, 1.7 mg/rat	3	13.2	152.9	6	9.6[5]	129.8[5]	9	10.4[5]	87.6[5]

[1] Vitamin A source for trial 1 was vitamin A palmitate dissolved in sesame oil. Total dosage was equivalent to 294 μg of vitamin A alcohol/rat.
[2] Significantly different from values obtained from rats receiving similar treatment in control diet ($P < 0.01$).
[3] Vitamin A source for trial 2 was vitamin A palmitate in a carbohydrate base (Capstrees, Nopco Chemical Company) dispersed in water. Total dosage was equivalent to 210 μg of vitamin A alcohol/rat.
[4] Carotene source was pure β-carotene dissolved in corn oil.
[5] Significantly lower than values obtained from rats receiving similar treatment in control diet ($P < 0.05$).

vitamin A levels representing an average of 26.2 and 26.6% of the administered dose in the rats receiving the control and the nitrate diet, respectively. Vitamin A storage of the oral dose was significantly reduced ($P < 0.01$) in the nitrite-fed rats. In this instance, storage was reduced to 15.7% of the administered dose. Injection of the oil-vitamin A solution resulted in very small amounts of vitamin A appearing in the liver of the rats fed the control and nitrate diets. However, storage of the injected dose was significantly higher ($P < 0.01$) in the rats receiving nitrite. This variation remains unexplained at this time.

Oral administration of a water-dispersed source of vitamin A (trial 2) resulted in an increase in liver vitamin A levels for all groups. This amounted to 59.5, 57.8 and 48.2% of the administered dose in the control, nitrate and nitrite groups respectively (table 3). As with the orally administered oil solution in the previous trial, storage of the orally administered water-dispersed vitamin A was significantly reduced ($P < 0.01$) by nitrite, but not nitrate feeding. Liver storage of the injected water-dispersed vitamin A was considerably less than that obtained by oral administration, and amounted to only 9.9, 8.2 and 11.1% of the administered dose for groups 3, 6 and 9, respectively. Thus, neither nitrate nor nitrite had a significant effect on the liver storage of the injected vitamin A. The small increase in storage in the group fed nitrite (group 9), however, is consistent with the observations made in trial 1.

Results of trials 1 and 2 indicate that absorption and subsequent liver storage of vitamin A, during the short time allowed, was less when administered by injection than when administered orally. It must be recognized, however, that the data are not adequate for comparing the effectiveness of these two methods of administration in promoting growth or liver vitamin A storage, when longer periods of time are involved.

Administration of two levels of carotene to rats receiving the control diet (trial 3) resulted in increases of liver vitamin A levels equivalent to 9.5 and 8.8% of the respective 1.0- and 1.7-mg carotene dos-

ages (table 3). Liver storage of vitamin A in the rats dosed with carotene was significantly reduced by the feeding of nitrate ($P < 0.05$) and nitrite ($P < 0.01$). The respective increases in liver vitamin A levels resulting from the 1.0- and 1.7-mg carotene dosages amounted to 7.0 and 7.4% for the nitrate-fed groups (groups 5 and 6), and 6.1 and 5.0% for the nitrite-fed groups (groups 8 and 9). Liver accumulations of carotene in this trial were small, with both nitrate and nitrite causing significantly lower levels in most instances.

In these experiments, the feeding of nitrite, but not nitrate, resulted in a reduction in the amount of vitamin A stored in the liver when vitamin A was administered orally, but not when administered by subcutaneous injection. This suggests that the action of nitrite in reducing liver storage of the orally administered vitamin A was the result of either a decrease in absorption of the vitamin from the intestine, or an increase in its destruction within the digestive tract. A reduction in vitamin A absorption by nitrite has not been demonstrated. However, Olson et al.[8] observed that a destruction of the provitamin, carotene, occurred when nitrite was present under acid conditions. In this study, the decreased storage of vitamin A from orally administered carotene in the presence of dietary nitrite was of a magnitude that would suggest a possible implication of the same mechanisms whereby the detrimental action of nitrite on preformed vitamin A was accomplished. The effect of nitrate on vitamin A storage from a carotene source, however, cannot be explained on the same basis. Olson et al.[9] showed that nitrate itself caused no destruction of carotene under a variety of conditions. The finding that the nitrate ion has an effect on thyroid function (Wyngaarden et al., '52; Bloomfield et al., '61), and the observation of Johnson and Baumann ('47b) that a functioning thyroid gland is necessary for the conversion of carotene to vitamin A, suggest a possible mechanism for the effect of nitrate on vitamin A storage from a carotene source. The possibility, however, that the nitrate effect is the result of a lowered absorption of carotene should not be overlooked. The need for additional work in this area is indicated.

Although the data obtained in these experiments, indicating an effect of nitrate or nitrite on the vitamin A status of rats, differ from results reported in some cattle and sheep studies,[10,11,12] they are in agreement with the *in vitro* data obtained by Olson et al.[13] which showed that sodium nitrite mixed with acid stomach contents destroyed carotene rapidly. Since the reduction of nitrate and absorption of reduction products in the ruminant animal often occur under pH conditions approaching neutrality, the difference sometimes obtained between ruminants and nonruminants appears to be expected.

SUMMARY

Rats receiving diets with and without 3% of sodium nitrate or 0.5% of sodium nitrite were administered vitamin A palmitate in oil solution or in a water dispersion, either orally or by subcutaneous injection, or β-carotene in oil solution by stomach tube. In all instances, during the brief periods allowed in these studies, the water dispersed source of vitamin A contributed to liver storage to a much greater extent than the oil solution, and the orally administered sources to a much greater extent than the injected sources. The feeding of nitrite, but not nitrate, significantly lowered liver storage of vitamin A from orally administered sources of preformed vitamin A, but not from injected sources. Both nitrate and nitrite significantly lowered the liver storage of vitamin A from carotene with the greatest effect resulting from nitrite. Possible mechanisms were discussed.

LITERATURE CITED

Bloomfield, R. A., C. W. Welsch, G. B. Garner and M. E. Muhrer 1961 Effect of dietary nitrate on thyroid function. Science, *134:* 1690.

[8] Olson, O. E., D. L. Nelson and R. J. Emerick. Effect of nitrate and some of its reduction products on carotene stability. Manuscript in preparation.

[9] See footnote 8.

[10] See footnote 7.

[11] Hale, W. H., F. Hubbert, Jr. and R. E. Taylor 1961 Effect of concentrate level and nitrate addition on hepatic vitamin A stores and performance of fattening steers. J. Animal Sci., 20: 934 (abstract).

[12] Sokolowski, J. H., U. S. Garrigus and E. E. Hatfield 1960 Nitrate poisoning, a study of possible relationships between nitrate, vitamin A and carotene. Sheep Day Report, SH 46.

[13] See footnote 8.

Foy, J. R., and K. Morgareidge 1948 Biological assay of vitamin A. Liver storage test of Guggenheim and Koch. Anal. Chem., *20:* 301.

Garner, G. B. 1958 Learn to live with nitrate, bull. 708, Missouri Agricultural Experiment Station, Columbia, Missouri.

Johnson, R. M., and C. A. Baumann 1947a Storage and distribution of vitamin A in rats fed certain isomers of carotene. Arch. Biochem., *14:* 361.

——— 1947b The effect of thyroid on the conversion of carotene to vitamin A. J. Biol. Chem., *171:* 513.

Phillips, P. H., and E. B. Hart 1935 The effect of organic dietary constituents upon chronic fluorine toxicosis in the rat. J. Biol. Chem., *109:* 657.

Vavich, M. G., and A. R. Kemmerer 1950 Factors influencing the utilization of carotene for storage of vitamin A in the rat. J. Nutrition, *40:* 605.

Wyngaarden, J. B., B. M. Wright and P. Ways 1952 The effect of certain anions upon the accumulation and retention of iodide by the thyroid gland. Endocrinology, *50:* 537.

26

547

REFERENCES

Alford, J. A. and Pierce, D. A. 1963. Production of lipase by *Pseudomonas fragi* in a synthetic medium. *J. Bacteriol.* **86**, 29.

Baumann, P., Doudoroff, M. and Stanier, R. Y. 1968. Study of the *Moraxella* group. I. Genus *Moraxella* and the *Neisseria catarrhalis* group. *J. Bacteriol.* **95**, 58.

Castell, C. H. and Garrard, E. H. 1941. The action of microorganisms on fat. III. Oxidation and hydrolysis of triolein by pure cultures of bacteria. *Can. J. Res.*, Section C, **19**, 106.

Fukuba, H. 1952. Studies on lipoxidase. 2. Distribution of lipoxidase in plants and microorganisms. *J. Agr. Chem. Soc. Japan* **26**, 167.

Gaddis, A. M. and Ellis, R. 1959. Paper chromatography of 2,4-dinitrophenylhydrazones. Resolution of 2-alkanone, n-alkanal, alk-2-enal, and alk-2,4-dienal derivatives. *Anal. Chem.* **31**, 870.

Hawke, J. C. 1966. The formation and metabolism of methyl ketones and related compounds. *J. Dairy Res.* **33**, 243.

International Union of Biochemistry. 1965. "Enzyme Nomenclature." Elsevier Publ. Co., Amsterdam.

Jensen, L. B. 1954. "Microbiology of Meats," 3rd ed. Garrard Press, Champaign, Ill.

Kolmer, J. A., Spaulding, E. H. and Robinson, H. W. 1951. "Approved Laboratory Technique," 5th ed., p. 448. Appleton-Century-Crofts, Inc., New York, N. Y.

Lea, C. H. 1938. Rancidity in edible fats. Food Investigation Special Report No. 46, H. M. S. Office, London.

Lea, C. H. 1952. Methods for determining peroxide in lipids. *J. Sci. Food Agr.* **3**, 586.

Maier, V. P. and Tappel, A. L. 1959. Rate studies of unsaturated fatty acid oxidation catalyzed by hematin compounds. *J. Amer. Oil Chemists' Soc.* **36**, 8.

Mukherjee, S. 1951. Studies on degradation of fats by microorganisms. I. Preliminary investigations on enzyme systems involved in the spoilage of fats. *Arch. Biochem.* **33**, 364.

Schwartz, D. P., Johnson, A. R. and Parks, O. W. 1962. Use of ion-exchange resins in the micro analysis of 2,4-dinitrophenylhydrazones. *Microchem. J.* **6**, 37.

Schwartz, D. P., Haller, H. S. and Keeney, M. 1963. Direct quantitative isolation of monocarbonyl compounds from fats and oils. *Anal. Chem.* **35**, 2191.

Shimahara, K. 1966. Bacterial peroxidation of fats. III. Identification of lipoxygenase-forming bacteria. *J. Ferment. Technol.* **44**, 230.

Smith, J. L. and Alford, J. A. 1968. Action of microorganisms on the peroxides and carbonyls of rancid fat. *J. Food Sci.* **33**, 93.

Tappel, A. L. 1963. Lipoxidase. In "The Enzymes," Vol. 8, ed. Boyer, P. D., Lardy, H. and Myrbäck, K., pp. 275–283. Academic Press, New York, N. Y.

Ms. rec'd 4/22/68; accepted 11/6/68.

A. S. EMODI and R. V. LECHOWICH
Department of Food Science, Michigan State University
East Lansing, Michigan 48823

Low Temperature Growth of Type E Clostridium botulinum Spores. 1. Effects of Sodium Chloride, Sodium Nitrite and pH

SUMMARY—The effects of pH and the addition of sodium chloride (NaCl) or sodium nitrite ($NaNO_2$) to trypticase-peptone-glucose (TPG) and trypticase-peptone-sucrose-yeast extract (TPSY) media upon spore outgrowth were investigated using seven Type E *Clostridium botulinum* strains. An inoculum of 1.0×10^4 spores/ml was used and the pH was adjusted with hydrochloric acid to cover the range of 5.2 to 6.6. To define salt tolerance, NaCl was added to the media at intervals of 0.5% in the range of 2.0 to 5.0% and at 0.1% intervals in the 4.5 to 5.0% range. The effect of $NaNO_2$ was investigated with the addition of 100 and 200 ppm $NaNO_2$ to the media. Samples were incubated at 30, 15.6, 10, 7.2, 5.0 and 3.4°C. Outgrowth of all strains tested was inhibited at pH 5.2 at 15.6°C. Inhibition occurred at higher pH at lower temperatures. None of the strains showed outgrowth with 4.87% NaCl in the media at any of the incubation temperatures used. Addition of 100 and 200 ppm $NaNO_2$ to the media inhibited the outgrowth of the Minneapolis, 517, 26080 and A6247 strains but not the Kalamazoo and Seattle Forks strains.

INTRODUCTION

OUTBREAKS OF FOOD poisoning by Type E *Clostridium botulinum* in the United States during the past 5 years have been few in number, but have received wide publicity. Studies (Dolman, 1957) have indicated that Type E *Cl. botulinum* is distributed in marine sediments and fish in the Canadian Pacific coastal area. Recent studies by Bott, *et al.* (1965) have conclusively demonstrated the presence of Type E botulism organisms in at least four of the five Great Lakes. The percentage of the isolations shows that this organism may be found in relatively large numbers in certain areas of Lake Michigan. Craig *et al.* (1965) has also shown that Type E *Cl. botulinum* can be found in the Columbia River area of Washington and Oregon.

The demonstration of the organism in many areas of the United States coupled with low-temperature growth ability of this organism reported by Schmidt *et al.* (1961), prompted this study to examine means for controlling the growth of this organism. Effective means of inhibiting low-temperature growth of Type E *Cl. botulinum* are necessary to maintain the public health safety record of our processed food supply.

Segner *et al.* (1966) studied the effect of sodium chloride (NaCl) and pH on the outgrowth of Type E *Cl. botulinum spores*. They found that at near optimum temperature using an inoculum of 1.0×10^3 spores/ml, pH 5.03 was the minimum pH permitting outgrowth and that 5.0% NaCl was required to inhibit growth.

Toxin formation by Types A and B *Cl. botulinum* was reported by Greenberg *et al.* (1959) in inoculated perishable cured meat with no accompanying organoleptic breakdown of the product. They found that a mixed strain inoculum consisting of five strains each of Types A and B *Cl. botulinum* in meat incubated at 30 to 37°C produced both toxin and putrefactive changes when the NaCl content in the phase of the meat was less than 6.25%. Water phase NaCl contents ranging between 6.25 and 9% permitted only toxin production with no putrefaction, and growth was inhibited when NaCl was greater than 9%. The physiological differences between Types A, B and E do not permit extrapolation of these data to Type E *Cl. botulinum*.

In the present investigation, the inhibitory NaCl and sodium nitrite ($NaNO_2$) concentrations and the effect of pH on the outgrowth of seven Type E *Cl. botulinum* strains were studied. Incubation temperature ranged from near optimum 30°C to near minimum 3.4°C.

MATERIALS AND METHODS

THE KALAMAZOO (K), Vancouver Herring (VH), Seattle Forks (SF), Minneapolis (M), 517, A6247 and 26080 Type E *Cl. botulinum* strains used in this study were selected because of their isolation from fishery products or processing equipment used in fish processing plants. In addition the VH strain had been reported by Dolman *et al.* (1950) to grow at low temperature and low pH. Three of the strains used here were also involved in fatal type E botulism cases in the United States—the M strain from the 1960 outbreak, the K strain that caused two deaths in Michigan and the 517 strain that caused five deaths in Tennessee in October, 1963.

The K, 26080 and A6247 strains were obtained from Mr. R. W. Johnston, Food and Drug Administration, Detroit, Michigan. The VH and M strains were obtained from Dr. C. F. Schmidt, Continental Can Company, Chicago, Illinois, the SF strain was obtained from Dr. J. T. Graikoski, Bureau of Commercial Fisheries, Ann Arbor, Michigan, and the 517 strain was obtained from Mr. D. Kautter, Food and Drug Administration, Washington, D. C.

Spores were prepared in a medium containing 5.0% trypticase (BBL), 0.5% peptone, 0.5% sucrose and 1.0% Difco yeast extract (TPSY). Since the yeast extract markedly stimulated sporulation of the K, 517, 26080 and A6247 strains, it was incorporated into the basal medium formula. The medium also contained 0.1% sodium thioglycollate and was adjusted to pH 7.2 prior to sterilization. The medium was sterilized in 1 L screw-cap Erlenmeyer flasks. During incubation at 30°C the course of sporulation was followed for approximately 24 to 28 hr by phase contrast microscopy. After sporulation was judged complete, the cultures were harvested and treated with lysozyme by the procedure of Brown *et al.* (1957). The spores were then alternately centrifuged and washed three times with cold sterile deionized water prior to suspending to a final concentration of 1.0×10^8 to 1.0×10^9 refractile spores/ml.

Viable counts of the stock spore suspensions were obtained using both Graikoski's beef infusion medium (personal communication, 1964) and trypticase-peptone-0.1% glucose medium. The counting media also were adjusted to pH 7.2 and contained 0.1% sodium thioglycollate. Appropriate dilutions were prepared so that aliquots of the dilutions would produce 10 to 50 colonies in each 16 × 150 mm screw-cap test tube. Colonies were counted after 24 to 36 hr incubation at 30°C using triplicate tubes per dilution.

The pH of the media was adjusted with hydrochloric acid with a Beckman Model 76 pH meter. The pH was recorded prior to inoculation and again at the end of 180 days at each incubation temperature. The pH had varied only about ± 0.1 of a pH unit even after the long incubation period. Media used were the trypticase-peptone-glucose medium (TPG) of Wagenaar *et al.* (1958) and the TPSY medium. All low-temperature growth media contained only 0.02% sodium thioglycollate since higher concentrations were found to inhibit outgrowth at the low temperatures employed.

After inoculation with about 1.5×10^6 viable unheated Type E spores per 15 ml of medium, the three replicate tubes for each pH level of each strain were layered with sterilized petrolatum-paraffin mixture. Growth was noted by formation of turbidity and gas production under the seal. Toxin formation was detected by trypsin digestion of culture aliquots, and toxin assays were performed with specific type E antitoxin protection tests in white mice weighing 15 to 20 g.

Incubation temperatures were 30, 15.6, 10, 7.2, 5.0 and 3.4°C and the total incubation time was 180 days.

RESULTS AND DISCUSSION

THE RESULTS presented in Table 1 indicate that pH tolerance is a function of incubation temperature and that minor variations in acid tolerance exist among Type E strains. At 5°C the minimum pH permitting outgrowth of different strains was either 6.2 or 6.4; at 7.2°C it decreased to 5.8 or 6.0, while at 10°C only five of the strains grew at pH 5.6 or 5.8. At 10°C the SF strain showed outgrowth at pH 6.0. At 15.6° four strains grew at pH 5.4 and the K and SF strains showed less acid tolerance and grew at pH 5.6 only. None of these strains showed outgrowth at pH 5.2.

The pH of the medium was rechecked after 180 days of incubation at which time the experiment was terminated. The change in pH was no more than ± 0.1 pH unit.

Table 1. Outgrowth of Type E *Cl. botulinum* strains in TPG and TPSY media at different pH levels.

Strain	Medium	Incubation temperature (C)			
		15.6°	10°	7.2°	5°
		Lowest pH permitting outgrowth[1]			
Kalamazoo	TPG	5.6	5.8	6.0	6.2
Seattle Forks	TPG	5.6	6.0	6.0	6.4
Minneapolis	TPSY	5.4	5.6	5.8	6.2
517	TPSY	5.4	5.8	5.8	6.2
26080	TPSY	5.4	5.8	6.0	6.2
A6247	TPSY	5.4	5.6	5.8	6.2

[1] No growth at next lowest pH (0.2 pH unit less).

The inhibitory effect of 100 and 200 ppm $NaNO_2$ added to the TPG and TPSY media in the pH range of 6.2 and 6.6 is presented in Table 2. The data show that in the pH range 6.2 to 6.6 the outgrowth of the M, 517, 26080 and A6247 strains was inhibited by the addition of 100 or 200 ppm of $NaNO_2$ to the media at 15.6 and 10°C. Only the K and SF strains exhibited outgrowth in the presence of nitrite. The SF strain grew in 4 days at 15.6°C in all three pH ranges tested when 100 ppm of nitrite was added to the medium. Sixty-seven days were required for outgrowth at pH 6.2 with 200 ppm nitrite sterilized in the medium. The K strain showed outgrowth after 25 days of incubation with 100 ppm nitrite in the medium, and after 34 days of incubation with 200 ppm nitrite in the medium at 10°C and pH 6.6. The medium was analyzed for nitrite after 180 days of incubation and 37.0 and 88.0 ppm $NaNO_2$ were recovered.

Sodium chloride has an inhibitory effect on the outgrowth of Type E *Cl. botulinum* spores, and a 5% NaCl concentration had been reported as the minimum concentration that prevented outgrowth.

Accordingly, the Type E strains were inoculated into TPSY medium at pH 7.2 containing 0 and 5.0% salt and were incubated at 15.6, 10.0, 7.2, 5.0 and 3.4°C. The results were summarized in Table 3. It appears that these strains are relatively tolerant to salt at 15.6 and 10.0°C since growth was obtained in the 4.5% but not in the 5.0% salt medium. At 7.2°C and 5.0°C the 517 strain was slightly less tolerant to salt. Average outgrowth time in the presence of 4.5% salt was considerably delayed at 10°C (31 days) and at 15.6°C (17 days). Salt tolerance was reduced somewhat at 7.2°C and reduced considerably at 5.0°C, where growth occurred only in 2.0 or 2.5% salt. No growth in 0 to 2.0% salt medium was evident at 3.4°C within 180 days of incubation.

To define the salt tolerance of Type E strains more critically, TPSY medium (pH 7.2) containing 4.5 to 5.0% salt at 0.1% intervals was incubated at 15.6 and 30°C. These temperatures were chosen to represent the temperatures the fish product might reach if the product was improperly handled. These results (Table 4) indicate that three of the six strains could grow in the presence of 4.8% salt when incubated at 15.6°C. Since these strains indicated somewhat greater salt tolerance than was found in the previous experiment, titrimetric analyses for chloride were performed. The positive tubes of the VH and of the 26080 strains at 4.8% salt were respectively found to contain 4.72 and 4.77% NaCl, while the negative tubes at 4.9% contained 4.87% NaCl.

To simulate the pH of some food substrates such as smoked fish more closely, a salt tolerance experiment was performed in TPSY medium at pH 6.5. The data in Table 4 show that tolerance to salt was generally lower at pH 6.5 than at 7.2. The M and A6247 strains were the least acid tolerant. The outgrowth of the M strain was inhibited by 4.5% salt at both temperatures while the outgrowth of the A6247 strain was inhibited only at 15.6°C. Since no lower salt concentrations were used

Table 2. Outgrowth time in days of six Type E *Clostridium botulinum* strains incubated 180 days in laboratory media at various pH levels with 100 and 200 ppm $NaNO_2$.

			Incubation temperature (C)								
			15.6			10			7.2		
			pH								
Strain	Medium	$NaNO_2$ ppm	6.2	6.4	6.6	6.2	6.4	6.6	6.2	6.4	6.6
K	TPG	100	....[1]	....	....	....	....	25	....	....	....
		200	....	....	....	....	....	34	....	....	....
SF	TPG	100	4	4	4	....	....	....	....	....	....
		200	67	....	....	....	....	....	....	....	....
Minn. 517 26080 A6247	TPSY	100	....	....	....	....	....	....	....	....	....
		200	....	....	....	....	....	....	....	....	....

[1] No growth.

Table 3. Outgrowth of Type E *Cl. botulinum* strains in TPSY + NaCl medium at pH 7.2.

	Incubation temperature (C)							
	15.6		10.0		7.2		5.0	
Strain	G[1]	NG[2]	G	NG	G	NG	G	NG
Minneapolis	4.5[3]	5.0	4.5	5.0	4.5	5.0	2.5	3.0
Kalamazoo	4.5	5.0	4.5	5.0	4.0	4.5	2.5	3.0
Vancouver Herring	4.5	5.0	4.5	5.0	4.5	5.0	2.5	3.0
517	4.5	5.0	4.5	5.0	3.5	4.0	2.0	2.5
26080	4.5	5.0	4.5	5.0	4.0	4.5	2.0	2.5

[1] Growth.
[2] No growth.
[3] Percent of NaCl in TPSY medium.

Table 4. Salt tolerance of six Type E *Cl. botulinum* strains in TPSY + NaCl medium at pH 6.5 and at pH 7.2.

	Incubation temperature (C)							
	30				15.6			
	pH							
	6.5		7.2		6.5		7.2	
	G[1]	NG[2]	G	NG	G	NG	G	NG
Minneapolis	....	4.5[3]	4.6	4.7	....	4.5	4.7	4.8
Kalamazoo	4.7	4.5	4.6	4.7	4.8	4.9	4.8	4.9
Vancouver Herring	4.7	4.8	4.7	4.8	4.7	4.8	4.8	4.9
26080	4.5	4.6	4.6	4.7	4.8	4.9	4.8	4.9
A6247	4.5	4.6	4.6	4.7	....	4.5	4.6	4.7
517	4.3	4.4	4.7	4.8	4.6	4.7	4.7	4.8

[1] Growth.
[2] No growth.
[3] Percent of NaCl in TPSY medium.

in this experiment the inhibitory salt concentration at pH 6.5 for these two strains were not determined. The K and 26080 strains exhibited the same salt tolerance at pH 6.5 and at pH 7.2 and the VH strain tolerated 0.1 to 0.2% less salt at the more acidic condition.

The results of these experiments show that outgrowth of Type E strains was inhibited at pH 5.2 and 15.6°C. At higher temperatures outgrowth may occur at lower pH levels since Segner *et al.* (1966) showed outgrowth at pH 5.03 and 30°C.

The nitrite experiment indicated that 100 or 200 ppm $NaNO_2$ in the media inhibited the outgrowth of only four of the six strains tested, while 5.0% NaCl inhibited all strains.

These findings show quite a variation among Type E strains in acid or nitrite tolerance. Sodium chloride is the only additive which in 5.0% concentration inhibited all strains tested. Segner *et al.* (1966) in a similar experiment using TPG medium and preheated spores of the Beluga, NE, Alaska and M strains showed that 5.0% salt was required to inhibit outgrowth at 16°C and higher temperatures, while at 8 and 10°C 4.5% was inhibitory. The inhibitory pH value was 5.21 for these strains at these temperatures except for the Beluga strain. Spores of the Beluga strain exhibited outgrowth even at pH 5.03 when 10×10^5 spores/ml was used.

Our results show good agreement with the study of Segner *et al.* (1966) even though different Type E strains, media and temperatures were employed. Some variation is indicated among Type E strains in salt, acid and nitrite tolerance and a marked interaction of these additives with incubation temperatures.

Extension of these data to fish preservation would indicate that a combination of acidification to pH 6.5, coupled with the use of 5% NaCl and up to 200 ppm $NaNO_2$ would render a fish product held at 10°C or below a poor substrate for the growth of Type E *Cl. botulinum*. Treatment of fish fillets with $NaNO_2$ has been practiced commercially in Canada where up to 200 ppm are permitted to be added to fresh fish, preserved fish and preserved meat products. Additional studies are needed to determine the relationships of nitrite content, storage temperatures of less than 10°C, and acidification of fishery products to pH 6.5 or less upon growth and toxin production by Type E *Cl. botulinum*.

REFERENCES

Bott, T. L., Deffner, J. S., McCoy, E. and Foster, E. M. 1965. Occurrence of *Clostridium botulinum* Type E in fish of the Great Lakes. *Bacteriol. Proc.* p. 1.

Brown, W. L., Ordal, Z. J. and Halvorson, H. O. 1957. Production and cleaning of spores of Putrefactive Anaerobe 3679. *Appl. Microbiol.* **5**, 156.

Craig, J. M. and Pilcher, K. S. 1965. Ecological survey of *Clostridium botulinum* in the Pacific Northwest. *Bacteriol. Proc.* p. 1.

Dolman, C. E., Chang, H., Kerr, D. E. and Shearer, A. R. 1950. Fish-borne and Type E botulism: Two cases due to home-pickled herring. *Can. J. Publ. Health.* **41**, 215.

Dolman, C. E. 1957. Type E (fish-borne) botulism. A review. *Japan J. Med. Sci.* (Biol.) **10**, 383.

Greenberg, R. A., Silliker, J. H. and Fata, L. D. 1959. The influence of sodium chloride on toxin production and organoleptic breakdown in perishable cured meat inoculated with *Clostridium botulinum*. *Food Technol.* **13**, 509.

Schmidt, C. F., Lechowich, R. V. and Folinazzo, J. F. 1961. Growth and toxin production by Type E *Clostridium botulinum* below 40°F. *J. Food Sci.* **26**, 626.

Segner, W. P., Schmidt, C. F. and Boltz, J. K. 1966. Effect of sodium chloride and pH on the outgrowth of spores of Type E *Clostridium botulinum* at optimal and suboptimal temperatures. *Appl. Microbiol.* **14**, 49.

Wagenaar, R. O. and Dack, G. M. 1958. Factors influencing growth and toxin production in cheese with spores of *Clostridium botulinum* Types A and B. I. Studies with surface ripened cheese Type I. *J. Dairy Sci.* **41**, 1182.

Ms. rec'd 12/14/67; revised 4/5/68; accepted 9/5/68.

This investigation was supported in part by the U. S. Department of the Interior, Bureau of Commercial Fisheries, Grant No. 14-17-0007-200.

Mich. Agr. Expt. Sta. Jour. Art. No. 4244.

Food Cosmet Toxic 6: 569-571 (1968)

550

μg/kg, which is 500 times as much as its concentration in the soil of old Moscow districts. Soil pollution depends on air pollution and may serve as an indicator of the overall pollution of the human environment with carcinogenic hydrocarbons.

Benzo[*a*]pyrene that has accumulated in the soil can penetrate into the vegetation. This was proved by a special study of plants gathered from the polluted territory.

Some strains of soil bacteria were isolated from the polluted soil and cultivated. All these cultures absorbed benzo[*a*]pyrene more or less actively. Some of them destroyed it, as was shown by the quantitative difference between the amount of hydrocarbon introduced into the nutrient medium and the amount remaining after 2–4 days of cultivation.

In further experiments the soil-bacteria cultures were added to samples of sterilized soil containing about 200 μg benzo[*a*]pyrene. After cultivation for 8 days 55–84% of the substance was destroyed.

The results show a kind of circulation of carcinogenic hydrocarbons in the human environment. They can fall from the air on to soil, can be destroyed by soil micro-organisms or pass into plants. This problem is of great interest and its study forms the basis of cancer prevention.

