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The Effects of Dietary Nitrate on Rabbits and Rats[1]

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The growing trend toward the use of fertilizers of high nitrogen content in the production of vegetables leads to the question of possible injurious effects upon those who consume the food thus grown; for under some conditions nitrogen in abundant supply in the soil may accumulate in the form of nitrate in the plant up to levels as high as 6.4% in Swiss chard and 11.1% (as KNO_3) in turnips (Gilbert et al., '46). Nitrate as such is a diuretic, but otherwise has no untoward effects in non-ruminant mammals. Varying amounts of it, however, are probably reduced to nitrite in the system, and this ion is known to exert a number of undesirable effects. Of these the conversion of some of the hemoglobin to methemoglobin is the most easily measured, and is probably potentially the most deleterious. In ruminants, toxic effects from this cause have sometimes proved fatal (Bradley et al., '40; Campbell et al., '54; Davidson et al., '41; Lindner et al., '48; Olson and Moxon, '42; Savage, '49). Effects of this kind in humans are much rarer, but methemoglobinemia and occasional deaths have been reported in infants ingesting well water high in nitrates (Walton, '51). No injury of any degree has been reported for non-ruminants attributable to nitrate in plant food. But as far as we have been able to discover no investigation of marginal toxicity has been published.

The work here reported is concerned primarily with levels of methemoglobin compared with levels of nitrate added to or naturally present in the food of laboratory rabbits and rats. Evidence for the compound responsible for the changes in the blood has been sought by means of nitrate balance studies.

Levels of methemoglobin compared with levels of sodium nitrate added to food

Rabbits and rats were chosen as test animals. There were 10 animals of each species in each test group. Rabbits were of mixed breed, half males, half females, and ranged in age from 11 to 15 months during this experiment. Their weight range was from 3150 to 3750 gm. Male rats were used and they ranged in age from 11 to 13 weeks, and in weight from 170 to 217 gm. They were housed in individual cages and all animals remained healthy throughout the experiments.

A double 5 by 5 interlaced Latin square design was used to minimize the effects due to animals and day sequences. In this design, day sequences were rows, animals were columns, nitrate levels were the dietary treatments, and in the rabbit experiments the time interval of bleeding would be represented by three sub-units in each square (see table 1). The rest period between the day sequences averaged three days since preliminary studies had indicated that a time interval of this duration was adequate in removing carry-over effects of previous treatment.

Trial determinations of methemoglobin were undertaken with rats and rabbits to determine the interval after feeding

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TABLE 2

Methemoglobin, in grams per 100 ml of blood, in animals consuming increasing levels of nitrate. Means of 10 animals. See table 1 for description of rations

	Rations in gm NO_3/kg				
	A 0.007	B 0.15	C 0.15	D 0.29	E[1] 0.73
1. Rabbits—$NaNO_3$ CV[2]—48	0.07	0.11	0.12	0.14	0.16
2. Rats—$NaNO_3$ CV—55	0.11	0.19	0.24	0.26	0.38
3. Rabbits—plant NO_3 CV—46	0.12	0.16	0.18	0.19	0.18
4. Rats—plant NO_3 CV—60	0.04	0.09	0.12	0.19	0.23

Values not underscored by the same line differ significantly from each other at the 1% level.

[1] Possibly due to the taste the rabbits would not always consume all of the ration containing 1 gm $NaNO_3$/kg body weight in a short period of time and the intake was slightly less than 1 gm $NaNO_3$/kg body weight. Also the C, D and E ration for rabbits on plant nitrate were not the same nitrate/body weight ratio as other C, D and E rations. They were limited by amount of collards the rabbit would eat, see table 1, section III.

[2] Coefficient of variability.

of a significant effect on methemoglobin levels of increase in nitrate in the diet.

To determine whether levels of methemoglobin of this order, or associated effects, could be harmful would require more intensive physiological measurements. Gross discomfort as evidenced by abnormal behavior was not apparent. None of the values in table 2 approached the level where cyanosis would appear in humans (Finch, '48) and no cyanosis was seen in the animals. The transitory nature of methemoglobin in the living animal in the absence of a continuing stimulus (Geiinsky, '40; Neill, '25; Spicer, '50) mitigates against injury.

Levels of methemoglobin compared with amount of nitrate fed in plant food

In order to see whether nitrates as they occur in plants would cause a rise in methemoglobin levels of the same order as those accompanying the $NaNO_3$ additions as reported, a similar experiment was performed using collards for rabbits and turnip greens for rats. The collards were grown with no special intent of producing a high nitrate content and contained 0.61% nitrate (dry basis). The turnip greens were grown using cultural conditions known to cause nitrate accumulation and contained 8.85% of nitrate (dry basis).

The methods and design used were the same as those described in the previous experiment. The same rabbits were used after a rest period. The ages ranged from 14 to 18 months and weights from 3150 to 3800 gm. The rats ranged in age from 39 to 43 weeks and in weight from 300 to 340 gm.

The results given in table 2, lines 3 and 4, are similar to those reported in lines 1 and 2 in that highly significant rises in methemoglobin levels accompanied certain ranges of rise in nitrate consumed. The generally higher methemoglobin levels of the rabbits in this study as compared with the previous one, including the higher level on the oat ration, may have been an effect of temperature. We obtained higher methemoglobin levels regularly during hot weather. Differences in temperature between the time of the two rat experiments were not marked and the levels of methemoglobin in the second experiment were lower than in the first one.

Mindful of the relation of methemoglobin to total hemoglobin and the fact that nitrates are known to have a diuretic ef-

fect on humans and animals, hematocrits and methemoglobin were determined simultaneously on rats being fed varied rations. The correlation coefficient was not significant [$r = 0.16$, $r^2 = 0.025$]. Less than 3% of the change in methemoglobin can be accounted for by the change in hematocrit.

Highest levels of methemoglobin found

Since the levels of the methemoglobin reported in the foregoing experiments were not manifestly injurious, an effort was made to discover how high the methemoglobin levels could be caused to go, for this is the crux of our interest in this problem. Some individual animals would eat nitrate more readily than others, and for the purposes of this determination these animals were fed as much sodium nitrate as they would consume. The highest level of methemoglobin recorded under these circumstances was 2.49 gm per 100 ml of blood. This occurred in a rat which was eating nitrate at the rate of 10 gm per kilogram of body weight. A high level of methemoglobin was also obtained in one rabbit owing to a peculiarity which caused its levels to rise above the levels found in other rabbits on the same regimen. This occurred during the preliminary studies and by the time the experiment proper was conducted this animal did not show this difference. During the time when the methemoglobin levels were higher than those of the other rabbits a methemoglobin value of 3.2 gm per 100 ml was obtained with the animal on a ration containing $NaNO_3$ at a level of 3 gm per kilogram of body weight. Some cyanosis was evident in the albino rat at the time of the high methemoglobin. No such observation could be made on the rabbit because of its natural pigment. Methemoglobin levels of the order just cited represent reduction in oxygen carrying hemoglobin of approximately 20%. In non-anemic animals not subject to undue exertion this is probably not serious but in animals already anemic the effects could well be critical.

Balance studies

The foregoing results showing rise in methemoglobin with rise in ingestion of nitrates pose the problem of the mechanism of the reaction. Since methemoglobin formation is known to result from the presence of nitrites in the circulation, the assumption has been made throughout the literature that the toxic effects of nitrate manifested by cyanosis are due to the reduction of nitrate to nitrite ions. Tracer studies involving some analyses on blood and tissue would be the best means of obtaining evidence bearing on this problem. Lacking facilities for such studies, we turned to balance studies to supply some evidence.

Only rabbits were used in the balance studies. The urinary excretion of nitrate at various intake levels was measured. Percentages recovered are given in table 3 and agree in general with the findings of Greene and Hiatt ('54).

TABLE 3

Urinary nitrate recovery in rabbits in 24 hours (10 rabbits, 4 24-hour periods)

Supplement to 90 gm of Purina Chow	Recovery in 24 hours
	%
None (Av. equivalent 22 mg $NaNO_3$/kg body wt.)	49.2
22 mg $NaNO_3$/kg body wt.	35.3
44 mg $NaNO_3$/kg body wt.	35.4
66 mg $NaNO_3$/kg body wt.	41.1
132 mg $NaNO_3$/kg body wt.	48.4
198 mg $NaNO_3$/kg body wt.	56.1

Total nitrogen in the feces was determined at two levels of nitrate intake (protein intake being constant), in order to rule out the possibility of nitrate being excreted in the feces.

In an effort to answer the question, "Is any nitrite excreted, and if so how much?," a modification of the alpha-naphthylamine method of the AOAC was used (Dawson, '56) for the determination of nitrite in the urine of 5 rabbits collected for 4 24-hour periods, while the daily ingestion of each rabbit was 90 gm of commercial chow[4] with 200 mg of NaNO. Nitrates were determined also. Then after a rest period the same 5 rabbits were fed 90 gm of oats for 9 days and nitrite and nitrates were determined in the urine for the last 7 days of this 9 day period. The results of these analyses are shown in table 4.

[4] Purina Rabbit Chow.

TABLE 4

Urinary nitrate and nitrite recovery in rabbits. Mean values of several consecutive 24-hour collections (5 animals per group)

Ration	Mean total nitrate fed per rabbit	Mean total urinary nitrate per rabbit	Mean total urinary nitrite per rabbit	Recovery[1]
	mg	*mg*	*mg*	*%*
90 gm oats	21	9	0.10	44
90 gm Purina Rabbit Chow + 200 mg $NaNO_3$/kg body wt.	2023	774	7.61	39

[1] The percentage recovery was calculated by adding the total urinary nitrate excreted to the total nitrate equivalent of the urinary nitrite and dividing by the total nitrate fed. In each case the nitrate equivalent of the nitrite in the urine represents 0.5% of the amount recovered.

The chow and nitrate solution were analyzed for nitrite and this small amount of nitrite in the ration was subtracted from the amount of nitrite excreted in urine to obtain the urinary nitrite figures in the table. These results agree with those in table 3 and with those reported by Kameoka and Morimoto ('53), who found that when nitrate was fed to rabbits as 2% of their diet it was completely absorbed but only about 50% excreted. These workers also found an increase in the amount of nitrite in the urine where nitrate in the food was increased. This increase in nitrite excretion following increased nitrate ingestion supports the hypothesis of some conversion of nitrate to nitrite.

SUMMARY

Methemoglobin rises significantly in the circulation of rabbits and rats with increases in the levels of nitrate ingested either as sodium nitrate or as it occurs naturally in plant food.

The levels to which methemoglobin rises are not high enough to cause abnormal behavior or appearance except in occasional animals temporarily aberrant due to unexplained causes, or in a larger number of animals ingesting amounts of nitrate greater than would likely occur under natural conditions.

The fact that large amounts of nitrate ingested cannot be recovered in that form in the excreta permits the assumption that some of it is reduced in the body. The recovery of small amounts of nitrite in the urine substantiates this assumption and provides evidence for its existence in the blood where it could act on the hemoglobin. Some nitrate remains unaccounted for and this raises the question of its fate and the reaction in the system of any other products which might be formed.

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Nitrate Content of Beets, Collards, Turnip Greens[1]

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The authors have included a discussion of factors involved in nitrate toxicity when foods containing large amounts are eaten.

HIGH NITRATE fertilization of plants is a common agricultural practice used to improve both the appearance and the yield of crops. Many plants accumulate nitrate when it is available in excess of their current needs. Plants containing high concentrations of nitrates have been known for many years to cause poisoning of livestock (1-4). The mechanism of this poisoning involves a reduction of ingested nitrate ions to nitrite ions which react with the hemoglobin to form methemoglobin. Suffocation and death may result from this methemoglobinemia.

Feeding studies on livestock have shown that increases in methemoglobin are related directly to the amount of potassium nitrate fed (5) and that the minimum lethal dose for intake in calves is in excess of 30 gm. per 100 lb. body weight (6). Simon *et al.* have reported (7), however, that 700-lb. pregnant heifers receiving 140 gm. potassium nitrate per day died several hours after the second dose. Of three pregnant heifers given daily doses of 100 gm. potassium nitrate, one aborted after three doses, one after five doses, and one after thirteen doses. Heifers given 70 gm. daily for forty-seven days calved at the normal time. According to Steward and Merilan (5), increasing levels of potassium nitrate intake were correlated with decreasing levels of milk production, although the composition of the milk was unchanged. The lethal dose of potassium nitrate for cattle appears to range, then, from 0.44 to 0.67 gm. per kilogram body weight per day, with pregnant heifers being particularly sensitive.

Although most reported cases of nitrate poisoning in livestock have occurred in cattle or sheep, the problem is not exclusively related to ruminant animals. Pigs, horses, and turkeys have been affected also (3, 8). Ruminant animals appear to be more sensitive than non-ruminant to nitrate toxicity, however. Dogs fed potassium nitrate in single doses of as much as 20 mEq per kilogram body weight showed no evidence of toxicity (9). Rats fed a single dose of 1 gm. sodium nitrate per kilogram body weight showed no toxic symptoms of methemoglobinemia. Rabbits fed 200 mg. sodium nitrate per day excreted about 50 per cent of this as nitrate in the urine within a period of 24 hr. Small amounts of nitrite were also found in the urine, showing that some conversion of nitrate to nitrite occurred (10). Methemoglobin is transitory in animals, which prevents cumulative effects in the absence of a continuing stimulus for its formation (11, 12).

The only specific cases of methemoglobinemia from excessive ingestion of nitrates to be reported in human beings have occurred in infants fed milk formulas made with well water containing high concentrations of nitrate ions (13). Most of the literature dealing with such cases implicates water containing 60 p.p.m. or more nitrate-nitrogen (14). Cases of methemoglobinemia and asphyxia from water have been reported in children up to three years old (15). Sollmann has reported (16) that 1 gm. potassium nitrate is a safe single dose for humans, whereas 5 gm. per day (as the inorganic nitrate salt) is dangerous for adults (17). According to Steyn (17), infants are 100 times more susceptible

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than adults. Comly indicates (18) that biochemical and physiologic differences in infants and young children are responsible for their increased susceptibility.

Some speculation exists as to whether high nitrate fertilization of plants grown for human consumption might result in foods sufficiently high in nitrate concentration to cause methemoglobinemia (19). However, no evidence has been reported for the occurrence of methemoglobinemia in human beings due to ingestion of foods containing large amounts of nitrates. Analyses have shown that fairly high concentrations of nitrate may be present in some vegetables (20). Turnip greens (21) and beets (22) are known to accumulate excessive concentrations of nitrates. These vegetables, as well as collards, are commonly grown and eaten in Mississippi. Therefore, the following investigation was undertaken to determine the nitrate concentration of these vegetables grown under conditions known to allow accumulation of nitrates, and the effect of various cooking methods on the nitrate concentration remaining in portions of these vegetables as served.

Experimental Procedure

Early Blood beets, Georgia collards, and Shogoin and Purple Top White Glove turnips were grown during 1959 and 1960 with high sodium nitrate fertilization. Plastic screening material was used to give sufficient shade to favor accumulation of nitrates in the plants. Plants were harvested at periodic intervals of about five days.

TURNIP GREENS AND COLLARDS

In preparation for analysis, turnip greens and collard leaves were washed thoroughly, blotted dry, and cut lengthwise through the mid-ribs. To minimize variation between leaves, half of each leaf was cooked and the other half was used as a raw sample. Three hundred-gm. samples were cooked by each of the following methods:

(a) Seven min. at 15 lb. pressure with ⅔ cup water.

(b) Twenty min. in a covered pan with 2 cups water.

(c) Two hr. in a covered pan with 8 cups water.

One-half tsp. salt was added in each case. After cooking, samples were drained for 2 min. and the liquor saved as a separate sample. Both cooked and raw vegetables were blended with water to make a homogeneous mixture. Aliquots of this mixture were used as samples. The 1959 samples were analyzed for nitrates by the xylenol method of the Association of Official Agricultural Chemists (23) as modified by U. S. Plant, Soil and Nutrition Laboratory (24), and the 1960 samples by the reduction and distillation method of Whitehead (25).

BEETS

To avoid cutting the beets, it was decided to use every third beet picked as the raw sample. Beets were prepared for analysis by washing and trimming off tops and spare roots. Eight to ten beets were then weighed and cooked whole by either of the following methods:

(a) Fifteen min. at 15 lb. pressure with 3 cups water.

(b) One hr. 15 min. in a covered pan with 6 cups water.

After cooking, the beets were drained and the liquor saved as a separate sample. Raw as well as cooked beets were peeled before blending to make the analyses comparable.

Aliquot samples were analyzed as above for nitrates.

TABLE 1 Paired comparison of mean total nitrate between raw vegetables and those cooked by various methods

COOKING TIME	MEAN TOTAL NITRATE		t*
	Before cooking	After cooking	
Collards			
	mg./100 gm. raw wt.		
7 min.	248.3	264.3	1.30
20 min.	276.1	281.9	.38
120 min.	293.3	288.0	.80
Turnip Greens			
7 min.	136.9	141.6	.38
20 min.	157.5	151.2	.94
120 min.	146.8	155.3	1.02
Beets			
20 min.	224.4	252.0	1.40
75 min.	224.6	230.5	.25

*t value required for significance at the 5% level is 2.36 for collards, 2.57 for turnip greens, and 2.45 for beets.

Results and Discussion

The nitrate content of the vegetables grown in 1960 was calculated as milligrams nitrate in a 100 gm. raw sample and in the resultant cooked vegetable. To determine whether there was any over-all loss of nitrates, a paired comparison method of analysis was made on the data for nitrates found in the raw and cooked (including cooking liquor) vegetables. The t values were computed based on the following number of paired observations for each cooking method: 8 for collards, 6 for turnip greens, and 7 for beets. These t values, given in Table 1, show that none of the cooking methods used produced a change in total nitrate content significantly different from that found in the fresh vegetable. Apparently, then, there is no destruction of nitrates due to cooking.

Since nitrate salts are water soluble, it would

be expected that the amount of nitrate removed from the vegetable to the cooking liquor would increase with the amount of cooking water. Collards and turnip greens cooked by Method c and beets cooked by Method b should, therefore, contain a smaller concentration of nitrates than those cooked by the other methods.

With all three vegetables, those cooked by Method a retained higher concentrations of nitrates in the vegetable itself than those cooked by the other methods. This difference was significant for beets and highly significant for collards and turnip greens. The difference between cooking Method b and cooking Method c was highly significant for collards but not significant for turnip greens. Apparently the difference between the use of 2 cups and 8 cups water had a greater effect on the collards than on the turnip greens. This effect may have been due to the fact that collards are more bulky than an equal weight of turnip greens and therefore require more water, or more time, for thorough soaking of the leaves. Beets showed the same effect but to a lesser extent. This finding would have been expected, also, since the beets were cooked whole and, therefore, had less surface area exposed to the cooking water than the other vegetables. Apparently there was considerable transfer of nitrate from the vegetable to the cooking liquor.

The amount of nitrate contained in 1-cup portions, double the average serving, was calculated. One cup turnip greens was assumed to weigh 145 gm.; 1 cup collards, 190 gm.; and 1 cup beets, 165 gm. (26). To further simulate actual use, 2 Tbsp. (25 ml.) cooking liquor were considered as added to the collards and turnip greens and none to the beets. Results of analyses of vegetables harvested during 1959 and 1960 were used for the calculations. Data from twelve harvests of collards, ten harvests of turnip greens, and thirteen harvests of beets were analyzed for variance. The data obtained with each cooking method were tested by Tukey's Honestly Significant Difference Test (27). The results are shown in Table 2.

Even though the particular vegetables analyzed here were grown under special conditions to increase the nitrate content, the amounts of nitrate found were not unusually high. Several purchases of canned greens gave values for nitrates even higher in some cases than those found in our vegetables. A purchased sample of fresh turnip greens was also found to be higher in nitrate than our specially treated samples. The results reported here would tend toward a low average value, since the harvests were made periodically during the whole growing season and the nitrate in leaves decreases considerably as the leaves grow older. Individual samples from another source might contain different amounts of nitrate depending, among other things, on the age of the leaves.

One gm. potassium nitrate is considered a safe single dose for human beings. This amount of potassium nitrate is contained in 1.1 cups collards or 1.7 cups each of beets and turnip greens and, therefore, could be ingested fairly easily. These calculations disregard the fact that additional nitrate may be present in the diet if meat containing potassium nitrate or nitrite as a preservative or coloring agent is cooked with the turnip greens or eaten at the same meal.

Water containing 60 p.p.m. nitrate nitrogen is considered hazardous for use with infants and small children. An infant receiving 16 oz. of such water would be ingesting only 0.20 gm. potassium nitrate, an amount contained in approximately ⅓ cup turnip greens or beets or ¼ cup collards. Small children (up to three years old) could easily ingest more than these amounts of vegetables, with subsequent occurrence of methemoglobinemia. Little is known concerning the effects of feeding diets high in nitrate content to infants and small children, persons suffering from various illnesses, or to pregnant or lactating women. The amounts of nitrate found in vegetables do not appear to be great enough in themselves to cause harm to adults in the absence of such complications as anemia, renal dysfunctions, or intestinal diseases. Since infants and small children are especially sensitive to nitrate toxicity, and, since as little as 0.20 gm. potassium nitrate in well water can be toxic to infants, a note of caution appears advisable in the feeding of vegetables containing this amount, or more, of potassium nitrate.

However, since no cases of methemoglobinemia from ingestion of such vegetables have been re-

TABLE 2 Mean nitrates per cup of vegetables cooked by various methods†

COOKING TIME	MEAN NITRATE IN COOKED VEGETABLE
Turnip Greens	
	mg./c.
7 min.	357.6
20 min.	238.8**
120 min.	178.8**
Collards*	
7 min.	571.7
20 min.	445.2
120 min.	262.5
Beets*	
20 min.	358.1
75 min.	293.3

*Beet samples differed from each other at the 5% level; collard samples differed from each other at the 1% level.

**Samples differed from 7-min. cooking samples at the 1% level.

†Mean nitrate in 1 cup represented mean nitrate in 145 gm. turnip greens plus 25 ml. cooking liquor for ten harvests; in 190 gm. collards + 25 ml. cooking liquor for twelve harvests; or in 165 gm. beets for thirteen harvests.

ported in infants or small children, there may be unknown factors which affect nitrate toxicity. Perhaps when vegetables containing high nitrate concentrations are ingested, other nutrients ingested simultaneously act as protective agents to prevent reduction of nitrate to nitrite. The presence of oxidizing agents, such as the tocopherols, in vegetables may prevent nitrate reduction. Results of feeding studies on cattle extrapolated to man show that 30.8 gm. potassium nitrate per day (for a 70-kg. man) would cause death. Results of feeding studies on non-ruminant animals extrapolated to man indicate that ingestion of 140 gm. potassium nitrate per day would cause no appearance of symptoms of methemoglobinemia. Either of these extrapolated values is well above the accepted standard for safety of 1 gm. potassium nitrate per day, which may indicate that nitrate in foods is less toxic than nitrate as the inorganic salt.

Further work is required on the effects of other nutrients in vegetables on the reduction of nitrate to nitrite before any conclusions, particularly with regard to the feeding of small children and infants, can be drawn.

Summary

Collards, turnip greens, and beets were grown under conditions known to allow high nitrate accumulation in the plants. Collards and turnip greens, cooked by three, and beets cooked by two methods were analyzed for nitrate before and after cooking. Although no over-all loss of nitrate occurred during cooking, some nitrate was transferred from the vegetable to the cooking liquor, the amount apparently being dependent to some extent on the amount of water used in cooking and to the cooking time. Discussed are some factors involved in nitrate toxicity as well as some factors which may prevent nitrate toxicity when foods containing fairly large amounts of nitrate are ingested.

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Relation of Ascorbic Acid to Nitrate Content of Turnip Greens and to Methemoglobin Formation

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HIGH NITRATE fertilization coupled with low light intensity can result in an increase in the nitrate content of turnip greens and a concomitant decrease in ascorbic acid content.[1,2] Since both ascorbic acid and nitrate are leached into cooking water, an opportunity is presented for reduction of nitrate by ascorbic acid. If oxidative loss of ascorbic acid occurs in this manner, the presence of considerable nitrate could decrease the nutritive value of cooked greens. These experiments were initiated to study the relationship of nitrate concentration to both the ascorbic acid content of raw greens and to the loss of ascorbic acid during cooking.

The amounts of nitrate and ascorbic acid present in cooked turnip greens must be considered from another standpoint also. Many cases of methemoglobinemia have been reported in infants drinking formulas prepared with water containing a large concentration of nitrates.[3,4] Even though levels found in vegetables[5,6] are often high enough to be a potential cause of methemoglobinemia, no evidence of nitrate toxicity from eating vegetables has been reported. In fact, Kübler[7] fed spinach containing nitrates to infants for two weeks without finding conclusive symptoms of methemoglobinemia. Since ascorbic acid has been used to reduce methemoglobinemia in animals and human beings, its presence in vegetables may be the protective agent against nitrate toxicity. This possibility was investigated using human blood and guinea pigs.

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MATERIALS AND METHODS

Relation of Nitrate and Ascorbic Acid

Shogoin and Purple Top White Glove turnips were grown on three plots. Plot 1 received a basic fertilization of 2,000 pounds 8-8-8 per acre and was unshaded. Plot 2, which was unshaded, and Plot 3, shaded to reduce light intensity 45 per cent, received the basic fertilization plus 1,800 pounds 8-8-8 and 900 pounds sodium nitrate per acre. Turnip greens were harvested weekly from each plot. All harvests for this study were made at 7:30 A.M., since the harvest hour is known to have an effect on plant constituents.[8] After thorough washing (three times), the leaves were cut in half through the mid-ribs and blotted dry. A sufficient number of half leaves to make a total weight of 300 gm. was cooked by the following methods: (1) seven minutes at 15 pounds pressure with two-thirds cup water; (2) twenty minutes in a covered pan with two cups water; and (3) two hours in a covered pan with eight cups water.

Half a teaspoonful of salt was added in each case. After cooking, the greens were drained two minutes and the liquor saved for a separate analysis. Both cooked greens and raw samples (other halves of cooked leaves) were blended with oxalic acid solution to make a homogenous mixture. Aliquots were analyzed for nitrates by a modification[9] of the Devarda Method of the Association of Official Agricultural Chemists[10] and for total ascorbic acid by the method of Schaffert and Kingsley.[11]

Relation of Ascorbic Acid and Methemoglobin

In order to investigate any deterrent effect that

ascorbic acid might have on methemoglobin formation, fifteen human subjects (eight male and seven female subjects varying in age from fifteen to forty-four years) were selected. They were put on diets relatively free of ascorbic acid for sixty hours. After twelve hours of fasting, 18 ml. of blood was taken from each subject. All blood was oxalated to prevent coagulation, and a 5 ml. aliquot was immediately added to 0.05 mg. ascorbic acid. The 0.05 mg. of ascorbic acid was added to get the blood level up to or above the amount found in blood when the human body is fully saturated with ascorbic acid.[12] Added to 0.02, 0.04, 0.06 and 0.08 mg. of sodium nitrite were 1 ml. aliquots of this mixture. Methemoglobin was determined by the method of Evelyn and Malloy[13] at intervals of five minutes, thirty minutes and two hours after addition of nitrite. Treated in the same manner was 4 ml. of blood without additional ascorbic acid. The remainder of the blood was used for ascorbic acid analysis.

To determine whether or not ascorbic acid was protective *in vivo*, the effect of an oral dose of nitrite was investigated in four guinea pigs. Guinea pigs were chosen as the test animal because they require ascorbic acid and cannot synthesize it. All animals were maintained on a rabbit pellet ration.* Two of the animals were given supplemental green leafy vegetables *ad libitum*. After twenty days, all animals were given an oral dose of sodium nitrite equivalent to 50 mg. per kg. body weight. Blood samples were taken one and a half hours later and assayed for methemoglobin. After a long rest period, two of these pigs were again paired off with one pig ingesting greens, and at the end of twenty days an oral dose of sodium nitrite equivalent to 100 mg. per kg. body weight was given.

RESULTS AND COMMENTS

Nitrates and Ascorbic Acid

Average amounts of nitrate and ascorbic acid found in 100 gm. raw turnip greens from each plot are given in Table I. Analysis of variance and testing the means by Duncan's Multiple Range Test[14] indicates that there was a highly significant increase in nitrates with increased fertilization and also with shading. The ascorbic acid decreased with extra fertilization and shading. A highly significant negative correlation ($r = -0.747$; r above 0.641 required for significance at 1 per cent level) was found between the amounts of nitrate and total ascorbic acid in the raw greens.

* Purina Rabbit Chow.

TABLE I

Mean Values for Nitrate and Total Ascorbic Acid in Fifteen Samples of Raw Turnip Greens

Plot	Nitrate (mg./100 gm.)	Total Ascorbic Acid (mg./100 gm.)
Basic fertilization, unshaded	159.3 (a)	135.1 (a)
High nitrate fertilization		
Unshaded	350.7 (b)	115.2 (ab)
Shaded	470.7 (c)	83.3 (b)

NOTE: Values not followed by the same letter differ at the 1 per cent level of probability from other values in the same column.

To determine the actual loss of ascorbic acid during cooking, the ascorbic acid content of the raw greens was compared with that found in cooked greens plus the cooking liquor. The average loss of ascorbic acid for each plot, for each of the three cooking methods, is given in Table II. The greatest loss of ascorbic acid was found to occur in the pressure cooked greens. Since cooking caused no destruction of nitrates in these experiments or in previous experiments conducted in this laboratory,[15] the raw nitrate content of the turnip greens was correlated with the decrease in ascorbic acid for each cooking method. None of these correlations was significant. The lack of correlation between the ascorbic acid loss and

TABLE II

Average Loss of Total Ascorbic Acid in Turnip Greens by Different Cooking Methods

Plot	Pressure Cooked (%)	Boiled 20 Minutes (%)	Boiled 2 Hours (%)
Basic fertilization, unshaded	49.6	20.6	18.6
High nitrate fertilization			
Unshaded	55.2	28.3	25.0
Shaded	48.7	26.9	19.1

TABLE III
Mean Values of Sixty Methemoglobin Determinations on Human Blood*

Time After Nitrite Addition (min.)	Untreated Blood (gm./100 ml.)	Blood with Ascorbic Acid Added (gm./100 ml.)
5	0.426	0.439
30	0.805	0.806
120	0.710	0.709

* Fifteen human subjects, four nitrite levels.

nitrate content, substantiated by the fact that an inspection of Table II shows that the greatest cooking loss of ascorbic acid did not occur in the plot containing the highest nitrate concentration, indicates that cooking loss of ascorbic acid is not related to the amount of nitrate present.

In order to get maximum ascorbic acid content, turnip greens should get a maximum amount of sunlight, and the use of excessive fertilizer should be avoided. Although maximum ascorbic acid was lost in the pressure cooked greens, this method of cooking should not be condemned. If the cooking liquor is discarded and only the greens ingested, the losses revert and are 55.7, 54.1 and 67.5 per cent for greens pressure cooked, boiled twenty minutes and boiled two hours, respectively.

Ascorbic Acid and Methemoglobin

Analysis of variance of the methemoglobin content of the human blood treated with nitrite showed no difference whether ascorbic acid was added to the blood or not. The average methemoglobin for all nitrate levels at each time interval is presented in Table III. Differences in methemoglobin with each increase of nitrite were highly significant. Also, there were some highly significant differences between subjects. Since the ascorbic acid analyses of the untreated blood samples revealed extensive variation also, the ascorbic acid content of the blood was correlated with the mean methemoglobin levels for all four nitrite additions. None of these correlations was significant (five minutes, $r = 0.257$; thirty minutes, $r = -0.102$; two hours, $r = +0.310$; r above 0.532 required for significance at 5 per cent level). At the fasting levels and with addition of 1 mg. ascorbic acid per 100 ml. of blood, ascorbic acid had no measurable effect on methemoglobin formation in human blood *in vitro*.

When the effect of an oral dose of sodium nitrite equivalent to 50 mg. per kg. body weight was explored, blood drawn from two guinea pigs receiving ascorbic acid had methemoglobin levels of 2.60 and 2.54 gm. per 100 ml. Blood taken from two guinea pigs that were on ascorbic acid-deficient diets contained 2.88 gm. and 2.23 gm. methemoglobin per 100 ml. One of the pigs, on the deficient diet, died two hours after the dose. After a rest period, two of the surviving guinea pigs were again paired off with only one getting ascorbic acid supplement. This time the pigs were given 100 mg. sodium nitrite per kg., and the methemoglobin in the blood was 2.75 gm. per 100 ml. for the guinea pig not receiving ascorbic acid supplement and 3.34 gm. per 100 ml. for the guinea pig receiving the ascorbic acid supplement. The guinea pig receiving ascorbic acid supplement died in less than two hours after nitrite ingestion. This death and the one mentioned previously appeared to be due to massive hemorrhages, particularly around the heart and groin. These deaths evidently were precipitated by vasodilation due to orally administered nitrite and showed no relation to the amount of ascorbic acid ingested. Since the methemoglobin showed a slight though insignificant increase when ascorbic acid was added to the diet when a decrease was expected if ascorbic acid had any protective influence, the animal studies were discontinued.

SUMMARY

Turnip greens that received shading and excessive fertilization had higher nitrate contents and 39 per cent less ascorbic acid than those grown with less fertilizer and without shade.

Since ascorbic acid decreases in plants as nitrate content increases, the effect of the nitrate content of turnip greens was studied in relation to ascorbic acid loss during cooking. The greatest losses of ascorbic acid did not

occur in turnip greens containing the largest concentration of nitrate, nor did loss of ascorbic acid correlate with the amount of nitrate present. Therefore, the presence of nitrate in turnip greens is not a significant factor in loss of ascorbic acid during cooking.

Although ascorbic acid has been used as an antidote for methemoglobinemia, the addition of small amounts (equal to or greater than those found normally) to human blood had no protective effect against methemoglobin formation. Guinea pigs, which had been kept for twenty days on a diet low in ascorbic acid, were no more susceptible to nitrite toxicity than those which had been fed supplements of ascorbic acid.

Accidental or climatic shading of highly fertilized turnip greens introduces no health hazard even though the greens have low ascorbic acid and high nitrate content. The retention of ascorbic acid during cooking was not affected. The unknown protective agent in vegetables, that has confined the nitrate toxicity reported in the literature to babies drinking formulas prepared with water containing large amounts of nitrates, is apparently not ascorbic acid.

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DIMETHYLNITROSAMINE FORMATION FROM SODIUM NITRITE AND DIMETHYLAMINE BY BACTERIAL FLORA OF RAT INTESTINE

by

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ABSTRACT: The incubation of rat caecal contents with (^{14}C)-labeled dimethylamine and sodium nitrite at neutral pH under anaerobic conditions leads to the formation of (^{14}C)-dimethylnitrosamine, detectable with thin layer chromatography. Glucose is required for the formation of dimethylnitrosamine by the intestinal flora. This suggests that the formation of the carcinogen from dimethylamine and sodium nitrite is dependent on the metabolic activity of the intestinal microorganisms.

INTRODUCTION: Nitrates and nitrites are included in the curing mixtures for meats to fix an attractive appearance of the meat (Wilson, 1960; Chichester and Tanner, 1968) through interaction with haem pigments. Nitrites also have antimicrobial action and retard spoilage of meats and fish (Tarr and Sunderland, 1940a; Tarr, 1941a, b; Tarr and Sunderland, 1941; Castellani and Niven, 1955; Chichester and Tanner, 1968). Spoilage of fish has been equated with the production of objectionable volatile materials such as trimethylamine and dimethylamine (Tarr, 1940; Tarr and Sunderland, 1940b). Dimethylamine also occurs in mammalian intestine as a result of bacterial degradation of ingested lecithin and choline (Asatoor and Simenhoff, 1965).

It is now known that nitrites may react non-enzymatically with alkylamines such as dimethylamine under acid conditions to yield dimethylnitrosamine (Mirvish, 1970), a potent carcinogen (Magee and Barnes, 1967; Magee, 1969) and hepatotoxic agent (Sakshaug et al., 1965).

We were interested in seeing whether the intestinal flora of the rat could convert precursor dimethylamine and nitrite into dimethylnitrosamine under neutral anaerobic conditions, which would indicate whether the addition of nitrite to foods might not present a hitherto underestimated toxicological hazard.

MATERIALS AND METHODS: Dimethylamine hydrochloride, choline chloride and dimethylnitrosamine were obtained from Eastman Organic Chemicals, Rochester, N.Y. (^{14}C)-Labeled dimethylamine hydrochloride and choline chloride (^{14}C)-methyl) were purchased from Amersham/Searle Corporation, Silver Spring, Md. Female Sprague-Dawley rats (Sprague-Dawley Co., Madison, Wis.), weighing 200 gm, maintained under standard conditions on unlimited Purina Chow rat cubes (Ralston Purina Co., St. Louis, Mo.) and water were used throughout. The animals were stunned and decapitated. The entire caecum was removed from each donor rat, and the contents were expressed, weighed and suspended with vigorous stirring in 4 volumes of ice-cold 0.14M sodium phosphate buffer pH 7.0. Animals were routinely killed at the same hour each day to minimize diurnal variation of caecal contents dependent on feeding patterns.

Incubations were performed in duplicate at 37° for 20 hr under an atmosphere of oxygen-free nitrogen in 30 ml brown screw-top bottles equipped with rubber diaphragm caps to which were added the following components: glucose (100 μmoles); sodium nitrite (400 μmoles); (^{14}C)-dimethylamine hydrochloride (100 μmoles, 1 μCi) or (^{14}C)-choline (200 μmoles, 2 μCi); caecal contents suspension (0.5 ml, containing approximately 0.12 gm caecal contents); 0.14M sodium phosphate buffer pH 7.0 to give final volumes of 2.0 ml. Control flasks contained the same components except for the bacterial suspension which was autoclaved at 20 lbs pressure for 15 min before addition to the incubation vessels. Some incubations were performed without glucose or sodium

nitrite.

Reactions were terminated by chilling the closed flasks and injecting 20% w/v zinc sulfate (0.1 ml) and saturated barium hydroxide (0.1 ml) into the vessels. After adding carrier dimethylnitrosamine (0.5 mmole) to each sample the suspensions were subjected to centrifugation.

Aliquots (.05 ml) of the deproteinized supernatant material containing approximately 40,000 DPM in experiments with (^{14}C)-dimethylamine and 80,000 DPM in corresponding experiments with (^{14}C)-choline were then examined for dimethylnitrosamine formation by ascending thin-layer chromatography using sheets of silicic acid chromatography medium ITLC type SA (Gelman Instrument Co., Ann Arbor, Mich.) and a benzene-95% ethanol (10:1 v/v) solvent system.

In this system standard dimethylamine did not migrate (Rf = 0) and was further characterized as a blue spot at the origin when sprayed with a sodium nitroprusside-acetaldehyde reagent (Stahl, 1969). In this same system, choline did not migrate (Rf = 0) and was detected by a dark blue color-reaction when sprayed with potassium iodoplatinate reagent (Stahl, 1969). Standard dimethylnitrosamine had an Rf value of 0.60 corresponding to a dark absorbing spot under ultraviolet light (240 mμ) which could also be rendered visible as a blue spot by spraying with a diphenylamine-palladium chloride reagent (Preussmann et al., 1964) and irradiation at 240 mμ.

Dimethylamine could also be separated from dimethylnitrosamine quite readily by using a solvent system prepared from n-hexane:diethyl ether:dichloromethane (5:3:1 v/v) modified from Preussmann et al. (1964). The Rf values for dimethylamine and dimethylnitrosamine in this system were 0 and 0.40 respectively.

After development of an experimental chromatogram, the dimethylnitrosamine

was detected by examination under ultraviolet light. The chromatogram was then appropriately sectioned so that each section could be separately assayed for radioactivity when cut into small pieces, placed into vials containing 10 ml scintillation solution (Bray, 1960) and counted in a liquid scintillation counter (Beckman, Model DPM-100) with an external standard. The formation of dimethylnitrosamine was equated with the DPM associated with the section of the chromatogram corresponding to the dark-absorbing spot under ultraviolet light where Rf = 0.60 or 0.40 depending on the solvent system used. Unchanged dimethylamine or choline was equated with the radioactivity remaining at the origin. Radioactivity in all other sections of the chromatogram was comparable to background levels.

In another type of experiment in which we measured the anaerobic reduction of nitrate to nitrite by intestinal microorganisms, caecal contents from donor rats were suspended in 8 volumes of buffer. The following components were then incubated in duplicate in final volumes of 30 ml under the general conditions already described: glucose (1 mmole); sodium nitrate (300 μmoles); buffered caecal contents suspension (5.0 ml, equivalent to approximately 0.60 gm caecal contents); 0.14M sodium phosphate buffer pH 7.0 to adjust volume to 30 ml. Duplicate aliquots (1 ml) were removed from incubation vessels at 0 and 20 hrs for the colorimetric determination of nitrite by a coupling reaction with sulfanilamide and N-(1-naphthyl)ethylenediamine (Hackenthal and Hackenthal, 1965).

RESULTS: As shown in Table 1, dimethylnitrosamine is formed when rat caecal contents are incubated anaerobically with dimethylamine and sodium nitrite, and the reaction mixtures are subsequently analyzed by ascending thin layer chromatography on silicic acid. The results shown in Table 1 refer to chromatographic separations achieved with

Table 1

FORMATION OF (^{14}C)-DIMETHYLNITROSAMINE BY BACTERIAL FLORA OF RAT CAECUM INCUBATED ANAEROBICALLY FOR 20 HRS WITH SODIUM NITRITE AND (^{14}C)-DIMETHYLAMINE OR (^{14}C)-CHOLINE

		Micromoles (^{14}C)-Dimethylnitrosamine Formed/Incubation* per 20 Hrs			
Radioactive Precursor	Expt. No.	Complete System	-Glucose	-Sodium Nitrite	Autoclaved Caecum Contents
(^{14}C)-Dimethyl-amine	1	3.77,4.02	1.13,1.24	0.34,0.39	1.19,1.29
	2	2.67,2.61	1.18,1.22	0.36,0.18	1.02,1.29
	3	2.85,2.84	0.96,0.83	0.16,0.27	0.92,0.80
(^{14}C)-Choline	4	0,0	0,0	0,0	0,0

*Aliquots of caecal contents incubated were equivalent to 0.12±0.01 gms. Total caecal contents per 200 gm rat weighed 3.20±0.18 gms. Total dimethylnitrosamine formation per rat equivalent can be obtained on multiplying by a factor, 26.7.

Experimental conditions are as indicated under Methods.

the solvent system benzene:95% ethanol (10:1 v/v). Identical results are achieved with the other solvent system, n-hexane:diethyl ether:dichloromethane (5:3:1 v/v).

The extrapolation of the experimental figures to whole caecum activity would indicate that the bacterial flora in the caecum of a 200 gm rat were capable of synthesizing between 70 and 108 μmoles of nitrosamine in 20 hrs.

The omission of glucose from the system diminishes the yield of dimethylnitrosamine which suggests that the formation of the carcinogen is dependent on the metabolic activity of the intestinal microorganisms. When the caecal suspensions were autoclaved prior to incubation under the same conditions, the yield of dimethylnitrosamine was comparable with that of the system when glucose was omitted. This seems to indicate that the formation of dimethylnitrosamine from its precursors can also

Table 2

THE FORMATION OF NITRITE FROM NITRATE BY BACTERIAL FLORA OF RAT CAECUM INCUBATED ANAEROBICALLY FOR 20 HRS WITH SODIUM NITRATE

Time (hrs)	Experiment No.	Micromoles nitrite/Incubation*
0	1	0.51,0.53
20	1	52.0,64.0
0	2	0.53,0.45
20	2	54.0,21.0

*Aliquots of caecal contents incubated were equivalent to 0.60 gms. Total caecal contents per 200 gm rat weighed 3.0 gms. Total nitrate formation per rat equivalent can be calculated on multiplying by a factor 4.

Conditions of incubation are as described in Methods section.

take place non-enzymatically to a limited extent, even at neutral pH. Previous studies (Sander, 1968; Sen et al., 1969; Mirvish, 1970) on the non-enzymatic formation of dimethylnitrosamine by interaction of dimethylamine and sodium nitrite have stressed that this occurs under acid conditions.

When sodium nitrite was omitted from the system, a small amount of radioactivity was associated with the spot corresponding to dimethylnitrosamine. This may be a reflection of trace amounts of nitrite that are inherently present in the system as food residues rather than alternative pathways of metabolizing (^{14}C)-dimethylamine, since the same results were obtained with two different chromatographic solvent systems.

Rat intestinal flora have been shown to be involved in the production of urinary dimethylamine from dietary choline (Asatoor and Simenhoff, 1965). However, as shown in Table 1 the anaerobic incubation of rat caecal contents with (^{14}C)-choline and sodium nitrite did not give rise to any detectable formation of (^{14}C)-dimethyl-

nitrosamine under the conditions of our experiments, which suggests that the caecal bacteria were not breaking down choline in a way that leads to the accumulation of (^{14}C)-dimethylamine capable of reacting with nitrite. It remains to be determined whether incubation of the bacterial flora of other portions of the intestine, with choline and sodium nitrite, can lead to dimethylnitrosamine formation through the intermediate formation of dimethylamine.

From Table 2 we can see that the anaerobic incubation of rat caecal contents with sodium nitrate, in the presence of glucose, leads to the accumulation of nitrite. This implies that under appropriate conditions and in the presence of dimethylamine, dietary nitrate which could be substantially reduced to nitrite by intestinal microorganisms, might contribute to the formation of dimethylnitrosamine.

DISCUSSION: We were able to show that dimethylnitrosamine can be formed from dimethylamine and sodium nitrite when these substances, which may be present in human food sources, are incubated at neutral pH under anaerobic conditions with rat intestinal microorganisms.

Nitrosamine formation from nitrite and secondary amines was previously demonstrated to occur spontaneously in the absence of bacteria, under acid conditions (Sander, 1968; Mirvish, 1970) prevailing in gastric juice (Sen et al., 1969). Moreover, Sander (1968) incubated a number of species of nitrate-reducing Enterobacteria with nitrate and several secondary amines, not including dimethylamine, and was able to demonstrate nitrosamine formation dependent on bacterial metabolism and the structure of the secondary amines. The highest yields of nitrosamines under these conditions were obtained with arylamines.

Our main purpose in this study was to concentrate on the formation of dimethyl-

nitrosamine, a potent carcinogen for the rat (Magee and Barnes, 1967; Magee, 1969), for mice (Takayama and Oota, 1963; Mirvish and Kaufman, 1970) and possibly for man (Montesano and Magee, 1970), from components that could occur in human diet, with particular reference to dimethylamine and sodium nitrite (Sen et al., 1969).

Dimethylamine occurs in various fish products (Ender et al., 1967; Wick et al., 1967) where it could interact spontaneously with any nitrite added as preservative (Tarr and Sunderland, 1940a,b) under acid conditions (Mirvish, 1970) to yield the nitrosamine (Sakshaug et al., 1965; Ender et al., 1967; Howard et al., 1970). Dimethylamine may also be a constituent of a variety of other foods (Sen et al., 1969). Furthermore, dimethylamine may be formed by the intestinal bacteria, from choline or lecithin (Asatoor and Simenhoff, 1965).

With regard to sodium nitrite, this is a permitted additive to meat and fish products, as is sodium nitrate (Chichester and Tanner, 1968). We measured the bacterial reduction of nitrate to nitrite and found that there is substantial reduction of nitrate to nitrite by the bacterial rat caecal contents. Since the kinetics of dimethylnitrosamine formation are proportional to the concentration of dimethylamine and the square of the concentration of nitrite (Mirvish, 1970), the bacterial mechanism for enhancing nitrite concentration in the intestine might compound the toxicological hazard attendant on the dietary intake of dimethylamine or its formation by intestinal bacteria from precursors such as choline or lecithin (Asatoor and Simenhoff, 1965). In this series of experiments, however, although we found evidence that rat caecal contents when incubated with dimethylamine and nitrite, led to the formation of dimethylnitrosamine, we did not detect any dimethylnitrosamine in corresponding incubations with choline and nitrite.

It remains to be investigated whether intestinal bacteria from other species, including humans, can give rise to dimethylnitrosamine when appropriately incubated with nitrite and dimethylamine or choline.

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PATHOGENESIS, INCIDENCE, AND POSSIBILITIES OF PREVENTING ALIMENTARY NITRATE METHEMOGLOBINEMIA IN INFANTS

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The Institute of Hygiene, Prague (Director: Prof. MUDr. K. Symon), and the First Paediatric Clinic of the Charles University, Prague (Chief: Prof. MUDr. J. Švejcar, D.Sc.)

SINCE 1945 when Comly[1] discovered that nitrates in water given to infants could cause them to become ill with methemoglobinemia, attention has been turned to the etiological relationship between intestinal bacterial flora and the origin of the methemoglobinemia.[2] In Czechoslovakian regions where water contains a high concentration of nitrates the disease became more frequent with the use of dried milk products for artificial feeding of infants. For this reason we concentrated our attention on the dried milks and succeeded in proving their content of spores of the *B. subtilis* group, almost in pure culture. These sporulating microbes intensively reduce nitrates to nitrites.[3,4,5,6] In our previous publications we were able to demonstrate that the bacteria increase on incubating full-cream milk ("Sunar"), skimmed milk ("Eviko"), and milk acidified with lactic acid ("Relakton").[3-6] Organisms do not grow, however, in dried buttermilk ("Lakton"),* nor does any reduction of nitrates to nitrites occur, contrary to the other milks. For reduction to occur it is necessary for the organisms to proliferate up to 10.[7] The amount of nitrites reaches its peak at the 16th hour after incubation, whereas formation of nitrite begins after 8 hours. We also demonstrated in experiments with animals that the response *in vivo* is the same as *in vitro*. When the dried milk products named above were diluted with water containing nitrates, the amount of nitrites in the blood of suckling rats and calves increased still more rapidly than *in vitro*, the rise of nitrites in blood being followed by an increase of methemoglobin estimated with Cruz's method.[7] As occurs *in vitro*, dried buttermilk ("Lakton") prevented bacterial growth and reduction of nitrates to nitrites in experiments with suckling rats and calves; and neither increase of nitrites nor methemoglobinemia appeared when dried buttermilk was fed.

In view of the foregoing results we have investigated: (1) Whether the same mechanism acts in infants as in experimental animals and whether the sporulating microbes contained in dried milk necessitate examination for elevated levels of methemoglobin in all areas with high amounts of nitrates in the water, and in infants in which no clinical symptoms are visible. (2) Can methemoglobinemia be provoked in infants carefully fed dried milk diluted with water containing nitrates, as it is provoked in experimental animals? (3) What is the cause of the preventive effects of dried buttermilk ("Lakton") when milk acidified with lactic acid ("Relakton") has no such property of prevention?

1. Statistics on the incidence of methemoglobinemia showed that from 1948 to 1960 a total of 314 cases was reported for the whole state. In our investigation of 40 communities in the vicinity of Prague we ascertained 115 cases of methemoglobinemia from a total of 5,800 children born in the period from 1953 to 1962, i.e., 2%.

Of the 115 cases, 69 were severe (60%), 46 light (40%). In the light clinical form of the disease, substitution of pure water for that containing nitrates proved to be sufficient treatment. In the more severe cases hospitalization and clinical treatment were

* The trade names for these products are used.

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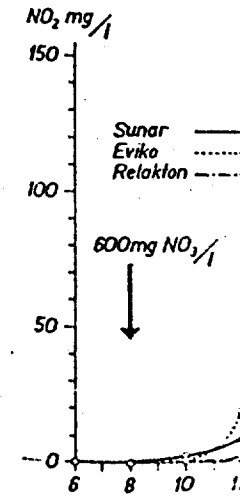

FIG. 1. Reduction of
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necessary. Out of the 115 cases of the disease 10 infants died (8%).

Besides these cases of apparent methemoglobinemia there were a great number of infants without any clinical symptoms, but with levels of methemoglobin exceeding the physiological.

We selected three districts with most frequent incidence of clinical methemoglobinemia where increased concentration of nitrates in water was ascertained. In these areas (Prague-West, Břeclav, and Písek) the level of methemoglobin in blood was examined in 328 infants. Methemoglobin elevated above the physiological level was found in 76, or 23.7% of the group. In control children in a territory without any incidence of clinical methemoglobinemia the average amount of methemoglobin in blood, ascertained with the Cruz method,[7] amounted to 0.86 ± 0.3%.

With amounts of methemoglobin under 5% no clinical signs were found. With amounts of methemoglobin exceeding 5%, light cyanosis was obvious.

A distinct effect of age on incidence of methemoglobinemia was shown: (*a*) In 112 infants aged 0–3 months, elevated levels of methemoglobin were found in 58, or 52%. (*b*) In 138 infants aged 3–12 months, elevated levels of methemoglobin were found in 18, or 13%. (*c*) In 78 infants over 12 months no elevated methemoglobin was found.

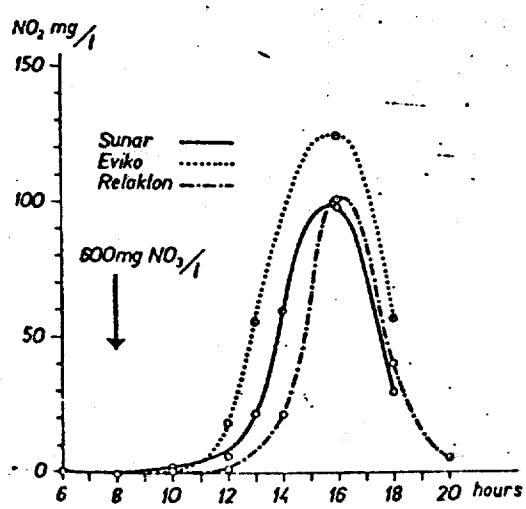

Fig. 1. Reduction of NO_3 to NO_2 (in relation to time), when NO_3 was added to 3 milk preparations after 8 hours of cultivation. ("Lakton" is not mentioned as it does not reduce nitrates to nitrites.)

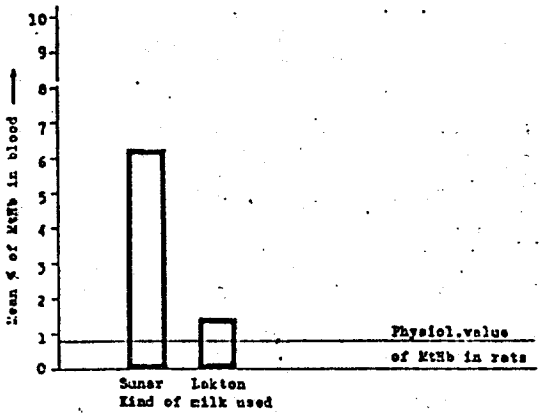

Fig. 2. Average levels of methemoglobin in suckling rats fed "Sunar" and "Lakton." ($t = 5.00$; $t = (0.01) = 3.13$. Difference highly significant.)

The finding that all infants with clinical or inapparent methemoglobinemia were fed on the dried-milk preparations of the trademarks "Sunar," "Eviko," and "Relakton" was important. These dried milks—commonly used for artificial feeding of infants—contain, as all the other dried-milk products do, the sporulating microorganisms of *B. subtilis*. It is not possible to suppress these microbes in the production of the dried milk to avoid deterioration of the product.[5,6]

None of the infants with clinical or inapparent methemoglobinemia was fed on the dried buttermilk "Lakton"; as we have proved, this preparation does not reduce nitrates to nitrates.[5,6]

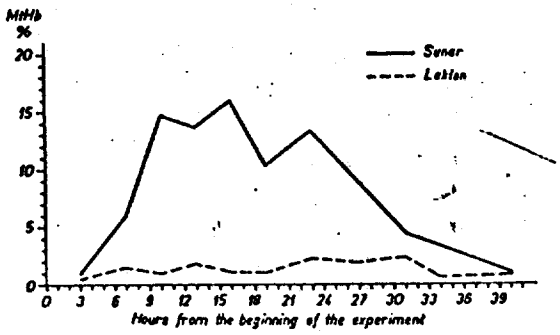

Fig. 3. Comparison of the level of methemoglobin in blood in calves fed "Sunar" and "Lakton."

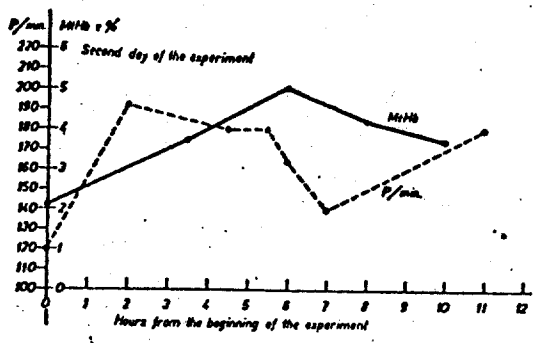

FIG. 4. Methemoglobin and pulse rate in experimentally developed nitrate methemoglobinemia.

2. Two infants hospitalized with severe anomalies of the nervous system were studied. The first infant, of female sex, aged 35 days, body weight 2,500 gm, was fed 6 times a day on the 10% solution of the dried milk, "Sunar." The infant was examined clinically and blood samples were taken for methemoglobin.[7] The level of methemoglobin in the infant observed had already risen to 2.11% (probably because of reduction in the enzyme activity in the infant since this methemoglobin concentration was ascertained before the infant became ill, and was found to be the same after recovery). Apparently, for the infant observed, this methemoglobin concentration was at a physiological level and constant. Afterward, 500 mg $NaNO_3$ in tea were given and, as indicated in Figure 5, blood samples were taken for the examination of methemoglobin and the infant was also clinically examined. On the following day another 500 mg $NaNO_3$ was administered. On the first day of $NaNO_3$ intake the amount of methemoglobin reached 3.83% after 6 hours and after 12 hours it returned to the original value. On the second day it increased after 6 hours following another dose of nitrate to 5.03% as is seen on Figure 5, and after 12 hours it returned again to the original value. The infant was slightly cyanotic around the mouth and in the acral portions of the body; before the rise of methemoglobin its pulse rate increased (see Fig. 4).

It can be seen from Table II that nitrates were not converted to nitrites in the doses of 200–1000 units/10 ml milk. It can be supposed that efficiency of nisin will be greater if the condition of its maximal solubility (low pH) is observed, as was not the case in our experiments.

On the third day the infant was given dried buttermilk, "Lakton," and after a week of this artificial feeding 3 more doses of 500 mg $NaNO_3$ in 3 days were given. When the feeding was changed to the dried buttermilk, "Lakton," repeated administration of nitrates was not accompanied by any clinical signs of methemoglobinemia and the level of methemoglobin ranged between the limits of the original values.

The second infant (of male sex, 72 days old, body weight 3,700 gm, fed 6 times a day on the 10% solution of the dried buttermilk, "Lakton") was given daily doses of 50 mg $NaNO_3$ in tea for 4 days, and the methemoglobin level in blood was observed. It did not exceed the original value of 0.86%.

Thus, when the infants were fed on dried buttermilk and given nitrates the methemoglobin remained at physiological levels.

This fact was confirmed by the case of an infant in an institution in the vicinity of Prague, who became ill with nitrate alimentary methemoglobinemia when fed on the dried milk "Eviko," diluted with water of nitrate concentration exceeding 50 mg/lit. On the 15th day of hospitalization, ni-

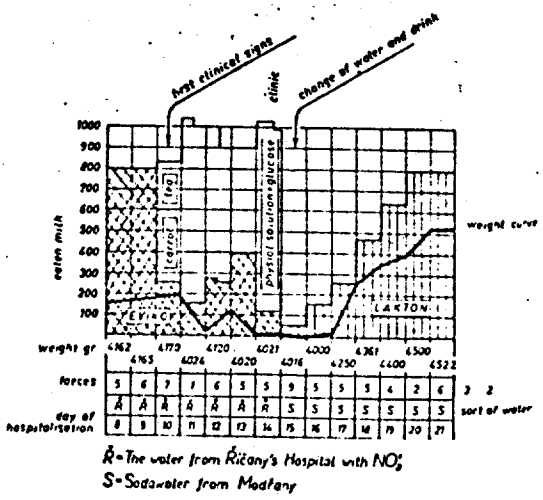

FIG. 5. Intake and weight gain of an infant artificially fed on "Eviko" after change to "Lakton."

trate methemoglobinemia with dyspepsia was evident. For treatment of dyspepsia, the dried buttermilk, "Lakton," diluted with soda water, was introduced. The infant began to consume larger doses of artificial feeding, its body weight increased and the signs of the disease disappeared. This infant was treated without any knowledge of our results and without our participation. When we examined the case afterward we ascertained that the soda water contained 140 mg NO_3/lit. Although the infant was thus given far larger amounts of nitrates than in the original water, which had provoked the disease, and that at the time of most severe clinical signs, it recovered quickly.