Occurrence of Nitrosamines in Foodstuffs for Human and Animal Consumption

F. ENDER and L. ČEH

Department of Biochemistry, The Veterinary College of Norway, Oslo 4, Norway

Since the discovery of the carcinogenic properties of various alkylnitrosamines (Magee & Barnes, *Br. J. Cancer* 1956, 10, 114; Druckrey *et al. Naturwissenschaften* 1961, 48, 134; Druckrey *et al. Acta Un. int. Cancr.* 1963, 19, 510), the question of the occurrence of various nitrosamines in nature has received great attention. Ender *et al.* (*Naturwissenschaften* 1964, 51, 637) reported the isolation and identification of *N*-nitrosodimethylamine in poisonous herring meal caused by the uncritical use of sodium nitrite as a preservative. The formation of *N*-nitrosodimethylamine in herring meal is caused by various methylamines which occur normally in marine fish. Dimethylamine, the most potent producer of *N*-nitrosodimethylamine, reacts with nitrite even at temperatures below 0°C (Ender *et al. Z. Tierphysiol. Tierernähr. Futtermittelk.* 1967, 22, 189), emphasizing the great risk of using nitrite as a preserving agent.

A preliminary study of the possible occurrence of nitrosamines in human food was started at our Institute in 1965. Such studies are difficult to accomplish because reliable methods for the determination of nitrosamines at extremely low levels are not at present available. Only a few reports about the occurrence of nitrosamines in food designed for human consumption have hitherto been published (Hermann, *Hoppe-Seyler's Z. physiol. Chem.* 1961, 326, 13; Laqueur *et al. J. natn. Cancer. Inst.* 1963, 31, 919; Marquardt & Hedler, *Arzneimittel-Forsch.* 1966, 16, 778).

We especially drew attention to the wide use of nitrate and nitrite in the food industry. Nitrate in fish and meat is readily converted to nitrite. The practice of dipping fish fillets in dilute sodium nitrite solutions must be considered extremely risky. The industrial curing method, which involves treatment of meat with brines containing nitrate or nitrite followed

by a smoking process needs to be considered. The detection of carcinogenic compounds, such as benzo[*a*]pyrene, in smoked food seems well established (Dungal, *J. Am. med. Ass.* 1961, 179, 789; Gorelova & Dikun, *Vop. Onkol.* 1958, 4, 417; Gorelova *et al. ibid.* 1960, 6, 39).

Dungal (*loc. cit.*) reported a wide occurrence of stomach cancer in Iceland, which he suggested was caused by the high consumption of smoked fish and meat. Though Dungal's report has been criticized, a re-evaluation of his paper seems pertinent in the light of the strange smoking procedure which has been used in rural districts in Iceland and in which the dried faeces and urine of sheep are used for smoke generation. Sheep dung contains detectable amounts of methylamines and low levels of nitrite.

Our preliminary studies have revealed that volatile nitrosamines are present in smoked fish and meat. Mushrooms also contain low levels of nitrosamines (Table 1). The quantity of nitrosamines in smoked fish and meat shows great variation (Table 1), the amount probably depending on the mode of the smoking procedure and the method of handling used. Precautions were taken in the isolation method used in these studies to avoid artefact-formation of nitrosamines. Recovery values for small levels of added *N*-nitrosodimethylamine amounted to about 70%.

The nitrosamines detected in mushrooms and smoked fish and meat products seem to be identical with *N*-nitrosodimethylamine, the amount of which was measured by the method of Daiber & Preussmann (*Z. analyt. Chem.* 1965, 206, 344) and more exactly by the sensitive

Table 1. *Levels of nitrosamine in foodstuffs*

Type of Food	Nitrosamine (μg/kg*)	No. of samples analysed
Fish		
Smoked herring	0·5–9·5	5
Kippers	0·5–2·4	4
do.	40	1
Smoked haddock (from Iceland)	15	1
Smoked mackerel	0·6	1
Meat		
Smoked sausage	0·8, 1·1, 2·4	3
Bacon	0·6, 1·2, 6·5	3
Smoked ham (from Iceland)	5·7	1
Mushrooms		
Polyporus ovinus	11·6	1
Boletus scaber	1·4	1
Amanita muscaria	30	1
Champignon	0·4, 5	2
Hydnum imbricatum	3, 15	2
Armillaria mellea	12	1
Lactarius trivialis	9·2	1
Russula emetica	10·2	1
A mixture of various edible mushrooms	14	1

*Determined by the hydrazine method.

hydrazine method elaborated in our own Institute (Ender, IV. Internationale Tagung der Weltgesellschaft für Buiatrik. 4–9 August 1966, Zürich). The identity with *N*-nitrosodimethylamine was based on methods described by Neurath & Doerk (*Chem. Ber.* 1964, **97**, 172) and Neurath *et al.* (*ibid.* 1964, **97**, 1631). The best proof was obtained with the first method, using thin-layer chromatography with silica-gel plates and chloroform as a solvent. Details of this study will be published elsewhere.

Experimental Studies on the Organotropic Effect of Nitrosamines in the Respiratory Tract

W. DONTENWILL

Forschungsinstitut, Verband d. Cigaretten-Industrie, Gazellenkamp 38, 2 Hamburg 54, Germany

In order to elucidate the reasons for the organotropic effect of nitrosamines in the respiratory tract of the Syrian golden hamster several investigations have been carried out. Polarographic determination of the elimination of diethylnitrosamine by various organs (trachea, lungs, liver and kidney) of this species (Barkemeyer & Elmenhorst, personal communication), like earlier investigations with dimethylnitrosamine in rats (Magee, in *Alkylierend wirkende Verbindungen.* First Conference on *N*-Nitroso-compounds and Lactones, Hamburg, 1963, p. 23), showed that nitrosamines are not present in higher concentration in the trachea and lungs of the hamster. The supposition by Herrold (*Proc. Am. Ass. Cancer Res.* 1964, **5**, 26) that greater elimination of nitrosamines via the respiratory tract is the reason for the organotropy could not be confirmed in the hamster.

Comparative studies on the effect of diethylnitrosamine in the transplanted trachea (Dontenwill & Rücker, *Z. Krebsforsch.* 1967, **69**, 270) showed quite clearly that this compound is converted from the 'transport form' into the 'effective form' (as defined by Druckrey & Raabe, *Klin. Wschr.* 1952, **30**, 882) by enzymic processes in the cells of the mucosa of the trachea. Papillomas occurred in the trachea implanted in the spleen or implanted subcutaneously in hamsters and in the trachea *in situ.* The organotropic carcinogenic effect is therefore not essentially dependent on the dose or the position of the organ in the circulation, but on organ-specific factors.

In subcutaneous injection studies in hamsters carried out in this laboratory* small amounts of diethylnitrosamine (4·6–9·3 mg/kg as the minimum dose) produced papillomas of the trachea in 10% of the animals. The organotropic effect of various nitrosamines was also demonstrated by the inhalation route using cigarettes impregnated with methanolic solutions of dimethyl-, diethyl- or butylmethylnitrosamine, nitrosopyrrolidine or nitrosopiperidine. With the exception of dimethylnitrosamine and nitrosopyrrolidine each of these nitrosamines also produced tumours in the trachea or in the nose cavity. No effect was seen in similar inhalation tests in rats. Control hamsters and rats inhaling fresh air showed no spontaneous papillomas of the trachea.

* Since this communication was delivered we have found that nitrosopyrrolidine and butylmethylnitrosamine show organotropic carcinogenic effects in the lungs of hamsters following subcutaneous injections. This work has revealed important species differences in the organotropic effect of nitrosamines given by the subcutaneous route.

Z. Lebensmittel-Untersuch. u. -Forsch., Band 145, Heft 3, 1971

551

Conditions and Chemical Reaction Mechanisms by which Nitrosamines may be Formed in Biological Products with Reference to their Possible Occurrence in Food Products*

F. Ender and L. Čeh**

Department of Biochemistry, Veterinary College of Norway, Oslo

Received Juli 1970

Summary. Experiments have shown that formation of volatile nitrosamines occurs when nitrite and alkylamines are present in the same media. Heterocyclic amines such as pyrrolidine and piperidine contribute to nitrosamine formation as well. Precursor substances leading to nitrosamine formation are, besides various biogenic amines, certain amino acids and proteins from meat and fish if nitrite is present in sufficient quantities and high temperature treatment is used. In order to prevent the formed nitrosamines from escaping at high temperatures a protecting colloid must be present. The amount and type of nitrosamine formed under various experimental conditions have been studied by quantitative and qualitative methods. In cases where large amounts of nitrosamines were formed, the toxicity of these was demonstrated with mink as test animals.

Zusammenfassung. Untersuchungen haben ergeben, daß die Bildung flüchtiger Nitrosamine auftritt, wenn Nitrite und Alkylamine in demselben Stoff vorhanden sind. Ebenso tragen heterozyklische Amine wie Pyrrolidin und Piperidin zur Bildung von Nitrosaminen bei. Vorläufer der Nitrosaminbildung sind neben verschiedenen biogenischen Aminen bestimmte Aminosäuren und Proteine aus Fleisch und Fisch, vorausgesetzt Nitrit ist in ausreichender Menge vorhanden und vorausgesetzt, daß hohe Erhitzungstemperaturen angewendet wurden. Um das Entweichen von gebildeten Nitrosaminen bei hohen Temperaturen zu vermeiden, muß eine Schutzhülle verwendet werden. Mit Hilfe quantitativer und qualitativer Methoden wurden die Menge und die Art der unter verschieden experimentellen Bedingungen auftretenden Nitrosaminen untersucht. Bei Auftreten stärkerer Mengen an Nitrosaminen wurde deren Toxicität an Versuchstieren nachgewiesen.

Introduction

The present paper is intended as an approach to the problem of the biogenesis of various alkyl- and other nitrosamines in nature. The occurrence and formation of nitrosamines in nature have received attention from specialists in cancer research since the first discovery of the hepatotoxic effect of dimethylnitrosamine [1] and the demonstration of a pronounced carcinogenic effect in various organs of rats by administration of a great number of different dialkylnitrosamines [2].

Since our first report in 1964 [3] on the identification and isolation of N-nitrosodimethylamine (DMNA) in poisonous herring meal, caused by uncritical use of $NaNO_2$ as a preservative agent, studies have been carried out on the biogenesis of various volatile nitrosamines as well as on the conditions under which the use of nitrite can lead to formation of nitrosamines. In a preliminary publication [4] we studied the problem of how far nitrosamines were formed by using ordinary smoking methods and nitrite as food additive, since such methods have been highly recommended by nutritionists in the food industry. Formation of DMNA has also been studied in model experiments in which trimethylamine-oxide, trimethyl-, dimethyl-, and monomethylamine were brought into reaction with $NaNO_2$ [5].

The present paper deals with studies of the conditions under which biogenic amines, various amino acids, and proteins by reaction with nitrite in small and large quantities may contribute to formation of nitrosamines. Of special interest is the fact that nitrosamine formation based on interaction between $NaNO_2$ and various biogenic amines increases greatly at high temperatures. The peculiar pattern of nitrosamine formation at freezing temperatures is of special interest as it must be caused by a concentration of nitrite and the amine in the liquid phase when the water crystallizes out. This finding probably explains the artificial production of methylbutyl nitrosamine in cigarette smoke concentrate by the use of cold traps at temperatures of about —80° C [6].

* Revised version of lecture given at a WHO meeting on nitrosamines in London, Oct. 1969.

** The authors wish to express their most sincere thanks to Docent A. Helgebostad for his valuable help by carrying out toxicological studies on mink.

Experimental

Formation of Nitrosamines[1] by Nitrosation

Alkylamines as Precursor Substances. Alkylnitrosamines in addition to the heterocyclic N-nitrosopyrrolidine and N-nitrosopiperidine are produced when the corresponding amines are brought into contact with $NaNO_2$ in watery solution under physiological conditions of pH and temperature. Of interest is the fact that nitrosamine formation at low and ordinary temperatures increases practically linearily with time passes. Maximum formation of dimethylnitrosamine occurs at a pH of about 3.5 (Table 1).

Table 1. *Formation of DMNA during 24 hrs at 22° C by 1 mmol Dimethylamine HCl and 1 mmol $NaNO_2$ in 100 ml H_2O at different pH values. Levels of DMNA were measured spectrophotometrically at λ max 226 nm*

pH	DMNA mg	Buffer used
1.0	0.026	KCl + HCl
2.0	0.040	
3.0	0.468	K-H-phtalate
3.5	0.523	
4.0	0.389	
4.5	0.173	
5.0	0.060	
5.5	0.023	
6.0	0.007	
7.0	0.001	phosphate

Table 2. *Production of nitrosamines from secondary amines and $NaNO_2$ in watery solution at pH 6.0. Abbreviations: S = Spectrophotometric method 228—233 nm; DP = Daiber and Preussmann method; mmol $NaNO_2$: 1 = 69 mg, 2 = 138 mg, 5 = 345 mg*

Nitrosamine	Sec. amine mg	mg nitrosamine produced in 100 ml solution; mmol amine/mmol $NaNO_2$											
		1/1		1/5		1/2		1/2		1/2		1/2	
		storage time in days											
		10		10		10		30		10		30	
		storage temperatures											
		+ 22 to + 24° C								— 18° C			
		methods											
		S	DP	S	DP	S	DP	S	DP	S	DP	S	DP
Diethyl-	73.1	0.016	0.019	0.26	0.28	0.044	0.047	0.11	0.12	0.54	0.59	1.61	1.85
Dipropyl-	101.2	0.018	0.018	0.33	0.35	0.055	0.057	0.18	0.19	0.60	0.66	2.15	2.36
Diisopropyl-	101.2	0.007		0.046	0.046	0.011	0.01	0.018	0.013	0.034	0.041	0.13	0.14
Dibutyl-	129.2	0.021	0.021	0.35	0.35	0.052	0.052	0.13	0.13	0.37	0.40	1.40	1.57
Diisobutyl-	129.2	0.046	0.042	0.73	0.72	0.10	0.092	0.33	0.35	0.54	0.51	2.20	2.26
Diamyl-	157.3	0.042		0.36	0.23	0.071	0.038	0.15	0.077			0.11	0.11
Diallyl-	97.2	0.39	0.39	10.9	7.32	1.49	1.36	3.16	2.49	5.00	5.16	15.23	14.28
N-nitroso-pyrrolidine	71.1	0.035	0.028	0.47	0.37	0.11	0.074	0.24	0.17	1.31	1.14	4.66	3.90
N-nitroso-piperidine	85.2	0.025	0.016	0.41	0.27	0.11	0.076	0.29	0.20	1.19	0.85	3.66	3.05

1 DMN[illegible] Dimethylnitrosamine, DBNA: Dibutylnitrosamine, DENA: Diethylnitrosa[illegible] DIBNA: Diisobutylnitrosamine, DPNA: Dipropylnitrosamine, DANA: Diamylnitrosa[illegible] [illegible]PNA: N-nitrosopyrrolidinitrosamine, DAlNA: Diallylnitrosamine.

Tables 2,3 survey nitrosamine production in model experiments and show the influence of variations in concentrations of the corresponding amines and nitrite and in temperature and pH values. The same tables also reveal that nitrosamine production will occur at physiological pH values and at temperatures from —18° C to +22° C. At higher temperatures from 50° C upwards, formation of nitrosamines increases markedly.

Table 3. *Production of nitrosamines from secondary amines and $NaNO_2$ in watery solution at pH 6.0, 6.5 and 7.0. Abbreviations: S = Spectrophotometric method 228—233 nm; DP = Daiber and Preussmann method; mmol $NaNO_2$: 1 = 69 mg, 2 = 138 mg, 5 = 345 mg. λ = spectralabsorption maxima in H_2O in the 230 nm and the 330 nm range, λ max (nm), boiled = boiled under reflux*

Nitrosamine	Sec. amine mg	λmax-230nm range / λmax-330nm range	mg nitrosamine produced in 100 ml solution; mmol amine/mmol $NaNO_2$							
			1/1 boiled 15 min	1/5	1/2		1/2		1/2	
			storage time in days							
					20		40		30	
			storage temperature							
			pH 6.0		+ 22 to 25° C pH 6.5				25° C pH 7.0	
			methods							
			S	S	S	DP	S	DP	S	DP
Dimethyl-	45.1	226/331								
Diethyl-	73.1	228/336	0.020	0.32	0.043	0.045	0.074	0.075	0.012	0.012
Dipropyl-	101.2	230/337	0.12	1.63	0.057	0.070	0.11	0.10	0.015	0.019
Diisopropyl-	101.2	230/345	0.020	0.23	0.008	0.010	0.012	0.018	0.0085	0.0085
Dibutyl-	129.2	229/330	0.084	1.23	0.050	0.054	0.091	0.10	0.029	0.038
Diisobutyl-	129.2	233/339	0.25	3.44	0.089	0.083	0.21	0.20	0.034	0.035
Diamyl-	157.3	230/338	0.029	0.57	0.099	0.059	0.14	0.074	0.073	0.033
Diallyl-	97.2	230/—	0.51	9.55	0.70	0.63	1.67	1.45	0.28	0.26
N-nitrosopyrrolidine	71.1	228/331	0.040	0.38	0.095	0.069	0.16	0.12	0.040	0.036
N-nitrosopiperidine	85.2	230/336	0.031	0.35	0.11	0.072	0.22	0.17	0.040	0.24

Amino Acids as Precursor Substances. A study of how organic substances with reactive amino groups other than alkylamines and other amines may participate in nitrosamine formation when treated analogously with nitrite and rice starch gave the interesting result that various amino acids under special reaction conditions produced relatively large amounts of nitrosamines when exposed in a dry environment to high temperature treatment. The heating process leads to a decarboxylation of the amino acid by which the corresponding amine is formed. This reacts in turn with nitrite to form the nitrosamine. Formation of great amounts of various nitrosamines in these products depends largely on the presence of a protecting colloid, such as starch, which prevents the formed nitrosamines from evaporation at temperatures from 100° C to 170° C. The use of high temperatures generally leads to production of large amounts of nitrosamines. No nitrosamine production occurs, however, if nitrite is not present.

Amino acids such as glycine, methylglycine (sarcosine), and valine produced mainly DMNA. L-alanine produced DMNA and DENA, whereas L-proline gave N-nitrosopyrrolidine. Small amounts of methyl-ethyl-nitrosamine (MENA)[2] were also formed by L-alanine. Whether other amino acids form nitrosamines when treated analogously with nitrite is at present not settled. Experiments in which arginine, histidine, and tryptophan were treated in a similar way gave no volatile

2 The true nature of this nitrosamine was proved after we receiv[illegible] an authentic sample of MENA from Dr. R. Preussmann of „Die Forschergruppe für Präve[illegible]-Medizin", Freiburg, Ger[illegible], W[illegible] grat[illegible] Dr [illegible]ssmann for this delivery.

nitrosamine formation with the investigative methods used. The yield of formed nitrosamines decreases markedly if the heating temperature is lowered (Table 5). A reduction of the reacting temperature from 170° C to 130° C reduced the dimethylnitrosamine production from glycine to nearly one-hundredth.

Table 4. *Production of nitrosamines from primary, secondary and tertiary amines and $NaNO_2$ in 100 ml water under different conditions of the reaction. Abbreviations: A/N = mmol amine/mmol $NaNO_2$; S = Spectrophotometric method, DP = Daiber and Preussmann method*

Amines	A/N	condition of the reaction	mg nitrosamine/100 ml method S	DP
Monomethyl-				
	2/5	boiled under reflux/30 min/pH 6.0		0.003
		autoclaved 120° C/30 min/pH 6.0		0.009
		stored 45 days/— 18° C/pH6.0		0.018
Monoethyl-				
	2/5	boiled under reflux/30 min/pH 6.0		0.002
		autoclaved 120° C/30 min/pH 6.0		0.008
		stored 45 days/— 18° C/pH 6.0		0.024
Diethyl-				
	1/2	thermostate oven 50° C/1 hour/pH 6.0		0.008
	2/1			0.007
	1/2	boiled under reflux/1 hour/pH 6.0		0.15
	2/1			0.13
	1/2	autoclaved/120° C/1 hour/pH 6.0		0.45
	2/1			0.31
Dipropyl-				
	1/2	thermostate oven 50° C/1 hour/pH 6.0		0.006
	2/1			0.006
	1/2	boiled under reflux/1 hour/pH 6.0		0.50
	2/1			0.32
	1/2	autoclaved/120° C 1 hour/pH 6.0		0.59
	2/1			0.33
Trimethyl-				
	2/5	boiled under reflux/30 min pH 6.0	0.023	0.029
		pH 5.0	0.123	0.130
		pH 4.0	0.440	0.450
		autoclaved 120° C/30 min pH 6.0	0.440	0.430
		stored 45 days/— 18° C pH 6.0		0.013
Triethyl-				
	2/5	boiled under reflux/30 min pH 6.0		0.023
		pH 5.0		0.075
		pH 4.0		0.128
		pH 3.0		0.066
		autoclaved 120° C/30 min pH 6.0		0.085
		stored 45 days/— 18° C pH 6.0		0.017

Proteins as Precursor Substances. Of nutritional interest is the fact that great amounts of volatile alkylnitrosamines can also be formed when meat and fresh-water fish not containing methylamines such as powan *(Coregonus lavaretus)* are treated with nitrite and starch in a similar way. It also seems likely that proteins from wheat and casein may react with nitrite under similar conditions, as seen from the preliminary investigations, though the actual nitrosamine produced is very low. The precursor substances must in these cases be nitrite and special amino acids which are released from proteins by destruction at high temperatures.

Table 5. *Production of nitrosamines from various amino acids and $NaNO_2$ capsuled in rice starch. (150 ml H_2O), DP = Daiber and Preussmann method*

Amino acid g	$NaNO_2$ g	Rice starch g	pH	mg nitrosamines produced per g amino acid, DP method heating temperatures (2 hrs), 130° C	150° C	170° C	Thin layer chrom.
Glycine	0.5	20	6.0	0.014	0.28	1.3	DMNA
Sarcosine	0.78	10	6.2	2.40	20.00	81.00	DMNA
L-Proline	0.6	10	6.2	3.47	18.7	72.50	Nitrosopyrrolidine*
L-Alanine	0.4	10	6.6	0.013	0.079	0.18	DMNA, DENA, MENA
L-Valine	0.4	10	6.0	0.054			DENA, DMNA, DBNA

* Measured by the spectrophotometric method at 228 nm.

Boiling of fish and meat leads to formation of glutineous substances such as fish solubles and gelatine (from collagen) which to some extent may prevent the formed nitrosamines from escaping. Heating to high temperatures causes denaturation combined with protein break-down and destruction of amino acids. Maximum nitrosamine production, predominantly DMNA, formed by these reactions was at a temperature of 170° C in a dry environment. The formation of alkylnitrosamines by interaction between amino acids and proteins, on one hand, and nitrite, on the other, was proved by quantitative and qualitative methods (Tables 5 and 6).

Table 6. *Production of nitrosamines in meat and fish treated with $NaNO_2$ under addition of rice starch (150 g), 1 l H_2O; DP = Daiber and Preussmann, Hyd. = Hydrazine method*

Material (1 kg)	$NaNO_2$ g	pH	mg nitrosamines produced per kg meat and fish, Heating temperatures 130° C DP	130° C Hyd.	150° C DP	150° C Hyd.	170° C DP	170° C Hyd.	Thin layer chrom.
Meat	25	5.6	3.64	3.08	13.60	11.50	15.60	13.30	DMNA
	12.5	5.6	1.90	1.72	6.60	5.48	7.45	6.86	DMNA
	5	5.6	0.62	0.42	0.92	0.64	2.0	1.01	DMNA
	50	5.6	0.52	0.65 autoclaved for 2 hrs at 120° C with water					DMNA
Fish	25	6.5	4.16	3.7	9.00		12.30	8.7	DMNA
	12.5	6.5	2.56		4.48		11.00	7.6	DMNA
	5	6.5	1.68		2.68		4.50	3.1	DMNA

Materials

Production of Nitrosamines from Various Amines

All amines provided by Fluka; formation of nitrosamines was carried out in solutions buffered with sodium acetate and acetic acid; concentration of each nitrosamine was made by repeated distillations in alkaline solutions (3 n-NaOH). The distillates were freed of non-reacting amines by acid distillation, except for nitrosopyrrolidine which was destructed up to 45% by distillation in H_2SO_4 solution. The other nitrosamines studied could be distilled quantitatively from H_2SO_4 solutions under atmospheric pressure, diamylnitrosamine after addition of 20% NaCl.

Elimination of disturbing amines (Tables 2–4) was not strictly necessary for quantitative determination of nitrosamines by the spectrophotometric method, except for nitrosopiperidine which absorbs at 260 nm. For the other nitrosamines neutralization was sufficient to avoid disturbancies in absorption at 228 nm.

Production of Nitrosamines from Amino Acids. Amino acids purchased from Fluka, Merck, and BDH; rice starch from BDH.

To a watery solution of various amino acids were added $NaNO_2$ and starch (Table 5). The suspension heated at 90° C in a boiling waterbath for 45 min, then evaporated to dryness at 40° C, ground in a mortar and then heated in a thermostate oven for 2 hrs at various temperatures. The resulting product was distilled quantitatively in vacuum from 3 n-NaOH, and the final distillate was tested quantitatively by 1. the Daiber and Preussmann method [7], 2. the hydrazine method, and 3. the spectrophotometric method. Identification of the nitrosamines formed was performed by thin layer chromatography using the Griess Hosway's reagent [8]. Toxicological studies with mink were carried out as described below.

Production of Nitrosamines from Meat and Fish. Watery suspensions of 1 kg ground meat or eviscerated fish to which $NaNO_2$ and rice starch were added in amounts described (Table 6) were heated on a hotplate at 90° C for 45 min, then evaporated to dryness at low temperature (40° C) and finally heated in a thermostate oven at temperatures between 130–170° C. The oven dried products were after grinding examined as described above.

Determination of Nitrosamines by Colorimetric Methods

Concentration of the Nitrosamine. The dried reaction products described above were first suspended in alkaline solution (3 n-NaOH) from which the nitrosamine was distilled in vacuum. The distillate was then concentrated by repeated distillations after addition of NaOH to give a 3 n-NaOH. The volume of each distillate should by this procedure correspond to half the previous one, except for DANA and nitrosopyrrolidine where a 75% distillate was taken.

Purification. Renewed distillation in a H_2SO_4 milieu, pH 1.5–2, was used in order to remove interfering substances such as bases, including aromatic amines. Further purification with activated charcoal was undertaken in cases where disturbing substances such as pyrrole derivatives were present. The nitrosamine was then regained quantitatively by steam distillation of the watery suspension of the carbon, which left the bulk of the impurities in the charcoal. The last distillate was used for determination and identification of the actual nitrosamine. Determination was generally carried out by means of one or two of the following methods:

Under practical analytical conditions it is possible to determine far lower levels by the hydrazine method [2] than by the Daiber and Preussmann [7] procedure, as the concentration technique is easier to complete and the specificity is greater. For analysis in the range of 0.001—0.01 mg/kg the hydrazine method should be preferred. A fairly good correspondence between these three methods was obtained in this study.

For higher nitrosamines the spectrophotometric method [3] was the only one which gave quantitative values with purified solutions. Reliable values were obtained by the Daiber and Preussmann method for lower nitrosamines up to dibutylnitrosamine. This method should, however, not be used for nitrosopyrrolidine and -piperidine as the yield corresponds only to 70—75%. The hydrazine method gives quantitative values for DMNA, DENA, and MENA, but only about 60% for higher alkylnitrosamines. The spectrophotometric method by which UV absorption at 230 or 330 nm is used for quantitative determination is only possible for measuring practically pure nitrosamine solutions not containing interferring substances which absorb in the 200—400 nm range.

The Hydrazine Method[2]

Not more than 10 µg DMNA transfer to a 100 ml Erlenmeyer flask, add HCl and dist. water to obtain 100 ml of a 0.2 n-HCl[c]) solution, simultaneously 1 g granulated zinc[f]); stand over night (15—20 hrs) at room temperature for reduction. Filter through a glass filter funnel[g]) into a 100 ml-beaker (wide type); pH exactly 2.0 (with n-HCl), if necessary, re-adjusted with n-NaOH. Place the beaker on a hotplate[h]), evaporate the solution at 90–95° C until 3–5 ml is left. During the last period of evaporation lower the temperature by placing an asbestos sheet between the beaker and the hotplate; cool to room temperature and add 5 ml para-dimethyl-amino-benzaldehyde[i]) solution, let stand for 10 min before dilution to 10 ml. The yellow color from the formed aldazine (typical absorption spectrum with max. at 458 nm) is measured in a spectrophotometer[j]) against a blank (98 ml dist. water + 2 ml 10 n-HCl, plus 1 g of pure zinc treated in the same way as the sample). The standard curve is prepared by treating authentic DMNA in exactly the same way as the sample. A suitable standard curve may also be prepared with pure symmetric or asymmetric dimethylhydrazine, but we prefer to use a standard curve based on DMNA[k]) reduced with zinc and hydrochloric acid as described above. Recovery tests are carried out in most cases for control and especially in cases where a new foodstuff is being analysed, if the DMNA content is extremely low. The analysis should be carried out as soon as possible after reduction of DMNA to the hydrazine derivative. The method for hydrazine determination is based on procedures worked out by Watt and Crisp [9].

[2] A detailed description of the hydrazine method will be published elsewhere.

The Daiber and Preussmann Method Used for Control

Pipette 1 ml of the final purified distillate into a Coleman cuvette[l]), add 0.5 ml of a 0.5% Na_2CO_3 solution[m]), place the cuvettes under a UV-lamp[n]) for 15 min at a distance of 30 cm from the light source. 2 ml of Reagent A (sulphanilic acid)[o]) and 2 ml of Reagent B (alpha-naphtylamine)[p] are added subsequently; mix the solution, let stand in darkness for 15 min, the red color is measured in a spectrophotometer at 520 nm. A blank is simultaneously prepared in the same way.

Reagents and Apparatuses Used for the Analysis. a) Phosphotungstic acid reagent: 100 g phosphotungstic acid (Baker's analysed reagent) and 50 g conc. sulphuric acid are diluted to 1 l with distilled water. — b) Sulphuric acid (Merck pro anal.). — c) Distilled water. NB! Fresh distilled water *free of traces of* NO_2. — d) Sodium hydroxide (Elektrokemiska AB). — e) Hydrochloric acid (Merck pro anal.). — f) Zinc (Matheson, Coleman & Bell: Reagent A. C. S. CB 825, Z 10, 20 mesh). (Suitable grain size is very important.). — g) Glass filter funnel (Jenaglass 3 Gl). — h) Hotplate (Type E 71 R/XIV 220 V, 12 amp.). — i) 2 g para-dimethyl-amino-benzaldehyde (Merck) and 10 ml conc. hydrochloric acid are diluted to 100 ml with 96% ethanol[q]). — j) Beckman DB spectrophotometer. The use of a recorder is necessary, as the shape of the absorption curve may indicate possible contamination which may disturbe the exact spectral measurements. — k) Dimethylnitrosamine (BDH). — l) Coleman cuvettes, 1.8 cm wide, 5 cm high. — m) Na_2CO_3 (Merck pro anal.). — n) UV-lamp (Hanau Q 600). — o) 1% sulphanilic acid sol. in 30% CH_3COOH. — p) 0.1% alpha-naphtylamine sol. in 30% CH_3COOH. — q) Rectified 96% ethanol. — r) Activated charcoal granules, 1.5 mm (Merck 2514). — Ground to smaller particles, but not to powder.