3. To explain the preventive effects of the buttermilk against the development of methemoglobinemia, only microbial flora need be taken into consideration as the buttermilk differs from the other three types of milk only in this regard. In order to ascertain whether such an effect occurs only *in vivo*, specimens of all the four types of dried milk diluted with water containing 600 mg nitrates in 1 liter were autoclaved for 2 hours to destroy their microbial flora. Table 1 shows that change of nitrates into nitrites occurred in all three types of milk without the bacteria of lactic fermentation, but not in "Lakton."

It was evident that the substance effective in preventing methemoglobinemia in dried buttermilk ("Lakton") must be independent of the vitality of the microbes, as it persists

TABLE I

PRESENCE OF NITRITES IN AUTOCLAVED SPECIMENS OF MILK WITH ADDITION OF 24 HOURS CULTURE OF MICROORGANISMS FROM THE MILK PREPARATION "EVIKO"

	Sunar	*Eviko*	*Relakton*	*Lakton*
Analysis carried out after 13 hours	+	+	±	0
Analysis carried out after 44 hours	+	+	+	0
Autoclaved control after 13 and 44 hours	0	0	0	0

TABLE II

EFFECT OF NISIN ON THE REDUCTION OF NITRATES TO NITRITES

Amount of nisin added (in units)	*Experiment*		
	1	*2*	*3*
200,000	−	−	−
20,000	−	−	−
2,000	−	−	−
1,000	−	−	−
200	+	−	−
100	+	−	+
30	+	+	+

also after their destruction. It is known that *S. lactis* contained in the buttermilk produces nisins[8] exerting an antibiotic effect and able to prevent the growth of sporulating microorganisms. For this reason we carried out another experiment in which 10 ml dried milk was diluted with water containing 600 mg NO_3 and nisin was added (English preparation Nisaplin-Research, Dir. Alin and Barret, England, 1 gm = 1,000,000 units), diluted in the physiological solution; after 15 hours reduction of nitrates to nitrites was estimated with the Griese reagent.

COMMENT

Our studies carried out *in vitro* and *in vivo*, indicate that the microorganisms of *B. subtilis* get into the digestive tract almost in pure culture with the artificial feeding on types of dried milk capable of reducing nitrates to nitrites. Growth of these organisms is not prevented when the milk is diluted directly before the feeding of the infant. Their growth can occur during the course of digestion and is supported by continually new intake of sporulating microorganisms. Nitrites, thus formed from nitrates in water, are absorbed and retained in the blood. The consequence of their presence in blood is the rise of methemoglobin over normal values, as was shown by our examinations in the areas with water containing high concentrations of nitrates. The blood methemoglobin level of all infants

getting such unwholesome water does not rise, and only in a certain number of cases are clinical signs found. This phenomenon we are not yet able to explain. It appears from our examinations that infants under 3 months of life are most affected. In older infants the disease is rare. This difference might be explained by two circumstances: (1) the presence of fetal hemoglobin, which can be more easily oxidized,[9] in the first 3 months of life, and (2) the different capacity of the oxidation-reduction system in different individuals.[10] In this study we are not yet able to appreciate the importance of these factors or perhaps also of some other factors.

The finding of the preventive effect of the buttermilk is important; our experiments with nisin suggest that this is related to the amount of microbes of lactic fermentation and the antibiotic effect of their production of nisin in the dried buttermilk. This finding explains the effects of the dried buttermilk on the intestinal bacterial flora in infants and suggests other dietary effects of this food.

It follows from our examinations that artificially fed infants in areas with high concentrations of nitrates in the water are rather often affected with inapparent methemoglobinemia. Consequently it is necessary to assume that artificially fed infants in such areas are in the state of intoxication with nitrites. For this reason medical treatment of the methemoglobinemia does not suffice and it is necessary to prevent the formation of nitrites in the ingestion tract of infants. It would be logical to use water of lower nitrate content for the dilution of the milk. But in the affected areas this is often impossible, as they are relatively large and all wells contain nitrates. Another method of prevention would be by feeding sterile evaporated milk, but this is not available in our country. We have proved that the use of dried buttermilk as a basis for artificial feeding in the first 3 months of life is a suitable preventive measure. As the buttermilk used in our country contains 1.4% fat, we need not fear any lack of fat in such a food intake.

SUMMARY

It was shown in clinical experiments that alimentary methemoglobinemia in artificially fed infants is provoked by nitrites originating from nitrates contained in water used for diluting dried-milk preparations. The reduction of nitrates is induced by the influence of sporulating germs of *B. subtilis* contained as spores in the dried milk. In areas where water contains high concentrations of nitrates a great percentage of artificially fed infants are affected, even if the methemoglobinemia does not manifest itself clinically. Dried buttermilk contains microbes of lactic fermentation S. *lactis*, producing the antibiotic nisin. This prevents the growth of the sporulating microbes of *B. subtilis*. Consequently, prevention of this disease, caused by intoxication with nitrites, is possible, by the use of dried buttermilk for the artificial feeding of infants up to 3 months of age.

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Growth from Spores of *Clostridium perfringens* in the Presence of Sodium Nitrite[1]

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Received for publication 10 November 1969

The method by which sodium nitrite may act to prevent germination or outgrowth, or both, of heat-injured spores in canned cured meats was investigated by using *Clostridium perfringens* spores. Four possible mechanisms were tested: (i) prevention of germination of the heat-injured spores, (ii) prior combination with a component in a complex medium to prevent germination of heat-injured spores, (iii) inhibition of outgrowth of heat-injured spores, and (iv) induction of germination (which would render the spore susceptible to thermal inactivation). Only the third mechanism was effective with the entire spore population when levels of sodium nitrite commercially acceptable in canned cured meats were used. Concentrations of 0.02 and 0.01% prevented outgrowth of heat-sensitive and heat-resistant spores, respectively. Nitrite-induced germination occurred with higher sodium nitrite concentrations.

Although sodium nitrite plays an important [illegible] in the preservation of canned cured meats, [illegible] actual mechanism by which it prevents spoil[illegible] is obscure. It is believed that the stability of [illegible] meat products is due to the ability of the [illegible]ing salts, sodium nitrite and sodium chloride, [illegible] block some stage in the outgrowth or germina[illegible], or both, of the indigenous bacterial spores [illegible]ch have been heat-injured by the brief thermal [illegible]cessing that these canned meats undergo. The [illegible]l thermal processing presumably renders the [illegible]res more sensitive to the curing salts; however, [illegible] transitional stages of spore germination or [illegible]growth, or both, at which sensitization occurs [illegible]in unclear.

[illegible]odium nitrite, when heated in a laboratory [illegible]dium, forms a bacterial inhibitor which has [illegible]n found to be highly effective in preventing the [illegible]th of several clostridial species (11, 12). [illegible]ever, recent evidence indicates that such an [illegible]itory product may not be active in cured [illegible] products (9).

It is known that commercially acceptable con[illegible]trations of sodium nitrite may interfere with [illegible] growth of bacterial cells (2). Another postu[illegible]d role of sodium nitrite in the preservative [illegible]m of cured meats may be its ability to induce [illegible] germination, thus making the spores sus[illegible]ble to subsequent heat processing (4). Indeed, nitrite-induced germination has been shown to occur with putrefactive anaerobe 3679h (5), *Clostridium butyricum* (1), and *Bacillus cereus* (S. H. Black, Bacterial Proc., p. 36, 1964).

The present study was initiated to determine whether the inhibition of microbial growth from spores by commercially acceptable levels of nitrite (0.02% maximum residual nitrite) occurs at both the level of germination and outgrowth.

MATERIALS AND METHODS

Preparation of spore suspensions. Spores of *C. perfringens* strain FD1, obtained from the Food and Drug Administration, and *C. perfringens* NCTC 8798 (Hobbs type 9) were used. Strain FD1 produces heat-sensitive spores that require no prior heat shock for germination, and strain NCTC 8798 produces heat-resistant spores that require heat shock for optimal germination (Labbe and Duncan, *unpublished data*).

The organisms initially were grown in 15 ml of Fluid Thioglycollate Medium (BBL); successive 10% inocula were made at 16-hr intervals until a 1,500-ml culture was obtained. The latter culture was inoculated into 15 liters of D-S sporulation medium (5). All incubations were at 37 C. When the number of free spores reached a maximum (about 15 hr for FD1 and 20 hr for NCTC 8798), the culture was harvested by continuous-flow centrifugation. The spores were resuspended in distilled deionized water and were subjected to ultrasonic treatment to remove spores still in their sporangia. Subsequently, the spores were washed several times with cold deionized water until a suspension of clean spores was obtained. During

[1] [illegible]ished with the approval of the Director of the Research [illegible] of the College of Agricultural and Life Sciences.

cleaning and storage in deionized water, about 3 to 4% of the spores became phase dark. A stock spore suspension adjusted to an optical density (OD) of about 325 Klett units was prepared, and the remainder of the spores was lyophilized.

Assessment of initiation of germination and outgrowth. Initiation of germination was measured as a decrease in OD at 660 nm by using a Bausch & Lomb Spectronic-20 colorimeter; initiation was confirmed by determining loss of spore-phase brightness by using a Zeiss phase-contrast microscope.

Some of the data are presented as per cent germination with the decrease in OD of the positive control taken as 100% germination.

Spore outgrowth was determined by the increase in OD that occurred subsequent to an initial decrease in OD due to initiation of germination. Outgrowth was confirmed by phase-contrast microscopy.

In studying the inhibitory effect of sodium nitrite upon germination and outgrowth, a complex medium was used which contained a final concentration of 2.5% Brain Heart Infusion (Difco) plus 0.27% added yeast extract. The *p*H was adjusted to give a final *p*H of 7.0 or 6.0. In this medium, outgrowth of non-heat-injured spores began after 15 to 20 min. Reagents for the nitrite-induced germination experiments were prepared as concentrated stock solutions in deionized water and diluted as necessary. A final reaction mixture volume of 6 ml was used. Fresh stock solutions of sodium nitrite were prepared before each experiment.

Strain FD1 spores initiated germination and grew without prior heat shock; strain NCTC 8798 spores were heated at 70 C for 15 min to initiate germination. In all germination and outgrowth studies, the reaction mixtures, contained in screw-cap tubes (16 by 125 mm), were preheated to the appropriate temperature in a thermostatically controlled water bath; spores were then added to a final concentration of about 3×10^7/ml.

Preparation of heat-injured spores. Heat-injured spores were prepared by heating 5 ml of a stock spore suspension containing about 2×10^8 spores/ml in a screw-cap tube (16 by 125 mm); the spores were heated either at 90 C for 5 min in the case of FD1 or at 100 C for 18 min in the case of strain NCTC 8798. These times and temperatures resulted in an approximately 0.33 *D* value (time in minutes for inactivation of 90% of the spores) for spores of both strains.

Viable counts of heat-injured spores were made by means of pour plates by using TSN agar (10). Incubation was at 37 C for 24 hr (strain FD1) or 48 hr (strain NCTC 8798) under a gas mixture of 90% nitrogen and 10% carbon dioxide.

RESULTS

Four mechanisms for possible inhibition of microbial growth from spores by sodium nitrite were investigated. These included (i) prevention of germination of heat-injured spores in a complex medium, (ii) prevention of germination of heat-injured spores in a complex medium by an inhibitory product formed by prior combination of sodium nitrite with a component in the medium

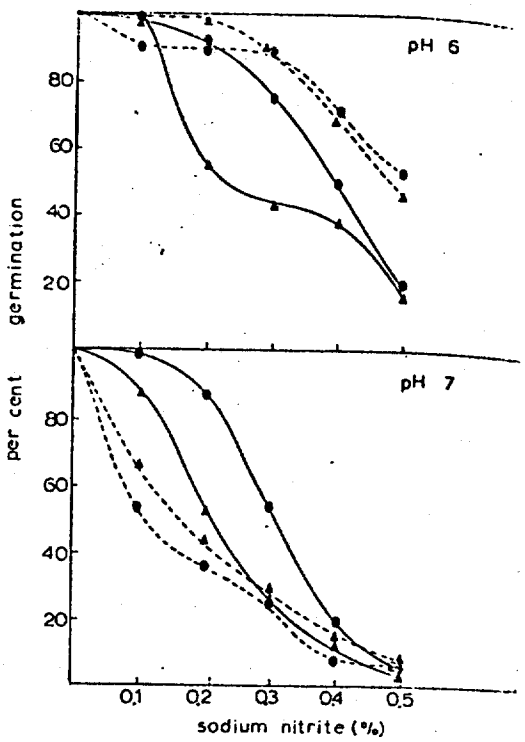
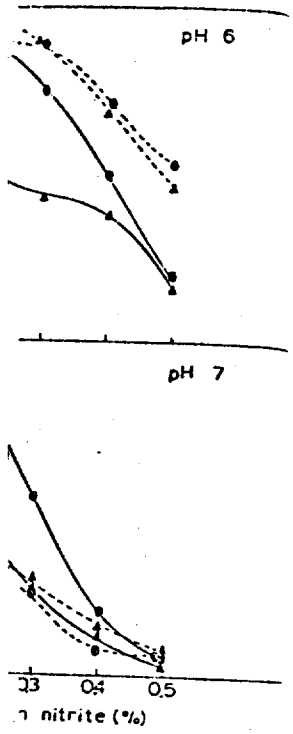


FIG. 1. *Effect of sodium nitrite on the germination of Clostridium perfringens strain FD1 spores. Spores were incubated at 45 C for 15 min in a germination medium consisting of 2.5% Brain Heart Infusion and 0.27% yeast extract. Sodium nitrite was added to the medium in the concentrations indicated either before or after autoclaving for 15 min at 121 C. Broken lines, heat-injured spores; solid lines, unheated spores;* ▲, *sodium nitrite added before autoclaving;* ●, *sodium nitrite added after autoclaving.*

during autoclaving, (iii) inhibition of outgrowth of heat-injured spores, and (iv) induction of germination (which would render the spore susceptible to thermal inactivation).

Inhibition of germination. The effect of adding sodium nitrite to a complex medium before or after autoclaving (15 min at 121 C) on the germination of heat-injured or non-heat-injured FD1 spores is shown in Fig. 1. At *p*H 7 and at sodium nitrite concentrations less than about 0.3%, heat-injured spores were more susceptible to inhibition of germination by nitrite regardless of whether nitrite was added before or after autoclaving. Addition of sodium nitrite before autoclaving the medium did not reduce the per cent germination of heat-injured spores, as compared to the germination obtained when nitrite was added after autoclaving. However, germination of non-heat-injured spores was reduced when nitrite was added before autoclaving. At *p*H 6, the results were nearly the reverse of those obtained at *p*H 7.0. At



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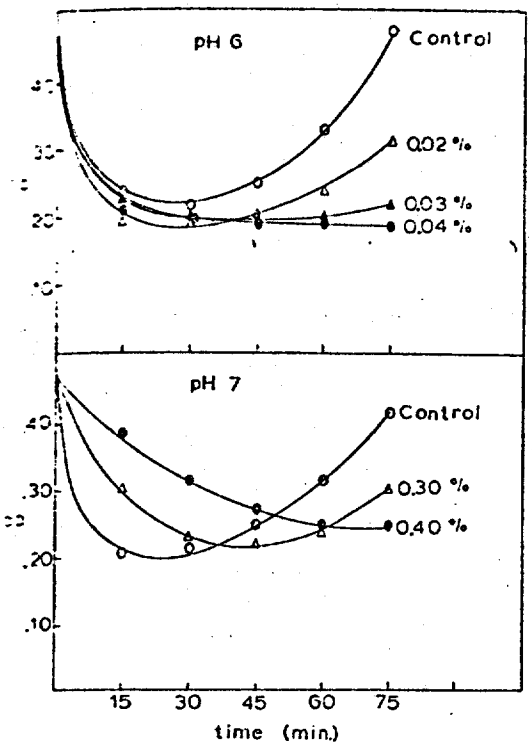

FIG. 2. *Effect of sodium nitrite on outgrowth of non-heat-injured Clostridium perfringens strain FD1 spores. Spores were incubated at 45 C in medium consisting of 2.5% Brain Heart Infusion and 0.27% yeast extract. Sodium nitrite was added to the sterile medium to give the concentrations indicated in the figure.*

sodium nitrite concentrations of 0.2% or more, the per cent germination of heat-injured spores was greater than that of non-heat-injured spores regardless of whether nitrite was added before or after autoclaving. The addition of sodium nitrite to the medium before autoclaving markedly reduced the germination of non-heat-injured spores at *p*H 6.0 as compared to the germination obtained when nitrite was added after autoclaving the medium. At concentrations greater than 0.3%, the germination of heat-injured spores was only slightly reduced when nitrite was added before autoclaving. Heat-injured or non-heat-injured NCTC 8798 spores were not prevented from germinating when sodium nitrite concentrations as high as 2% were added to the complex media after autoclaving.

Duncan and Foster (3) observed that even as much as 4% sodium nitrite does not prevent loss of refractility of PA 3679h spores in liver-veal-agar. Gould (7), on the other hand, noted inhibition of several *Bacillus* species in yeast-glucose-agar by 0.075 to 0.25% nitrite. The various effects of sodium nitrite on germination are probably due primarily to species differences. Considering the maximum residual sodium nitrite concentration of 0.02% that is actually permissible in practice, it is unlikely that either sodium nitrite or an inhibitory product formed by heating nitrite with a complex medium contributes to the preservation of canned cured meat by blocking germination of all contaminating spores. The possibility that nitrite may block germination of a fraction of the heat-injured spore population is discussed later.

Inhibition of outgrowth. The effect of nitrite upon outgrowth of unheated *C. perfringens* spores is shown in Fig. 2. At *p*H 6, 0.04% sodium nitrite was required to inhibit outgrowth of unheated strain FD1 spores. Ten times that amount was required at *p*H 7. Also at *p*H 7, a concentration of 0.4% nitrite decreased the rate of germination.

Heat-injured FD1 spores were much more sensitive to nitrite than non-heat-injured spores, with a concentration of 0.02% preventing outgrowth at *p*H 6 and 0.1% preventing outgrowth at *p*H 7 (Fig. 3). Small amounts of sodium nitrite apparently stimulated outgrowth of heated spores

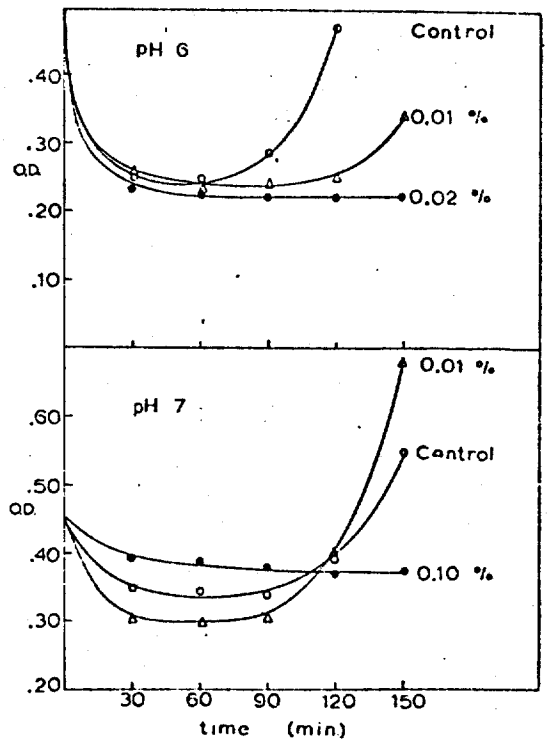

FIG. 3. *Effect of sodium nitrite on the outgrowth of heat-injured Clostridium perfringens strain FD1 spores. Incubation conditions were the same as those described in Fig. 2, except for the concentrations of sodium nitrite used.*

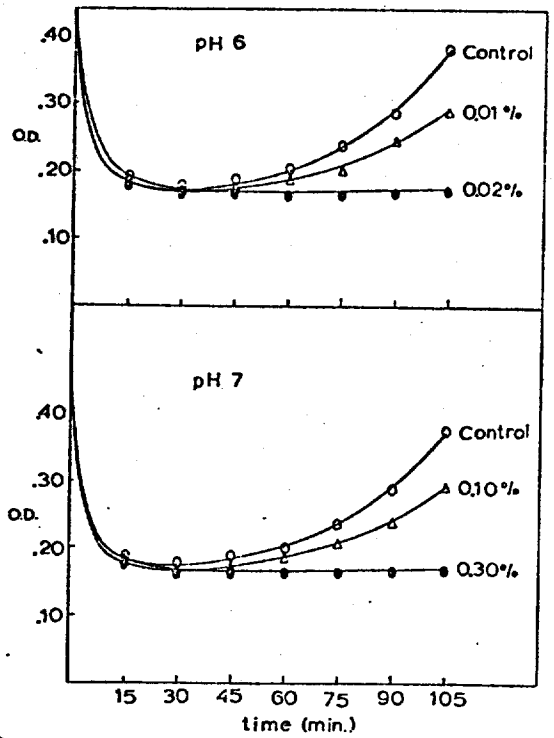

FIG. 4. *Effect of sodium nitrite on the outgrowth of non-heat-injured Clostridium perfringens strain NCTC 8798 spores. Spores were heat-shocked at 70 C for 15 min and incubated under the same conditions as those described in Fig. 2, except for the concentrations of sodium nitrite used.*

at *p*H 7, since the OD at concentrations of 0.01% was greater after 150 min than that of the control.

Similar experiments were conducted with NCTC 8798, a heat-resistant strain. This strain was more sensitive to nitrite than was FD1; concentrations of 0.02 and 0.3% were required for inhibition of outgrowth of non-heat-injured spores at *p*H 6 and 7, respectively (Fig. 4). As with strain FD1, heat-injured spores were more susceptible to inhibition of outgrowth by sodium nitrite both at *p*H 6 (0.01%) and *p*H 7 (0.04%; Fig. 5).

Although commercial heat treatments employed in the processing of canned cured meats may destroy heat-sensitive spores, it is unlikely that all spores of resistant strains are so affected. That the latter may be more sensitive to inhibition of outgrowth by sodium nitrite is therefore of added importance.

Sodium nitrite-induced Germination. There have been several reports on induction of spore germination by sodium nitrite (1, 5; S. H. Black, Bacterial. Proc., p. 36, 1964). Generally, the temperatures of incubation in these studies were below that to which spores are subjected during the thermal processing of canned cured meats. We have confirmed that nitrite induction of germination does occur with *C. perfringens* and have investigated the possibility that this may be a factor in preservation of canned cured meats. If commercially acceptable concentrations of nitrite induced germination of the spores in a meat product during processing, the spores would become heat-labile and would therefore be inactivated by the thermal process.

The effect of nitrite concentration on the induction of germination of strain FD1 spores is shown in Fig. 6. There is a limiting concentration (about 1.5%) above which the level of initiation of germination will not increase under the stated conditions. The effects of temperature and *p*H are shown in Table 1. As the temperature increased, the per cent initiation of germination increased at *p*H 6. Complete germination occurred in 90 min at low temperatures (25 C) and at *p*H 6 only if the nitrite concentration was sufficiently high (6%). Essentially no germination occurred at *p*H 8. Nitrous acid appears to be the effective agent, since the extent of initiation of germination was greatest at *p*H 6. No germination occurred after 2 hr at *p*H 6 when the spores were incubated at temperatures of 25, 37, 45, 60, 75, or 90 C with as much as 0.02% sodium nitrite, the maximum

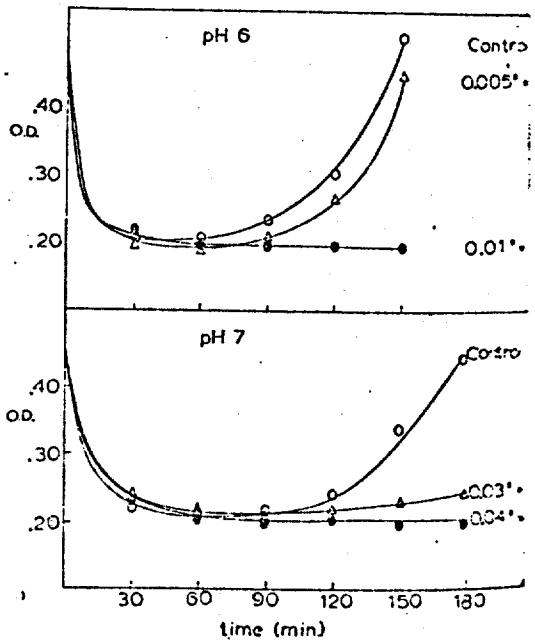

FIG. 5. *Effect of sodium nitrite on the outgrowth of heat-injured Clostridium perfringens strain NCTC 8798 spores. Incubation conditions were the same as those described in Fig. 2, except for the concentrations of sodium nitrite used.*

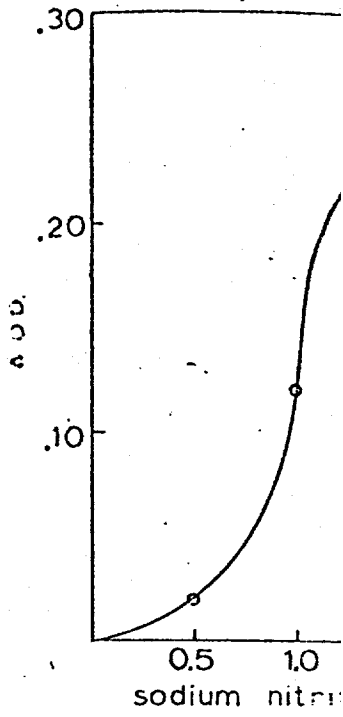

FIG. 6. *Effect of $NaNO_2$ concen-tration of Clostridium perfringens were incubated in 33 mM phosphate plus added sodium nitrite. Germination change (△) in OD that occurred incubation at 45 C.*

TABLE 1. *Effects of temperature induced germination of Clostridium FD1 spores*[a]

Temp and *p*H
45 C, *p*H 8
45 C, *p*H 7
45 C, *p*H 6
60 C, *p*H 8
60 C, *p*H 7
60 C, *p*H 6
75 C, *p*H 8
75 C, *p*H 7
75 C, *p*H 6

[a] Germination mixtures con and 33 mM phosphate buffer. determined after 1 hr of incub

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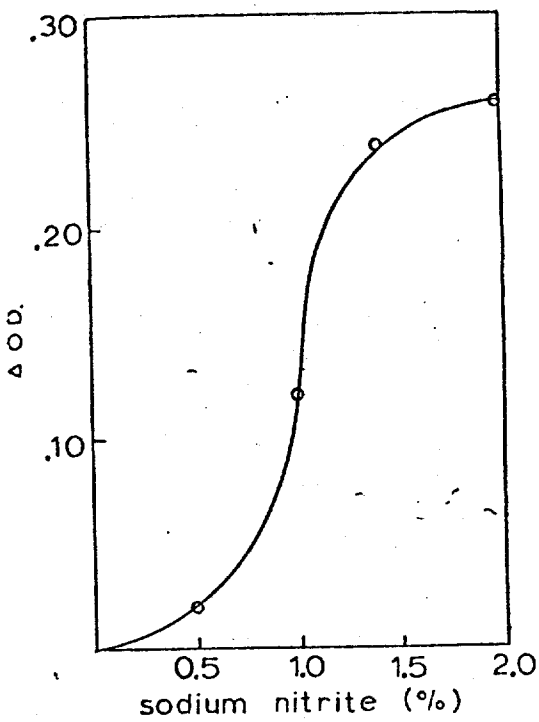

FIG. 6. *Effect of $NaNO_2$ concentration upon germination of Clostridium perfringens FD1 spores. Spores were incubated in 33 mM phosphate buffer (pH 6.0) plus added sodium nitrite. Germination is expressed as the change (Δ) in OD that occurred after 1 hr of incubation at 45 C.*

TABLE 1. *Effects of temperature and pH on nitrite-induced germination of Clostridium perfringens FD1 spores*[a]

Temp and pH	Germination
	%
45 C, pH 8	4
45 C, pH 7	7
45 C, pH 6	18
60 C, pH 8	4
60 C, pH 7	11
60 C, pH 6	46
75 C, pH 8	0
75 C, pH 7	4
75 C, pH 6	96

[a] Germination mixtures contained 1% $NaNO_2$ and 33 mM phosphate buffer. Germination was determined after 1 hr of incubation.

allowable concentration in canned cured meats. Slight decreases in OD in the control tubes were observed at 75 and 90 C after 24 hr. Microscopic examination of these controls revealed spores with a phase-dark periphery but a refractile core. It is unlikely that most of these spores remained viable since this strain, FD1, is itself heat-sensitive with a D_{90} equal to about 15 min.

It may be concluded that, although sodium nitrite may induce bacterial spore germination, concentrations that are commercially acceptable will not.

DISCUSSION

The method by which sodium nitrite may act to prevent microbial growth of heat-injured spores in canned cured meats was investigated by using *C. perfringens*. Four possible mechanisms were tested: (i) prevention of germination of heat-injured spores, (ii) prior combination with a component in a complex medium to prevent germination of heat-injured spores, (iii) inhibition of outgrowth of heat-injured spores, (iv) induction of germination (which would render the spore susceptible to subsequent heat treatments).

When sodium nitrite is heated in a complex medium, it combines with some component in the medium to yield an unknown but extremely potent inhibitory agent; e.g., autoclaving 3.5 μg/ml (0.00035%) in a medium for 20 min at 109 C produces enough inhibitor to inhibit 8×10^5 vegetative cells in 50% of the trials (12). This product has been shown to be effective against vegetative cells of several *Clostridium* species (11). However, the present results indicate that, even when using concentrations of sodium nitrite up to 10 times greater than commercially acceptable levels, the inhibitory agent, if indeed produced, is not effective in preventing initiation of germination of most of the intact or heat-injured *C. perfringens* spores. For instance, at pH 6, about 0.5% nitrite was required to reduce the per cent germination of heat-injured strain FD1 spores to 50% of the control, whether sodium nitrite was added before or after autoclaving.

It has been found (9) that the inhibitory agent formed by heating nitrite in a complex medium may have little effect in the preservation of canned cured meats. A meat suspension heated with 150 μg (0.015%) of sodium nitrite per ml was inhibitory to *C. botulinum*; however, the inhibition was thought to be due to residual inorganic nitrite since dialysis removed the inhibitory activity. Furthermore, the inhibitory factor produced by heating nitrite in a complex medium could be inactivated by nonfat meat solids.

The present results show that sodium nitrite at greater than commercially acceptable concentrations does not prevent initiation of germination of the majority of non-heat-injured or heat-injured *C. perfringens* spores, regardless of whether it is added before or after autoclaving the complex

germination medium. However, a small number of the spore population may actually have been blocked from initiating germination. These blocked spores would not have been detected by the technique used for assessment of spore germination, since extinction measurements are of little value in estimating germination above about 90% (8). It would be difficult to distinguish a low number of nitrite-blocked spores from the "superdormant" spores (8) that may be present in a normal spore population.

The fact that a small number of spores may be found by postprocessing analysis of canned cured meats (13) indicates that either inhibition of the initiation of germination of the heat-injured spores has occurred or that the spores represent a superdormant fraction. Indeed, superdormancy may have been enhanced by heat injury.

Although sodium nitrite alone did not prevent germination of the majority of the spore population, it may have inhibited germination of small numbers of spores. This inhibition should be greater in combination with the curing salt sodium chloride.

Sodium nitrite and sodium chloride are not solely responsible for the preservation of canned cured meats. Heat plays an important role, although it is not sufficient to destroy all bacterial spores. The thermal treatment is thought to injure the spores, making them more sensitive to curing salts in the outgrowth medium (3, 14). This phenomenon was observed in the present studies with heat-damaged *C. perfringens* spores. At *p*H 6.0, concentrations of 0.02 and 0.01% sodium nitrite inhibited outgrowth of heat-damaged spores of a heat-sensitive and a heat-resistant strain, respectively. Unheated spores required 0.04 and 0.02%, respectively. It should be pointed out that the incubation temperature was 45 C for 3 hr. It is conceivable that, with additional incubation, inhibition might have been overcome. A temperature of 45 C was used since it is near the optimum for growth of *C. perfringens*. Roberts and Ingram (14) pointed out that the injured spore may be affected by the temperature prevailing while it is struggling against the inhibitory salts. Undoubtedly, inhibition of outgrowth of germinated spores by sodium nitrite is an important aspect of canned meat preservation, since commercially acceptable concentrations were effective.

Nitrite-induced germination has been observed with several organisms. We confirmed its occurrence with *C. perfringens*. High temperature, low *p*H, and high sodium nitrite concentrations yielded optimal germination. Undissociated nitrous acid presumably was the effective agent. Duncan and Foster (4) postulated that the phenomenon of nitrite-induced germination may be involved in the stability of the preservative system. Once germinated by sodium nitrite, the spores would be destroyed by the heat treatment rendered the particular product. This seems unlikely, since, in the present experiments, germination was not obtained after 1 hr at 90 C with as much as 0.02% sodium nitrite, the maximum concentration allowable in commercial use. Whether spores of other organisms would be similarly affected is not known.

An important factor in the safety and stability of canned cured meats which must be mentioned is that of cell numbers (13). In the present experiments, concentrations of about 10^7 spores per ml were used; this is much greater than the actual spore load in meats. Concentrations of sodium nitrite lower than those used here may have been equally effective against germination and outgrowth had we employed a smaller number of spores.

In these experiments no attempt was made to investigate the additive effect that a combination of sodium chloride plus sodium nitrite might have in inhibiting growth from spores. Conceivably, the combined inhibitory effect would be greater than that reported for nitrite alone.

ACKNOWLEDGMENTS

This investigation was supported by a grant from the Graduate School of The University of Wisconsin and by contributions from the food industry to the Food Research Institute.

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Table 2. Absorptivities of isomerized fats from mixtures of horse meat and meat products

Composition of Samples	Ratio of Extracted Fat	Absorptivity
Pork Sausage/Horse Meat		
100 0	100/0	0.76
90 10	98.5/1.5	0.86
80 20	96.6/3.4	1.03
50 50	87.6/12.4	1.35
20 80	64.0/36.0	2.56
10 90	44.1/55.9	2.99
0 100	0/100	4.12
Frankfurters/Horse Meat		
100 0	100/0	0.86
90 10	97.7/2.3	1.15
80 20	94.5/5.5	1.18
50 50	82.2/17.8	1.46
20 80	53.6/46.4	2.81
10 90	33.9/66.1	3.46
0 100	0/100	4.12

sage contained 6.3, 29.1, and 44.8% fat, respectively.

The data in Tables 1 and 2 show that the presence of horse fat can be detected at low levels in the presence of other animal fats, with the possible exception of deer fat. However, when horse meat is mixed with commercial cooked meat products, its detection becomes difficult because of the relatively low content of fat in horse meat. No attempt was made at quantitative determinations of the fatty acids, but a study of the literature indicates that this could improve the detection of horse meat in mixtures. The assumption that a high absorptivity shows the presence of horse meat is erroneous unless certain vegetable oils are known to be absent.

The Associate Referee recommends[1] that the study be continued.

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[1] This recommendation was approved by the General Referee and by Subcommittee C, and was adopted by the Association. See *This Journal*, 45, 125 (1962).

1207

The Determination of Nitrate and Nitrite in Meat and Meat Products*

WENDELL A. LANDMANN (American Meat Institute Foundation, University of Chicago, Chicago 37, Ill.)

The 1960 report of Subcommittee C (*This Journal*, 44, 68 (1961)) recommended that Goals 23.010–23.015 for nitrates in meat be given further study. These are the ferrous sulfate and the xylenol procedures for nitrite. Neither has attained official status; they are still listed as first action.

The amount of nitrate in meat-curing mixtures has been reduced in recent years, and levels of nitrate likely to be found in cured meats are at the lower limits of detection by both these methods. Furthermore, since both methods determine nitrate plus nitrite, nitrite must be determined by an independent procedure and the results adjusted accordingly. In the presence of larger amounts of nitrite, the determination of small amounts of nitrate is subject to considerable error. Although these methods are actually more suitable for pickling brines and curing mixtures, they are still being used for analyses of cured meats.

* Presented as the report of the Associate Referee at the Seventy-fifth Annual Meeting of the Association of Official Agricultural Chemists, 30 Nov. 1, 1961, at Washington, D.C. Journal Series No. 223, American Meat Institute Foundation.

Revision or re-evaluation is therefore necessary.

In such a study it was deemed advisable to investigate newer methods which are sensitive at low ranges of nitrate concentration and which give a direct value for nitrate, uninfluenced by nitrite. Methods for the direct determination of nitrite should also be investigated, and the first action method, 23.016, should be compared with more recent methods so that one could be selected for official study.

With these objectives the Associate Referee has attempted to evaluate the ferrous chloride and xylenol methods and to examine, in a preliminary way, applicable published methods for the direct determination of nitrate. Results of a previous collaborative study (1) of these methods were re-examined. Although values were reported by only 4 laboratories, they were clear-cut enough to indicate the need for revision of the existing xylenol method, 23.015, or its replacement by a new one. The statistical evaluation is shown below. The sample submitted to each laboratory was a solution containing sodium chloride, sugar, 0.075% sodium nitrate, and 0.275% sodium nitrite:

Method	*Mean % $NaNO_3$ Found*	*Std Dev.*
23.010 ($FeCl_2$)	0.080	0.0197
23.015 (Xylenol)	0.022	0.0045

On the basis of this study it appears that any new method should be compared to the ferrous chloride method rather than to the xylenol method. The cooperating laboratories generally agreed that the xylenol method was unreliable, tedious, and cumbersome, and should be replaced by a more rapid direct method.

Several recent methods for the direct colorimetric determination of nitrate are available. Those based on the nitration of aromatic compounds to form chromophores are quite sensitive and give accurate results, but have the disadvantage that concentrated sulfuric acid must be used as the solvent and reaction medium. Two such methods which have been found to work well are the brucine procedure of Landmann, *et al.* (2), and the chromotropic acid procedure of West and Lyles (3). The brucine procedure, when subjected to a preliminary collaborative study, compared favorably with the ferrous chloride method.

Other methods for determining nitrate involve the reduction of nitrate to ammonia (4) and the direct spectrophotometric estimation of nitrate (5). These do not appear to be suitable for meat products.

Several methods have been developed for the determination of nitrate by reduction to nitrite. These are essentially nitrite methods (6–9), since nitrate is determined by difference before and after reduction. However, some of these methods appear to be promising as a single method for both nitrate and nitrite in meats and will be investigated for this purpose.

In addition to nitrate-nitrite procedures already mentioned, an iodometric method for the direct determination of nitrite has been found to be quite rapid and suitable for application to meat (10). A modification of this method has long been used with satisfactory results for the determination of nitrite in curing mixtures and pickling brines; its application to meat should be investigated as an alternative for method 23.016–23.017.

Another colorimetric procedure for nitrite which appears to be applicable to meat employs phenothiazine as the chromogenic material (11). This method will also be investigated.

In the coming year the brucine and chromotropic acid methods for nitrate will be studied collaboratively, and the results will be compared to those obtained by the ferrous chloride method, 23.010. Further investigations will be conducted on the applicability of simultaneous nitrite-nitrate methods to cured meat. Studies on the two direct nitrite methods will be continued, with the hope that one of these will prove suitable for collaborative work.

It is recommended[1] that work on the determination of nitrates and nitrites in meat and meat products be continued.

[1] This recommendation was approved by the General Referee and by Subcommittee C and was adopted by the Association. See *This Journal*, 45, 125 (1962).

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The "Banco" Test: A Rapid Method for Fat in Meat and Edible Meat Products*

By B. B. ANDERSON (Anderson Laboratories, Inc., Fort Worth, Texas), L. LEON ROBINSON (Armour and Co. Control Laboratory, Forth Worth, Texas), and JOE E. HODGKINS (Chemistry Department, Texas Christian University, Fort Worth, Texas)

A simple, rapid, and accurate method is needed for determining the fat content of edible meats and meat products such as hamburger, pork trimmings, and various types of sausage and meat loaf. Conventional solvent extraction methods are too time consuming to be satisfactory for blending purposes. The modified Babcock method is hazardous, often chars the sample, and is generally unsatisfactory for meat products containing additives such as cereal, milk powder, and sodium caseinate.

A quantitative method, based on the principle of the "Detergent Test" (1) for the determination of fat in milk and milk products, has been developed for the determination of fat in meat and meat products. The method is rapid and non-hazardous, and has proved to have an excellent degree of accuracy and reproducibility. The usual application requires about 30 minutes, compared to about 6 hours for the standard AOAC method (2).

* Presented at the Seventy-fifth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 30-Nov. 1, 1961, at Washington, D.C.

METHOD

Apparatus

(a) *Centrifuge.*—Standard Babcock-type centrifuge which, however driven, must be constructed throughout and so mounted as to be capable, when filled to capacity, of rotating at necessary speed with minimum vibration and without liability of causing injury or accident. Proper rate of rotation may be ascertained by reference to table below. ("Diam. of wheel" signifies the inside bottoms of opposite cups measured through center of rotation of centrifuge wheel while cups are horizontally extended (3)).

Diam. of Wheel, in.	*rpm*
14	909
16	848
18	800
20	759
22	724
24	693

(b) *Reaction bath.*—Any device for maintaining water at boiling point and at level at 1.5-2 inches.

(c) *Tempering bath.*—Any device for maintaining water at 55-60° C and at level of approximately 6 inches.

(d) *Reagent measure.*—Any device graduated to deliver 15 ml.

Sec. 26.060(a), fourth line, after "0.1N NaOH" delete "42.032-42.033." In last sentence, after "0.25N NaOH," add "42.032-42.033." Sec. 26.060(b), fourth line, after "0.1N NaOH" add "42.032-42.033."

Sec. 42.032, last sentence: Add "or 42.034."

The recommendations of the Associate Referee were approved by the General Referee and by Subcommittee A and were adopted by the Association. See *This Journal*, 49, 161-165 (1966).

REFERENCES

(1) *Official Methods of Analysis*, 10th Ed., Association of Official Agricultural Chemists, Washington, D.C., 1965.

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(3) Subcommittee A's supplementary report submitted November 5, 1964. (The recommended changes in methods 42.031(c) and 42.033, were inadvertently omitted from the published report.)

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1208

MEAT AND MEAT PRODUCTS

Determination of Nitrite and Nitrate in Meat Products

WENDELL A. LANDMANN (Agricultural Science Department, Texas A&M University, College Station, Texas 77843)

A study was initiated on a method for the simultaneous determination of nitrate and nitrite in meat products. Nitrate and nitrite are determined by difference before and after the reduction of nitrate with metallic cadmium. Nitrate is diazotized, coupled with sulfanilic acid and 1-naphthol, and measured. From 93 to 95% of nitrate and nitrite added to meat can be recovered. The nitrate and nitrite do not interfere with each other in the determination. Ruggedness tests were satisfactory, and the method should be studied collaboratively.

According to the approved recommendations of the Associate Referee on Nitrate and Nitrite in Meat Products in 1963 (1), methods for nitrate were to be investigated and further investigations of an iodometric determination of nitrite in meat were carried out. Continuing studies on the application to meat of the thiosulfate-iodometric method for nitrite in dry cure mix continued over a period of several years and then were abandoned. The results obtained with smaller levels of nitrite were highly erratic, and the necessity of working with extremely dilute standard solutions of sodium thiosulfate presented problems which made the method impractical for use in a control laboratory. Consequently, a study was begun on a new method by Follett and Ratcliff (2) for the simultaneous determination of nitrite and nitrate. In this method, nitrate and nitrite in meat products are determined by difference, before and after the nitrate is reduced to nitrite by a column of metallic cadmium. The nitrite is determined by measuring the color developed when the diazotized sulfanilic acid couples with 1-naphthol to form the dye Orange I.

Experimental

The procedure described by Follett and Ratcliff was followed closely, except for the design of the cadmium columns and the use of a pH meter to adjust the pH of the protein-free filtrate.

Nitrite Determination

A meat sample in hot water and buffer (pH 9.6) was blended or homogenized. Celite was added and the mixture was filtered into a volumetric flask. The filtrate was heated and its pH adjusted to 5.5-6.5 with potassium aluminum sulfate; then it was cooled and filtered. The acidic filtrate was diluted to

volume with buffer and water, an aliquot was reacted with Orange I reagent, and the developed color was measured spectrophotometrically at 474 mμ. The concentration of sodium nitrite was determined by reference to a standard curve.

Nitrate Determination

A solution of protein-free filtrate (prepared as above) was put through a column consisting of glass wool, washed sea sand, and spongy cadmium; the solution was followed by distilled water. The eluate was then analyzed colorimetrically as for nitrite with Orange I reagent. The column was washed with dilute HCl, water, and then diluted buffer before subsequent determinations.

Recovery Experiments and Ruggedness Test

The method was subjected to recovery experiments and a ruggedness test. For the recovery experiments, a factorial design with two levels of sodium nitrite (20 ppm and 200 ppm in 10 g meat) and three levels of sodium nitrite (20 ppm, 100 ppm, and 500 ppm) was employed. Two replicate experiments were carried out on meat samples to which the appropriate aliquots of each solution were added after the sample was ground. Recoveries of sodium nitrite were 95 ± 5% of the amounts present and were not affected by the amount or level of sodium nitrate present. Recoveries of sodium nitrate were 93 ± 5% when more than 100 ppm was present. When less than 100 ppm was present, recoveries were higher, and at 20 ppm, they were sometimes greater than 100%. The analysis of variance indicated that the amount of nitrate recovered was not influenced by the level of nitrite present and was elevated only at the 20 ppm level of nitrate. This effect was probably due to the meat blank, which would show up at the low level only and which was not corrected for. However, the results were consistent enough to warrant further testing of the method.

A ruggedness test was carried out according to Youden (3) to determine the importance of variations in operating conditions and to predict the behavior of collaborative tests on this method. The sam[illegible] consisted of 10.0 g fresh meat to whi[illegible] amount of sodium nitrite equivalent to [illegible] ppm was added. The following factors [illegible] examined:

A. Nitrite added prior to grinding [illegible] a. nitrite added to ground meat slurry.

B. Sample filtered immediately [illegible] grinding; b. sample slurry allowed to s[illegible] in refrigerator overnight before filtering

C. Initial temperature of slurry 75 [illegible] initial temperature of slurry 60°.

D. Filtrate temperature 90°; d. filtr[illegible] temperature 80°.

E. Final de-proteinized solution, pH 7 [illegible] e. final de-proteinized solution, pH 5.0.

F. 10.0 ml Orange I reagent added; f. [illegible] ml Orange I reagent plus 0.2 ml H_2O add[illegible]

G. Color development, 45 min.; g. c[illegible] development, 20 min.

Results of the ruggedness test showed [illegible] only factors A and E had any pronoun[illegible] effect on the final results. Factor E, pH [illegible] the final solution before color developm[illegible] influenced the result as expected, since t[illegible] authors of the method point out the necess[illegible] for careful control of the pH for color d[illegible] velopment. Factor A, time of addition [illegible] nitrite to the meat, is not a factor wh[illegible] would be encountered in actual practice [illegible] it exerted the largest effect on the resul[illegible] This factor was included in deciding [illegible] procedure to be followed in a future c[illegible] laborative study in which changes in [illegible] nitrite content of a sample prior to analy[illegible] are to be avoided. The standard deviat[illegible] estimating the analytical error to be [illegible] pected from typical variations in the pr[illegible] cedure, in addition to Factor A, was 12 p[illegible] for these samples containing 200 ppm sodi[illegible] nitrite. Thus the procedure appears to [illegible] rugged as well as accurate.

Discussion and Recommendation

The procedure outlined above and giv[illegible] in detail by Follett and Ratcliff appear[illegible] be useful for the determination of nitr[illegible] and nitrate in meat products. It has [illegible] advantage of allowing both compounds [illegible] be determined by one procedure, simply a[illegible] rapidly. It seems to be at least as accur[illegible] as the *m*-xylenol procedure for nitrate a[illegible]

This report of the Associate Referee was presented at the Seventy-ninth Annual Meeting of the Association of Official Agricultural Chemists, Oct. 11-14, 1965, at Washington, D.C.

[illegible]h easier. It also appears to be [illegible] accuracy to the Griess method fo[illegible] [illegible]te and is as simple to perform. [illegible] It is recommended that the proc[illegible] [illegible]llett and Ratcliff be studied collab[illegible]

[illegible] recommendation of the Associate Refe[illegible] [illegible]ed by the General Referee and by S[illegible] [illegible] and was accepted by the Association. [illegible]*nal*, 49, 167-172 (1966).

Chemical Identification [illegible]

[illegible] H. R. COOK and JOHN D. S[illegible]
[illegible]al Marketing Service, Meat Insp[illegible]

Horse, pork, and beef fat are [illegible] by gas chromatography of the [illegible]fiable matter which is first fractio[illegible] column chromatography. Fractions [illegible] three give peaks which identify th[illegible]

Last year the Association reco[illegible] that further study be made on the [illegible]dentification of horse meat by u[illegible] [illegible]hromatography (1). This paper is [illegible] to show progress in carrying out th[illegible] [illegible]mendation.

Our primary concern is the ide[illegible] [illegible] cooked horse meat. Biological p[illegible] for species identification are not [illegible] to meats in which the protein has [illegible]natured by cooking or other proces[illegible] [illegible]ver, since the fat appears to be [illegible]ffected by cooking, it was used fo[illegible] [illegible]examination.

Some, but not all, horse fat [illegible] [illegible]hen heated with alcoholic KO[illegible] [illegible]milar approach, which is essen[illegible] [illegible]pendent upon the relative amoun[illegible] [illegible]enic acid in the fat for species ide[illegible] [illegible]as pursued by Cook (3). Howeve[illegible] [illegible] varying amounts of linolenic ac[illegible] [illegible]dentification of horse fat, especial[illegible] [illegible]tures, was questionable.

In our method the unsaponifiab[illegible] [illegible] the fat samples was first sep[illegible] [illegible]ording to sec. 26.049, *Official A[illegible] [illegible]nalysis*, 10th Ed., with some min[illegible]

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be encountered in actual practic
erted the largest effect on the re
actor was included in deciding
ure to be followed in a future
ative study in which changes in
content of a sample prior to an
be avoided. The standard devi
ating the analytical error to be
from typical variations in the
, in addition to Factor A, was 12
iese samples containing 200 ppm
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Discussion and Recommendation

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ch easier. It also appears to be superior
ccuracy to the Griess method for sodium
rite and is as simple to perform.

It is recommended that the procedure of
llett and Ratcliff be studied collaboratively
as a method for both nitrite and nitrate in
meat products.

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(3) Youden, W. J., *This Journal*, 46, 55–62 (1963).

The recommendation of the Associate Referee was ap-
ed by the General Referee and by Subcommittee
and was accepted by the Association. See *This*
al, 49, 167–172 (1966).

Chemical Identification of Meat

H. R. COOK and JOHN D. STURGEON (U.S. Department of Agriculture, Consumer
Marketing Service, Meat Inspection Division, Washington, D.C. 20250)

Horse, pork, and beef fat are identified by gas chromatography of the unsaponifiable matter which is first fractionated by column chromatography. Fractions two and three give peaks which identify the fats.

Last year the Association recommended
t further study be made on the chemical
ntification of horse meat by use of gas
omatography (1). This paper is presented
show progress in carrying out this recom-
ndation.

Our primary concern is the identification
cooked horse meat. Biological procedures
species identification are not applicable
meats in which the protein has been de-
ured by cooking or other processes. How-
r, since the fat appears to be the least
cted by cooking, it was used for chemical
mination.

Some, but not all, horse fat turns red
n heated with alcoholic KOH (2). A
lar approach, which is essentially de-
ndent upon the relative amount of lino-
e acid in the fat for species identification,
pursued by Cook (3). However, because
varying amounts of linolenic acid in fats,
ntification of horse fat, especially in mix-
s, was questionable.

In our method the unsaponifiable matter
the fat samples was first separated ac-
ding to sec. 26.049, *Official Methods of*
lysis, 10th Ed., with some minor changes
as follows: Because animal fats are lower in
unsaponifiable matter than vegetable fats, it
was necessary to use a quantity of sample
and KOH, each about 25 times greater than
in the original method, to obtain a weigh-
able amount of each fraction after column
chromatography; 600 ml ether was used in
each extraction; 250 ml water and 250 ml
KOH were used to wash the combined ether
extracts.

Florisil chromatography was then used as
described by Eisner, *et al.* (4), except that
the total weight of the unsaponifiable mat-
ter of the fats was used instead of about 40
mg. All fractions separated were examined
except the sterol, which contained only
cholesterol.

The fractions were examined on a Barber-
Colman Model 5000 gas chromatograph with
a flame detector and a 5 mv recorder. Glass
columns, 8 ft by 5 mm, were used with
about 25 lb nitrogen carrier gas. The detec-
tor was operated at 235°C and the sampler
at 220°C. Various Applied Science Labora-
tories' coatings were used on a Gas Chrom
Q support.

The sample fractions were first chromato-
graphed isothermally on nonpolar substrates
such as SE-52 and JXR. Temperature pro-
gramming and polar substrates were used
later. This technique gave better separation
of the complex mixture of compounds of
varying molecular weights. Significant differ-

Journal of the AOAC 43 (1960)

ference into a small disposable aluminum dish, add sand or asbestos, and mix, spreading on bottom of dish with glass or aluminum paddle. Dry as above. Roll edges of aluminum dish and insert with paddle into thimble. Proceed as in 22.26, using petroleum ether, 10.83, if desired." That the modified method be adopted as official.

(3) That the serological method for the detection of horsemeat, as described by the Associate Referee in 1954,[3] be studied collaboratively.

(4) That the method for lactose be further studied with a view toward identifying the reducing substance in corn sirup which affects the determination, and eliminating the source of interference.

[3] Brandly, P. J., *This Journal*, 37, 843 (1954).

1209

The Determination of Nitrate in Meat and Meat Products*

By W. A. LANDMANN, MOHAMMED SAEED, KATHERINE PIH, and D. M. DOTY
(American Meat Institute Foundation, University of Chicago, Chicago, Ill.)

During the past three years the American Meat Institute Foundation has conducted a collaborative testing program to evaluate methods of meat analysis. Among the analyses made, the nitrate determination showed the greatest variation in results, particularly in cured meat samples. The reason for this, in part, is that the methods involve the total determination of nitrate plus nitrite, thereby compounding the error in the former determination with that from the latter. A direct nitrate determination would therefore be preferable.

A number of direct colorimetric methods for nitrate have appeared in the literature, the most prominent of which are the phenoldisulfonic acid method, the *m*-xylenol method, and the brucine method. In every case, the presence of nitrite interferes or, as in the *m*-xylenol procedure, changes the procedure to a total nitrite-nitrate method subject to the same problems already mentioned. Furthermore, the phenoldisulfonic acid and *m*-xylenol methods are cumbersome and tedious.

The brucine method, however, is rapid and simple, and is applicable to water and soil analysis (1, 2). A critical study has recently been carried out by Fisher, *et al.* (1) in conjunction with the development of a rapid colorimetric determination of nitrate, nitrite, or both, in water and soil samples. The brucine method for nitrate has been used by Niinivaara (3) and MacDougall and Roadhouse (4). In all of these procedures large volumes of reagent are required. It was felt that if the same accuracy could be obtained on a smaller scale, the reaction could be carried out in test tubes to permit closer temperature control and better reproducibility. To this end, investigations of the brucine-nitrate reaction were made. A number of recommendations from earlier studies were utilized in this work, and have been incorporated into the procedure finally developed. The differences between the existing procedures and that recommended here are chiefly the scaled-down volumes and the more closely controlled conditions.

Temperature Effects in Color Development.—In the previous procedures the heat necessary for the reaction was supplied by the heat of mixing sulfuric acid and water. This gave widely varying temperatures which affected the intensity of the color developed by the brucine. This variation could largely be eliminated by using an ice bath to cool solutions both prior to and

* Presented at the Seventy-third Annual Meeting of the Association of Official Agricultural Chemists, Oct. 12-14, 1959, at Washington, D.C. American Meat Institute Foundation journal paper No. 198.

during mixing, and by using a boiling water bath to develop the color. The time required to develop maximum color, measured from the instant the cooled reaction mixture was immersed in a boiling water bath, was two minutes. If the tubes were immediately cooled to room temperature in an ice bath, the color remained stable and constant for 24 hours.

Interference From Organic Materials, Chlorides, and Nitrite.—Exposure to boiling water for longer than two minutes produced interfering color due to charring of organic material, especially carbohydrates. In order to minimize this interference, which was quite pronounced in the concentrated sulfuric acid medium generally used, phosphoric acid was substituted for the sulfuric acid. While the color development in phosphoric acid was excellent, and the charring almost nil, the high viscosity of the phosphoric acid caused some difficulty in measuring volume. However, a mixture of equal volumes of phosphoric and sulfuric acids, as recommended by Gad (5), was quite satisfactory.

Chloride ions, even in large excess, did not interfere with the determination under the conditions of the reaction as finally established. Ascorbic acid and isoascorbic acid also did not interfere.

Nitrite ions, the chief interfering substance, could easily be removed by the urea treatment. A study of the reaction showed that all nitrite, in concentrations normally encountered in meat and curing salts, could be removed by adding a drop of saturated urea solution to the acidified sample and allowing the reaction to proceed at room temperature for four to six minutes. While shorter time intervals were not sufficient to remove the nitrite completely, reaction times longer than six minutes caused a slight loss of nitrate.

Effect of Brucine Reagent Solution on Color Development.—The composition of the brucine reagent solution itself affected the standard curve markedly (Fig. 1). A chloroform solution of brucine was used during the early investigations, but produced erratic color development. Furthermore, Beer's law was not obeyed over the entire concentration range employed. The 10% brucine solution in 91% ethanol, as used by MacDougall and Roadhouse (4) gave a much more satisfactory standard curve. The color reaction also proceeded much more smoothly, possibly because of the complete miscibility of the reagent and sample solution. Fisher and co-workers have recommended the use of a 2% solution of brucine in alcohol. While this solution gives a satisfactory standard curve in the low range, the limit of the reagent's ability to develop a color proportional to nitrate concentration is apparently reached at about 60 or 80 mmg/ml of sodium nitrate.

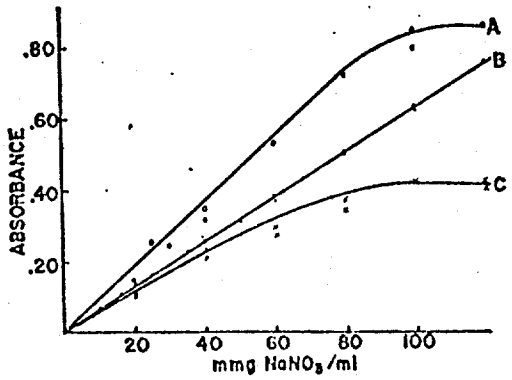

Fig. 1—Effect of brucine reagent composition on color development of standard nitrate solutions.
A: 8.0% brucine in chloroform. B: 10.0% brucine in ethanol. C: 2.0% brucine in ethanol.

METHOD

Apparatus

(a) *Buret.*—50 ml, with delivery tip ground to an opening of 2 mm.

(b) *Boiling water bath.*—Large enough to contain test tube rack and tubes.

(c) *Safety pipet.*

(d) *Spectrophotometer.*—B & L Spectronic 20, Coleman Junior, or equivalent.