Identification of Nitrosamines. Thin layer chromatographic measurements with silica gel plates, using either Griess Hosway's reagent or $PdCl_2$ reagent after irradiation with UV-light as described by Preussmann *et al.* [8, 10]. Methylene chloride was used for extraction of nitrosamines from purified distillates and identical pure nitrosamines were used for controls. Known derivatives of nitrosamines have also, in some cases, been used for identification by a method described by Neurath and Doerk [11]. A further proof of the presence of nitrosamines was based on toxicological studies with mink as test animals, as described in the following section.

Toxicological Studies. The toxic effect of nitrosamines was studied with mink as test animals. DMNA prepared from glycine, sarcosine, meat, and fish[4] induced lethal effect in mink after single doses given via stomach tube.

Dose of DMNA mg/kg live weight	Prepared from	Time within death occurred Days	Number of mink tested
12.8—29	glycine	4—2	2
16.5—21	sarcosine	7—2	2
16.5—17.5	meat	2—1	2
17	fish	4	1

550 mg nitrosopyrrolidine prepared form L-proline caused death 1 hr after application in one animal. Abnormally high liver-fat content was found in 2 mink tested after lethal doses of DMNA and nitrosopyrrolidine were given. Macroscopic signs of fatty liver degeneration were observed in all animals. Icterus was seen in animals treated with DMNA prepared from meat, glycine and sarcosine.

Results and Discussion

Nitrosamine formation has been demonstrated for a large number of alkylamines, other amines, and various amino acids, such as glycine, methyl glycine or sarcosine, L-alanine, L-valine, and L-proline when treated with nitrite and starch as described before (see Tables 2–5). Nitrosamine formation based on interaction between proteins from fresh meat and fish and nitrite was studied by analogous procedures as described for the amino acids (Table 6).

The present paper discusses nitrosamine formation in model experiments when $NaNO_2$ in great quantities is brought into reaction with alkylamines, amino acids, and proteins from meat and fresh water fish, in this case powan. It is shown that

[4] DMNA prepared from fish and used in this study was purified by adsorption on charcoal as described in the section dealing with analytical procedures.

arge amounts of carcinogenic nitrosamines, especially DMNA, can be produced rom amino acids, fish, and meat under special reaction conditions in the presence f uncritically high levels of $NaNO_2$ and starch. Other nitrosamines are also rmed as shown in the tables.

That large amounts of nitrosamines, especially dimethylnitrosamine, were formed rom meat and fish protein is revealed from the fact that 1 kg raw meat and 1 kg raw sh produced 16 and 12 mg DMNA, respectively, under the reaction conditions used.

Toxicological studies with mink as test animals revealed lethal effects and ypical liver lesions and fatty liver degeneration — characterized by high fat content — within a few days after single doses of DMNA corresponding to 13 and 17 mg ere given. The observed toxic effect was identical in all trials whether DMNA was erived from amino acids, fish, or meat. In cases where N-nitrosopyrrolidine, formed rom proline treatment, was administered to mink, a single dose of about 600 mg as necessary to produce lethal effects. Pathological-anatomical liver symptoms ue to DMNA poisoning in fur-bearing animals, cattle, and sheep have been de-cribed by Koppang [12, 13], and Koppang and Helgebostad [14].

The wide occurrence of biogenic amines in nature [15] indicates the importance f the question of how far the presence of nitrite in biological products may lead o formation of nitrosamines due to the ease with which amines, especially secondary mines, but also, to a smaller extent, tertiary, and primary amines react with sodium itrite even at physiological temperatures and pH conditions. Tertiary amines pro-uced higher levels than the corresponding primary amines.

Formation of large quantities of nitrosamines in biological products depends ainly on the following factors and conditions: (1) the presence of nitrite and e actual alkylamine, (2) pH and temperature, (3) time of reaction, (4) the presence a protecting colloid — such as starch or other glutineous substances — which pre-nts the formed nitrosamines from evaporation at high temperatures.

A preliminary study has revealed that most alkylamines form nitrosamines when ought into contact with $NaNO_2$. The occurrence of traces of nitrosamines in edible d non-edible mushrooms as verified by Ender and Čeh [4] is caused by nitrite and xylamines found in these species.

The fact that nitrate in biological products, i.e. meat, fish, and plant products, converted to nitrite suggests that suspicion must also be directed against nitrate a possible potent nitrosamine producer in the presence of one or another alkyl-nine.

The formation of nitrite and nitrate in biological products by microorganisms ould also be considered in this connection. Though the occurrence of nitrite in rious food products is generally low and seldom greater than 60 mg/kg, its presence a potent nitrosamine former must be regarded. Of interest in this connection is paper by Sander [16] dealing with the question: can nitrite in human diet be a use of carcinogenesis through formation of nitrosamines? Sander has presented list (Table 7) showing the wide variation of nitrite in various groups of food. latively high levels of nitrite were found in smoked fish, sausages, and ham. Similar vels of nitrite in food have been found in our laboratory.

We have intentionally used high concentrations of $NaNO_2$ which are neither countered nor legal in the food industry. The production of high levels of rosamines must therefore be regarded as a typical artefact. The intention with ing uncritically high levels of nitrite was to study the conditions under which *maxi-um production of nitrosamines* occurs, especially in those experiments in which sh and meat have been treated with sodium nitrite and starch at high tempera-ures. This was necessary as the first step towards elucidating the pattern and ode of nitrosamine formation in biological products.

Table 7. *Presence of nitrite in groups of foods (quoted from Sander [16]); a = average level; l = lowest level; h = highest level; N = Number of samples*

Groups of foods	mg/kg nitrite a	l	h	N
Milk (incl. condensed milk)	0	0	0	5
Fresh meat	0	0	0	3
Bread and baked products	0.1	0	0.5	8
Flour	0.5	0	1	3
Vegetables, fruit (also canned products)	0.2	0	0.7	15
Frozen vegetables	1.5	0.6	4.0	6
Fish, fresh and canned	0.9	0	1.9	12
Smoked fish	9.7	2.3	14.5	3
Cheese	2.1	0.3	8.1	22
Sausages and ham	18.5	0.5	61.5	80

As mentioned [4] the use of nitrite in the meat and fish industry may generally give rise to small amounts of nitrosamines, from about 0.5 up to 9.5 µg/kg. The levels were generally lower in fish products than in meat products.

Consideration should also be given to the fact that smoking methods commonly employed in the food industry may also contribute to nitrosamine formation. The practical use of $NaNO_2$ for preservation of meat products in Norway presupposes — as fixed by law — that $NaNO_2$ is only used in combination with salt brines where the actual $NaNO_2$ concentration is limited to a maximum of 0.6% of the total quantity of NaCl in the brine.

Further studies on nitrosamine formation in sterilization processes by canning and the modern roasting, broiling, and grilling treatment of fish and meat should be carried out to elucidate whether such treatment of food can lead to formation of larger amounts of nitrosamines, especially when high temperature treatment is used. Treatment in autoclave leads to increased nitrosamine production from methylamines (Table 4). Nitrosamine contamination of meat and fish due to the use of firing and smoking gases, a common practice in the food industry, is also possible [4]. Assumption of nitrosamine formation is reasonable since firing gases and smoke used for preservation of meat and fish contain traces of nitrogen oxide. The detection of methylamine in dry-cured smoked ham [17] has been considered to be unique as raw meat does not contain measurable quantities of methylamines. If this is correct, it gives further support to the nitrosamine-forming theory. The fact that the use of firing gases in the herring meal industry, as stressed in previous papers, leads to higher levels of nitrosamines may possibly be explained by the high temperatures of these gases, up to 900° C.

Studies should be carried out to ascertain the maximum nitrosamine production under the various techniques used by the food processing industry as well as in ordinary housekeeping. In a further work we have decided to study formation of nitrosamines in firing gases from the petrol industry as it is likely to suppose that traces may occur in exhaust gases from motor vehicles as well as in waste gases from the coal and gas industry.

Literatur

1. Magee, P. N., Barnes, J. M.: Brit. J. Cancer 10, 114 (1956).
2. Druckrey, H., Preussmann, R., Ivankovic, S., Schmähl, D.: Z. Krebsforsch. 69, 103 (1967).
3. Ender, F., Havre, G. N., Helgebostad, A., Koppang, N., Madsen, R., Čeh, L.: Naturwissenschaften 51, 637 (1964).
4. Ender, F., Čeh, L.: Alkylierend wirkende Verbindungen. 2nd Conference on Tobacco Research, Salzburg 1967, 83.

5. Ender, F., Havre, G. N., Madsen, R., Čeh, L., Helgebostad, A.: Z. Tierphysiol., Tierernäh. u. Futtermittelk. 22, 181 (1967).
6. Neurath, G.: Alkylierend wirkende Verbindungen. 2nd Conference on Tobacco Research, Freiburg 1967, 95.
7. Daiber, D., Preussmann, R.: Z. Anal. Chem. 206, 344 (1964).
8. Preussmann, R., Neurath, G., Wulf-Lorentzen, G., Daiber, D., Hengy, H.: Z. Anal. Chem. 202, 187 (1964b).
9. Watt, S. W., Crisp, J. D.: Anal. Chem. 24, 2006 (1952).
10. Preussmann, R., Daiber, D., Hengy, H.: Nature 201, 502 (1964).
11. Neurath, G., Doerk, E.: Chem. Ber. 97, 172 (1964b).
12. Koppang, N.: Nord. Veterinärmed. 16, 305 (1964).
13. Koppang, N.: Nord. Veterinärmed. 18, 205 (1966).
14. Koppang, N., Helgebostad, A.: Nord. Veterinärmed. 18, 210 (1966).
15. Guggenheim, M.: Die biogenen Amine. Basel: Karger 1951.
16. Sander, J.: Arch. Hyg. Bakteriol. 151, 22 (1967).
17. Landmann, W. A., Batzer, O. F.: J. Agr. Food Chem. 14, 210 (1966).

Prof. F. Ender
Department of Biochemistry
Veterinary College of Norway
Postboks 8146, Oslo dep.
Oslo 1 (Norway)

Nichtenzymatische Bräunung

I. Mitteilung

Reaktionen aliphatischer Carbonylderivate mit Aminen in Modellsystemen

G. Janíček und J. Pokorný

Mitteilung aus dem Institut für Lebensmittelchemie der Chemischtechnologischen Hochschule zu Prag (ČSSR)

Eingegangen am 20. Mai 1970

Non-Enzymatic Browning

I. Reactions of Aliphatic Carbonyl Derivatives with Amines in Model Systems

Summary. Carbonyl derivatives produced by lipid oxidation, e. g. aldehydes and hydroxyketones, can react with free amino groups with formation of various coloured condensation products. The reaction is rather slow at room temperature, but it is accelerated by moderate increase of temperature so that it may be finished within one hour. The main products formed in the beginning of the reaction have the composition corresponding to imines. In presence of excess amine groups, these products probably predominate even after longer reaction times. In the case of media where aldehydes are present in excess, and, particularly, in acid media, compounds containing two or even more aldehydic radicals for each amino group are formed by further aldolization of primary reaction products. In the case of hydroxy ketones, no reactions analogous to aldolization take place, on the contrary, one hydroxyketo group can bind two amino derivatives.

Zusammenfassung. Die durch die Autoxydation der Fette und anderer Lipide entstandenen Carbonylderivate (Aldehyde und Hydroxyketone) können mit freien Aminogruppen unter der Bildung verschiedener gefärbter Kondensationsprodukte reagieren. Die Reaktion verläuft langsam bei Zimmertemperatur, sie ist jedoch durch mäßiges Erhitzen so beschleunigt, daß sie schon binnen 1 Std beendet werden kann. Am Anfang bilden sich meistens Produkte, deren Zusammensetzung der Anwesenheit von Iminogruppen entspricht. In einem an Aminogruppen reichen Milieu überwiegen diese Produkte auch nach einer längeren Einwirkung. In aldehydreichen und namentlich in sauren Milieus werden durch eine nachfolgende Aldolisierung die zwei oder mehr Aldehydradikale je eine Aminogruppe enthaltenden Produkte gebildet. Im Falle von Hydroxyketonen verlaufen keine der Aldolisierung analogische Reaktionen, im Gegensatz kann sich eine Hydroxyketogruppe mit zwei Aminogruppen binden.

Einleitung

Die nichtenzymatische Bräunung der Lebensmittel ist hauptsächlich verursacht durch die sog. Maillard-Reaktion [1], d. h. durch einen Komplex von Reaktionen reduzierender Zucker mit freien Aminogruppen der Eiweißstoffe, Peptide, Aminosäuren und verschiedenen Aminoderiva-

Ein neues Alkaloid aus Catharanthus roseus G. Don

Über 50 Alkaloide sind bisher aus *Catharanthus roseus* G. Don. (*Vinca rosea* L.) insbesondere durch den Arbeitskreis von SVOBODA[1]) isoliert worden. Eine Anzahl von ihnen konnte in den letzten Jahren strukturell aufgeklärt werden. Besondere Bedeutung kommt den vier pharmakologisch hochaktiven dimeren Indolalkaloiden zu, die eine beträchtliche neolytische Aktivität zeigen: Vincaleukoblastin[1]), Leurosin[2]), Leurocristin[3]) und Leurosidin[3]).

Bei der Untersuchung von *C. roseus* konnten wir ein Alkaloid isolieren, welches offenbar mit den bisher beschriebenen nicht identisch ist. Die Aufarbeitung des Pflanzenmaterials erfolgte in Anlehnung an das von SVOBODA et al.[4]) angegebene Fraktionierungs-Schema: 0,8 kg getrocknete Blätter von im Gewächshaus kultivierten Pflanzen (Samen aus Portugal) wurden erschöpfend mit Petrolbenzin Kp. 70° extrahiert. Der eingeengte Extrakt wurde mehrmals mit 2n HCl ausgeschüttelt. Die vereinigten salzsauren Fraktionen wurden mit NH_4OH auf pH 9 eingestellt und danach mit CH_2Cl_2 extrahiert. Die vereinigten Ausschüttelungen haben wir zur Trockne eingeengt und erhielten 1,35 g Rohalkaloid.

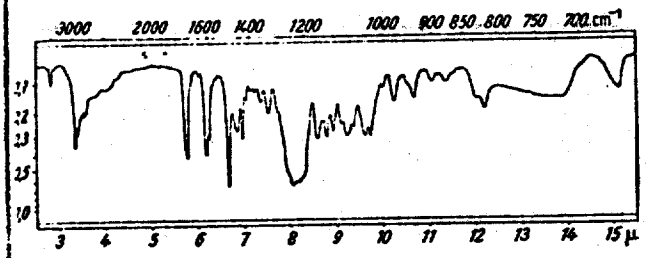


Fig. 1. IR-Spektrum von Alkaloid X in Chloroform

Die chromatographische Analyse zeigte die Anwesenheit von fünf Alkaloiden: Tetrahydroalstonin, Catharanthin, Vindolin (Hauptalkaloid), Vindolinin und ein unbekanntes Alkaloid = Alkaloid X. Die Auftrennung des Alkaloidgemisches erfolgte durch Säulenchromatographie unter Verwendung von Al_2O_3 Woelm basisch, das durch 10%ige Essigsäure desaktiviert worden war. Es wurde nacheinander eluiert mit Benzin, Benzin/Benzol-Mischungen und anschließend mit Methylenchlorid. Die das Alkaloid X enthaltenden CH_2Cl_2-Fraktionen wurden vereinigt und zur Trockne eingeengt. Der Rückstand wurde erneut an einer Al_2O_3-Säule chromatographiert und das Alkaloid X aus Petrolbenzin/Aceton (4:2) kristallin erhalten. Ausbeute: 8 mg, Schmp: 163 bis 165° (Boetius-Heiztisch).

Aus dem UV-Spektrum, Maxima bei 214, 252 und 304 mμ, kann man schließen, daß es sich um ein Alkaloid vom Dihydroindol-Typ handelt. Das IR-Spektrum (s. Fig. 1) ist dem des Vindolins[5]) sehr ähnlich. Alkaloid X unterscheidet sich vom Vindolin lediglich durch das Fehlen der Bande bei 1370 cm^{-1}.

Alkaloid X wurde in acht verschiedenen Lösungsmittelgemischen sowohl ein- als auch zweidimensional auf Dünnschichtplatten chromatographiert. Es konnte jeweils nach Besprühen mit Cerammoniumsulfatlösung[6]) nur ein Fleck nachgewiesen werden. Ähnlich wie Vindolin färbt sich Alkaloid X mit dem Reagenz karmesinrot. R_F-Werte auf Kieselgel G-Merck-Platten (Adsorbens angerührt mit 0,5 n KOH) mobile Phase: Äthylacetat/Äthanol (3:1). Alkaloid X: 0,25; Vindolin: 0,57.

Weitere Untersuchungen zur Strukturaufklärung sind im Gange.

Für die großzügige Unterstützung sei Herrn Dr. G. H. Svoboda, Eli Lilly a. Co., Indianapolis, Ind. U.S.A. auch an dieser Stelle herzlich gedankt.

Deutsche Akademie der Wissenschaften, Institut für Biochemie der Pflanzen in Halle/Saale

D. GRÖGER und K. STOLLE

Eingegangen am 18. Juli 1964

[1]) NOBLE, R. L., C. T. BEER u. J. H. CUTTS: Ann. N.Y. Acad. Sci. 76, 882 (1958). — [2]) SVOBODA, G. H.: J. Amer. Pharm. Assoc., Sci. Ed. 47, 834 (1958). — [3]) SVOBODA, G. H.: Lloydia 24, 173 (1961). — [4]) SVOBODA, G. H., N. NEUSS u. M. GORMAN: J. Amer. Pharm. Assoc., Sci. Ed. 48, 659 (1959). — [5]) NEUSS, N.: Lilly Collection of Physical Data of Indole and Dihydroindole Alkaloids, 1 ed. Indianapolis: Eli Lilly & Co. 1960. — [6]) CONE, N. J., R. MILLER u. N. NEUSS: J. pharm. Sci. 52, 688 (1963).

Isolation and Identification of a Hepatotoxic Factor in Herring Meal Produced from Sodium Nitrite Preserved Herring

Demonstration of a hepatotoxic disorder in domestic animals fed on herring meal derived from one or two Norwegian factories has been published[1–7]).

We studied the problem of genesis of disorder and the nature of the toxic principle in this meal — which is not nitrite itself. By use of large amounts of $NaNO_2$ and especially if the preserved herring was worked up immediately after addition of nitrite — as opposed to the factory method — we produced toxic herring meal containing nitrite below the permissible limit. Drying and heating of solubles — and herring-meal at 115—130° C gave more toxic products than drying at 40° C. Extraction with ether, acetone and chloroform failed to remove the toxic agent, which remained in the herring meal. Extraction methods using watery solutions were ineffective as all products lost toxicity. We therefore first isolated from fish the "mother-substances" which in reaction with $NaNO_2$ produced the toxic factor, then the factor itself. Another difficulty was that the concentrated mother-substances during treatment with $NaNO_2$ lost activity. This was prevented by adding rice-starch to the solution of the mother-substances before being dried and heated at 115° C for 2 hours. The starch itself, presumably acting as a "Schutzkolloid", did not become toxic when treated analogously with H_2O and $NaNO_2$. Vertebrae and skin of herring contained large amounts of the mother-substances and still larger amounts were found in identical organs from cod. Extremely toxic products resulted when codskin was worked up immediately after addition of the $NaNO_2$-solution, whereas storage of codskin for 38 days in the same solution at 13—21° C reduced the level of nitrite in the skin almost to zero and gave a practically nontoxic material after drying and heating. For isolation of the mother-substances we used precipitation and extraction methods, dialysis, column chromatography with cellulose as adsorbent, followed by elution with 67% ethanol acidified with acetic acid to pH 5.0. As spot test analyses[8]) of the chromatographic fractions revealed the occurrence of aliphatic amines, the amines were distilled over (at pH 8.0) in 0.2 N HCl and the amine-hydrochlorides were extracted with hot chloroform[9]). The amines were identified as di- and trimethylamines based on paperchromatographic studies (dimethylamine) and melting point determination of the chloroplatinate of trimethylamine using authentic preparations for control.

The lowest dose of the toxic codskin preparations which induced lethal effect in mink was about 17—25 mg, when applied with stomach tube. The same was observed when pure N-nitrosodimethylamine was given in doses down to 17—30 mg. With toxic fractions prepared from herring solubles by a revised method, lethal effect was induced when dosed with 50—70 mg. The pathological-anatomical picture in the test animals was of a special type and, depending on dosage and duration of exposure to the toxic substances, lesions in the liver appeared, similar to those observed in field cases of herring meal intoxication[7]). The formation of N-nitrosodimethylamine in the fish products was further verified by the fact that pure di- and trimethylamines, which occur in a variety of marine fishes[10]),[11]), when being treated separately with $NaNO_2$ and starch in the described manner, produced the same toxic substances. Doses of 20 mg and 60 mg resp. induced lethal effect and identical pathological symptoms. N-nitrosodimethylamine was also proved directly in a steam distillate of a soluble meal prepared from $NaNO_2$-preserved herring. That the various toxic substances described above are nitrosodimethylamine was proved by the absorption spectrum in U.V. (max. 225 mμ and 330 mμ in H_2O and 350 mμ in $CHCl_3$) and in I.R. A further proof was based on gaschromatograms of the most toxic fish preparations.

Further details will be published elsewhere.

The authors are indebted to the Central Institute for Industrial Research, Oslo, for the G. C. and I. R. analyses, and to the Norw. Herring Meal Industry for financial support.

Dept. of Biochemistry, Dept. of Pathology, Res. St. for Fur-bearing Animals — The Veterenary College of Norway

F. ENDER, G. HAVRE, A. HELGEBOSTAD, N. KOPPANG, R. MADSEN and L. CEH

O. BJÖRNSON participated 8 mths. in the work.

Eingegangen am 16. November 1964

[1]) BOHLER, N.: 9th Nordic Vet. Congr., Copenhagen 1962, p. 774. — [2]) KOPPANG, N., and A. HELGEBOSTAD: Norsk Pelsdyrblad

Rev. 27, 686 (1957). — 9. KUMMER, H., und v. POHLHEIM, P., 1960: Bewertung und Klassifizierung der Weizen- und Roggennachprodukte im Hinblick auf die Neufassung des Futtermittelgesetzes. Kraftfutter, 43, 428. — 10. LINDNER, K., 1964: Aminosäurenzusammensetzung und Nährwert der Weizeneiweißfraktionen. Qual. Plant. Mater. Veg. XI, 31. — 11. LYMAN, C. M., KUIKEN, H. A., and HALE, F., 1956: Amino acids in feedstuffs. Essential amino acid content of farm feeds. J. Agric. Food Chem. 4, 1008. — 12. MOORE, S., 1963: On the determination of cystine as cysteic acid. J. Biol. Chem. 238, 235. — 13. MORAN, T., 1959: Nutritional significance of recent work on wheat flour and bred. Nutr. Abstr. Rev. 29, 1. — 14. MORRISON, F. B., 1959: Feeds and feeding, 22ND Edition. The Morrison Publishing Company. Appendix. — 15. POMERANZ, Y., and MILLER, B. S., 1963: Comparison of methods for determination of lysine in cereals. J. of the A. O. A. C., 46, 399. — 16. POZSAR, B. J., und KISS, B., 1965: Die Wirkung der Mineraldünger auf die Veränderung des Aminosäureverhältnisses im Eiweißgehalt bei der Weizensorte Besostaia-1. Naturwissenschaften 52, 350. — 17. SCHUPHAN, W., und POSTEL, W., 1956: Studien über exogene Aminosäuren im Eiweiß von Cerstencaryopsen unter dem Einfluß genetischer und ökologischer Faktoren. Naturwissenschaften 43, 473. — 18. SCHUPHAN, W., 1963: Beziehungen zwischen Eiweißqualität und Backeigenschaften des Weizens in Abhängigkeit von der Düngung. Qual. Plant. et Mater. Veg. IX, 165. — 19. SPACKMAN, D. H., STEIN, W. H., and MOORE, S., 1958: Automatic recording apparatus for use in the chromatography of amino acids. Analytical Chemistry 30, 1190.

Anschrift der Autoren: Prof. Dr. Dr. J. BRÜGGEMANN und Dr. H. ERBERSDOBLER, Institut für Tierphysiologie der Universität München, 8 München 22, Veterinärstraße 13

553

From the Department of Biochemistry and the Research Station for Fur-bearing Animals, The Veterinary College of Norway, Oslo

Studies on conditions under which N-nitrosodimethylamine is formed in herring meal produced from nitrite-preserved herring

The risk of using nitrite uncritically as a preservative agent

By F. ENDER, G. N. HAVRE, R. MADSEN, L. ČEH and A. HELGEBOSTAD

Herring meal has for many years been used in Norway as an excellent protein feed for livestock. Injurious effects caused by the feeding of herring meal were unknown before 1957 when the first cases of a hitherto peculiar disorder in mink characterized by intensive liver damage was first detected by BÖHLER (1), who gave a description of the disease in 1962.

Because of the obscure nature of the aetiology of this disorder at that time, the designation "a new X-disease" seemed justified.

In 1962 KOPPANG (2) verified the occurence of this disorder in fur-bearing animals, and stated that he also observed a disease with similar pathological-anatomical liver symptoms in ruminants, to which the name "toxic hepatosis" was given.

KOPPANG drew attention to the fact that this disorder occurred in livestock for which a special type of herring meal produced from nitrite-preserved herring by a single factory in North Norway was used as a protein feed for ruminants. Based on experiments KOPPANG (3) succeeded in proving that the feeding of batches of toxic

herring meal purchased mainly from this particular factory gave rise to the development of the hepatotoxic disorder in cattle and sheep. A more detailed describition of the clinical as well as the pathological-anatomical symptoms of herring meal poisoning in cattle, sheep, and goats has been given by SVENKERUD and KOPPANG (4).

In 1963 feeding experiments of the toxic herring meal to mink were carried out by the present authors. Various types of toxic herring meal based on nitrite-preserved herring were produced on a laboratory scale at the Department of Biochemistry. Before entering into the present research problem, we suggested that the use of nitrite as a preservative agent might give occasion to formation of toxic substances in the meal, especially when the meal was dried at high temperatures, an assumption that was fully verified as demonstrated in *Table 1*.

Table 1

The influence of temperature on the toxicity of whole meals and soluble meals as indicated by determination of the lethal doses for mink

Type of herring meal and final heating temperature	Number of feeding days before death occurred	Total dose of herring meal administered before death or before pelting time g	
Whole meal temp. 50 ° C	250	2540	Hepatosis, died
" " " "	160	1640	" "
Whole meal temp. 100 ° C	105	1090	Hepatosis, died
" " " "	104	1080	" "
Whole meal temp. 135 ° C	80	830	Hepatosis, died
" " " "	78	810	" "
Whole meal temp. 175 ° C	85	890	Hepatosis, died
" " " "	134	1380	" "
Soluble meal temp. 50 ° C	193	390	Hepatosis, died
" " " "	193	390	Hepatosis, pelted
Solubles temp. 100 ° C	92	460	Diagnosis uncertain
Boiled not dried	178	890	Hepatosis, pelted
Soluble meal temp. 115 ° C	39	80	Hepatosis, died
" " " "	44	90	" "
Soluble meal temp. 150 ° C	193	390	Hepatosis, died
" " " "	193	390	Hepatosis, pelted
Soluble meal temp. 175 ° C	193	390	No hepatosis, pelted
" " " "	193	390	" " "

Amount of sodium nitrite added to the raw fish: 630 g per 100 kg.

By production of these toxic types of meals we tripled the quantity of nitrite which was earlier used by the herring-meal industry. 630 g $NaNO_2$ was added to 100 kg herring and the herring mass was worked up to meal immediately after addition of nitrite, as we observed that in cases in which the nitrite-preserved fish was stored for a long time before processing, only traces of toxic substances were formed in the prepared meal. This observation has caused the herring-meal industry to reduce the amount of nitrite heavily as mentioned later in this paper.

The purpose of our tests was to study conditions under which the use of nitrite may result in formation of great quantities of toxic substances in the produced meal. Further studies aimed at isolating and identifying the toxic factor, which is not nitrite itself.

In 1964 ENDER
methylamine (in th
stated that methyla
These authors gave
is formed in the me
heavily by rising th
in *Table 2*.

Number of experiment	
1.	Fresh fre
2.	Fresh fre
3.	Fresh fre
4.	Fresh fre
5.	Winter he
6.	Winter he
7.	Winter he
8.	Winter he

[1] DMNA = N-nitr

Determination o
by a modified DAIS
method, a new proc
tatively reduced to
measured colourime
method a yellow al
method permits exa
1 p.p.m.

Great interest ha
since it was shown t

As a consequen
necessity to provide
have shown that fo
various methylamin

The assumption
the formation of th
belonging to the fr
traces of methylami
products showing n
over a long period.

The identity of
based upon the follo
1. Gas-chromatogr
2. Maximum of sp

In 1964 ENDER et al. (5) reported the isolation and identification of N-nitrosodimethylamine (in the following called DMNA) from poisonous herring meal and stated that methylamines in the fish react with $NaNO_2$ under formation of DMNA. These authors gave a description of the more special conditions under which DMNA is formed in the meal and pointed to the fact that the level of DMNA is increased heavily by rising the drying temperature as well as the quantity of nitrite as shown in *Table 2*.

Table 2

Number of experiment	Quality of herring	Amount of $NaNO_2$ added per 100 kg herring mass g	Level of DMNA [1] in herring meals after drying at following temperatures: below 50 ° C p.p.m.	at 130 ° C for 2 hours p.p.m.
1.	Fresh from Oslo Fjord	0	—	0
2.	Fresh from Oslo Fjord	7	—	2.3
3.	Fresh from Oslo Fjord	23.3	—	7.7
4.	Fresh from Oslo Fjord	70	—	14.1
5.	Winter herring stored by refrigeration	70	2.3	14.0
6.	Winter herring stored by refrigeration	210	5.1	46.4
7.	Winter herring stored by refrigeration	630	13,9	174
8.	Winter herring stored by refrigeration	1260	29.6	421

[1] DMNA = N-nitrosodimethylamine.