Reagents

(a) *Brucine solution.*—Dissolve 10 g brucine alkaloid in approximately 80 ml 95% ethanol with the addition of 3 ml distilled water. Bring to volume of 100 ml with 95% ethanol.

(b) *Phosphoric acid-sulfuric acid mixture.*—Mix with care equal volumes of *o*-phosphoric acid, 85%, reagent grade, and concentrated sulfuric acid, reagent grade, sp. gr. 1.84. Cool before using.

(c) *Urea solution.*—Saturated solution of reagent grade urea in water.

(d) *Sodium nitrate stock solution.*—Dissolve 1.000 g dried $NaNO_3$ in 1 L recently boiled distilled water. For preparation of standard curve, dilute this solution 1:10, to contain 100 mmg $NaNO_3$ per ml.

Preparation of Standard Curve

From a 10.0 ml buret transfer aliquots of 0.50, 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 ml of the diluted stock solution into a series of 10.0 ml volumetric flasks. Add distilled water to the mark and mix. Treat 1.0 ml of each solution with brucine as described below.

Preparation of Sample

(a) *Meat or meat products.*—Thoroughly mix 10.0 g meat with 40 ml distilled water for 3 minutes in a Waring Blendor. Transfer the slurry quantitatively to a 250 ml beaker, using a maximum of 20 ml water. Heat over a steam bath for one hour, cool, and filter the extract through Whatman #42 (or similar porosity) filter paper into a 100 ml volumetric flask. Rinse the beaker, filter, and precipitate 3 times, using 10 ml portions of water each time. Bring the volume to the 100 ml mark with water and mix. This final solution should contain between 10 and 50 mmg $NaNO_3$ per ml.

(b) *Pickle solution.*—Dilute 1:10 with water.

(c) *Dry cure mix.*—Dissolve 1 g in 100 ml water. Dilute 10.0 ml of this solution to 100 ml or use an appropriate aliquot to bring the nitrate concentration into proper range.

Determination

Pipet aliquots of 1.0 ml of each sample or standard solution into test tubes for analysis. Prepare a reagent blank and sample blank by using 1.0 ml water and 1.0 ml sample solution, respectively. To each of the tubes add 0.1 ml saturated urea solution and 1.0 ml phosphoric-sulfuric acid mixture. Allow to stand at room temperature for 5 minutes, then cool in a water bath at about 10°C. With the tubes in the water bath, add 1.0 ml brucine reagent to all solutions except the sample blank. To the sample blank, add 1.0 ml 95% ethanol. From the special 50 ml buret, add 9.0 ml of the concentrated acid mixture to each tube. Mix the contents of each tube thoroughly with a stirring rod after the addition of the acid and allow to stand for 1 minute. (The temperature of the solutions in the tubes will not rise above room temperature if the addition of the acid and the stirring are carried out with the tubes in the cold water bath.)

Place all the tubes simultaneously into the vigorously boiling water bath (of sufficient size to allow boiling to resume within a few seconds after the tubes are introduced). After 2 minutes remove all tubes at the same time and immediately place them into the cold water bath (10°C) until they reach room temperature (3–5 minutes). (These manipulations can be carried out conveniently by using a test tube rack with handles.)

Set the spectrophotometer to read zero absorbance with distilled water at a wavelength of 410 to 425 mμ. Read the absorbance of all solutions against this setting. Correct the readings of the standard solutions for the reagent blank and the readings of the sample solutions for both the reagent and the sample blank. The sodium nitrate concentration in the sample aliquot may be obtained from the standard curve and the original concentration calculated by applying the appropriate dilution factors.

Comments

While the standard curve is usually quite reproducible from day to day, variations sometimes occur because of the highly empirical nature of the reaction upon which this procedure is based. To minimize this difficulty, it is advisable to include three nitrate standards with each group of nitrate determinations as a check on the standard curve.

The handling of concentrated acid solutions and the toxic brucine reagent should present no great problem, since the procedure calls for no precautions beyond those inherent in good laboratory technique. However, a safety pipettor for use with the brucine reagent is essential.

Results and Discussion

Recovery studies were carried out on a large number of meat samples. For preliminary work, fresh meat samples containing known amounts of sodium nitrite only, sodium nitrate only, and equal amounts of both were prepared. The effect of the amount of meat sample present was also studied. Results are shown in Table 1. Recovery was quite good, and nitrite equivalent to 400 ppm in a meat sample did not interfere.

The results with cured meat samples are

Table 1. Recovery of nitrate from fresh meat by the brucine procedure

Sample (mg/ml)	Added $NaNO_2$ (mmg)	Added $NaNO_3$ (mmg)	Found $NaNO_3$ (mmg)	Recovery (%)
10	10	0	0.	—
20	20	0	0	—
30	30	0	0	—
40	40	0	1.0	—
100	0	0	0	—
5	0	5.0	5.0	100
10	0	10.0	9.5	95
20	0	20.0	20.5	102
30	0	30.0	30.5	102
40	0	40.0	39.5	98.7
10	10	10	10	100
20	20	20	21	105
30	30	30	30	100
40	40	40	40	100

shown in Table 2. The ham sample contained both nitrite and nitrate originally present as a result of curing. Varying quantities of both nitrite and nitrate were added to the ground ham extract as indicated. Samples containing more than 100 mg of meat per ml tended to give high absorbance values, but when these were corrected for the absorbance of the corresponding blank, correct nitrate values were obtained.

A sample of wieners cured with nitrite, and having no nitrate originally present, was also studied by adding $NaNO_2$ and $NaNO_3$ to the extract. Recoveries with these samples were excellent over the entire range. In no case was there any indication of nitrite interference.

The precision of the method was found to be ±2% by statistical analysis of a range of actual laboratory samples.

In order to compare methods, seven to thirteen samples of curing pickle and meat were analyzed for nitrate by three methods: gasometric ferrous chloride, *m*-xylenol, and brucine. Results were compiled in the categories of pickle and meat samples, and the methods compared statistically through the Student "*t*" test. As can be seen from Table 3, all methods differ significantly from one another, *i.e.*, results from one method do not necessarily agree with those from another method on the same sample. The method of choice must therefore be based on other considerations such as degree of precision and accuracy. These aspects have been examined statistically by comparing results obtained on a standard pickle sample prepared and distributed by the Analytical Methods Committee of the American Meat Institute Foundation and analyzed by the three methods in four different laboratories. Although the reliability of the statistics is undoubtedly limited by the small number of

Table 2. Recovery of nitrate from cured meat by the brucine procedure

Sample (mg/ml)	Added $NaNO_2$ (mmg/ml)	Added $NaNO_3$ (mmg/ml)	$NaNO_3$ Found Total (mmg/ml)	$NaNO_3$ Found Net (mmg/ml)	Recovery (%)
Lunch Meat					
100	0	0	6.0		
200	0	0	10.0		
300	0	0	15.0		
400	0	0	20.0		
100	0	10	17.0	11.0	110
100	0	20	25.0	19.0	95
100	0	30	35.0	29.0	97
100	0	40	45.5	39.5	99
Wieners[a]					
100	0	0	0		
10	10	10	10.5		105
20	20	20	21.5		107
30	30	30	30.5		101
40	40	40	39.0		97

[a] Nitrite cure only.

Table 3. Comparison of methods by Student's "t" test (7-13 samples, each analyzed by three methods)

Method	Pickle	Meat
Xylenol vs brucine	P > .001[a]	P > .01[b]
Xylenol vs gasometric	.02 > P > .05[c]	P > .001
Gasometric vs brucine	P = .05	P > .001

[a] .001, very highly significant.
[b] .01, highly significant.
[c] .05, significant.

Table 4. Comparison of methods. "Standard" pickle containing 0.075% $NaNO_3$

Method	Lab	$NaNO_3$ Found (mean, %)	Std. Dev.	Coefficient of Variation %	Recovery %
Gasometric	1	0.105	0.03	28.5*	140
	2	0.083	0.008	9.65	110
	3	0.067	0.008	11.9	89
	4	0.063	0.012	19.1	84
	Among labs		0.0117[a] or 0.0197		
Xylenol	1	0.088	0.013	14.8	117
	2	0.080	0.005	6.25	106
	3	−0.050	0.125	—*	—
	4	−0.030	0.105	—*	—
	Among labs		0.0139[a] or 0.0945		
Brucine	1	0.070	0.005	6.65	96
	2	0.086	0.011	12.8	114
	3	0.078	0.003	3.85	104
	4	0.030	0.045	150*	40
	Among labs		0.0089[a] or 0.0270		

[a] Standard deviation calculated by omitting values marked*.

measurements available, a pattern becomes evident from the data (Table 4). The coefficient of variation (standard deviation divided by the mean) is an index of reproducibility, while the per cent recovery is an index of accuracy. The "among laboratories" standard deviation was calculated in two ways. The values marked with footnote [a] were derived by omitting the data which appear to be most out of range, and which represent the least likely deviation among laboratories. The second value includes all data, and represents the total deviation of the data considered here. It is obvious at once that the xylenol method is least reliable. If the starred values in the gasometric and brucine procedures are not considered, the brucine method appears best for precision, accuracy, and interlaboratory variation. If all values are considered, the two are about equivalent.

An additional point in favor of the brucine method is its rapidity and simplicity. Ten samples along with the standard checks can easily be handled at one time. The total time from sample preparation to the completion of calculations is two hours.

Summary

A direct colorimetric determination of nitrate in meat products and curing pickle by means of brucine has been developed. By proper choice of reagents and careful control of reaction conditions, precise and accurate results can be obtained. The volumes involved are small enough to allow running a number of tests simultaneously in test tubes in a shorter time than that required by present methods.

Interlaboratory tests of the method show it is at least equivalent, and possibly superior, to methods used at this time for determining nitrate in meats.

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Effect of Curing Ingredients and Procedures on the Survival and Growth of Staphylococci in and on Cured Meats[1]

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Cured meats, as a result of the selective bacteriostatic activity of the curing salts, have a bacterial flora that is quite different from that of fresh meats. The major selective action is due to the sodium chloride. However, one of the major groups of microorganisms that are not inhibited by any palatable salt concentration is the staphylococci, including the food-poisoning variety.

Castellani and Niven (1955) found that the anaerobic growth of food-poisoning staphylococci in bacteriologic broth media could be inhibited by very low levels of sodium nitrite when glucose was autoclaved in the medium. This raised the interesting possibility that a suitable combination of curing methods and vacuum-packaging might result in a product that would not support growth of food-poisoning staphylococci.

It is also known (Tarr, 1941; Castellani and Niven, 1955) that the pH of the environment has a pronounced effect on the bacteriostatic action of sodium nitrite. Various phosphate compounds and ascorbic acid recently have been added to the curing ingredients permitted under federal inspection, and these may result in shifting the pH of the finished product, thus influencing bacterial growth for better or worse.

Numerous other investigators have also investigated the effect of acids and environmental conditions on the survival and growth of food-poisoning staphylococci (Levine and Fellers, 1940; Nunheimer and Fabian, 1940; Castellani *et al.*, 1953; and Weiser *et al.*, 1954). However, none of this information is directly applicable to conditions encountered in the curing and processing of hams.

Materials and Methods

Four typical coagulase-positive, enterotoxin-producing strains of staphylococci were used throughout this work. Strains 161, 196, and 210 were isolated from food-poisoning cases involving beef tongue, ham, and potato salad, respectively. Strain S-6 was isolated from wholesome frozen shrimp.

Studies on factors affecting the nitrite tolerance of food-poisoning staphylococci were carried out using variations of the following medium: tryptone, 1.0 per cent; yeast extract, 0.5 per cent; salt (NaCl), 5.0 per cent; sugar (glucose or sucrose), 1.5 per cent; phosphate (KH_2PO_4), 0.5 per cent; nitrate ($NaNO_3$), 0.25 per cent; and of nitrite ($NaNO_2$), varying amounts; pH 6.0. The concentration of the curing ingredients, and the pH, is that which might be found in the moisture phase of a commercially cured ham (Fields and Dunker, 1952).

The general procedure was to make up double-strength basal medium and dispense in 5-ml amounts into 18-mm test tubes for the anaerobic experiments and into 50-ml Erlenmeyer flasks for the aerobic experiments. These were then sterilized for 15 min at 250 F.

From a stock solution of 10 per cent $NaNO_2$ that was previously sterilized by Seitz filtration, suitable dilutions were prepared aseptically in sterile distilled water and added to the tubes and flasks. Sterile distilled water was added aseptically to bring the final volume to 10 ml. One series of tubes and flasks was used to determine the pH of the media at the time of inoculation. Inoculation was with one drop of a 1:100 dilution of a 24-hr culture grown in tryptose-phosphate broth (Difco). The tubes for anaerobic incubation were mixed gently and sealed with melted vaspar. Incubation was at 86 F.

For studies using ground pork muscle, pork loin was purchased in a local retail market, the fat trimmed off, and the lean meat ground through a sterile grinder into a sterile pan. Curing salts were added, mixed in with sterile spatulas, and the meat passed through the grinder again. The meat then was inoculated with an appropriate dilution of a 24-hr culture, mixed, and put through the grinder again. The meat was weighed out in 100-g lots in sterile 250-ml beakers, nitrite or other variables mixed in, tamped down, and covered with sterile vaspar. Incubation was at 86 F. The curing ingredients were added in the following amounts: NaCl, 5.0 per cent; glucose, 1.5 per cent; $NaNO_3$

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where indicated), 0.25 per cent; and $NaNO_2$, varying levels.

Samples were plated quantitatively on APT agar (Evans and Niven, 1951), and phenol red mannitol agar (Difco) with an additional 7.0 per cent NaCl. The pH of the meat was measured by mixing a 10-g sample with 10 ml of distilled water and using a Beckman Model H pH meter.

The amount of nitrite in the meat samples was determined by a slight modification of the method described in the official methods of the Association of Official Agricultural Chemists (A.O.A.C., 1950). Twenty-five-g samples were extracted on the steam bath at 176 F for 30 min, and the color developed with the Griess reagent was determined with a Coleman Junior spectrophotometer at a wave length of 522 mμ.

For the majority of the experiments involving ham curing, a commercial-type curing pickle was made up to 63 salometer brine, using distilled water and CP salt. To each liter of brine was added sucrose, 35.8 g; $NaNO_3$, 1.8 g; and $NaNO_2$, 1.2 g. The bacterial inoculum was grown in APT broth and diluted to the desired concentration in curing pickle. When hams were pumped with inoculated curing pickle, they were handled inside a large polyethylene bag to prevent formation of an aerosol containing pathogenic staphylococci. The hams were pumped to a 10 per cent increase in weight and then placed in uninoculated cover pickle of the same composition. After two weeks in cover pickle, the hams were heated in a commercial-type smokehouse, but no smoke was generated.

Results and Discussion

The effect of heating glucose in a tryptone-yeast extract broth upon the nitrite tolerance of food-poisoning staphylococci is illustrated by table 1. At a pH of approximately 6.0, aerobic growth occurred in the presence of 500 ppm of nitrite; whereas, anaerobically, growth was inhibited by as little as 2 ppm of nitrite. Omission of the other curing salts did not affect appreciably the inhibitory level of nitrite.

When glucose was sterilized separately and added aseptically to the medium, or when sucrose was used rather than glucose, anaerobic tolerance to nitrite was greatly increased, aerobic tolerance was slightly increased, and considerably increased anaerobic growth occurred in the control media (without nitrite). Anaerobic nitrite tolerance was in the range of 100–200 ppm.

When sterile glucose was added aseptically to the sterile medium and heated at 212 F for 1 hr or at 175 F for 3 hr, anaerobic growth also occurred in the presence of 100–200 ppm of nitrite. In ground pork muscle, the growth of food-poisoning staphylococci was only partially inhibited by as much as 500 ppm of added nitrite. As shown in table 2, the presence of 2500 ppm of nitrate had a slightly inhibitory effect on the staphylococci, and the combination of 2500 ppm of nitrate and 500 ppm of nitrite prevented any appreciable multiplication of the inoculum. The additon of either 5000 ppm of sodium hexametaphosphate or 400 ppm of ascorbic acid had little or no effect on anaerobic nitrite toxicity. Furthermore, the omission of all sugar from the curing formula did not affect the anaerobic nitrite tolerance of these staphylococci in ground pork muscle.

Since the nitrite level in the meat decreased greatly within 48 hr, even when bacterial growth was sub-

TABLE 1. *Nitrite tolerance of food-poisoning staphylococci in a medium containing glucose and curing salts**

Strain No.	Aerobic Growth				Anaerobic Growth	
	Nitrite (ppm)					
	0	50	200	500	0	2
161	+++	+++	++	+	+	−
196	+++	+++	++	+	+	±
S 6	+++	+++	++	±	+	−
210	+++	+++	++	±	+	−

* Growth recorded after 48 hr at 86 F. Glucose autoclaved in the medium. pH 6.0 at time of inoculation. Growth was estimated visually: +++ = maximum growth, − = no growth.

TABLE 2. *Nitrite and nitrate tolerance of a Staphylococcus (strain 210) in ground pork muscle**

Nitrite Added	Nitrate Added	Nitrite Recovered	Staphylococcus Count	Total Count
ppm	*ppm*	*ppm*	× 10^5/g	× 10^5/g
0	0	1	135	87
100	0	18	1210	2500
200	0	27	10	17
500	0	54	10	12
0	2500	34	8	32
100	2500	34	16	7
200	2500	33	12	16
500	2500	55	0.4	0.5

* The meat also contained 5.0 per cent NaCl and 1.5 per cent glucose. The pH was 5.6, the inoculum was 0.13 × 10^5 staphylococci per g, and incubation was anaerobic at 86 F for 4 days.

TABLE 3. *Nitrite stability in ground pork muscle**

Nitrite Added	Nitrite Recovered After			
	1 Hr	18 Hr	42 Hr	66 Hr
ppm	*ppm*	*ppm*	*ppm*	*ppm*
0	0	0	0	0
100	86	47	25	17
200	149	88	52	45
500	341	225	138	102

* To the meat was added 5.0 per cent NaCl, 1.5 per cent glucose, 5 ppm Aureomycin (Lederle), 10 ppm Rimocidin (Pfizer), and the indicated amount of nitrite. Samples were incubated at 86 F under vaspar seals. Total bacterial counts at 1 hr and 66 hr showed fewer than 10 viable microorganisms per g.

TABLE 4. *Effect of pH and nitrate on aerobic and anaerobic growth of a food-poisoning Staphylococcus in broth*

pH†	Growth Without Nitrate*		Growth With 2500 ppm of Nitrate*	
	Aerobic	Anaerobic	Aerobic	Anaerobic
6.9	130	97	101	98
5.6	79	5	64	6
5.4	58	0	51	0
5.2	40	0	36	0
5.0	10	0	20	0
4.8	0	0	0	0

* Growth was measured turbidimetrically and is recorded as Optical Density × 100 after 72 hr at 86 F. Two strains (161 and 210) were tested and gave virtually identical results.

† The pH was measured before inoculation and after incubation. In no case did the pH change more than 0.1 during incubation.

stantially inhibited, an experiment was set up with no bacteria added and the indigenous flora controlled by antibiotics. The results given in table 3 show that meat constituents rather rapidly destroyed nitrite in the absence of any bacterial growth. This is in agreement with the results of Rose and Peterson (1953).

Lowering the pH of ground pork muscle, or of ground commercial ham, to 4.8 to 5.0 with lactic acid completely prevented anaerobic multiplication of the staphylococci for at least one week at 86 F, even in the absence of nitrite.

These results prompted a careful study of the pH limits of growth of food-poisoning staphylococci in a broth medium. The test medium contained sucrose as the sugar and did not contain any nitrite. Nitrate was added to one series and omitted from the other. As shown in table 4, anaerobic growth was virtually prevented at pH 5.6. Aerobic growth was diminished at pH 5.6 and prevented at 4.8. The presence of nitrate had no effect on the pH tolerance, either aerobically or anaerobically, even though these organisms are capable of reducing nitrate to nitrite. As noted in table 2, food-poisoning staphylococci are able to grow anaerobically in ground pork muscle at pH 5.6.

When moderate numbers of staphylococci were sprayed on the surface of commercially cured, ready-to-eat hams and the hams incubated at room temperature, surface growth was extensive, but the microorganisms failed to penetrate into the interior of the hams until after gross spoilage had taken place.

When moderate numbers of staphylococci were inoculated into chilled curing pickle solutions and held at 40 F, at least 50 per cent of the inoculum died off during the first 24 hr, and the pickle was usually free of these organisms within 72 hr. The results of one such experiment are given in table 5. As also shown in this table, the addition of an aqueous extract of raw pork to the pickle greatly extended the survival time of the inoculated bacteria. Other experiments showed that omission of nitrite and nitrate from the pickle did not change these results.

Moderate numbers of food-poisoning staphylococci were added to curing pickle, which was then pumped into hams through the vascular system. The hams were cured for 2 weeks under uninoculated cover pickle at 40 F. Viable staphylococci were found in the interior of the cured hams, on the surface, and in the cover pickle. Quantitative estimations indicated that most, if not all, of the organisms injected into the hams with the pump pickle remained viable.

Two such hams were heated in a smokehouse to an internal temperature of 137 F, using the following smokehouse temperatures: 110 F for 2½ hr, 120 F for 2 hr, 130 F for 1½ hr, 140 F for 2 hr, and 150 F until the hams reached an internal temperature of 137 F (5½ hr). These were allowed to cool in the smokehouse for about 9 hr. One of the hams was sampled, and no viable staphylococci were found on the surface or in the interior. The second ham was held at 78 to 81 F

TABLE 5. *Effect of meat juices on survival of a Staphylococcus (strain 210) in curing pickle*

Incubation Time at 40 F	Suspended in Distilled Water	Curing Pickle		Curing Pickle Plus Meat Juices*	
		Aerobic	Anaerobic	Aerobic	Anaerobic
1 hr	19,500†	16,500	17,000	17,700	18,000
4 hr	16,500	12,000	19,300	16,200	16,000
8 hr	16,500	9,300	19,100	16,600	16,000
24 hr	—‡	7,000	9,000	18,000	16,500
48 hr	—	3,200	5,600	13,100	16,400
72 hr	—	0	0	11,500	13,000
7 days	—	0	0	3,300	6,900

* Contained 5 per cent of an aqueous extract of raw pork.

† Staphylococci per ml. The inoculum was approximately 20,000 staphylococci per ml.

‡ This flask was broken after the 8-hr incubation. However, in another similar experiment the count was 246 after 24 hr and 0 after 48 hr.

TABLE 6. *Survival of a Staphylococcus (strain 210) in and on hams during curing, heating, and subsequent storage**

Sampled	Internal Temperature When Sampled	pH	Staphylococcus Count	
			Interior	Surface
Hr at F	*F*			
Before heating	40	6.6	46×10^3	39×10^3
12 at 120	108	6.4	30×10^3	43×10^4
16 at 120	112	6.5	50×10^3	35×10^5
24 at 120	116	6.4	50×10^2	261×10^2
48 at 120	118	6.5	14×10^2†	135×10^6
48 at 120 plus 72 at 86	86	6.3	90†	95×10^7

* These hams were inoculated with approximately 50×10^3 staphylococci per g, held in cover pickle for 2 weeks at 40 F, placed in a smokehouse for 48 hr at 120 F, and held for 72 hr at 86 F. Each sample represents a different ham.

† Most of the colonies on these plates appeared to be sporeformers.

for 3 days before sampling, and there were still no viable staphylococci on or in this ham. Thus, it seems certain that this processing schedule killed all of the staphylococci that survived the cure.

When such hams were heated at a smokehouse temperature of 120 F, the staphylococci in the interior were slowly killed, as shown in table 6. In the experiment recorded in this table, the staphylococci on the surface multiplied extensively. In another experiment, under supposedly identical conditions, the surface count was greatly reduced during the 2 days of heating, and even after subsequent incubation for 4 days at 86 F the surface had only 8500 staphylococci per g and the interior only 160 staphylococci per g. It would appear that a smokehouse temperature of 120 F is so near the upper temperature limit of growth of these organisms that small variations in temperature and relative humidity may mean the difference between multiplication or destruction of the surface flora. The most interesting point is the consistent destruction of the staphylococci in the interior of these hams. Even though the interior of the hams was in the temperature range of 50–115 F for a period of 7½ hr, there was no evidence of any staphylococcal multiplication inside the hams during this period.

Summary

Food-poisoning staphylococci were able to grow vigorously in ground pork muscle containing any combination of curing ingredients that is permissible and palatable. However, if the pH of the meat was lowered, to 4.8 to 5.0, anaerobic growth of the staphylococci was prevented even in the absence of nitrite.

In a broth medium, anaerobic growth of staphylococci was inhibited at pH 5.6, although growth occurred in meat at this pH. Aerobic growth in broth was diminished at pH 5.6 and prevented at pH 4.8.

Staphylococci died rather rapidly in curing pickle, but were protected by added meat juices. Most, if not all, staphylococci injected into hams with the pump pickle survived a normal curing operation until the smoking operation. Heating in a smokehouse to an internal temperature of 137 F killed all surviving staphylococci. Heating in a smokehouse at 120 F for 48 hr slowly killed the staphylococci in the interior of the ham, but they occasionally multiplied on the surface. There was no evidence of any staphylococcal multiplication in the interior of the hams at any time during this heating period.

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Nitrates, Nitrites, and Methemoglobinemia

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This review is primarily concerned with the relationships of the clinical condition of methemoglobinemia, particularly in infants, to the presence of nitrates and derived nitrites in food and water consumed. For a proper understanding of the likelihood of occurrence of potentially hazardous quantities in the food and water, the ecology of nitrate is briefly considered. Effects on domestic and other animals are included only to the extent that they shed light on the toxicologic mechanisms in man. Effects on plant life and problems of eutrophication are omitted. Nitrogen oxides in air are mentioned only as they contribute to the nitrate content of soil; the direct effects of inhaled oxides are not considered.

Authors are mentioned in the text where they bring out a specific point. To avoid the disrupting effect of long citations, individual names are not given in the text where several different authors support a statement; they can generally be identified from the titles given in the bibliography.

CLINICAL PICTURE

The present concern over the possibility of methemoglobinemia being produced in infants as a result of nitrate ingestion may be said to have started with Comly's report in 1945 of two cases seen by him, and of others described by his colleagues. Several reports followed on cases seen in the U. S. A. (see References). Cases were reported in England in 1950. By 1966, according to Schmidt and Knotek, 1060 cases had been reported, with a fatal result in 83 of them, but they give no references. They discussed 115 cases that they had attended. Both food and water were incriminated in the causation. (Marienfeld attributes substantially the same data to Sattelmacher with the date of 1962.)

Attention was shifted from water to food as a source of intoxication by Simon, Schupan, and others (see References) who gave graphic accounts of cases brought about in Europe by the ingestion of nitrite from preparations of spinach. The victim, usually under 6 months of age, exhibits cyanosis, tachycardia, dyspnea, and marked restlessness. Examination fails to reveal respiratory or cardiac causes. The blood, when drawn, has a chocolate brown color, and reveals the presence of abnormal amounts of methemoglobin on spectroscopic examination. If relief is not provided, the symptoms deepen and lethargy punctuated by muscle spasm develops. A fatal outcome is then likely.

Dramatic relief is provided by the intravenous injection of methylene blue or of thionene. Removal of the stomach contents by lavage speeds recovery by diminishing further intoxication.

The history of such cases in Europe usually reveals the feeding of one of the suspect vegetables (see later) under conditions that promote the formation of nitrite from the original nitrate, or the use of nitrate-containing water in the preparation of the infant's formula. In the U. S. A., food seems not to have been reported as a source of infant poisoning, but over 320 cases have been reported from the use of nitrate-containing well water. The Public Health Service Drinking Water Standards of 1962 stated that, from 1947 to 1950, 139 cases of methemoglobinemia, including 14 deaths, were reported in Minnesota alone from nitrate in farm well-water supplies. The authors go on to say "there are no reports of methemoglobinemia in infants fed water from public water supplies in the United States, although levels of nitrate in some may be routinely in excess of 45 mg/l." In 1965, however, Vigil and Warburton reported a case in which a municipal water supply drawn from wells was incriminated. The levels of nitrate in the water supply were not very high (up to 62 mg/l.), but the concentration may have been increased by boiling. Numerous reports in the last 20 years (see References and later) have confirmed the risk to infants of nitrate-containing well water in the U. S. A. The risk increases with the amount of water consumed, as in hot weather.

As pointed out in a recent editorial (*Lancet*, 1968), salt has been used for centuries in the curing of meat. The efficacy of nitrate impurities in salt led to the deliberate addition of nitrates; the discovery that much of their action was due to nitrite formation led in turn to the further substitution of nitrite. A limit of 200 ppm of nitrite (or nitrate) in corned products has been set by Federal regulation on the basis of the amount of conversion of hemoglobin to methemoglobin that it could produce in adults (Lehman). No harmful effects have been reported in adults from these normal amounts, although cases have occurred from the accidental addition of excessive amounts (Orgeron *et al.*). (Corned beef is fortunately a rare ingredient in the diet of infants!)

TOXICOLOGY

The toxicity of nitrates, as such, is relatively low; the fatal adult dose being 7–35 g (Burden). It is the nitrite, formed by reduction from the nitrate, that presents a primary hazard (lethal dose 20 mg/kg). This reduction may be brought about by the action of organisms, particularly of the coliform and clostridia species, in plants or food during storage, or by contamination of containers of sterile food after they have been opened (see later). Ruminal flora readily bring about the conversion, making ruminants particularly liable to toxic effects. In the healthy human alimentary canal nitrates are rapidly absorbed in the upper intestine, but disturbances of alimentary function may delay absorption and permit greater opportunity for conversion (Kübler). Conversion is more likely in infants by reason of low gastric acidity and frequency of gastroenteric infections.

The nitrite ion, after absorption, can convert the divalent iron of hemoglobin to the ferric form, which is incapable of picking up and giving off oxygen readily in accordance with the partial pressure of the gas in the lung and tissue capillaries, respectively.

The small amount of methemoglobin normally produced [normal level—0.03–

0.13 g/100 ml (Sunderman and Sunderman)] is readily reconverted to hemoglobin by the reducing action of enzymes. But if the capacity for reconversion is exceeded, abnormal concentrations will build up. Proportions of methemoglobin up to 5% (0.75 g/100 ml) may not result in symptoms, but the transformation of 70% of the hemoglobin is likely to be rapidly fatal. Pregnancy has continued, however, in dairy cattle with maintained methemoglobin levels of 40–50% (Winter *et al.*).

Numerous reports show that methemoglobin accumulation as the result of nitrite action is much more likely to occur in infants. Two explanations are given: the first, that fetal hemoglobin, which may constitute up to 80% of pigment in the newborn, is more easily converted; the second, that infants may have a temporary deficiency of methemoglobin reductase, or of its coenzyme reduced DPNH-dependent diphosphopyridine nucleotide, which is normally generated by glycolysis in the red cell (Metcalf; Editorial, *British Medical Journal*, 1966; Bartos and Desforges; Ross and Desforges; Ross). Except in rare cases of genetic enzyme deficiency (Balsamo *et al.*), this undue susceptibility seems largely to have disappeared by the end of the second trimester. The rate of conversion to methemoglobin, however, is reported to remain high until puberty, when a marked slowing appears (Keohane and Metcalf). This sensitivity may also appear in connection with pregnancy, carcinoma, and dietary aberrations (Metcalf).

Another possible adverse effect of dietary nitrite has recently gained some attention (Druckrey *et al.*, 1963; Editorial, *Lancet*, 1958; Lijinsky and Epstein, 1970). The red color of cured meat is largely due to the formation of nitroso-compounds of myoglobin and hemoglobin. It is entirely conceivable that a comparable reaction of nitrites with secondary amines would produce nitrosamines, a family of compounds that contains many known powerful carcinogens. Herring meal which produced toxic liver reactions in cattle was found to contain dimethylnitrosamine in concentrations up to 30–100 ppm. The acute toxic symptoms were reproduced by the experimental feeding of dimethylnitrosamine (Sakshaug *et al.*). The feeding of 5 or even 2 ppm can induce liver tumors in rats (Terracini *et al.*). Malignant tumors have appeared in rats fed nitrate and secondary amines (Sander and Bürkle). Diethylnitrosamine has been reported in food plants in the Transkei where a molybdenum deficiency is believed to favor a high nitrite content in plants, and esophageal cancer is common (Burrell *et al.*, Duplessis *et al.*). In the words of Lijinsky and Epstein, however, "it should be stressed that the individual occurrence of nitrites and secondary amines is not necessarily hazardous; both reactants must be present simultaneously in the stomach to form nitrosamine. Even so, not all secondary amines react with nitrite to form nitrosamines in conditions prevailing in the mammalian stomach, and not all nitrosamines are carcinogenic." Another possible variant on this story that needs to be checked out is the formation of hydroxylamines, some of which are carcinogenic.

Other effects that may be produced by nitrites include tachycardia, peripheral vasodilatation, vomiting and diarrhea (Aussannaire *et al.*). The inclusion of nitrite in animal diets has on occasion been shown to degrade carotene and vitamin A in the alimentary canal, and thus to reduce the amount available for absorption (Phillips, 1966). Increased weight of the thyroid gland on the one hand, and

decreased I uptake on the other, have been reported experimentally, but with doses that can be regarded as excessive (Fassett; Bloomfield *et al.*). The interaction of nitrate and thyroxin appears complex (Cline *et al.*). Indeed, the effect of sublethal levels of nitrate in animal feed remains an open question (Davison). The question of human hypersensitivity to nitrate has recently been raised (Epstein). Mental retardation has been reported (Fialkow *et al.*) in familial diaphorase deficiency, but the causative mechanism is obscure.

NITRATES IN FOOD

As far back as 1907 Richardson reported data on the nitrate content of vegetables and cured meats, with the following comment:

> ". . . . nitrates are regularly taken into the system with the food and regularly eliminated by the kidneys, and when we consider that there is present of nitrates in various fresh vegetables, including beets, squash, parsley, turnips, radishes, celery, cabbage, lettuce, cucumbers, spinach (sic), string beans, and egg plant the equivalent or more than the equivalent of the quantity of saltpeter ordinarily present in cured meats, and considering also the relatively small quantity of meat and particularly of salt meat included in the average diet, we must conclude that the average person obtains a larger quantity of nitrates from his vegetable than from his meat foods."

The modern use of nitrates and nitrites in the curing of meat has already been mentioned. It remains to add that in Britain their use is permitted not only in cured and pickled meat products but also in some cheeses (Editorial, *Lancet*, 1968).

Wilson published further data on the nitrate content of foods in 1943. The juice of spinach and of cauliflower had particularly high concentrations of nitrate, that of lettuce and of celery somewhat less but still high. Analyses made by Smith in Columbia, Mo., of various vegetables as grown and as purchased, gave high values for radishes, lettuce, celery, beets, spinach, kale, and mustard. Jackson *et al.* showed that fresh spinach may contain up to 740 ppm of nitrate, and canned spinach up to 550 ppm. The data reported by the authors mentioned above are given in condensed form in Table 1.

Clinical experience with methemoglobinemia in infants led Simon to ascertain the nitrate content of various spinach preparations, with the results given in Table 2. Analyses of commercial baby foods are summarized in Table 3. Kamm *et al.* in Canada found spinach preparations particularly high in nitrate, with beets the next highest among the foods examined. Later analyses by Brabent and Boulerice, quoted by Commoner, gave approximately half these concentrations for spinach and beets. The values found by Commoner himself are fairly moderate. Analyses conducted at the National Institute of Environmental Health Sciences (Fishbein *et al.*) on commercial baby foods show beets again at the top of the list, but with somewhat lower values than some of those cited by most others. Spinach, it should be noted, had concentrations only about one-quarter of those in the beet samples.

There is considerable variation in the concentrations quoted. Analytical tech-

TABLE 1
NITRATE CONTENT OF VEGETABLES AND MEATS

Authors	Year	Type, etc.	No. of samples	ppm NO_3 High	Mean	Low
Radish						
Richardson, W. D.	1907	Market	6	3056	1804	531
Wilson, J. K.	1949	Market		1176		303
Smith, G. E.	1966	Grown		9000		2250
Smith, G. E.	1966	Market		6500		850
Jackson, W. A., *et al.*	1967	Fresh	2	1734	1492	1250
Beets						
Richardson, W. D.	1907	Market	7	8062	2552	930
Wilson, J. K.	1949	Grown			1333	
Smith, G. E.	1966	Grown		4500		600
Jackson, W. A., *et al.*	1967	Fresh and canned	3	1654	1197	671
Egg plant						
Richardson, W. D.	1907	Market	4	4430	1474	354
Kale						
Smith, G. E.	1966	Grown		4750		650
Jackson, W. A., *et al.*	1967	Fresh	1		1857	
Jackson, W. A., *et al.*	1967	Canned	2	2174		1542
Broccoli						
Wilson, J. K.	1949	Grown		3846		785
Smith, G. E.	1966	Market		850		400
Jackson, W. A., *et al.*	1967	Frozen	2	594		506
Spinach						
Richardson, W. D.	1907	Market	5	3809	1892	310
Richardson, W. D.	1907	Canned	5	1949	1100	266
Wilson, J. K.	1949	Market	3	2353		1600
Wilson, J. K.	1949	Quick frozen			3636	
Jackson, W. A., *et al.*	1967	Fresh and frozen	3	739	524	238
Jackson, W. A., *et al.*	1967	Canned	2	550	473	396
Phillips, W. E. J.	1968	Canned	3	1435	886	505
Phillips, W. E. J.	1968	Frozen	3	2463		429
Phillips, W. E. J.	1968	Fresh	3	2330		1501
Lettuce						
Richardson, W. D.	1907	Market	5	3544		399
Wilson, J. K.	1949	Grown (head)		1250		870
Wilson, J. K.	1949	Market (curly)		1818		400
Smith, G. E.	1966	Grown		2250		150
Smith, G. E.	1966	Market		6000		300
Jackson, W. A., *et al.*	1967	Fresh	5	893	664	488
Celery						
Richardson, W. D.	1907	Market	5	2879	1496	797
Richardson, W. D.	1907	Market	2		190	
Wilson, J. K.	1949	Grown			1212	
Wilson, J. K.	1949	Market			1240	
Wilson, J. K.	1949	Market			3232	
Smith, G. E.	1966	Market		3250		500
Jackson, W. A., *et al.*	1967	Fresh	2	2957	2786	2614
Turnip						
Richardson, W. D.	1907	Market	5	2879	1012	88
Smith, G. E.	1966	Grown		4500		250

TABLE 1 (*Continued*)

Authors	Year	Type, etc.	No. of samples	ppm NO_3 High	Mean	Low
Smith, G. E.	1966	Market		3500		50
Jackson, W. A., *et al.*	1967	Frozen (greens)	1		1619	
Jackson, W. A., *et al.*	1967	Canned (greens)	3	2168		1199
Parsley						
Richardson, W. D.	1907	Market	2	1417	1056	753
Jackson, W. A., *et al.*	1967	Fresh	1		1698	
Squash						
Richardson, W. D.	1907	Market	4	1373	660	310
Smith, G. E.	1966	Market		2250		400
Jackson, W. A., *et al.*	1967	Fresh	1		299	
Cabbage (green)						
Richardson, W. D.	1907	Market	5	487	176	35
Wilson, J. K.	1949	Grown			2222	
Wilson, J. K.	1949	Market		1200		186
Smith, G. E.	1966	Market		1650		150
Jackson, W. A., *et al.*	1967	Fresh	2	471	315	158
Cauliflower						
Richardson, W. D.	1907	Market	3	398	132	26
Wilson, J. K.	1949	Grown			2000	
Smith, G. E.	1966	Market		1250		100
Jackson, W. A., *et al.*	1967	Fresh	1		53	
Carrots						
Richardson W. D.	1907	Market		89	66	40
Wilson, J. K.	1949	Grown			442	
Wilson, J. K.	1949	Market			325	
Wilson, J. K.	1949	Quick frozen			181	
Smith, G. E.	1966	Grown		500		100
Smith, G. E.	1966	Market		500		50
Jackson, W. A., *et al.*	1967	Fresh, frozen, and canned	4	194	102	18
Ham						
Richardson, W. D.	1907		20		845	
Bacon						
Richardson, W. D.	1907		19		383	
Dried beef						
Richardson, W. D.	1907		12		968	

niques may differ greatly in their efficiency (PHS Drinking Water Standards), but not all of the variation in reported data is due to differences in analytical techniques. There is a very great real variability in the nitrate content of both raw vegetables and processed products, as indicated by the high standard errors of the samples analysed by Fishbein *et al.*, both between and within lots. This variability is largely a reflection of the conditions under which the plants are grown (see later; Table 10). The implication is clear that control by monitoring of content would be a most formidable task.

The nitrite, as opposed to the nitrate, content of fresh vegetables is low, but the nitrate may be converted to nitrite as already mentioned by the action of

TABLE 2
LEVEL OF NITRATE IN COOKED SPINACH, SALAD PURÉE, SPINACH JUICE, FROZEN SPINACH, AND CANNED SPINACH (AFTER SIMON, 1966)

Kind	Firm	Mg of nitrate/kilo in a series of products			
		1	2	3	4
Spinach purée	A	770	710	720	530
Spinach purée	B	830	1,080	960	820
Spinach purée	C	1,090	1,040	1,210	1,100
Spinach purée	D	660	900	750	580
Spinach purée	E	430	840	1,020	470
Spinach purée	F	1,080	1,070	930	—
Spinach purée	G	210	120	135	82
Salad purée	G	310	260	320	310
Spinach juice	H	2,800	2,000	1,320	2,100
Frozen spinach	I	320	630	390	800
Canned spinach	J	780	930	1,220	980

various organisms during storage. Table 4 from Simon and Fig. 1 from Schupan illustrate this clearly. The experiments of Phillips (1968) provide further examples. That nitrate may be converted to nitrite in sterile preparations exposed to ambient contaminants has been confirmed at NIEHS for baby foods, but not for squash, carrots, or green beans. (In spinach the conversion was equivocal.) The exact circumstances of exposure apparently affect the outcome.

The formation of nitrite from nitrate is offset to a certain extent by further degradation of the nitrite, presumably to ammonia and nitrogen, and by a certain amount of leaching out in cooking water. Where the cooking water is added back to the preparation, of course, the toxic ion is apt to be restored (Phillips; Simon).

The presence of nitrate and nitrite in cured meats (U. S. limit 200 ppm) has already been mentioned. Accidental addition of large amounts has resulted in poisoning of children (Orgeron *et al.*; Singley).

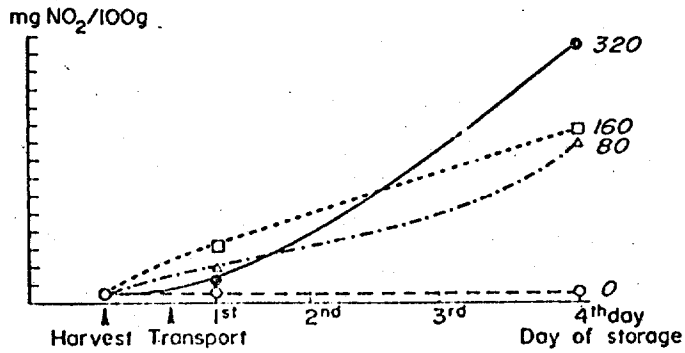

FIG. 1. Formation of nitrite after transportation and storage of spinach (after Schupan). The amount of nitrite produced depends on the quantity of fertilizer nitrogen used (kg/hectare).

TABLE 3
NITRATE CONTENT OF BABY FOODS

Authors	Year	Type, etc.	No. of samples	ppm NO_3		
				High	Mean	Low
Wilson, J. K.	1949	Beets		750		333
Kamm, L., *et al.*	1965	Beets	6	2165	977	634
Commoner, B.	1968	Beets	5		370	
NIEHS	1969	Beets			245 ± 97	
Wilson, J. K.	1949	Spinach		833		616
Kamm, L., *et al.*	1965	Spinach	5	1668	1373	1074
Commoner, B.	1968	Spinach	4		56	
NIEHS	1969	Spinach			66 ± 21	
Wilson, J. K.	1949	Green beans			37	
Kamm, L., *et al.*	1965	Wax beans	2	568	444	321
Kamm, L., *et al.*	1965	Green beans	3		163	
Commoner, B.	1968	Beans	8		48	
NIEHS	1969	Green beans			43 ± 10	
Wilson, J. K.	1949	Squash			0	
Kamm, L., *et al.*	1965	Squash	5	409	282	44
Commoner, B.	1968	Squash	3		126	
NIEHS	1969	Squash			84 ± 28	
Wilson, J. K.	1949	Carrots		3		0
Kamm, L., *et al.*	1965	Carrots	8	167	101	66
Commoner, B.	1968	Carrots	7		26	
NIEHS	1969	Carrots			38 ± 10	
Kamm, L., *et al.*	1965	Mixed vegetables	2	106		92
Kamm, L., *et al.*	1965	Garden vegetables	5		180	
Wilson, J. K.	1949	Peas			0	
Commoner, B.	1968	Peas	3		2	
Kamm, L., *et al.*	1965	Meats		48		4
NIEHS	1969	Meats			5.4 ± 3.4	
Commoner, B.	1968	Corn	2		2	
Commoner, B.	1968	Sweet potatoes	1		16	
Kamm, L., *et al.*	1965	Graham crackers	1		211	
Kamm, L., *et al.*	1965	Pre-cooked cereals		211		18
Kamm, L., *et al.*	1965	Dairy products		31		0
Kamm, L., *et al.*	1965	Fruits		31		0

NITRATES IN DRINKING WATER

The International Drinking Water Standards of 1958 noted that a concentration in excess of 50 mg/l. may result in infantile methemoglobinemia, but set no limit. The Public Health Service Drinking Water Standards of 1962 contained the following paragraph:

> "In light of the above information and because of the uncertainty introduced by tardy analyses, the frequent lack of attention to possible interfering factors in the analysis, the health of the infant, and the uncertain influence of associated bacterial pollution, 10 mg nitrate nitrogen (or 45 mg nitrate) per liter of water is a limit which should not be exceeded."

TABLE 4
CONTENT OF NITRATE AND OF NITRITE IN COOKED SPINACH JUICE
(FROM SIMON, 1966)

Holding temperature	Duration of holding (hr)	Spinach juice	
		mg NO_3/l.	mg NO_2/l.
4°	3	1,860	0
	6	1,860	0
	24	1,960	10
20°	3	1,850	2
	6	1,960	8
	24	1,550	197
37°	6[a]	1,860	8
	24	1,660	12
	3	1,730	188

[a] Presumably a misprint for 3, 6, and 24 hr as in the previous sections.

They concluded with the warning:

> "It is important, therefore, for health authorities in areas in which nitrate content of water is known to be in excess of the recommended limit to warn the population of the potential dangers of using the water for infant feeding and to inform them of alternate sources of water that may be used with safety."

The Federal Water Pollution Control Administration Recommended Water Quality Standards agree with the PHS Standards, and take matters a step further by suggesting permissible values at the point of use for process water, in food processing plants, as 10 mg/l. of nitrate (not nitrate nitrogen). Burden gives the following calculated limits based on age and climate.

PERMISSIBLE LIMITS FOR NITRATE-NITROGEN IN DRINKING WATER

Consumer	Assumed weight, kg	Region	Maximum permissible dose of nitrate-nitrogen, mg	Approx. daily water intake, liters	Permissible limit of nitrate-nitrogen in water, ppm
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

Drinking water taken from lake and river sources seldom exceeds the PHS limits. The highest level reported in the finished water supplies of the 100 largest cities in 1962 was 23 ppm of nitrate, and the median level 0.7 ppm (Durfor and Becker). Supplies drawn from wells, however, frequently exceed the limits, as indicated by the following.

(a) Comly's original report for Iowa, in 1934–35, which remains the most succinct and dramatic, reads:

> "The nitrate content of water taken from wells in Iowa varied from zero to 125 parts per million (as nitrate nitrogen) in a survey made of 2,000 samples taken from domestic and municipal wells in 1934 and 1935. The highest nitrate nitrogen content on record in the State Hygienic Laboratory is 567 parts per million. The nitrate nitrogen of the water given to the infants varied from 64 to 140 parts per million, and the severity of symptoms seemed to parallel roughly the amount of nitrate present."

(b) Bosch *et al.* indicate the state of affairs in Minnesota in 1950.

> "A compilation was made of all the water supply data obtained in the school-farm surveys and the methemoglobinemia cases. Of the 389 wells investigated, 244 (63 per cent) were dug, 125 (32 per cent) drilled, and 20 (5 per cent) driven. A total of 206 wells, more than 60 per cent of which were drilled, contained less than 10 ppm. nitrate nitrogen. Of the remaining 183 wells, 23 contained between 10 and 20 ppm.; 43, between 21 and 50 ppm.; 66, between 51 and 100 ppm.; and 51, over 100 ppm. All but 16 of these 183 wells were dug.
>
> "Of the 389 wells investigated, 188 were located satisfactorily. Fifteen of the 188 were also constructed satisfactorily. Twelve other wells were constructed satisfactorily but were located within 50 ft. of a source of contamination. Of the satisfactorily constructed wells, all were drilled; only three contained over 10 ppm. nitrate nitrogen; two of the wells were also located satisfactorily.
>
> "Of the 283 wells on which data were available, 169 (60 per cent) were less than 60 ft. in depth. Fifty-five (60 per cent) of the 91 wells containing over 100 ppm. nitrate nitrogen were less than 40 ft. deep.
>
> "During this study 28 (5.4 per cent) of the 514 municipal supplies in the state were found to contain over 5 ppm. nitrate nitrogen; 16 (3.1 per cent) of these contained 10 ppm. or more. The highest concentration was 27 ppm., in a dug well used as the source for municipal supply in the section of the state from which most of the methemoglobinemia cases have been reported."

(c) Tables 5 and 6, given by Walton, indicate the widespread occurrence in the United States, in 1951, of high nitrate content in water associated with infantile methemoglobinemia. Numerous reports from specific localities confirm the situation.

(d) The incidence of high concentrations in Nebraska is relatively mild (University of Nebraska Extension Service). For 1961: 3% over 10 ppm of nitrate N, with an average of 15.5. In Kansas, however, 33% of 454 dug wells in 1939 and 51% of 243 dug wells in 1945 showed concentrations of over 10 ppm nitrate N. Fifteen per cent of municipal supplies drawn from wells in 1950 exceeded this limit (Metzler and Stoltenberg).

(e) In Southern California, of 800 wells examined in 1960–61, 182 showed concentrations of at least 22 ppm of nitrate, and 88 exceeded 45 ppm. The highest value was 330 ppm (Editorial, *Environment*). Present values may well be higher

TABLE 5

HIGH NITRATE WATERS, THEIR GEOGRAPH. DISTRIB. & TOTAL REPORTED CASES OF INFANT METHEMOGLOBINEMIA (FROM WALTON)

State	Total no. methemo. cases reported[a]	Year	Type of supply	No. of samples examined	% Samples examined within indicated NO_3–N (ppm) range			
					0–10	11–20	21–50	50+
Calif.	1	—	—	—	—	—	—	—
Ga.	6	—	—	—	—	—	—	—
Ill.	75	1946		7,000	80±	←	20±	→
Ind.	1	—	—	146	←	98.7	→	← 1.3 →
Iowa	69	1939	Dug wells	454	66.5	10.6	14.3	8.6
			Drilled wells	647	95.5	2.5	1.5	0.4
			Bored wells	278	79.5	7.9	7.2	5.4
		1943	Dug wells	243	48.5	11.1	12.4	28.0
			Private wells					
			<100′ deep	1,943	←	72±	→	← 28± →
			>100′ deep	370	←	96.5	→	← 3.5 →
Kansas	13	—	Rural wells	—	67±	←	33±	→
Mass.	0	1948	Private	—	—	←	47 samples	→
Mich.	7	—	—	2,847	93.0	←	7.0	→
Minn.	139	1947–9	Municipal	514	96.9	←	3.1	→
				2,912	84.9	←	15.1	→
Mo.	2	—	—	—	—	—	—	—
Neb.	22	1948–9	Municipal	416	95.0	←	5.0	→
			Non-municipal	2,687	70.8	←	20.2	→
			School	258	95.0	←	5.0	→
			Farm	275	78.0	←	22.0	→
N.Y.	2	1943	—	2,864	96.1	3.3	0.6	0.0
		1944	—	3,528	96.9	2.5	0.6	0.0
		1945	—	5,117	97.4	1.8	0.7	0.1
		1946	—	6,524	96.4	2.1	1.3	0.2
N.D.	9	—	—	509	84.9	←	15.1	→
		—	—	287	84.0	←	16.0	→
Ohio	0		Rural wells	10,500	90.5	5.0	3.5	1.0
			State park wells	400	100.0	0.0	0.0	0.0
Okla.	0	—	—	415	Av. NO_3–N		Conc. = 28 ppm	
S.D.	Several	—	—	—	—	—	—	—
Texas	0	—	Rural wells				Numerous	
Va.	1	—	Rural wells	—	—	—	—	—

[a] Includes all reported cases irrespective of the date of their occurrence or the type of water with which they were associated.

as a rising water table overtakes nitrate deposits from fertilizers. A PHS study conducted in 1969 reported levels up to 111 ppm of nitrate in the Riverside and San Bernardino counties.

(f) Smith, from whom Fig. 2 is taken, states as follows:

"Analyses of nearly 5000 water samples for nitrate and nitrite in 45 counties in Missouri during the past year (1963) show that about 42 percent of them contained over 5 ppm of nitrate–nitrogen, a concentration high

TABLE 6

REPORTED CASES OF NITRATE WATER-INDUCED INFANT METHEMOGLOBINEMIA CLASSIFIED ACCORDING TO NITRATE-NITROGEN CONCENTRATION OF WATER USED IN FEEDING FORMULA (FROM WALTON)

State	Methemoglobinemia		Number of cases associated with indicated ranges of NO_3-N concn. (ppm)						No. of cases for which data are available
	Reported cases	Reported deaths	0–10	11–20	21–30	31–50	51–100	100+	
Calif.	1	0	0	0	0	0	1	0	1
Ga.	6	3	—	—	—	—	—	—	0
Ill.	75	6	0	1	2	2	12	11	28
Ind.	1	0	0	0	0	0	1	0	1
Iowa	Several	11	0	0	0	0	1	1	2
Kansas	13	3	0	0	1	1	2	8	12
Mich.	7	0	0	0	0	0	0	7	7
Minn.	139	14	0	2	25		53	49	129
Mo.	2	0	0	0	0	0	0	2	2
Neb.	22	1	0	1	0	4	9	8	22
N.Y.	2	0	0	0	0	0	1	0	1
N.D.	9	1	0	1	1	0	0	6	8
Ohio	0	0	0	0	0	0	0	0	0
Okla.	0	0	0	0	0	0	0	0	0
S.D.	Several	0	—	—	—	—	—	—	—
Texas	0	0	0	0	0	0	0	0	0
Va.	1	0	0	0	0	0	1	0	1
	278+	39	0	5		36	81	92	214

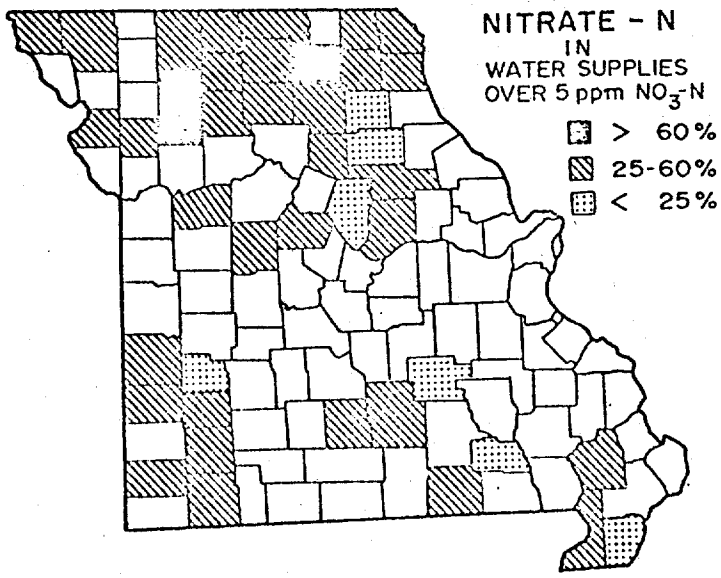

FIG. 2. Counties in Missouri where over 5000 water supplies have been analyzed for nitrates. Counties were selected on the basis of geologic and soil regions (from Smith, 1961).

enough to be considered important in livestock production and in infant feeding. The number of water supplies containing nitrate varied from a low of 12 percent to over 75 percent in individual counties. Nitrites were found in only 1 to 2 percent of rural wells during the winter months but this increased to 3 to 4 percent in warm weather. Highest contamination was found in areas with the largest livestock production. There was good correlation between the nitrate content in well water and hydrologic-geologic areas, but only a limited relationship with soil types."

Table 7, also from Smith, illustrates the extent to which rural sources of water other than wells may contain nitrates.

(g) The situation in Illinois in 1969 has been summarized by Walker.

"Although only four of the 789 public ground water supplies in Illinois contain more than 45 ppm of nitrate, recent studies indicate that several thousand domestic and farm wells . . . may produce water containing more than the recommended safe nitrate level."

TABLE 7
NITRATES IN WATER SUPPLIES—COOPER COUNTY, MO., SPRING 1964 (FROM SMITH, 1964)

Type	No. sampled	Number over 5 ppm NO_3-N	Per cent
Drilled	255	126	49
Cisterns	28	18	64
Dug	31	27	87
Spring	37	31	84
Ponds	15	2	17
Driven	3	3	100
Artesian	2	0	0

It is clear that concentrations above the limits recommended by the Public Health Service are common in rural well waters, and that this has been the state of affairs for a long time.

That the situation is not confined to the U. S. A. is indicated by the data that Wood gives for Canada and England.

Reporters	Nitrate nitrogen	
	Over 20 ppm (%)	Over 50 ppm (%)
Robertson and Draycott (1949), 2000 wells, Canada	25.5	18.8 (5.3% over 300)
Heather (1951), 600 wells, Essex	21.7	4.5 (0.5% over 100)
Wood (1952/3), 1311 wells, E. Suffolk	41.0	13.1 (2.3% over 100)

Reports from continental Europe extend the occurrence (Bachmann; Werner *et al.*).

ECOLOGY OF NITRATES

Food and drinking water are simply the two points in a complex ecological network at which nitrates can pass into man. An appreciation of that network is necessary if one is to have a proper understanding of the circumstances which determine the occurrence of nitrate in food and drinking water.

Agricultural soil constitutes a natural center to the ecological net and provides a convenient departure point for its consideration. A simplified list of inputs to and outputs from the soil is given as Table 8. The more significant items will be mentioned in turn.

TABLE 8
BALANCE OF NITRATE IN THE SOIL

Inputs	Outputs
Atmospheric NO_3 in rain and snow	Uptake by plants
N_2 fixation by soil bacteria	N_2 to air following bacterial action
Mineral breakdown	Runoff
Breakdown of plant and animal tissue	Erosion and leaching
Human and animal wastes	Percolation to deeper strata, rivers, and ocean
Industrial wastes	Mineral deposition
Nitrogenous fertilizers	

It has been estimated (Harr *et al.*) that precipitation, in a moderately industrialized region, may add as much at 55 lb/acre, year of nitrate nitrogen from the atmosphere. One-half of this atmospheric source would be derived from auto exhaust and one-third from heating fuels.

The important effect of legume root nodules in fixing atmospheric nitrogen has long been utilized by agriculturalists. Some idea of the additions to be obtained is given by the data in Table 9 derived from Miller. Fixation by algae in freshwater lakes has received some attention lately (Howard *et al.*).