Determination of the level of DMNA in poisonous herring meal was carried out by a modified DAIBER and PREUSSMANN (6) method and verified by the hydrazine method, a new procedure worked out in our laboratory by which DMNA is quantitatively reduced to dimethylhydrazine by Zn and HCl, the quantity of which is measured colourimetrically by reaction with p-dimethylaminobenzaldehyde. By this method a yellow aldazine is formed with maximum extinction at 458 mμ. The last method permits exact determination of DMNA levels in herring meal of less than 1 p.p.m.

Great interest has in recent years been paid to N-nitrosodimethylamine in nature, since it was shown that this substance exerts a carcinogenic effect in rats (7, 8, 9).

As a consequence of the strongly toxic effect of the DMNA it was an absolute necessity to provide a secure proof that it was in fact the toxic factor. Our studies have shown that formation of DMNA is caused by the reaction of $NaNO_2$ with various methylamines present in the fish.

The assumption that methylamines in the raw fish material might contribute to the formation of the toxic factor was -- inter alia – supported since the ide, a fish belonging to the fresh water species which generally are claimed to contain only traces of methylamines, when exposed to the same treatment with $NaNO_2$ gave meal products showing no detectable toxicity when tested on mink in large daily doses over a long period.

The identity of N-nitrosodimethylamine isolated from the toxic herring meal was based upon the following qualifications:

1. Gas-chromatographic studies.
2. Maximum of spectrophotometric absorption in H_2O at 226 and 330 mμ.

3. Maximum of spectrophotometric absorption in $CHCl_3$ at 350 mμ.
4. Identity of the infra-red spectrum when compared with pure N-nitrosodimethylamine purchased from the British Drughouses.
5. Oral application of a single dose of 17 mg DMNA to mink resulted in death after the elapse of 24 hours whether the toxic substance was derived in pure state from herring meal or was purchased from a drug house. The carcasses gave in both cases the same pathological-anatomical picture.
6. Dimethylamine and trimethylamine, the presence of which we detected in our most toxic basic products after extensive chromatographic fractionating, gave huge quantities of DMNA by treatment with $NaNO_2$, whose identity was again secured by the measures mentioned above under Points 2, 3, 4, and 5. This is perhaps the most secure proof that the substance formed in herring meal is really DMNA.

Reference to these studies has briefly been given by ENDER et al. (5) in 1964. More detailed information of the detection of methylamines in herring and cod preparations, which contributes to formation of the toxic factor DMNA, was given by ENDER (10) at the VIII International Buiatric Congress in Zürich in 1966.

Table 3

Production of dimethylnitrosamine from dimethylamine hydrochloride and sodium nitrite in watery solutions at varying temperatures

(The solutions were buffered to pH 6.5 with Na-acetate and acetic acid)

Temperature °C	DMNA mg/l
50°	0.2
	0.2
60°	0.4
	0.4
70°	0.75
	0.75
80°	1.6
	1.5
90°	3.2
	2.8
100°[1]	8.2
	8.4

[1] Taken from Table 5.
All solutions contained 40 milli mole dimethylamine/l and 40 milli mole sodium nitrite/l. After heating under reflux for 15 minutes the solutions were immediately cooled to room temperature by running tap water.

Table 4

Production of dimethylnitrosamine from dimethylamine hydrochloride and sodium nitrite in watery solutions at low temperature

Storage at + 4 °C	
Time stored Days	DMNA mg/l in stored sample
2	1.6
	1.5
8	6.3
	6.0
18	16
	11
30	26
	24
52	42
	42
76	69
	68
104	88
	88
128	110
	110
157	136
	131

All solutions contained 40 milli mole dimethylamine per litre and 40 milli mole sodium nitrite per litre. pH in the solutions: 6.3.

Table 5

Production of dimethylnitrosamine (DMNA) from watery solutions of dimethylamine hydrochloride and sodium nitrite at different pH

(Temperature: Constant at 100 ° C)

Concentration of $NaNO_2$: constant 40 m.mol./litre			Concentration of dimethylamine-HCl: constant 40 m.mol./litre		
Dimethylamine-HCl m.mol./l	DMNA mg/l		$NaNO_2$ m.mol.	DMNA mg/l	
	pH 6.5	pH 6.0		pH 6.5	pH 6.0
4	0.75 0.75	2.3 2.5	4	0.2 0.2	0.4 0.4
20	4.1 4.5	11 10	20	2.6 2.6	5.2 5.6
40	8.2 8.4	19 19	40	8.2 8.4	19 19
60	13 13	28 31	60	17 16	39 43
80	17 17	38 38	80	24 26	64 60

All solutions were buffered with Na-acetate and acetic acid. – 250 ml of the solutions were boiled under reflux for 15 min, set aside for 10 min, and cooled to room temperature by running tap water. – To the cooled solution was added NaOH, and DMNA was distilled into a receiver.

The formation of DMNA is in model experiments studied under varying conditions. By this procedure various methylamines applied as hydrochlorides in watery solutions were brought into reaction with sodium nitrite. In order to prevent the formed DMNA from escaping under the following drying process, rice starch was added to the watery solution because of its incapsulating effect as demonstrated earlier. In herring meal a similar effect is obtained by the denaturated proteins and gluteneous material, as illustrated by ENDER et al. (5) in 1964. The theory about the incapsulating effect provides that DMNA in herring meal is formed under pressure. Temperature and pH are varied within the usual limits for herring-meal production.

Levels of DMNA are in most experiments determined by a spectrophotometric method, using the maximum extinction at 226 mμ as a measure. In cases in which this method gave unreliable values, the DAIBER-PREUSSMANN (6) method has been used, by which the formed DMNA is quantitatively decomposed by ultra-violet light into nitrite-ions, the quantity of which is measured using the GRIESS (11) reagent with sulphanilic acid and α-naphthylamine.

The model experiments are hardly directly comparable with ordinary herring-meal production. The experiments reveal, however, which reaction mechanisms will be present in herring-meal production and clearly demonstrate that all of the four occurring amines may give rise to DMNA. The result from our model test may appear from Tables 3–7.

Table 6

Produktion of dimethylnitrosamine (DMNA) from methylamine hydrochlorides and sodium nitrite "capsuled" in rice starch

Methylamine hydrochlorides	Initial mixture					Products D_1 Reaction temp. 50 ° C Reaction time 24 h.			Products D_2 Reaction temp. 100 ° C Reaction time 2 h.			Products D_3 R. temp. 115 ° C R. time 2 h.			Products D_4 R. temp. 135 ° C R. time 10 min.			Products D_5 Reaction temp. 135 ° C Reaction time 2 h.		
	Methylamine hydrochlorides g	$NaNO_2$ g	Rice-starch g	H_2O g	pH [1]	pH [2]	$NaNO_2$ ‰	DMNA mg/g	pH [2]	$NaNO_2$ ‰	DMNA mg/g	$NaNO_2$ ‰	DMNA mg/g	$NaNO_2$ ‰	DMNA mg/g	pH [2]	$NaNO_2$ ‰	DMNA mg/g
Mono-	4	1.2	76	800	6.5	7.5	15	0	8.2	8.0	0.4	1.5	1.1	2.2	0.9	7.2	0.05	1.4
Di-	2	1.5	38	400	6.5	7.4	35	3.3	7.7	15	26	7.0	30	6.0	34	7.5	1.2	38
Tri-	2	1.04	38	400	6.5	7.1	26	0.3	8.8	14	3.8	6.0	6.2	5.0	7.5	8.7	0.3	7.3
Trimethyl-oxide	2	1.4	38	400	4.5	5.5	8.0	0.95	7.1	2.5	2.7	0.7	3.7	1.2	3.5	7.6	0.1	4.0
Trimethyl-oxide	2	0.2	38	400	5.5	5.6	5.0	0.05	5.8	3.0	0.8	2.3	1.6	2.0	1.3	5.8	0.35	2.8
Trimethyl-oxide	3	0.3	57	600	6.5	7.2	5.0	0	—	—	—	5.0	0.1	5.0	0.1	7.5	3.5	0.7

[1] pH was adjusted by addition of 1 N CH_3COOH and 1 N NaOH. – [2] pH was measured in a suspension of 0.2 g product in 5 ml water. Product D_1 was obtained by heating the initial mixture at 90 ° C for 45 min and evaporating to dryness at 50 ° C. The other products were obtained by heating the D_1 product, except for product D_3, which was obtained by heating the D_2 product. – Levels of DMNA and nitrite refer to the final products (D_1–D_5).

A brief outline of these experiments is given in the following:

The experiments presented in *Table 3* demonstrate the formation of DMNA from equimolecular levels of $NaNO_2$ and dimethylamine in watery solutions at a pH of 6.5 and at temperature between 50° and 100° C.

DMNA will also be formed at low temperature (+ 4° C) if the reaction takes place during a longer period as shown in *Table 4*.

The figures presented in *Table 5* illustrate the influence of the concentration of the 2 reactants and the pH as to formation of DMNA.

The extent to which the four methylamines, i. e. mono-, di-, and trimethylamine, besides trimethylamine oxide contribute to formation of DMNA is shown in *Table 6*. In these experiments rice starch was added to the reaction mixture in order to prevent the formed DMNA form escaping under the following drying and heating temperature as described earlier in this paper. The figures presented in this Table clearly demonstrate that the stronger contribution to formation of DMNA is afforded by dimethylamine. Trimethylamine

comes in the second place. Monomethylamine and trimethylamine oxide contribute only a little to development of DMNA. Consideration needs, however, to be taken that trimethylamine oxide during boiling of the herring in transformed to di- and trimethylamine, which both react with nitrite to form DMNA. By raising the drying temperature from 50° C to 135° C, the output of DMNA increased markedly.

The output of the formed DMNA is demonstrated in *Table 7* under conditions in which the 4 methylamines react with $NaNO_2$ as dry salts at atmospheric pressure as well as in a closed glass tube (under pressure). Formation of DMNA from dimethylamine and nitrite expire near the theoretical output, whereas trimethylamine and its oxide under these special conditions give about 1/3 of the theoretical output. With monomethylamine the greatest output was achieved when the reaction occurred in a sealed system.

Table 7

Production of dimethylnitrosamine (DMNA) from a mixture of methylamine hydrochlorides and sodium nitrite as dry and pure salts

Methylamine hydrochlorides	Heated at 135° C under reflux for 1 hour		Heated at 135° C in a sealed glass tube for 1 hour	
	mg DMNA per 100 mg methylamine-HCl	Average [1] % DMNA of theoretical	mg DMNA per 100 mg methylamine-HCl	Average [1] % DMNA of theoretical
Mono-[2]	1.0		4.7	
	1.0	1.8	5.1	9.0
Di-	86		91	
	89	97	86	98
Tri-	22		21	
	21	28	21	27
Trimeth. oxide	22		23	
	22	33	21	33

One part of amine hydrochloride was mixed with two parts of sodium nitrite and heated. [1] It is assumed that 2 moles monomethylamine hydrochloride and 1 mole $NaNO_2$ will yield 1 mole DMNA and that 1 mole of three other methylamine hydrochlorides with 1 mole $NaNO_2$ will also yield 1 mole DMNA. – [2] Monomethylamine hydrochloride was purified by repeated re-crystallizations.

If the DMNA-containing reaction products mentioned in Table 6 were repeatedly treated with water, redried, and heated again, the DMNA content decreased gradually to very low levels. This treatment leads to evaporation of DMNA, as rice starch has only a negligible capsulating effect when moist. A similar effect is observed when toxic herring meal is treated in the same way. By this method it is possible to detoxify a poisonous herring meal.

The procedure used by the industry today aimes at lowering the amount of DMNA in herring meal at its outmost. A standardized solution of $NaNO_2$ and formalin is added to the raw fish in quantities corresponding to a maximum of 0.06 per cent of sodium nitrite by weight of fish. The raw material is stored and processing of the herring meal can first take place when the content of nitrite in the raw fish is less than 0.02 per cent. This procedure leads to formation of minute quantities of DMNA as illustrated by an experiment conducted under laboratory conditions (Table 8).

Table 8

Formation of DMNA in herring meal produced from nitrite-formalin-preserved winter herring

Dates	$NaNO_2$ in herring during storage g/100 kg	Dried at 50° C DMNA p.p.m.	Heated 2 hours at 100° C DMNA p.p.m.
22/4	69	2.8	15
28/4	50	3.2	9.7
6/5	35	1.3	6.5
12/5	30	0.8	6.6
20/5	20	0.5	6.3
26/5	2.5	0.2	0.5

The herring was preserved on 22/4.

Concentration of preserving agent: 60 g $NaNO_2$+240 g 35 % formaldehyde per 100 kg herring. During storage, parts of the preserved herring were worked up to meal at named dates. The temperature during storage was 7—16° C.

Acknowledgement

The authors are indebted to the Norwegian Herring Meal Industry and the Royal Norwegian Council for Scientific and Industrial Research for financial support.

Summary

In the introductory work a description is given of how it was first recognized that the use of nitrite as a preservative agent in the production of herring meal led to disorders in livestock. In the present paper the authors describe the various conditions under which DMNA – the toxic agent – is formed in herring meal. The reaction mechanism by which various methylamines present in herring react with sodium nitrite has been outlined by model experiments, the results of which have been briefly described.

The scope of our studies has been to gain insight into the mode of formation of toxic substances in herring meal, with a view to the general risk of using sodium nitrite as a preservative agent for food products.

Zusammenfassung

In der vorliegenden Arbeit wird eine Beschreibung darüber gegeben, wie die Verwendung von Nitrit als Konservierungsmittel bei der Heringsmehlherstellung zu Erkrankungen bei Tierbeständen führt. In der hier vorliegenden Schrift beschreiben die Verfasser die verschiedenen Bedingungen, unter denen DMNA – das giftige Agens – im Heringsmehl gebildet wird. Der Reaktionsmechanismus, durch den verschiedene Methylamine, die in dem Hering vorhanden sind und mit dem Natriumnitrit reagieren, ist durch Modellversuche, deren Ergebnisse kurz beschrieben wurden, in groben Zügen dargestellt.

Der Umfang unserer Studien war darauf ausgerichtet, einen Einblick in die Art der Bildung von giftigen Substanzen im Heringsmehl zu gewinnen im Hinblick auf das allgemeine Risiko, Natriumnitrit als Konservierungsmittel für Futtermittel zu verwenden.

Literature

1. Böhler, N., 1962: Proc. IX Nordic Vet. Congr., Copenhagen, p. 774. — 2. Koppang, N.: Ibid. p. 777. — 3. Koppang, N., 1964: Nord. Vet.-Med. 16, 305. — 4. Svenkerud, R., and Koppang, N., 1964: Proc. XIV Scand. Congr. Path. and Microbiol., Oslo, p. 151. — 5. Ender, F., Havre, G., Helgebostad, A., Koppang, N., Madsen, R., and Ceh, L., 1964: Naturwissenschaften 51, 637. — 6. Daiber, D., and Preussmann, R., 1964: Z. anal. Chem. 206, 344. — 7. Barnes, J. M., and Magee, P. N., 1954: Brit. J. Industr. Med. 11, 167. — 8. Magee, P. N., and Barnes, J. M., 1956: Brit. J. Cancer 10, 114. — 9. Druckrey, H. R., Preussmann, R., and Schmähl, D., 1963: Extrait de Acta International Contre Le Cancer 19, 510. — 10. Ender, F., 1966: VIII International Buiatric Congress, Zürich. — 11. Griess, P., 1879/1899: Ber. 12, 427 (1879); Z. angew. Chem. 12, 666 (1899).

Anschrift der Autoren: Norges Veterinærhogskole, Institutt for Biokjemi, Oslo, Norwegen

Aus dem Veterinär-Physiologischen Institut der Justus-Liebig-Universität Gießen
Direktor: Prof. Dr. Dr. h. c. V. Horn

Ein einfaches Verfahren zur Körper-N-Bestimmung bei Ratten, zugleich ein Beitrag zur Verbesserung der Kjeldahl-Analyse

Von H. Rufeger

Seit mehreren Jahren werden N-Zunahmen bei Ratten sowohl in Versuchen zur Wertermittlung von Futter- und Nahrungsproteinen (5) als auch zur Prüfung der Zuverlässigkeit von N-Bilanzversuchen (2) in größerem Umfange nach der Körper-N-Methode ermittelt. Hierbei wird zu Beginn der Körper-N der Versuchstiere aus deren Gewicht und dem analysierten durchschnittlichen N-Gehalt einer Vergleichsgruppe berechnet und von dem zu Versuchsende analysierten Körper-N der Versuchstiere abgezogen.

Zur Bestimmung des Körper-N von Ratten sind bereits sehr verschiedenartige Verfahren beschrieben worden. Sie sind m. E. mit gewissen Mängeln behaftet, die ihre Anwendung bei den einleitend genannten Aufgaben in unterschiedlichem Maße beeinträchtigen.

Der Aufschluß der ganzen Tierkörper (12, 1) mit Hilfe des Kjeldahl-Verfahrens ist außerordentlich langwierig und außerdem auch unsicher, weil er sich im Falle seines Mißlingens oder zur Kontrolle nicht wiederholen läßt. Somit ergibt sich die Notwendigkeit, die Tierkörper vor dem N-Aufschluß in einen homogenen, zur Probenentnahme geeigneten Zustand zu bringen. Hierzu ist vorgeschlagen worden, die Tierkörper unzerkleinert (15, 20), homogenisiert (1) oder tiefgefroren gemahlen und ent-

560

JOHN M. ESSIGMANN and PHILLIP ISSENBERG[1]
Dept. of Nutrition & Food Science, Massachusetts Institute of Technology, Cambridge, MA 02139

GAS CHROMATOGRAPHIC DETERMINATION OF VOLATILE NITROSAMINES IN FOODS

INTRODUCTION

THE ROLE OF N-nitroso compounds in the etiology of human cancer has not been established, but these compounds are a cause of increasing concern as potential environmental carcinogens (Lijinsky and Epstein, 1970). Since nitrosamine concentrations in the nanogram per gram range may be formed by reaction of naturally occurring amines and added nitrites in fish (Sen et al., 1970; Fazio et al., 1971a), it is essential that conditions under which formation occurs by investigated. Rates and mechanisms of formation and destruction of nitrosamines in foods must be evaluated in order to eliminate any potential public health hazard from this source in processed foods. Screening methods are required for application in epidemiological studies of the relationship between exposure of human populations to nitrosamines and cancer risk.

Systematic investigation of nitrosamine formation during processing, storage and cooking of foods is limited by the analytical methods currently available. Wasserman (1971) has recently reviewed the literature and reported a survey of analytical procedures. Nitrosamines are ordinarily removed from water and nonvolatile materials by distillation and extraction, though not necessarily in that order (Fazio et al., 1971a, b; Sen, 1971). Concentration is required because of the very small quantities (ng/g) which must be detected. Clean-up is a critical step because most foods contain relatively large quantities of volatile material which may interfere with nitrosamine detection during gas chromatographic (GC) analysis. Methods having sufficient specificity are limited to those using mass spectrometry (MS) for confirmation of identity of volatile nitrosamines (Fazio et al., 1971a; Telling et al., 1971), after quantitative estimation by GC. The available procedures are time consuming, and the number of steps and extreme care required to minimize losses limit the number of samples which can be examined simultaneously.

Our investigations were undertaken to determine the extent to which analytical procedures for determination of volatile nitrosamines could be simplified without loss of sensitivity and specificity. Distillation and extraction were combined into a continuous process using a technique which allows large volumes of water to be distilled from a food sample and the distillate effectively extracted with large volumes of solvent without dilution of the extract. Some factors influencing recovery of volatile nitrosamines from foods were examined.

A sensitive, specific GC detector was desirable for efficient screening and routine quantitative determination of nitrosamines in food extracts. The detector described by Rhoades and Johnson (1970) was evaluated.

Confirmation of identity by MS is essential to establish the presence of nitrosamines unequivocally. Problems associated with adequate sample clean-up for combined GCMS were studied.

A chromatographic solvent stripping technique which permits GC analysis of dilute solutions was evaluated.

EXPERIMENTAL

CAUTION: Many nitrosamines are highly potent carcinogens. In the work reported below, nitrosamine solutions, spiked samples, and concentrates were handled in efficient fume hoods. Gloves were worn and precautions taken to avoid spills, skin contact and inhalation. Gas chromatographs used for nitrosamine determination should be equipped with destructive detectors or be vented to a hood.

Reagents

Pesticide grade dicholoromethane (DCM) and chromatoquality pentane were obtained from Matheson, Coleman and Bell, Norwood, Ohio. All other reagents were of analytical grade.

Distillation-extraction of nitrosamines from aqueous solutions

Nitrosamines were recovered from aqueous solutions (200 ml) containing 10–50 ng/ml dimethylnitrosamine (DMN), 10–50 ng/ml diethyl nitrosamine (DEN), 0.1g/ml NaCl, and 0.2g/ml K_2CO_3. Approximately 25 ml of DCM were used as the extracting solvent in the distillation-extraction apparatus described by Likens and Nickerson (1964). The vapor tubes above the aqueous solution were wrapped with heating tape and the distillation rate of each liquid was adjusted to approximately 1 ml/min. Distillation times ranged from 1–5 hr after initial

In later studies, a Kuderna-Danish apparatus was used for initial concentration; 50–μl aliquots were analyzed by GC.

Distillation-extraction of food materials

A number of foods was examined to determine the nature and amounts of interfering compounds. We examined smoked fish, canned chopped ham, bologna, summer sausage, canned "potted meat," "old fashioned loaf," corned beef spread, Vienna sausage and frankfurters. A 100-g food sample was homogenized with 100 ml distilled H_2O, 20g NaCl and 4g K_2CO_3 and then subjected to distillation-extraction for 4 hr. concentration in a nitrogen stream and GC analysis. If necessary, 1 ml concentrates were treated by a simple clean-up procedure involving a single stage extraction with an equal volume of 0.01N HCl in Na_2SO_4-saturated distilled H_2O.

Further modification of the procedure for samples of canned corned beef was necessary to remove compounds interfering with GC determinations. 100g of sample were homogenized with 200 ml of H_2O, 100g NaCl, 10g K_2CO_3 and after distillation-extraction, concentrated using the Kuderna-Danish apparatus. The concentrate was acid extracted and then subjected to a liquid column chromatography clean-up step using both 0.05–0.2 mm chromatographic grade silica gel (E. Merck) and alumina (Fisher Scientific Co.) packed columns. Each column was packed with 5g of material and rinsed with 30 ml of pentane. The concentrated corned beef extract (1 ml) was transferred onto the alumina column which was then eluted successively with 20 ml of pentane and 25 ml of 25% DCM in pentane. The fraction which would have contained nitrosamines was eluted with 15 ml of 30% diethyl ether in DCM which was

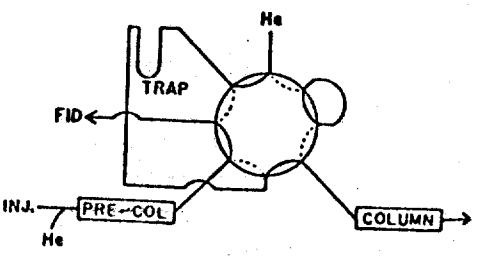


Fig. 1—Gas chromatographic solvent stripping system. Precolumn, 5.3 mm ID x 46 cm 20% Carbowax 20M on 80/100 mesh Chromosorb G. Solid lines indicate valve ports connected during solvent stripping and during analysis.

[1] Present address: Fundex Institute for the

then concentrated to 1 ml in a nitrogen stream. This concentrate was then treated identically on the silica gel column. The silica-gel column clean-up technique was adapted from the procedure described by Fazio et al. (1971b). This chromatographic clean-up was developed using both DCM solutions of nitrosamines and corned beef extracts.

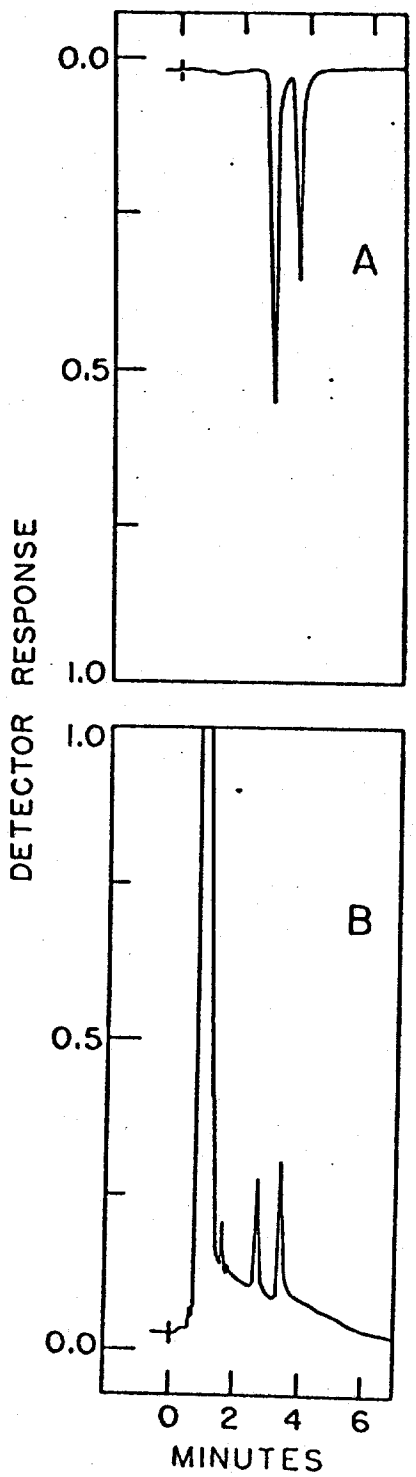


Fig 2—Chromatograms of 50 µl of a 1 ng/µl solution of DMN and DEN. The retention times of DMN and DEN are 2.9 and 3.7 min, respectively, in the CECD chromatogram (A) and 2.8 and 3.5 min in the FID chromatogram (B).

GC analysis

The chromatographic system used is shown schematically in Figure 1. 50–100 µl aliquots of solution were injected into a 5.3-mm ID × 46-cm long precolumn packed with 20% Carbowax 20M on 80/100 mesh Chromosorb W. After elution of solvent (1.5 min for DCM) to the atmosphere (solid lines), the effluent from the precolumn was diverted by a valve (Carle model 2012) to the trap (broken lines) which was cooled with liquid nitrogen (−196°C). Trapping was continued for a period sufficient to collect all of the nitrosamines of interest (7.0 min for DEN, 15 min for nitrosopyrrolidine). The valve was then returned to its original position (solid lines) and the trap immediately heated to approximately 90°C. This procedure rapidly transferred the nitrosamines from the trap to the analytical column. An identical solvent stripping system was installed on the combined GCMS instrument.

As a safety precaution, material eluted from the columns was transferred either to a fume hood or to destructive detectors.

Analytical columns used with the solvent stripping system included a 2.3-mm ID × 3.2-m long column packed with 3% Carbowax 4000 on 80/100 mesh Chromosorb G, a 0.5-mm ID × 160-m long open tubular column coated with Carbowax 20M, and a 0.5-mm ID × 15.2-m long SCOT column coated with Carbowax 1540 (Perkin-Elmer Corp., Norwalk, Conn.). The packed column and precolumn were installed in an F & M model 810 GC equipped with a flame ionization detector (FID) and a Coulson electrolytic conductivity detector (CECD). Helium flow rates for both columns were 35 ml per min. Injector and FID temperatures were 220 and 270°C, respectively. Column temperature was 140°C for both columns. The Carbowax 1540 SCOT column and the Carbowax 20M open tubular column were used in the inlet chromatograph of the GCMS system (Issenberg et al., 1969).

Detection of nitrosamines: comparison of the CECD with the FID

Sensitivity and selectivity of the CECD (Tracor, Inc., Model C321, Austin, Texas) were compared with the FID for the determination of some alkyl and some heterocyclic nitrosamines. The CECD was connected to the GC oven by a 0.75-mm ID heated transfer line, maintained at 125°C. The detector was operated in the pyrolytic mode as described by Rhoades and Johnson (1970) and an auxillary carrier flow of 95 ml per min was used. In these studies, small volumes (1 µl) of DCM solutions containing approximately 1 µg of each nitrosamine were injected onto the Carbowax 4000 analytical column which was directly connected to either the CECD or the FID. Peak areas were measured by using a digital integrator (Problematics, Inc., model CDA100A, Waltham, Mass.).

RESULTS & DISCUSSION

THE DISTILLATION-EXTRACTION apparatus has a number of theoretical advantages. Most volatile alkyl nitrosamines have partition ratios that favor extraction with DCM (Nunn and du Plessis, 1971). The combined distillation-extraction technique permits continuous recycling of the aqueous and organic phases without dilution of the extract. A number of distillation-extractions can be performed simultaneously because each apparatus requires a minimum amount of attention. Early use of this method with food homogenates was encumbered by severe foaming problems. This problem was controlled when the homogenate was distilled from a large (2-liter) flask with careful stirring. It was also found helpful to heat the vapor tubes above the homogenate and solvent flasks.

Table 1—Relative response of the Coulson electrolytic conductivity detector (CECD), operated in the pyrolytic mode, and the flame ionization detector (FID) to selected nitrosamines

Nitrosamine	Retention time[a] (min)	Peak area relative to dimethylnitrosamine	
		CECD	FID
Dimethylnitrosamine	2.1	1.0	1.0
Methylethylnitrosamine	2.4	0.81	1.5
Diethylnitrosamine	2.6	0.53	1.8
Methylpropylnitrosamine	3.2	0.60	1.7
Dipropylnitrosamine	4.6	0.29	2.1
Diallylnitrosamine	4.8	0.28	2.3
Methylbutylnitrosamine	4.6	0.53	1.8
Ethylbutylnitrosamine	5.1	0.46	2.1
Propylbutylnitrosamine	6.4	0.22	2.2
Dibutylnitrosamine	9.3	0.18	2.3
Nitrosopyrrolidine	12.0	0.12	1.6
Ethylbenzylnitrosamine	58.7	0.42	2.3
Ethylphenylnitrosamine	9.7	0.0	0.61
Butylphenylnitrosamine	19.5	0.0	0.54
Nitrosomorpholine	15.2	0.03	0.43

[a]Column: 2.3 mm ID × 3.2m packed with 3% Carbowax 4000 on 80/100 mesh Chromosorb G; helium flow rate 35 ml/min; temperature 140°C

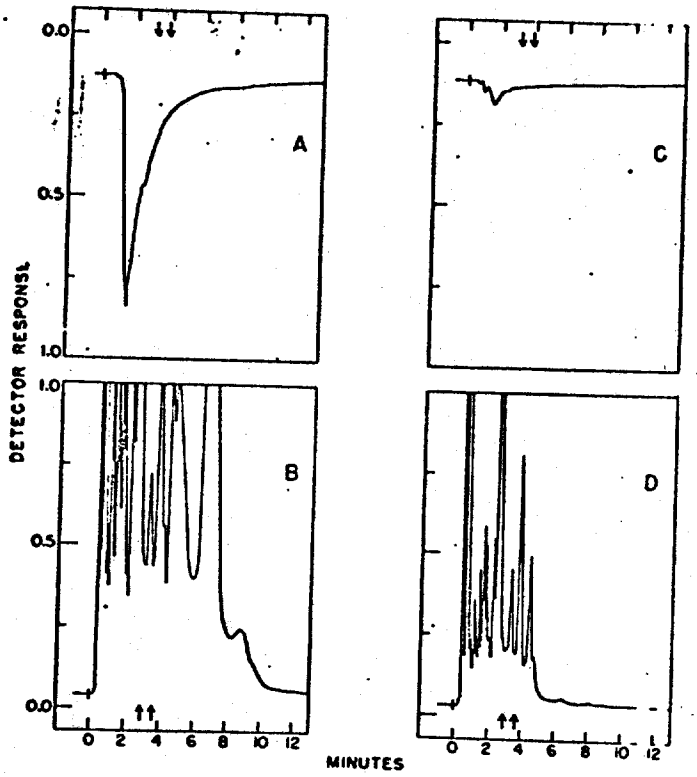


Fig. 3–Chromatograms of 50 µl of concentrate prepared from 100g of smoked fish. Arrows indicate retention times of DMN and DEN. (A) CECD, untreated concentrate; (B) FID, untreated concentrate; (C) CECD, concentrate extracted with 0.01N HCl; (D) FID, concentrate extracted with 0.01N HCl.