Organic matter from dead plants and animals, as well as animal and human wastes, liberates many nitrogenous compounds such as urea, ammonia, urates,

TABLE 9
CONTRIBUTION OF LEGUMES TO SOIL NITROGEN, 1917–1941 (AFTER MILLER, 1947)

Cropping system	N in crops, lb/acre	N turned under, lb/acre	N gain, lb/acre
Corn, wheat, clover	850	0	20
Corn, wheat, clover manured	900	280	140
Rye, turned under	760	760	−640
Rye, cowpeas turned under	2150	2150	200
Red clover taken off	670	0	340
Red clover turned under	760	760	680
Alfalfa, taken off	1790	0	440
Sweet clover turned under	560	560	1080

etc., all of which contribute heavily, through subsequent conversion, to the nitrate content of the soil in the immediate locality. Smith reports, for example, that cores taken where livestock have been fed for more than 50 years, often contain as much as 500–600 lb/acre, ft (250 ppm) of nitrate nitrogen, at 5–10 ft depth. With such a soluble and mobile ion as NO_3, the turnover must be very large to permit such a high equilibrium concentration. It has been estimated (Garman) that 1.7 billion tons of animal excreta must be disposed of annually in the U. S., but much of this bypasses agricultural soil into rivers, lakes, and oceans.

Breakdown of nitrate-containing minerals may add to the soil content, but the amount is likely to be small except in a few special situations.

Industrial wastes may add their quota of nitrate in certain localities, but this is more likely to be indirect through contamination of surface water (see later).

Impressive though the above more or less natural additions to the soil content may be, they do not make up for the loss of nitrogen from cultivated soils. They are, moreover, irregular in distribution and erratic in time. Many farmers are worried that the nitrogen reserves of cultivable soils, and therefore the nitrate contents, are decreasing.

A major drain on soil nitrogen is the removal of crops. It was estimated, for example, that 8.8 million tons of nitrogen were removed in this way from U. S. soil in 1965 (White). The rate of removal is almost certainly increasing. Eventually, of course, a large part of this loss finds its way back to the soil through the breakdown of organic wastes, fixation of atmospheric nitrogen, or recovery of atmospheric content through rain; but a significant part is lost into the oceanic sink or deposited in noncultivated areas. In the meantime the soil reserves are progressively diminished.

Surface run-off, leaching, and erosion produce still greater losses of nitrate, a large part of which are nonrecoverable. Duley and Miller (1923) reported annual losses of nitrogen ranging up to 75 lb/acre from agricultural land. Only a small proportion of this is actually in the form of nitrate, but the reserves from which future nitrate may be formed are depleted. It is said (Smith) that "the losses of essential mineral nutrients during the past half century in the United States have been much greater from erosion than from crop removal," itself a very substantial figure.

The total loss of organic nitrogen over the last 70 years has been estimated at 20 million tons/year (Stanford, quoted by Garman). It is in the light of this large and possibly increasingly negative balance that the effect of the addition of fertilizer needs to be seen.

From very low figures in 1945, the annual application of nitrogen in the form of fertilizer has risen steeply. Figure 3 indicates a present rate of about 7 million tons. Even this quantity does not meet the annual removal by crops of 9 million tons or more, let alone compensate for continuing losses by other routes. The following excerpt from Allison illustrates the point.

> "The chief channel of loss in normal agricultural practice is probably leaching, which usually occurs chiefly in the fall and spring months. The

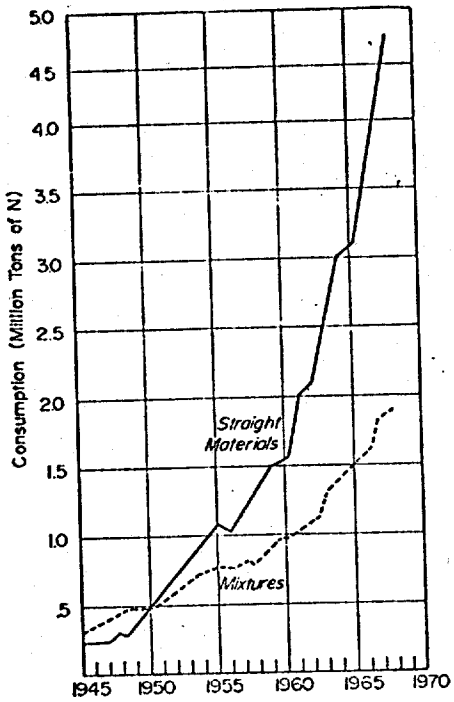

FIG. 3. Annual consumption of nitrogen. (Farm Chemicals Handbook).

major gaseous loss is believed to be molecular nitrogen formed from nitrates and nitrites by biological denitrification. Loss as ammonia is usually an important channel only in alkaline soils or where ammonia, or ammonia sources, are supplied by unsatisfactory methods or in amounts beyond the capacity of the soil to absorb it. Much smaller losses may occur as a result of the decomposition of nitrous acid to nitric oxide in acid soils, or possibly through the interaction of nitrous acid with ammonia or amino acids. There is also some evidence for slow and continuing losses of nitrogen to the air in the finer-textured soils as a result of denitrification in soil aggregates or anaerobic pockets .... Where heavy applications of urea or ammonia are made under conditions that lead to much nitrite and inhibited nitrate formation, large losses of nitrogen, chiefly as molecular nitrogen, may occur in slightly acid soils."

The gap is being closed by rising fertilizer use, but only slowly; a considerable annual deficit still occurs. As attempts are made to close the gap, however, there are bound to be localized "hot spots," where an excess of nitrate may develop. There is the possibility that an anxious grower may add more nitrogenous fertilizer than the soil requires, and that abnormally high concentrations of nitrate may consequently accumulate in plants, particularly where other factors such as temperature, rapid growth rate, mineral imbalance, and soil moisture are favorable to the uptake (Table 10) (Simon; Smith). In modern, large-scale agricultural practice, however, cost accountants are not likely to let excessive use of

TABLE 10

FACTORS FAVORING ACCUMULATION OF NITRATE IN PLANTS (FROM SMITH, 1966)

Drought
High temperatures
Shading and cloudiness
Deficiency of plant food nutrients (P, K, Ca)
Excessive soil nitrogen (manures, legume residues, nitrogenous fertilizers)
Plant damage from insects and certain weed control chemicals

fertilizer continue for long. The possibility of accumulation from neighboring livestock feeding has been mentioned earlier. In irrigated land, nitrates and other minerals may be carried down and deposited in the soil, but this is less likely to occur than with ions such as Ca that form insoluble compounds.

Water constitutes a second center which interlocks with that of soil in the nitrate ecology. Ammonia and nitrates enter surface and underground water from a variety of sources, with sewage providing 78,000 lb/sq mile, year on the average in the United States, as against about 5,000 lb from rainfall.

> "The primary sources of nitrogen in domestic waste waters are feces, urine, and waste food. An ASCE committee in 1937 gave a range of total nitrogen contribution of 8–12 lb with an average of about 11 lb N per capita per year. This was about equally distributed between NH_3–N and organic N. About 50 per cent of the organic nitrogen in domestic wastes can be enzymatically hydrolyzed to NH_3–N either during transport of wastes to the treatment plant or during treatment. Fresh domestic waste contains little or no NO_2–N or NO_3–N, but these may be formed from the biologic oxidation of NH_3–N during aerobic biologic treatment.
>
> "Some removal of nutrients does take place during treatment. Much of the organic nitrogen is contained as suspended solids, which can be more or less removed with the sludge obtained by settling. In many treatment plants, however, this sludge is treated anerobically, and a good portion of the nitrogen released by this process is returned to the treatment plant to pass to the stream. In general, about 20–50 per cent of the total nitrogen in domestic waste is removed by treatment. The higher figure applies when fresh wastes are treated by complete treatment with no return of sludge nutrients to the effluent streams" (American Water Works Association Task Group).

The concentration of nitrogen (in all forms) in municipal sewage varies from 35–50 ppm in raw sewage to 13–34 ppm in the tertiary effluent from treatment plants when alum is used. The latter figure may be reduced to 3.5 ppm or less when lime is used. If, as has been proposed, nitrilotriacetic acid (NTA) is substituted for phosphates in detergents, a rise in the nitrate content of municipal sewage can be expected. Domestic sewage may be a major source of nitrates for the ground water in rural communities, as may animal feeding lots. [A cow is said to generate as much waste as 16.4 humans, a pig as 1.9, and a chicken as one-seventh (AWAA Task Group).] Animal remains and burial grounds are

among the more arcane sources cited. Substantial amounts may be discharged from industrial waste treatment plants, but little information is available on the absolute amounts.

Water distributed in soil has every opportunity to acquire quasi-equilibrium concentrations of soluble substances. This water in turn contributes to the composition of water in the aquifers. High concentrations of nitrate acquired through the interchanges described above will be passed on to the aquifers. Well water will reflect the nitrate concentration of the percolation area or aquifer that the well taps. The concentration will vary as the water table moves into or out of nitrate-rich zones with season and water use.

The situation is well described in an Iowa State University report (Hanway *et al.*).

> "Nitrates in solution move downward through soil, sand layers and porous rock formations toward the water table. The nitrates may then appear in the tile drainage water or ground water.
>
> "Nitrates are more concentrated below or near the area of waste accumulation or disposal such as manure piles, feedlots, septic tank disposal fields, cesspools, privies, etc.
>
> "Excess nitrates are also more apt to be found in ground water under low areas and waterways that collect or convey runoff from higher ground.
>
> "Water percolating through decomposing peat soils or through mineral soils which have received a heavy application of nitrogen fertilizer or manure will move nitrates to the ground water table.
>
> "Nitrates leached by percolating water tend to accumulate in the upper portion of the ground water table in the absence of rapid ground water movement. They remain there an indefinite period of time. Nitrate concentration is reduced through dilution by ground water containing little or no nitrate and by ground water movement.
>
> "Nitrates move with the ground water. Ground water pumped from a well may contain excess nitrates even though the decomposing organic waste or other nitrogen source may be located a considerable distance away from the well. If the ground water moves rapidly through a porous sand or creviced rock formation with little opportunity for dilution to occur, a well several miles from the nitrogen source may contain high-nitrate water.
>
> "Leaching occurs only when the ground is not frozen and when excess rainfall, snow melt, or irrigation water causes surface runoff and percolation below the plant rooting zone. Consequently, the nitrate concentration in a water supply will vary according to the season of the year and the amount of excess water."

The situation in Southern California presents an interesting case in point. For many years the water table fell as the volume withdrawn by pumping exceeded the resupply. But at some time after 1950, apparently, the balance was reversed, so that a rising water table may well be catching up with nitrates deposited from fertilizer in the meantime (Editorial, *Environment*).

TABLE 11
ESTIMATE OF NITROGEN CONTRIBUTIONS FROM VARIOUS SOURCES
(FROM AWWA TASK GROUP REPORT)

Source	1,000,000 lb/year	Usual concentration in discharge, mg/l.
Domestic waste	1,100–1,600	18–20
Industrial waste	>1,000	0–10,000
Rural runoff		
Agricultural land	1,500–15,000	1–70
Nonagricultural land	400–1,900	0.1–0.5
Farm animal waste	>1,000	†
Urban runoff	110–1,100	1–10
Rainfall[a]	30–590	0.1–2.0

[a] Considers rainfall contributed directly to water surface.
† Insufficient data for estimate.

Relative contributions to ground water of nitrogen from various sources are indicated by Table 11.

Removal of nitrate from waste waters is a difficult problem. The PHS Drinking Water Standards, 1962, says flatly: "At present there is no method of economically removing excessive amounts of nitrate from water." Since then there have been some improvements, but the economic feasibility is uncertain. A biological method involves the addition to a highly nitrified secondary effluent of organic material as food for denitrifying bacteria. One pilot-scale method using contact stabilization sludge as food produced an 86% reduction of nitrates to elemental nitrogen at an estimated cost of 5–6¢ per 1000 gal (Federal Water Pollution Control Administration).

Laboratory tests indicate that denitrification can be obtained in carbon and sand packed beds, using methanol as a food source, at an estimated cost of 2¢ per 1000 gal for the methanol. Nitrates were reported reduced to 1 mg/l. or less.

For the present, the moral seems to be—keep nitrates from entering the water supply, or use water that has a low nitrate content for critical uses such as preparation of infant formulas.

SUMMARY AND CONCLUSIONS

Spinach and beets may have fairly high concentrations of nitrate (1000 ppm and over) in their natural state. Several other vegetables may show high concentrations on occasion. Cured meats are permitted to contain up to 200 ppm of nitrate and nitrite.

Domestic or commercial preparations, including baby foods, made from plants with a high content, are likely to have concentrations that are almost as high.

Monitoring of vegetables or commercial products for their nitrate content would seem to be impracticable in view of the very large variability both between and within lots.

The nitrate contained in the raw vegetables may be converted into nitrite by

accompanying organisms in the course of storage for several days, particularly at higher temperatures.

The nitrate content of sterile commercial preparations may similarly be converted if they are kept for several days after opening and exposure to contaminating organisms.

Cooking may do something to speed up the rate of conversion of nitrate into nitrite, but some of the salt content is likely to be leached out in the cooking water. This removal would, of course, be undone if the cooking water were subsequently added to the diet.

Well waters frequently exceed the USPHS standard of 10 ppm of nitrate nitrogen (45 ppm of nitrate). These situations seem to have been of long standing. The increasing use of nitrogenous fertilizers seems not, so far, to have affected the levels significantly, except where a previously low water table rises to mobilize deposited fertilizer (or other nitrogenous materials).

Organisms capable of converting nitrate to nitrite may occur in the alimentary canal, particularly in the course of digestive upsets. It is the nitrite derived in any of the above ways, and not the nitrate, that constitutes a hazard. Absorbed into the blood, it converts hemoglobin into methemoglobin, destroying its power of carrying oxygen. This conversion will tend to happen more readily in infants because of the presence of the more susceptible fetal hemoglobin and a relative lack of enzymes that reconvert the pigment to hemoglobin.

The cases reported in the U. S. A. have been almost exclusively due to the use of nitrate-containing water, but many cases have been reported in Europe due to the use of baby food preparations in which nitrite has been formed from the original nitrate, either in storage of the vegetable or in preparations kept at room temperature. The clinical condition, although alarming and potentially fatal, is fairly easily diagnosed and remedied.

The condition can be prevented by: (a) using only fresh or continuously frozen vegetables in the domestic preparation of baby food; (b) discarding the cooking water; (c) preparing only enough, or opening only enough of a commercial preparation, for one meal and discarding any remainder; (d) avoiding the use of water that contains nitrate in the preparation of baby formulas or food.

A possible additional hazard to be considered is the action of nitrate on secondary amines, also contained in or derived from certain foods, to form nitrosamines with carcinogenic potentiality.

SUGGESTED ACTIONS

1. Continued improvement in protection of wells from contamination by surface water containing nitrogenous material.
2. Increased systematic sampling of rural well waters and clear identification of those that exceed recommended limits.
3. Continuing surveillance of wells in areas where the water table is rising, seasonally or continuously, to the point that deposited nitrates are likely to be mobilized.
4. Periodic reminder to communities dependent on nitrate-containing well waters about the dangers to infants.

5. Critical research into the possibility of formation of carcinogenic nitrosamines and allied substances in cured meats.

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The Metabolism of Nitrate and Nitrite in the Sheep

1. THE REDUCTION OF NITRATE IN THE RUMEN OF THE SHEEP

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A disease of cattle following the ingestion of young oat hay or straw, referred to as 'oat hay poisoning', was reported in the U.S.A. by Newsom, Stout, Thorp, Barber & Groth (1937). Subsequently, Bradley, Eppson & Beath (1939) were able to produce the disease in the laboratory by feeding a steer on the affected hay. Analysis of a number of samples of toxic hay revealed that in all cases nitrate was present in abnormally high concentrations—3·2–7·2% of the dry matter. Bradley, Eppson & Beath (1940) showed that the ingestion of potassium nitrate produced the same symptoms, and simultaneously observed a severe methaemoglobinaemia. It was found that 1 g. potassium nitrate kg. body weight was a lethal dose, and in a few hours resulted in the conversion of 70–80% of the haemoglobin into methaemoglobin. Sheep were affected in the same way. Since nitrite is known to convert haemoglobin into methaemoglobin, it was concluded that in the alimentary tract of the ruminant nitrate was reduced to nitrite and that the latter gave rise to the methaemoglobinaemia. A similar disorder, 'tribulosis', had been reported many years previously in South Africa (Rimington & Quin, 1933; quoted by Russell, 1944). Symptoms resembling those of 'tribulosis' were produced by dosing sheep with the expressed juice of species of *Tribulus* or with potassium nitrate itself. While the present work was in progress the South African group reported that incubation of untreated rumen contents *in vitro* with nitrate resulted in the formation of nitrite (Sapiro, Hoflund, Clark & Quin, 1949).

The present investigations were designed to provide information concerning (*a*) the reduction of nitrate to nitrite *in vivo* in the rumen of the sheep, and the further reduction of nitrite to ammonia; (*b*) the changes, if any, in the ammonia content of the rumen fluid subsequent to dosing with nitrate; (*c*) the degree of methaemoglobinaemia produced after different amounts of nitrate or nitrite are placed in the rumen, and after the intravenous injection of known amounts of nitrite.

METHODS

The experimental sheep were Oxford Down × Halfbred 3-year-old wethers, fitted with a permanent rumen fistula as described by Phillipson & Innes (1939). They were kept in metabolism cages and fed 3·5 lb. (1·6 kg.) good meadow hay daily, with water *ad lib.* The weights of the sheep remained constant, ±1 kg., throughout the period of study. Urine samples, free from faeces, were collected under toluene. Blood samples were taken from the jugular vein, and clotting was prevented by sodium oxalate.

Samples of rumen contents were withdrawn through the fistula and the material so obtained filtered through muslin. The filtrate is termed rumen liquor (RL). All analyses of RL were carried out on a clear filtrate obtained by the following procedure: 2 ml. saturated lead acetate were added to 10 ml. of RL followed by 0·5 g. decolorizing char-

Table 1. *Recovery of nitrate added to rumen liquor, urine and water*

Material analysed	Nitrate added (mg. NO_3-N/100 ml.)	Colorimetric method		Conway method		Fe/H_2SO_4 reduction	
		Found (mg./100 ml.)	Recovery (%)	Found (mg./100 ml.)	Recovery (%)	Found (mg./100 ml.)	Recovery (%)
Rumen liquor	0	0·36	—	0·33	—	0·32	—
	10	9·7	93	9·8	95	9·3	90
	20	19·4	95	19·8	97	18·5	91
	30	29·2	96	29·4	97	28·5	94
Urine	0	0·45	—	0·24	—	0·20	—
	20	22·9	112	19·6	97	18·6	92
	40	34·7	83	38·2	95	37·4	93
Water	0	—	—	—	—	—	—
	5	4·95	99	4·90	98	5·05	101
	10	9·85	98	10·00	100	9·73	97

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coal; after shaking, 3 ml. saturated Na_2SO_4 were added to precipitate the excess lead, and the mixture filtered through a Whatman no. 1 filter paper. This procedure yielded a colourless clear filtrate. Urine samples were clarified in the same way, but the solutions were always very faintly coloured. All doses of $NaNO_3$ and $NaNO_2$ were administered to the sheep as 10% (w/v) aqueous solutions, unless otherwise stated.

Nitrite plus nitrate in RL filtrates were determined by the colorimetric phenoldisulphonic acid method (Association of Official Agricultural Chemists, 1947) using a Spekker photoelectric absorptiometer with a spectral violet Ilford filter no. 601 (maximum transmission 430 mμ.). Urine filtrates were unsuitable for the colorimetric method and were therefore analysed by the following volumetric methods. Preformed NH_3 and total nitrite and nitrate were determined by NH_3 estimations before and after reduction either by Devarda's alloy (Conway, 1947) or by powdered Fe and 8% (w/v) H_2SO_4 (Lees & Quastel, 1946). Table 1 shows that the three methods gave comparable results, with recoveries of nitrate added to normal RL consistently above 90%.

Nitrite was estimated by the Griess-Ilosvay method (Association of Official Agricultural Chemists, 1947) using a King photoelectric colorimeter with a green filter (maximum transmission 520 mμ.). Recoveries of nitrite added to RL or urine were within the limits of 91–99% and duplicates agreed ±3%.

Methaemoglobin and total haemoglobin were determined by the method of Evelyn & Malloy (1938) using the Beckman spectrophotometer.

RESULTS

Metabolism of nitrate in the rumen

The nitrate and nitrite concentrations in the rumen of the untreated animal are uniformly low throughout a 24 hr. period. However, the ammonia concentration rises after feeding (McDonald, 1948), a fact which complicates the interpretation of the ammonia values if the animal is dosed with nitrate at the time of feeding.

McDonald (1949), however, has shown that the ammonia content of rumen liquor is relatively constant during the last 8 hr. before feeding. This observation suggested an experimental procedure which would permit an assessment of the ammonia formation from nitrate. The animal was fed at 6 p.m., and samples of RL were collected from 8 a.m. the following morning. This experiment served as a control; it may be seen from Fig. 1 that the ammonia level remained constant, thus confirming McDonald's observation. The procedure was repeated save that 12 g. sodium nitrate were introduced into the rumen 16 hr. after feeding.

The results (Fig. 1) show that there was a rapid fall in the nitrate, coincident with the production of nitrite which, however, disappeared during the succeeding 5 hr. In the control experiment the ammonia concentration remained constant throughout, whilst after the addition of nitrate there was a definite rise lasting 5–7 hr. This may be due to a stimulation of ammonia production by the nitrate or a conversion of the nitrate to ammonia. The latter is the more probable, since only a small proportion of the nitrate is accounted for in the urine or blood, and, as will be shown later, it cannot all be absorbed as nitrite, for such a quantity would be far in excess of the lethal dose. In this experiment excretion in the urine accounted for less than 8% of the nitrate introduced into the rumen: the nitrite content was very low, and the ammonia values variable.

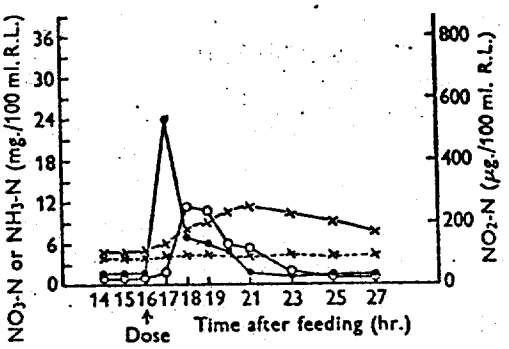

Fig. 1. Nitrate, nitrite and ammonia in RL after placing 12 g. $NaNO_3$ in the rumen, 16 hr. after feeding. ×---×, control NH_3-N (mg./100 ml. RL); control NO_3-N and NO_2-N negligible; ×—×, experimental NH_3-N (mg./100 ml. RL); ●—●, experimental NO_3-N (mg./100 ml. RL); ○—○, experimental NO_2-N (μg./100 ml. RL).

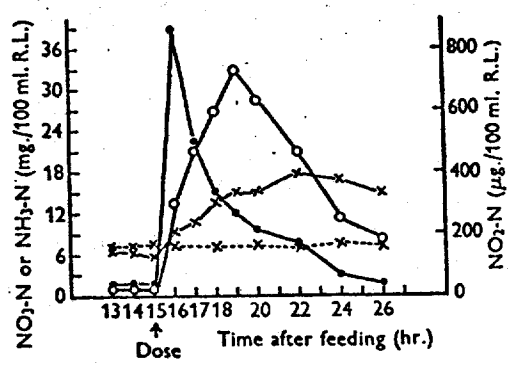

Fig. 2. Nitrate, nitrite and ammonia in RL after introducing 25 g. $NaNO_3$ into the rumen, 15 hr. after feeding. ×---×, control NH_3-N (mg./100 ml. RL); control NO_3-N and NO_2-N negligible; ×—×, experimental NH_3-N (mg./100 ml. RL); ●—●, experimental NO_3-N (mg./100 ml. RL); ○—○, experimental NO_2-N (μg./100 ml. RL).

The changes in the nitrate, nitrite and ammonia of the RL, following a dose of sodium nitrate sufficient to produce a severe methaemoglobinaemia, were next investigated, 25 g. sodium nitrate being given to the sheep 15 hr. after feeding. A control experiment was carried out in which no nitrate was administered. The results are presented in Fig. 2. The methaemoglobin content of the blood had

reached its maximum 7 hr. after dosing and amounted to 63 % of the total haemoglobin.

The results show the same general tendencies as in Fig. 1, when half this quantity of sodium nitrate was administered, but the increase in nitrite was much more marked. The nitrate, nitrite and ammonia concentrations in the RL were constant in the control experiment. The nitrate concentration 1 hr. after dosing was 39 mg. NO_3-N/100 ml. RL (25 g. sodium nitrate in 6 l. RL is equivalent to 68·6 mg. NO_3-N/100 ml. RL). Whilst the nitrate concentration fell rapidly there was still an appreciable amount present 6 hr. after dosing. The nitrite concentration rose steadily for 4 hr. and fell uniformly almost to normal during the next 6 hr., but there was still a considerable amount of nitrite (460 μg. NO_2-N/100 ml. RL) present in the RL 7 hr. after dosing, at which time the methaemoglobin content of the blood was at its maximum. There was a pronounced rise in the ammonia concentration, which presumably accounted for a considerable proportion of the nitrate disappearing at the later stages. Only a small proportion (4 %) of the nitrate was lost in the urine.

Nitrate and methaemoglobin formation

A series of experiments was carried out in which the methaemoglobin content of the blood was determined after increasing quantities of sodium nitrate (0–25 g.) had been placed in the rumen. A different quantity was administered daily, and control experiments, in which no nitrate was administered, were carried out at the beginning and at the end of the series. The results of these control experiments showed excellent agreement. After dosing, samples of blood were collected at intervals over the succeeding 8 hr. period and analysed for total haemoglobin and methaemoglobin. The haemoglobin contents of all samples lay within the range of 11–13 % (w/v) of the blood. The results are given in Fig. 3.

As the quantity of sodium nitrate administered was increased from 12 to 22·5 g. the extent of the methaemoglobin production gradually became greater, and the time between administration and the maximum value attained became longer. When the dose was increased to 25 g. sodium nitrate there was a sharp rise in the maximum methaemoglobin. This may be due to the fact that above a certain concentration of nitrate the rate of reduction of nitrite to ammonia becomes limiting, and in consequence nitrite accumulates in the rumen. This nitrite passes into the blood in increasing concentration and gives rise to a severe methaemoglobinaemia.

Nitrite and methaemoglobin formation

A further series of experiments was carried out in which sodium nitrite was placed in the rumen. The quantity administered was increased daily as with nitrate. Samples of blood were collected and methaemoglobin estimated (Fig. 4). The administration of less than 5 g. sodium nitrite (5 % (w/v) solution) had but little effect on the methaemoglobin content of the blood. There was a significant methaemoglobinaemia when 7 or 9 g. sodium nitrite were placed in the rumen, with a further increase in the maximum methaemoglobinaemia attained when the dose was increased to 10 g. sodium nitrite. The length of time between the administration and the peak values again became greater as the dose was raised.

The results of the analysis of samples of RL taken at intervals after 10 g. sodium nitrite were placed in the rumen are presented in Table 2, and the results obtained following an equivalent dose of sodium nitrate, are included for comparison. The nitrite concentration remained above 500 μg. NO_2-N/100 ml.

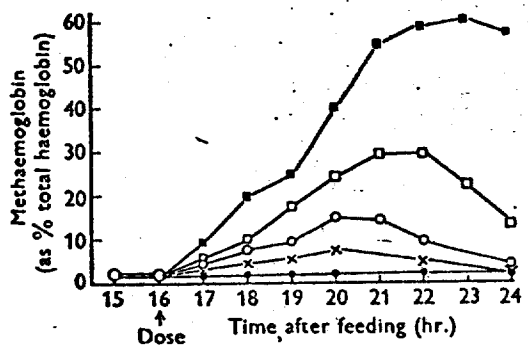

Fig. 3. Methaemoglobin production when various amounts of $NaNO_3$ are placed in the rumen, 16 hr. after the sheep was fed. ⊙—⊙, Exp. 3, 0 g. $NaNO_3$; ×—×, Exp. 4, 12 g. $NaNO_3$; ○—○, Exp. 5, 17·5 g. $NaNO_3$; □—□, Exp. 6, 22·5 g. $NaNO_3$; ■—■, Exp. 7, 25 g. $NaNO_3$.

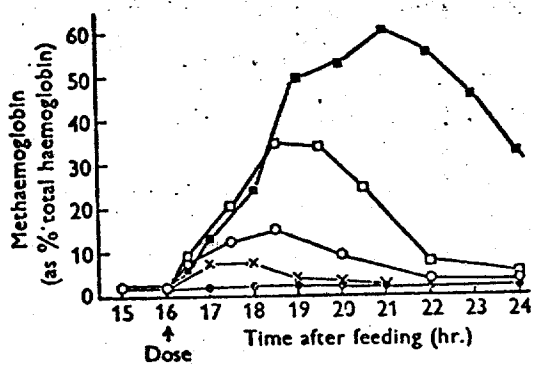

Fig. 4. Methaemoglobin production when various amounts of $NaNO_2$ are placed in the rumen, 16 hr. after the sheep was fed. ⊙—⊙, Exp. 10, 1 g. $NaNO_2$; ×—×, Exp. 11, 5 g. $NaNO_2$; ○—○, Exp. 12, 7 g. $NaNO_2$; □—□, Exp. 13, 9 g. $NaNO_2$; ■—■, Exp. 14, 10 g. $NaNO_2$.

Table 2. NH_3-N *and* NO_2-N *in rumen liquor, and methaemoglobin in blood, after placing* 10 *g.* $NaNO_2$ *or* 12 *g.* $NaNO_3$ *in the rumen*

(The dose was given 16 hr. after feeding.)

Time after feeding (hr.)	Exp. 16, 10 g. $NaNO_2$ in rumen			Exp. 4, 12 g. $NaNO_3$ in rumen		
	NH_3-N (mg./100 ml. RL)	NO_2-N (μg./100 ml. RL)	Met. Hb as % total Hb	NH_3-N (mg./100 ml. RL)	NO_2-N (μg./100 ml. RL)	Met. Hb as % total Hb
15	4·5	12·5	2·0	4·0	10	1·5
16	4·6	10·0	2·3	4·0	11	1·7
17	5·4	17,500	13	4·9	20	2·5
18	7·0	8,100	24	9·8	248	4·0
19	9·4	2,750	49	10·8	232	5·5
20	9·9	1,250	53	10·9	122	8·0
21	9·3	570	61	11·1	100	—
23	8·1	120	46	10·2	26	4·9
25	5·7	30	32	9·0	18	—
27	4·9	15	—	8·0	16	—

RL for 5 hr. after the administration of the sodium nitrite while the concentration after administration of 12 g. sodium nitrate was negligible by comparison. However, the changes in ammonia concentrations were very similar, supporting the concept that nitrate is reduced in the rumen to ammonia, with nitrite as an intermediate. Nitrite and nitrate excretion was negligible.

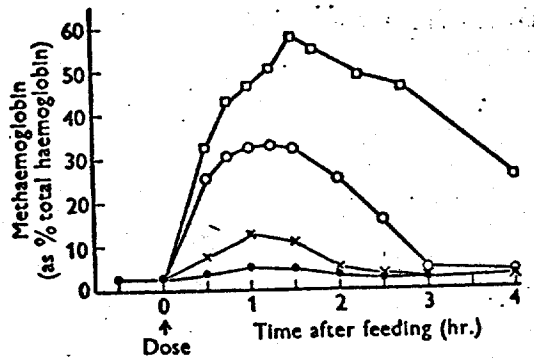

Fig. 5. Methaemoglobin production on the intravenous injection of $NaNO_2$. ●—●, Exp. 17, 0·5 g. $NaNO_2$; ×—×, Exp. 18, 1 g. $NaNO_2$; ○—○, Exp. 19, 1·5 g. $NaNO_2$; □—□, Exp. 20, 2 g. $NaNO_2$.

For comparison a series of experiments was carried out in which sodium nitrate was injected into the jugular vein in order to determine the amount of methaemoglobin produced by the interaction of known amounts of nitrite and the haemoglobin of the circulating blood. Samples of blood and urine were collected at intervals. The nitrite excreted in the urine was again negligible and in no case accounted for more than 1–2 % of the sodium nitrite given. A conversion of 60 % of the blood haemoglobin into methaemoglobin resulted when 2 g. sodium nitrite were injected (Fig. 5). The rise of methaemoglobin to its maximum was rapid and there was also a sharp increase in the methaemoglobin maximum as the dose was increased (Fig. 6). This is to be expected since in this experiment the nitrite and haemoglobin were brought into immediate contact, and the rate of methaemoglobin formation was independent of the rates of reduction processes or of passage through the rumen wall.

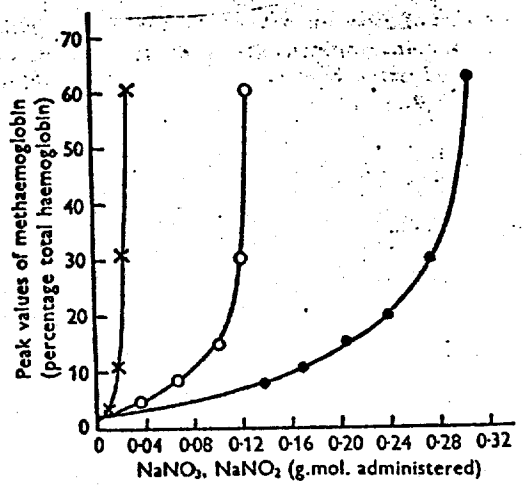

Fig. 6. Peak values of methaemoglobin plotted against dosage. ●—●, nitrate placed in the rumen; ○—○, nitrite placed in the rumen; ×—×, nitrite injected intravenously.

The peak methaemoglobin values produced on introducing various amounts of nitrate or nitrite into the rumen are compared in Fig. 6 with those resulting from the intravenous injection of nitrite. When less than 0·25 mol. nitrate or 0·11 mol. nitrite was added to the rumen, there was an approximately linear relationship between the amount administered and the extent of the methaemoglobinaemia. Greater quantities resulted in a disproportionately large formation of methaemo-

globin. This suggests that above a certain concentration of nitrite the rate of reduction of nitrite to ammonia is limited, and not as rapid as the reduction of nitrate to nitrite. In both cases nitrite would thus accumulate in the rumen, and presumably pass into the blood stream.

DISCUSSION

It is evident that nitrate is reduced to ammonia by the micro-organisms in the rumen of the sheep, and that nitrite is an intermediate in this reaction. The nitrite accumulates under certain conditions, and may lead to a production of methaemoglobin in the blood. A dose of 25 g. sodium nitrate or 10 g. sodium nitrite introduced into the rumen of the sheep (60 kg.) produces the same degree of methaemoglobinaemia (60 % of the total haemoglobin), as 2 g. sodium nitrite injected intravenously. The amount of nitrite injected intravenously may be expressed quantitatively in relation to the haemoglobin converted into methaemoglobin. The live weight of the sheep was 60 kg. and the blood haemoglobin level was 12 % (w/v). It is assumed that the sheep has a blood volume equivalent to 10 % of the live weight and that the iron content of the haemoglobin is 0·34 %. Knowing that 2 g. sodium nitrite administered intravenously converted 60 % of the haemoglobin to methaemoglobin it can be shown that approximately 4 mol. sodium nitrite are required to produce 1 mol. methaemoglobin. These figures are relevant to *in vivo* conditions and to the particular sheep employed.

The fact that 10 g. sodium nitrite introduced into the rumen has a similar effect suggested that only 20 % of the nitrite passed to the blood stream. Sodium nitrate (25 g.) placed in the rumen results in a similar degree of methaemoglobinaemia, but there are probably so many factors governing the methaemoglobin production in this instance that it is only possible to say that this is effectively equivalent to 10 g. sodium nitrite.

The bulk of the nitrate placed in the rumen is undoubtedly reduced to ammonia, and nitrite is an intermediate in this reaction. This nitrite can pass, to a greater or less extent, into the blood stream, where it leads to the conversion of part of the haemoglobin to methaemoglobin. Increasing the dose of nitrate to 22·5 g. sodium nitrate or more, led to a marked accumulation of nitrite in the rumen and a sharp increase in the extent of methaemoglobinaemia. It is concluded that at this level the rate of reduction of nitrite to ammonia becomes a limiting factor in the removal of nitrite, whereas further added nitrate is still reduced to nitrite. In the same way when nitrite is introduced into the rumen the reduction to ammonia becomes limited above a certain concentration of nitrite. In both cases, following doses of nitrate or nitrite greater than certain critical amounts the sheep will suffer from a methaemoglobinaemia that rapidly becomes more severe with larger doses, as was found experimentally (Fig. 6).

It is possible to correlate the peak methaemoglobin values with the changes in the nitrate, nitrite and ammonia in the rumen liquor after administration of nitrate. When 12 g. sodium nitrate was administered to the sheep (Fig. 1) the nitrate concentration fell off rapidly at first and then more slowly, while the ammonia values rose steadily for 5 hr. Nitrite was present in appreciable amount only for a short period during the initial rapid fall in the quantity of the nitrate present. It is possible that most of the ammonia, produced initially, enters the normal synthetic mechanisms in the rumen. The ammonia produced later would possibly be less utilized in this way owing to saturation of this mechanism and the rise in the concentration would be greater in proportion to the nitrate disappearing, on the whole fitting in with the observed steady rise in ammonia over several hours. This situation is still more obvious when 25 g. sodium nitrate were given to the sheep. Since the amount of nitrate present was still appreciable 5–6 hr. after administration the ammonia values increased over a longer period and to a higher level. The quantity of nitrite present in this case was greater, and persisted in the rumen for several hours. The extent of the methaemoglobinaemia increased to a peak of 60 % of the total haemoglobin at 7 hr. after dosing with nitrate.

SUMMARY

1. Nitrate introduced into the rumen of the sheep is reduced to ammonia.

2. Nitrite is an intermediate in this reaction and may accumulate under certain conditions and lead to a partial conversion of blood haemoglobin to methaemoglobin.

3. Sodium nitrate (25 g.) or sodium nitrite (10 g.) placed in the rumen, or 2 g. sodium nitrite injected intravenously, result in a methaemoglobinaemia corresponding to 60 % conversion of the total haemoglobin.

The author wishes to thank Dr I. W. McDonald and Dr S. R. Elsden for their constant help and advice, and the Agricultural Research Council for a Scholarship.

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The Use of Radioactive Isotopes in Immunological Investigations

5. QUANTITATIVE ASPECTS OF THE ANTIBODY-ANTIGEN REACTION

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The reaction between antigen and antibody which results in precipitation has been studied quantitatively by Heidelberger, Marrack, Haurowitz, Kabat, Hooker and Boyd, and many other investigators (see reviews by Heidelberger, 1939, 1946; Marrack, 1938; Boyd, 1947; Kabat & Mayer, 1948; Wormall, 1948). These investigations have been based mainly on the determination of small amounts of nitrogen in the precipitates. The amount of antibody can be readily calculated from these determinations if the antigen contains little or no nitrogen (e.g. polysaccharides) or alternatively, the amounts of both antigen and antibody can be calculated, if the antigen contains some special group or element (e.g. dyestuff, iron, copper, etc.) which can be determined separately. Few natural proteins, however, contain a suitable label, and the introduction of foreign groups, leading to the production of unnatural proteins or protein complexes, is frequently undesirable. When unlabelled protein antigens are used, the analysis of the specific precipitates for both antigen and antibody is complicated, and may sometimes be impossible.

We have used antigens and antibodies labelled with radioactive isotopes to study the precipitin test and certain other serological reactions. At first our choice of antigen was limited to biologically prepared labelled proteins such as lipovitellin and vitellin (Francis & Wormall, 1948), and chemically altered proteins such as phosphorylated or mustard gas sulphone-treated proteins containing appreciable quantities of introduced phosphorus or sulphur (Boursnell, Dewey, Francis & Wormall, 1947; Banks, Boursnell, Dewey, Francis, Tupper & Wormall, 1948). With an improved supply of certain carrier-free radio-isotopes it later became possible, however, to use such small amounts of the element that the protein molecule could be adequately labelled without significant change in the properties of the protein other than the acquirement of radioactivity. The experiments described here were carried out in order (*a*) to obtain information about the best methods for labelling protein antigens and antibodies with radio-isotopes for serological investigations, and (*b*) to use these labelled compounds for quantitative studies on certain precipitin reactions. In most of these experiments chemically altered protein antigens have been used, e.g. those labelled with 8–12 % radio-iodine; but where antibodies have been labelled with ^{131}I this has been effected with small amounts of radio-iodine of high specific activity. Preliminary notes of a few of these investigations have been published elsewhere (Boursnell *et al.* 1947; Banks, Francis, Mulligan & Wormall, 1949, 1950).

EXPERIMENTAL

Methods

Precipitin reactions. Since we usually varied the technique of these tests to suit the particular experiment undertaken, only the general details will be given here; exact details, where they are required, are given in the appropriate table or parts of the text. The antigen and antibody solutions, plus

another organophosphorus insecticide. Populations D and E had been, respectively, 24 and 31 times as resistant to malathion as population A, and the mixed population proved to be 15 times more resistant to dichlorvos than population A.

Table 3 Response of Two Whitefly Populations to Dichlorvos, 1972

Population	Slope ± s.e.	Log LC_{50} ± s.e.	LC_{50} (p.p.m.)
A	1.44 ± 0.162	1.53 ± 0.058	34
D/E	0.90 ± 0.138	2.71 ± 0.106	513

Our observations show that resistance to at least three chemicals occurs in glasshouse whitefly and that the level of resistance varies in different populations. It seems likely that resistance will become widespread through the distribution of plants infested with resistant whiteflies. Work is continuing on the assessment of other chemicals for the control of this pest.

We thank Mr K. G. Gostick for his advice and help with analyses.

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Selective Feeding of *Anopheles gambiae* according to ABO Blood Group Status

In investigations of physiological factors affecting the selection of human hosts for feeding by the mosquito *Anopheles gambiae* (species A from Nkolmekok[2], Cameroons), pairs of subjects were chosen who contrasted in ABO blood group status. The experiments were conducted under conditions which simulated as closely as possible those under which females of *A. gambiae* normally bite. Because illumination was limited to a dim red light, counting how many of the twenty female insects used took a blood meal from the arm of a particular host proved difficult. We found, however, that even if only a very small blood meal was taken during the ten minutes' testing time, the ABO status of this meal could be reliably established within an hour, by extracting the blood from the mosquito's gut and performing direct agglutination tests on it with anti-A and anti-B sera. In over a hundred blind tests there were only two cases of possible error.

Table 1 Mean Number of Bites in Each Group

Blood group	No. of subjects	Mean No. of bites*
O	42	5.045
A	41	3.276
B	14	4.250
AB	5	3.280
A+B+AB	60	3.503

* For each subject exposed more than once the average value was used.

Analysis of the blood group data reveals that the mosquitoes preferentially selected hosts of blood group O. The mean numbers of bites in the different groups are shown in Table 1. For statistical analysis the ratio of bites in individuals of one blood group to those of another group were used. The standardized normal deviate (s.n.d.) is calculated for each test, with continuity correction. For individuals of O and A blood group (the most common test situation) this is expressed as

$$\text{s.n.d.} = \frac{\log_e[(r_O + \tfrac{1}{2})/(r_A + \tfrac{1}{2})]}{[(r_A + \tfrac{1}{2})^{-1} + (r_O + \tfrac{1}{2})^{-1}]^{\frac{1}{2}}}$$

where r_O is the number of mosquitoes biting the O subject and r_A the number biting the A subject. This procedure obviates the variation in total number of mosquitoes choosing to feed from test to test and the logit transformation effectively normalizes the distribution of the ratio as is indicated by a normal plot of the s.n.d.s. Some subjects took part in several experiments in different combinations of pairs, but singling out for analysis only those cases where a subject was first used produces a normal plot with the same mean and slope as when using all the experiments, and this slope is very close to the theoretical value. All the variation is therefore accounted for by the blood group difference. On the null hypothesis that mosquitoes feed randomly with respect to ABO status, the s.n.d.s have a mean of O and the standard deviation of the mean equals $1/n^{\frac{1}{2}}$. In the 97 pairs of O and A individuals in these experiments, the sum of the s.n.d.s is 38.3 and t_{96} equals 3.9 which is significant at the 0.1% probability level. In O and B pairs $t_{25} = 3.3$, significant at the 0.5% probability level. When O subjects are compared with all non-O subjects, the sum of the s.n.d.s is 59.2 and $t_{125} = 5.1$, also significant at the 0.1% probability level.

These experiments strongly suggest that *A. gambiae* recognizes ABO blood group variation, and is selective in its feeding, with a preference for blood group O. The basis for this recognition and selection is not obvious, although ABH blood group substances do occur on skin cells[1-3] and have been reported in sweat[4].

This finding could be of great importance in infectivity rates, the development of acquired immunity and natural selection for ABO blood groups in malarious regions. It needs, however, to be confirmed and extended to other strains of *A. gambiae* and other Anophelines.

We thank Dr G. Davidson and Professor L. Bruce-Chwatt for advice.

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Carcinogenic Nitrosamines formed by Drug/Nitrite Interactions

NITROSAMINES are compounds with broad carcinogenic activity in many animal species, but have seldom been detected in the environment in significant quantities. Man may nevertheless be exposed to nitrosamines formed *in vivo* from ingested nitrite and secondary amines[1-3]. Nitrite is a common constituent of the diet, as are several secondary amines[4]. Since tertiary amines can also react with nitrite[5] in the mildly acid conditions of the

mammalian stomach, the exposure of man to the many tertiary nitrogen compounds which are drugs[6] must also be considered.

We have examined the interaction of nitrite with six tertiary nitrogen compounds representative of different types of chemical structure: oxytetracycline (antibiotic), aminopyrine (analgesic), disulphiram (antialcoholic), nikethamide (respiratory stimulant), tolazamide (oral hypoglycaemic), and piperine (found in pepper), all of which gave measurable yields of nitrosamine.

We did not study these reactions exhaustively, but examined formation of nitrosamine products in varying conditions. Reactions were carried out in aqueous acid solutions with an excess of sodium nitrite, usually at 37° C and at a *p*H between 3.5 and 5.5. Reactions were terminated after 4 h or less by addition of KOH to *p*H 11, and the nitrosamines were extracted with methylene chloride and estimated by gas chromatography on columns of diethyleneglycol succinate on 'Chromosorb W'. The identity of the nitrosamine formed was established by mass spectrometry and in some cases by infrared spectrometry. The reaction conditions and yields of nitrosamines are given in Table 1.

All compounds tested gave measurable yields of nitrosamines at body temperature. The two tertiary amines, oxytetracycline and aminopyrine, gave high yields of dimethylnitrosamine (DMN) and were studied in more detail. In the aminopyrine reactions, the effect on nitrosamine formation of reactant concentration and *p*H is shown in Table 2. It appeared that DMN was formed in good yields over a wide range of *p*H, and that a large excess of nitrite did not increase the yield. The death from acute DMN hepatotoxicity of rats given small doses of aminopyrine and nitrite by stomach tube has been reported[7].

Similar studies with oxytetracycline, however, showed a marked dependence of DMN yield on *p*H and on nitrite concentration (Table 3). The optimum *p*H was 3, and a lower yield of DMN at lower molar ratios of nitrite was found.

The dialkylamides, nikethamide, piperine, and disulphiram gave rather lower yields of the respective nitrosamines, diethylnitrosamine (DEN) and nitrosopiperidine (NP), than did the two tertiary amines. With these compounds as well, formation of nitrosamine was favoured at *p*H greater than 3, the yield of nitrosamine being quite small near *p*H 2. In the case of piperine and disulphiram, the percentage yield of nitrosamine might have been greater than indicated, because much of the starting material remained undissolved.

Tolazamide, which is both a substituted urea and a dialkylhydrazine, was converted readily to N-nitrosohexamethyleneimine (NHM), which has been reported as a potent liver carcinogen in rats[8]. The reaction of N′,N′-dialkylhydrazides with nitrous acid was studied[9], but nitrosamines were not positively identified in the products.

Although these reactions were conducted with higher concentrations of drug and nitrite than would be found in the human stomach, the yields of nitrosamine expected in normal use of the drugs may still be predicted from the course of the reactions. In the case of aminopyrine, a large proportion of the amine is converted to DMN regardless of concentration. For example, a dose of 100 mg would be expected to yield several mg of DMN, a significant amount of such a potent carcinogen. The reaction of disulphiram and tolazamide with nitrite is so rapid that mg quantities of nitrosamine would be expected, in the presence of sufficient nitrite. The reaction of oxytetracycline, however, is markedly dependent on concentration and *p*H and the amount of DMN produced might well be negligible

Table 1 Reaction of Drugs with Nitrite

Compound	Wt.	Nitrite	Acid	Vol.	*p*H	Temp.	Time	Yield of nitrosamine
Oxytetracycline	250 mg	0.5 g	1 ml. AcOH	12 ml.	4.0	37° C	4 h	24 mg DMN (65%)
	250 mg	0.5 g	2.3 g NaH_2PO_4	12 ml.	4.2	37° C	4 h	4.2 mg DMN (11%)
Aminopyrine	1.0 g	2.0 g	4 ml. AcOH	25 ml.	4.2	37° C	4 h	234 mg DMN (73%)
	1.0 g	2.0 g	8.6 g NaH_2PO_4	25 ml.	4.7	37° C	4 h	106 mg DMN (33%)
Disulphiram	0.74 g	1.8 g	1.5 ml. AcOH	12 ml.	4.0	37° C	2 h	22 mg DEN (4.4%)
	0.74 g	1.8 g	3.4 g NaH_2PO_4	12 ml.	4.6	37° C	2 h	31 mg DEN (6.2%)
	0.74 g	1.8 g	0.01 M HCl	12 ml.	2.0	37° C	2 h	3 mg DEN (0.6%)
Nikethamide	1.0 g	1.7 g	1.5 ml. AcOH	12 ml.	3.8	37° C	2 h	0.8 mg DEN (0.13%)
	1.0 g	1.7 g	0.01 M HCl	12 ml.	2.1	37° C	20 h	0.02 mg DEN (0.003%)
	1.0 g	1.7 g	0.01 M HCl	12 ml.	3.0	37° C	20 h	0.06 mg DEN (0.01%)
	1.0 g	1.7 g	0.01 M HCl	12 ml.	3.6	37° C	20 h	1.65 mg DEN (0.28%)
	1.0 g	1.7 g	0.01 M HCl	12 ml.	4.2	37° C	20 h	1.65 mg DEN (0.28%)
Tolazamide	4.0 g	3.5 g	3 ml. AcOH	25 ml.	4.4	37° C	4 h	445 mg NHM (28%)
	4.0 g	3.5 g	8.6 g NaH_2PO_4	25 ml.	4.2	37° C	4 h	367 mg NHM (23%)
Piperine	100 mg	1.0 g	1.5 ml. AcOH	12 ml.	3.6	90° C	4 h	11.2 mg NP (27%)
	100 mg	1.0 g	1.5 ml. AcOH	12 ml.	3.6	37° C	4 h	2.2 mg NP (5%)
	100 mg	1.0 g	0.01 M HCl	12 ml.	2.3	37° C	4 h	0.05 NP (0.1%)

Table 2 Reaction of Aminopyrine with Nitrite

Wt. of aminopyrine	Vol.	Wt. of $NaNO_2$	Acid	*p*H	Temp.	Time	Yield of DMN
1.0 g	25 ml.	2.0 g (6 mol)	4 ml. AcOH	4.2	37° C	4 h	234 mg (73%)
1.0 g	25 ml.	2.0 g (6 mol)	8.6 g NaH_2PO_4	4.7	37° C	4 h	106 mg (33%)
1.0 g	25 ml.	2.0 g (6 mol)	8.6 g NaH_2PO_4+3.7 g NaCl	4.2	37° C	4 h	109 mg (34%)
0.36 g	12 ml.	0.42 g (4 mol)	HCl	2.5	37° C	3 h	66 mg (57%)
0.36 g	12 ml.	0.42 g (4 mol)	HCl	3.2	37° C	3 h	77 mg (67%)
0.36 g	12 ml.	0.42 g (4 mol)	HCl	3.9	37° C	3 h	61 mg (53%)
0.36 g	12 ml.	0.42 g (4 mol)	HCl	4.6	37° C	3 h	45 mg (39%)
62 mg	10 ml.	56 mg (3 mol)	0.01 M HCl	5.3	37° C	3 h	6.6 mg (33%)
62 mg	10 ml.	56 mg (3 mol)	0.01 M HCl+0.12 ml. AcOH	3.5	37° C	3 h	7.0 mg (35%)
62 mg	10 ml.	100 mg (5 mol)	0.01 M HCl	5.5	37° C	3 h	6.2 mg (31%)
62 mg	10 ml.	100 mg (5 mol)	0.01 M HCl+0.12 ml. AcOH	3.5	37° C	3 h	6.9 mg (35%)
62 mg	10 ml.	200 mg (10 mol)	0.01 M HCl	5.7	37° C	3 h	6.8 mg (34%)
62 mg	10 ml.	200 mg (10 mol)	0.01 M HCl+0.12 ml. AcOH	3.7	37° C	3 h	7.1 mg (36%)

Table 3 Reaction of Oxytetracycline with Nitrite

Wt. of OTC	Vol.	Wt. of $NaNO_2$	Acid	pH	Temp.	Time	Yield of DMN
100 mg	20 ml.	1.0 g	3 ml. AcOH	4.2	90° C	16 h	74 mg (100%)
250 mg	10 ml.	0.5 g	1 ml. AcOH	3.5	37° C	4 h	24 mg (65%)
250 mg	12 ml.	0.5 g	2.3 g NaH_2PO_4	4.2	37° C	4 h	4.2 mg (11%)
250 mg	12 ml.	0.5 g	2.3 g NaH_2PO_4 +2.5 g NaCl	3.7	37° C	4 h	7.4 mg (20%)
100 mg	4 ml.	56 mg	0.05 ml. AcOH	3.6	37° C	1 h	0.9 mg (6%)
100 mg	4 ml.	56 mg	0.05 ml. AcOH	3.6	37° C	2 h	1.9 mg (13%)
100 mg	4 ml.	56 mg	0.05 ml. AcOH	3.6	37° C	2.5 h	2.6 mg (18%)
100 mg	12 ml.	56 mg	HCl	2.0	37° C	4 h	0.03 mg (0.2%)
100 mg	12 ml.	56 mg	HCl	3.0	37° C	4 h	0.3 mg (2.0%)
100 mg	12 ml.	56 mg	HCl	4.0	37° C	4 h	0.03 mg (0.2%)
100 mg	12 ml.	200 mg	HCl	2.0	37° C	4 h	0.1 mg (0.7%)
100 mg	12 ml.	200 mg	HCl	3.0	37° C	4 h	2.2 mg (15%)
100 mg	12 ml.	200 mg	HCl	4.0	37° C	4 h	1.0 mg (7%)

The hazard of nitrosamine formation from various drugs is therefore variable, but no exposure to a carcinogen can be considered insignificant.

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Mechanism of the Antitumour Effect of Interferon in Mice

REPEATED inoculation of mouse interferon preparations inhibits the growth of several murine transplantable tumours and increases the survival of tumour inoculated mice[1-6]. The mechanism of this antitumour action of interferon *in vivo* is unknown. Since comparable interferon preparations inhibited the division of murine leukaemia L 1210 and Ehrlich ascites (EA) cells in suspension cultures[7-9] it is possible that interferon also acts *in vivo* by inhibiting tumour cell multiplication. It is also possible, however, that interferon enhances in some manner the capacity of the host to reject the transplanted tumour.

We have used murine leukaemia L 1210 cells which are sensitive to the antiviral effect of interferon and to the inhibitory effect of interferon on cell division[7-9]. We refer to these cells as L 1210 S cells to distinguish them from cells of a mutant cell subline, L 1210 R, resistant to both the antiviral and cell division inhibitory activities of interferon[8,9].

We reasoned that if the antitumour effect resulted from a direct inhibition of the multiplication of tumour cells by interferon, interferon would protect mice inoculated with interferon-sensitive cells but would not protect mice inoculated with interferon-resistant cells. On the other hand if the antitumour effect of interferon were in part host mediated, interferon might protect mice inoculated either with interferon-sensitive or interferon-resistant cells.

We therefore determined the effect of interferon treatment on DBA/2 mice inoculated with interferon-sensitive or interferon-resistant L 1210 cells. The origins of L 1210 S and L 1210 R cells and the techniques of cultivation *in vitro* have been previously described[7-9]. One to two-month-old male or female DBA/2 mice were inoculated intraperitoneally with either L 1210 S or L 1210 R cells. Twenty-four hours later, mice of each group were inoculated with 50,000–64,000 NIH international reference units of mouse brain or cell culture interferon, and treatment was continued once daily for one month. Control mice were inoculated with a mock interferon or left untreated. The techniques used in the preparation and assay of concentrated mouse brain or cell culture interferon have been previously described[2,5,8,9].

Daily interferon treatment was associated with an increase in the mean survival time of DBA/2 mice inoculated with interferon-sensitive and with interferon-resistant L 1210 cells (Table 1). A protective effect was observed in mice treated with mouse interferon derived from brain or from cell culture medium, and in mice inoculated with varying numbers of tumour cells (Table 1). There was no protection in mice treated with control preparations or with a heterologous

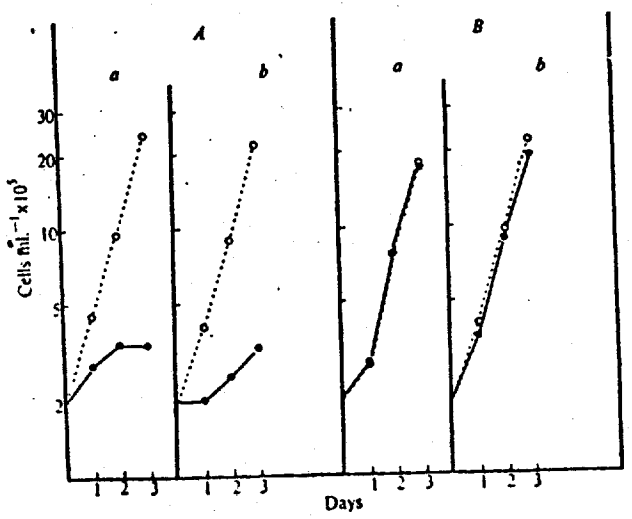

Fig. 1 Effect of interferon on the multiplication *in vitro* of L 1210 S (*A*) or L 1210 R (*B*) cells obtained from the peritoneal cavities of DBA/2 mice. *a*, Control; *b*, interferon treated. "Interferon treated" refers to the treatment of a mouse inoculated with L 1210 S or R cells. Cells cultivated in non-agitated suspension culture: O, no interferon; ⊙, 1,600 units mouse cell culture interferon/0.2 ml.

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Nitrosamines as Environmental Carcinogens

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Human cancer might be caused by nitrosamines formed in the body from ingested nitrites and secondary amines. Cooking could be a source of secondary amines.

ALTHOUGH several groups of chemicals have been demonstrated to be carcinogenic during the past few decades, there are only few cases in which exposure to such chemicals has been causally related to cancer in man. For example, skin cancer in workers in the coal tar and creosote industries is clearly associated with exposure to polynuclear compounds; similarly, bladder cancer among workers in the dyestuff and rubber industries is related to exposure to 2-naphthylamine, benzidine and other aromatic amines. Apart from such restricted cases of occupationally induced cancer, there is little evidence relating the high incidence of human cancer to particular chemical carcinogens.

The recent discovery of the highly carcinogenic aflatoxins prompted the speculation that liver cancer in the tropics might be caused by ingestion of nuts and grains contaminated with mycotoxin as a result of improper harvesting and storage. Even so, mycotoxins can hardly be implicated in cancer in modern industrialized society.

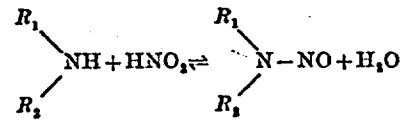
In our view, any group of chemical carcinogens significantly implicated in human cancer must be both widespread and multipotent. Such chemicals are the nitrosamines, intensively studied since the first report of hepatic cancer induced by nitrosodimethylamine in rats[1]. In a large series of nitrosamines recently tested, most were carcinogenic in a wide range of organs of various species[2]. The most interesting attributes of carcinogenic nitrosamines are their systemic action and organ specificity. Thus, nitrosopiperidine produces tumours of the oesophagus in rats, as well as in other sites, whether administered orally, intravenously or subcutaneously[3]. Nitrosoheptamethyleneimine and nitrosooctamethyleneimine both induce squamous carcinomas of the lung and oesophagus when given to rats in drinking water[4]; by the same route in hamsters, however, nitrosoheptamethyleneimine produces cancer of the forestomach only (unpublished results of W. L.).

Nitrosamines in the Environment

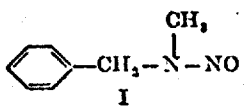
It thus seemed logical to look for nitrosamines in the environment in an attempt to account for some epidemiological aspects of human cancer. Unfortunately, nitrosamines are very difficult to detect, especially in low concentrations. They neither fluoresce nor have well defined absorption spectra, and have a wide range of physical characteristics, particularly in volatility and solubility. A group analysis is of limited value, for not all nitrosamines are carcinogenic. Nitrosamines have been found in tobacco smoke, grains and alcoholic beverages in concentrations of less than 5 p.p.m.[5]. The significance of such low concentrations to man is unknown[6]. Higher concentrations of nitrosamines have been found in nitrite-preserved fish meal intended for animal feeds; these fish meals were highly toxic to ruminants[7].

Precursors of Nitrosamines

There has been increasing interest in the possibility that nitrosamines can be formed *in vivo* and so give rise to tumours in individuals not apparently exposed to nitrosamines *per se*. Secondary amines have recently been shown to react with nitrite to form nitrosamines in conditions of defined *p*H and in other conditions similar to those in the mammalian stomach[8], and also *in vitro* in gastric juice[9,10].



Rats given nitrite and a secondary amine, methylbenzylamine, together in their diet developed oesophageal tumours[11], which are also induced by nitrosomethylbenzylamine (I), the corresponding nitrosamine[8].



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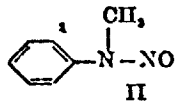


II

It has been suggested that such *in vitro* and *in vivo* interactions might be significant to man[6,9]. These considerations have directed our attention to the environmental distribution of nitrites and secondary amines as essential precursors of nitrosamines.

Nitrates and Nitrites

Nitrates are widely distributed in nature, particularly in plants and forage, and nitrites are readily formed from them. Excessive use of nitrate fertilizers, use of the herbicide 2,4-dichlorophenoxyacetic acid, and molybdenum deficiency in soil result in accumulation of nitrate in plants and forage[12] and methaemoglobinaemia in cattle as a consequence of nitrite poisoning. A close association has been demonstrated recently between molybdenum deficiency in garden plants and the occurrence of oesophageal cancer in localized areas of the Transkei; dimethylnitrosamine has been isolated from food plants in these areas[13]. Nitrates can occur in significant concentrations in water, particularly in agricultural areas, and produce toxic or fatal methaemoglobinaemia in infants after conversion to nitrite[14]. Contamination of water with nitrates results from sewage discharges, intensive use of nitrate fertilizers, and rising water tables which leach sub-soil nitrates into well waters. Nitrites are intermediate in the reduction of nitrates in plants and can be produced from nitrates in plants and other sources by nitroreductases present in a wide range of saprophytic and parasitic bacteria[15]. Large concentrations of nitrites are found in stored green vegetables, especially spinach, celery and green salad, as a result of bacterial reduction of nitrate[16]. Furthermore, nitrates and nitrites are widely used as preservatives in meat and fish, in permissible concentrations of 500 and 200 p.p.m. in the United States[17]. It seems likely, therefore, that nitrite ions are not infrequently present in the stomach of man. The average daily intake of nitrite has been estimated as 22 μmoles[9], equivalent to 1·5 mg of $NaNO_2$; some individuals undoubtedly consume much more nitrite.

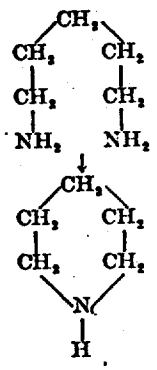
Secondary Amines

The limiting factor, then, in the formation of nitrosamines seems to be the availability of nitrosatable secondary amines. Because secondary amines have seldom been sought in the environment, information about their distribution is scanty. They occur, particularly as dimethylamine and diethylamine, in fish meal and fish products, cereals and tea[18] and in tobacco and tobacco smoke[19]. In such sources, secondary amines are usually found together with primary and tertiary amines.

Methods of identifying secondary amines are not well developed and those usually measured are diethylamine and dimethylamine; the remaining bases are conventionally grouped as other unidentified amines. Conversion of dimethylamine and diethylamine into nitrosamines is not favoured in dilute acid conditions, as prevail in the stomach, because of the relatively strong basicity of these amines[11,20]; this conversion has, however, been demonstrated with diethylamine[10]. In this connexion, rats fed a mixture of dimethylamine or diethylamine and sodium nitrite failed to yield tumours[11,20]. Conversion of other secondary amines to nitrosamines is favoured when the nitrosamine, such as nitrosomethylbenzylamine (I) and nitrosomethylaniline (II), tends to be less soluble. Cyclic amines, such as piperidine (III), are in this category and the derived nitrosamines are potent carcinogens[3]. Because the feeding of nitrite alone did not give rise to tumours[11,20], the formation of nitrosamine from either endogenous secondary amines, such as epinephrine or proline, or from secondary amines in rat food is unlikely.

Secondary Amines in Cooking

Man is the only species which cooks and preserves its food, and cooking possibly affords a source of secondary amines. Pyrolysis of protein and cooking of protein food might produce free amino-acids such as proline, arginine and hydroxyproline, and nitrosatable secondary amines such as pyrrolidine and piperidine. The diamines cadaverine and putrescine present in partially decayed meat and fish are converted by heating into piperidine (III) and pyrrolidine (IV), respectively; these two amines could therefore be formed when meat or fish is cooked.



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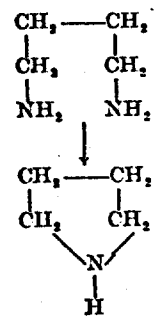
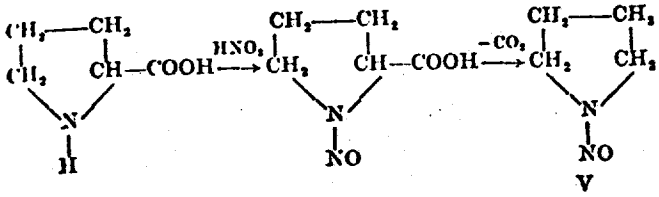


IV

Nitrosoproline has been synthesized recently[21]. It has been further shown that both proline and hydroxyproline are nitrosated in dilute acid solution at 35°–40° C in the presence of an excess of sodium nitrite; in each case the yield exceeded 50 per cent of the theoretical value; the crystalline nitrosamino-acids were identified by mass and nuclear magnetic resonance spectrometry. It has not been possible to isolate a nitroso derivative of arginine. In the presence of dilute alkali at ambient temperature, nitrosoproline undergoes decarboxylation with formation of nitrosopyrrolidine (V), as identified by mass spectrometry. The preparation and properties of nitrosoproline

and nitrosohydroxyproline will be reported elsewhere (unpublished results of W. L.).



The possibility therefore exists that proline ingested in food can be converted into nitrosoproline by nitrite present in the stomach. The nitrosoproline could then be decarboxylated, possibly bacterially, in the alkaline conditions prevailing in the duodenum and small intestine, yielding the highly carcinogenic nitrosopyrrolidine. It is likely that hydroxyproline undergoes parallel reactions *in vivo*.

Uses of Secondary Amines

Secondary and other amines are formed during alcoholic fermentation. A mixture of crude organic bases was at one time made commercially by distillation of the residues of fermented molasses with lime; nitrogenous bases have been found in spirits and several amines, including pyrrolidine, in wine[22]. It is therefore possible that the high incidence of oesophageal cancer associated with the consumption of crude beers and other liquors in Zambia[5] is due to secondary amines, in addition to nitrosamines.

Various flavouring agents are prepared from secondary amines[23], including bread flavourings prepared from pyrrolidine or piperidine, and meat flavourings prepared from free amino-acids including proline. These additives could be nitrosatable themselves or contain the unreacted amines. Some toothpastes contain derivatives of sarcosine; nitrososarcosine is carcinogenic[3]. Some drugs are secondary amines, such as piperazine which is used in the treatment of worms; dinitrosopiperazine produces tumours of the oesophagus and liver when fed to rats[3].

Tobacco contains several secondary amines, particularly pyrrolidine to the extent of 0·01 per cent[19]. These amines could be released and ingested when tobacco is chewed, particularly when mixed with lime. Cigarette smoke contains many secondary amines, including pyrrolidine and piperidine[19]. These amines could dissolve in the saliva during smoking and be converted to nitrosamines in the stomach. Such nitrosamines could be absorbed and give rise to tumours of the lung systemically, a property of several nitrosamines.

In vivo formation of nitrosamines from ingested nitrite and certain secondary amines is potentially a serious problem, especially because nitrosamines are possibly more significant in human cancer in industrialized society than are polynuclear compounds, azo dyes or aflatoxins. Epidemiological data on the carcinogenicity of nitrosamines should be re-evaluated in the light of these considerations. It should, however, be stressed that the individual occurrence of nitrites and secondary amines is not necessarily hazardous; both reactants must be present simultaneously in the stomach to form nitrosamine. Even so, not all secondary amines react with nitrite to form nitrosamines in conditions prevailing in the mammalian stomach, and not all nitrosamines are carcinogenic.

Consideration should be given to the reduction or elimination of one or other nitrosamine precursors, nitrites and certain secondary amines, from the diet. For this purpose, more information is needed on the quantitative distribution of these compounds in the environment. On the immediate practical level, however, it should not be difficult to reduce the amount of nitrite and nitrate added as preservatives to food, particularly meat and fish.

Removal from Diet

In summary, nitrosamines seem to be a major candidate class of carcinogens that are likely to be causally related to human cancer in industrialized society. Nitrosamines act systemically and produce cancer in a wide variety of organs of many species. Additionally, individual nitrosamines exhibit marked organ specificity. Although methods for detection of nitrosamines lack sensitivity, their widespread environmental distribution has been demonstrated recently. Even more widespread and of possibly greater significance are the precursors of nitrosamines—nitrites and secondary amines. These precursors react in defined conditions *in vitro* and probably also *in vivo* to form nitrosamines. Reduction of human exposure to nitrites and certain secondary amines, particularly in foods, may result in a decrease in the incidence of human cancer.

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To determine if protective immunity resulted from the injections of irradiated sporozoites, the two monkeys (T-944, T-951) plus a control animal (T-984) were bitten by five, six and five mosquitoes, respectively, all of which were found to be heavily infected with the homologous strain of the parasite.

The results indicated that the prepatent periods in the immunized animals were delayed from 2 to 5 days over that of the control animal (Fig. 1). The parasitaemia curve in T-951 was similar to that of the control with a maximum parasitaemia of 360,000/mm^3 on the tenth day of patent parasitaemia versus a maximum parasitaemia of 320,000/mm^3 on the ninth day of patent parasitaemia in the control. The parasitaemia curve in T-944 was considerably less, or flattened, with a maximum of 69,000 parasites/mm^3 on the eighth day of patent parasitaemia.

These preliminary results indicate that immunization of *M. mulatta* monkeys with sporozoites irradiated with 13 krad of X-irradiation failed to protect them completely from malaria infection. However, the delays in prepatent period for the two immunized monkeys suggest that the number of sporozoites which were able to initiate tissue stage development was reduced.

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Carcinogen Dimethylnitrosamine produced *in vivo* from Nitrite and Aminopyrine

IN experimental conditions, nitrite can interact with secondary amines in the rodent stomach at mildly acid *p*H to form nitrosamines[1–4], a large number of which are potent carcinogens[5]. As tertiary amines also react in this way[6], we have examined the analgesic aminopyrine, 4-dimethylaminoantipyrine (pyramidon), as being representative of many commonly used tertiary amine drugs.

Aminopyrine reacts readily with sodium nitrite at *p*H 2.5 to 5.5, giving a high yield (up to 70% of theoretical) of dimethylnitrosamine (DMN). Since the liver of the rat, and all mammals (including man) that have been studied, is susceptible to the acute toxic effect of DMN administration[7], the formation of DMN *in vivo* could be inferred by demonstrating liver damage in rats given aminopyrine and nitrite together.

Groups of six male MRC (Wistar-derived) rats weighing 250±25 g were given 1 ml. by gavage of solutions containing (*a*) 35 mg of aminopyrine, (*b*) 40 mg of sodium nitrite, and (*c*) 35 mg of aminopyrine+40 mg of sodium nitrite. After 68 h all the animals were killed with CO_2 vapour and the livers examined. The livers of rats in group (*c*) all showed severe centrilobular liver necrosis (Fig. 1), typical of the effect of DMN itself, while the livers of the rats in the other two groups showed no such change. Microscopically, the amount of liver necrosis corresponded to that seen after an exposure of approximately [illegible] mg of DMN per rat (12–21 mg/kg) similarly administered.

In another experiment, groups of four male MRC rats were treated as before for 3 consecutive days. All animals treated with aminopyrine and nitrite, but none of the other animals, died 1 h after the last gavage. On autopsy, the liver was swollen and dark red with a "nutmeg" appearance. Microscopically, there were bloody ascites and severe liver necrosis in all four animals but no significant hepatic alteration in the animals receiving aminopyrine or nitrite alone. Serum glutamic pyruvic transaminase (SGPT) levels, an index of liver necrosis, were increased two to four-fold in rats receiving the combined treatment (40–100 Reitman–Franklin units), when compared with untreated controls or animals receiving either compound alone (18–21 U).

Fig. 1 Severe liver necrosis in a rat given 35 mg of aminopyrine+40 mg $NaNO_2$ 68 h previously by gavage. Only islands of viable liver parenchyma remain. (Haematoxylin and eosin, ×88.)

To evaluate dose response effects when the nitrite concentration was decreased, groups of four male MRC rats weighing 200±25 g were treated with the indicated solutions by intragastric intubation (Table 1). Animals were killed 48 h after

Table 1 Dose Response Effects

Treatment	No. of treatments	Hepatic necrosis	SGPT*, mean values
Aminopyrine 35 mg+$NaNO_2$ 40 mg	1	+++	90
Aminopyrine 35 mg+$NaNO_2$ 20 mg	1	++	64
Aminopyrine 35 mg+$NaNO_2$ 10 mg	1	+	32
Aminopyrine 35 mg+$NaNO_2$ 5 mg	1	0	28
Aminopyrine 35 mg+$NaNO_2$ 40 mg	3	++++	175
Aminopyrine 35 mg+$NaNO_2$ 20 mg	3	+++	117
Aminopyrine 35 mg+$NaNO_2$ 10 mg	3	++	78
Aminopyrine 35 mg+$NaNO_2$ 5 mg	3	+	30
Aminopyrine 35 mg	1	0	25
Aminopyrine 35 mg	3	0	27
$NaNO_2$ 40 mg	3	0	25
$NaNO_2$ 20 mg	3	0	30
$NaNO_2$ 40 mg	1	0	25
Untreated controls		0	25

+, Individual cells necrotic around central vein.
++, All cells around central vein necrotic; one-third of the lobule involved.
+++, Pronounced centrilobular necrosis involving more than one-third of the lobule.
++++, Extensive centrilobular necrosis linking adjacent central vein structures.
* Reitman–Franklin units.

the last treatment, the livers examined histologically and SGPT levels determined.

The results indicate that a significant amount of DMN was formed even with a dose of 10 mg of sodium nitrite, since toxic manifestations were observed after three such treatments.

It was possible that the formation of DMN from aminopyrine and nitrite was due to nitrosation of dimethylamine produced from aminopyrine by hydrolysis. Intubation of four MRC rats for three successive days with a solution containing 15 mg of dimethylamine (as hydrochloride) and 35 mg of sodium nitrite in 1 ml. water did not, however, produce any liver alterations in animals killed 2 days after the last dose. However, dimethylamine hydrochloride and nitrite at much higher concentrations do produce liver necrosis in rats[8].

The demonstration that aminopyrine reacts with sodium nitrite in weakly acid solution to give a high yield of the potent carcinogen dimethylnitrosamine, both in the test tube and *in vivo*, may indicate a possible hazard to man of ingestion of aminopyrine when nitrite is present in the stomach. In the chemical studies, a 30% conversion to DMN was obtained using 62 mg of aminopyrine and 56 mg (3 mol) of sodium nitrite. Although these concentrations are higher than those that would be experienced by man, there is no reason to believe that the production of DMN would be negligible at lower concentrations. If the experience were repeated over a long period, the formation of DMN from aminopyrine, taken as a drug, and sodium nitrite, in the food, might be a considerable carcinogenic stimulus to an individual.

Aminopyrine is only one of many drugs that might lead to the formation of carcinogenic nitrosamines by interaction with nitrite[9]. Studies of the nitrosation of some of these compounds are in progress.

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Magnetic Resonance of Isotope Hybrid Flavoprotein ^{2}H-Flavoprotein (^{1}H-Flavin Mononucleotide)

FLAVOPROTEINS lend themselves particularly well to study by the combined techniques of magnetic resonance and isotope substitution[1-3]. Recent electron spin resonance (ESR) and electron-nuclear double resonance (ENDOR) investigations have already indicated the utility of this approach to the study of the structure and function of flavin and flavoproteins[4-8]. We have now prepared an unusual isotope hybrid flavoprotein

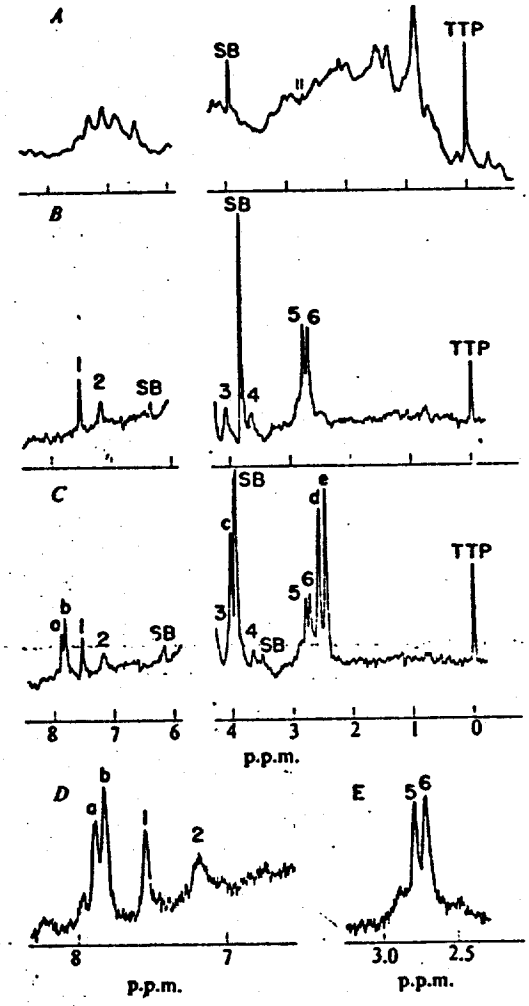

Fig. 1 PMR spectra (220 MHz) of fully deuterated flavoprotein substituted with ^{1}H-flavin mononucleotide by heating in the presence of ^{1}H-FMN and nicotinamide adenine dinucleotide phosphate. The flavoprotein, about 0.0015 M, was dissolved in neutral 0.05 M phosphate buffer in ^2H_2O. All spectra were obtained by repetitive scanning. *A*, ^{1}H-flavoprotein. *B*, ^{2}H-flavoprotein (^{1}H-FMN). Peaks 1 and 2 are protons at positions 6 and 9 of the isoalloxazine ring, peaks 3 and 4 are assigned to the ribityl-CH_2-groups, and peaks 5 and 6 to isoalloxazine methyl groups. TTP is internal trisilyltetradeuteriopropionate and SB is a sideband. The sweep rate was 10 Hz s^{-1} and 256 scans were collected. *C*, The same sample as in *A*, but with added 0.0025 M FMN. The peaks at *a*, *b*, *d* and *e* represent the 6 and 9 protons and the methyl groups of the isoalloxazine moiety of the free FMN, and peak *c*, a ribityl CH_2. Sweep rate as in *A*, 219 scans. *D*, Downfield portion of *B*, expanded. Scans, 545, at 5 Hz s^{-1}. *E*, Upfield portion of *A*, expanded. Scans, 512, at 2.5 Hz s^{-1}.

that gives promise of great value for magnetic resonance studies. This substance is obtained by the introduction (by exchange) of an ^{1}H-flavin mononucleotide (^{1}H-FMN) prosthetic group into (fully deuterated) ^{2}H-flavoprotein that can be isolated from the blue-green thermophilic alga *Synechococcus lividus*. This isotope hybrid protein makes it possible to apply in a unique way proton magnetic resonance (PMR) and ESR to the chemistry of protein-bound FMN.

The flavoprotein was isolated from *S. lividus* grown in 99.8% ^2H_2O in our usual culture conditions[9-10]. Our initial examination indicated that this protein is metal-free, has a molecular weight of about 18,000, and has one FMN prosthetic group. PMR studies with the fully deuterated protein showed that even the difficulty exchangeable hydrogen in the protein was completely exchanged with the solvent at 75°–80° C in a few minutes, indicating an unfolding of the protein at this temperature. Because it is known that the prosthetic groups of many flavoenzymes are noncovalently bound[1], we heated a mixture of ^{2}H-flavoprotein in D_2O buffer ($pD = 7.4$) with a

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THE PRODUCTION OF HYDROXYLAMINE BY THE REDUCTION OF NITRATES AND NITRITES BY VARIOUS PURE CULTURES OF BACTERIA[1]

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Hydroxylamine can be prepared chemically by the oxidation of ammonia or by the reduction of nitrates or nitrites. This intermediary position upon the oxidation-reduction scale has led several workers to postulate that hydroxylamine is also an intermediary product in the oxidation of ammonia or in the reduction of nitrates by biological means. Proof of the presence of hydroxylamine in biological oxidations or reductions has, however, been lacking until the recent work of Blom (1928). He was able to demonstrate the production of hydroxylamine in several synthetic media in which the nitrate radical was the only source of nitrogen. The bacteria involved were an unknown mixture obtained from a large inoculum of soil. The present study was made in order to determine, by Blom's technic, if the ability to reduce nitrates to hydroxylamine is characteristic of a variety of organisms in pure culture.

Blom's test for hydroxylamine consists essentially of the determination of the nitrites formed by the oxidation of hydroxylamine with iodine. It is apparent that the medium must be free of nitrites before the presence of minute amounts of hydroxylamine can be demonstrated. None of the organisms used in the present study were able to remove all of the nitrites produced from nitrates when synthetic media similar to those of Blom's were used. In nutrient broth cultures, however, all of the nitrites formed were removed by the bacteria whenever the amounts of nitrates

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were sufficiently small. Some of the cultures reduced the nitrates and the nitrites with surprising rapidity, especially in the presence of d-glucose, as is shown by the following experiment. Sodium nitrate was added to twelve-hour d-glucose-broth cultures of *A. aerogenes* and *Esch. coli* at the rate of 27.5 mgm. per liter. At intervals of ten minutes the amount of sodium nitrite was determined colorimetrically by means of the sulfanilic acid-dimethyl-alpha-naphthylamine test. When the nitrites disappeared, the absence of nitrates was demonstrated by the diphenylamine and by the phenol-disulphonic acid methods. The results are shown in table 1.

TABLE 1

TIME	$NaNO_3$	
	A. aerogenes	*Esch. coli*
minutes	*mgm. per liter*	*mgm. per liter*
0	0	0
10	1.7	1.6
20	2.7	5.1
30	3.8	9.5
40	6.5	11.9
50	8.0	6.4
60	3.1	2.6
70	0	0

The test used for detecting small amounts of hydroxylamine was the same as the one used by Blom, except that dimethyl-alpha-naphthylamine was substituted for alpha-naphthylamine in accordance with the suggestion of Wallace and Neave (1927). The necessary reagents are as follows:

(1) 10 grams sulfanilic acid dissolved in 1000 cc. of 5 N acetic acid
(2) 6 cc. dimethyl-alpha-naphthylamine dissolved in 1000 cc. of 5 N acetic acid
(3) 1.3 grams iodine dissolved in 100 cc. glacial acetic acid
(4) 2.5 grams $Na_2S_2O_3 \cdot 5H_2O$ dissolved in 100 cc. of water

The procedure for the determination is as follows: Two cubic centimeters of solution 1 and a few drops of solution 3 are added to 10 cc. of the culture. After a period of five minutes, the excess

iodine is removed with solution 4 and then 1 cc. of solution 2 is added. The development of a pink color is considered a positive test for hydroxylamine. About twenty minutes is usually required for the development of the maximum coloration.

The following organisms were studied: *A. aerogenes*, *Esch. coli*, *Staph. aureus*, *B. subtilis*, *B. ramosus*, *B. vulgatus*, *B. anthracoides*, *B. cereus*, *B. mycoides*, and *B. megatherium*. In all cases a sterile solution of sodium nitrate was added to actively growing broth cultures. At frequent intervals a nitrite test was made. When the last trace of nitrites disappeared, a hydroxylamine test was made. Frequently, in the cases where hydroxylamine was detected, a positive test could be obtained only during a period of a very few minutes after the nitrites disappeared. The time required for the reduction of the nitrates and nitrites varied with the amounts present and with the activity of the culture. Repeated trials with *Esch. coli* and *A. aerogenes* consistently gave positive tests for hydroxylamine when the tests were made immediately after the disappearance of the nitrites. The results were the same whether nitrates or nitrites were used. All of the other organisms which reduced nitrates and nitrites did so much more slowly than did *Esch. coli* or *A. aerogenes*. Positive results were obtained with cultures of *Staph. aureus*, *B. subtilis*, *B. vulgatus*, *B. ramosus*, and *B. anthracoides*, but not with perfect regularity. *B. cereus* and *B. mycoides* showed extreme weakness in their ability to reduce either nitrates or nitrites and no positive test for hydroxylamine was obtained. *B. megatherium* alone showed no ability to reduce nitrates or nitrites and always gave a negative test for hydroxylamine.

When hydroxylamine was added to the cultures in the form of the hydrochloride, it always disappeared very rapidly. In view of this fact, it seems probable that the cultures which reduce nitrites very slowly produce hydroxylamine at such a slow rate that it never accumulates in sufficient quantities to be demonstrated by the test used.

It is usually assumed that whenever the nitrite radical is reduced by bacteria, ammonia is formed. In a synthetic medium with sodium nitrate as the only source of nitrogen a luxuriant

growth of *A. aerogenes* was obtained in twelve hours, yet the culture was ammonia-free, as judged by the Nessler test, until the fourth day. Since sodium nitrate was the only source of nitrogen, it naturally follows that the ammonia was produced by the reduction of the nitrates. Nevertheless, the intermediary stages in the process are not established. It is possible that during the first three days ammonia was formed by the reduction of nitrates through the intermediary steps of nitrites and hydroxylamine, but that it was utilized by the bacteria as rapidly as it was formed. On the other hand, it is also possible that no ammonia was formed during the first three days; and that the ammonia which appeared in the old cultures was a result of the degradation of the bacterial protoplasm.

CONCLUSIONS

Hydroxylamine has been demonstrated as a reduction product of nitrates and nitrites in broth cultures of *A. aerogenes*, *Esch. coli*, *Staph. aureus*, *B. subtilis*, *B. vulgatus*, *B. ramosus*, and *B. anthracoides*. The fact that these organisms represent such a diversity of types strongly suggests that the production of hydroxylamine is characteristic of the bacterial reduction of nitrites.

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Toxic Liver Injury

THE METABOLISM OF DIMETHYLNITROSAMINE

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In a previous paper (Barnes & Magee, 1954) dimethylnitrosamine was shown to cause acute centrilobular necrosis of the liver in the rat, mouse, guinea pig, rabbit and dog, when given in doses of the order of 25 mg./kg. body weight. No gross abnormality was found in organs or tissues other than the liver. In long-term feeding experiments, when dimethylnitrosamine was added to the diet of rats at a level of 50 parts per million, a high incidence of malignant hepatic tumours occurred, with no evidence of primary tumours elsewhere. In similar experiments with rabbits no tumours were produced (Magee & Barnes, 1956).

It appears that dimethylnitrosamine is a highly specific liver poison, and the present paper describes experiments which were performed in the hope of throwing some light on the mechanism of this specificity. A polarographic method for the estimation of dimethylnitrosamine in tissues and body fluids has been developed in this Laboratory (Heath & Jarvis, 1955) and this method was used to study the distribution of the compound in the animal body after administration, and its excretion.

METHODS

The animals used were albino rats of the Porton strain, mice of the C. 57 Chester Beatty strain and commercially obtained albino mice, and cross-bred rabbits. The rats and mice were maintained on M.R.C. diet 41 (Bruce & Parkes, 1949) and the rabbits on M.R.C. diet 18 (Bruce, 1947). Dimethylnitrosamine, when given orally, was diluted with water and administered by a metal cannula attached to a syringe. For parenteral administration dilution was with 0·9% NaCl. Intravenous injection was by tail vein in rat and by ear vein in rabbits, both procedures being without anaesthesia, unless otherwise stated. The compound was estimated in tissues and body fluids by the polarographic method of Heath & Jarvis (1955). In determinations of the total body content of dimethylnitrosamine in rats the animals were killed by intraperitoneal injection of Na pentobarbital (Veterinary Nembutal, Abbott) and immediately frozen by immersion in liquid N_2. The rats were then transferred to a bucket and fragmented by percussion with a heavy brass ram-rod. The fragments were disintegrated further in 2·5% (w/v) sulphosalicylic acid by means of an Atomix blender (Measuring and Scientific Equipment Ltd., London, S.W. 1), and the resulting tissue suspension was made up to a known volume and centrifuged. Dimethylnitrosamine was estimated in a suitable portion of the supernatant fluid. A similar procedure was used for whole mice except that they were killed by cervical dislocation, and it was found possible to disintegrate them in the blender without preliminary freezing. In experiments on the excretion of dimethylnitrosamine, rats were placed in conventional metabolism cages constructed of metal coated with bakelite. Urine and faeces were collected separately by means of a glass separator. Total hepatectomy was performed on rats by Dr K. K. Cheng, using a two-stage technique (Cheng, 1951). The second stage of the operation on the previously prepared animals was carried out under ether anaesthesia. Control animals of similar weight to that of the hepatectomized rats underwent a sham operation, in which the abdomen was opened and closed. Dimethylnitrosamine was injected intravenously into the hepatectomized and the sham-operated control rats after operation, while they were still under anaesthesia. Total nephrectomy on rats was performed by Dr J. M. Barnes, and again control animals underwent a sham operation, both

procedures being carried out under ether anaesthesia. Blood samples were obtained from an ear vein in rabbits without anaesthesia and from the inferior vena cava, by means of a syringe, in rats immediately after they had been killed with coal gas. Heparin was always used as anticoagulant. Plasma was separated from red cells by centrifuging, and dimethylnitrosamine was determined in known volumes of plasma, red cells and whole blood respectively. Dialysis of 3 ml. samples of rat plasma was carried out in cellophan sacs against 5 l. of 0·9% NaCl, for 22 hr. at 2°, with mechanical stirring.

RESULTS

Metabolism of dimethylnitrosamine

Recovery of dimethylnitrosamine from the whole animal. Twelve mice weighing 20 g. were given 1 mg. of dimethylnitrosamine (purified by distillation, b.p. 151°) orally. Three were killed immediately and analysed for dimethylnitrosamine. The remaining nine animals were placed in glass beakers so that all excreta could be collected and were killed in groups of 3 at 0·5, 1 and 4 hr. after injection. Immediately after death each animal, together with any excreta produced during the experimental period, was analysed. As shown in Table 1, good recovery of the total dose was obtained at zero time, and the amounts of dimethylnitrosamine recovered fell off quite uniformly with time, until at 4 hr. none was recovered.

In a second experiment, 4 rats were given orally 50 mg. of dimethylnitrosamine/kg. body wt. One was killed immediately by intraperitoneal Nembutal and the remainder at 4, 8 and 24 hr. after injection. Again good recovery was obtained at zero time, the amount of the compound recovered fell off with time, and none was found at 24 hr. (Table 1).

Excretion of dimethylnitrosamine. Two rats were each given 10 mg. of dimethylnitrosamine orally (50 mg./kg. body wt.) and were placed in the same metabolism cage. Urine and faeces were collected at known times after injection and analysed for dimethylnitrosamine. In a similar experiment two pairs of rats were given 100 mg. of dimethylnitrosamine (500 mg./kg. body wt.) intravenously, and the combined urine from each pair was analysed after 24 hr. The results are shown in Table 2.

These results suggested either that the compound was metabolized by the animal or that it became combined with a tissue constituent in such a way that it could no longer be extracted with sulphosalicylic acid. The latter possibility was not easily

Table 1. *Recovery of dimethylnitrosamine from the whole animal*

Rats and mice received 50 mg. of dimethylnitrosamine/kg. body wt. orally. In all cases, except the rat killed 24 hr. after administration, excreta produced during the experimental period were included with the whole animal for analysis.

Mice				Rats		
No. of animals	Time after injection (min.)	Dimethylnitrosamine recovered, as percentage of injected dose: Mean	Range	No. of animals	Time after injection (hr.)	Dimethylnitrosamine recovered, as percentage of injected dose
3	0	97·1	96·0–99·5	1	0	94
3	30	65·4	57·5–70·0	1	4	57
3	60	33·6	31·2–36·0	1	8	34
3	240	0	—	1	24	0

Table 2. *Excretion of dimethylnitrosamine*

In Expt. *A*, one pair of rats received 50 mg. of dimethylnitrosamine/kg. body wt. orally, providing a total of 20 mg. for excretion. In Expt. *B*, two pairs of rats received 500 mg. of dimethylnitrosamine/kg. body wt. intravenously, providing 200 mg. for excretion from each pair.

Expt. *A*			Expt. *B*		
Time after injection (hr.)	Dimethylnitrosamine recovered (μg.): Urine	Faeces	Time after injection (hr.)	Dimethylnitrosamine recovered (from urine only): First pair (mg.)	Second pair (mg.)
3·5	96	No sample	24	9·45	21·87
4·5	30	No sample	—	—	—
23	208	Nil	—	—	—
24	Nil	Nil	—	—	—
Total	334	0	—	—	—
Percentage of total injected dose	1·7	0	Percentage of total injected dose	4·7	11

Table 3. *Recovery of dimethylnitrosamine from rats which had undergone various experimental procedures*

All animals received 50 mg. of dimethylnitrosamine/kg. body wt. intravenously.

Experimental procedure	Time after injection of dimethylnitrosamine (hr.)	Percentage of injected dose recovered from whole animal	
		Experimental	Control
Total hepatectomy. Controls sham-operated. Dimethylnitrosamine injected at operation	4	99	75
	6	98	73
Total nephrectomy 24 hr. before injection of dimethylnitrosamine. Control sham-operated	4	71	62
Total nephrectomy 7 hr. before injection of dimethylnitrosamine. Control sham-operated	19	4	2
Dimethylnitrosamine 50 mg./kg. body wt. orally 24 hr. before injection of a second dose intravenously. Two normal rats as controls	6	74	35 25

investigated, since procedures adequate to degrade the main tissue components would also cause chemical breakdown of dimethylnitrosamine. Alkaline hydrolysis of the acid-extracted tissue residue was the method of choice, since dimethylnitrosamine is more resistant to alkali than to acid. Liver from a rat killed 4 hr. after an oral dose of 50 mg./kg. body wt. was extracted with sulphosalicylic acid in the usual way, and the residue was re-extracted until the washings no longer contained detectable dimethylnitrosamine. This residue was then suspended in 50 ml. of water, to which was added 50 g. of KOH, and the mixture was refluxed for 2·5 hr. The resulting digest was analysed. A similar experiment was done with two mice, which were killed 45 min. after receiving 50 mg. of dimethylnitrosamine/kg. body wt. by intraperitoneal injection. In this experiment the livers and also the rest of the bodies were acid-extracted separately and the residue was refluxed with KOH for 4 hr. Small quantities of dimethylnitrosamine were recovered from the acid extracts, but none from the alkaline digests.

In a control experiment duplicate samples containing 50 μg. of dimethylnitrosamine were refluxed with KOH of the same concentration for 4 hr., after which 39 and 38 μg. respectively were recovered. Since as little as 0·25 μg. of dimethylnitrosamine/ml. of sample can be detected by the polarographic method, under the conditions of the experiment not more than 10 μg. could have been present in a combination resistant to acid extraction. These results suggested that the greater part, if not all, of the injected dose was metabolized, apart from the small amount excreted in the urine.

Experiments to investigate the site of metabolism of dimethylnitrosamine. Two rats which had undergone total hepatectomy were given 50 mg. of dimethylnitrosamine/kg. body wt. intravenously, and two other animals received the same dose after sham operations. The hepatectomized rats were killed at 4 and 6 hr. respectively after injection of dimethylnitrosamine, and the controls were killed at the same times. The total body content of the compound in the four animals was determined. As shown in Table 3 the amount of the compound recovered from the hepatectomized animals suggested that none had been metabolized, as it was well within the range of error of the method. The control animals, on the other hand, showed substantial loss in spite of the immediately preceding anaesthetic and abdominal incision. The effect of previous damage to the liver was investigated by giving a rat 50 mg. of dimethylnitrosamine/kg. body wt. orally, a dose sufficient to produce extensive necrosis (Barnes & Magee, 1954), and then, 24 hr. later, 50 mg./kg. intravenously. Two control animals were given the same dose intravenously, and all three were killed 6 hr. later. The percentage recoveries shown in Table 3 indicate that previous damage to the liver causes considerable reduction in the rate at which dimethylnitrosamine disappears from the body. The effect of total nephrectomy was then tried. One

Table 4. *Effect of SKF 525A on the metabolism of dimethylnitrosamine by the mouse*

The animals received an oral dose of SKF 525A (mg./kg. body wt.) as indicated, followed by intraperitoneal injection of 50 mg. of dimethylnitrosamine/kg. body wt. 45 min. later, and were killed after a further 90 min.

Dose of SKF 525A	Recovery of dimethylnitrosamine from whole mouse (% of injected dose)
100	19
100	17
—	9
—	11
200	48
—	0
400	58
—	0

nephrectomized and one control animal were given 50 mg. of dimethylnitrosamine/kg. body wt. intravenously 7 hr. after the operation and killed 19 hr. after the injection. The other nephrectomized animal and its control received the same dose 24 hr. after operation, and were killed 4 hr. later. It will be seen from Table 3 that total nephrectomy reduces the rate of disappearance of dimethylnitrosamine only slightly, and that virtually none was recovered 24 hr. after administration. These experiments strongly suggested that the liver is the most important, perhaps the only, site of metabolism of dimethylnitrosamine.

Effect of 2-diethylaminoethyl diphenylpropylacetate hydrochloride (SKF 525A) on the metabolism of dimethylnitrosamine by the whole animal. Two mice were given 100 mg. of SKF 525A/kg. body wt. in water orally and 40 min. later 50 mg. of dimethylnitrosamine/kg. body wt. intraperitoneally. Two control mice received the same treatment except that water replaced the SKF 525A. In another experiment two mice received 200 and 400 mg. of SKF 525A/kg. body wt. respectively, followed by the same dose of dimethylnitrosamine as before. The amounts recovered from the whole mice are shown in Table 4.

Distribution of dimethylnitrosamine in the body

Recovery of dimethylnitrosamine from tissues and organs. Four rats were given 50 mg. of dimethylnitrosamine/kg. body wt. and were killed by intraperitoneal Nembutal 0·5, 1, 2 and 4 hr. respectively after injection. Various organs, as indicated in Table 5, were removed and analysed individually. The remainder of the carcass was also analysed. From the results shown in Table 5 it was concluded that the compound was distributed throughout the organs and tissues of the body. In another experiment, two rats were given 50 mg./kg. body wt. and the concentration in some organs was compared with that in the blood 4 hr. after injection. The results are also shown in Table 5, where it can be seen that the concentrations are again very similar, including that in the blood. This experiment indicated that the dimethylnitrosamine found in the various organs could not have been due merely to their content of blood.

The distribution between plasma and red cells was studied in 4 rats and 1 rabbit, all of which received 500 mg. of dimethylnitrosamine/kg. body wt. intravenously. Blood was taken from the rats at 2 hr. and from the rabbit at 30 min. and 2 hr. after injection. The concentrations of dimethylnitrosamine in red cells, plasma and whole blood were about equal.

Recovery of dimethylnitrosamine from the circulating blood. A rabbit weighing 1·6 kg. was given 80 mg. of dimethylnitrosamine by intravenous injection. Blood for analysis was withdrawn at intervals from an ear vein. Fig. 1 shows log (concentration of dimethylnitrosamine) plotted against time. There is a rapid initial reduction in concentra-

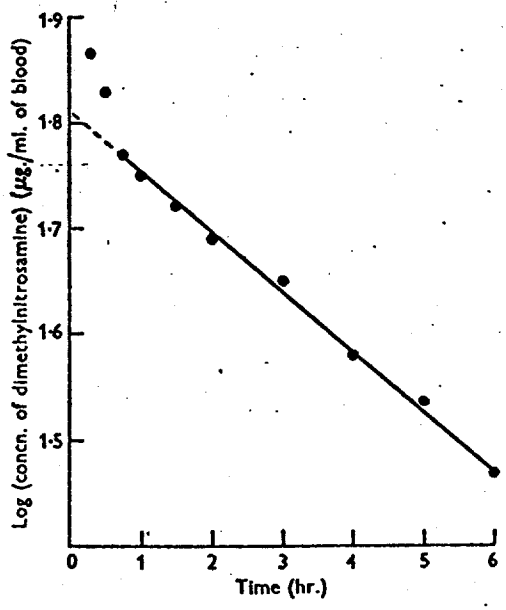

Fig. 1. Fall in concentration of dimethylnitrosamine in the circulating blood of a rabbit weighing 1·6 kg. after the intravenous injection of 80 mg. of the compound.

Table 5. *Distribution of dimethylnitrosamine in tissues and organs*

Rats were given 50 mg. of dimethylnitrosamine/kg. body wt. intravenously.

Expt.	No. of animals	Time after injection (hr.)	Concn. of dimethylnitrosamine in tissues (μg./g. wet wt.)							
			Blood	Liver	Kidney	Spleen	Brain	Heart and lungs	Intestinal tract	Carcass
1	1	0·5	—	78	82	62	39	74	—	62
	1	1	—	35	37	33	32	38	28	28
	1	2	—	33	39	31	34	33	14	22
	1	4	—	35	39	33	40	37	—	25
2	2	4	23	25	28	20	—	26	—	—
			20	20	26	25	—	27	—	—

tion followed by a more gradual and linear fall. On extrapolation to zero time this line cuts the ordinate at a point corresponding to 64 μg. of dimethylnitrosamine/ml. of blood.

Possibility of binding of dimethylnitrosamine to plasma protein. Rat plasma (3 ml.) was dialysed for 22 hr. at 2° against 5 l. of 0·9 % NaCl to which dimethylnitrosamine had been added to make a concentration of 40 μg./ml. Analysis showed that the final concentrations of dimethylnitrosamine in the fluid in the sac and in the surrounding fluid were 41 and 43 μg./ml. respectively. In another experiment, blood was taken from a rat which had been given 500 mg. of dimethylnitrosamine/kg. body wt. intravenously 2 hr. previously. The plasma was separated and dialysed against 0·9 % NaCl under the same conditions as before. The initial concentration of dimethylnitrosamine found inside the sac was 672 μg./ml. and the final concentration was zero. It was concluded that dimethylnitrosamine is not bound to plasma protein.

DISCUSSION

The results of the experiments on the recovery and excretion of dimethylnitrosamine from the whole animal strongly suggest that the compound is rapidly metabolized. The assumption that satisfactory recovery of dimethylnitrosamine from the tissues can be obtained by extraction with sulphosalicylic acid is based on the work of Heath & Jarvis (1955), who studied the addition of the compound to tissue minces *in vitro* and also recovery from the whole animal immediately after injection. A possible explanation for the progressive decrease in the amount recovered from the whole animal is that it becomes bound to a tissue constituent in such a way as to be no longer extractable with the acid. When mouse liver and remaining carcass were refluxed with strong alkali after thorough extraction with sulphosalicylic acid, no further recovery was obtained. The conditions used would cause saponification of the fat and considerable, if not complete, hydrolysis of the protein, as well as some destruction of the dimethylnitrosamine itself. In the nature of the experiment, conclusions can only be drawn within the limits of sensitivity of the method, and it may be said that probably not more than 1 % of the injected dose can have been bound to tissue constituents. This, of course, would not apply to a form of combination resistant to the alkali treatment. Probably some dimethylnitrosamine is lost through the lungs, but it seems unlikely that the proportion of the total dose excreted in this way can be very large. This assumption is supported by the very high recovery obtained from the rats whose livers had been removed (Table 3).

Dutton & Heath (1956), in this Laboratory, have treated rats and mice with dimethylnitrosamine labelled with ^{14}C and have shown that a large fraction of the radioactivity can be recovered as expired carbon dioxide. This provides conclusive evidence that the compound is, in fact, metabolized.

The almost complete recovery of the injected dose from the totally hepatectomized rats suggests a dominant, if not an exclusive, role for the liver in the metabolism of the compound. Such an experiment is, of course, impossible to control adequately, and it might be that removal of the whole liver could lead to such general depression of the animal that extrahepatic metabolism of dimethylnitrosamine would be reduced to undetectable levels. More dimethylnitrosamine was recovered from the controls to the hepatectomized rats than from normal animals. This slower rate of disappearance of the compound is probably related to the immediately preceding general anaesthetic and abdominal incision. Birnie & Grayson (1952) have shown that general anaesthesia causes reduction in liver temperature and liver blood flow in the rat, and that several hours elapse before normal conditions return. Dr H. B. Stoner (unpublished work) has shown that abdominal incision exacerbates the changes due to anaesthesia. The finding of marked reduction in the rate of disappearance of the poison from rats with previously damaged livers, coupled with the almost negligible effect of removal of the kidneys, is consistent with the idea that the liver is mainly responsible for its metabolic alteration. Support for this conclusion is provided by the work of Dr M. Vandekar (unpublished work) in this Laboratory. He has found that dimethylnitrosamine disappears after addition to rat-liver slices respiring *in vitro*, but not after addition to kidney, spleen or brain slices.

The experiments in which dimethylnitrosamine was measured in different organs and tissues do not indicate any concentration of the compound in the liver which could be held responsible for its toxic action (Table 5). In fact the amounts per unit weight of tissue are remarkably similar in most of the viscera. The lower figures found in the gastrointestinal tract can probably be explained by the effect of the intestinal contents, which might add to the weight of the sample without increasing the amount of dimethylnitrosamine proportionately. This view is supported by the absence of the compound in the faeces. Similar reasoning could be applied to the effect of bone and hair in the carcass. The concentration in the brain is lower than in the other viscera at first, but later reaches the general level (Table 5); this is probably due to the 'blood brain barrier'. The concentration in the blood was found to be closely similar to that in several of the organs (Table 5) and it is therefore apparent that the blood in a tissue could not account for the amount of the compound found in it. Further conclusions on the distribution in the body can be drawn from the

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curve of disappearance of dimethylnitrosamine from the circulating blood of the rabbit (Fig. 1). The initial rapid fall presumably represents equilibration of the compound in the body as well as loss by metabolism, and the second linear phase further metabolic loss. Both phases, of course, must include the small losses due to excretion. Extrapolation of the second part of the curve to zero time gives a figure of 64 μg. of dimethylnitrosamine/ml. of blood. Since the concentrations in blood and in plasma are about equal, this indicates a concentration of 69 μg./ml. of plasma water, if a water content in plasma of 93 % is assumed (Soberman, 1950). This figure may be regarded as the initial concentration in plasma water if equilibration had been instantaneous, and it may be compared with the concentration that would be expected if the compound was uniformly distributed in the total body water. If a value for total water in the rabbit of 74 % of the body wt. (Soberman, 1950) is taken and again immediate equilibration is assumed, the concentration would be 67 μg./ml. after injection of 80 mg. of dimethylnitrosamine into the rabbit weighing 1·6 kg. This is in excellent agreement with the value found by extrapolation. Similar curves have been reported by Soberman (1950) after the intravenous injection of antipyrine into rabbits. This compound was shown to be rapidly distributed throughout the total body water and, like dimethylnitrosamine, it is metabolized by the animal. It appears, therefore, that dimethylnitrosamine is uniformly distributed throughout the body water and consequently that it must penetrate into the tissue cells. If this is so some explanation is required for the occurrence of selective damage to the liver, since the compound must be relatively innocuous to the other cells. A possible explanation is that a metabolite of dimethylnitrosamine, formed only inside the liver cell, is the toxic substance. This would be present in the liver in higher concentration than elsewhere. Such a metabolic process has been proposed by Miller & Miller (1953) for *p*-dimethylaminoazobenzene in liver carcinogenesis, and also by Nygaard, Eldjarn & Nakken (1954) to explain the specificity of action of thioacetamide on the liver. In a previous paper (Magee & Barnes, 1956) attention has been drawn to similarities in the pathological effects due to *p*-dimethylaminoazobenzene, thioacetamide and dimethylnitrosamine, both in acute and chronic experiments. It is well known that many substances foreign to the body are metabolized only by the liver, and much recent work by Brodie *et al.* (1955) has shown that many of these metabolic transformations, including several demethylation reactions, are inhibited by the compound SKF 525A (2-diethylaminoethyl diphenylpropylacetate hydrochloride) (Cook, Toner & Fellows, 1954). The finding that this compound inhibits the rate of disappearance of dimethylnitrosamine may be significant (Table 4), but the dose required was high and it may be an unspecific effect.

In recent years doubt has been cast on the suitability of the term 'detoxication' when applied to the metabolism of foreign substances. Thus Peters (1952) has developed the idea of a 'lethal synthesis' in which an innocuous substance may be transformed into a toxic one, rather than the reverse. For example, it has been shown that the liver can convert schradan (OMPA) and parathion from weak into strong inhibitors of cholinesterase (Myers, Mendel, Gersmann & Ketelaar, 1952; Dubois, Doull & Coon, 1950; Cheng, 1951). Similar ideas have been advanced by Popper, de la Huerga & Koch-Weser (1954), who suggested that the mechanism of the liver necrosis due to bromobenzene may be a 'conditioned amino acid deficiency' in which the liver is depleted of sulphur-containing amino acids by the excretion of mercapturic acid, and that 'the process of detoxication as such may become detrimental to the liver'.

The goal of any investigation into pathological processes should be the elucidation of the 'biochemical lesion', as defined by Peters (1936). It seems likely that the train of chemical events leading to the death of the liver cell after administration of dimethylnitrosamine is related, in time at least, to the mechanism of metabolism of the compound. Since metabolism of the compound probably starts immediately after injection (Table 2), the biochemical lesion may well occur equally early.

SUMMARY

1. The metabolism, distribution in the body and excretion of dimethylnitrosamine were studied after its administration to rats, mice and rabbits. It has been shown previously that this compound is a selective liver poison in these species, and that it can produce malignant primary liver tumours in the rat.

2. Evidence is presented which suggests that dimethylnitrosamine is rapidly metabolized, and that the liver is the main, probably the only, organ concerned.

3. The compound is distributed uniformly in most tissues of the body, and there is reason to believe that it penetrates into the intracellular space. There is no selective concentration in the liver.

4. SKF 525A (2-diethylaminoethyl diphenylpropylacetate hydrochloride) inhibits the metabolism of dimethylnitrosamine in the mouse, but only when given in large doses.

5. The findings are discussed in relation to the pathogenesis of the acute liver necrosis caused by dimethylnitrosamine.

I would like to thank Professor G. R. Cameron, F.R.S., for very valuable discussion, Dr J. M. Barnes and Dr K. K. Cheng for performing the surgical procedures, and Mr J. A. E. Jarvis for invaluable technical assistance. The compound SKF 525A was a gift from Dr K. Carter, of Smith, Klyne and French Ltd.

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The Activity for Chicks of some Vitamin B_{12}-like Compounds

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In the last few years several compounds similar to the cobalamins have been found in nature; in addition to these, new analogues of vitamin B_{12} have been produced by guiding the biosynthetic activities of certain micro-organisms. The preparation, chemical nature and properties of these substances have been reviewed by Kon (1955); they differ from the cobalamins in the nature of the nucleotide part of the molecule. Since many of them have growth-promoting activity for micro-organisms, it was of interest to determine whether or not they were of use to higher animals. The comparatively large amount of material necessary for an animal test limited the number of samples examined to those which could be relatively easily prepared, but we have been able to test on chicks five of the benziminazole series, three purine derivatives, factor B (which has no nucleotide) and factor D (of which the chemical constitution is unknown).

EXPERIMENTAL

Substances tested

Naturally occurring vitamin B_{12}-like compounds. Factors A, B, D and pseudovitamin B_{12} were prepared from calf faeces. Vitamin $B_{12\,III}$, later identified as the 5-hydroxybenziminazole analogue of vitamin B_{12} (Robinson, Miller, McPherson & Folkers, 1955; Friedrich & Bernhauer, 1956), was isolated from sewage by Friedrich & Bernhauer (1953).

Unnatural analogues of vitamin B_{12}. These were prepared by 'shunting' the biosynthesis of vitamin B_{12} by the addition to the medium of the appropriate intermediate (Fantes & O'Callaghan, 1954; Ford, Holdsworth & Kon, 1954). The samples tested were the analogues containing benziminazole, 5 (or 6)-methylbenziminazole, 5:6-dichlorobenziminazole, 2:3-naphthiminazole or 2:6-diaminopurine in the nucleotide.

Preparation for test. All these substances, with the exception of factor D, were purified by repeated ionophoresis and chromatography and contained less than 0·5% of vitamin B_{12}. Factor D and some early samples of factor A were purified by chromatography only. Solutions of the analogues were prepared and standardized by measuring the absorption at 361 mμ.; the $E^{1\%}_{1\,cm.}$ value was assumed to be the same as for cyanocobalamin.

Biological tests

Method and diet. In general, the method of Coates, Harrison & Kon (1951) was followed. Rhode Island Red × Light Sussex chicks were used, bred from hens given a diet low in vitamin B_{12}. Each experimental group consisted of about 10–15 chicks of mixed sexes. The test materials were given either by intramuscular injection twice weekly or were added to the basal diet, which had the following percentage composition: ground maize, 37·8; ground barley, 20; defatted soya grits, 35; dried grass, 3; bone meal, 1·5; limestone flour, 1; arachis oil, 1; salt mixture, 0·7 (NaCl, 0·672 part; $MnSO_4,4H_2O$, 0·028 part). To each 100 g. of diet were added vitamin A (680 i.u.) and vitamin D_3 (64 i.u.) dissolved in the arachis oil, and a vitamin supplement consisting of riboflavin (0·77 mg.), nicotinic acid (5·5 mg.), biotin (0·02 mg.), folic acid (0·08 mg.), thiamine hydrochloride (0·33 mg.), pyridoxine hydrochloride (0·44 mg.) and calcium pantothenate (1·65 mg.).

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The Metabolism of Carcinogenic Nitrosamines and the Metabolic Fate of their Alkyl Groups

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Many alkyl *N*-nitroso compounds are powerfully carcinogenic and can cause acute cellular injury to the liver and other organs. Some of these compounds are also strongly mutagenic and teratogenic [1-3]. The dialkylnitrosamines, as exemplified by dimethylnitrosamine (*N*-nitrosodimethylamine) are relatively stable chemically, while the nitrosamides, for example, *N*-nitrosomethylurea, may be chemically unstable and decompose quite rapidly at neutral pH. The balance of the available evidence suggests that the nitrosamines only become biologically active after metabolic decomposition in the cells of affected organs, while the nitrosamides probably decompose in the tissues without metabolic activation, although, in some cases, their decomposition may be accelerated by sulphydryl compounds [1, 2]. Both types of nitroso compound have been shown to alkylate cellular constituents of the tissues of treated animals [2] and it has been concluded that an active alkylating compound must be formed by the decomposition of the nitroso compounds in the body. The nature of the alkylating intermediate is not known and various suggestions have been made including the corresponding diazoalkane and the carbonium ion. Recent work by Lijinsky *et al.*, using fully deuterated dimethylnitrosamine, has shown that the alkyl group reacts as $-CH_3$, which suggests that the diazoalkane is not involved [4]. The formation of an active methylating agent from dimethylnitrosamine *in vivo* was inferred from the evidence of methylation of RNA and DNA in the livers of rats treated with dimethylnitrosamine [5]. As with alkylation of nucleic acids *in vitro* by chemical alkylating agents, the main site of alkylation was on the 7-position of guanine [5], but recent work has shown that reaction may also occur on adenine and cytosine [6]. The biological significance of the cellular alkylations following exposure of animals to carcinogenic nitroso compounds is not clear, nor is the role of possible alkylations in mutagenesis and teratogenesis by these compounds. The formation of other biologically active metabolites from nitroso compounds cannot be excluded and a number of potent mutagens

might be formed, including nitrous acid and derivatives of hydroxylamine and hydrazine.

Each nitroso carcinogen tends to show a characteristic distribution of tumour induction in the different organs [3]. The factors determining the tumour distribution are not well understood but there is some correlation between the extent of reaction with cellular components as measured by alkylation of nucleic acids in the different organs and the incidence of tumours. This is evident with dimethylnitrosamine in the rat [7, 8], where methylation of DNA and RNA occurs mainly in the liver and to a lesser extent in the kidney and lungs, which are organs in which tumours are induced in this species. Methylation is extremely low in the small intestine and dimethylnitrosamine has never been reported to induce tumours of this organ in the rat. Metabolism of dimethylnitrosamine occurs relatively rapidly with rat liver slices *in vitro* [9] and at a lower rate with kidney slices. Rat small intestine slices with oxygen uptake about equal to that of liver slices showed no detectable metabolism of dimethylnitrosamine as measured by production of radioactive CO_2 from [^{14}C] dimethylnitrosamine (P. N. Magee, unpublished results). These findings suggest that organ specificity of the nitrosamines may be determined to some extent by the level of activity of the enzymes involved in their decomposition.

Following the administration of dimethylnitrosamine to rats, the level of methylation of the DNA and RNA, as measured by formation of 7-methylguanine in the liver and kidney rises to a maximum during the first 24 hours and then falls [10]. The presence of 7-methylguanine with the methyl group derived from dimethylnitrosamine was still detectable 12 days after treatment with the carcinogen but could not be detected at 61 days (J. Frei and P. N. Magee, unpublished results).

Radioactive 7-methylguanine is present in the urine of rats 5 hours after administration of ^{14}C- or ^{3}H-labelled dimethylnitrosamine (30 mg per kg body wt.) and, following this dose, the excretion of 7-methylguanine is raised above the normal urinary level by a factor of about 2 to 3 [11].

Treatment of rats with diethylnitrosamine is followed by ethylation of liver RNA with the formation of 7-ethylguanine [12]. Subsequent work has shown that DNA is also ethylated and that 7-ethylguanine, with the ethyl group derived from the diethylnitrosamine, is excreted in the urine (P. N. Magee, unpublished results). There is some evidence that some of the labelled 7-methylguanine appearing in the urine after treatment with [^{14}C] dimethylnitrosamine may have been derived from methylated nucleic acids [11].

The function of nucleic acids might be affected in at least two ways in organs of animals treated with nitroso carcinogens. The presence of foreign or excessive alkyl groups on guanine, adenine and cytosine might lead to impairment of template function, and the possible excision of alkylated guanines, and perhaps

other bases, from
genetic material, as

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4. Lijinsky, W., L
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13. Brookes, P. and

other bases, from DNA might be followed by chain breakage and deletion of genetic material, as suggested by Brookes and Lawley [13].