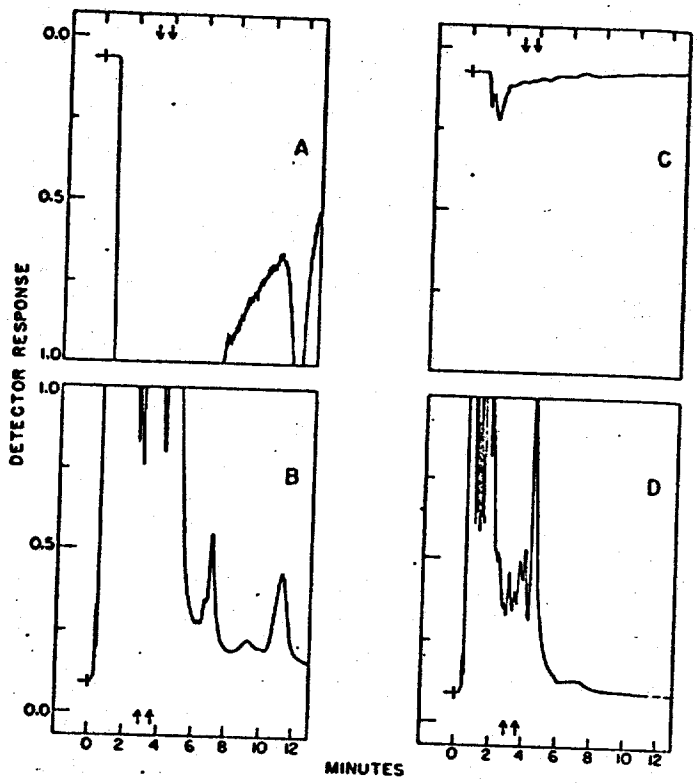


Fig. 4–Chromatograms of 50 µl of concentrate prepared from 100g of canned "potted meat." Arrows indicate retention times of DMN and DEN. (A) CECD, untreated concentrate; (B) FID, untreated concentrate; (C) CECD, concentrate extracted with 0.01N HCl; (D) FID, concentrate extracted with 0.01N HCl.

Recoveries of DMN and DEN from water solutions in initial studies were about 30 and 40%, respectively. Part of this loss was found to be associated with concentration of the large volume of organic extract in a nitrogen stream. This step was replaced by concentration in a Kuderna-Danish apparatus to approximately 5 ml and final concentration to 1 ml in a nitrogen stream. Recovery was also increased by adding a condenser to the top of the distillation-extraction apparatus and by cooling all condensers with ethylene glycol chilled to 0–5°C. With these modifications, recoveries for 10 ng/g aqueous solutions of DMN and DEN were approximately 60 and 70%, respectively, with a distillation-extraction time of 4 hr.

The solvent stripping system permits injection of large volumes (50–100 µl) of dilute solutions without solvent damage to high efficiency GC columns. The precolumn is a high capacity column with efficiency adequate for separating nitrosamines of interest from the solvent (DCM). The system has been used successfully with support-coated open tubular, capillary and conventional packed columns. Considerably less than 1 µl of solvent reaches the analytical column for a 50-µl injection. This technique has proven particularly useful in analysis of dilute solutions by GCMS, where injection of large volumes of solvent must be avoided.

The CECD, operated in the pyrolytic mode, exhibited sensitivity and selectivity necessary to determine small quantities of volatile nitrosamines in food extract concentrates. Figure 2 compares response of the CECD and the FID to a sample containing 50 ng each of DMN and DEN. Levels of approximately 10 ng can be measured routinely with the CECD. Table 1 indicates that under the appropriate analytical conditions the response to nitrosopyrrolidine would be approximately one-tenth that to DMN. With satisfactory recovery from food homogenates, a 50-µl aliquot of a concentrate representing 10 ng/g nitrosopyrrolidine in the original sample would produce an easily measured peak in the chromatogram. It would probably be necessary to

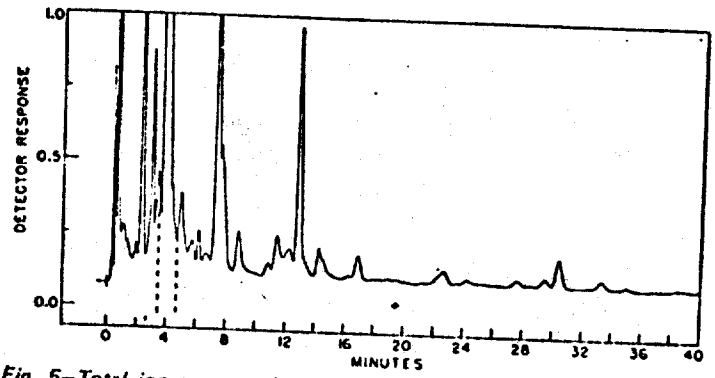


Fig. 5–Total ion current chromatogram of 50 µl of concentrate prepared from chopped ham to which 66 ng/g DMN and DEN were added

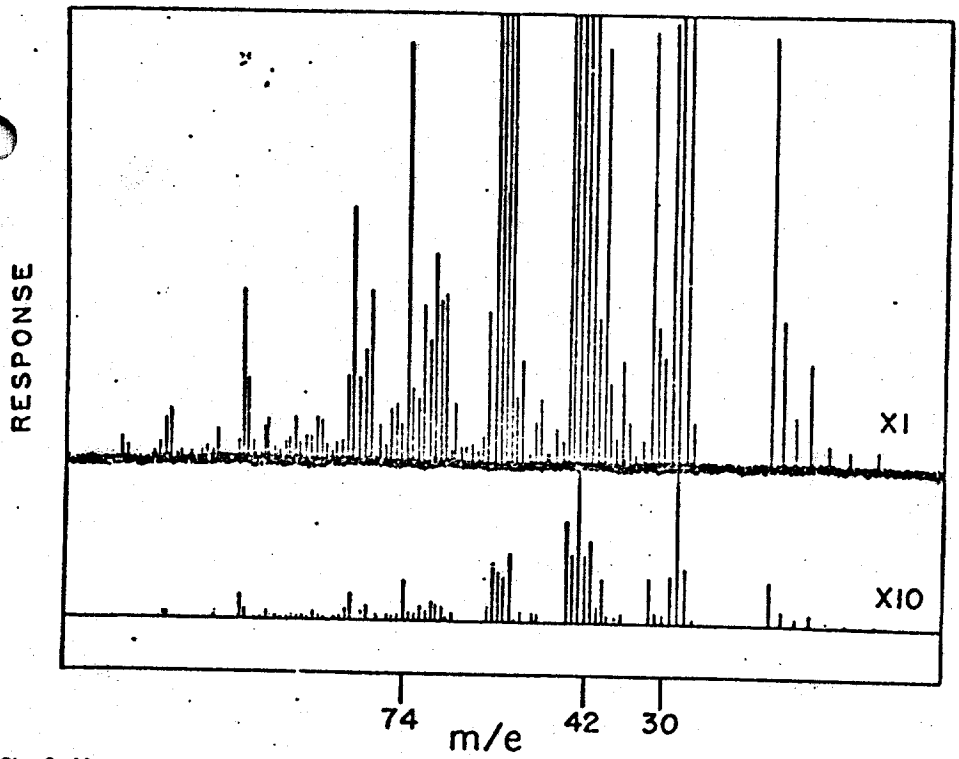


Fig. 6—Mass spectrum recorded at the retention time of DMN from concentrate prepared from canned chopped ham to which 66 ng/g DMN were added.

se high efficiency columns or temperature programming with packed columns to provide GC peaks of sufficient sharpness for routine determination of nitrosopyrrolidine.

The complexity of concentrates prepared from foods by the distillation-extraction procedure is illustrated by Figure 3. A 50-μl sample of a concentrated distillate from smoked fish was analyzed using a splitter to divide the column effluent equally between the CECD (3A) and FID (3B). The effect of a single contact extraction with 0.01N HCl on the CECD and FID chromatograms is shown in Figures 3C and 3D. The times at which DMN and DEN, if present, would elute are indicated on these chromatograms. Figure 4 shows chromatograms of a similarly prepared concentrate of a 100-g sample of "potted meat." In this case, the efficacy of the acid extraction clean-up is clearly shown. Removal of basic compounds from extracts of smoked fish, canned chopped ham, bologna, summer sausage, canned "potted meat," "old fashioned loaf," corned beef spread, Vienna sausage and frankfurters provided sufficient clean-up for specific detection of alkyl nitrosamines with the CECD. GC analysis of DCM solutions of nitrosamines before and after clean-up by acid extraction showed that no DMN or DEN was lost to the aqueous phase. Neither DMN nor DEN was detected at the 10-ng/g level in the limited number of commercial food products examined.

In Figures 3 and 4, CECD sensitivity is such that 50 ng of DMN, corresponding to 100% recovery of 10 ng/g in the original sample would yield a peak amplitude of approximately 30% of full scale. The detection limit is approximately 2 ng/g.

The most difficult interference problem encountered was with a concentrate from distillation-extraction of a sample of canned corned beef. The simple acid extraction was not sufficient to remove interferences detected using both the FID and the CECD. GCMS analysis demonstrated that the chromatographic peaks were not nitrosamines. The inclusion of a column chromatography step using both silica gel and alumina columns produced a concentrate which permitted quantitative determination of alkyl nitrosamines added to the sample. Recovery of DMN and DEN through the column chromatography procedure was greater than 80%.

Figure 5 shows the total ion current chromatogram recorded during GCMS analysis of a concentrate prepared from canned chopped ham to which 66 ng/g DMN and DEN were added. For this analysis, the precolumn was operated at 130°C and the Carbowax 1540 SCOT analytical column was operated at 100°C. A mass spectrum, recorded at the retention time of DMN, is shown in Figure 6. The spectrum indicates that DMN is indeed present, even though the major spectral peaks arise from a mixture of interfering compounds that constitute the GC peak. Repetitive scanning of spectra and plotting of specific ion peaks during elution of GC peaks provides stronger evidence for confirmation of the presence of DMN. Figure 7 shows a series of such plots recorded during elution of the DMN added to the sample. The peaks at m/e 30, 42 and 74 are major peaks in the DMN spectrum. These all attain maximum intensity at the retention time of DMN, while the other more intense peaks (m/e 75 and 71) reach maximum values earlier. Identification of the nitrosamine is limited, in this case, by the extent of

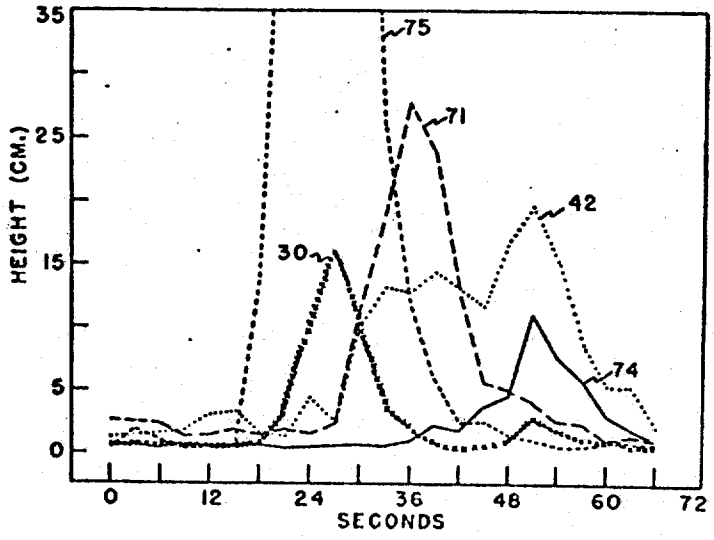


Fig. 7—Plot of individual mass peaks appearing around the retention time of DMN from concentrate prepared from canned chopped ham to which 66 ng/g DMN were added. The peaks at m/e 30, 42 and 74 are major peaks in the DMN spectrum.

separation from interfering compounds. If these data were recorded with an unspiked sample, identification of DMN would have to be considered tentative. Improved separation is required during the GCMS confirmation stage. Open tubular columns may prove helpful for this purpose in the future. Multiple column GC clean-up is feasible and may be necessary if low resolution MS is used for confirmation of identity. Less clean-up is required when high resolution MS is employed (Telling et al., 1971).

The need for unambiguous confirmation of identity of chromatographic peaks tentatively identified as nitrosamines must be emphasized. The approach described provides flexibility so that foods containing no interfering compounds may be examined rapidly and easily. Clean-up steps, consisting of simple extraction, liquid chromatography, or preparative GC may be added as required by the nature of the materials present. A standard analytical method appropriate for all volatile nitrosamines and all foods is not yet available.

REFERENCES

Fazio, T., Damico, J.N., Howard, J.W., White, R.H. and Watts, J.O. 1971a. Gas chromatographic determination and mass spectrometric confirmation of N-nitrosodimethylamine in smoke-processed marine fish. J. Agr. Food Chem. 19: 250.

Fazio, T., Howard, J.W. and White, R. 1971b. Multidetection method for analysis of volatile nitrosamines in foods. Presented at the International Agency for Research on Cancer and the Deutsches Krebsforschungszentrum Joint Meeting on the Analysis and Formation of Nitrosamines, Heidelberg, October.

Issenberg, P., Kobayashi, A. and Mysliwy, T.J. 1969. Combined gas chromatography-mass spectrometry in flavor research: Methods and applications. J. Agr. Food Chem. 17: 1377.

Lijinsky, W. and Epstein, S.S. 1970. Nitrosamines as environmental carcinogens. Nature 225: 21.

Likens, S.T. and Nickerson, G.B. 1964. Detection of certain hop oil constituents in brewing products. Proc. Am. Soc. Brewing Chemists, p. 5.

Nunn, J.R. and du Plessis, L.S. 1971. N-nitrosamine analysis. Part 1. Estimation of low molecular weight alkyl N-nitrosamines. Presented at the International Agency for Research on Cancer and the Deutsches Krebsforschungszentrum Joint Meeting on the Analysis and Formation of Nitrosamines, Heidelberg, October.

Rhoades, J.W. and Johnson, D.E. 1970. Gas chromatography and selective detection of N-nitrosamines. J. Chromatog. Sci. 8: 616.

Sen, N.P. 1971. Multidetection methods for determining volatile nitrosamines in foods. Presented at the International Agency for Research on Cancer and the Deutsches Krebsforschungszentrum Joint Meeting on the Analysis and Formation of Nitrosamines, Heidelberg, October.

Sen, N.P., Smith, D.C., Schwinghamer, L. and Howsam, B. 1970. Formation of nitrosamines in nitrite treated fish. Can. Inst. Food Technol. J. 3: 66.

Telling, G.M., Bryce, T.A. and Althorpe, J. 1971. Use of vacuum distillation and gas chromatography-mass spectrometry for determination of low levels of volatile nitrosamines in meat products. J. Agr. Food Chem. 19: 937.

Wasserman, A.E. 1971. A survey of analytical procedures for N-nitrosamines. Presented at the International Agency for Research on Cancer and the Deutsches Krebsforschungszentrum Joint Meeting on the Analysis and Formation of Nitrosamines, Heidelberg, October.

Ms received 4/21/72; revised 6/3/72; accepted 6/5/72.

Presented in part at the 31st Annual Meeting of the Institute of Food Technologists in New York. Parts of this work were reported at the IARC/DKZ Joint Meeting on Analysis and Formation of Nitrosamines in Heidelberg, Oct. 1971.

Contribution Number 1935 from the Dept. of Nutrition & Food Science, Massachuetts Institute of Technology, Cambridge, MA 02139.

This work was supported in part by Contract No. 70-2180 from the National Cancer Institute, by a grant from the American Meat Institute Foundation, by a fellowship to the senior author from the Campbell Soup Company and by USPHS Training Grant Number FD-00003 from the Food and Drug Administration.

We are grateful to Barbara Klaubert for preparation of the food extracts and to T.J. Mysliwy for the mass spectral analyses. The nitrosamine samples other than DMN, DEN and nitrosopyrrolidine were provided by Dr. W. Fiddler, USDA Eastern Utilization R & D Div., Philadelphia, Pa.

BRUCELLOSIS IN NORTH DAKOTA SWINE

Alice I. Goldsby

Department of Veterinary Science

North Dakota Agricultural Experiment Station

Brucellosis in swine was first described by Traum.[1] Since the original description of the disease there have been numerous reports of the prevelance of swine brucellosis and at the present time there are numerous investigations in progress concerning the economic and public health aspects of swine brucellosis.

It has been quite generally accepted that the swine organism *Brucella suis* is quite pathogenic to man. Evidence also indicates that *Brucella suis* may be pathogenic to cattle.

During the past year or so the livestock producers, public health authorities and veterinarians have been summarizing and reevaluating the existing information on the general problem of brucellosis. The ultimate aim is the complete eradication of this disease.

The diagnosis of brucellosis is based on isolation of the organism from blood or tissues of suspected cases or by the agglutination method. Crawford and Manthei[2] regard an agglutination titer of 1:100 as indicative of brucella infection in swine. It has, however, been shown in numerous investigations that titers of 1:50 or 1:25 may be found in swine with active brucellosis.

In a previous study if was found that North Dakota white tailed deer appear to be very resistant to brucellosis since only 0.22% showed a significant agglutination titer.[3]

The North Dakota Livestock Sanitary Board recently passed a regulation requiring breeding swine to pass a negative brucellosis test before entrance into the state.

It seemed that a survey of the incidence of brucellosis in North Dakota and western Minnesota swine would furnish information of value in the preliminary phase of a program directed against swine brucellosis.

Blood samples of swine were collected on the killing floor of the Armour and Company packing plant at West Fargo. One day's kill was selected as a more or less representative sample of the swine marketed. The serum agglutination test was conducted using U.S.D.A. Bureau of Animal Industry antigen.

Five-hundred-forty-three samples of blood were collected and the serum tested at a 1:25 dilution. One-hundred-twenty-one or 22.3% were either positive or suspicious for the presence of brucella agglutinins. One-hundred-six of these positive sera were retested at

[1] Traum, Jacob, Report of the Chief of the Bureau of Animal Industry, U.S. Department of Agriculture, page 30 (1914).

[2] Crawford, A. B. and Manthei, C. A., Brucellosis of Swine, U.S.D.A. Circular 781 (1950).

[3] Bolin, F. M., Goldsby, Alice I., Rheault, Pat and Eveleth, D. F., Brucellosis in North Dakota Deer, North Dakota Agricultural Experiment Station Bimonthly Bulletin, Volume XI, No. 3, pages 102-103 (1949).

various dilutions. The results are as follows: Of the 106 samples 90 were at least suspicious in the 1:50 dilution and 39 were positive or suspicious in the 1:100 dilution and 10 were positive or suspicious in the 1:200 dilution. At the 1.100 dilution 7.4% of the samples showed complete or partial agglutination.

These data collected on only a small number of swine indicate that 7.4% of the swine killed in a single day at the West Fargo packing plant may be suspected as being infected with brucella organisms.

The incidence of swine brucellosis found in this study is higher than that reported for cattle in the state.[4,5,6]

The findings in this investigation indicate that a vigorous program for the eradication of swine brucellosis is needed.

Summary

Swine killed at the West Fargo packing plant of Armour and Company showed agglutination titers of 1:100 or higher in 7.4% of the animals.

The author is indebted to the officials of Armour and Company and to Dr. J. L. Meyers for assistance in making this study. To Mrs. Pat Rheault and Mrs. Muriel Stringer are due thanks for collecting the blood and aid in completing the tests.

THE TOXICITY OF NITRATES FOR BABY CHICKS

D. F. Eveleth

Department of Veterinary Science

North Dakota Agricultural Experiment Station

Poisoning of chickens by potassium nitrate was investigated by Guberlet[1] who found that the toxic dose was about one gram of the salt per pound of body weight. In these studies single large doses of potassium nitrate in water were given. This investigator lists thirst, anorexia, vomition, diarrhea, retarded heart action, subnormal temperature and cyanosis as symptoms.

Biester and Schwarte[2] in summarizing the literature on sodium chloride poisoning in birds list loss of appetite, severe depression and progressive paralysis followed by respiratory failure as the outstanding symptoms.

[4,5,6] Brandenburg, T. O., Report of the North Dakota Livestock Sanitary Board (1946-47-48).

[1] Guberlet, John E., Potassium Nitrate Poisoning in Chickens, with a Note on its Toxicity, Journal of the American Veterinary Medical Association, Vol. 62, pp. 362-365 (1922).

[2] Biester, H. E., and Schwarte, L. H., Diseases of Poultry, Second Edition (1948).

Abbott[3] has reported on the high nitrate content of well water of some North Dakota wells and pointed out the danger of the use of such water in infant feeding.

The formation of methemaglobin by nitrites produced in the gastrointestinal tract from nitrates has been stressed by numerous investigators of nitrate poisoning in mammals. Guberlet does not mention methemaglobin in his report on nitrate poisoning in chickens.

In some flocks of chickens there are yearly losses of young chicks from unknown causes. It appeared worth while to determine if some of these losses could be due to nitrate poisoning.

In the first trial (Table I) day old chicks were given a standard growing ration containing 1% added sodium chloride. The drinking water was distilled with or without added sodium nitrate or sodium chloride. In these trials all of the chicks did well in the ten day trial except those receiving the water containing 1% sodium chloride. Eighteen of the twenty birds used died with a generalized edema.

Since in this trial there was no evidence of toxicity from the nitrates a second trial was made (Trial II, Table I).

In this case the deaths in the two groups receiving 0.073 molal sodium chloride and sodium nitrate were nearly the same and both groups presented similar pathology.

Chicks dying from both sodium chloride and sodium nitrate showed a generalized edema with or without a deposition of urates on the visceral organs. In neither case was there evidence of methemaglobin formation.

Since chicks did not appear to be capable of reducing nitrates to nitrites and since sodium chloride was equally as toxic as sodium nitrate a different explanation of these death losses was needed.

It has been shown by Scrivner[4] and confirmed by us[5] that by decreasing the "salt" intake of chicks and poults that the incidence of generalized edema can be decreased.

There are also certain similarities in the generalized edema from kidney damage in birds to the formation of urinary calculi in mammals which may or may not cause edema. Some animals often show edema but calculi cannot be grossly demonstrated. Beeson, Pence and Holm[6] have shown that in many cases the primary calculi are

[3] Abbott, G. A., High Nitrates in Drinking Waters, and Their Toxicity to Infants. Proceedings of the North Dakota Academy of Science, Vol. 2 (1949).

[4] Scrivner, L. H., Experimental Edema and Ascites in Poults. Journal of the American Veterinary Medical Association, Vol. 108, pp. 27-32 (1946).

[5] Eveleth, D. F., Bolin, F. M. and Goldsby, Alice I., "Mushy" Chick or Poult Disease. N. Dak. Agricultural Experiment Station Bimonthly Bulletin, Vol. 9, No. 6, pp. 159 (1947).

[6] Beeson, W. M., Pence, J. W. and Holm, G. C., Urinary Calculi in Sheep. American Journal of Veterinary Research, Vol. 4, No. 11, pp. 120-126 (1943).

formed in the kidney tubules of lambs. This appears to be identical to the condition in fowls where uric acid deposits form in the tubules and prevent water elimination thus causing edema.

Cases observed in post mortem examination of "visceral gout" present deposits of uric acid on the visceral organs without edema. It is theorized that the deposition of uric acid in tissues is the result of a failure in kidney function, but which in absence of excessive sodium salts does not produce an effusion of fluid into the tissues.

Summary

Water containing 4000 PPM of nitrate is nontoxic to day old chicks. Water containing 0.073 molal concentrations of either sodium chloride or sodium nitrate has a tendency to produce kidney damage and generalized edema in chicks.

TABLE I.

Toxicity Trials with Sodium Nitrate and Sodium Chloride

Trial I.

	Initial No.	Final No.	Initial Weight	Final Weight
NaCl 1%	20	2	41.5	Edematous
Distilled water	20	20	41.4	94.1
NO_3—				
100 PPM	20	20	42.3	90.5
250 PPM	20	18	41.2	83.2
500 PPM	20	18	40.1	86.2
1000 PPM	20	20	41.0	84.7

Trial II.

Comparison of Toxicity of Sodium Nitrate and Sodium Chloride

	Initial No.	Final No.	Initial Weight	Final Weight
Distilled water	10	10	36.4	98.2
*NaCl .43%	10	7	34.4	102.2
NO_3—				
1:2000 PPM	15	14	34.3	89.3
*1:4000 PPM	15	12	36.2	93.5

* 0.073 molal solutions.

J. Food Sci. 37 (2), 274-276 (1972)

578

T. Y. FAN and S. R. TANNENBAUM
Dept. of Nutrition & Food Science, Massachusetts Institute of Technology, Cambridge, MA 02139

STABILITY OF N-NITROSO COMPOUNDS

INTRODUCTION

VARIOUS ENVIRONMENTAL factors reportedly lead to the formation of carcinogenic nitrosamines in foods and animal feeds, but analytical methodology is inadequate to measure low concentrations of many of these compounds. In fact, no method exists to determine or detect nonvolatile compounds such as the nitrosamino acids. Since little is known about the stability of N-nitroso compounds, some types of structures could be stable enough to survive intact in the highly complex food system under such extreme conditions as thermal processing. We initiated this study to measure the stability of some representative compounds in model systems with which the analytical problem was avoided by choice of the system. The compounds selected for study include nitrosamines and nitrosamino acids; the experimental variables include pH, temperature, and chemical composition of the reacting medium. Although the results are not conclusive for foods, they provide direction for further study.

EXPERIMENTAL

Reagents and chemicals

Dimethylnitrosamine (DMN) was obtained from Eastman Organic Chemicals, Rochester, N.Y. N-nitrosopyrrolidine (NPy), N-nitrososarcosine (NSar) and N-nitrosoproline (NPr) were synthesized from the parent amines and nitrite (White, 1955).

The buffers for pH 2.2–9.2 were prepared from 0.1M citric acid and 0.2M K_2HPO_4. Buffers for pH 9.2–12.5 were prepared from 0.1M NaOH and 0.2M K_2HPO_4. All other chemicals were reagent grade.

Kinetics of decomposition of nitroso compounds

Nitroso compounds were diluted in buffers of desired pH to a concentration of 1 mM. Then 5 ml of the solution was sealed in ampules and heated in an oil bath at the desired temperature. At predetermined time intervals, ampules were withdrawn from the oil bath for analysis.

The kinetics of the decomposition of nitroso compounds was followed by the disappearance of the parent compound. After heating, ampules were opened and diluted 15-fold with pH 7 buffer, and analyzed for nitrite and N-nitroso compound using an automated variation of the photolytic dissociation procedure (Daiber and Preussmann, 1964; Fan and Tannenbaum, 1971).

Reaction of nitroso compound with food constituents

The reaction between nitroso compounds and other food constituents was studied as follows: 1-mM solutions of nitroso compounds were incubated with 0.1% chemical compounds in the appropriate buffer; 5 ml of the reaction mixture was sealed in ampules and heated in an oil bath at desired temperature; samples were withdrawn from oil bath; the content of the ampule was diluted 15-fold and the concentration of N-nitroso compounds determined as previously described.

Separation of DMN and NPy from NSar and NPr

A simple lyophilization apparatus separated DMN (and NPy) from NSar (and NPr) on the basis of differential volatility. Over the pH range 2.2–12.5, DMN and NPy were completely recovered in the distillate and NSar and NPr were completely recovered in the residue.

RESULTS

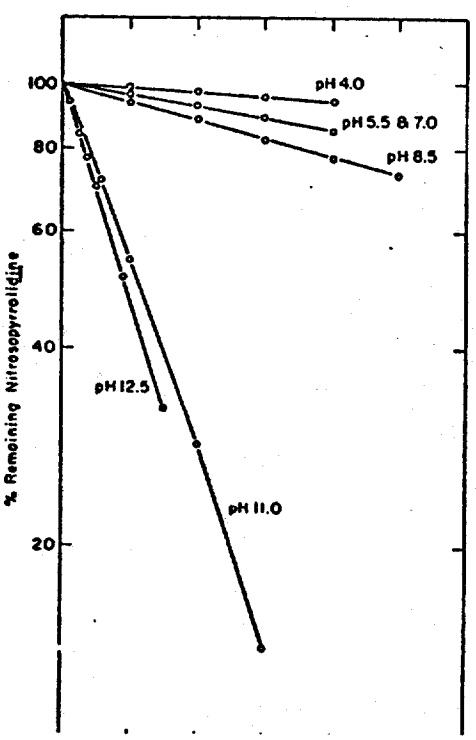
Kinetics of decomposition of nitroso compounds

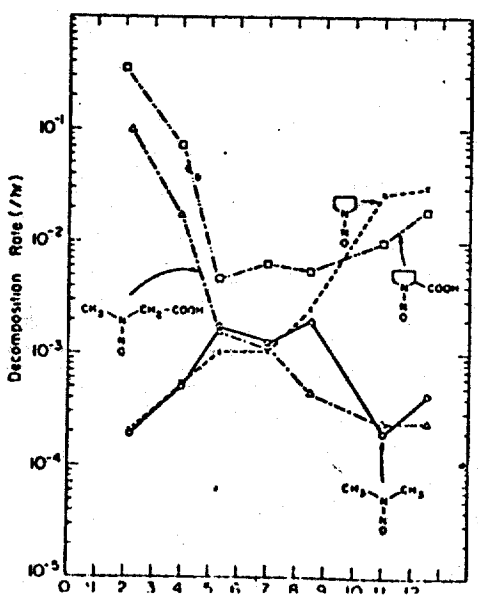
The decomposition of each of the compounds studied follows first-order reaction kinetics. Figure 1 shows typical kinetic data for thermal decomposition of NPy at 110°C at pH 2.2–12.5. Because the decomposition was first order, its rate was easily calculated. The half-life of each compound is given in Table 1 as a function of pH, and the rate constants are shown in Figure 2. Three distinguishable trends are observable:

(1) At acidic pH (2.2–4), compounds with a carboxylic group adjacent to the amino nitrogen (NSar and NPr) have high decomposition rates, about one thousand times faster than compounds of similar structure without a carboxyl group.