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Toxicity of Nitrosamines: Their Possible Human Health Hazards*

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Introduction

Many *N*-nitroso compounds have powerful biological actions. These include the induction of acute and chronic toxic changes in animals—carcinogenicity, mutagenicity and embryopathic and teratogenic actions. Aspects of the biological actions of the *N*-nitroso compounds have recently been discussed (Magee, 1969) and their toxicity has been reviewed by Magee & Swann (1969). The carcinogenic action of the nitroso compounds has been studied very extensively by Druckrey and his colleagues who have presented a detailed review of their work up to 1967 (Druckrey, Preussmann, Ivankovic & Schmähl, 1967). Aspects of the carcinogenic action of the nitrosamines have also been reviewed by Magee & Schoental (1964) and Magee & Barnes (1967). The latter article contains a brief account of the mutagenic properties of some of the carcinogenic nitroso compounds.

Biological actions of some nitroso compounds

The *N*-nitroso compounds can be divided into two groups, one including the dialkyl, alkylaryl and diaryl nitrosamines and the other the alkyl and aryl nitrosamides. The biological actions of the two groups differ in several ways, probably because of their different chemical properties. The nitrosamines, as exemplified by dimethylnitrosamine, are chemically stable under physiological conditions, although they undergo photochemical decomposition in ultraviolet light. The nitrosamides, on the other hand, show varying degrees of instability depending on the pH of the medium. Many of these compounds yield diazoalkanes on treatment with alkali and they have been extensively used for this purpose in synthetic organic chemistry. Some nitrosamides decompose quite readily even at neutrality. The half-life of the potent carcinogen *N*-nitrosomethylurea, for example, is 125 hr at pH 4, 24 hr at pH 6·5, 1·2 hr at pH 7 and only 0·1 hr at pH 8 (Druckrey *et al.* 1967). The chemical structures of dimethylnitrosamine and *N*-nitrosomethylurea are shown in Fig. 1.

The nature of the toxicity of the nitroso compounds seems to be determined, at least in part, by these differences in chemical stability. The predominant effect of the dialkylnitrosamines is damage to the liver, the lesion produced being a rather characteristic haemorrhagic

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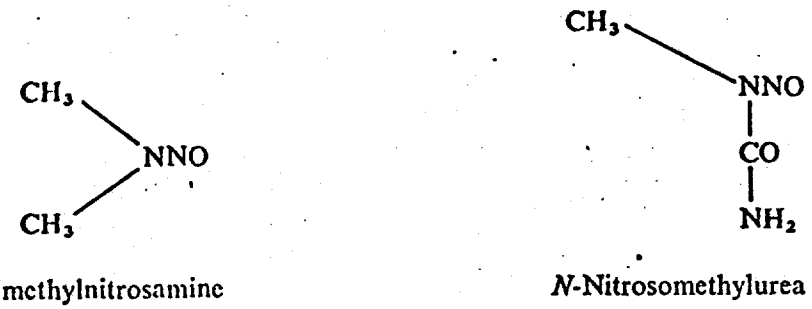

Dimethylnitrosamine *N*-Nitrosomethylurea

FIG. 1. Chemical structures of dimethylnitrosamine and *N*-nitrosomethylurea.

type of centrilobular necrosis. This specificity of action seems to be best explained by the assumption that these compounds require metabolism for their tissue-damaging action and that the active molecular species is a metabolite of the parent nitrosamine. This hypothesis would explain the relatively selective action on the liver since, generally speaking, the enzymes responsible for the metabolism of drugs and other foreign compounds are present in highest amount in the liver. The liver is not, however, the only organ capable of metabolizing the nitrosamines. Using the production of radioactive CO_2 from ^{14}C-labelled nitrosamine as an index of metabolism, Dr. R. Montesano, working at the Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, has recently shown that dimethylnitrosamine and diethylnitrosamine are metabolized *in vitro* to varying degrees by tissue slices from several organs other than the liver (R. Montesano, unpublished data 1970). Of the tissue slices from the rat, liver was the most active in metabolizing dimethylnitrosamine, kidney showed appreciable activity and lung and oesophagus were definitely active, while the activity of small intestine was extremely low. A similar general pattern was seen with the diethyl compound, but the rate of metabolism was lower in the liver slices and rather higher in the lung than with the dimethyl compound.

With hamster tissues the pattern of dimethylnitrosamine metabolism was similar to that found with rat tissues. The diethyl compound, however, showed a remarkable difference in behaviour, in that hamster-lung tissue had a greater capacity than liver for metabolizing the carcinogen, while the other organs were considerably less active. This contrasting metabolic capacity of hamster lung is consistent with the powerful carcinogenic action of diethylnitrosamine on the respiratory tract of the hamster and the lack of carcinogenicity of the dimethyl compound on the lung of this species (see later). It is possible that the greater cytopathological action of diethylnitrosamine, compared with dimethylnitrosamine, on the nasal epithelium of the Syrian hamster observed by Greenblatt & Rijhsinghani (1969) may be explained by a greater capacity of this tissue to metabolize the diethyl compound. In contrast to the nitrosamines, which do not appear to cause significant cellular injury at the site of application, several nitrosamides, chemically less stable, have marked local cytopathic actions, sometimes inducing very severe tissue necrosis. It is not known whether the rate of decomposition of the nitrosamides is dependent on the action of enzymes but, in some cases, it is catalysed by sulphydryl compounds (Schoental & Rive, 1965).

In addition to their local action, some of the nitrosamides may exert a so-called radiomimetic action, causing damage to organs with rapid cell turnover, such as the bone marrow, the lymphoid tissues and the crypts of the small intestine. There are striking parallels between the biological action of the *N*-nitroso compounds and the naturally occurring hepatotoxin and carcinogen, cycasin, which is found in cycad plants. Cycasin is

the glucoside of methylazoxymethanol. The biologically active component is methylazoxymethanol, and the glucose moiety must be split off before the compound can produce its pathological effects in the animal (see Spatz, 1969). It is therefore of interest that the ultrastructural changes induced in rat liver by methylazoxymethanol are reported to be indistinguishable from those of dimethylnitrosamine but clearly different from the changes produced by carbon tetrachloride or hydrazine (Ganote & Rosenthal, 1968). These findings provide additional evidence for the virtual biological equivalence of cycasin and dimethylnitrosamine and carry obvious implications for the possible additive effects of the natural and synthetic carcinogens. Apart from exceptional circumstances, however, such as occurred in the outbreak of acute liver poisoning in fur-bearing animals in Norway and elsewhere (see later), it is unlikely that nitrosamines could occur or be formed in sufficiently high concentration in the environment to reach acutely toxic levels. The possibility that potentially carcinogenic levels may occur, however, is far less unlikely and must be seriously considered.

The bulk of the published information on the carcinogenic *N*-nitroso compounds is available in the reviews quoted earlier (Magee & Schoental, 1964; Magee & Barnes, 1967; Druckrey *et al.* 1967). In what follows, certain selected newer data will be discussed. The topics to be considered will be some carcinogenic cyclic nitrosamines, the species susceptible to carcinogenesis by nitroso compounds, the possible effects of sex and hormones, transplacental carcinogenesis and possible synergistic effects of other agents.

Several cyclic nitrosamines, including *N*-nitrosopyrrolidine, *N*-nitrosopiperidine and *N*-nitrosomorpholine are carcinogenic, inducing tumours of the liver and other organs in the rat and mouse (Druckrey *et al.* 1967). Recently the induction of tumours with *N*-nitrosohexamethyleneimine, *N*-nitrosoheptamethyleneimine and *N*-nitrosooctamethyleneimine has been reported. A high incidence of tumours of the liver, tongue and oesophagus was induced in the rat by Goodall, Lijinsky & Tomatis (1968) who administered the hexamethylene compound continuously in the drinking water. Schmähl (1968), however, reported a much lower incidence of tumours, mainly in the liver, when the same material was given by repeated subcutaneous injection. The heptamethylene and octamethylene derivatives were given to rats in the drinking water by Lijinsky, Tomatis & Wenyon (1969) and produced many squamous tumours of the lungs and oesophagus. *N*-Nitrosoheptamethyleneimine was the more potent, producing lung and oesophageal tumours in as little as 7 and 16 wk respectively. These latter findings are interesting in view of the possible involvement of nitrosoamines in human lung cancer in cigarette smokers, a question which is briefly discussed later. Goodall, Lijinsky & Smillie (1968) have reported the oncogenicity of *p*-nitroso-*N*,*N*-dimethylaniline in rats. A low but significant incidence of tumours was obtained, notably in the oesophagus and stomach, and hyperplastic lesions were observed in the alimentary and urinary tracts. These findings are of particular interest since this appears to be the first occasion on which carcinogenesis by a *C*-nitroso compound has been reported.

Particularly from the work of Schmähl and his colleagues (see Schmähl & Osswald, 1967) it is now well established that a wide range of animal species is susceptible to the induction of liver tumours by diethylnitrosamine. These species include the rat, mouse, hamster, guinea-pig, rabbit, dog and monkey. The evidence of rapid induction of liver cancer in primates reported by Kelly, O'Gara, Adamson, Gadekar, Botkin, Reese & Kerber (1966), and the subsequent culture of a cell line derived from a monkey hepatocarcinoma (Dawe, Whang-Peng, Morgan, O'Gara & Kelly, 1968) are of particular interest. The presence of α-foetoprotein, an α-globulin which occurs in foetal serum but not in that of normal adults,

has been reported in the serum of 87% of a group of 23 monkeys with diethylnitrosamine-induced liver tumours but not in 19 without liver tumours (Hull, Carbone, Gitlin, O'Gara & Kelly, 1969). Since α-foetoprotein occurs in the serum of a high proportion of human subjects with liver cancer, the positive findings with nitrosamine-induced tumours in the monkey may have implications for the possible significance of environmental nitrosamines in the aetiology of human liver cancer (see later).

Additional reports of carcinogenesis by nitroso compounds in species other than rats and mice have appeared since the reviews mentioned above. In the guinea-pig, tumours of the liver and urinary bladder have been induced by di-*n*-butylnitrosamine (Ivankovic & Bücheler, 1968), of the stomach and pancreas by *N*-nitrosomethylurea and *N*-nitrosomethylurethane (Druckrey, Ivankovic, Bücheler, Preussmann & Thomas, 1968) and of the liver by dimethylnitrosamine (LePage & Christie, 1969a). Liver tumours have been induced by diethylnitrosamine in the pig (Schmähl, Osswald & Goerttler, 1969) and in the rabbit (LePage & Christie, 1969b), and in the rainbow trout by dimethylnitrosamine (Ashley & Halver, 1968). The wide range of species susceptible to carcinogenesis by nitroso carcinogens indicates that man is probably susceptible also. Proof of the sensitivity of man is lacking, however, and the only possible direct evidence, apart from deliberate exposure of human subjects, must be obtained from epidemiological studies. Some recent indirect evidence may be relevant, however. Dimethylnitrosamine, labelled with radioactive carbon, has been incubated with fresh human-liver slices and the rate of its metabolism measured by the production of radioactive CO_2. Although the rate of oxygen uptake of the human liver slices was lower than that with rat-liver slices, as previously reported by Booth, Boyland & Cooling (1967), the rate of $^{14}CO_2$ production from the [^{14}C]dimethylnitrosamine was nearly as high with the human as with the rat slices. Alkylation of the slice nucleic acids, as measured by the conversion of guanine to 7-methylguanine, was also nearly as great with the human as with the rat tissues (Montesano & Magee, 1970). From these results it was concluded that man is probably about as sensitive as the rat to the carcinogenic action of dimethylnitrosamine and, by extrapolation, perhaps to other nitroso carcinogens.

Earlier reports of nitrosamine carcinogenesis had not given any clear indication that sex was an important factor in the incidence of induced tumours. There was little information on hormonal effects until the work of Goodall and Lee, who showed that hypophysectomy in rats did not inhibit liver carcinogenesis by dimethylnitrosamine, in contrast to the well-known inhibitory action of this procedure on carcinogenesis by azo dyes, acetylaminofluorene and aflatoxin. Hypophysectomy also had no effect on the methylation of rat-liver nucleic acids by dimethylnitrosamine (Goodall, 1968; Lee & Goodall, 1968). Similarly, hormonal changes due to orchidectomy appeared to have no effect on the incidence of brain and peripheral-nerve tumours induced in rats by *N*-nitrosomethylurea (Jänisch, Warzok & Schreiber, 1969). There have, however, been some indications that hormonal effects may play a part in nitrosamine carcinogenesis in some experimental situations. Reuber & Lee (1968) studied the effect of age and sex on the hepatic lesions induced in Buffalo rats by diethylnitrosamine. They found that the incidence of liver tumours was greatest in animals in which treatment was started early in life (at 4 wk of age) and became progressively less as the age of the rats at the start of treatment increased. In the earlier age groups there was a higher incidence of liver carcinoma in females, but none of the females developed cirrhosis. A more clear-cut dependence on sex was observed by Vesselinovitch (1969) who treated 7-day-old C57BL x C3H/F hybrid mice with six injections of dimethylnitrosamine at 3-day intervals. He noted a positive dose–response relationship for the induction of liver and lung

tumours and found that the liver tumours were significantly more frequent in males than in females. The possibility of a hormonal influence is also suggested by the report of Druckrey & Ivankovic (1969) than 50 out of 124 rats treated during pregnancy with a single dose of *N*-nitrosoethylurea had malignant tumours of the ovaries, uterus or vagina, while treatment of non-pregnant rats with this carcinogen rarely caused tumours at these sites.

One of the most remarkable features of nitroso carcinogenesis is the capacity of the compounds to induce tumours in the progeny of animals treated during pregnancy. This transplacental carcinogenesis is particularly well illustrated by the work of Ivankovic & Druckrey (1968) on the induction of malignant tumours of the nervous system in the progeny of mothers treated with *N*-nitrosoethylurea on day 15 of pregnancy. In these experiments most of the known histological types of glial tumour were observed and it was possible to obtain a tumour incidence of 100% with the appropriate dose of carcinogen. An alarming feature of this work was the observation that some nervous-system tumours were seen in the offspring of mothers who had received doses of *N*-nitrosoethylurea which were well below the median lethal level and were not associated with obvious signs of toxicity. These observations have been confirmed in ten different strains of BD rats receiving single intravenous doses of 50 mg *N*-nitrosoethylurea/kg body weight on day 15 of pregnancy, when 162 of 179 offspring died of malignant tumours of the brain, trigeminal nerves, spinal cord or peripheral nervous system. Multiple tumours were frequent (Druckrey, Landshütz & Ivankovic, 1970). The histology and histopathogenesis of these nervous-system tumours have been studied in detail by Wechsler, Kleihues, Matsumoto, Zulch, Ivankovic, Preussmann & Druckrey (1969). The transplacental effects of the nitrosamines are less severe than those of the nitrosamides discussed above. Mohr and his colleagues have shown that diethylnitrosamine administered at the end of pregnancy can cause tumours in the progeny of mice and hamsters (see Mohr, 1968), and Napalkov & Alexandrov (1968) have reported transplacental carcinogenesis in rats with several nitroso compounds including methyl- and ethylnitrosoaniline. Thomas & Bollmann (1968), however, concluded that treatment of Wistar rats with diethylnitrosamine during pregnancy had little transplacental carcinogenic effect.

There have been several reports of synergistic and modifying effects on nitrosamine carcinogenesis and two recent examples involving respiratory and renal carcinogenesis are of particular interest. Montesano & Saffiotti (1968) have shown that systemic treatment of hamsters with a relatively low dose of diethylnitrosamine, which by itself would induce few, if any, tumours of the respiratory tract, can greatly increase the incidence of respiratory tumours induced by subsequent treatment with a polycyclic hydrocarbon which also, by itself, would only have very low activity. These authors have also shown that intratracheal instillation of inorganic particulate material such as ferric oxide particles, can exert a similar synergistic action on diethylnitrosamine carcinogenesis. The incidence of kidney tumours in the rat in response to a single dose of dimethylnitrosamine can be greatly increased by prior maintenance of the animals on a protein-deficient diet (Swann & McLean, 1968). These examples of modifying effects on nitrosamine carcinogenesis may have important implications for human populations exposed to low levels of nitrosamines in the environment.

The teratogenic and mutagenic effects of the nitroso compounds have been discussed previously (Magee & Barnes, 1967). As with the transplacental carcinogenic action, it appears that the nitrosamides are more effective teratogens than the nitrosamines, a finding which may be related to the relative chemical stability of the two types of compound and the

probable requirement for metabolic activation of the nitrosamines. Napalkov & Alexandrov (1968) failed to obtain teratogenic effects in the rat with dialkylnitrosamines, while Ehrentraut, Juhls, Kupfer, Kupfer, Zintzsch, Rommel, Wahmer, Schnurrbusch & Mockel (1969) succeeded in producing deformities in pig foetuses by injecting the pregnant mothers intravenously with the nitrosamide, *N*-nitrosoethylurea. The mutagenic actions of the nitrosamides in various micro-organisms are well known, and *N'*-nitro-*N*-nitroso-*N*-methylguanidine is among the most powerful of chemical mutagens. The question of possible hazards to man from mutagenic chemicals in the environment is under widespread discussion at the present time. Until recently tests for mutagenicity in the mammal have been few and have involved the use of very large numbers of animals. Since the introduction of the simplified dominant lethal tests (Bateman, 1966), however, it has been possible to screen many more compounds. This procedure has been applied by Epstein & Shafner (1968) to a number of carcinogens, food additives, pesticides and other chemicals. These authors found that dimethylnitrosamine and some other carcinogens were inactive in this test, a probable reflection of the failure of metabolism of the compound in the vicinity of the male germ cells. This failure to detect mutagenic activity by the dominant lethal test in compounds requiring metabolic activation has led to the development of the host-mediated assay for mutagenesis by Legator and his colleagues (see Gabridge & Legator, 1969). In this procedure, the mammal, during treatment with a potential chemical mutagen, is injected with an indicator micro-organism in which mutation frequencies can be measured. After sufficient time has elapsed, the micro-organisms are withdrawn from the animal and the induction of mutants is determined. Comparison of the mutagenic action of the test compound directly on the organism with that in the host-mediated assay indicates whether the host can detoxify the compound or whether mutagenic products can be formed as the result of host metabolism. The indicator organisms used currently by this group are histidine auxotrophs of *Salmonella typhimurium* and a heterokaryon of *Neurospora crassa*. A series of nitroso compounds was tested by this procedure, using Salmonella, and the active compounds included dimethylnitrosamine, streptozotocin, cycasin and *N'*-nitro-*N*-nitroso-*N*-methylguanidine (Legator, 1970). While these findings are of considerable interest, it is probably too early to assess their significance in relation to possible mutagenic hazards to man from nitroso compounds in the environment.

Possible occurrence of nitrosamines in food for human or animal consumption

Although research on nitrosamine toxicity and carcinogenesis was initiated by indications of human toxicity arising from the industrial use of dimethylnitrosamine (Barnes & Magee, 1954), it was not until considerably later that the possible occurrence of these compounds in situations other than the industrial environment was suspected. This possibility was emphasized rather dramatically in Norway by an outbreak of serious liver disease in sheep in 1960. At this time a number of sheep became seriously ill, sometimes fatally, and post-mortem examination revealed severe necrosis of the liver as the main pathological feature (Sakshaug, Sögnen, Hansen & Koppang, 1965). All the affected sheep were found to have been fed on a diet containing fish meal preserved with nitrite. This suggested that formation of nitrosamines might have occurred, since fish contain relatively large amounts of secondary and tertiary amines. The nature of the toxic principle in this meal was studied by Ender, Havre, Helgebostad, Koppang, Madsen & Ceh (1964), who showed that dimethylnitrosamine was present, sometimes at levels as high as 30–100 ppm. Pure dimethylnitrosamine

was shown to produce in sheep liver lesions closely similar to those observed in animals that had received the toxic fish meal. This clear indication that enough dimethylnitrosamine could be formed to kill the animals obviously raised the possibility that smaller amounts of this or other nitrosamines might occur in other foods, including those for human consumption, perhaps at concentrations not high enough to cause obvious toxic effects but sufficiently high to induce cancer if the consumption of such food were continued for a long enough period of time. The possible occurrence of nitrosamines in cigarette smoke was suggested by Druckrey & Preussmann (1962) since tobacco contains several secondary amines, including nornicotine and anabasine and may also contain nitrites. *N*-Nitrosoanabasine was subsequently shown to be carcinogenic by Boyland, Roe, Gorrod & Mitchley (1964).

A considerable amount of experimental work has been carried out to determine whether, in fact, any nitrosamines are present in food or tobacco smoke and, if present, in what concentration. These investigations have been hampered from the outset by the lack of sufficiently sensitive and specific analytical methods. A range of methods has been developed, including polarography, thin-layer chromatography, gas–liquid chromatography and mass spectometry, but it must probably be admitted that really adequate procedures are not yet available, although considerable progress has been made.

There have been several reports of the presence of nitrosamines in food and drink for human consumption but the methods of identification have not always been adequate and the results have sometimes been conflicting.

Ender & Čeh (1968a, b) have reported that nitrosamines are present in very small amounts in a variety of foodstuffs consumed by man and animals. The foods included smoked herring, kippers, smoked haddock (from Iceland), smoked sausage, bacon and smoked ham (also from Iceland). The concentrations varied within a range of about 0·5–9 μg/kg, with one sample of kipper containing 40 μg/kg and one of smoked haddock containing 15 μg/kg. In addition, a number of mushrooms were studied, most of which showed nitrosamine concentrations up to about 10 μg/kg. One sample of *Amanita muscaria* contained 30 μg/kg and a mixture of edible mushrooms 14 μg/kg. These figures refer to volatile nitrosamines without specific identification and the concentrations were determined by the hydrazine method of Ender which has been described (Ender & Čeh, 1968b). The presence of diethylnitrosamine in white flour has been claimed by Marquardt & Hedler (1966), denied by Thewlis (1967) and claimed again by Hedler & Marquardt (1968).

There has been much recent interest in the geographical pathology of cancer (see Doll, 1967 & 1969) and it appears highly probable that the observed differences in tumour incidence in different geographical areas may be related to aetiological factors in the environment. Notable examples of such localized distribution of tumours are the remarkably high incidence of liver cancer in parts of East Africa and of cancer of the oesophagus in the Transkei area of South Africa. In the latter area there appears to have been a marked increase in oesophageal cancer among the Bantu population since about 1940 and it has been suggested that a nitrosamine occurring in the environment might be responsible, since these carcinogens, especially the unsymmetrical dialkylnitrosamines (Druckrey, Preussmann, Blum, Ivankovic & Afkham, 1963), are among the very few which are known to induce tumours of the oesophagus in experimental animals. It is therefore of interest that the presence of nitrosamines has been reported in food and drink consumed by the local population. McGlashan, Walters & McLean (1968) have analysed samples of locally-distilled alcoholic drinks resembling gin from areas with a high incidence of oesophageal cancer and have reported the presence of material with the properties of nitrosamines at

levels as high as 3 ppm. Subsequently McGlashan (1969) has given an account of a geographical pathology survey of a large area of central Africa with neighbouring areas with apparently many and apparently few cases of oesophageal cancer. He noted a significant spatial correlation between the geographical patterns of incidence of oesophageal carcinoma and the drinking of sugar-based alcoholic spirit. Samples of spirits from eastern Zambia, central Kenya and the Transkei, although prepared in apparently dissimilar vessels, were all found to be contaminated in varying degree with zinc. Nitrosamine-like compounds in native spirits were also reported in all these areas.

More recently Du Plessis, Nunn & Roach (1969) have analysed the fruit of the plant *Solanum incanum*, since its juice is used in South Africa to curdle milk. These authors claim to have isolated dimethylnitrosamine from alcoholic extracts of this fruit and to have established its identity by a number of criteria, including thin-layer chromatography, gas chromatography, and nuclear magnetic resonance and infrared spectroscopy.

The position with regard to nitrosamines in tobacco and tobacco smoke is not entirely clear but their presence in very low concentrations has been reported in both (Serfontein & Smit, 1967; Neurath, 1967; Johnson, Millar & Rhoades, 1968).

It seems, therefore, that there is evidence for the occurrence of carcinogenic nitrosamines in the environment, apart from those from industrial sources, and the implications of this evidence must seriously be considered. It must be emphasized, however, that the available analytical methods for the detection and estimation of these compounds at the very low levels reported are not yet satisfactory and final conclusions on the possible hazards involved cannot yet be drawn.

Formation of nitroso carcinogens in the stomach

It is well known that nitrosamine formation from secondary amines and nitrites is strongly pH dependent and is favoured by the acid conditions prevailing in the mammalian stomach. There is now substantial evidence that nitrosamines can be formed under these conditions. Sander and his colleagues (Sander, 1967; Sander, Schweinsberg & Menz, 1968) demonstrated the formation of the corresponding *N*-nitroso compound by incubating several secondary amines, including diphenylamine, *N*-methylaniline and *N*-methylbenzylamine, with nitrite in the presence of gastric juice under various conditions.

The yield of nitrosamine was much greater with weakly basic than with strongly basic secondary amines and the optimum pH was in the range 1–3. Similar results were obtained by Sen, Smith & Schwinghamer (1969) who demonstrated the *in vitro* formation of diethylnitrosamine when diethylamine and sodium nitrite were incubated with gastric juice from rats, rabbits, cats, dogs and man. Human and rabbit gastric juice (pH 1–2) produced more diethylnitrosamine than juice from the rat (pH 4–5). The nitrosation reaction was demonstrated *in vivo* in cats and rabbits and the identity of the nitrosamine was established by thin-layer and gas chromatography. The authors suggest that the rabbit, the cat and the ferret may be more suitable test species than the rat for future work because their gastric juices are similar in pH to that of man. The kinetics of nitrosation of dimethylamine have been studied recently by Mirvish (1970) in buffered aqueous solutions using the tritium-labelled amine. The rate of reaction was maximal at pH 3·4 when formation of dimethylnitrosamine was proportional to dimethylamine concentration and to the square of the concentration of nitrite. Recently Dr. R. Montesano (unpublished data, 1970) has demonstrated methylation of nucleic acids of the stomach, liver and small intestine of rats given

[^{14}C]methylurea and sodium nitrite simultaneously by stomach tube. Since no evidence of nucleic-acid methylation was obtained when [^{14}C]methylurea was administered without the nitrite, these results were interpreted to indicate that the unstable and highly potent carcinogen *N*-nitrosomethylurea was probably formed in the stomach and absorbed into the circulation of the rat. The above evidence for the nitrosation of secondary amines under the conditions prevailing in the mammalian stomach strongly suggested that carcinogenic amounts of nitrosamines might be formed in this manner. Earlier work by Druckrey, Steinhoff, Beuthner, Schneider & Klärner (1963) failed to induce tumours in rats fed sodium nitrite and diethylamine or the nitrite alone. Recently, however, Sander & Bürkle (1969) have reported the induction of malignant tumours in rats as a result of the simultaneous feeding of morpholine or *N*-methylbenzylamine and sodium nitrite, the predominant sites of tumour formation being the liver and oesophagus, respectively. Sander (1968) has also shown that nitrosamines can be formed from secondary amines by several bacterial species, including *Escherichia coli*, *E. dispar*, *Proteus vulgaris* and *Serratia marcescens*. The nitrosation of aromatic secondary amines was considerably greater than that of aliphatic amines and the reaction occurred at pH values that did not permit spontaneous reaction between the amines and sodium nitrite (Sander, 1968). Subsequently the same author reported bacterial reduction of nitrate to nitrite in the human stomach (Sander & Seif, 1969). Thirty-one human subjects were given sodium nitrate and diphenylamine intragastrically and the presence of nitrosodiphenylamine was later demonstrated in the stomach contents. In view of the widespread distribution of alkali nitrates in the environment, this finding may have important implications since such a reductive mechanism could provide a source of nitrite for the nitrosation of any dietary secondary amines.

Conclusions

It is clear that many *N*-nitroso compounds are powerfully carcinogenic and it seems probable that more of this group of compounds will prove to be carcinogenic in the future. There appears to be no published report of any animal species that is resistant to carcinogenesis by these agents and no reason to believe that man is not susceptible. Experimental work in the rat indicates that most organs are sensitive to the carcinogenic action of one or more nitroso carcinogens and recent reports suggest that this may be true for other species. The nitroso compounds, therefore, have many of the properties that might be predicted for an environmental carcinogen to which man would be susceptible. There are several reports indicating that nitrosamines may be present in the human environment in a variety of situations but, apart from a few cases, the amounts reported have been low. Formation of carcinogenic nitrosamines from secondary amines and nitrites under the conditions existing in the human stomach has been demonstrated and simultaneous feeding to rats of secondary amines and sodium nitrite has resulted in the production of tumours. Nitrites are present in the environment and are used in many countries as deliberate food additives. Nitrates occur in massive amounts in some areas and are widely distributed in soils and plants throughout the world, and micro-organisms capable of catalysing the reduction of nitrates to nitrites have a wide distribution. There are, therefore, potential or actual sources of nitrite over wide areas. The position with regard to secondary amines is less clear but they may be present in various foods, including vegetables, fish, mushrooms, cheese and fruit, as well as in wine, beer and some drugs. Amines may also occur in tobacco and tobacco smoke. It appears, therefore, that man and animals may absorb variable small amounts of

nitroso carcinogens, either directly from the environment or after their formation in the alimentary tract, over long periods of time. These possibilities have been discussed in the *Lancet* (1968) and by Lijinsky & Epstein (1970). The possible hazard to man of exposure to very low concentrations of carcinogenic nitrosamines is extremely difficult to assess in the absence of reliable data on dose-response relationships at low dose levels.

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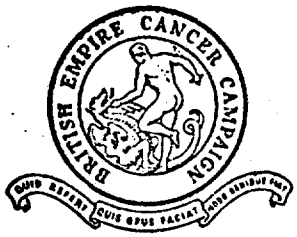

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THE PRODUCTION OF MALIGNANT PRIMARY HEPATIC TUMOURS IN THE RAT BY FEEDING DIMETHYLNITROSAMINE

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In a previous paper (Barnes and Magee, 1954) some toxic properties of dimethylnitrosamine (DMN) were described. It was found that rats and other animals suffer severe liver damage after the administration of DMN in doses of the order of 25 mg. per kilogram body weight, either orally or parenterally. The liver lesion was an extensive centrilobular type of necrosis, involving all lobules, followed by evidence of regeneration in surviving animals. An outstanding feature of the necrosis was its very haemorrhagic character and the liver lesion was frequently accompanied by massive haemorrhagic ascites and bleeding into the gastro intestinal tract.

These features of the acute liver necrosis were similar to those produced by the senecio alkaloids in rats (Davidson, 1935). A recent paper (Schoental, Head and Peacock, 1954) showed that senecio alkaloids also produced liver tumours if they were administered over longer periods. It seemed possible, therefore, that the chemically much simpler molecule DMN might also produce liver tumours in rats. The present paper describes the results of a long-term feeding experiment in which the compound was added to the diet of rats. A smaller and shorter experiment on rabbits was also performed. In the rats a very high incidence of hepatic tumours occurred, many with metastatic spread, but in the rabbits no tumours were observed. In no instance were primary tumours found in organs other than the liver.

MATERIAL AND METHODS

Male and female albino rats and cross-bred male rabbits were used. The basal diets were M.R.C. Diet 41 (Bruce and Parkes, 1949) for the rats and M.R.C. Diet 18 (Bruce, 1947) for the rabbits. The diet was given in powder form. DMN was made up as a solution in arachis oil so that 10 ml. added to 1 kg. of powder gave the required concentration.

The food intake of the rats was measured for each cage of 5 animals by filling the container each day and measuring the unconsumed residue.

The animals were weighed weekly and observed each day when their food was being measured. Post mortems were carried out on all animals that were either found dead or killed by coal gas when seriously ill. Tissues were fixed in Helly's fluid and formol-saline, embedded in paraffin and sectioned at 5μ. All sections were stained with haematoxylin and eosin as a routine and selected material was stained by Mallory's connective tissue method, Gomori's silver impregnation, and

Perl's prussian blue reaction for iron. Frozen sections of formol fixed material were stained for fat with Sudan IV.

RESULTS

First experiment

The results of this experiment have been briefly reported previously (Barnes and Magee, 1954) and are included here in greater detail for comparative purposes.

Groups of 6 rats were fed 200, 100 and 50 parts per million (ppm.) DMN respectively. Those receiving 200 ppm. died in less than 5–6 weeks, and at autopsy on 3 rats the livers were small and pale, but regular in outline. There was no intraperitoneal haemorrhage. In one rat the pancreas was oedematous and in another had a dead-white opaque appearance.

Rats on 100 ppm. DMN lived 9–14 weeks. One of the six had haemorrhage into the gut. The livers were smaller than normal but irregular in shape and some had "fatty" lobes. In several the pancreas was either oedematous or prominently white and opaque. Rats on 50 ppm. killed after 20 weeks in this first experiment showed no gross abnormality except a general rounding of the edges of the liver.

Histology.—In those rats dying after 5 weeks on 200 ppm. DMN, the liver was intersected by irregularly-shaped bands of tissue composed of hepatic cells in various degrees of degeneration, red cells, macrophages, with and without yellow pigment, fibroblasts and fine eosinophil fibrils (Fig. 1). Silver impregnation preparations showed increase and condensation of reticulin in the strands (Fig. 2). The parenchymal cells showed more large nuclei and binucleate forms than usual, but mitotic figures were very rare. Very early bile-duct proliferation was occasionally seen.

Rats dying after 9 weeks on a diet with 100 ppm. DMN had rather similar liver lesions with more fibrous tissue (Fig. 3). The parenchymal cells showed similar cytological changes, and were occasionally grouped into nodules surrounded by very fine fibrous capsules. Bile-duct proliferation was well established (Fig. 4).

In those dying later (13 weeks) bile-duct proliferation was rather more advanced and the new ducts were moderately dilated, with some flattening of the epithelium. These structures now had an obvious resemblance to the huge forms to be described later. Occasional hyperplastic parenchymal nodules occurred (Fig. 5) and there was evidence of diffuse hyperplasia of the parenchymal cells. The hyperplastic liver cells often showed very large nuclei with large, often multiple nucleoli. The term hyperplasia is used here in a purely descriptive sense, following Opie (1944) who defined it as being "characterised by changes in the size, staining, and arrangement of cells, and may occur diffusely with no sharp demarcation or in foci, so that well defined nodules are formed".

The rats on 50 ppm. DMN killed after 20 weeks had livers which showed much less general damage. The main difference from the normal was cytological. There was a generalised increase in cell size, with large nuclei and nucleoli. In one animal well established bile-duct proliferation was present.

Second experiment

Ten male and 10 female rats were given a diet containing 50 ppm. DMN with 5 males and 5 females as controls on the same diet with arachis oil alone. The

rats on DMN did not grow as well as their controls and their average weight, together with the mean weekly food intake for the 1st, 13th and 26th weeks are given in Table I. Up to this time rats had appeared well, but during the 27th week the first death took place. Deaths continued at intervals until the 42nd week, when the single survivor was killed. One male control rat died with a pulmonary abscess in the 6th week. The rest were healthy when killed at the end of the experiment. One was found to have a sarcoma of the mesenteric glands at autopsy. Of the 20 animals receiving DMN 11 died from effects of an acute haemorrhage into the peritoneal cavity. Bleeding had taken place from the surface of the liver at a site usually marked by adherent omentum. In every animal the liver showed a gross irregularity of its surface with a varying number of nodules scattered throughout. Damage was often more severe on the left side, where the lobes were shrunken and distorted to a marked extent. The irregular nodular appearance of the right side of the liver is shown in Fig. 6 while one of the smaller left lobes is distorted by extravasated blood. A normal liver (Fig. 7) is shown for comparison. Numerous small translucent cysts were frequently seen in addition to the solid nodules. The most striking feature of these livers was the irregularity in outline and variation in the shape of individual lobes (Fig. 8). Uniformly shrunken fibrotic livers were not seen.

TABLE I.—*Mean Weight with Standard Error and Weekly Food Intake in g. per 100 g. Rat for Rats on a Diet Containing 50 ppm. DMN. (Number of rats in brackets.)*

		Start.	13 weeks.	26 weeks.
Male:				
Control (4)	Body weight	111	293 ± 27	351 ± 17
	Food intake	78	48·5	45
DMN (10)	Body weight	103	245 ± 21	293 ± 32
	Food intake	74·5	47	37
Female:				
Control (5)	Body weight	104	212 ± 16	244 ± 18
	Food intake	67·5	49·5	49
DMN (10)	Body weight	110	184 ± 22	209 ± 20
	Food intake	71	52·5	48

In those animals dying later in the experiment there was usually a mass of red lymph nodes in the portal fissure. The pancreas in two rats had an opaque dead-white appearance, but otherwise the abdominal viscera appeared normal. In the chest the mediastinal glands were usually red and the lungs showed either a general congestion or more commonly, a number of red petechiae scattered over the pleural surface. Pleural exudates were not encountered.

Of the 9 animals not dying from an acute internal haemorrhage, one rat, the first to die, had a peritoneal cavity filled with orange coloured exudate, and the liver distorted by a large rubbery mass on its proximal surface resembling a sarcoma. No primary tumour could be found elsewhere. Of the other 8 rats, one only was killed in good condition as the sole survivor after 40 weeks. Its liver contained several nodules. The others were killed because they were ill. All had nodular livers and in three omentum was adherent to parts of the liver with indications of a small haemorrhage from the liver surface. Another had a

peritoneal cavity full of clear fluid and two had serious involvement of the lungs. No rat appeared to have died solely from liver failure due to necrosis or destruction of the organ by fibrosis.

Male and female rats appeared to respond equally to DMN with the same mean survival time and similar lesions in their livers.

Histology.—With one exception the animals killed or dying at different times during the experiment showed liver lesions of a similar character, therefore a general description of the histological changes will be given.

In harmony with the gross appearance there was great variation in the histological picture seen in sections taken from different parts of the same liver. The more grossly damaged areas showed complete absence of the normal architecture and were hardly recognisable as hepatic (Fig. 9).

Parenchymal nodules were seen in every liver, many being similar to those described in the first experiment. They were roughly circular, surrounded by a fibrous capsule, and appeared to be compressing the surrounding structures (Fig. 10 and 11). They were composed of closely packed parenchymal cells, which lacked the normal trabecular arrangement, and there was great variation in cell and nuclear size, but mitotic figures were infrequent. Occasional small bile ducts and fairly frequent sinusoids were seen. Some of the nodules showed necrosis and haemorrhage in their central parts. In the extreme cases almost the whole structure was replaced by blood clot, which was sometimes undergoing organisation. Diffuse parenchymal hyperplasia was also present. No sharp distinction could be drawn between advanced hyperplasia and true neoplasia of the parenchymal cells. Changes characteristic of neoplasia were considered to be the presence of cells varying extremely in size and shape with bizarre multinucleated forms. These cells had large nuclei with either a single large or multiple deeply basophilic nucleoli. Mitotic figures were common and frequently abnormal (Fig. 12). Neoplastic change of this type was seen in areas of hyperplasia, most frequently in the nodules. Typically, the lesion consisted of an outer zone of neoplastic cells surrounding a central haemorrhagic necrotic mass mass (Fig. 13). This appearance was also typical of that seen in metastatic tumours (Fig. 14). The tumours were mainly anaplastic with varying degrees of pseudo-acinar formation.

Bile-duct proliferation was always present and showed much variation in the form of the proliferated ducts and in the amount of the accompanying fibrous tissue. The most frequent appearance was of large multilocular cystic structures, which corresponded to the yellow translucent cysts seen in the gross specimens. They were lined by a thin layer of flattened epithelium with only a sparse fibrous framework between the loculi, and with little or no surrounding fibrosis (Fig. 15). In other areas the cystic dilatation was less marked or absent and the epithelium cubical or low columnar. Here the interstitial and surrounding fibrous tissue was more abundant. No continuity with normal bile-ducts was seen, but the presence of a wide range of intermediate forms together with the appearance of the lining epithelium suggested a common biliary origin for the lesions (serial sections were not made). In some places, the structural irregularity was very marked and the epithelial nuclei were hyperchromatic (Fig. 16), but mitotic figures were rare and this type of lesion was not found in the metastases.

A third type of lesion seen in only one animal had the appearance of a fibrosarcoma. The tumour mass consisted of large numbers of fibroblasts, many

abnormal, with frequent mitotic figures. Preparations stained by Mallory's method showed much collagen production (Fig. 17). There were occasional tubular structures within the tumour resembling bile ducts. No metastases were found in this animal, but the tumour was locally invasive.

Intravascular ante-mortem thrombosis was occasionally found (Fig. 18), the type of vessel being difficult to identify because of the gross general distortion of the liver. Some fibrosis was present in all the livers, but this was never a very prominent feature and again no precise anatomical distribution could be assigned.

Nineteen out of the 20 rats developed primary hepatic tumours. Metastases were present in 7 of the animals, 4 being pulmonary and the remainder intra-abdominal. No histological evidence of primary neoplasia was found except in the liver. The sole surviving rat, which was killed in apparent good health, showed slight diffuse and nodular hyperplastic change in its liver parenchyma.

Third experiment

Six male rabbits (2·1–2·8 kg.) were given a diet containing 20 ppm. DMN. A lower level was used because rabbits are more sensitive than rats to the acute effects of DMN. After 10 weeks five of the rabbits had lost a little weight, but otherwise appeared normal, the sixth had lost more weight and appeared ill. The concentration of DMN was raised to 30 ppm. for another 4 weeks and to 50 ppm. for a further 8 weeks, by which time all the rabbits had died. One was killed during the 11th week. Four others became ill during the 18th–20th weeks and were killed and the last animal was killed in the 22nd week. They showed no signs of poisoning beyond a gradual loss of weight and increased listlessness and general weakness. In every case the liver was small (25–35 g.) dark and slightly, but not grossly, fibrosed, as judged by the ease with which it was cut. Nodules, cysts and haemorrhages were not seen. The other abdominal organs appeared normal and the loss of condition of the animals could only be attributed to a progressive loss of liver tissue.

Histology.—No evidence of neoplasia was found in the livers which showed occasional areas of early bile-duct proliferation and parenchymal hyperplasia, also some slight irregular fibrosis.

DISCUSSION

As far as we are aware, the experimental production of liver tumours by dimethylnitrosamine has not been reported previously, therefore our findings will be discussed in relation to those of other workers using different agents.

Several substances have been shown to produce primary liver tumours after repeated small doses, and there is a definite similarity in the character of the lesions produced. Thus some of the compounds when administered in single larger doses give rise to an acute centrilobular type of necrosis involving all lobules, followed by evidence of parenchymal cell regeneration. Butter yellow (Orr and Price, 1948); carbon tetrachloride (Cameron and Karunaratne, 1936); thioacetamide (Ambrose, De Eds and Rather, 1949; Gupta, 1955) and senecio alkaloids (Davidson, 1935) all have this property and DMN falls into the same group. It must be emphasized that with the exception of senecio, DMN causes a much greater amount of haemorrhage associated with the necrosis than the other compounds.

Chronic choline deficiency (Salmon and Copeland, 1954) and feeding with bentonite, which is believed to induce the same condition (Wilson, 1954) on the other hand do not produce centrilobular necrosis, but diffuse fatty change which is followed by fibrosis and tumour formation. In chronic ethionine feeding (Popper, de la Huerga and Koch-Weser, 1954), fatty change and central necrosis precede lesions which are probably neoplastic.

It appears, therefore, that the behaviour of DMN is comparable to that of the tumour-producing agents which are able to cause acute centrilobular necrosis. The early morphological changes in the liver, as shown by the rats receiving 200 ppm. for 5 weeks, are consistent with such a comparison. The formation of strands of tissue destruction with macrophage infiltration and early fibrosis (Fig. 1) is similar to that described by Orr (1940) using butter yellow. At the same time many of the parenchymal cells showed increased size, including enlargement of the nuclei and nucleoli, and there was early but recognisable bile-duct hyperplasia. Similar changes in parenchymal cell size have been noted by Ambrose, De Eds and Rather (1949) using thioacetamide, and have been the subject of special investigations by Rather (1951) and Kleinfeld and Lessler (1954) who agree that they are the earliest morphological abnormality. In the present work no animals were studied earlier than five weeks after the start of DMN administration, therefore no opinion can be expressed on the exact chronology of the appearance of the lesions.

The rats on 50 ppm. DMN which were examined before the 26th week were from the first experiment and they showed minimal liver changes except one with characteristic liver damage. The rats in both experiments were from the same stock, but the first experiment was carried out during the winter and the second started in the summer.

In discussing the tumours, problems of nomenclature arise. The term hepatoma is defined in Blakiston's *New Gould Medical Dictionary* (1949) as " Any tumour originating in the liver; applied more particularly to nodular foci of regeneration, to adenomas, and to that form of primary hepatic carcinoma made up of cells which, in arrangement and form, resemble the cells of the hepatic cords ". Both the restricted and the general sense of the word have been used by writers on experimental hepatic tumours. A further difficulty is created by nomenclature implying pathogenesis, thus such terms as bile-duct carcinoma and cholangioma indicate the origin of the neoplasm in biliary epithelium, while hepatoma may imply that it arises in hepatic cells. In the case of tumours induced by butter yellow, there is considerable disagreement on the cell of origin. Opie (1944); Orr (1940); Cortell (1947) and Richardson and Borsos-Nachtnebel (1951) maintain that tumours can arise from both hepatic cells and biliary cells. Edwards and White (1941) and Dalton and Edwards (1942), however, believe that all true neoplasia in the liver due to butter yellow has its origin in the hepatic cell only, and that changes in the bile-ducts are to be interpreted as hyperplastic. A third group of workers (Price, Harman, Miller and Miller, 1952) working with derivatives of butter yellow have concluded that most, if not all, of the neoplasms, regardless of their histological pattern, arise from areas of cholangiofibrosis and thus have a common pathogenesis.

As far as possible, therefore, in the present paper terms which imply a definite pathogenesis have been avoided.

The distinction between advanced hyperplasia and true neoplasia must

ultimately be subjective, but the existence of metastatic spread provides objective evidence of malignancy. As Opie (1944) has pointed out "histological structure is an uncertain index of malignancy, tumours with metastases have been regarded as decisively malignant, they reproduce these distinctive characteristics in the metastases that formed". We have made no attempt to distinguish between advanced hyperplasia and benign neoplasia, but in some instances a lesion has been regarded as malignant in the absence of observed metastasis, if its histological and cytological structure was indistinguishable from that found in tumours with definite evidence of distant spread. A significant number of tumours did in fact show metastases, and the histological and cytological characteristics of the secondary tumours have been regarded as criteria for the diagnosis of neoplasia in the lesions occurring in the liver. With these criteria in mind it can be said that the tumours caused by DMN are predominantly anaplastic with some tendency to the formation of structures resembling glands. The difficulties of assigning a cell of origin have been discussed above. However, these tumours appear to arise either in hyperplastic parenchymal nodules or in regions of diffuse

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EXPLANATION OF PLATES.

FIG. 1.—Rat Liver. First experiment. DMN 200 ppm. for 5 weeks. Shows strands of damaged tissue described in the text. H. & E. × 35.

FIG. 2.—Rat Liver. First experiment. DMN 200 ppm. for 5 weeks. Shows reticulin in the strands. Silver impregnation. × 70.

FIG. 3.—Rat Liver. First experiment. DMN 100 ppm. for 9 weeks. Shows the formation of fibrous tissue. Mallory. × 70.

FIG. 4.—Rat Liver. First experiment. DMN 100 ppm. for 9 weeks. Shows early bile-duct proliferation. H. & E. × 70.

FIG. 5.—Rat Liver. First experiment. DMN 100 ppm. for 13 weeks. Shows a hyperplastic nodule. H. & E. × 70.

FIG. 6.—Rat Liver. Second experiment. DMN 50 ppm. Shows the irregular nodular appearance of the liver, with a lobe distorted by extravasated blood. The scale is graduated in centimetres.

FIG. 7.—Rat Liver. Second experiment. Control liver for comparison.

FIG. 8.—Rat Liver. Second experiment. To show irregularity in outline and variation in shape of individual lobes.

FIG. 9.—Rat Liver. Second experiment. To show the extremely severe structural change in the liver. H. & E. × 3.

FIG. 10.—Rat Liver. Second experiment. A typical hyperplastic nodule. H. & E. × 60.

FIG. 11.—Rat Liver. Second experiment. The same nodule as Fig. 10, Mallory. × 75.

FIG. 12.—Rat Liver. Second experiment. Part of a tumour under higher magnification showing extreme anaplasia, huge nucleoli and a large abnormal mitotic figure. H. & E. × 300.

FIG. 13.—Rat Liver. Second experiment. Typical tumour consisting of a rim of neoplastic tissue surrounding a massive central core of necrotic debris and blood clot. H. & E. × 3.

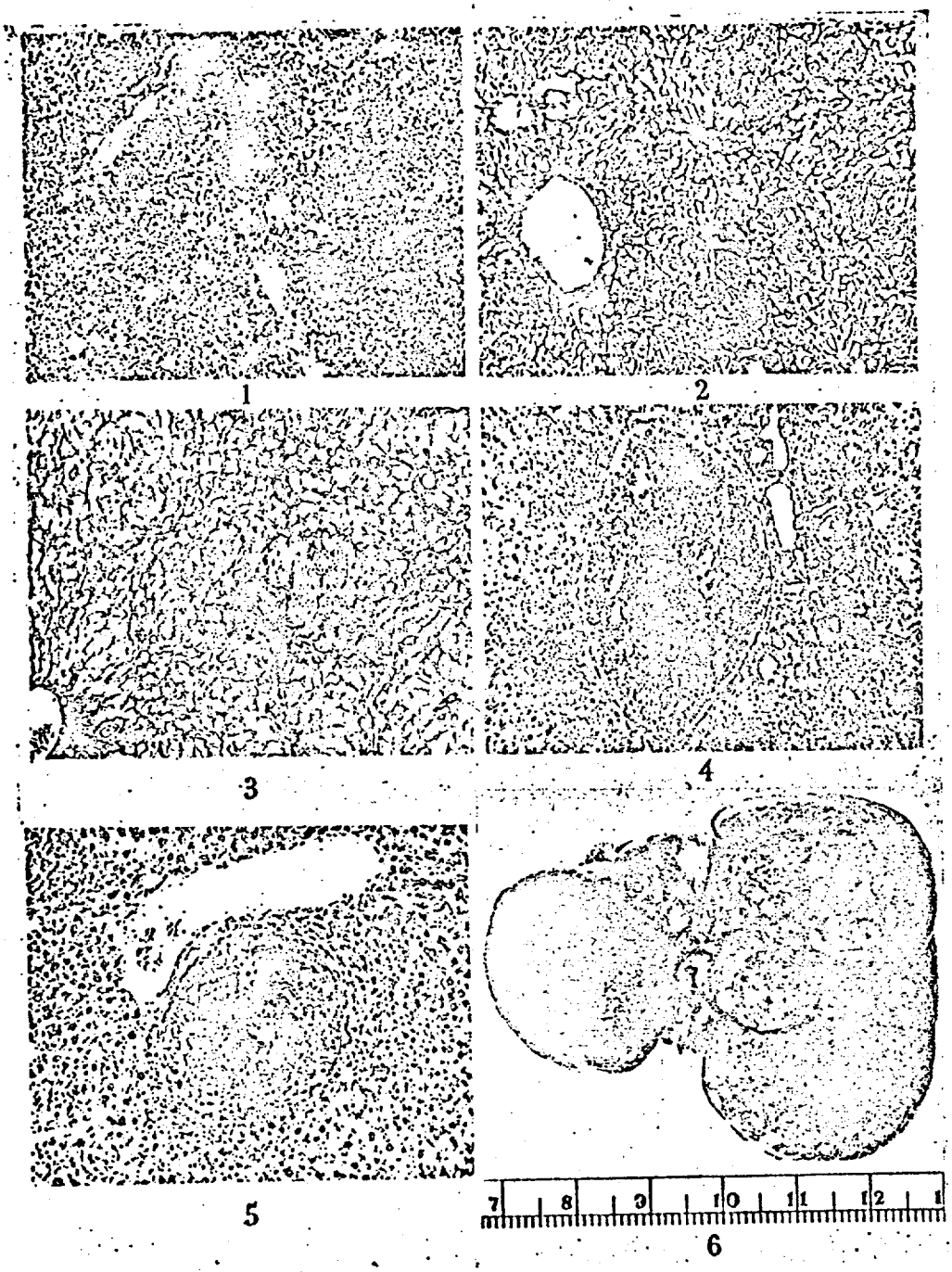
FIG. 14.—Rat Lung. Second experiment. Shows a pulmonary metastasis. H. & E. × 30.

FIG. 15.—Rat Liver. Second experiment. Shows very severe cystic bile-duct proliferation. H. & E. × 43.

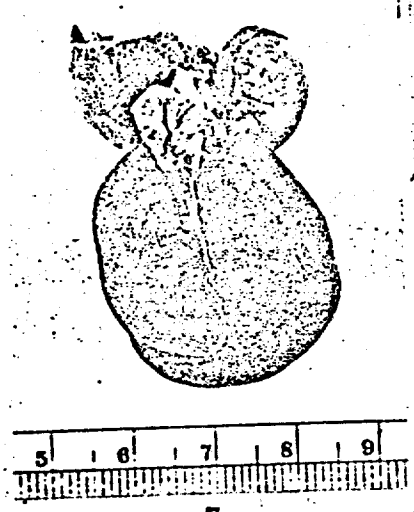
FIG. 16.—Rat Liver. Second experiment. Very irregular bile-duct hyperplasia with hyperchromatic nuclei. H. & E. × 85.

FIG. 17.—Rat Liver. Second experiment. Sarcoma, to show the general structure and production of collagen. Mallory connective tissue method. × 85.

FIG. 18.—Rat Liver. Second experiment. Showing ante-mortem intravascular thrombosis. H. & E. × 85.



Magee and Barnes.



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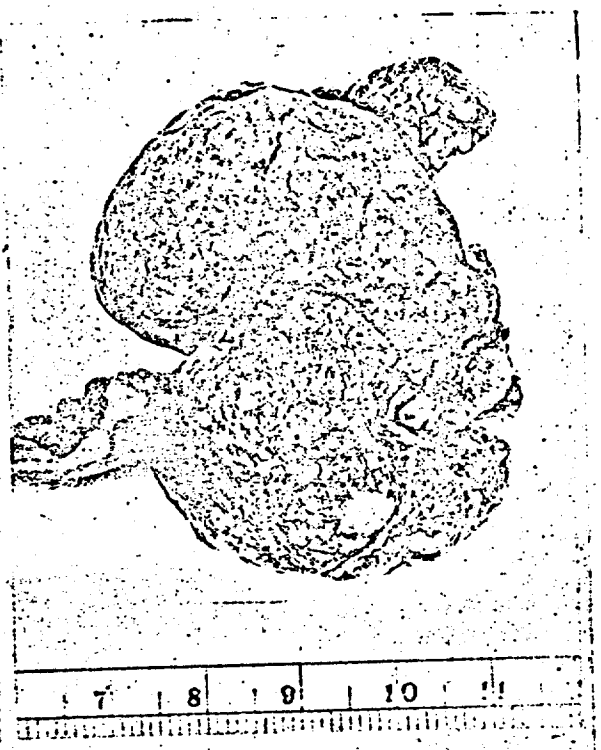

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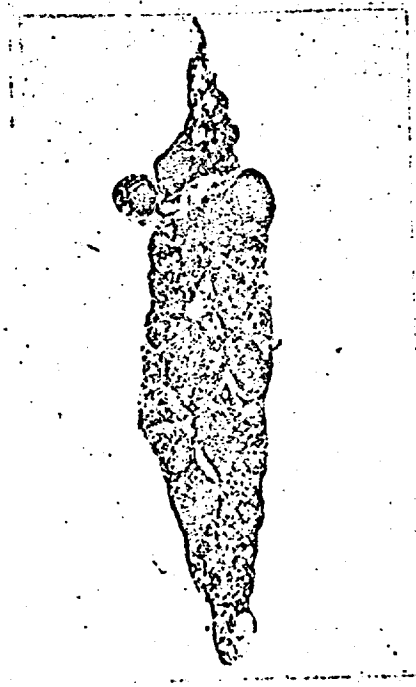

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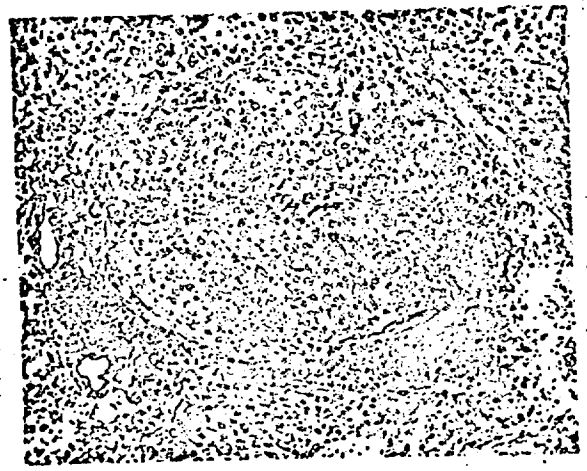

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parenchymal hyperplasia and their cytology shows more resemblance to hepatic than to biliary cells. The invariable accompaniment of massive necrosis and haemorrhage increases the difficulty of exact classification.

The enormous cystic structures so commonly observed are regarded as extreme forms of bile-duct proliferation, as all intermediate degrees from normal ducts can be found. They appear to correspond to the cystadenomata of Orr (1940) and Opie (1944). The neoplastic character of this type of lesion has been contested by Edwards and White (1941). Occasionally, in areas of duct proliferation, the structure was much more irregular and the cells contained larger and hyperchromatic nuclei. Mitotic figures were not common however, and this type of structure was never seen in metastatic growths, therefore its classification remains in doubt.

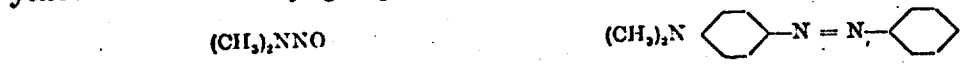
The single sarcomatous tumour remains. Here again no metastasis was found, but the invasive character and the frequent mitotic figures suggested malignancy. More important is the question whether this tumour was caused by DMN or whether it arose spontaneously. No similar tumour was observed in the livers of control animals, nor indeed in any rat in our colony. Richardson and Borsos-Nachtnebel (1951) have noted fibrosarcoma and angiosarcoma in rats fed 3-methyl-4-dimethylamino azobenzene, and Firminger and Mulay (1952) described apparent sarcomatous change in the stroma of an adenocarcinoma induced by azo dye.

As already emphasized, haemorrhage and necrosis are constant and prominent features in acute and chronic DMN poisoning. Ischaemic factors played a part in the production of necrosis, notably in the hyperplastic nodules. A disordered vascular system has been shown to be present in malignant neoplasms of the liver (including butter yellow tumours in rats) by Breedis and Young (1949), who state that these tumours tend to acquire an exclusively arterial blood supply.

Apart from its intrinsic interest, a knowledge of the cell of origin in experimental hepatic tumours is of some importance in relation to chemical investigation. Mueller and Miller (1953) have shown that butter yellow and certain of its derivatives are metabolised *in vivo* and *in vitro*, and unpublished work in our laboratory indicated that DMN also undergoes rapid metabolic alteration. These metabolic reactions may be concerned in the carcinogenic process, and there may be some correlation between the cells which mediate them and those which ultimately become neoplastic.

DMN may prove a useful agent in the study of experimental hepatic carcinogenesis. It is miscible with water in all proportions and can be readily estimated in tissues by a simple polarographic method (Heath and Jarvis, 1955).

At present no hypothesis can be advanced on the mechanism of action of DMN in the production of tumours. It may be noted that both DMN and butter yellow have N-dimethyl groups.



Dimethylnitrosamine

p-dimethyl-amino-azobenzene (butter yellow)

It has been shown that at least one N-methyl group is required for the carcinogenic activity of dyes related to butter yellow in the liver of the rat (Miller and Miller, 1953).

SUMMARY

Twenty rats, divided into two groups according to sex, were fed a normal diet (M.R.C. diet 41) to which had been added dimethylnitrosamine at a level of 50 parts per million. Between the 26th and 40th week of this treatment 19 of the animals developed primary hepatic tumours, metastatic spread being present in 7 cases. An attempt to produce tumours in rabbits by the same agent was unsuccessful. Dimethylnitrosamine, by virtue of its chemical and physical properties, may be of some value in the investigation of hepatic carcinogenesis.

We would like to thank Professor G. R. Cameron, F.R.S., for very valuable discussion and criticism. Mr. R. Legg, A.I.M.L.T., made the histological preparations and photomicrographs.

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1309

THE EXPERIMENTAL PRODUCTION OF TUMOURS IN THE RAT

by

DIMETHYLNITROSAMINE (N - NITROSO DIMETHYLAMINE)

BY

P. N. MAGEE and J. M. BARNES,

(Toxicology Research Unit., M. R. C. Laboratories, Woodmansterne Road, Carshalton, Surrey).

A single oral dose of 20 to 30 mg. per Kg. body wt. of dimethylnitrosamine (DMN) has been shown to produce a severe liver necrosis in a number of animal species (BARNES and MAGEE, 1954). As we have already reported, when it is fed to rats at a level of 50 ppm. in a normal diet 19 out of 20 animals both male and female developed liver tumours within 42 weeks. These tumours had points of similarity to those produced by the azo dyes and other liver carcinogens (MAGEE and BARNES, 1956). This paper describes the incidence of tumours in rats living 40 weeks or longer from the start of experiments in which DMN was given at different dose levels for different periods, starting when the rats were about 100 gm. in weight.

Our findings are summarised in *table 1* and will be described in a little more detail.

The first three groups, numbers 1, 2 and 3 in the left hand column of *table 1* are rats which received DMN in their diet throughout the experiment, and the first, Experiment 1, is our original observation of the effect of 50 ppm. At lower concentrations, experiment number 2, there were some liver tumours but no kidney tumours, while at 5 ppm, that is experiment 3, there were no tumours, and indeed hardly any liver damage.

In the next 6 experiments DMN at different levels was given for limited periods. At 50 ppm for 1 month, experiment 4, there were no tumours but when this level of DMN was fed for 2 and 3 months, experiment 5, there were 5 liver tumours and two kidney tumours.

In the next group of experiments higher levels of DMN were fed for shorter periods. Indeed at these levels, 100 and 200 ppm, a severe and fatal liver necrosis develops if the administration is prolonged.

At 100 ppm for 1 week, experiment 6, there were no tumours but feeding at this level for 2, 3 and 4 weeks, experiment 7, resulted in a single (unusual) liver tumour but an incidence of over 50 % of kidney tumours. At 200 ppm for 1 week, experiment 8, there was

Fig. 1.

Female rat. Dimethylnitrosamine 30 mg. per Kg. body wt. Single oral dose. Killed 2 yr. later. Dissection showing one large lobulated tumour arising from left kidney and smaller tumour on opposite side.

an even higher incidence of kidney tumours.

Finally, the late effects of a single treatment with DMN were observed in the survivors from a group which had been given an approximate LD 50 dose. This experiment was originally done in order to see whether a single does of DMN could produce a chronic progressive liver lesion similar to that seen after a single dose of some of the senecio alkaloids (SCHOENTAL and MAGEE, 1957). No progressive liver lesions nor liver tumours were seen but several kidney tumours developed. This was experiment 9 in *table 1*.

TABLE I.

The incidence of liver and kidney tumours in rats given dimethylnitrosamine.

Exp.	Dose ppm.	Duration (weeks)	Liver	Kidney
1.	50	42	19/20	0/20
2.	10 & 20	102	7/11	0/11
3.	5	104	0/6	0/6
4.	50	4	0/6	0/6
5.	50	8 & 12	5/11	2/11
6.	100	1	0/6	0/6
7.	100	2,3 & 4	1/16	10/16
8.	200	1	0/4	4/4
9.	mg/Kg. 30	—	0/15	3/15

Macroscopically the kidney tumours varied in size from barely visible to enormous growths and they were often bilateral. A typical appearance is seen in *Fig. 1* where there is a large lobulated tumour arising from the lower pole of the right kidney and a smaller one on the opposite side. Areas of haemorrhage and necrosis were common in the larger tumours. Microscopically, the earliest lesions, histologically speaking, were areas of tubular hyperplasia in the renal cortex with glomeruli persisting in their midst.

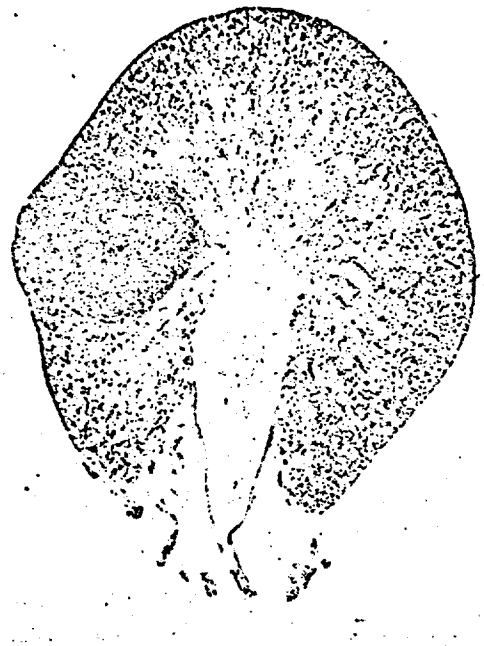

Fig. 2.

Female rat. Dimethylnitrosamine 30 mg. per kg. body wt. Single oral dose. Killed 2 yr. later. Section of kidney showing sharply demarcated tumour. Haematoxylin and Eosin. x 6

A rather more advanced lesion is shown in *Fig. 2*. Here the lesion is very definite and clear cut. It is raising the capsular surface of the kidney and extends through the cortex into the medulla almost to the base of the renal papilla. The same lesion under higher magnification is seen in *Fig. 3* which shows the boundary of the tumour with the kidney tissue. The lesion is made up of well differentiated tubular structures, and although the line of demarcation is very clear, there is no capsule. The clear outline of the lesion is partly due to the dark basophil staining of the cells. Much larger tumours with similar microscopic structures have been observed, but in these extensive breakdown of the tumour substance is the rule. In spite of the great size of some of these lesions they have had the characteristics of benign tumours and we have classified them as adenomas.

In several other tumours the structure was far less well differentiated. The boundary of such a tumour is shown in *Fig. 4*. Here there is little attempt at tubule formation and the

cells are often massed together in sheets. The same tumour under higher magnification is shown in *Fig. 5*. The contrast with the regular tubular structure shown in *Fig. 3* is apparent and mitoses are common. Although metastases have not been seen these latter tumours are regarded as malignant because

Fig. 3.

Same kidney as Fig. 2. Note the very well differentiated structure of the tumour and absence of capsule. Haematoxylin and Eosin.

of the extreme anaplasia and rapid growth. We have classified them as adenocarcinomas. It is tempting to postulate a progression in the development of these tumours from a very well differentiated stage through intermediate stages of less differentiation to a final picture of marked anaplasia. However, the smaller tumours tended to occur in older rats and the more anaplastic ones tended to be found earlier so it cannot be said whether the carcinomas developed from adenomas or not. Further work on the early development of these tumours is in progress.

These observations raise interesting questions on the effect of dose and duration of exposure on the response of animals to carcinogens. In the case of DMN we do not know

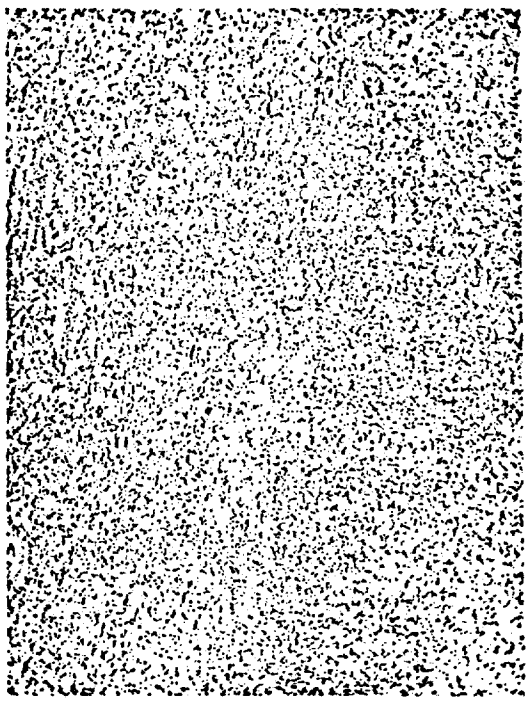

Fig. 4.

Male rat. Dimethylnitrosamine 200 p.p.m. in normal diet 1 week. Killed after 47 weeks. Note poorly differentiated tumour and compare with Fig. 3.

the nature of the actual carcinogen but it may be the product of a reaction between DMN and a normal cell constituent. In the rat, metabolism of DMN occurs almost exclusively in the liver (MAGEE, 1956) and it is possible that when large doses are given some of this metabolite spills over into the circulation to be picked out and perhaps concentrated prior to excretion by the kidney tubules. Alternatively, the kidney lesion may result from metabolism of DMN by the renal epithelium itself.

Two other interesting points can be noted. The first is that the liver and kidney tumours have never occurred together in the same animal. The second is that the rate of growth of the kidney tumours seems to be much more variable than that of the liver tumours. However, it is not yet possible to say whether the small kidney tumours seen in old rats killed many months after the dose of DMN are of recent origin or represent an early lesion that failed to develop into a large tumour. Very much less experimental work has been done on tumours of the kidney than on

Fig. 5.

Same section as Fig. 4. Tumour under higher magnification showing anaplasia and frequent mitoses. Haematoxylin and Eosin .

liver tumours and we hope that dimethylnitrosamine may prove to be a useful agent in this respect.

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SUMMARY

Previous observations (Magee and Barnes, Brit. J. Cancer 1956, 10, 114) has shown that dimethylnitrosamine fed at a level of 50 p.p.m. in the diet of rats produced malignant liver tumours in almost every animal within 40 weeks. The effect of higher and lower concentrations of dimethylnitrosamine fed over longer and shorter periods have now been examined. In addition, rats surviving the single LD50 dose have been observed for over two years. As well as affecting the incidence of liver damage and the occurrence of liver tumours, the changes in dose schedule have led to the appearance of kidney tumours in several rats including those that had only a single dose of dimethylnitrosamine. The findings are presented in detail and discussed.

RESUME

Des observations antérieures (Magee et Barnes, Brit. J. Cancer 1956, 10, 114) avaient montré que le diméthylnitrosamine; donné à raison de 50 millionièmes dans la nourriture de rats, provoquait endéans les 40 semaines des tumeurs malignes du foie chez presque tous les animaux.

L'effet de concentrations plus et moins élevées de diméthylnitrosamine, données pendant des périodes plus et moins longues, a été examiné. On a de plus observé, pendant deux ans, les rats survivant à une dose unique de LD50.

Les variations dans les doses ont affecté l'incidence des lésions hépatiques et des tumeurs du foie, mais ont également provoqué l'apparition de tumeurs rénales chez plusieurs rats dont certains n'ont reçu qu'une dose unique de diméthylnitrosamine. Les résultats sont présentés en détail et discutés.

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1310

INDUCTION OF KIDNEY TUMOURS IN THE RAT WITH DIMETHYLNITROSAMINE (*N*-NITROSODIMETHYLAMINE)

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(PLATES VII–X)

THE production of liver necrosis and malignant liver tumours in rats with dimethylnitrosamine (DMN) has already been described (Barnes and Magee, 1954; Magee and Barnes, 1956). As a part of a study of the mechanism of action of DMN, the effect of varying the dose schedule was examined. It was found that short intensive feeding with DMN, which produced considerable liver damage initially, was not followed by progressive liver damage, but rather the liver tended to heal and return to normal. However, tumours appeared in the kidneys. This paper describes the conditions under which a high incidence of kidney tumours can be produced and includes a detailed description of their histology. A preliminary account of some of this work has already appeared (Magee and Barnes, 1959).

The induction of hepatocellular carcinoma in the rat with DMN was confirmed by Schmähl and Preussmann (1959), who also produced similar liver tumours with diethylnitrosamine (Schmähl, Preussmann and Hamperl, 1960). Tumours of the kidney and lung in rats fed with DMN were reported by Zak, Holzner, Singer and Popper (1960) and Argus (1961) observed tumours of the liver, kidney and lung after feeding with DMN and diethylnitrosamine. Squamous carcinoma of the trachea and lung occurred in golden hamsters fed with diethylnitrosamine by Dontenwill and Mohr (1961). Cirrhosis of the liver was observed in this species, but no hepatic cancer. Studies on the relation of chemical constitution to biological activity by Druckrey *et al.* (1961*a*) have shown that several other *N*-nitrosamines, including the range of dialkyl compounds from dimethyl to di-amyl nitrosamine and the heterocyclic compounds *N*-nitroso morpholine and *N*-nitroso piperidine, are also carcinogenic. The last compound also induced squamous carcinoma of the oesophagus. Tumours of the kidney, but not of the liver, were induced by dimethylnitramine, an oxidation product of dimethylnitrosamine. The related compound *N*-nitrosomethylurethane induces squamous carcinoma of the stomach after oral administration to rats (Schoental, 1960; Druckrey *et al.*, 1961*b*). This work of Druckrey and his colleagues was considered to support the idea that the dialkylnitrosamines are carcinogenic by virtue of a metabolic conversion to active alkylating agents, possibly diazoalkanes. Alkylation (methylation) of nucleic acids and proteins of rat liver and probably other tissues *in vivo* has recently been demonstrated (Farber and Magee, 1960; Magee and Farber, 1962; Magee and Hultin, 1962).

MATERIALS AND METHODS

Male and female white rats (Porton strain) were given MRC Diet 41B. Dimethylnitrosamine was dissolved in arachis oil which was added at the rate of

10 ml. per kg. diet powder and mixed in a Hobart mixer. The concentrations in the diet were checked occasionally by the method of Heath and Jarvis (1955); the concentration was usually about 20 per cent. below the calculated level. When the DMN administration was for a limited period the rats were returned to Diet 41B cubes for the rest of the experiment. The rats were started at 100–150 g. body weight.

Necropsies were carried out on all rats, and where death occurred naturally histological examination was included if autolysis was not too advanced. Tissues were fixed by immersion in Helly's fluid or 10 per cent. formol-calcium, embedded in paraffin and sectioned at 5 μ. Sections were stained with haematoxylin and eosin as a routine method and frequently by the periodic acid-Schiff (P.A.S.) method (Lillie, 1954). Frozen sections of formol-calcium-fixed material were stained with sudan IV for fat.

Results

A summary of the experiments in which the development of kidney tumours was observed is given in table I. Some material provided by Dr D. F. Heath is included in the general description of the kidney tumours. All these rats received only one dose of DMN, some received in addition one dose of dimethylformamide (DMF), which had no apparent effect on the incidence of tumours. Whilst no kidney tumours were seen in rats receiving DMN in doses low enough to permit their survival for a normal life span (2 yr), their incidence was high in rats receiving the compound in toxic doses for a short period, and there were a few in survivors from an LD50 dose. If feeding at 200 and 100 parts per million (p.p.m.) is continued for longer than 1 or 4 weeks respectively the rats die from liver necrosis within the ensuing 7–14 days. If DMN is withdrawn at this time the majority of rats make a fairly rapid recovery and in a few weeks appear healthy. Those that were observed to have kidney tumours had died, or been killed because they were ill, 47–112 weeks after DMN administration had been stopped. The cause of death was in some cases attributable to the mass of tumour and associated destruction of the kidney tissue; in others illness was due to lung infection or other causes and the kidney tumours were found at necropsy. A surprising amount of kidney destruction seemed to be compatible with survival of the rat.

From these observations it was clear that kidney tumours were best produced in our rats by feeding DMN at high levels for a short period, and so a further experiment was done in which males and females were fed with DMN at 100 p.p.m. for 4 weeks. Some rats were killed immediately after DMN feeding was stopped to see whether any acute renal lesions had been produced by the DMN. At this time the animals all had obvious liver damage, though it varied in severity from rat to rat, but no renal lesions were detected. Other rats were killed at intervals and a careful naked-eye and microscopic examination was made of the kidney in order to study the initial stages of the lesion. The findings are summarised in table II. There was a high incidence of kidney tumours in both sexes, though they appeared to develop earlier in the females than in the males. Of the 18 rats examined

at 26 weeks or more, 13 had ki
that showed anything more tha

T

Summary of experiments in which rats
a range of concentrations for diff
became very ill and were killed

Expt	DMN	No.	Sex
1	50 p.p.m.* diet	20	M. and
2	20 ,,	6	F.
	10 ,,	6	F.
	5 ,,	6	F.
3	50 ,,	6	F.
	50 ,,	6	F.
	50 ,,	6	F.
	50 ,,	6	F.
4	100 ,,	6	M.
	100 ,,	6	M.
	100 ,,	6	M.
	100 ,,	6	M.
	200 ,,	6	M.
5	30 mg. per kg. body weight	36	24 M. 12

* Parts per million.

T

Kidney tumours found in 17 male
p.p.m. in their food for 4 weeks
rats were killed at 4 weeks and 5
2 weeks

Time from start of expt (wk)	No. kill
12	4
26	4
36	4
38	6
48	4

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suggestive of neoplasia, but th
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Tumou

The tumours of the kidney
into two groups: well-differ

n a part mixer. The concentrations in
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ment. The rats were started at 100–150 g.

rats, and where death occurred naturally
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SULTS

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general description of the kidney
nly one dose of DMN, some received in
mide (DMF), which had no apparent
. Whilst no kidney tumours were
s low enough to permit their survival
incidence was high in rats receiving
short period, and there were a few
If feeding at 200 and 100 parts per
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ration had been stopped. The cause
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the males. Of the 18 rats examined

at 26 weeks or more, 13 had kidney tumours, but only 5 had livers that showed anything more than rounded edges and some fusion of

TABLE I

Summary of experiments in which rats were given dimethylnitrosamine (DMN) in a range of concentrations for different periods and observed until they died or became very ill and were killed

Expt	DMN	No.	Sex	Duration of DMN administration (wk)	Incidence of tumours in rats surviving 30 wk or more	
					Liver	Kidney
1	50 p.p.m.* diet	20	M. and F.	42	19/20	0/20†
2	20 ,,	6	F.	102	5/5	0/5
	10 ,,	6	F.	102	2/6	0/6
	5 ,,	6	F.	104	0/6	0/6
3	50 ,,	6	F.	4	1/6	0/6
	50 ,,	6	F.	8	4/5	1/5
	50 ,,	6	F.	12	4/5	1/5
	50 ,,	6	F.	24	1/1	0/1
4	100 ,,	6	M.	1	0/6	0/6
	100 ,,	6	M.	2	0/6	2/6
	100 ,,	6	M.	3	0/6	3/6
	100 ,,	6	M.	4	0/5	4/5
	200 ,,	6	M.	1	0/4	4/4
5	30 mg. per kg. body weight	36	24 M. and 12 F.	(one dose only)	0/15	3/15

* Parts per million. † Magee and Barnes (1956).

TABLE II

Kidney tumours found in 17 male and 18 female rats which received DMN 100 p.p.m. in their food for 4 weeks and were then returned to normal diet. Eight rats were killed at 4 weeks and 5 died from subacute liver damage in the following 2 weeks

Time from start of expt (wk)	No. killed	Proportion of	
		male rats developing kidney tumours	*female* rats developing kidney tumours
12	4	0/2	0/2
26	4	0/2	2/2
36	4	2/2	2/2
38	6	—	5/6
48	4	2/4	—

lobes. In one liver there was a mass of hyperplastic parenchyma suggestive of neoplasia, but the others showed gross shrinkage of the left lobes with multiple cystic lesions.

Tumours of the kidney

The tumours of the kidney can be divided for descriptive purposes into two groups: well-differentiated, obviously epithelial tumours

whose origin from renal tubules seems reasonably certain, and highly anaplastic tumours whose histogenesis is not established. Both forms of tumour can be traced back to very early stages and they sometimes occur together in the same kidney. Sometimes the same type of tumour occurs bilaterally, sometimes each type occurs on one side. A general description of the typical form of each tumour type, followed by descriptions of their earlier development will be given.

Well-differentiated tumours

Macroscopically, these tumours appear as firm pinkish-grey lobulated masses arising from the surface of the kidney (fig. 1). Sometimes the tumour masses are very large (fig. 2) occupying much of the abdominal cavity, and they may become adherent to adjacent organs; sometimes they are so small as to be barely visible on the surface. Many intermediate sizes are seen (fig. 1, tumour of right kidney; fig. 3). There is no evidence of growth along the renal vein.

Microscopically, these tumours are usually very well differentiated. Sometimes the lesions have not attained a great size when the animal is killed and in some the darker-stained tumour can be readily seen against the lighter surrounding renal tissue (fig. 4). The tumour extends from the renal capsule, which it raises slightly, throughout the width of the cortex into the medulla, almost reaching the base of the renal papilla. Although sharply demarcated from the renal parenchyma by its darker cells, it has no capsule. In one small segment of its circumference there is evidence of compression of adjacent tubules and glomeruli suggestive of early formation of a pseudo-capsule. The structure of the tumour mimics that of the renal tubules very closely (fig. 5). The neoplastic tubular formations are closely packed, with only a rather scanty fine fibrous framework, and although they are quite definite they usually have no lumina. The tumour cell has a small round or oval vesicular nucleus with rather scanty chromatin and a central basophilic nucleolus. The cytoplasm is more deeply basophilic than that of the renal tubular cells and is very finely granular without vacuoles; the cell boundaries can often be seen quite clearly. Mitotic figures in this type of tumour are uncommon.

Another type of differentiated tumour occurs which is more palely stained (fig. 6). Tubular differentiation is less definite in this type and intersecting fine fibrous strands are more in evidence, giving an impression of lobulation. The cells are larger and cell boundaries are seen with difficulty or not at all. The nuclei are rather variable in size and the cytoplasm is abundant, often containing large vacuoles (fig. 7).

These tumours grow by expansion; rarely they remain confined within the kidney causing atrophy of renal parenchyma, so that the final appearance may be of a tumour surrounded by a rim of kidney tissue, the whole not much bigger than a normal rat kidney. More

Fig. 1.—Large lobulated tumour arising from left kidney, and smaller tumour on right side.

Fig. 2. ki

Figs. 1, 2 and 3.—Female rat. Dime weight; single oral d

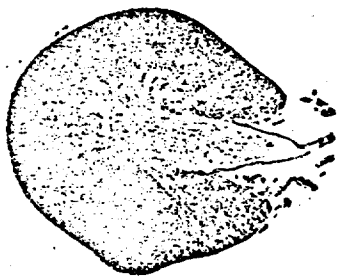

Fig. 4.—×3·5.

Figs. 4 and 5.—Female rat. DMN 3 Sharply demarcated well-differe

Fig. 6. - Male rat. DMN 200 parts per m (p.p.m.) in diet for 1 week, follow normal diet for 98 weeks. Killed. stained well-differentiated tumour. fig. 4.) ×35.

Figs. 2-7 from preparatio

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DIMETHYLNITROSAMINE RAT KIDNEY TUMOURS

FIG. 1.—Large lobulated tumour arising from left kidney, and smaller tumour on right side.

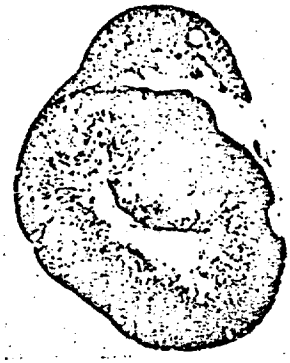

FIG. 2.—Tumour of left kidney. ×2¼.

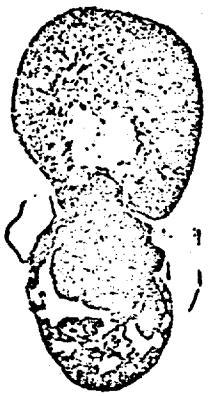

FIG. 3.—Tumour of right kidney. ×3.

FIGS. 1, 2 and 3.—Female rat. Dimethylnitrosamine (DMN) 30 mg. per kg. body weight; single oral dose. Killed 104 weeks later.

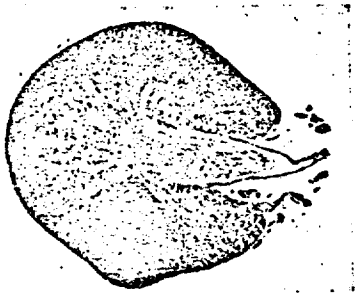

FIG. 4.—×3·5.

FIG. 5.—×65.

FIGS. 4 and 5.—Female rat. DMN 30 mg. per kg. body weight; single oral dose. Sharply demarcated well-differentiated tumour of kidney without capsule.

FIG. 6.—Male rat. DMN 200 parts per million (p.p.m.) in diet for 1 week, followed by normal diet for 98 weeks. Killed. Palely stained well-differentiated tumour. (Cf. fig. 4.) ×35.

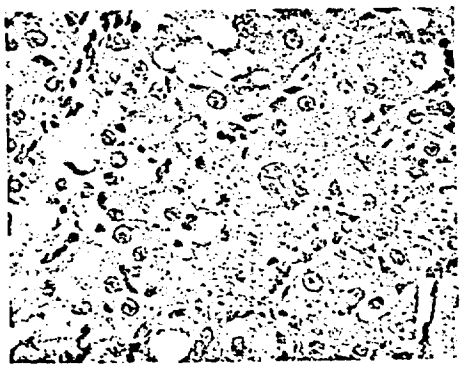

FIG. 7.—Male rat. DMN 200 p.p.m. for 1 week, followed by normal diet for 86 weeks. Killed. Palely staining kidney tumour; variation in nuclear size, vacuoles in cytoplasm and indistinct nuclear boundaries. ×265.

FIGS. 2–7 from preparations stained with hæmatoxylin and eosin.

commonly the tumours expand outwards until they reach the renal capsule, which they raise, and then appear to grow more easily. An extreme case of this type of growth is shown in fig. 3. Here the growth has started in the lower pole of the kidney (fig. 1, tumour of right kidney), and the intrarenal part is well circumscribed by the renal tissue, but the extrarenal part has expanded in fungating fashion to produce a fairly large tumour. It appears to have lifted and stretched the renal capsule, but has nowhere penetrated it. The majority of tumours of this type, some of which become enormous, fall between these extremes. A typical example of a very large well-differentiated tumour is shown in fig. 2. The surviving kidney tissue is stretched over the tumour, rather in the form of a cap, and there is massive breakdown of tumour tissue. Even the very large growths are still limited by a capsule, often greatly thickened, which appears to be derived from the renal capsule. More commonly the cells are the darker-staining type with well-differentiated tubular formations, sometimes with a marked papillary arrangement (fig. 8). Differentiation may be less good in some areas where the cells are arranged in trabecular fashion. Some of the tumours are of the paler-cell type with more abundant, sometimes almost foamy cytoplasm. Occasionally this appearance is sufficiently marked to resemble the typical clear cell of human renal carcinoma (fig. 9). The fibrous stroma is variable in amount, and occasionally undergoes cartilaginous metaplasia (fig. 10). A rare variant is much less well differentiated and grows by invasion rather than by expansion (fig. 11). This type of tumour appears to be of epithelial origin on histological and cytological grounds, and can be distinguished from the very common anaplastic type to be described later.

The probable course of development of these well-differentiated tumours can be traced from a very early stage. The first change in the sequence leading to the tumours seems to be the formation of large tubules with irregular epithelium, sometimes with slight papillary projections into the lumen (fig. 12). Enlarged cortical epithelial cells with big atypical nuclei (Zak *et al.*, 1960) have been observed, and these may represent an even earlier stage. This process of tubular epithelial proliferation continues, so that the lumen of the original tubule may become filled with cells, or the lesion may develop in a more cystic fashion with progressive increase in size, with or without intracystic papillary epithelial proliferation (fig. 13). This process presumably continues until a minute but quite typical adenoma-like lesion is formed in the renal cortex.

Anaplastic tumours

Macroscopically these tumours are variable in appearance depending on the degree of tissue breakdown and hæmorrhage (fig. 14). The tumour tissue is pale and pinkish-white, of a fleshy, sometimes rather gelatinous and friable consistency. It is best seen in small tumours

where degeneration and hæmorrhage are not prominent (fig. 15). Like the well-differentiated tumours, these growths vary considerably in size and may be enormous (fig. 16). The largest tumours occupy much of the abdominal cavity, displacing the abdominal viscera, and may weigh 100 g. or more.

Microscopically the anaplastic tumours are more complex than the well differentiated because they grow by infiltration rather than expansion and thus cause greater distortion of the renal architecture. The larger tumours may be partially encapsulated where they have grown outwards, retaining a covering of thickened renal capsule.

The histological appearance of these lesions is very variable in different areas. The tumour tissue is typically anaplastic (fig. 17) and often highly vascular. In large areas the cells occur in masses with no definite arrangement and they are closely packed together with oval, elongated or spindle-shaped vesicular nuclei. The nuclear membrane is well stained and sharp and the chromatin is rather scanty and scattered. One or two small nucleoli are commonly present. The cytoplasm is basophilic, but not very deeply stained, and the cell boundaries are not easily seen. Mitotic figures are usually frequent. The stroma is delicate in the central part of the tumour masses, and scattered tubular structures are quite often seen in the midst of densely packed tumour cells. These structures resemble renal tubules (fig. 17). The junction between the tumour and the renal tissue is much less sharply defined than it is in the well-differentiated tumours. The invading tumour cells are usually more elongated, resembling actively proliferating fibroblasts or sarcoma cells and they infiltrate between the renal tubules (fig. 18). As this process advances, the tubules gradually become engulfed in the main tumour mass and many appear to be strangulated by concentric layers of tumour cells (fig. 19) giving an appearance of whorls with degenerate tubular debris in their centres. The tumours may provoke a very severe fibrous reaction with the formation of areas of densely scirrhous tissue containing scattered tubules and cysts of varying size, and even surviving glomeruli, separated from the kidney by a narrow region of typically elongated invading neoplastic cells. The nature of this scirrhous tissue varies. In some areas it is densely collagenous; in others it is much more cellular, and is composed of the characteristic anaplastic elongated tumour cells. Sometimes the stroma may be quite loose, with a myxomatous appearance, and cartilage formation is not uncommon (fig. 20). The tubular and cystic structures, often very large, are a frequent feature (figs. 16 and 21). They are lined with epithelium, sometimes flattened, sometimes cubical or even columnar. It is usually in a single layer, but may be stratified. These cysts seem to originate in the renal tubules engulfed in the tumour, since active epithelial proliferation of such tubules is seen from time to time. Surviving glomeruli are less commonly found in the scirrhous or more cellular masses, and may show bizarre cellular proliferation.

8.—Differentiated tumour showing papillary structure. ×65.

FIGS. 8 and 9.—Male rat. DMN 100 p.p.m. 44 weeks.

10.—Female rat. DMN 100 p.p.m. for 4 weeks, followed by normal diet for 39 weeks. Killed. Cartilage formation in stroma of differentiated papillary tumour of kidney. ×65.

12.—Female rat. DMN 100 p.p.m. for 4 weeks, followed by normal diet for 3 weeks. Large renal tubule with papillary ingrowth of epithelium. ×265.

FIG. 12 from section stained by period stained

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DIMETHYLNITROSAMINE RAT KIDNEY TUMOURS

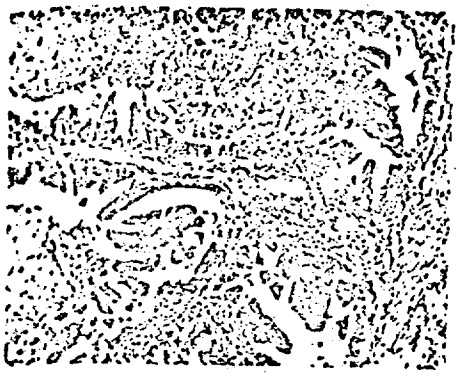

FIG. 8.—Differentiated tumour showing papillary structure. ×65.

FIG. 9.—Kidney tumour with clear cells resembling human renal adenocarcinoma. ×65.

FIGS. 8 and 9.—Male rat. DMN 100 p.p.m. for 4 weeks, followed by normal diet for 44 weeks. Killed.

FIG. 10.—Female rat. DMN 100 p.p.m. for 4 weeks, followed by normal diet for 39 weeks. Killed. Cartilage formation in stroma of differentiated papillary tumour of kidney. ×65.

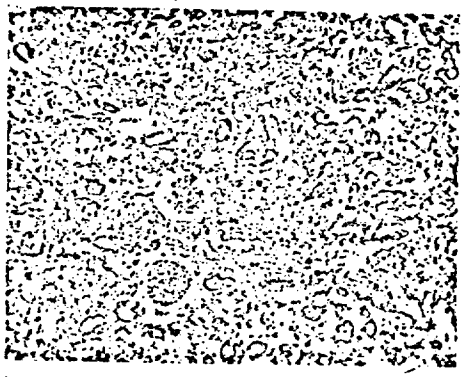

FIG. 11.—Male rat. DMN 100 p.p.m. for 2 weeks, followed by normal diet for 104 weeks. Poorly differentiated, probably epithelial, tumour invading kidney. ×65.

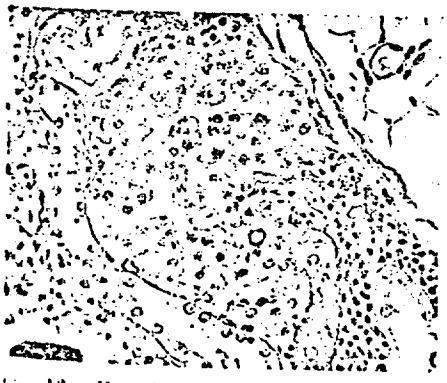

FIG. 12.—Female rat. DMN 100 p.p.m. for 4 weeks, followed by normal diet for 32 weeks. Large renal tubule with papillary ingrowth of epithelium. ×265.

FIG. 13.—Male rat. DMN 100 p.p.m. for 4 weeks, followed by normal diet for 80 weeks. Killed. Intratubular epithelial proliferation and early cyst formation. ×65.

FIG. 12 from section stained by periodic acid-Schiff technique; others from sections stained with H. and E.

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DIMETHYLNITROSAMINE RAT KIDNEY TUMOURS

FIG. 14.—Male rat. DMN 100 p.p.m. 4 weeks, followed by normal diet 64 weeks. Killed. Large tumour of right kidney with hæmorrhagic areas and smaller tumour of left kidney.

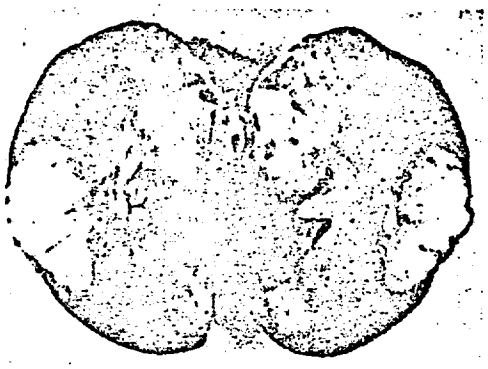


FIG. 15.—Female rat. DMN 100 p.p.m. 4 weeks, followed by normal diet 34 weeks. Killed. Macroscopic appearance of early anaplastic tumours of the kidney.

FIG. 16.—Female rat. DMN 50 p.p.m. 12 weeks, followed by normal diet 57 weeks. Killed. Large anaplastic tumour: cystic and hæmorrhagic areas with small kidney remnant at one end. ×3.

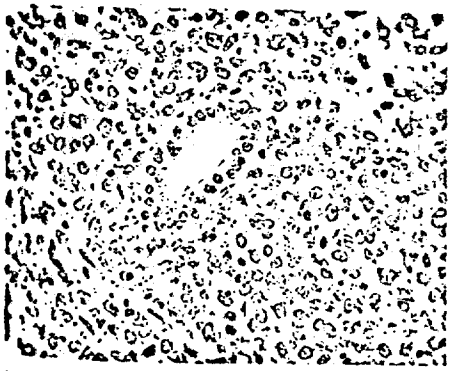


FIG. 17.—Same rat as fig. 16. Typical area: tubular structure in mass of anaplastic tumour cells. ×265.

FIG. 18.—Male rat. DMN 100 p.p.m. 4 weeks, followed by normal diet 44 weeks. Junction between anaplastic tumour and renal tissue: elongated tumour cells invading between tubules. ×265.

FIGS. 16–18 from sections stained with H. and E.

As with the well-differentiated tumours, extensive necrosis, often accompanied by massive hæmorrhage, is common. These degenerative changes are probably ischæmic; the tumours are very large and thrombosis is occasionally seen in large vessels supplying them.

The cell of origin of the anaplastic tumours has not been determined. Regions suggestive of differentiation into tubular structures are frequently encountered, but interpretation of these is difficult because of the occurrence of pre-existing renal tubules in the midst of masses of tumour cells. Very early anaplastic tumours have been studied, where the complications of stromal and cystic reaction are minimal or absent. Serial sections show the typical oval and elongated cells as in figs. 17 and 18, forming the little tumour and actively invading. Sometimes the appearances are suggestive of derivation from renal tubular epithelium, but they are not conclusive. It seems unlikely that the anaplastic tumours are derived from the well-differentiated ones, since they frequently occur alone. When the two types coexist in the same kidney they may approach one another very closely, and the differentiated tumours may even be invaded by the anaplastic, but there is nothing to suggest transformation of one into the other. Conversely, differentiated papillary epithelial proliferation may be found in the cysts occurring in the anaplastic tumours.

Tumours of the liver

All but one of the tumours in this series were hepatocellular carcinomas. They were very similar to those reported previously by us (Magee and Barnes, 1956) and to those described by Schmähl and Preussmann (1959), who used dimethylnitrosamine and by Schmähl, Preussmann and Hamperl (1960), who used diethylnitrosamine. Their main interest in the present context is for comparison of their pulmonary metastases with the primary lung tumours. The one remaining liver tumour is of interest because it seems reasonably certain that it is a carcinoma of the intrahepatic bile-ducts. Macroscopically the lesion was a firm whitish mass occupying and replacing a large part of the liver and there were numerous nodules of a similar appearance and consistency scattered throughout the peritoneal cavity. Microscopically the liver mass is composed of neoplastic glandular structures, some fairly regular, some grossly irregular, with varying amounts of fibrous stroma, sometimes scanty, but predominantly dense. The structure of the peritoneal nodules is closely similar to that of the tumour mass in the liver (fig. 22). Microscopic examination of the kidney of the same rat revealed a small well-differentiated tumour, resembling an adenoma, which was not visible macroscopically. Because of the smallness of the kidney tumour, its high degree of differentiation, and the failure to find secondaries in other animals with this type of tumour, it was concluded that the peritoneal nodules were secondary deposits from a primary carcinoma of the intra-hepatic bile-ducts.

Changes in the liver in rats killed or dying during the earlier stages of the experiments. A systematic study of the development of the liver tumours was not made, but some of the changes observed in the rats killed after short periods of exposure to the carcinogen are interesting. A striking lesion was observed in animals exposed to 100 p.p.m. of DMN for 4 weeks before they were returned to normal diet. The livers during the following month commonly have enormous parenchymal cells with few of normal size. The lesion is very like that seen at similar periods after single doses of the pyrrolizidine alkaloids (Schoental and

Magee, 1957, 1959). These lesions have not been observed after single doses of DMN or higher dietary levels for shorter periods (e.g. 200 p.p.m. for one week). Under these conditions an initial hæmorrhagic parenchymal necrosis is followed by almost complete recovery of the liver, with only minimal fibrosis and adherence of the lobes.

Tumours of the lung

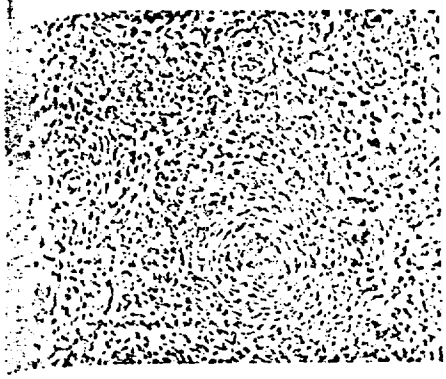
Tumours in the lung have not been common. They are primary pulmonary adenomas and secondary tumours from the kidney and liver. The pulmonary adenomas are not remarkable, resembling those more commonly found in the mouse. In one rat bearing a typical anaplastic kidney tumour the lungs showed an actively growing tumour (fig. 23), whose structure is closely similar to that of the kidney lesion and quite different from that of the pulmonary adenomas; this tumour is regarded as a metastasis from the kidney. The remaining tumours in the lung occurred in rats with hepatocellular carcinomas. In severe cases the greater part of the lung tissue is replaced by rapidly growing highly anaplastic tumour closely resembling the primary in the liver and quite different from the other types of tumour found in the lung. These are considered to be secondaries from the liver.

Lesions occurring in control rats and in rats dying of intercurrent disease

Rats dying during the earlier stages of the experiments usually had lung infections as the main or contributory cause of death. Treated and control animals that lived to old age showed about the expected incidence of the spontaneous diseases of ageing rats, including chronic inflammatory lung lesions with bronchiectasis, chronic kidney disease, sometimes with mesenteric arteritis, pituitary hyperplasia and tumours with hæmorrhages, and a variety of the usual spontaneous tumours. The pathology of these diseases of old rats has been discussed recently (Magee, 1959). Neither in these rats nor in any others of our colony have spontaneous tumours of the kidney been observed at any time.

Discussion

The resemblance between the differentiated kidney tumours induced by dimethylnitrosamine in the rat and the human renal adenocarcinoma (hypernephroma) is very close indeed. The great structural variations of these tumours in man is well known (Evans, 1956; Lucké and Schlumberger, 1957; Willis, 1960). The darker-staining, very well-differentiated tumours with granular cytoplasm (fig. 5) and the lighter-staining types (fig. 6) both have their human counterparts, and the classical human variant with clear cells can be very closely replicated in the rat (fig. 9), though it is less frequently seen in this species. The frequent occurrence of areas of poor differentiation in adult human renal tumours, often with the formation of spindle-cells, gives rise to an appearance suggestive of sarcoma, but most authorities regard these as anaplastic variants of an epithelial tumour. Anaplastic tumours are common in our rat material, but their cell of origin is uncertain. Other features common to both species are the occurrence of cysts, areas of necrosis, often with massive hæmorrhages, and the formation of a false capsule by compression of renal tissue. It seems, therefore, that conclusions drawn from our material may be



19. Male rat. DMN 100 p.p.m. 4 weeks, followed by normal diet 56 weeks. Renal tubules engulfed and surrounded by concentric layers of anaplastic tumour cells. ×165.

Fig. 21.—Same rat as fig. 19. Epithelium-lined kidney tumour.

22. Male rat. DMN 100 p.p.m. 4 weeks, followed by normal diet 73 weeks. Peritoneal nodule: structure closely similar to that of tumour in liver. ×65.

All figures from sections st

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rter [illegible]ls (e.g. 200 p.p.m. for one week).
orrhagic parenchymal necrosis is followed
, with only minimal fibrosis and adherence

of the lung

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y of these diseases of old rats has been
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SCUSSION

the differentiated kidney tumours
e in the rat and the human renal
a) is very close indeed. The great
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, 1957; Willis, 1960). The darker-
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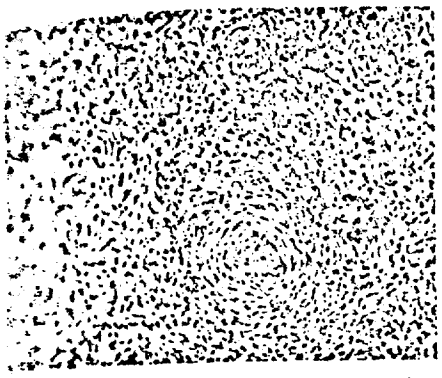
DIMETHYLNITROSAMINE RAT KIDNEY TUMOURS



Male rat. DMN 100 p.p.m. 4 weeks, followed by normal diet 56 weeks. Renal [illegible] engulfed and surrounded by con[illegible] layers of anaplastic tumour cells. [illegible]

FIG. 20.—Male rat. DMN 100 p.p.m. 4 weeks, followed by normal diet 56 weeks. Killed. Cartilage formation in anaplastic kidney tumour. ×65.

[illegible] 21.—Same rat as fig. 19. Epithelium-lined cysts of varying size in anaplastic kidney tumour. ×35.

Male rat. DMN 100 p.p.m. 4 [illegible] followed by normal diet 73 weeks. [illegible] Peritoneal nodule: structure [illegible] similar to that of tumour in liver. [illegible]

FIG. 23.—Female rat. DMN 100 p.p.m. for 4 weeks, followed by normal diet for 42 weeks. Found dead. Lung: metastasis from anaplastic kidney tumour. ×65.

All figures from sections stained with H. and E.

relevant to problems of the human tumours. The history of renal carcinoma has been dominated by the well-known controversy about their derivation since Grawitz' original claim that the tumours arise in adrenal rests (Grawitz, 1883, cited by Willis, 1960). In our rat material it has been possible to trace the development of the well-differentiated tumours from minute proliferative lesions, almost certainly of renal tubular cells, up to large typical adenocarcinomas, apparently histologically identical with their human counterparts. We cannot exclude the possibility that some of these tumours may have arisen from adrenal rests, but have no evidence to support it. However, the predictability with which the tumours arise after dosing with DMN may make a more comprehensive search worth-while to anyone anxious to check this hypothesis.

A major feature of difference from the tumours occurring in man is the failure of the well-differentiated rat tumours to metastasise, and, surprisingly, the occurrence of only one pulmonary metastasis from the anaplastic types. This may be related to the lack of renal vein involvement in the rat material, since there is some correlation between this and frequency of metastasis in man (Riches, Griffiths and Thackray, 1951). Strictly, therefore, the "malignancy" of the well-differentiated tumours remains in doubt. Most writers maintain that no histological distinction can be drawn between human renal adenomas and adenocarcinomas, and take size as a rather arbitrary division between innocence and malignancy, with the reservation that all adenomas are potentially adenocarcinomatous. The size of such tumours as that in fig. 2 coupled with the close structural resemblance to the human lesions suggests that our well-differentiated tumours fall into the same group. The invasive character of the anaplastic tumours, as well as the one pulmonary metastasis (fig. 23), leaves no doubt of their malignancy.

The cell of origin of the anaplastic tumours has not been determined. Early examples of this type of tumour have been encountered and some studied in serial section, but unequivocal evidence of their origin has not been found. A possible derivation is from renal tubular epithelium, since regions suggestive of neoplastic proliferation of these cells with apparent penetration of the tubular basement membrane and infiltrative growth between surrounding tubules may be seen in the early tumours. Origin from the epithelium lining the renal papilla is also possible, since masses of tumour cells are often seen in close apposition to this layer. A third possibility is neoplastic change induced in cells of the renal stroma. The characteristic resemblance to sarcoma and the difficulty of distinguishing between the neoplastic and stromal cells would support this. The cartilaginous areas in the anaplastic tumours (fig. 20) may arise by metaplastic change in the stroma of the tumour, as is probably the case in the well-differentiated tumours (fig. 10). Such stromal metaplasias are well known (e.g. Evans, 1956). On the other hand if the cartilaginous changes are arising in the tumour cells,

sarcoma rather than anaplastic carcinoma is suggested. The kidney containing well-differentiated and anaplastic growth are very like the mixed tumours (carcinosarcomas) which occasionally occur in adult human kidney (R. A. Willis, personal communication). The anaplastic tumours must also be compared with the human nephroblastoma or Wilms' tumour. A transplantable nephroblastoma of the rat has been described by Olcott (1950). The structure of this tumour appears to have been rather similar to that of the anaplastic neoplasms of our animals. The tubular and cystic structures seen in our material (fig. 18) appear probably to arise from pre-existing renal tubules that have been engulfed by the advance of the infiltrating tumour cells but they certainly exhibit much proliferative activity, sometimes with the formation of multiple epithelial layers, which might indicate neoplastic transformation. No evidence of plain or striated muscle was found in the tumours. Whatever the nature of the anaplastic tumours they certainly can occur alone, and there is no evidence to suggest that they ever arise from the well-differentiated ones. The poorly differentiated but more certainly epithelial variety of tumour as shown in fig. 11 is much less common, and the earliest typical anaplastic tumours do not seem to have arisen from this type.

The well-differentiated tumours induced by dimethylnitrosamine resemble those induced by 4′-fluoro-4-aminobiphenyl, reported by Matthews and Walpole (1958). Not only do the histological features appear similar, but these authors comment on the absence of severe pathological changes in the kidney preceding the appearance of neoplastic changes. This is in contrast with the renal adenomas and adenocarcinomas induced in the rat by chronic lead poisoning (Zollinger, 1953) where there is severe renal damage before the tumours appear. Anaplastic tumours similar to those induced by dimethylnitrosamine were not described in either case. A closer structural resemblance to these may be found in the kidney tumours induced by oestrogens in the male golden hamster (Matthews, Kirkman and Bacon, 1947; Horning and Whittick, 1954). These tumours are usually adenomatous, but often have areas of spindle cells resembling sarcoma. Kirkman and Bacon (1950) were unable to determine the cell of origin of the hamster tumours, discussing possible derivation from tubular, renal pelvic and interstitial tissue cells. Horning and Whittick, on the other hand, claimed clear recognition of their origin from the epithelium of cortical tubules. Spontaneous tumours of the kidney do occur in the rat and have been discussed by Eker (1954). They have not been observed in our colony.

With one exception, the liver tumours have been similar to those described previously (Magee and Barnes, 1956; Schmähl and Preussmann, 1959; Schmähl, Preussmann and Hamperl, 1960). The exceptional tumour has the features of a carcinoma of the intrahepatic bile-ducts (Evans, 1956; Edmondson, 1958; Willis, 1960) and there seems little reason to doubt its nature, which is supported by the

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occurrence of mucus-secreting cells in the glandular acini. A detailed study of the histogenesis of the liver tumours has not been made, but the appearance of the livers from rats maintained from 4–8 weeks on the diet containing dimethylnitrosamine is worthy of comment. The extensive replacement of the normal liver parenchyma by very large hepatic cells, with varying degrees of interstitial infiltration by elongated cells and nodular hyperplasia of more normal parenchyma resembles a similar stage in the chronic liver lesion produced by the pyrrolizidine (senecio) alkaloids. However, the alkaloids can induce subacute and chronic liver lesions in the rat after only one dose (Schoental and Magee, 1957), but recovery of the liver from a single dose of dimethylnitrosamine appears almost complete in surviving rats. The pyrrolizidine alkaloids have not been reported to induce tumours of the kidney.

The pulmonary adenomas appeared to be identical with those induced in the rat with acetylaminofluorene by Orr and Bielschowsky (1947) and by Zak *et al.* (1960) with dimethylnitrosamine. They closely resemble the more common pulmonary adenoma of the mouse and we agree with Zak *et al.* that they are primary lung tumours and not metastases from the kidney. Since pulmonary adenomas rarely occur spontaneously in the rat it seems likely that they are caused by dimethylnitrosamine, but a larger number of animals would be necessary to establish this. Quite distinct from these were the metastatic tumours from the anaplastic kidney tumour (fig. 23) and the hepatocellular carcinomas which resembled the primary tumours.

The relation between concentration of dimethylnitrosamine in the diet, time of administration and incidence of liver and kidney tumours summarised in table I may be relevant to current discussion on dose-response in carcinogenesis (Druckrey, Schmähl and Schildbach, 1961). The incidence of liver cancer in the animals fed with the compound continuously in their diet (19 out of 20) is higher than that recorded by Schmähl and Preussmann (1959), who recorded 8 rats with hepatocellular carcinoma and 2 with liver sarcoma out of a total of 19. This may reflect a greater sensitivity in our strain of rats or more stringent diagnostic criteria of malignancy on their part. There is no doubt, however, that dimethylnitrosamine is powerfully carcinogenic at a level of 50 p.p.m. in the diet. Whilst, in our experience, no tumours are induced at 5 p.p.m., it would be quite unjustifiable to draw conclusions from these figures, since the numbers of animals in most of the groups are small. The table does suggest, however, that dimethylnitrosamine may be a useful carcinogen in experiments on incidence of tumours in relationship to dose. The occurrence of kidney tumours in rats many months after they have received only one dose of dimethylnitrosamine should be emphasised, especially as there is evidence that most or even all of the compound, as such, has been metabolised or excreted within 24 hours (Dutton and Heath, 1956; Magee, 1956).

Recent biochemical work suggests that dimethylnitrosamine may cause its acute damaging effect on the liver by methylation of cell constituents, notably proteins and nucleic acids (Magee and Farber, 1962; Magee and Hultin, 1962). It may be that the induction of cancer in the liver and kidney is also related to methylation of nucleic acids. This is discussed more fully elsewhere (Magee, 1962).

Summary

Tumours of the kidney were induced by feeding rats with diets containing dimethylnitrosamine at levels from 50 to 200 p.p.m. for periods varying from 1 week to 3 months, and then returning them to a normal diet. Kidney tumours also appeared in some rats that had survived a single oral LD50 dose of dimethylnitrosamine. Histologically the tumours were of two types: one was well-differentiated, closely resembling human renal adenocarcinoma, and obviously of epithelial origin; the other was highly anaplastic and its histogenesis was not determined. Tumours of the liver and lung were also observed.

We would like to thank Professor R. A. Willis for his advice on the histology of kidney material and for his help in the preparation of this paper. We also thank Dr Cuthbert Dukes for valuable discussion on the kidney tumours. We are indebted to Mr R. F. Legg for the photomicrographs.

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A. Willis for his advice on the histology the preparation of this paper. We also discu n on the kidney tumours. We are omi raphs.

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Toxic Liver Injury and Carcinogenesis

METHYLATION OF RAT-LIVER NUCLEIC ACIDS BY DIMETHYLNITROSAMINE *IN VIVO*

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In the preceding paper (Magee & Hultin, 1962) it was shown that methylation of proteins of rat-liver slices occurred during incubation of the slices with the hepatotoxin and carcinogen dimethylnitrosamine. This finding supports the hypothesis that dimethylnitrosamine undergoes enzymic oxidative *N*-demethylation in the liver with subsequent intracellular formation of a methylating agent, possibly diazomethane. Evidence for reaction with nucleic acids was also obtained. The reactions of nucleic acids and their components with alkylating agents have been studied very extensively *in vitro*

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(Timmis, 1960); there is a marked chemical specificity for alkylation on the 7-position of the guanine moieties. This has been shown with dimethyl sulphate (Lawley & Wallick, 1957; Reiner & Zamenhof, 1957), diazomethane (E. Shaw, personal communication) and mustard gas (Brookes & Lawley, 1960). The last-named authors showed further that similar alkylation occurs *in vivo* when ascites tumour cells are exposed to mustard gas.

It seemed probable, therefore, that methylation of nucleic acids might occur in the livers of animals treated with dimethylnitrosamine and in any other organs capable of *N*-demethylation of the compound. This paper describes experiments in which rats were injected with [^{14}C]dimethylnitrosamine and the incorporation of radioactivity into proteins and nucleic acids of liver, kidney and other tissues was measured. The detailed labelling of the liver RNA was studied, after hydrolysis, by ion-exchange and paper chromatography. The dose of dimethylnitrosamine was sufficient to cause acute centrilobular necrosis of the liver in all the injected rats and to induce tumours of the kidney in a small proportion of the animals.

MATERIALS AND METHODS

Chemical reagents

Dimethylnitrosamine. This was obtained commercially and purified by distillation (b.p. 151°).

Isotopic materials. [^{14}C]Dimethylnitrosamine was prepared by Dr D. F. Heath by a previously published method (Dutton & Heath, 1956). Sodium [^{14}C]formate, L-[*Me*-^{14}C]-methionine, L-[^{14}C]leucine and DL-[^{14}C]valine were obtained from The Radiochemical Centre, Amersham, Bucks.

Special chemicals. Samples of 1-methylguanine, 7-methylguanine, 6-hydroxy-2-methylaminopurine, 2-dimethylamino-6-hydroxypurine, 2-amino-6-methoxypurine and 7-methylxanthine were gifts from Dr G. H. Hitchings (Wellcome Research Laboratories, Tuckahoe, N.J., U.S.A.).

Animals. Wistar albino rats of the Porton strain were maintained on M.R.C. diet 41B (Bruce & Parkes, 1956). Female rats weighing about 200 g. were used.

Conduct of animal experiments

[^{14}C]Dimethylnitrosamine, suitably diluted with the non-radioactive compound, was dissolved in 0·9% (w/v) NaCl and injected intraperitoneally. The dose was 30 mg./kg. body wt., containing 10–35 μc of radioactivity. The other radioactive compounds were given in doses containing the same amount of radioactivity after solution in 0·9% (w/v) NaCl without the addition of non-radioactive carrier. Specific activities were: valine, 1 mc/m-mole; formate 2 mc/m-mole; methionine, 5 mc/m-mole; and leucine 6 mc/m-mole. The rats were kept during the experimental period in a cage placed in a fume cupboard. At 5 hr. after injection of the isotopic material, the rats were anaesthetized by intraperitoneal injection of sodium pentobarbital (Veterinary Nembutal, Abbott), and blood was withdrawn from the abdominal aorta into a syringe moistened with heparin solution. In most experiments the liver, kidneys, spleen and pancreas were then rapidly dissected out and immediately frozen by immersion in liquid nitrogen.

Preparation of nucleic acid, protein, lipid and acid-soluble fractions

Ribonucleic acid. RNA was prepared by a slight modification (K. S. Kirby, personal communication) of the phenol method of Kirby (1956). The tissues were disintegrated in 0·5% (w/v) naphthalene-1,5-disulphonic acid (disodium salt, pH 6·5), followed by phenol treatment and polysaccharide removal, but dialysis was omitted and the RNA was precipitated from the methylCellosolve–phosphate buffer mixture by the addition of 10 vol. acetic acid. The RNA precipitate was washed with 80% acetic acid, ethanol and ether, twice. In some experiments sodium nucleate was extracted according to Davidson & Smellie (1952), and the RNA nucleotides were separated from the DNA by the modification of the Schmidt & Thannhauser (1945) procedure described previously (Magee & Hultin, 1962). RNA was determined by the method of Webb (1956).

Deoxyribonucleic acid. DNA was prepared essentially by the phenolphthalein diphosphate method of Kirby (1961). After removal of polysaccharide material, the dialysis step was omitted and the DNA was precipitated by adding an equal volume of ethylCellosolve. This precipitate contained a large amount of buffer salts which were removed by repeated solution in water, addition of sodium acetate to a final concentration of 2% and precipitation by 1 vol. of ethylCellosolve. The white fibrous product was washed with ethanol and ether and dried over phosphorus pentoxide *in vacuo*.

Protein. Proteins were isolated by a slight modification of the conventional Schneider (1945) procedure. The tissues were disintegrated in 5% (w/v) trichloroacetic acid containing non-radioactive carrier (10 mg./ml.). After extraction with cold and hot trichloroacetic acid, the tissue residues were washed with water, acetone, ethanol, chloroform–methanol (2:1, v/v), chloroform–methanol–conc. hydrochloric acid (200:100:1, by vol.) (Huggins & Cohn, 1959), ethanol–ether (3:1, v/v) twice and ether twice.

Lipid. The lipid fraction was obtained by evaporation of the pooled lipid extracts to dryness at 60° in an atmosphere of nitrogen, solution of the residue in chloroform–methanol (2:1, v/v) and purification according to Folch, Ascoli, Lees, Meath & LeBaron (1951). The product was dissolved in chloroform and the solution was evaporated to dryness under nitrogen.