(2) At intermediate pH (5.5–8.5), decomposition rate constants for all compounds were similar at approximately 10^{-3} hr^{-1} with NPr being slightly more labile at 5×10^{-3} hr^{-1}.

(3) At alkaline pH (11.0–12.5), compounds with cyclic structure (NPy and NPr) had high decomposition rates: about one hundred times those of compounds without a cyclic structure at similar pH





mpounds. In Figure 3 all the curves ow faster net formation of nitrite in the tial reaction stages. However, the ount of nitrite produced is less than e amount of nitroso compounds decomposed, indicating a complex mechanism of decomposition and/or re-reaction nitrite.

Figure 3 also shows that the amount nitrite formed was dependent on the I at which nitroso compounds were ated, more nitrite being formed in ore alkaline media. Differences in the rmation of nitrite at different pH's are t due only to differences in the decomsition rates of the parent nitroso compounds. For example, at pH 5.5 and 7.0 r NPy, the decomposition rates were entical; however, more nitrite was produced at 7.0 than at 5.5. The alkalinity of e medium apparently affects the ount of nitrite produced or recovered, d, in this respect, is similar to the pH fect observed upon UV-irradiation of trosamines (Fan and Tannenbaum, 71).

onversion of NPr to NPy or NSar to MN

Considering the chemical structures of oth NPr and NSar, one might postulate hat decarboxylation leads to NPy and MN respectively, as suggested by Lijiny et al. (1970). Therefore, solutions of NPr were heated for periods up to 3 hr at H 2.2, 4, 5, 6, 7, 9 and 12.5 and mperatures of 70, 90 and 110°C. No Py was detectable in any case, although ow yields (about 1%) of nitrite appeared the distillate for samples heated at pH -6 and 110°C. Similarly, solutions of Sar were heated at 110°C up to 9 hr at H 2.2, 7 and 12.5. No DMN was found any of the distillates.

eaction between nitroso compounds and her food constituents

The reaction between nitroso compounds and some representative compounds found commonly in foods (glucose, ascorbic acid, cysteine, pepsin and NA) has also been investigated. After 3 r of heating at 70, 90 and 110°C at pH we found no acceleration in the deomposition of NPr. Similar results were und for NPr at pH 9 and NPy at both H 5 and 9.

DISCUSSION

E STUDIED four nitroso compounds hich represent four different structural aracteristics. NPy is a heterocyclic nitrosamine. NSar is a simple dialkylnitrosamine with a carboxylic substitute on the rbon α to the amino group. NPr has th cyclic structure and a carboxylic up. DMN has neither cyclic structure carboxylic group. From this study decomposition of these four com- we see how the introduction of a carboxylic group affects the stability of nitroso compounds to thermal treatment, and we can compare the stability of cyclic and noncyclic nitroso compounds.

Generally, the compounds under study are quite stable. At intermediate pH, their average half-life is 497 hr (21 days) at 110°C. However, the introduction of a carboxylic group weakens the stability at acidic pH. At pH 2.2, the half-life is only 2 hr for NPr and 7 hr for NSar (Table 1). At the same pH, DMN and NPy have a half-life of 3600 hr (150 days). Since the only structural difference for these two classes of compounds is the carboxylic group, we may conclude that this group is responsible for the instability of these compounds. Also, if decarboxylation is a primary decomposition reaction, then NPr could be converted to NPy and NSar to DMN. The significance of this type of conversion would be due to the following:

(1) NPr is a noncarcinogenic compound and NSar shows only weak carcinogenic activity. On the other hand, NPy and DMN are known to be very strong carcinogens (Druckrey et al., 1967; Magee and Barnes, 1967). The conversion of NPr to NPy or NSar to DMN by decarboxylation would be the conversion of a weak or a noncarcinogen to a highly carcinogenic compound.

(2) Under acidic conditions, NPr and NSar have a significant decomposition rate. The mammalian stomach is sufficiently acidic to effect some decomposition, although the temperature is probably too low.

As previously indicated, neither NPy nor DMN could be detected in the reaction mixture when NPr and NSar were subjected to thermal decomposition over a wide pH range. Therefore, if decarboxylation does occur, loss of the nitroso group may occur simultaneously, since the expected products, NPy and DMN, are stable under the reaction conditions.

Denitrosation was a primary reaction during the decomposition of N-nitroso compounds by UV irradiation (Chow, 1967). The product is the short-lived species NO or NO^+, which may combine with hydroxide ions to form nitrite. During thermal decomposition of nitroso compounds, nitrite was released to the reaction medium, therefore it is reasonable that denitrosation is a primary reaction in this process.

Nitrite was not detected at acidic pH (2.2 and 4.0) where the hydroxide ion concentration may be too low to form nitrite. Alternatively, the nitrite may be inherently unstable at these conditions of high temperature and low pH (Taylor, 1928) or may re-react to form more stable organic structures (Chow, 1967).

Many food constituents such as sugars, amino acids, reducing compounds and nucleic acids do not appear to accelerate the decomposition of nitrosamines at elevated temperatures, whereas nitrosoguanidine reacts with both cysteine and protein under physiological conditions (Sugimura et al., 1968; Schultz and McCalla, 1969; McCalla and Reuvers, 1968). Nitrosamines decompose at ambient temperatures with Udenfriend's hydroxylation system (Malling, 1966). Since ascorbic acid is a component of this system, apparently the combination of ascorbate, Fe^{++} and oxygen causes the conversion. Therefore, in the complex milieu of a food, nitrosamines may be less stable than in our model system. However, experiments with foods are compli-

Table 1—Half-lives of N-nitroso compounds at 110°C

	Half-life, days			
pH	NPy[a]	DMN[b]	NSar[c]	NPr[d]
2.2	150	150	0.3	0.08
4.0	55	55	1.7	0.4
5.5	28	16	18	6.0
7.0	28	24	25	4.5
8.5	11.4	15	67	5.3
11.0	1.1	150	120	3.0
12.2	1.0	67	120	1.5

[a]N-nitrosopyrrolidine
[b]dimethylnitrosamine
[c]N-nitrososarcosine
[d]N-nitrosoproline

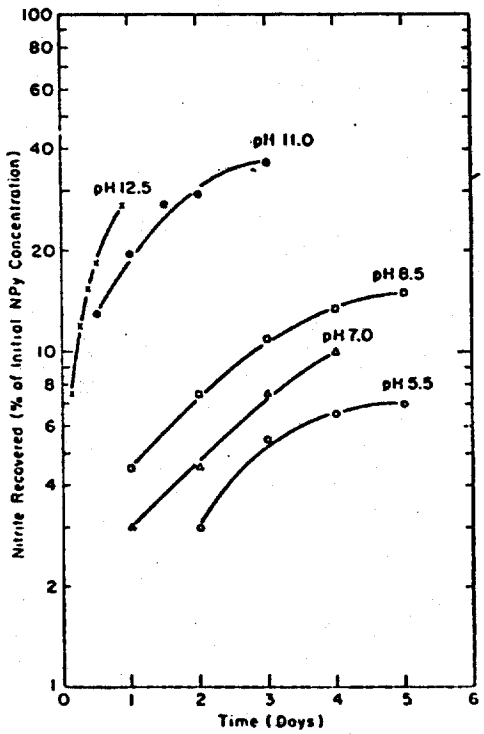


Fig. 3—Kinetics of formation of nitrite during decomposition of N-nitrosopyrrolidine at 110°C.

cated by lack of suitable analytical procedures for some of the compounds investigated.

The behavior of some other nitroso compounds should now be partially predictable on the basis of our experiments. Thus, simple dialkylnitrosamines should behave similarly to DMN, heterocyclic compounds, such as N-nitrosopiperidine, similarly to NPy, and N-nitrosamino acids similarly to NSar.

REFERENCES

Chow, Y.L. 1967. Photochemistry of nitroso compounds in solution. 5. Photolysis of N-nitrosodialkylamines. Canadian J. Chem. 45: 53.

Daiber, D. and Preussmann, R. 1964. Quantitative colorimetrische Bestimmung organischer N-Nitroso-Verbindungen durch Photochemische Spaltung der Nitrosaminbindung. Z. Anal. Chem. 206: 344.

Druckrey, H., Preussmann, R., Isankovic, S. and Schmahl, D. 1967. Organotrope carcinogene wirkungen bei 65 verschiedenen N-Nitroso-Verbindungen and BD-Ratten. Z. Krebsforsch. 69: 103.

Fan, T.Y. and Tannenbaum, S.R. 1971. Automatic colorimetric determination of N-nitroso compounds. J. Agr. Food Chem. 19: 1267.

Lijinsky, W., Keefer, L. and Loo, J. 1970. The preparation and properties of some nitrosamino acids. Tetrahedron 26: 5137.

Magee, P.N. and Barnes, J.M. 1967. Carcinogenic nitroso compounds. Advan. Cancer Res. 10: 168.

Malling, H.V. 1966. Mutagenicity of two potent carcinogens, dimethylnitrosamine and diethylnitrosamine, in Neurospora crassa. Mutation Res. 3: 537.

McCalla, D.R. and Reuvers, A. 1968. Reaction of N-methyl-N'-nitro-N-nitrosoguanidine with protein. Formation of nitrosoguanido derivatives. Can. J. Biochem. 46: 1411.

Schultz, U. and McCalla, D.R. 1969. Reaction of cysteine with N-methyl-N-nitroso-p-toluenesulfonamide and N-methyl-N'-nitro-N-nitrosoguanidine. Can. J. Chem. 47: 2021.

Sugimura, T., Fujimura, S., Nagao, M., Yokoshima, T. and Hasegawa, M. 1968. Reaction of N-methyl-N'-nitro-N-nitroso-guanidine with protein. Biochim. Biophys. Acta 170 427.

Taylor, T.W.J. 1928. The action of nitrous acid on amino compounds. Part 1. Methylamine and ammonia. J. Chem. Soc. 1928: 1099.

White, E.H. 1955. The chemistry of the N-alkyl-N-nitrosoamides. 1. Method of preparation. J. Am. Chem. Soc. 77: 6008.

Ms received 11/14/71; revised 12/28/71; accepted 12/29/71.

This paper is Contribution No. 1878 from the Department of Nutrition & Food Science, Massachusetts Institute of Technology, Cambridge, MA 02139.

The work for this paper was supported by Contract No. NIH-70-2180 from the National Cancer Institute, N.I.H., and a grant from the American Meat Institute Foundation.

Automatic Colorimetric Determination of *N*-Nitroso Compounds

(579)

In this paper, a procedure is described for automatic analysis of any type of *N*-nitroso structure. The new method has been adapted from the classical approach of cleavage of the compound by uv irradiation followed by analysis of released nitrite by diazotization and coupling to form a dyestuff. Automation of the procedure has increased the sensitivity relative to background noise level and eliminated numerous sources of analytical error. When the irradiation used is in the region of 250 nm, the method is not entirely specific and problems arise when samples contain high concentrations of nitrate or *C*-nitroso structures. However, if the irradiation is in the region of 360 nm, the specificity is greatly increased with only a small sacrifice in yield, and the problem of interference by nitrate is eliminated. The analyzer is sufficiently sensitive for individual samples and chromatographic eluents, and has already proved extremely useful in studies of formation and decomposition mechanisms of several *N*-nitroso compounds.

The toxicity and carcinogenicity of most compounds with an *N*-nitroso structure are well established (Druckery *et al.*, 1967; Magee and Barnes, 1967); these compounds have recently been indicated as potential environmental health hazards. Thus it would be useful to have a method to screen large numbers of food and beverage samples at a reasonably low concentration level and in a convenient sample size.

The most specific, sensitive, and quantitive methods available are those which utilize gas chromatography (gc) and mass spectrometry after a suitable cleanup procedure (Howard *et al.*, 1970); the possibility also exists for highly specific gc detectors (Rhoades and Johnson, 1970). These methods, however, are tedious and are applicable only to volatile compounds.

Methods which may be more suitable for repetitive analysis, although they may be equally time-consuming, include the thin-layer chromatographic procedures modified from the initial work of Preussmann and coworkers (1964; modified by Sen *et al.*, 1969); the polarographic procedures (Heath and Jarvis, 1955; Walters *et al.*, 1970); and possible combinations. These methods are also useful for determination of nonvolatile compounds.

Since we found the polarographic procedures relatively nonspecific and the half-wave potential varied considerably according to the structure of the compound, we decided to modify the procedure introduced by Daiber and Preussmann (1964) which utilized the Griess Reagent to measure the amount of nitrite released from a compound after uv irradiation. This paper describes such a procedure which can be used either to process large numbers of individual samples or to monitor the eluate of a chromatographic column (Eisenbrand *et al.*, 1970). An automatic method for determining nitrate and nitrite in water and soil (Henriksen and Selmer-Olsen, 1970) and a comparison of 52 spectrophotometric methods for the determination of nitrite (Sawicki *et al.*, 1963) have been previously reported.

EXPERIMENTAL

Reagents and Chemicals. Dimethylnitrosamine (DMN) and diethylnitrosamine (DEN) were obtained from commercial sources (Eastman Organic Chemicals, Rochester, N.Y.). *N*-Nitrosopyrrolidine (NPy), *N*-nitrosoproline (NPr), and *N*-nitrososarcosine (NS) were synthesized from the parent amines and nitrite (White, 1955).

Sulfanilic acid reagent was prepared by adding 1 g to 50 ml of aqueous acetic acid (30% v/v), stirring for 1 min, and filtering. *N*-1-Naphthylethylenediamine dihydrochloride (0.2 g) was dissolved in 100 ml of 30% acetic acid.

All buffers were prepared from 0.1 *M* citric acid and 0.2 *M* K_2HPO_4. All other chemicals were reagent grade.

Automatic Analyzer. The flow diagram for the analyzer is shown in Figure 1. All equipment (except irradiator) and all tubing are of standard variety as supplied by the Technicon Co. (Tarrytown, N.Y.) and correspond to models listed under Autoanalyzer I. The uv irradiator consists of a black box fitted with two germicidal lamps (General Electric Co., Model G 15T8: short-wave; or Model F 15T8: long-wave). In the irradiator, the sample passes through a quartz coil (Syncor, Inc., Malden, Mass.) of the following dimensions: length, 6.5 ft; coil diameter, 2 in.; tubing inside diameter, 0.11 in.; tubing outside diameter, 0.19 in. The residence time in the irradiator was fixed at 30 min and the distance from the coil to the uv lamps was variable, although nominally 2 in. above the coil (energy density of 2.7 mW/cm^2).

To obtain standard curves, solutions of *N*-nitroso compounds were prepared in buffers or in water over the concentration range 1 to 100 $\times$ 10^{-6} mol/l. (1–100 μM). The solutions were then placed in sample cups for introduction into the analyzer. The sample volume was approximately 0.25 ml.

The uv irradiation process converts *N*-nitroso compounds to a variety of products, depending upon conditions (Chow, 1967), but nitrite is a major final reaction product under conditions of sufficiently high hydroxide ion concentration (Daiber and Preussmann, 1964). The irradiated stream is then mixed with sulfanilic acid and naphthylethylenediamine to form the coupled azo dye. The absorbance of the solution is then measured at the absorption maximum, 550 nm.

The analysis of nitrite and some other types of compounds (*e.g.*, alkyl nitrites) can be accomplished with the same system by simply bypassing the uv irradiator.

RESULTS

Standard Curves. Each of the *N*-nitroso compounds gave linear calibration curves of absorbance *vs.* concentration over the entire concentration range explored. The yields, however, were considerably less than 100% and were influenced by the pH of the sample solution.

The yield of each compound after short-wave irradiation in water and in pH 7 buffer is compared to that of nitrite in Table I. The results are expressed as absorptivities on a molar basis, and the yields can be calculated by comparison to the molar absorptivity of nitrite.

The complex pH behavior of nitrite and dimethylnitrosamine is given in Figures 2 and 3. The nitrite curves in Figure 2 are derived from irradiated and nonirradiated samples and indicate a constant pH-independent loss of nitrite due to

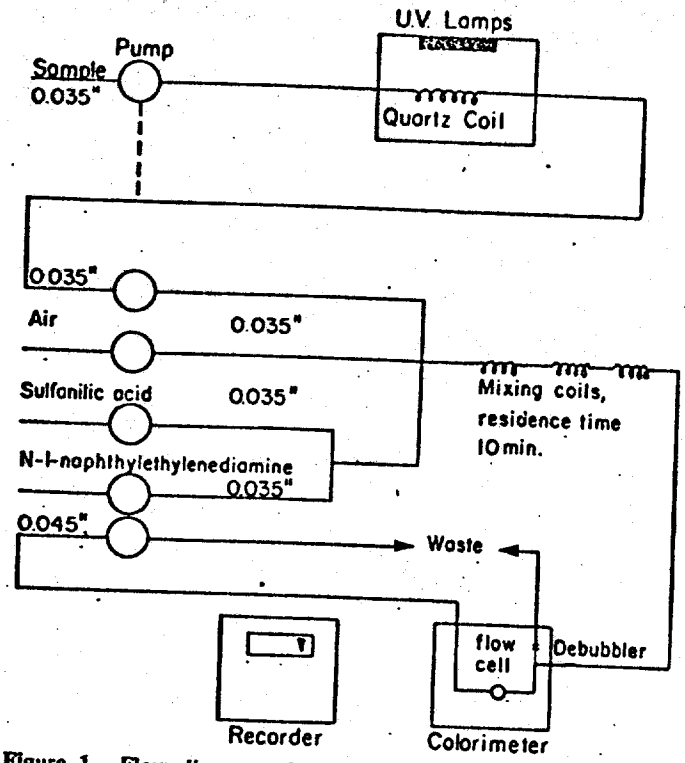


Figure 1. Flow diagram of apparatus for automatic analysis of *N*-nitroso compounds

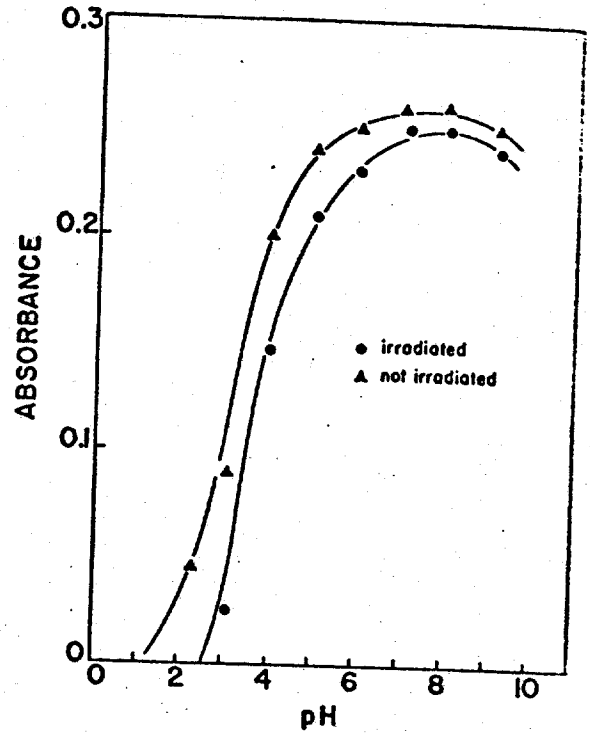


Figure 2. Influence of pH on analysis of nitrite

uv irradiation. The curve for DMN is typical of that of the other *N*-nitroso compounds, which all have linear portions in the pH range 5 to 8. For this reason, the Δ absorbance/Δ pH for each compound studied is given in Table II. Although increased sensitivity could be gained by using pH 8 buffers, a working condition of pH 7 was chosen to give leeway for possible pH variations in the samples.

Interfering Compounds. To determine whether salts of organic and inorganic acids, sugars, nucleoside bases, amino acids, and other compounds would interfere with the analysis, 0.1% solutions of the following compounds were tested: (salts and buffers) KH phthalate, Na acetate, KH_2PO_4, K_2HPO_4, K_2SO_4, Na tartrate, $NaNO_3$, NH_4 oxalate, NaCl, Na citrate, Na formate; (other biochemicals) glucose, fructose, proline, creatine, methionine, cysteine, histidine, tryptophan, *N*-acetylglucosamine, *N*-acetylarginine, arginine, sarcosine, and uracil. None of the compounds listed gave a color reaction with short-wave irradiation, or interfered with the reaction of *N*-nitroso compounds, with the exception of nitrate. Nitrate gives a color reaction only if the sample is introduced through the irradiator, equivalent to a 0.0167% yield of nitrite in distilled water and 0.157% in pH 7 buffer. Therefore, high concentrations of nitrates would be of significance, and the ubiquity of these salts would require special precautionary procedures.

Following the recommendation of Sander (1967), we converted the irradiator to long-wave uv (short-wave ≃250 nm; long-wave ≃360 nm) to eliminate nitrate interference. Under these conditions no detectable nitrite was formed from sodi-

Table I. Molar Absorptivity Yields of *N*-Nitroso Compounds[a]

Compound	Absorptivity, l./mol × 10^3	
	Water	pH 7 Buffer
Dimethylnitrosamine	3.3	3.5
Diethylnitrosamine	2.3	3.2
N-Nitrosopyrrolidine	2.1	2.9
N-Nitrosoproline	2.0	3.1
Sodium nitrite (irradiated)	5.3	5.5

[a] Short-wave uv.

Table II. Influence of pH on Absorbance of *N*-Nitroso Compounds[a]

Compound	Δ A/Δ pH
Dimethylnitrosamine	0.035
Diethylnitrosamine	0.020
N-Nitrosopyrrolidine	0.045
N-Nitrosoproline	0.030

[a] Only over pH range 5 to 8.

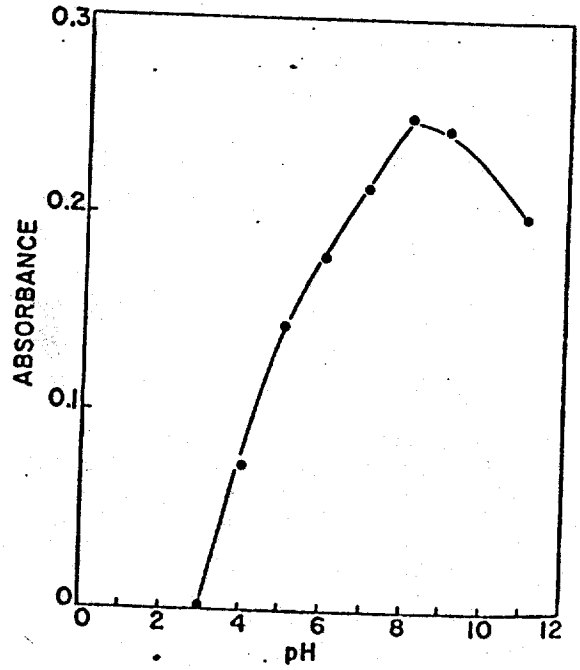


Figure 3. Influence of pH on analysis of dimethylnitrosamine

tions that contained 1000 ppm of nitrate; the molar absorptivity yield of DMN was reduced to 1.5×10^3. Nitrososarcosine also yielded 1.5×10^3.

Application of the Analyzer to the Analysis of Nitrite and *N*-Nitroso Compounds. CASE 1: *N*-NITROSO COMPOUNDS, NO INTERFERENCE BY NITRITE. The sample pH should be known and be in the pH range 5 to 8. The sample is introduced through the irradiator section and its concentration determined with the appropriate calibration curves.

CASE 2: NITRITE, NO *N*-NITROSO COMPOUNDS. The pH of the sample is measured and controlled as above and the sample is introduced after the irradiator.

CASE 3: *N*-NITROSO COMPOUNDS AND NITRITE. The sample is analyzed with and without irradiation, and the sample pH is controlled as in Case 1. The absorbance recorded for the irradiated sample is the sum of that yielded from the *N*-nitroso compound and that from the nitrite. Since some nitrite is lost in the irradiation process, the actual nitrite contribution to the absorbance of the irradiated sample must be corrected for this loss (Figure 2) calculated from the absorbance of the unirradiated sample. Appropriate pH corrections can be made, if necessary, and the concentration of *N*-nitroso compound can be calculated from the corrected absorbance.

DISCUSSION

Several significant improvements in the colorimetric procedure for *N*-nitroso compounds are provided by conversion to automatic analysis. No corrections are necessary for evaporative weight losses in the sample. Problems of unevenness of the irradiation field are eliminated. Since the samples are irradiated in the absence of air, background absorbance is reduced almost to zero (in the presence of air, nitrogen oxides are formed which leads to introduction of extraneous nitrite into the irradiated sample). Irradiation time is controlled, so that yields and background noise are constant.

In addition, problems of variable sample pH and of possible interference by nitrite in the sample had not been previously considered. The pH behavior is readily interpretable from the chemistry of the process. In the irradiation process, the *N*-nitroso compound is usually postulated to yield a short-lived species, NO or NO^+. Under acidic conditions, this species is thought to be recaptured by the elimination product of the parent compound (Chow, 1967), but under conditions of lower acidity, there will be competition from hydroxide ions to yield HONO. Since the p*K* for that acid is approximately 3.4, at pH's above 4, the acid will exist almost entirely as NO_2^-, effectively driving the reaction to form nitrite.

The final pH of the mixture of sample and color reagents also determines the rate of color formation for a fixed nitrite concentration. Although the diazotization reaction of sulfanilic acid proceeds at low pH, the rate of the coupling reaction (with the aromatic amine) increases with increasing pH until the concentration of free amine is a maximum (Weiner and Fieser, 1956). Thus the yield for a fixed reaction time would increase over the pH range 2 to 6. Similar arguments can be used to explain the decreased yields at sample pH above 9, due to effects on the rate of the diazotization reaction.

Certain problems of interference have not been solved, and may require chromatographic separation or cleanup. Walters *et al.* (1970) have demonstrated interference qualitatively by a variety of compounds containing the NO group (nitramines, *C*-nitro compounds, alkyl nitrites, and nitrates). Interference by alkyl nitrites can be corrected by procedures similar to those used for nitrite salts. If the other types of compounds prove to be fairly ubiquitous, one might resort again either to chromatographic procedures or to the use of long-wave uv irradiation, which we have confirmed eliminates the problem of interference by nitrate. Sander (1967) has also indicated that long-wave uv is relatively specific for compounds having the N–NO structure, and is therefore insensitive to C–NO compounds.

The present method provides a linear response to concentrations of *N*-nitroso compounds of less than 1 ppm. This is also the approximate sample concentration required for gas chromatographic analysis with a flame ionization detector (Issenberg, 1971), although the total volume of sample required for gc may be five times less. The selective gc detector of Rhoades and Johnson (1970) would provide even more sensitivity at a lower background noise level. Although a range-expander on the recorder could increase the sensitivity of our analyzer, in practice it is the signal/noise ratio that establishes the actual detection limit. On this basis, it appears that combining the present method with an appropriate liquid chromatographic separation would allow analyses of nonvolatile *N*-nitroso compounds at concentration levels similar to those achievable for volatile compounds with gc and an ionization detector. At the same time, the method will be immediately useful for studies of the kinetics of *N*-nitroso compound formation and decomposition.

LITERATURE CITED

Chow, Y. L., *Can. J. Chem.* 45, 53 (1967).
Daiber, D., Preussmann, R., *Z. Anal. Chem.* 206, 344 (1964).
Druckery, H., Preussmann, R., Ivankovic, S., Schmahl, D., *Z. Krebsforsch.* 69, 103 (1967).
Eisenbrand, G., Spaczynski, K., Preussmann, R., *J. Chromatogr.* 51, 304 (1970).
Fieser, L. F., Fieser, M., "Organic Chemistry," D. C. Heath and Co., Boston, 1956, p 619.
Heath, D. F., Jarvis, J. A. E., *Analyst* 80, 613 (1955).
Henriksen, A., Selmer-Olsen, A. R., *Analyst* 95, 514 (1970).
Howard, J. W., Fazio, T., Watts, J. O., *J. Ass. Offic. Anal. Chem.* 53, 269 (1970).
Issenberg, P., personal communication, 1971.
Magee, P. N., Barnes, J. M., *Advan. Cancer Res.* 10, 168 (1967).
Preussmann, R., Neurath, G., Wulf-Lorentzen, G., Daiber, D., Hengy, H., *Z. Anal. Chem.* 202, 187 (1964).
Rhoades, J. W., Johnson, D. E., *J. Chromatogr. Sci.* 8, 616 (1970).
Sander, J., *Z. Physiol. Chem.* 348, 852 (1967).
Sawicki, E., Stanley, T. W., Pfaff, J., D'Amico, A., *Talanta* 10, 641 (1963).
Sen, N. P., Smith, D. C., Schwinghamen, L., Marleau, J. J., *J. Ass. Offic. Anal. Chem.* 52, 47 (1969).
Walters, C. L., Johnson, E. M., Ray, N., *Analyst* 95, 485 (1970).
White, E. H., *J. Amer. Chem. Soc.* 77, 6008 (1955).

Tsai-Yi Fan
Steven R. Tannenbaum*

Department of Nutrition and Food Science
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Received for review March 31, 1971. Accepted May 13, 1971. The work for this paper was supported by Contract No. NIH-70-2180 from the National Cancer Institute, NIH, and a grant from the American Meat Institute Foundation. This is Contribution Number 1778 from the Department of Nutrition and Food Science, M.I.T.

Toxicants Occurring Naturally in Food. National Academy of Sciences - National Research Council Pub. No. 1354. 1966

585

Nitrates and Nitrites

The occurrence of inorganic nitrates in animal forage and occasionally in some water supplies has, at times, given rise to serious acute toxic effects, especially those resulting from methemoglobin formation. The accidental use of excessive amounts of nitrate–nitrite mixtures in the preservation of the color of meat products has also been associated with this type of injury. Many investigations have been concerned with the factors influencing the nitrate content of plants and the mechanism of toxic action, especially in ruminants. Some authors have considered the possibility that, under some conditions, the intake of sufficient quantities of vegetables of high nitrate content by human beings might result in methemoglobin formation secondary to reduction of the nitrate to nitrite.

As early as 1895, an investigation by Mayo[12] of acute deaths in a herd of cattle in Kansas established clearly that the cause was due to the unusually large quantities of potassium nitrate present in the corn stalks used as fodder. The corn was grown near a barn on land with a high organic nitrogen content and contained as much as 25 percent dry weight of potassium nitrate. The toxic manifestations of tremors, diuresis, collapse, and later of cyanosis were reproduced by dosing cattle with potassium nitrate at about 1.3 g/kg. Although methemoglobin formation due to nitrites was not clearly understood at the time, particular mention was made of the very dark color of the blood, and chemical studies showed evidence for conversion of some of the nitrate to nitrite in the blood and especially in the bile.

This remarkable study thus established the now well-recognized essential elements in the acute toxicity of plant nitrates to livestock,

[illegible] nitrogen fertilization and the reduction of nitrate to nitrite *in vivo* by bacterial action in the rumen.[13] Although most work on the problem has been done with cattle, the same type of acute effects have been seen in sheep,[18] although they appear less sensitive than cattle. Turkeys[17] also show nitrite toxicity after ingestion of high nitrate feeds.

In addition to acute effects, there has been some concern in recent years as to the possible relationship of high nitrate animal diets to vitamin A deficiency, conversion of carotene to vitamin A, and possible effects on the thyroid.[4] Experimentally, some interference by nitrates or nitrites with carotene utilization or vitamin A shortage has been found with calves,[13] rats,[4,15,20] lambs,[6] and chicks.[19] When comparisons were made, nitrites were more potent in these respects than nitrates. Thyroid weight was increased in chicks[19] and rats.[24] Since several thousand parts per million of nitrites and much more of nitrates were used in these experiments, the practical nutritional significance of the findings, although suggestive, does not seem to be clear at present.

The hazard of excessive nitrates in water supplies has been generally recognized since Comly[2] described cyanosis in two infants resulting from the use of well waters containing 64 to 140 ppm of nitrate nitrogen. These waters contained only traces of nitrite ion (0.4 to 1.3 mg/liter). The total intake of nitrate ion was of the order of 0.2 g/day. The possible role of coliform contamination and digestive disturbances was recognized. Many outbreaks have occurred and the subject has been reviewed by Burden,[1] Steyn,[21] and Walton.[23] It is generally agreed that nitrates in water supplies are especially hazardous to young infants because of their relatively higher gastric pH, which facilitates the reduction to nitrite by bacteria. In addition, an infant has a relatively high fluid intake compared with an adult. Water containing in excess of 10 ppm nitrate nitrogen (equals 45 mg of nitrate ion/liter) is considered hazardous for use in infant feeding.[22] Livestock have been affected by nitrate nitrogen in water at levels of 70–150 ppm.

Sodium nitrate and nitrite are permitted in a limited number of meat and fish products at levels of 500 and 200 ppm, respectively. Bacterial reduction of nitrate in these products causes the formation of nitric oxide hemoglobin or myoglobin and results in a comparatively stable red or pink color. Outbreaks of poisoning from the accidental incorporation of excessive amounts of nitrate–nitrite mixtures in meat products have occurred.[16] Two of the ten cases described by Orgeron *et al.*[16] were in children 2 and 3 years old and followed the eating of wieners containing over 5,000 ppm of nitrite.

Lehman[11] does not consider the usual 200 ppm residual nitrite in meats such as corned beef hazardous for adults, but he stresses the lower margin of safety in children because of their lower total hemoglobin content. In addition, the molar ratio between nitrite and hemoglobin was noted to be such that 1 g of sodium nitrite could theoretically produce as much as 460 to 1,800 g of methemoglobin. He advises that the use of nitrates be carefully controlled and that extension of use to other products seems undesirable. Lehman also mentions some unpublished studies that showed sodium nitrate to be tolerated in the diet of rats for a lifetime at levels of 1 percent with no effects and only growth depression at 10 percent. Dogs tolerated 2 percent for 3–4 months. Sodium nitrite was tolerated by rats for 121 days at 93 ppm and by a cat for 105 days at 390 ppm.

Druckrey *et al.*[3] recently considered the possibility that nitrites in foods might cause teratogenic or carcinogenic effects. They point out the possibility of formation of nitrosamines resulting from formation of the nitrosyl cation, NO^+, in the highly acid conditions in the stomach. Long-term studies with rats at levels as high as 100 mg/kg/day of sodium nitrite did not give evidence for a nitrosamine type of carcinogenic effect nor was there evidence of teratogenic action. Some shortening of life span and reduction in hemoglobin concentration were noted. They conclude that the present use is safe but that it might be desirable to reduce it because of the narrow safety margin. The need for better chemical methods of detecting nitrosamines in foods was mentioned.

The possibility of methemoglobin formation in human beings from ingestion of vegetables containing high levels of nitrate has been considered in a number of reports. Wilson,[26] while studying the nitrate content of plants in relation to nitrate fertilization, noted the increased nitrate content of a number of leafy vegetables such as beets, broccoli, cabbage, cauliflower, and lettuce that results from high levels of fertilization. He suggested the possibility that some reduction to nitrite in the digestive tract might occur, although the quantities usually eaten would make it unlikely that nitrate from this source alone would prove hazardous. In a later paper,[25] added information was given on the nitrate content of certain vegetables and baby foods. The content varied considerably but was found to be from about 1,000 to 5,000 ppm for such vegetables as spinach, broccoli, rhubarb, cabbage, and lettuce, but very low for some vegetables such as tomatoes. Strained baby foods contained lower concentrations, for example, two samples of spinach contained only [illegible] ppm. Concern was expressed that

the increasing nitrate content associated with high nitrate fertilization might be hazardous to babies, especially if nitrate intake in some waters was considered.

Gilbert *et al.*[5] reported the high nitrate content of some vegetables but felt it was unlikely that any acute effects would result from normal intakes. Neither Gilbert nor Wilson were aware of any reports of actual cases in human beings. Kilgore *et al.*[9] considered the possibility that the ascorbic acid present in such foods might act as a protective agent against nitrate toxicity. No evidence could be found for this in guinea pigs, however. An earlier study by Kilgore and her associates[8] demonstrated that rats and rabbits could develop some methemoglobin (about 20 percent) if given large amounts of inorganic nitrate in the diet (3–10 g/kg/day). Feeding lower doses of nitrate ion or collard greens containing 0.6 percent NO_3^- (dry weight basis) produced slight amounts of methemoglobin. A nitrate balance study showed only small amounts of nitrite in urine and some of the nitrate remained unaccounted for.

In a study using human subjects, Kübler[10] fed seven infants a diet in which one fifth of the food intake was replaced by spinach containing 68 mg of NO_3^-/100 g of prepared vegetable. The daily intake of NO_3^- ion was about 16–21 mg/kg/day. Feedings continued for 1 week, followed by a week of low NO_3^- intake. Urines were studied for NO_3^- content, creatinine, chloride, and blood nitrite (NO_2^-), and methemoglobin contents were determined. The urinary nitrate increased rapidly with high NO_3^- intake and subsided quickly after cessation of intake. No diuresis was seen. No nitrite or methemoglobin could be found in blood. Kübler felt that the nitrate ion is so rapidly absorbed in the upper gut that normally no bacterial reduction to nitrite occurs. He pointed out that this might not be the case if a gastrointestinal upset was present. He concluded that there was little evidence of a risk to infants from nitrate in vegetables and that the nitrate ion was rapidly excreted without reduction to nitrite.

Recently, Holscher and Natzschka[7] reported two cases of methemoglobinemia in infants (55 percent methemoglobin in one case) after eating spinach puree. The spinach contained large amounts of nitrite (about 218 mg of nitrite ion/100 g wet weight) and only small amounts of nitrate. It was assumed that for some unknown reason the normal nitrate content had been reduced to nitrite during storage. Also of importance in this situation is the sensitivity of fetal and infant hemoglobin to the action of methemoglobin-forming agents such as nitrites.[14]

It may be concluded that, in contrast to ruminants, the risk of either acute or chronic injury to human beings from the naturally occurring nitrate content in vegetables is very low indeed. It is difficult to conceive of any situation in which adults might be affected, and it would seem to require unusual circumstances for methemoglobin to be produced in infants from this source. Under normal conditions, the nitrate ion is very rapidly absorbed and excreted as such with little opportunity for reduction to nitrite. Some conditions under which a high vegetable nitrate intake might impose some added risk to infants are the simultaneous ingestion of high nitrate water or meat, intestinal disturbances, and anemias. While past experience indicates that such situations must have been rare, it is also clear that the unusual sensitivity of infants to methemoglobin-forming agents requires continued study and control of the total nitrate–nitrite content of their food and water. The possibility of accidental reduction of the relatively harmless nitrate ion during storage or processing of vegetables obviously needs further investigation as suggested by the cases described above.[7] Continued attention needs to be given to vegetable nitrate content in relation to fertilization and growing conditions. The nature of any "protective" agents in vegetables as suggested by Kilgore and the existence of any significant thyroid or vitamin A effects are areas for further investigation. Additional information on the metabolism of nitrates and nitrites in human beings, especially infants, seems desirable.

NOTE: In a recent letter to the editor [*Lancet*, *i*, 872 (1966)] Claus Simon of the Pediatric Department of the University of Kiel reports additional cases of nitrite toxicity from spinach. He believes that, if spinach with a high nitrate content from excessive fertilization is allowed to stand at room temperature after preparation, bacterial reduction may occur. This may even occur in frozen spinach. It probably does not occur with canned spinach. He suggests that prepared spinach should not be kept at room temperature, excessive fertilization should be avoided, the nitrate content of spinach used for infant feeding should not be more than 300 ppm, and spinach should not be given in the first 3 months of life.

A useful summary of current conclusions regarding the effect of nitrate fertilization on nitrate concentrations in plants is provided in Agriculture Information Bulletin No. 299 of the Agriculture Research Service, U.S. Department of Agriculture [*The Effect of Soils and Fertilizers on the Nutritional Quality of Plants*, U.S. Govt. Printing Office, Washington, D.C. (Oct. 1965)].

References

1. E. H. W. J. Burden, "The Toxicology of Nitrates and Nitrites with Particular Reference to the Potability of Water Supplies," *Analyst*, *86*, 429 (1961).
2. H. H. Comly, "Cyanosis in Infants Caused by Nitrates in Well Water," *J. Am. Med. Assoc.*, *129*, 112 (1945).
3. H. Druckrey, D. Steinhoff, H. Beuthner, H. Schneider, and P. Klärner, "Prufung von Nitrit auf chronisch toxische Wirkung an Ratten," *Arzneimittel-Forsch.*, *13*, 320 (1963).
4. R. J. Emerick and O. E. Olson, "Effect of Nitrate and Nitrite on Vitamin A Storage in the Rat," *J. Nutr.*, *78*, 73 (1962).
5. C. S. Gilbert, H. E. Eppson, W. B. Bradley, and O. A. Beath, "Nitrate Accumulation in Cultivated Plants and Weeds," *Wyoming Agr. Exp. Sta. Bull. No. 277*, Laramie, Wyoming (1946).
6. E. E. Hatfield, G. S. Smith, A. L. Neumann, R. M. Forbes, U. S. Garrigus, and O. B. Ross, "Interactions of Nitrite, alpha-Tocopherol and 1-Methyl-2-mercaptoimidazole upon the Vitamin A Nutrition of Lambs Fed 'High Nitrate' Silage," *Proc. Soc. Animal Prod., Western Sec.*, *12*, 41 (1961).
7. P. M. Hölscher and J. Natzschka, "Methämoglobinämie bei jungen Säuglingen durch nitrithaltigen Spinat," *Deut. Med. Wochschr.*, *89*, 1751 (1964).
8. L. Kilgore, L. Almon, and M. Gieger, "The Effects of Dietary Nitrate on Rabbits and Rats," *J. Nutr.*, *69*, 39 (1959).
9. L. Kilgore, A. R. Stasch, and B. F. Barrentine, "Relation of Ascorbic Acid to Nitrate Content of Turnip Greens and to Methemoglobin Formation," *Am. J. Clin. Nutr.*, *14*, 52 (1964).
10. W. Kübler, "Die Bedeutung des Nitratgehaltes von Gemüse in der Ernährung des Säuglings," *Z. Kinderheilk.*, *81*, 405 (1958).
11. A. J. Lehman, "Nitrates and Nitrites in Meat Products," *Assoc. Food Drug Officials U.S. Quart. Bull.*, *22*, 136 (1958).
12. N. S. Mayo, "Cattle Poisoning by Potassium Nitrate," *Kansas Agr. Expt. Sta. Bull. 49*, Manhattan, Kansas (1895).
13. P. K. McIlwain and I. A. Schipper, "Toxicity of Nitrate Nitrogen to Cattle," *J. Am. Vet. Med. Assoc.*, *142*, 502 (1963).
14. W. K. Metcalf, "The Sensitivity of Intracorpuscular Haemoglobin to Oxidation by Nitrite Ions. I. The Effect of Growth, Starvation and Diet," *Phys. Med. Biol.*, *6*, 427 (1962).
15. B. L. O'Dell, Z. Erek, L. Flynn, G. B. Garner, and M. E. Muhrer, "Effects of Nitrite Containing Rations in Producing Vitamin A and Vitamin E Deficiencies in Rats," *J. Animal Sci.*, *19*, 1280 (1960).
16. J. D. Orgeron, J. D. Martin, C. T. Caraway, R. M. Martine, and G. H. Hauser, "Methemoglobinemia from Eating Meat with High Nitrite Content," *Public Health Rept. (U.S.)*, *72*, 189 (1957).
17. C. W. Riggs, "Nitrite Poisoning from Ingestion of Plants High in Nitrate," *Am. J. Vet. Res.*, *6*, 194 (1945).
18. K. B. Sinclair and D. I. H. Jones, "Nitrate Toxicity in Sheep," *J. Sci. Food Agr.*, *15*, 717 (1964).
19. J. L. Sell and W. K. Roberts, "Effects of Dietary Nitrite on the Chick: Growth, Liver Vitamin A Stores and Thyroid Weight," *J. Nutr.*, *79*, 171 (1963).

20. G. S. Smith, A. L. Neumann, and E. E. Hatfield, "Carotene Utilization and Vitamin A Nutrition as Influenced by Dietary Nitrite and 'High Nitrate' Silage: Laboratory Studies," *Proc. Soc. Animal Prod., Western Sec.*, *12*, 42 (1961).
21. D. G. Steyn, *The Problem of Methaemoglobinaemia in Man with Special Reference to Poisoning with Nitrates and Nitrites in Infants and Children*, Publ. No. 11, University of Pretoria, Pretoria, Union of South Africa (1960).
22. *U.S. Public Health Service Drinking Water Standards* (1962), p. 47.
23. G. Walton, "Survey of Literature Relating to Infant Methemoglobinemia Due to Nitrate-Contaminated Water," *Am. J. Pub. Health*, *41*, 986 (1951).
24. C. Welsch, R. A. Bloomfield, G. B. Garner, and M. E. Muhrer, "Effect of Nitrate in The Diet on Weight of Thyroid and Adrenal Gland," *J. Animal Sci.*, *20*, 981 (1961).
25. J. K. Wilson, "Nitrate in Foods and Its Relation to Health," *Agron. J.*, *41*, 20 (1949).
26. J. K. Wilson, "Nitrate in Plants: Its Relation to Fertilizer Injury, Changes During Silage Making, and Indirect Toxicity to Animals," *J. Am. Soc. Agron.*, *35*, 279 (1943).

Oxalates

For many years case reports
literature of acute poisoning
particularly those in the *P*
species rhubarb (*Rheum rh*
acetosa L.). Because of the fa
somewhat higher oxalate co
the symptoms has almost in
number of textbooks on pois
of oxalic acid.[3–5,17,18,23,31] S
noted in human beings inges
occurrence of corrosive effec
hemorrhage, renal colic or
Other authors have expressed
rhubarb poisoning. For exa
content of rhubarb leaves is
states that fatal poisoning by
that a person would need to
recorded fatal dose of oxali
content of vegetables such as
unless an unlikely degree of i
that this may not be the cas
vegetable matter.

Although there is no quest
5 g or more) oxalic acid as cr
be fatal with associated co
symptoms, and renal damag

Gas Chromatographic Determination and Mass Spectrometric Confirmation of *N*-Nitrosodimethylamine in Smoke-Processed Marine Fish

588

Thomas Fazio,* Joseph N. Damico, John W. Howard, Richard H. White, and James O. Watts

The gas chromatographic method for the determination of *N*-nitrosodimethylamine in smoked nitrite-treated chub fish has been modified to include other species of fish. The nitrosamine, confirmed by mass spectrometry, was isolated from samples of raw, smoked, and smoked nitrite- and/or nitrate-treated sable, salmon, and shad. Levels of *N*-nitrosodimethylamine found ranged from 4 to 26 ppb (μg/kg).

Magee and Barnes (1967) have described *N*-nitrosamines as one of the most formidable and versatile groups of carcinogens yet discovered. Their role as environmental hazards in the etiology of human cancer has caused growing apprehension among experts (*Brit. Ind. Biol. Res. Ass. Inform. Bull.*, 1968; Eisenbrand *et al.*, 1969; *Lancet*, 1968; Lijinsky and Epstein, 1970). Much of this concern is related to the widespread occurrence of nitrosamine precursors (nitrates, nitrites, and secondary amines) in foodstuffs and their possible interactions to form the *N*-nitroso compounds. The possibility that such reactions could take place in the mammalian stomach after ingestion of foods containing nitrites and secondary amines has also received attention (Sander *et al.*, 1968; Sen *et al.*, 1969). Nitrates are widely distributed in the human environment and are easily reduced to nitrites. In addition, nitrates and/or nitrites are utilized in this country as preservatives and color additives in smoked fish and meats under prescribed limitations (*Federal Register*, 1961, 1962, 1964, 1969). Only meager information is available on the environmental distribution of secondary amines. The presence of dimethylamine and diethylamine, along with some primary and tertiary types, has been noted in fish and other products, but the characterization of other secondary amines has not as yet been accomplished (Lijinsky and Epstein, 1970).

Unfortunately, the question of whether the aforementioned nitrosation reactions do occur in foods containing nitrites and secondary amines and/or in the mammalian stomach after ingestion of such products has not been sufficiently resolved to permit any definitive conclusions. The major obstacle to progress in the area has been the lack of reliable analytical techniques to provide quantitative determination as well as unequivocal identification of the *N*-nitrosamines. Such identifications are mandatory when dealing with the suspected presence of carcinogenic compounds.

Aside from two reports in the literature which are concerned with relatively high levels of *N*-nitrosodimethylamine formed under unusual conditions, substantive analytical evidence for the presence of nitrosamines in foods has not been presented. Ender *et al.* (1967) utilized various spectrophotometric procedures to characterize *N*-nitrosodimethylamine in herring meal treated with excessive levels of sodium nitrite. Duplessis *et al.* (1968) employed nuclear magnetic resonance and infrared spectrophotometry to confirm the presence of the same compound in the fruit of a submarine bush used as food in the Transkei region of South Africa. Ender and Ceh [illegible] have also reported low levels of nitrosamines (range, [illegible] 40 μg/kg) in smoked fish, meat, and mushrooms. However, these authors indicate that it is not possible to guarantee [illegible] exact levels and types of nitrosamines, although [illegible] methods have been used for detection. They also noted [illegible] the possibility of artifacts as well as the risk of contamination during the analysis must be considered. This is in contrast to their earlier studies on fish meal in which, as previously indicated, much larger quantities of *N*-nitrosodimethylamine were involved and confirmation was achieved by infrared spectrophotometry.

Howard *et al.* (1970) reported a gas chromatographic [illegible] procedure for the determination of *N*-nitrosodimethylamine in smoked nitrite-treated chub, which had a demonstrated sensitivity of 10 ppb (μg/kg). They also indicated that mass spectrometry could be utilized to confirm the identity of the nitrosamine at levels as low as the aforementioned 10 [illegible]. Since publication of this work, studies have continued [illegible] other species of fish (sable, shad, and salmon) in which nitrites and nitrates are utilized as preservatives and [illegible] fixatives (*Federal Register*, 1961, 1962, 1964, 1969). These investigations were deemed necessary, since marine fish [illegible] reported to have a higher amine content than freshwater fish (Rea and Shewan, 1949) and, therefore, would present a greater potential for nitrosamine formation. This report describes the results of these studies in which *N*-nitrosodimethylamine has been isolated at levels up to 26 ppb and identification has been confirmed by mass spectrometry [illegible] samples of raw, smoked, and smoked nitrite- and/or nitrate-treated fish. Although confirmation of identity at levels [illegible] below 10 ppb involves an inordinate effort, it was undertaken for some samples to elucidate the extent of processing effects on nitrosamine levels.

EXPERIMENTAL

Materials. The apparatus and reagents used in [illegible] study have been described in detail by Howard *et al.* ([illegible]). The solvents, methylene chloride, methanol, and pentane and the Celite 545 were purified and tested prior to [illegible] assure the absence of interfering peaks.

Gas Chromatography. A Barber-Colman 5000 [illegible] Selectra-System gas chromatograph with a modified [illegible] thermionic detector was employed. A 9 ft × 4 mm [illegible] glass coiled column packed with 10% Carbowax [illegible] 5% KOH on 100/120 mesh Gas Chrom Q support was [illegible] as an alternative to the original column (10% Carbowax [illegible] 3% KOH on Gas Chrom P) with the same chromatographic parameters previously described (Howard *et al.*, 1970). [illegible]

Division of Food Chemistry and Technology, Bureau of Foods and Pesticides, Food and Drug Administration, Washington, D.C. 20204

der the conditions used, a retention time of 14.5 min was obtained for *N*-nitrosodimethylamine.

Gas-Liquid Chromatography–Mass Spectrometry Apparatus. The instrumentation setup for the glc–mass spectrometry combination was identical to the system described by Damico and Barron (1971) except for the following features. Electron impact ionization was utilized and a three-way valve (Hoke, 305 stainless steel tubing; maximum temperature 180° C) was installed between the exit of the column and the separator so that the column effluent could be isolated from the mass spectrometer system in order to vent the solvent front and thereby prevent the solvent from entering the spectrometer. The glc column was a 9 ft × 1/8 in. o.d. stainless steel column packed with 10% Carbowax 1540 + 5% KOH on 100/120 mesh Gas Chrom Q.

Analytical Procedures. During this investigation, the analytical procedure for the determination of *N*-nitrosodimethylamine in chub fish (Howard *et al.*, 1970) was modified to improve the cleanup and recovery values obtained for salmon, shad, and sable fish.

A 37.5-ml aliquot (equivalent to 25 g sample) of the filtered digest was added to a 2 l. boiling flask containing 175 ml of distilled water, 25 g of KOH, and boiling chips. Using the described apparatus (Howard *et al.*, 1970), the solution was then distilled and a total distillate of 75 ml was collected in a 250-ml separatory funnel. The distillate was acidified with 10 ml of 6 *N* HCl and extracted three times (3 min shaking time for each extraction) with 75 ml portions of methylene chloride. The methylene chloride extracts were pooled in a 500 ml separatory funnel and extracted once with 75 ml of 1 *N* NaOH (3 min shaking time). The lower methylene chloride layer was filtered through 35 g of anhydrous Na_2SO_4 held in a 60 ml coarse fritted-glass funnel and prewetted with 25 ml of methylene chloride) into a 500 ml Kuderna-Danish evaporator with a 4 ml concentrator tube attached. The Na_2SO_4 was washed with 50 ml of methylene chloride. The remainder of the procedure, including concentration, column chromatography, and gas chromatographic analysis, was as previously described (Howard *et al.*, 1970).

Preparation of Sample for Confirmatory Glc-Mass Spectrometric Analysis. A 300 ml aliquot (equivalent to 200 g of sample) of the filtered digest was transferred to a 5-l. boiling flask containing 1000 ml of distilled water, 136 g of KOH, and boiling chips. The distillation apparatus (Howard *et al.*, 1970) was connected to the boiling flask. The solution was distilled and 90 ml of distillate (principally methanol) was collected in a 500 ml separatory funnel. The funnel was shaken and replaced with a 1 l. separatory funnel in which 360 ml of distillate was collected.

The 90 ml methanolic distillate was diluted with an equal volume of distilled water and the solution was shaken for 3 min with 180 ml of methylene chloride. After separation of the layers, the lower layer was passed through 50 g of anhydrous Na_2SO_4 (held in a 60 ml coarse fritted-glass funnel and prewetted with 25 ml of methylene chloride) into a 1 l. Kuderna-Danish evaporator with a 4 ml concentrator tube attached. The extraction was repeated two times and the individual methylene chloride extracts were filtered through the Na_2SO_4. Finally, the Na_2SO_4 was washed with an additional 25 ml volume of methylene chloride. Carborundum grains were added in the concentrator tube, the distilling column was inserted, and the solvent was carefully concentrated to 1 ml as described by Howard *et al.* (1970).

The 1.0 ml concentrate and 360-ml distillate were transferred to a 5 l. boiling flask containing 61 g of KOH and boiling chips. The solution was then redistilled (as above); 120 ml of distillate was collected in a 500 ml separatory funnel. The distillate was shaken three times (3 min each time) with 120 ml aliquots of methylene chloride. The extracts were passed successively through 50 g of Na_2SO_4 into the Kuderna-Danish evaporator and evaporated to 1 ml, as described above. The 1 ml concentrate was quantitatively transferred to an acid-Celite column (Howard *et al.*, 1970) and the same procedure was followed subsequently. The methylene chloride was concentrated to a final volume of 0.5 ml, and an 8 μl aliquot was injected into the gas chromatograph, using the modified detector, to ascertain the quantity of *N*-nitrosodimethylamine present. The 0.5 ml concentrate was then carefully concentrated to not less than 100 μl, since further evaporation will result in substantial losses of *N*-nitrosodimethylamine. Prior to each confirmation the standard was injected at a concentration equivalent to that found in the sample. [Note: It was established that at least 1 μg of the *N*-nitrosodimethylamine (or a concentration of 10 ng/μl) must be present for confirmation.]

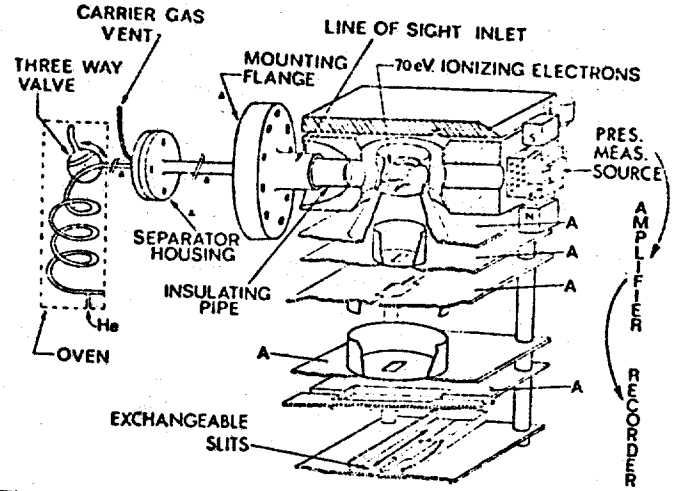


Figure 1. Instrumental setup for glc-mass spectrometric confirmation of *N*-nitrosodimethylamine in fish products. A, Ion optic components of the ion source

Because of these concentration limitations, a 5-μl aliquot (50 ng) of the sample concentrate must be injected on the column in order to obtain an identifiable mass spectrum for confirmation of *N*-nitrosodimethylamine in the fish samples. Accordingly, 5 μl of the sample solution (100 μl total) was injected on the glc column at 70° C with a carrier gas flow of 40 ml per min. The carrier gas vent (see Figure 1) was immediately capped with a silicone septum and the three-way valve was adjusted so that the column effluent was venting to atmosphere in the column compartment. After 3 min, the column was programmed to 120° C at a rate of 10° per min. The three-way valve was adjusted 6 min after the sample injection so that the column effluent was directed against the membrane and the silicone plug was then removed from the carrier gas vent. Initially, the response of the pressure measuring source was negative when the mass spectrometer was isolated from the column effluent. Sometimes, the amplifier must be recompensated during the run because the recorder pen was off scale and attenuating was unsatisfactory, since relatively high sensitivity was needed to detect the *N*-nitrosodimethylamine peak. As the peak eluted from the column, its mass spectrum was recorded at maximum glc response by magnetic scanning. (Note: A background mass spectrum was recorded just before the *N*-nitrosodimethylamine peak.) After the *N*-nitrosodimethylamine eluted from the column, the column effluent was isolated from the

Table III. Mass Spectral Data for *N*-Nitrosodimethylamine Isolated from Marine Fish Samples and 100 ng of *N*-Nitrosodimethylamine Standard

m/e	Relative Intensities (%) for Samples				
	A	B	C	D	E
26	4.5	3.0	...	3.4	7.5
27	17	20	1.0	20	2.5
28	45	35	...	...	...
29	...	...	...	9.3	...
30	9.0	10	1.0	13	20
38	...	8.0	...	...	10
39	12	19	...	17	15
40	9.0	10	3.1	7.6	12
41	23	17	50	42	20
42	100	100	100	100	100
43	9.0	15	62	34	50
53	...	...	...	...	7.5
54	3.4	...	...	...	2.5
55	...	40	59	43	7.5
56	6.0	...	...	2.5	...
58	...	...	...	25	...
59	1.0	0.50	...	4.2	5.0
69	14	8.0	...	14	...
70	14	10	9.4	12	5.0
74	39	47	62	40	90

[a] A, Raw sable; B, shad—smoked nitrite-treated; C, salmon—smoked nitrate-treated; D, sable—smoked nitrate-treated; and E, 100 ng of *N*-nitrosodimethylamine standard.

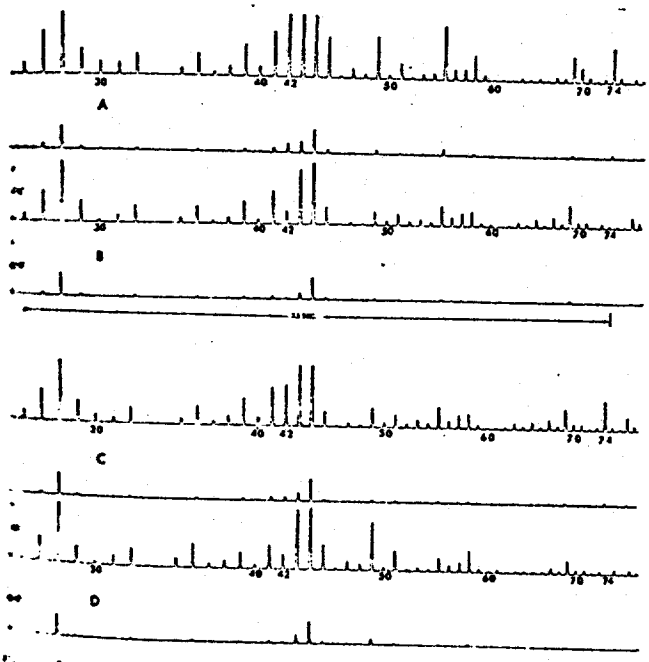


Figure 4. Mass spectra of fish samples and *N*-nitrosodimethylamine. A, *N*-Nitrosodimethylamine isolated from smoked nitrite-treated sable fish; B, background for A; C, 100 ng of *N*-nitrosodimethylamine standard; and D, background for C

The mass spectrum of the background recorded just prior to the elution of the *N*-nitrosodimethylamine peak (designated as 1, Figure 3) is shown in Figure 4 (B). Spectra for 100 ng of the *N*-nitrosodimethylamine standard (C) and background (residual methylene chloride and column bleed) are also shown. All spectra were recorded with the same instrumental parameters, using two recorder sensitivity ranges.

A comparison of the relative intensities obtained for the nitrosodimethylamine isolated from the various fish samples and the 100-ng nitrosamine standard after compensation is presented in Table III. An examination of the data reveals that the relative intensities of the *m/e* 74 ion and the other major mass peaks of the nitrosamine in the fish extracts show some variations from those obtained for the standard. With respect to the lower mass peaks, similar variations were observed in the spectra from successive runs for the 100-ng nitrosamine standard; however, the relative intensities of the *m/e* 74 ion varied only 10 to 17%. The variations in the *m/e* 74 ion may be attributed to the fish background which contributes to the ion current of the nitrosamine at *m/e* 42 (base peak) and, thereby, affects the relative intensity of the mass peak at *m/e* 74. Such variations were not observed in the absolute intensity of the *m/e* 74 ion as presented in Figure 4. The glc response from the pressure measuring source and the maximum peak height of the *m/e* 74 ion obtained for the nitrosamine in the standard and the fish extract, as shown in Table III, were found to be consistent. On the basis of the above data and identical glc retention times we concluded that the component is, in fact, *N*-nitrosodimethylamine.

ACKNOWLEDGMENT

We wish to express our appreciation to Edward O. Haenni, Chief, Additives and Instrumentation Branch, for general guidance, advice, and review of the manuscript.

LITERATURE CITED

Brit. Ind. Biol. Res. Ass. (B.I.B.R.A.) Inform. Bull. 7, 223 (1968).
Damico, J. N., Barron, R. P., *Anal. Chem.* 35, 17 (1971).
Duplessis, I., Nunne, I., Roach, N., *Nature* (London) 22, 1198 (1968).
Eisenbrand, G., Marquardt, P., Preussmann, R., *Z. Anal. Chem.* 247, 54 (1969).
Ender, F., Ceh, L., *Food Cosmet. Toxicol.* 6, 569 (1968).
Ender, F., Havre, G., Madsen, R., Ceh, L., Helgebostad, A., *Z. Tierphysiol. Tierernaehr. Futtermittelk.* 22, 181 (1967).
Federal Register 26, 8973 (1961).
Federal Register 27, 2090 (1962).
Federal Register 29, 14985 (1964).
Federal Register 34, 13659 (1969).
Howard, J. W., Fazio, T., Watts, J. O., *J. Ass. Offic. Anal. Chem.* 53, 269 (1970).
Lancet 1, 1071 (1968).
Lijinsky, W., Epstein, S., *Nature* (London) 225, 21 (1970).
Magee, P., Barnes, J., *Advan. Cancer Res.* 10, 163 (1967).
Rea, G. A., Shewan, J. M., *Advan. Food Res.* 2, 343 (1949).
Sander, J., Schweinsberg, F., Menz, H. P., *Z. Physiol. Chem.* 349, 1691 (1968).
Sen, N., Smith, D. C., Schwinghamer, L., *Food Cosmet. Toxicol.* 7, 301 (1969).

Received for review August 13, 1970. Accepted October 29, 1970.

589

FOOD ADDITIVES

Analysis of Nitrite- and/or Nitrate-Processed Meats for N-Nitrosodimethylamine

By T. FAZIO, R. H. WHITE, and J. W. HOWARD (Division of Chemistry and Physics, Food and Drug Administration, Washington, D.C. 20204)

A total of 51 samples of various meat products was analyzed for N-nitrosodimethylamine with a method demonstrated to be sensitive to 10 ppb. The identity of the amine in one ham sample was confirmed by mass spectrometry at the 5 ppb level, the highest quantity detected in the various samples by GLC analysis. The presence of N-nitrosodimethylamine in the other samples could not be definitely established, since the apparent levels were too low for confirmatory analysis.

In our prior publication (1) it was reported that N-nitrosodimethylamine (DMNA) was not found in the 5 experimental lots of smoked nitrite-treated chubs examined. After modification of this procedure to include other species of fish, trace quantities of DMNA (4–26 ppb (μg/kg)), confirmed by mass spectrometry, were isolated from samples of raw, smoked, and smoked nitrite- and/or nitrate-treated sable, salmon, and shad (2). As a result of these findings it was recommended that meat products processed with nitrite and/or nitrate should be analyzed to determine if DMNA is formed.

Möhler and Mayrhofer (3) have been engaged in the development of analytical procedures for the analysis of nitrite-treated meat products, nitrate-treated cheeses, and flour. The results of the studies reported thus far indicate that nitrosamines, if present, do not exceed the 1 ppm level. The authors conclude, however, that there is much uncertainty as to the positive identification of the amines with their present methodology, which is non-specific and lacking in sensitivity. More recently, Freimuth and Gläser (4) found a commercial sample of cured meat (Kasseler) to contain 40 ppb (μg/kg) of N-nitrosodiethylamine. Ender and Ceh (5) reported levels of nitrosamines ranging from 0.6 to 6.5 ppb in smoked sausage, bacon, and ham. However, these authors indicated that they could not establish the exact levels and types of nitrosamines found, although various methods were used for detection. They further noted that consideration must be given to the possible formation of artifacts as well as to contamination during the analysis.

In view of the dearth of information, it was concluded that factual data on the possible presence of DMNA in nitrite- and/or nitrate-processed meats are urgently needed. The purpose of this report is to describe the results of the analysis of DMNA obtained on 51 meat products.

Method and Description of Samples

The apparatus and reagents used in this study were described in detail in a previous report (1). The methodology employed for the determination of DMNA was that developed for marine fish (2). Briefly, the method is as follows: The comminuted food sample is digested in methanolic potassium hydroxide and the nitrosamine is distilled from alkaline solution, further isolated by extraction and column chromatographic techniques, and determined by GLC, using a modified thermionic detector.

The samples used in this study consisted of 31 meat products obtained on the retail market and 20 supplied by the American Meat Institute Foundation (AMIF). Categories included cold cuts, sausages, hams and bacons, canned meats, baby foods, and fresh and pickled (corned) meats. Some of the products were analyzed both as received and also after cooking according to recipes listed on the manufacturers' packaging material. As necessary, samples were cut into strips, cubed, and comminuted by passage twice through a Hobart chopper (Model 4612, Hobart Manufacturing Co., Troy, Ohio), using a coarse cutting plate.

Results and Discussion of Survey

Prior to the survey, recovery studies were conducted. Recoveries of DMNA added to 100 g meat at a level of 10 ppb ranged from 70 to 90%.

A total of 51 samples of various meat products,

Table 1. Apparent N-nitrosodimethylamine (DMNA) levels found in various meat products[a–c]

Code	Type	Apparent DMNA Found, ppb
	I. Cold Cuts	
1	Bologna	1
2	Bologna	1
3*	Bologna	2
4	Chicken breasts	2
5	Chipped beef	2
6*	Chipped beef	2, 2, 2
7	Chopped ham	1
8*	Cooked salami	2
9	Corned beef loaf	3
10	Liverwurst	4
11*	Liverwurst	2
12*	Old fashion loaf	2
13	Pastrami	1
14*	Pepperoni	3
15*	Pickle loaf	2
16	Salami	1
17	Salami	2
18	Spiced luncheon meat	0
19	Thuringer	2
20*	Thuringer	2
Range	0–4 ppb apparent DMNA	
	II. Sausages	
21	Beef stick	2, 3, 2
22	Frankfurters	1, 2, 1
23	Frankfurters	1
24*	Frankfurters	1
25	Polish sausage	1
26	Pork roll	1
27	Tangy sausage stick	3, 3
Range	1–3 ppb apparent DMNA	
	III. Baby Foods	
28	Beef	2
29	Beef liver	3
30	Chicken	2
31	Ham	2
Range	2–3 ppb apparent DMNA	
	IV. Canned Meats	
32	Chopped beef	2
33	Corned beef	3
34	"Spam"	3
35	"Treet"	3
36*	Vienna sausage	1
Range	1–3 ppb apparent DMNA	
	V. Bacon, Hams, Other Pork Products	
37	Bacon (slab, Smithfield)	2[d], 2[e]
38*	Bacon (slab)	2[e]
39	Canned cherry glaze ham	1
40	Canned ham	0[d], 1[e]
41	Country ham (Smithfield)	3[d], 2[e]
42	Picnic	3[d], 2[e]
43*	Smoked regular ham (bone-in)	5[d,f], 3[e]
44*	Smoked cottage roll (boneless butt)	2[d], 2[e]
45*	Smoked pork jowl	2[e]
Range	0–5 ppb apparent DMNA	
	VI. Miscellaneous Beef Products	
46[b]	Round, inside	2[d], 1[e]
47[b]	Round, outside	2[d], 2[e]
48[b]	Beef knuckle	2[d], 2[e]
49*	Corned brisket	1[d], 2[e]
50*	Smoked tongue	2[d], 2[e]
51*	Corned beef brisket	2[e]
Range	1–2 ppb apparent DMNA	

[a] *Asterisk indicates products of known history supplied by AMIF; all other products obtained on local retail market (Washington, D.C.).
[b] Samples No. 46, 47, and 48 were fresh beef samples, not treated with nitrite and/or nitrate.
[c] For convenience, the measurable peaks present at the DMNA retention time, but not confirmed as DMNA, are calculated as DMNA.
[d] Sample as received.
[e] Sample cooked according to directions.
[f] Sample confirmed by mass spectrometry.

including cold cuts, canned meats, bacon, hams and other pork products, and miscellaneous beef products, were analyzed during this survey. Minute gas chromatographic peaks (peak heights of approximately 3 to 15 mm) with the same retention time as DMNA were observed for the majority of the samples. As shown in Table 1, if these peaks are calculated as DMNA, the levels range from 0 to 5 ppb; however, only 1 of the 51 samples (No. 43, ham) showed an apparent content as high as 5 ppb. Also shown in Table 1 are analytical results obtained after cooking of bacon, hams and other pork products, and miscellaneous beef products. On the basis of these limited results, it would appear that cooking had little or no effect as observed by the GLC peaks obtained.

The only attempt to confirm the identity of the apparent DMNA levels concerned the one ham product (No. 43) found to contain 5 ppb. This sample was checked and the presence of DMNA was confirmed by mass spectrometry. No attempt was made to confirm the analytical results obtained on the other samples, since the majority were too low (1–2 ppb) for mass spectrometric confirmation. It should be noted that Samples No. 46, 47, and 48 were fresh beef samples which were received and analyzed 4 days after slaughter. These products were not treated with nitrite and,

or nitrate, yet they also showe
be calculated as equivalent to
Thus, no definitive conclusion
cerning the identity of the G
for those samples. During the
vestigation, blank runs (withc
through the entire procedure
ever, the minute GLC peaks a
of DMNA, observed with the
not apparent for these blanks

Acknowledgm

We wish to express our app
lowing members of the Divisi
Physics for the indicated as
Haenni, Acting Director, for
advice, Joseph N. Damico

Received February 23, 1971.

REMIND

In 1969, the AOAC
Meeting of the Associa
the convenience of att
green insert at the ba

of nitrate, yet they also showed peaks that could be calculated as equivalent to 1–2 ppb DMNA. Thus, no definitive conclusion can be drawn concerning the identity of the GLC peaks obtained for those samples. During the course of this investigation, blank runs (without a meat sample) through the entire procedure were made; however, the minute GLC peaks at the retention time of DMNA, observed with the meat samples, were not apparent for these blanks.

Acknowledgments

We wish to express our appreciation to the following members of the Division of Chemistry and Physics for the indicated assistance: Edward O. Haenni, Acting Director, for general guidance and advice, Joseph N. Damico for the mass spectrometric studies, and James O. Watts for gas-liquid chromatographic analyses. We also thank John L. Iverson, Division of Food Technology, for helping to perform some of the analyses.

We are also indebted to W. J. Auman and the American Meat Institute Foundation for some of the meat samples used in this investigation.

Received February 23, 1971.

References

(1) Howard, J. W., Fazio, T., and Watts, J. O., *JAOAC* 53, 269–274 (1970).
(2) Fazio, T., Damico, J. N., Howard, J. W., White, R. H., and Watts, J. O., *J. Agr. Food Chem.* 19, 250–255 (1971).
(3) Möhler, K., and Mayrhofer, O. L., *Z. Lebensm.-Unters. Forsch.* 135, 313–318 (1968).
(4) Freimuth, U., and Gläser, E., *Nahrung* 14, 357–361 (1970).
(5) Ender, F., and Ceh, L., *Second Conference on Tobacco Research, Freiburg, Germany* 83–91 (1967).

REMINDER . . .

REGISTRATION FEE

In 1969, the AOAC established a registration fee of $3 for attendance at the Annual Meeting of the Association. The fee has been instituted to help defray the meeting costs. For the convenience of attendees, special advance prepaid registration forms are printed on the green insert at the back of this issue of *JAOAC*.

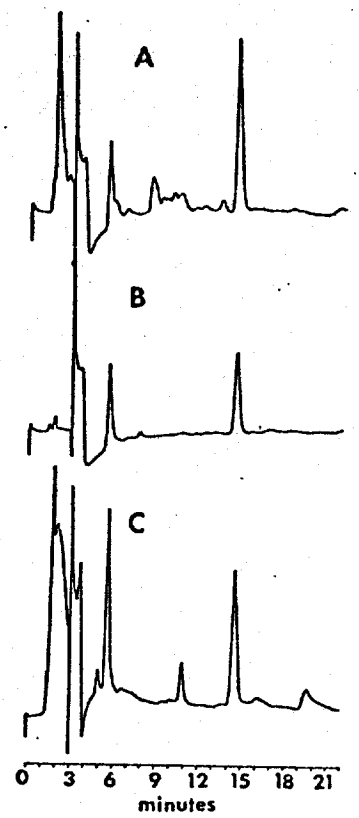


Figure 2. Gas chromatograms of *N*-nitrosodimethylamine extracted from marine fish using modified thermionic detector. A, Isolate from 400 g of smoked nitrite-treated sable; B, isolate from 200 g of smoked nitrate-treated shad; and C, isolate from 400 g of smoked nitrate-treated salmon

Table I. Recoveries of *N*-Nitrosodimethylamine Added at Levels of 10 ppb to 400 g of Fish

Species of Fish	No. of Runs	Range %	Av. %
Salmon	7	71–80	75
Shad	7	71–94	78
Sable	3	72–81	77

Table II. Results of Analysis of *N*-Nitrosodimethylamine in Fish[a]

Type of Processing	*N*-Nitrosodimethylamine Found (ppb) Processing Plant 1	Processing Plant 2
Sable		
Raw	4, 4	4, 4
Smoked	9	5, 4
Smoked, nitrate-treated[b]	14, 12	...
Smoked, nitrate-treated[b]	14, 13	...
Smoked, nitrite-treated	...	8, 9
Smoked, nitrite- and nitrate-treated	...	20, 26
Salmon		
Raw	0	0
Smoked	5	0
Smoked, nitrate-treated	16, 17	...
Smoked, nitrite-treated	...	4, 6
Shad		
Raw	0	...
Smoked, nitrate-treated[b]	10	...
Smoked, nitrate-treated[b]	12	...

[a] Skins and bones removed prior to analysis. [b] Identical samples with exception of packaging.

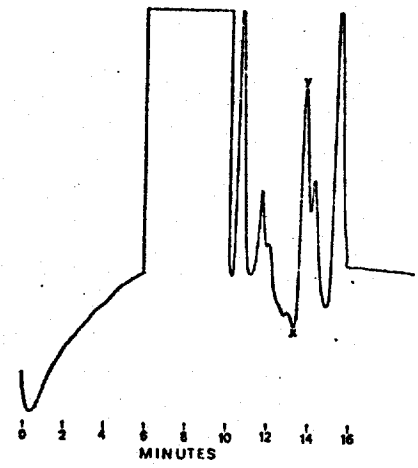


Figure 3. Gas chromatogram of an extract of smoked nitrite-treated sable fish containing *N*-nitrosodimethylamine, utilizing the pressure measuring source as the glc detector

mass spectrometer by adjusting the three-way valve to prevent contamination of the ion source by components with longer retention times than *N*-nitrosodimethylamine.

RESULTS AND DISCUSSION

The sable, salmon, and shad fish utilized in this study were obtained from two processing plants in the United States. The products included raw fish and fish smoked without prior treatment or treated with nitrate, nitrite, or both. All of the smoked fish were subjected to the so-called "hot-smoked process."

Prior to recovery runs, the individual sample lots of fish were comminuted and analyzed for *N*-nitrosodimethylamine by glc. In instances where the nitrosamine was found, the recovery values were adjusted appropriately. As shown in Table I, recoveries of *N*-nitrosodimethylamine added at levels of 10 ppb to 400 g of the sable, shad, and salmon samples ranged from 71 to 94%.

The results of the analyses of the various fish samples collected from the two processing plants are summarized in Table II.

N-Nitrosodimethylamine was found in all the sable samples analyzed at the following levels: raw, 4 ppb; smoked, 4 to 9 ppb; smoked nitrate-treated, 8 to 14 ppb; and smoked, nitrite- and nitrate-treated, 23 ppb (average value of two determinations of one lot). The nitrosamine was not detected in the raw salmon and shad. However, the compound was found in one of the smoked salmon samples at the 5 ppb level and in the smoked nitrate-treated salmon and shad products at levels ranging from 10 to 17 ppb. Representative chromatograms of extracts of smoked nitrite- or nitrate-treated sable, shad, and salmon obtained with the modified thermionic detector are shown in Figure 2.

The presence of *N*-nitrosodimethylamine was confirmed by mass spectrometry in all of the fish samples reported to contain it in Table II. The chromatogram obtained for the smoked nitrite-treated sable, utilizing the pressure measuring source as the glc detector, is presented in Figure 3. The amplifier of the pressure measuring source was recompensated 10 min after the injection of the sample in order to observe peaks with retention times in the region of the *N*-nitrosodimethylamine. This chromatogram contains extraneous peaks which are not observed in the chromatograms (Figure 2) obtained with the modified thermionic detector.

The mass spectrum obtained for the *N*-nitrosodimethylamine (designated as glc peak y, Figure 3) isolated from the smoked nitrite-treated sable is presented in Figure 4 (

treated smoked hake which was found to contain DMN in our previous studies[17]. A strong peak corresponding to DMNA (Fig. 1) thus confirms the identity of DMN.

Although we have only used the technique for the determination of DMN, it is anticipated that other volatile nitrosamines could be similarly converted to the nitramines and detected by GLC. It is hoped that the extreme sensitivity of the technique will be useful for studying the metabolism of various nitrosamines as well as for detecting their presence in the environment.

The author wishes to thank Mrs. BARBARA DONALDSON for technical assistance and Mr. R. C. O'BRIEN for taking the infrared spectrum.

Food and Drug Research Laboratories,
Department of National Health and Welfare,
Ottawa, Ontario (Canada)

N. P. SEN

1 P. N. MAGEE AND J. M. BARNES, *Brit. J. Cancer*, 10 (1956) 114.
2 D. SCHMÄHL AND H. OSSWALD, *Experientia*, 23 (1967) 497.
3 J. SAKSHAUG, E. SÖGNEN, M. A. HANSEN AND N. KOPPANG, *Nature*, 206 (1965) 1261.
4 E. KRÖLLER, *Deut. Lebensm.-Rundschau*, 10 (1967) 303.
5 F. ENDER AND L. ČEH, *Food. Cosmet. Toxicol.*, 6 (1968) 569.
6 L. S. DU PLESSIS AND J. R. NUNN, *Nature*, 222 (1969) 1198.
7 R. PREUSSMANN, G. NEURATH, G. WULF-LORENTZEN, D. DAIBER AND H. HENGY, *Z. Anal. Chem.*, 202 (1964) 187.
8 W. J. SERFONTEIN AND P. HURTER, *Nature*, 209 (1966) 1238.
9 F. ENDER AND L. ČEH, *Alkylierend Wirkende Verbindungen*, Second Conference on Tobacco Research, Freiburg, 1967, p. 83.
10 H.-J. PETROWITZ, *Arzneimittel-Forsch.*, 18 (1968) 1486.
11 N. P. SEN, D. C. SMITH, L. SCHWINGHAMER AND J. J. MARLEAU, *J. Ass. Offic. Anal. Chem.*, 52 (1969) 47.
12 J. K. FOREMAN, J. F. PALFRAMAN AND E. A. WALKER, *Nature*, 225 (1970) 554.
13 J. H. HOWARD, T. FAZIO AND J. O. WATTS, *J. Ass. Offic. Anal. Chem.*, 53 (1970) 269.
14 O. G. DEVIK, *Acta Chem. Scand.*, 21 (1967) 2302.
15 J. H. ROBSON, *J. Am. Chem. Soc.*, 77 (1955) 107.
16 W. D. EMMONS AND A. F. FERRIS, *J. Am. Chem. Soc.*, 75 (1953) 4623.
17 N. P. SEN, D. C. SMITH, L. SCHWINGHAMER AND B. HOWSAM, *Can. Inst. Food. Technol. J.*, 3 (1970) 66.

Received May 4th, 1970

J. Chromatog., 51 (1970) 301–304

530

CHROM. 4846

Separation of carcinogenic nitrosamines on Sephadex LH-20

Chromatographic separations are well-established methods for the analysis of N-nitroso compounds. Thin-layer chromatography on silica gel or alumina[1] and a variety of gas chromatographic methods have been successfully utilised for the separation and identification of these substances[2]. During our investigations of suitable methods for trace analysis of N-nitroso compounds[3–5] we examined whether gel chromatography could be employed as a separation technique for nitrosamines and possibly also for the removal of interfering contaminants.

Materials and me

All nitrosa
by known meth
nation. Fifty per
choice even for hi

A wheat flo
wheat flour with
(rotatory) and the
phase was evapor
adsorbed residue
50 ml were collec

Sephadex L
packed into a 1
eluate showed no

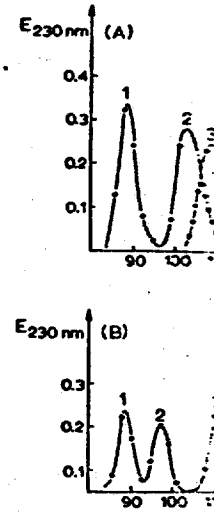


Fig. 1. Gel chroma
eluent, methanol-w
nitrosamine; 2 = 2
1 = 10.5 μg dimet
nitrosamine; 4 = 5
di-n-hexylnitrosami

of nitrosamines d
with black pap
was performed at
concentrations in
using a Zeiss DM

Results and disc

As can be
an efficient sepa

ur previous studies[17]. A
s the identity of DMN.
ermination of DMN, it is
y converted to the nitra-
nsitivity of the technique
rosamines as well as for

for technical assistance

N. P. SEN

re, 206 (1965) 1261.

AND H. HENGY, Z. Anal,

nd Conference on Tobacco

J. Ass. Offic. Anal. Chem.,

45 (1970) 554.
hem., 53 (1970) 269.

23.
an. Inst. Food. Technol. J.,

LH-20

ods for the analysis of
gel or alumina[1] and a
v utilised for the sepa-
estigations of suitable
examined whether gel
f trosamines and

Materials and methods

All nitrosamines investigated were synthesised in our laboratory and purified by known methods; their purity was established by a gas chromatographic examination. Fifty percent aqueous methanol, analytical grade, was the solvent system of choice even for highly lipophilic N-nitroso compounds such as di-*n*-hexylnitrosamine.

A wheat flour concentrate was prepared by extraction of 1000 g of commercial wheat flour with dichloromethane in a Soxhlet for 12 h. The solvent was evaporated (rotatory) and the residue was partitioned in *n*-heptane–acetonitrile[3]. The acetonitrile phase was evaporated together with 100 g of alumina (neutral). The alumina with the adsorbed residue was filled in a column and eluted with 50% aqueous methanol; 50 ml were collected.

Sephadex LH-20 was allowed to swell for 12 h in the solvent and was then packed into a 1 × 100 cm column. The gel was washed with the solvent until the eluate showed no absorption in UV light at 230 nm. To prevent photolytic cleavage

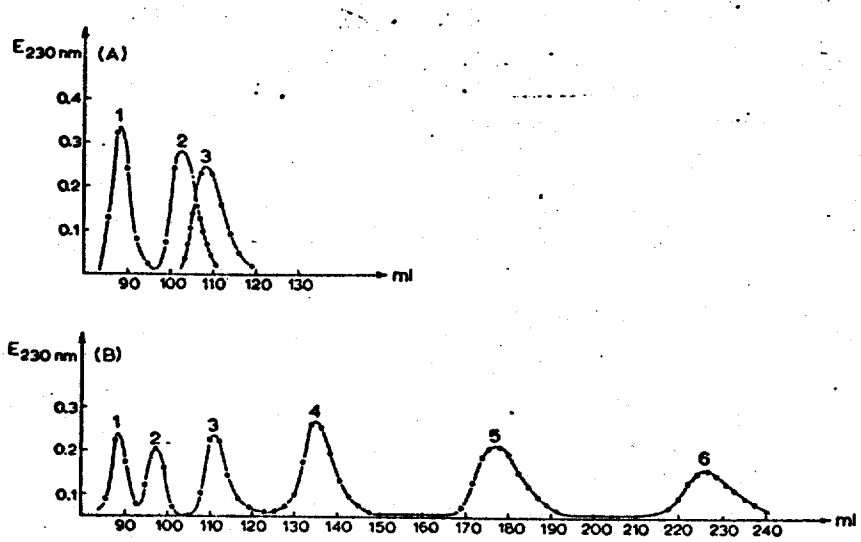


Fig. 1. Gel chromatography on Sephadex LH-20. Column, Sephadex LH-20, 100 × 100 mm; eluent, methanol–water (1 : 1), 4.3 ml/h; sample volume, 0.5 ml. (A) 1 = 19.4 μg methylethylnitrosamine; 2 = 26.4 μg methylbutylnitrosamine; 3 = 25.3 μg methylpentylnitrosamine. (B) 1 = 10.5 μg dimethylnitrosamine; 2 = 14 μg diethylnitrosamine; 3 = 21.5 μg di-*n*-propylnitrosamine; 4 = 50 μg di-*n*-butylnitrosamine; 5 = 74 μg di-*n*-pentylnitrosamine; 6 = 52 μg di-*n*-hexylnitrosamine.

of nitrosamines during chromatography, the column was screened from direct light with black paper. The compounds were applied in a sample volume of 0.5 ml, elution was performed at a rate of 4.3 ml/h and fractions of 2 or 3 ml were collected. Nitrosamine concentrations in the fractions were determined by UV spectroscopy at 230–235 nm, using a Zeiss DMR 21 recording spectrophotometer.

Results and discussion

As can be seen from Fig. 1, gel chromatography on Sephadex LH-20 resulted in an efficient separation of all six symmetrical di-*n*-alkylnitrosamines under the con-

ditions described above. Dimethylnitrosamine was eluted first, followed by diethylnitrosamine, di-*n*-propylnitrosamine and the higher homologues.

The elution sequence of the methylalkylnitrosamine homologues follows the same principle; thus methylethylnitrosamine is eluted before methylbutylnitrosamine which is followed by methylpentylnitrosamine. A similar separation as that shown in Fig. 1 was obtained with the application of 140–270 μg of symmetrical dialkylnitrosamines using a 2 × 100 cm column.

Whilst dialkylnitrosamine homologues which differ in two methylene groups were invariably well separated, the separation of nitrosamines with a difference of only one methylene group was incomplete (methylbutyl- from methylpentylnitrosamine).

The above results indicate that the separation is not based upon a gel filtration process. Apparently the elution behaviour of symmetrical and unsymmetrical nitrosamines under these conditions is determined primarily by absorption effects. The affinity of a given nitrosamine to the Sephadex LH-20 matrix increases with its lipophilic character and can be correlated with the distribution coefficient of the compounds in the system *n*-hexane–aqueous buffer (ref. 6, p. 115).

The usefulness of gel chromatography for the separation of micro-amounts of nitrosamines from biological materials was also investigated. A similar mixture of nitrosamines was dissolved in a 2-ml aliquot of the wheat flour concentrate and applied on the 2 × 100 cm column. Over the whole elution range in which nitrosamines were to be expected, UV-absorbing contaminants from the extract prevented the determination of the substances in the eluate by UV spectroscopy.

Although mixtures of pure nitrosamines can be successfully separated by gel chromatography on Sephadex LH-20, the method as described cannot be used for the separation of nitrosamines from interfering biological contaminants.

This investigation was carried out under contract with the International Agency for Research on Cancer.

Forschergruppe Praeventivmedizin and
Abtlg. für Exp. Therapie, Freiburg (G.F.R.)

G. EISENBRAND
K. SPACZYNSKI
R. PREUSSMANN

1 R. PREUSSMANN, G. NEURATH, G. WULF-LORENTZEN, D. DAIBER AND H. HENGY, *Z. Anal. Chem.*, 202 (1964) 187.
2 L. FISHBEIN AND H. FALK, *Chromatog. Rev.*, 11 (1969) 265.
3 G. EISENBRAND, P. MARQUARDT AND R. PREUSSMANN, *Z. Anal. Chem.*, 247 (1969) 54.
4 G. EISENBRAND, A. V. HODENBERG AND R. PREUSSMANN, *Z. Anal. Chem.*, 251 (1970) 22.
5 G. EISENBRAND, K. SPACZYNSKI AND R. PREUSSMANN, *J. Chromatog.*, 51 (1970) 503.
6 H. DRUCKREY, R. PREUSSMANN, S. IVANKOVIC AND D. SCHMÄHL, *Z. Krebsforsch.*, 69 (1967) 103.

Received May 26th, 1970

J. Chromatog., 51 (1970) 304–306

EN for 35 weeks.
atoxylin and eosin.

oma in pulmonary

EN for 35 weeks.
and eosin. × 150

pulmonary vessels