Acid-soluble fraction. This was taken as the supernatant obtained by centrifuging the first tissue suspension in 5% trichloroacetic acid. The concentration of [^{14}C]dimethylnitrosamine in this fraction was reduced to negligible levels by repeated extraction with chloroform containing excess of non-radioactive carrier dimethylnitrosamine. Remaining trichloroacetic acid was removed by repeated extraction with ether.

Hydrolysis and chromatography of nucleic acids

Acid hydrolysis. The RNA and DNA preparations were hydrolysed according to Vischer & Chargaff (1948) by heating with N-HCl for 1 hr. at 100°. Under these condi-

tions the purine bases and pyrimidine nucleotides are liberated. The acid-soluble fraction of liver was hydrolysed in the same way. Ion-exchange chromatography of the acid hydrolysates was carried out essentially by the method of Cohn (1949) as modified by Weissmann, Bromberg & Gutman (1957). Dowex 50 cation-exchange resin (H^+ form) was used with gradient elution by hydrochloric acid, increasing in concentration from 1 to 4 N. For smaller amounts of nucleic acid (up to 50 mg.), the column was 1 cm. × 10 cm. For larger amounts of nucleic acid (up to 500 mg.) the column was 2 cm. × 25 cm. The effluent fractions were collected automatically, 10 and 25 ml./hr. from the smaller and larger columns respectively. The extinction of each fraction was measured at 260 mμ against water. The guanine and adenine peaks were identified by their ultraviolet-absorption spectra in each experiment.

Alkaline hydrolysis. This was carried out as described previously (Magee & Hultin, 1962).

Paper chromatography. The products of hydrolysis of nucleic acids were separated on Whatman 3MM paper using the propan-2-ol–ammonia solvent of Markham & Smith (1952) or the propan-2-ol–hydrochloric acid solvent of Wyatt (1951). Ultraviolet-absorbing areas were detected according to Markham & Smith (1949), marked in pencil and eluted with water. Similar areas of paper were cut out and eluted as blanks against which the ultraviolet absorption of the eluates was read at different pH values according to Weissmann *et al.* (1957).

Spectrophotometry and radioactivity measurements

Ultraviolet spectrophotometry was carried out with a Unicam SP. 500 instrument. Solid and liquid samples were assayed for radioactivity as described previously (Magee & Hultin, 1962). Radioautographs were made by exposure of paper chromatograms to Ilford X-ray film.

RESULTS

Preliminary experiments showed that both the protein and the RNA of rat liver became labelled after the animals had been given [^{14}C]dimethylnitrosamine by intraperitoneal injection. As with the system *in vitro* (Magee & Hultin, 1962), the specific radioactivity of the RNA was considerably higher than that of the protein. The ratio of the radioactivity of RNA to that of protein in rats treated with [^{14}C]dimethylnitrosamine was then compared with that in animals receiving various normal metabolites, also labelled (Table 1). Only with dimethylnitrosamine did this ratio exceed unity. The precursors of biological methyl groups, methionine and formate, showed higher ratios than the typical amino acids leucine and valine. The radioactivity of the proteins and RNA of rat liver, kidney, spleen and pancreas was then measured in rats killed 5 hr. after injection of [^{14}C]dimethylnitrosamine (Table 2). The high radioactivity in the liver RNA is in contrast with the lower activity in the kidney and the almost negligible value in the pancreas. This very low activity of pancreatic RNA indicated that the washing and purification procedures were probably adequate to remove radioactivity not chemically combined or otherwise very firmly bound to the nucleic acid. The high radioactivity of the splenic RNA was unexpected but its probable significance became apparent later (Fig. 7).

The greater specific radioactivity of the RNA compared with that of the protein from the livers of the rats given [^{14}C]dimethylnitrosamine was in agreement with results with rat-liver slices *in vitro* (Magee & Hultin, 1962). Alkaline hydrolysis and ion-exchange chromatography of this RNA gave the elution pattern shown in Fig. 1. Nearly all of the radioactivity appeared just before guanylic acid and merged into it. As with the similar peak of radioactivity obtained *in vitro* (Magee & Hultin, 1962), this radioactivity could be well separated from the guanylic acid by paper chromatography with the propan-2-ol–ammonia solvent system (Markham & Smith, 1952). It appeared therefore that the major radioactive compound *in vivo* was the same as that *in vitro*. In contrast with the results *in vitro*, the bases guanine and adenine were almost unlabelled in the experiments *in vivo*.

Table 1. *Incorporation of radioactivity into RNA and proteins of liver in rats treated with* [^{14}C]*dimethylnitrosamine and with some labelled normal metabolites*

All rats received labelled compound containing the same amount of radioactivity (35 μc) by intaperitoneal injection and were killed 5 hr. later. The no. of rats is given in parentheses.

Labelled compound	Specific radioactivity of RNA/specific radioactivity of proteins
[^{14}C]Dimethylnitrosamine (3)	5·3
L-[*Me*-^{14}C]Methionine (3)	0·40
Sodium [^{14}C]formate (4)	0·32
L-[^{14}C]leucine (1)	0·046
DL-[^{14}C]valine (1)	0·040

Table 2. *Distribution of radioactivity in RNA and proteins of some organs of rats treated with* [^{14}C]*dimethylnitrosamine*

Female rats (4), of about 200 g. body wt., received [^{14}C]dimethylnitrosamine (30 mg./kg. body wt., 17·5 μc per rat) and were killed 5 hr. later. Specific radioactivities of proteins and RNA from pooled organs were measured at 'infinite thickness'.

Organ	Specific radioactivity of protein (counts/min./cm.^{2})	Specific radioactivity of RNA (counts/min./cm.^{2})
Liver	215	1063
Kidney	183	279
Pancreas	535	14
Spleen	106	336

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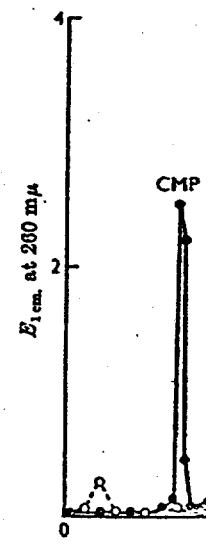

Fig. 1. Ion-exchange c
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After acid hydrolysis of the RNA under conditions yielding free purine bases and pyrimidine nucleotides (Vischer & Chargaff, 1948), ion-exchange chromatography again gave one major peak of radioactivity, now appearing between guanine and adenine (Fig. 2), and very much smaller peaks in the regions of the pyrimidine nucleotides and adenine respectively. The main radioactive peak corresponded exactly with a very small peak of ultraviolet-absorbing material which had the spectrum of a guanine derivative and, from the work of Lawley & Wallick (1957) and Reiner & Zamenhof (1957), seemed likely to be 7-methylguanine. This material was transferred to paper for chromatography with the propan-2-ol–ammonia solvent and comparison with an authentic sample of 7-methylguanine. The unknown and the authentic materials had nearly the same R_F values (0·28 and 0·26 respectively), and the ultraviolet-absorbing band of the unknown corresponded exactly with the radioactive band as shown by radioautography. Both materials were then eluted from the paper and their ultraviolet spectra were plotted against the appropriate blanks at pH 2, 6 and 9 respectively. The curves at pH 2 and pH 9 are shown in Fig. 3. The correspondence between the authentic and the unknown is very close and similar results were obtained at pH 6. The ultraviolet-absorption data also agree well with those given for 7-methylguanine by Weissmann *et al.* (1957). On paper chromatography with the propan-2-ol–hydrochloric acid solvent (Wyatt, 1951), the unknown material had the same R_F as authentic 7-methylguanine, and could be distinguished from

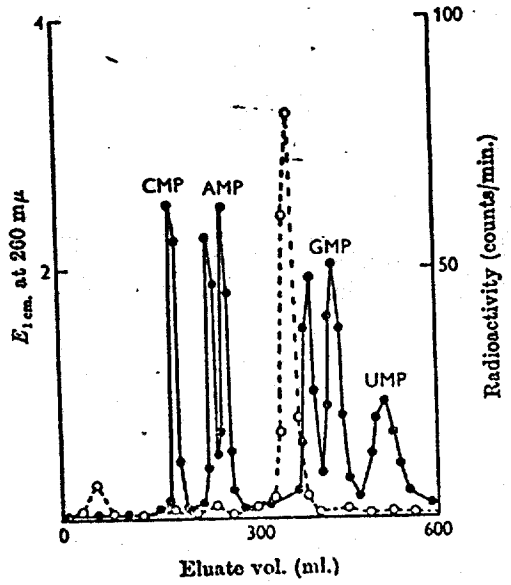


Fig. 1. Ion-exchange chromatography of alkaline hydrolysate of RNA from liver of rat treated with [^{14}C]dimethylnitrosamine (30 mg./kg. body wt., 20 μc) 5 hr. earlier. Dowex 1 (formate form) column (1·5 cm. × 21 cm.) with gradient elution from 1–4 N-formic acid. ●, $E_{1cm.}$ at 260 mμ; ○, radioactivity in counts/min.

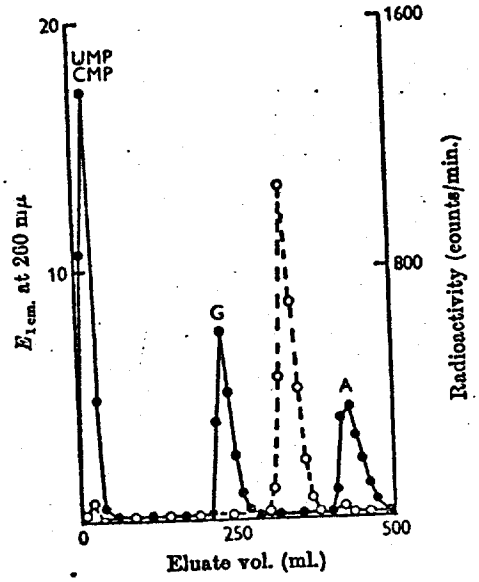


Fig. 2. Ion-exchange chromatography of acid hydrolysate of RNA (30 mg.) from liver of rat treated with [^{14}C]dimethylnitrosamine (30 mg./kg. body wt., 35 μc) 5 hr. earlier. Dowex 50 (H^+ form) column (1 cm. × 10 cm.) with gradient elution from 1–4 N-HCl. ●, $E_{1cm.}$ at 260 mμ; ○, radioactivity in counts/min.; G, guanine; A, adenine.

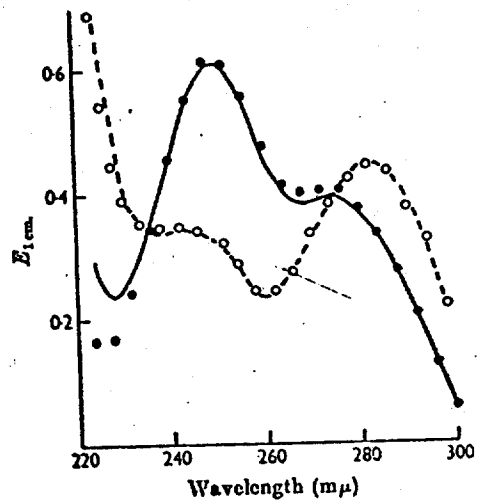


Fig. 3. Ultraviolet-absorption spectra of 7-methylguanine. Smooth curves are for an authentic sample and points for the major peak of unknown radioactive material isolated from the liver of a rat treated with [^{14}C]dimethylnitrosamine (Fig. 2). ●, pH 2; ○, pH 9.

1-methylguanine, 6-hydroxy-2-methylaminopurine, 2-dimethylamino-6-hydroxypurine and 2-amino-6-methoxypurine, which were chromatographed simultaneously on the same paper.

It appeared, therefore, that the unknown major radioactive compound was 7-methylguanine. This was confirmed by conversion of the unknown material into a xanthine derivative by treatment with nitrous acid. Ten rats were injected with non-radioactive dimethylnitrosamine (30 mg./kg. body wt.) in the usual way and RNA was prepared from their combined livers. Acid hydrolysis was followed by ion-exchange chromatography on the larger column and the material appearing between guanine and adenine was collected and added to the acid hydrolysate of the RNA obtained from the liver of one rat treated with [^{14}C]dimethylnitrosamine in the usual way. The combined materials were then applied to the smaller ion-exchange column and eluted as before. The pattern of elution was similar to the previous one (Fig. 2) but there was considerably more ultraviolet-absorbing material corresponding to the main radioactive peak which was derived from the livers of the ten rats treated with non-radioactive dimethylnitrosamine. These fractions were evaporated to dryness and the pooled material was treated with nitrous acid according to Weissman *et al.* (1957). After removal of the nitrous acid, small amounts of guanine and adenine were added as markers and the mixture was rechromatographed on the small column. The radioactivity now emerged considerably earlier, in the expected position of 7-methylxanthine, well before guanine, and was accompanied by an ultraviolet-absorbing peak (Fig. 4). There was now no radioactivity or ultraviolet absorption between guanine and adenine. This material was applied to paper and found to have nearly the same R_F (0·41) as a simultaneously chromatographed authentic sample of 7-methylxanthine (0·40). The unknown ultraviolet-absorbing band again corresponded exactly with the band of radioactivity shown by radioautography. Elution of the unknown and authentic materials and comparison of their ultraviolet spectra showed excellent correspondence between the curves, and the spectral data agreed well with those for 7-methylxanthine given by Weissmann *et al.* (1957). It is concluded that treatment of rats with dimethylnitrosamine under these conditions is followed by the appearance of 7-methylguanine in the RNA of the liver. The amount of 7-methylguanine present corresponds to about 2% of the guanine.

The presence of 7-methylguanine in RNA could be explained by incorporation of an appropriately methylated precursor, by methylation of the polynucleotides or by a combination of the two. If methylated precursors such as nucleosides or nucleotides were involved these should be detectable in the acid-soluble fraction of the liver. This fraction from the liver of a rat was freed from [^{14}C]dimethylnitrosamine and trichloroacetic acid by repeated extraction with chloroform followed by ether. The whole fraction was then hydrolysed with N-hydrochloric acid so that any nucleotide or nucleoside methylated on the 7-position of guanine would be broken down to give 7-methylguanine. The resulting mixture was chromatographed after the addition of small amounts of guanine and adenine as markers. The total amount of radioactivity was not high and several small radioactive peaks appeared as elution proceeded, but there was no significant radioactivity in the expected region of 7-methylguanine between guanine and adenine (Fig. 5).

The labelled RNA from several kidneys was combined and hydrolysed with acid followed by column chromatography. The elution pattern (Fig. 6) was similar to that obtained with liver RNA (Fig. 2) except that the total radioactivity was very much less (Table 2) and the proportion in guanine and adenine was higher. The main radioactive peak occurred in the expected position of 7-methylguanine but insufficient material was present for determination of the ultraviolet-absorption spectrum.

Ion-exchange chromatography of pancreatic RNA, whose total radioactivity was much lower than that of kidney (Table 2), showed only a trace of radioactivity between guanine and adenine and very little activity in these bases.

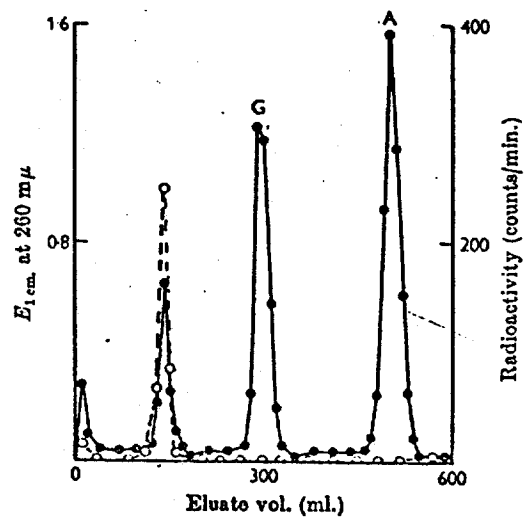
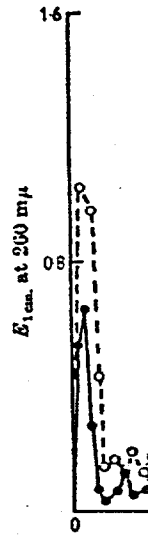


Fig. 4. Rechromatography of radioactive material appearing between guanine and adenine after it had been treated with nitrous acid (see text). Conditions and symbols as in Fig. 2, with guanine (G) and adenine (A) (500 μg. each) added as markers.



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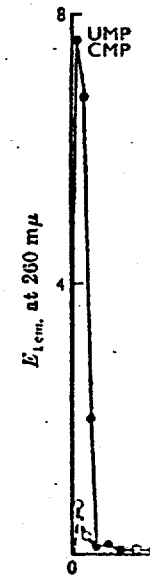

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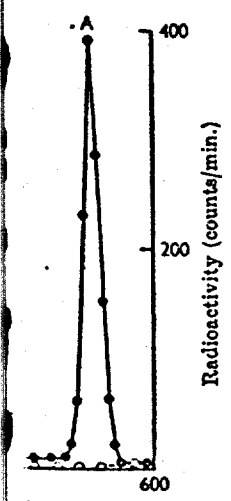

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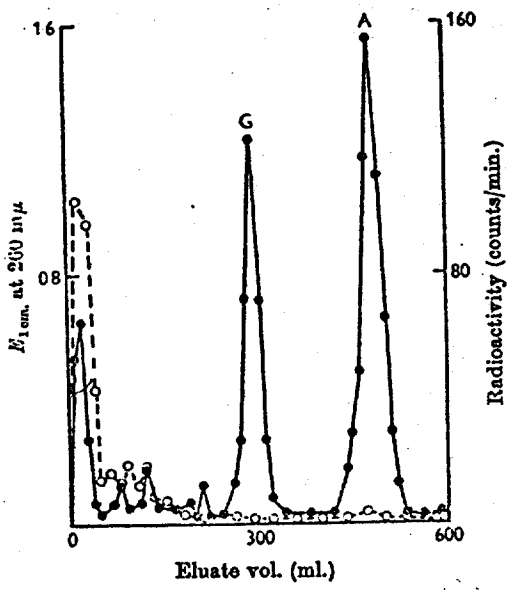

Fig. 5. Ion-exchange chromatography of the acid-soluble fraction of liver from a rat treated with [^{14}C]dimethylnitrosamine 5 hr. earlier. The fraction was hydrolysed with N-HCl and adenine and guanine markers (500 μg. each) were added before application to the column. Conditions of chromatography and symbols as in Fig. 2.

The RNA prepared from spleen was more radioactive than that from kidney or pancreas (Table 2) and its pattern of elution after acid hydrolysis and chromatography is shown in Fig. 7. Here almost all of the radioactivity corresponds exactly with the major normally occurring bases and there is again only a trace in the expected position of 7-methylguanine. A probable explanation of this finding is that the bases in the splenic RNA had been labelled by normal metabolic processes via the C_1 metabolic pool. Support for this was obtained by examination of RNA prepared from spleens of rats treated with sodium [^{14}C]formate. After acid hydrolysis and chromatography, the elution pattern was very similar to that obtained after treatment with [^{14}C]dimethylnitrosamine (Fig. 7) except that there was no detectable radioactivity at all in the position of 7-methylguanine.

The finding of 7-methylguanine in the RNA of rat liver suggested that it might also be present in the DNA. DNA, RNA and protein were isolated from the pooled livers of four rats which had each received 14 μc of [^{14}C]dimethylnitrosamine 5 hr. before being killed. The specific radioactivities of the RNA and protein were 810 and 160 counts/min./cm.^{2} respectively and that of the DNA was 239 counts/min./cm.^{2} before removal of polysac-

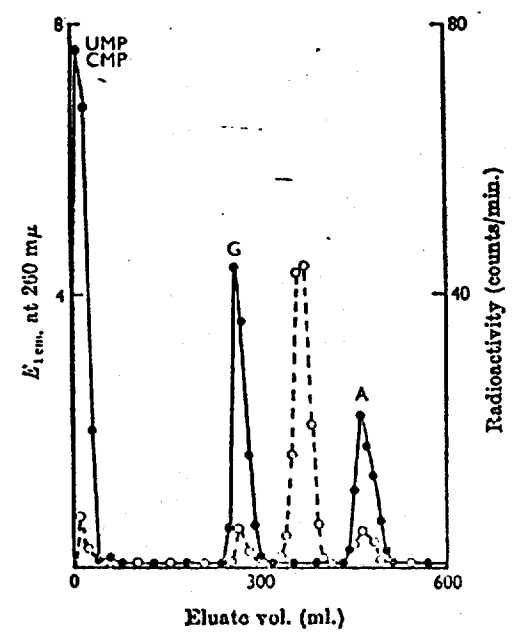

Fig. 6. Ion-exchange chromatography of pooled RNA from kidneys of rats treated with [^{14}C]dimethylnitrosamine (30 mg./kg. body wt.) 5 hr. earlier. Conditions and symbols as in Fig. 2.

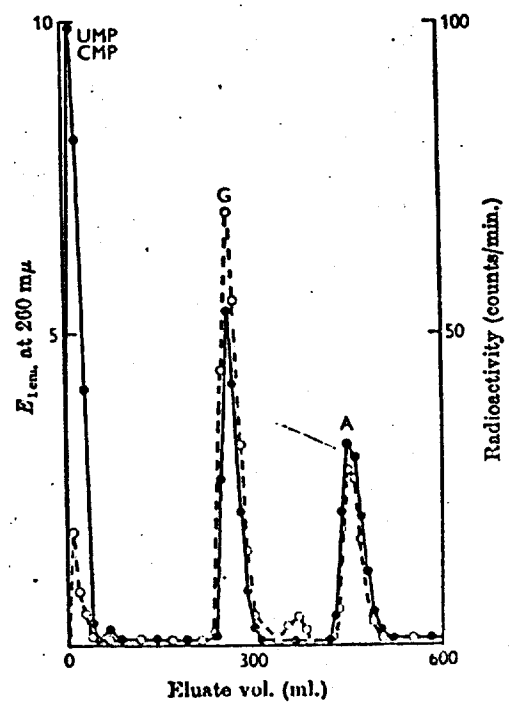

Fig. 7. Ion-exchange chromatography of pooled RNA from spleens of rats treated with [^{14}C]dimethylnitrosamine 5 hr. earlier. Dose and conditions as in Fig. 6.

charide material. This DNA was hydrolysed with N-hydrochloric acid and chromatographed on Dowex 50 (H^+ form) in the same way as the RNA. The elution pattern shown in Fig. 8, where there is a large single peak of radioactivity in the expected region of 7-methylguanine. The total radioactivity in this peak is 411 counts/min. It can be calculated from Fig. 2 that this amount of radioactivity could have been due to the presence of 13 mg. of RNA in the DNA sample. According to Kirby (1961), however, DNA prepared by the phenolphthalein diphosphate method is free from detectable RNA, and moreover determinations of RNA in our DNA samples indicate that not more than 1 mg. of RNA could have been present, and probably less. It appears, therefore, that the liver DNA like the RNA contains 7-methylguanine. DNA was also prepared from the kidneys of two rats which had received 20 μc of [^{14}C]dimethylnitrosamine 5 hr. earlier. It had low but definite radioactivity (30 counts/min./cm.^{2}) and contained no detectable RNA.

The total lipid material obtained from about 2 g. of liver pooled from four rats killed 5 hr. after receiving [^{14}C]dimethylnitrosamine was assayed for radioactivity after evaporation on a metal planchet. It was found to have 50 counts/min., indicating that the lipid, like the proteins and nucleic acids, contains radioactivity incorporated *in vivo* from dimethylnitrosamine.

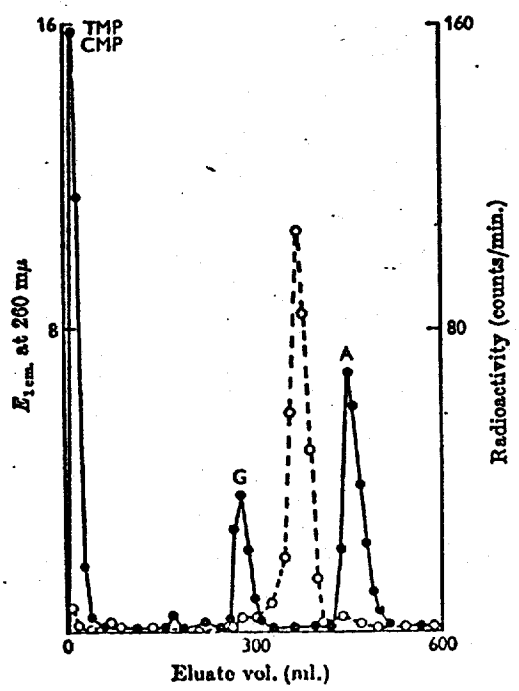

Fig. 8. Ion-exchange chromatography of pooled DNA from rats treated with [^{14}C]dimethylnitrosamine (30 mg./kg. body wt., 14 μc) 5 hr. earlier. Conditions and symbols as in Fig. 2.

DISCUSSION

The results of this investigation clearly show that the hepatotoxin and carcinogen dimethylnitrosamine is capable of methylating both RNA and DNA *in vivo* to produce the abnormal guanine derivative, 7-methylguanine. Although insufficient material was available to enable the identification of this compound by all the classical chemical procedures, the evidence presented leaves very little doubt about its nature. The position of the abnormal guanine in the column and paper chromatograms, its absorption spectrum virtually identical with that of an authentic sample of 7-methylguanine, and its conversion into 7-methylxanthine on deamination all strongly favour its identity with 7-methylguanine.

This alkylated guanine is the only unusual compound found in more than minute amounts in the nucleic acids labelled with dimethylnitrosamine. The other components labelled were guanine and adenine, no doubt due to the incorporation into the purine skeleton of a C_1 fragment derived from dimethylnitrosamine. These findings are chemically analogous to those observed in the protein labelled with dimethylnitrosamine (Magee & Hultin, 1962) where, apart from probable carboxyl methyl esters, histidines methylated in the 1- and 3-positions of the imidazole ring were among the major abnormal components found. It appears, therefore, that there is a special predilection of the methylating agent derived from dimethylnitrosamine for the imidazole ring of histidine or guanine. The similarity of the reaction with nucleic acids *in vivo* with that of alkylating agents *in vitro* (Timmis, 1960) is very striking. The methylation of guanine in liver RNA is considerably more active than is that of histidine in protein.

As discussed in the preceding paper, it was suggested by Schoental (personal communication) and by Rose (1958) that diazomethane may be the methylating agent derived from dimethylnitrosamine. A possible scheme for the metabolism of dimethylnitrosamine by rat liver incorporating this suggestion and based on previous work (Heath & Dutton, 1958; Brouwers & Emmelot, 1960; Magee & Hultin, 1962) and on the present findings is shown in Fig. 9. It should be emphasized that, in this scheme, the initial oxidative *N*-demethylation of dimethylnitrosamine is enzymic. Whether diazomethane is actually formed or whether methylation occurs by some other mechanism is not yet established. The recent demonstration of the existence of the very unstable monomethylnitros-

amino and its decomposition to diazomethane and water (Müller, Haiss & Rundel, 1960) supports the concept of the formation of the diazoalkane. On the other hand, Austin (1960) maintains that breakdown of the monomethylnitrosamine may lead to the formation of a carbonium ion, which may be responsible for methylation. Recent work on the relation of chemical structure to biological activity by Druckrey, Preussmann, Schmähl & Müller (1961) and by D. F. Heath (personal communication) is in agreement with the alkylation hypothesis. Heath has shown that *n*-butylmethylnitrosamine induces acute changes in rat liver similar to those induced by dimethylnitrosamine, but that *tert.*-butylmethylnitrosamine, which would not be expected to yield a diazoalkane metabolically, does not.

The occurrence of 7-methylguanine in liver RNA could be explained by chemical methylation of the intact RNA molecule or by incorporation of a methylated precursor. The second possibility is made unlikely by the absence of any radioactive material corresponding to 7-methylguanine in the acid-soluble fraction of the liver after hydrolysis (Fig. 5). The incorporation of a nucleoside or nucleotide methylated in the 7-position is also unlikely because the N-7 atom must become quaternary with the formation of the ribosyl bond on the N-9 atom. In addition to making the molecule unstable, such a change might alter the character of the molecule as a substrate for the enzyme systems involved in the incorporation of precursors into RNA.

The pattern of incorporation of ^{14}C into RNA and protein in the different tissues (Table 2) is interesting in relation to the pathological changes induced by dimethylnitrosamine. At the dose level given the liver is acutely damaged with severe haemorrhagic necrosis (Barnes & Magee, 1954) though severe damage in the kidney, pancreas and spleen is minimal or absent. In some of the rats, however, tumours of the kidney would be expected to appear after about a year (Magee & Barnes, 1959, 1962). There is a clear correlation between pathological action and incorporation into RNA of liver, kidney and pancreas but with spleen it only becomes apparent when it is seen that nearly all of of the radioactivity of the RNA is in the commonly occurring bases (Fig. 7). There is no correlation between the radioactivity of the tissue proteins and the distribution of the pathological lesions, but one might become apparent if the relative incorporation into normal and into abnormal protein constituents were known. The high figure in the pancreatic protein probably reflects the very rapid rate of protein synthesis in the pancreas. The radioactivity of the liver protein is rather low in relation to the other tissues, which may be explained by the

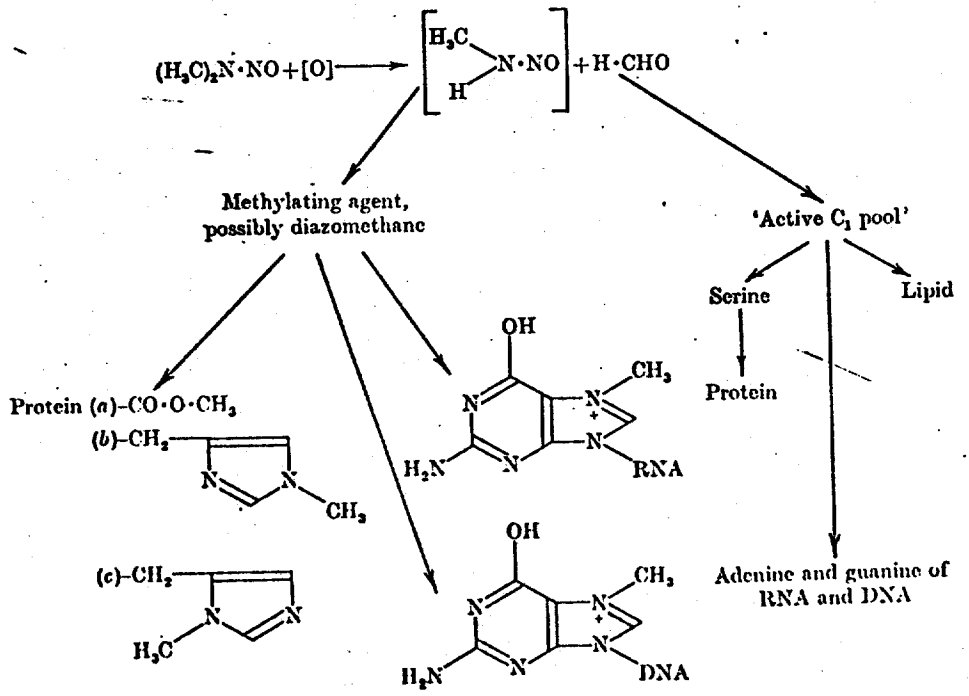

Fig. 9. Some postulated metabolic pathways of dimethylnitrosamine.

inhibitory effect of dimethylnitrosamine on amino acid incorporation into the liver (Magee, 1958; Hultin, Arrhenius, Löw & Magee, 1960).

Although insufficient material was available for ultraviolet spectroscopy, the position of the radioactive material in the chromatographic profile of the kidney RNA (Fig. 6), coupled with earlier evidence that dimethylnitrosamine is metabolized to a small extent in the kidney (Magee & Vandekar, 1958), support the conclusion that methylation similar to that in the liver has taken place, though to a much smaller degree. Similarities of the chromatographic profiles of the splenic RNA after administration of [^{14}C]dimethylnitrosamine (Fig. 7) and of [^{14}C]formate respectively are quite consistent with the predicted incorporation from the C_1 metabolic pool of a derivative of dimethylnitrosamine. The very low total radioactivity of pancreatic RNA is also consistent with this (Table 2). The traces of radioactivity in the 7-methylguanine position of the chromatograms of the splenic and pancreatic RNA emphasize the probable necessity of metabolic activation of dimethylnitrosamine before methylation of cell constituents can occur. Even though the compound is distributed in about the same concentration in all the viscera (Magee, 1956), the spleen and pancreas have a negligible capacity to metabolize dimethylnitrosamine *in vitro* (Magee & Vandekar, 1958).

The methylation of DNA in the liver (Fig. 8) seems reasonably well established, since the largest amount of possible RNA contaminant would not have been enough to account for the observed radioactivity unless this RNA was derived from a fraction with very high specific radioactivity. The same probably applies to the kidney DNA, which contained no detectable RNA. However, since the specific radioactivity was low, no firm conclusions on this matter can be drawn.

The possible importance of the methylation of RNA and DNA in carcinogenesis deserves brief consideration. The somatic-mutation hypothesis of the origin of cancer was introduced by Boveri (1914); the frequent occurrence of mitotic abnormalities in tumours led to the idea that neoplasia might originate in cells produced by such abnormal divisions. More recently, it has been suggested that changes in the molecule of DNA, not necessarily accompanied by chromosome changes, may be responsible for the production of mutations and for carcinogenesis (Boyland, 1954; Haddow, 1958). Although this concept has been supported by much work on reactions of carcinogens and mutagens with DNA *in vitro* (Boyland, 1954), evidence of any combination between a carcinogen and DNA occurring *in vivo* has appeared only very recently. Brookes & Lawley (1960) demonstrated alkylation of DNA in Ehrlich ascites-tumour cells by sulphur mustard but the mustards, although very cytotoxic, are not highly carcinogenic. In a preliminary communication (Farber & Magee, 1960) we reported the probable ethylation of the RNA of liver in rats treated with the hepatic carcinogen [^{14}C]-ethionine as well as the methylation of RNA by dimethylnitrosamine. This work with ethionine has been confirmed and extended by Stekol, Mody & Perry (1960) who also reported binding to DNA. The methylation of DNA in rat liver and probably also in kidney which has been demonstrated in the present paper may be relevant to the induction of cancer. As Lawley (1957) has pointed out, alkylation of DNA on the 7-position of guanine leads to instability with the alkylated base splitting out of the molecule. This might lead to breakage of the chain as with the apurinic acids (Brown & Todd, 1955). If the mechanism of carcinogenesis is similar to that of mutation it may also be relevant that Tessman (1959) has demonstrated the production of mutants of the $\phi \times 174$ bacteriophage following the alteration of only one base of the DNA by treatment of the phage with nitrous acid, which may also be a metabolite of dimethylnitrosamine (Heath & Dutton, 1958).

The suggestion that cancer may arise as an extrachromosomal event was made by Lewis (1935) and has received support from Haddow (1944, 1947), Darlington (1948) and Kaplan (1959). The possibility that RNA may be involved in this process is supported by the discovery of infective viral RNA (Fraenkel-Conrat, 1956; Gierer & Schramm, 1956), and by the finding that the nucleic acid of some tumour viruses is RNA (Epstein & Holt, 1958; Bonar & Beard, 1959). The methylation of liver and kidney RNA in rats treated with dimethylnitrosamine suggests the possibility that alterations in this nucleic acid may play a part in the induction of cancer by this compound.

Hems (1958) showed that the 7-position of the imidazole ring of guanosine is particularly vulnerable to ionizing radiation with subsequent ring opening and formation of a 5-formamidopyrimidine derivative. It is probable that a similar pyrimidine derivative is formed on alkaline hydrolysis of rat-liver RNA prepared from rats treated with dimethylnitrosamine (Fig. 1). It seems, therefore, that essentially similar changes in the cellular nucleic acids may occur in radiation injury and in chemical injury by dimethylnitrosamine, and also in viral mutagenesis.

The occurrence of 7-methylguanine in human urine has been known since the work of Krüger & Salomon (1898) and has been confirmed recently by Weissmann *et al.* (1957) and by Wyngaarden, Blair & Hilley (1958). Its presence in dog urine has also been reported (Jackson & Entenman, 1957). The origin of the urinary compound is still not clear but

presumably it is derived from the blood passing through the kidney. It also appears that it can occur naturally in the RNA of *Escherichia coli* (D. B. Dunn, personal communication). It is possible, therefore, that minute quantities of 7-methylguanine may be formed in human nucleic acids by methylating agents in our environment and this, perhaps, might play a part in the induction of cancer in man.

SUMMARY

1. Rats were injected intraperitoneally with the hepatotoxin and carcinogen dimethylnitrosamine labelled with radioactive carbon. The dose was adequate to cause acute necrosis of the liver in all the rats and to induce tumours of the kidney in some of the survivors about a year later. The animals were killed 5 hr. after injection when the liver injury was not far advanced.

2. Incorporation of radioactivity into RNA and protein of liver, kidney, spleen and pancreas was observed and compared with that following similar intraperitoneal injection of labelled methionine, leucine, valine and formate. Incorporation from dimethylnitrosamine occurred into DNA of liver and probably also of kidney. The lipid fraction of liver became labelled.

3. Alkaline hydrolysis of the labelled liver RNA followed by ion-exchange chromatography revealed nearly all of the radioactivity in a peak close to, but distinct from, guanylic acid. This material could be separated from guanylic acid by paper chromatography.

4. Acid hydrolysis of the labelled liver RNA, followed by ion-exchange chromatography, revealed almost all the radioactivity appearing between, and separate from, guanine and adenine, with a corresponding small peak of ultraviolet absorption. This material was identified as 7-methylguanine from its behaviour on ion-exchange and paper chromatography and from its ultraviolet-absorption spectrum. Treatment of this material with nitrous acid gave a product which was identified as 7-methylxanthine by the same criteria.

5. No evidence of 7-methylguanine was found in acid hydrolysates of the acid-soluble fraction of liver from rats treated with radioactive dimethylnitrosamine.

6. Radioactive material in the expected position of 7-methylguanine was found on ion-exchange chromatography of acid hydrolysates of kidney RNA and liver DNA but insufficient material was present for ultraviolet-absorption spectra. Traces only of radioactivity in the position of 7-methylguanine were found in hydrolysates of pancreas and spleen RNA. The latter showed considerable incorporation into guanine and adenine. The chromatographic profile of splenic RNA from rats treated with radioactive formate was very similar to that with dimethylnitrosamine but there was no measurable radioactivity in the position of 7-methylguanine.

7. These results are considered to support the hypothesis that an active methylating agent is formed metabolically from dimethylnitrosamine which methylates tissue components *in vivo*. This hypothesis is discussed in relation to current ideas on carcinogenesis, viral mutagenesis and cell injury by alkylating agents and radiation.

We would like to thank Dr D. F. Heath for the [^{14}C]-dimethylnitrosamine and Dr G. H. Hitchings for the methylated purines. We are grateful to Dr G. M. Timmis, Dr Elliot Shaw and many others for helpful discussion, and to Mr J. A. E. Jarvis and Mr R. Price for technical assistance. This work was supported in part by grants from the American Cancer Society and the U.S. Public Health Service (A 610).

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Citrate-Promoted Helix Formation in Gelatin

THE VISCOSITY–TIME EFFECT

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The changes which take place when native collagen is converted into the water-soluble protein, gelatin, are generally regarded as irreversible reactions. Veis, Anesey & Cohen (1958) describe the essential aspects as: (1) irreversible melting of the polypeptide helical coils; (2) irreversible severance of inter-peptide-chain bonds; (3) irreversible hydrolysis of peptide linkages. Courts (1958) showed that (3) involved only especially-labile peptide bonds and that (1) is likely to be the final stage, resulting from rupture of hydrogen bonds.

In special instances, however, evidence from the application of widely differing techniques shows that the random-coil structure of gelatin molecules appears partially to re-form a collagen-type configuration. Thus Robinson (1953) prepared gelatin films from a 5% solution by hot (55°) and cold (18°) evaporation. The 'cold form' of gelatin gave the characteristic infrared dichroism of collagen. Veis & Cohen (1960) obtained some unusual heat-precipitated gelatin fibres, which revealed in the electron microscope a banded structure with 610 Å spacings. Working with the heat-denatured soluble fraction of rat-tail-tendon collagen, Flory & Weaver (1960) have demonstrated that a relatively slow transition from a random-coil to a helical configuration seems likely to account for the increases in viscosity in dilute solutions and in optical-rotation values obtained with this material.

Results similar to those of Flory & Weaver (1960) were obtained in the present work, and show that the reversion behaviour is likely to be a general reaction for gelatin. A number of typical gelatins, with a wide variety of values for weight-average molecular weight and rigidity modulus, have been studied as dilute solutions in citrate and other buffers. The increase in viscosity of these solutions with time is clearly enhanced by aliphatic carboxylic acids. Since molecular reorientation of the gelatin is a plausible explanation of this behaviour, the degree to which a gelatin can demonstrate the viscosity–time effect is viewed in relation to some other physical properties, which are governed by the size and shape of the molecule. To examine a possible relationship with molecular weight, it was necessary to fractionate a series of gelatins into units with considerably enhanced weight-average molecular-weight homogeneity. No such relation-

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vate and α-oxoglutarate in the blood and urine but none in glyoxylate. In thiamine-deficient rats, the glyoxylate in blood and urine contained up to 6·9% of the radioactivity from the injected glycine, and radioactivity was not found in the pyruvate or α-oxoglutarate.

6. The present study suggests that the glyoxylate found in blood and urine of thiamine-deficient rats is derived from the glycine liberated by excessive tissue breakdown.

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Toxic Liver Injury and Carcinogenesis

METHYLATION OF PROTEINS OF RAT-LIVER SLICES BY DIMETHYLNITROSAMINE *IN VITRO*

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Dimethylnitrosamine is a powerful liver poison in several mammalian species (Barnes & Magee, 1954; Jacobsen, Wheelwright, Clem & Shannon, 1955). It is carcinogenic in the rat, inducing tumours of the liver (Magee & Barnes, 1956; Schmahl & Preussmann, 1959) and of the kidney (Magee & Barnes, 1959, 1962; Zak, Holzner &

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Popper, 1960). It has been suggested (Magee, 1956; Stoner & Magee, 1957; Brouwers & Emmelot, 1960; Hultin, Arrhenius, Löw & Magee, 1960) that dimethylnitrosamine must be metabolically activated before it becomes hepatotoxic and that a metabolite or metabolites may be responsible for toxicity and carcinogenicity. The possibility that the active molecule might be diazomethane was suggested by Dr Regina Schoental (personal communication) and by Rose (1958). Support for such an alkylation hypothesis has come from the work of Druckrey and his colleagues. After an earlier report (Schmähl, Preussmann & Hamperl, 1960) that diethylnitrosamine is carcinogenic in rat liver, the same group (Druckrey, Preussmann, Schmähl & Müller, 1961 *a*) have reported the results of carcinogenicity tests on a series of nitrosamines. Only those compounds which were expected to yield diazoalkanes proved to be carcinogenic, and those which were unlikely to do so, on chemical grounds, failed to induce tumours. Diazomethane itself has been shown to be carcinogenic in the lung in mice and rats by Schoental (1960), who also showed that nitroso-*N*-methylurethane induces stomach tumours in the rat. This latter result has been recently confirmed by Druckrey *et al.* (1961 *b*) in the rat. Brouwers & Emmelot (1960) have demonstrated the formation of formaldehyde from dimethylnitrosamine by a microsomal enzyme system in rat liver and have suggested that this may be the actively carcinogenic molecule. Reasons against this have been advanced by Hultin *et al.* (1960).

In the present paper experiments are described in which rat-liver slices were incubated with [^{14}C]dimethylnitrosamine *in vitro*, and the incorporation of radioactivity into proteins and nucleic acids was studied.

MATERIALS AND METHODS

Chemical reagents

Dimethylnitrosamine. This was obtained commercially and purified by distillation (b.p. 151°).

Radioactive compounds. [^{14}C]Dimethylnitrosamine was synthesized by Dr D. F. Heath as described by Dutton & Heath (1956). [^{14}C]Paraformaldehyde was supplied by The Radiochemical Centre, Amersham, Bucks.

Special reagents. A mixture containing 1-methyl-, 3-methyl-, and 1,3-dimethyl-histidine was prepared by methylation of histidine by Dr A. R. Mattocks according to Sheehan & Frank (1949) as modified by Tallan, Stein & Moore (1954). Authentic samples of 1-methyl- and 3-methyl-histidine were supplied by the California Corp. for Biochemical Research, Los Angeles 63, California, U.S.A.

Experiments on tissue slices

Albino rats of the Wistar strain were maintained on a diet of vitamin-enriched rat-bread (Lindberg, Älvsjö, Sweden) or on M.R.C. diet 41 B (Bruce & Parkes, 1956), and were killed by decapitation. Rats of either sex, usually weighing about 250 g., were starved overnight before use. Tissue slices were prepared and incubated essentially as described previously (Hultin *et al.* 1960) except that Krebs–Ringer phosphate medium (Umbreit, Burris & Stauffer, 1957) was sometimes used instead of the bicarbonate medium. At the end of the incubation period the vessels were plunged into ice-cold water and the medium was removed from the slices and discarded. The slices were washed twice with ice-cold medium containing a large excess of non-radioactive dimethylnitrosamine and were disintegrated in water, again containing excess of the unlabelled compound, in a homogenizer of the type described by Potter & Elvehjem (1936). An equal volume of 10% (w/v) trichloroacetic acid was then added to the tissue suspension with stirring and the precipitate was centrifuged down. In some experiments the supernatant acid-soluble fraction was retained for isolation of formaldehyde.

Isolation of [^{14}C]*formaldehyde and periodic acid oxidation of serine*

Isolation of [^{14}C]formaldehyde from the acid-soluble fraction was carried out according to Mitoma & Greenberg (1952). Formaldehyde (4 mg.) was added to 25 ml. of the acid-soluble fraction, made 25% (w/v) with respect to trichloroacetic acid. This mixture was distilled almost to dryness *in vacuo*, the distillate being trapped in 12 ml. of an aqueous solution containing 0·4% of dimedone (5,5-dimethylcyclohexane-1,3-dione) buffered at pH 7·4 (MacFadyen, 1945). The formaldehyde–dimedone compound, methylenebis-(5,5-dimethylcyclohexane-1,3-dione), was purified by repeated solution in alkali containing excess of dimethylnitrosamine and reprecipitation with acid, dried and assayed for radioactivity. Distillation of added carrier formaldehyde was repeated three times to remove residual formaldehyde, and a fourth time with collection of the distillate and preparation of the dimedone derivative to confirm that removal was complete. Serine (20 mg.) was then added to the residue in the distillation flask and periodic acid oxidation carried out as in the procedure of Mitoma & Greenberg (1952) but with the method of Boyd & Logan (1942). Distillation and collection of the formaldehyde–dimedone compound was followed by assay of its radioactivity, which was assumed to be derived from the β-carbon atom of serine.

Isolation of proteins and nucleic acids

Proteins were isolated essentially according to Schneider (1945). After centrifuging, the acid-soluble supernatant was discarded and the precipitate was washed three times with 5% (w/v) trichloroacetic acid on the centrifuge, followed by ethanol, ethanol–ether (3:1, v/v), twice with ether, and then dried. The dry powder was then suspended in 5 vol. of 10% (w/v) NaCl, and heated at 100° for 1 hr. to extract the nucleic acids (Davidson & Smellie, 1952). This procedure was repeated twice by using 2·5 vol. of 10% NaCl and heating for 0·5 hr., and the three extracts were pooled and set aside for precipitation and purification of the mixed sodium nucleates. The protein residue was then extracted twice with 5% (w/v) trichloroacetic acid at 90° for 20 min. to remove any remaining nucleic acids (Schneider, 1945), washed with ethanol and ether, and dried. This material was regarded as the mixed total protein of the

tissue. The nucleic acids were precipitated from the 10% NaCl extract by the addition of 2 vol. of ethanol, and were purified according to Davidson & Smellie (1952).

Hydrolysis and ion-exchange chromatography of proteins

Proteins were hydrolysed with 6 N-HCl either in a sealed tube at 110° for 16 hr. or by refluxing for 20 hr. The neutral and basic amino acids were separated by ion-exchange chromatography on a column (1 cm. × 150 cm.) of Dowex 50 resin (H^+ form) essentially according to Hirs, Moore & Stein (1954) after preliminary removal of the acidic amino acids on a column of Amberlite IR-4 B resin. The effluent from the columns was collected by a mechanical fraction collector. The basic amino acids were separated on a column (0·9 cm. × 50 cm.) of Dowex 50 (Na^+ form), eluting with buffers of increasing ionic strength according to Tallan *et al.* (1954) and Moore & Stein (1954). The presence of amino acids in the effluent fractions was detected by spotting on strips of Whatman no. 1 paper followed by development of colour with ninhydrin. Amino acids were determined quantitatively according to Yemm & Cocking (1955). Buffer salts were removed from fractions by the method of Dréze, Moore & Bigwood (1954), and the salt-free acid solutions of the amino acids were evaporated to dryness at room temperature by exposing the contents of the tubes to a stream of compressed air in a fume cupboard. The residual material in each tube was dissolved in about 2 ml. of water and transferred to plastic (polythene) planchets on which it was evaporated to dryness for assay of radioactivity.

Hydrolysis and ion-exchange chromatography of nucleic acids

In a preliminary experiment, the whole material extracted by 5% trichloroacetic acid at 90° (Schneider, 1945) was shaken repeatedly with ether until the aqueous phase was barely acid (pH about 5). Hydrochloric acid was then added to a final concentration of 1 N followed by heating at 100° for 1 hr., thus liberating purines but leaving pyrimidine nucleotides intact (Vischer & Chargaff, 1948). This hydrolysate was applied to a column of Dowex 50 (H^+ form) and eluted with increasing concentrations of hydrochloric acid. The extinction at 260 mμ of each fraction was measured and several fractions were combined, evaporated to dryness in a rotary evaporator at 50° and finally taken to dryness on glass planchets for assay of radioactivity. In later experiments the isolated mixed nucleic acids were hydrolysed with alkali by the modification of the method of Schmidt & Thannhauser (1945) due to Davidson & Smellie (1952). The RNA nucleotides, after precipitation of DNA and potassium perchlorate, were separated by chromatography on a column (1·5 cm. × 21 cm.) of Dowex 1 anion-exchange resin (formate form) with gradient elution from 1 to 4 N-formic acid, according to Brumm, Potter & Sickevitz (1956) as modified by Gilbert & Yemm (1958). After the extinction at 260 mμ had been recorded, the fractions were evaporated to dryness individually, as described above, and transferred to polythene planchets for radioactive assay.

Paper chromatography

Amino acids were identified by descending chromatography on Whatman no. 1 paper using phenol saturated with water as solvent. The position of amino acid spots was revealed by spraying with, or dipping in, 0·2% ninhydrin in acetone (Toennies & Kolt, 1951). Special colour reactions used were the Pauly reaction for histidine, as applied to paper chromatograms by Sanger & Tuppy (1951), and the Sakaguchi reaction for arginine according to Jepson & Smith (1953). Products of hydrolysis of RNA were separated by the propan-2-ol–ammonia solvent system of Markham & Smith (1952) on Whatman 3MM paper. The position of ultraviolet-absorbing material on the paper was detected according to Markham & Smith (1949). Aliphatic amines were separated by the method of Schwyzer (1952).

Radioactivity measurements and spectrophotometry

The radioactivity of solid materials was assayed on aluminium planchets (area 1 cm.²) and corrected, when necessary, to 'infinite thickness' with an empirical correction curve. Liquids were assayed as 'infinitely thin' samples after evaporation on to glass or plastic planchets as described above. Measurements of radioactivity were made with end-window counters and automatic sample changers (Tracerlab, or Phillips). Extinction measurements were made with Beckman or Unicam SP. 500 instruments.

RESULTS

Metabolic formation of formaldehyde from dimethylnitrosamine

Incubation of [^{14}C]dimethylnitrosamine with liver slices was shown to give [^{14}C]formaldehyde by the procedure of Mitoma & Greenberg (1952). Carrier formaldehyde added to the acid-soluble fraction of liver slices incubated with [^{14}C]dimethylnitrosamine was isolated as the formaldehyde–dimedone compound and found to be radioactive (422 counts/min.). After removal of almost all the residual formaldehyde, as shown by the very low radioactivity (9 counts/min.) of the dimedone compound obtained with the formaldehyde from the fourth redistillation (see Methods section), carrier serine was added, followed by periodic acid oxidation to convert the β-carbon atom into formaldehyde (Nicolet & Shinn, 1939). This was then isolated as before, and found to be radioactive (51 counts/min.), indicating the incorporation of carbon from [^{14}C]dimethylnitrosamine into serine. The metabolic formation of formaldehyde from dimethylnitrosamine by the liver (Brouwers & Emmelot, 1960) is therefore confirmed, and the presence of radioactivity in the β-carbon atom of serine indicates that it has entered the C_1 metabolic pool.

Incorporation of radioactivity from [^{14}C]dimethylnitrosamine into proteins of liver slices

Rat-liver slices were incubated aerobically and anaerobically with [^{14}C]dimethylnitrosamine and kidney slices were incubated anaerobically. After incubation for 3 hr., proteins were isolated from the

slices and assayed for radioactivity. The specific radioactivities were: liver protein (aerobic incubation), 39 counts/min.; liver protein (anaerobic incubation), 2 counts/min.; kidney protein (aerobic incubation), 4 counts/min. These results suggested that some form of incorporation or binding of dimethylnitrosamine or a metabolite to the liver protein had occurred, that this process required oxygen, and that there was a smaller, but probably significant, incorporation into kidney protein.

With [^{14}C]dimethylnitrosamine of higher specific radioactivity, the time course of incorporation of ^{14}C into protein of rat-liver slices was determined. The rate of incorporation was found to be linear for times of incubation up to 3 hr. Proteins isolated at zero time had no detectable radioactivity, indicating that the washing procedure was adequate to remove unchanged [^{14}C]dimethylnitrosamine. In this experiment the specific radioactivity of the residues before extraction of the total nucleic acids with hot 5% trichloroacetic acid was found to be considerably higher than that of the final nucleic acid-free protein powders. This suggested that incorporation had also occurred into the nucleic acids of the liver slices.

The nature of the binding of the radioactivity to the protein was then studied by dissolving samples of the protein in formic acid and in alkali, adding a large excess of non-radioactive dimethylnitrosamine to the solutions, and reprecipitation followed by determination of radioactivity. Protein with initial specific activity of 72 counts/min. gave 71 counts/min. after precipitation from solution in formic acid, indicating that the radioactivity was not due to unchanged [^{14}C]dimethylnitrosamine and probably not to other small molecular metabolites adsorbed on to the protein precipitate. Solution in alkali, on the other hand, caused reduction in the specific radioactivity of the protein to 19 counts/min. The conditions of hydrolysis were adequate to hydrolyse any methyl esters of carboxylic groups of the protein which might have been formed.

Since formaldehyde is produced by the metabolic breakdown of dimethylnitrosamine, the observed radioactivity in proteins and other cell constituents could be explained by incorporation via normal metabolic pathways. This possibility was explored by acid hydrolysis of the radioactive protein and subsequent ion-exchange chromatography, according to Hirs *et al.* (1954). The chromatographic profile is shown in Fig. 1. There are three main peaks of radioactivity. The first, in the region of serine, and the second smaller peak, in the region of methionine, could probably be explained by normal incorporation via [^{14}C]formaldehyde. The large peak of radioactivity in the region of the basic amino acids lysine and histidine could not be explained in this way. For example, rat-liver slices were incubated with [^{14}C]formaldehyde with subsequent isolation, hydrolysis and chromatography of the proteins. Peaks of radioactivity were again observed in the regions of serine and of methionine but there was no radioactivity in relation to the basic amino acids. The possibility that some unchanged dimethylnitrosamine remained occluded in the protein was further tested by the addition of a small amount of [^{14}C]dimethylnitrosamine to non-radioactive normal rat-liver protein, followed by hydrolysis and chromatography in the usual way. Almost all the radioactivity appeared in the region between methionine and leucine and there was none with the basic amino acids. This radioactive material was found to be volatile in alkali and to migrate with carrier dimethylamine on paper chromatography by the method of Schwyzer (1952). Since dimethylnitrosamine is converted into dimethylamine by concentrated hydrochloric acid (Hickinbottom, 1948), it seems probable that the radioactive material under examination was in fact dimethylamine.

Methylation of histidine residues of wheat proteins by methyl bromide was demonstrated by Bridges (1955), and Alexander & Cousens (1958) showed that the imidazole group of histidine was rather readily, and to some extent preferentially, alkylated in native proteins by several biologically active alkylating agents. A preliminary experiment suggested that a similar reaction might occur after metabolism of [^{14}C]dimethylnitros-

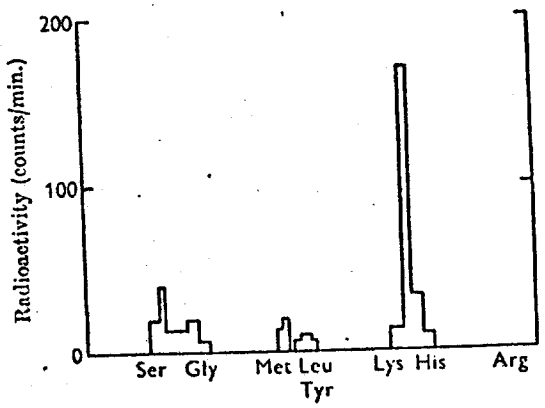

Fig. 1. Ion-exchange chromatography of an acid hydrolysate of proteins (293 mg.) prepared from rat-liver slices incubated *in vitro* with [^{14}C]dimethylnitrosamine. Dowex 50 (H^+ form) column (1 cm. × 150 cm.) essentially according to Hirs, Moore & Stein (1954). Elution sequence, first 1000 ml. with N-HCl, followed by 4N-HCl. Position of amino acids shown by conventional abbreviations, radioactivity as histogram. Acidic amino acids were removed initially on a column (1 cm. × 30 cm.) of Amberlite IR-4B resin.

amino, with the formation of radioactive methylated histidines. Radioactive protein from slices incubated with [^{14}C]dimethylnitrosamine was hydrolysed and subjected to ion-exchange chromatography of the basic amino acids according to Tallan *et al.* (1954). Three peaks of radioactivity emerged with the basic amino acids, one merging into histidine, one after lysine and well separated from it, and one emerging later just after ammonia. It seemed likely that the first two peaks were 3[^{14}C]-methyl- and 1[^{14}C]-methyl-histidine respectively, but there was no reaction with ninhydrin, probably because the amounts present were too small. When a small amount of a synthetic mixture of methylated histidines was added to the protein hydrolysate to act as carriers, the chromatographic profile shown in Fig. 2 was obtained. In this experiment the 3-methylhistidine merged into the histidine, but the 1-methylhistidine was well separated. The first radioactive peak appeared to correspond to the 3-methylhistidine and was not associated with histidine itself. Lysine was free from radioactivity and the small peak of 1-methylhistidine corresponded closely with the second radioactive peak. The third radioactive peak emerging after ammonia was not identified and the fourth very small peak coincided with a small ninhydrin-positive one which was probably 1,3-dimethylhistidine, since this is its expected position (R. Bridges, personal communication). The final peak of radioactivity coincided with arginine. The radioactive material apparently coinciding with 3-methylhistidine and overlapping histidine was rechromatographed under different conditions according to Moore & Stein (1954) to obtain separation of the two amino acids. This was achieved, as shown in Fig. 3, where all the radioactivity was found to be associated with 3-methylhistidine, the profiles of amino nitrogen concentration and radioactivity coinciding well apart from one point. No radioactivity was detected in the histidine. Similar rechromatography of the 1-methylhistidine showed very good coincidence between the profiles. Rechromatography of the arginine on Dowex 50 (H^+ form), with hydrochloric acid as eluent, also showed close coincidence of the profiles of concentration and radioactivity. It was concluded that a large part of the radioactive material appearing in the region of the basic amino acids was made up of 1-methyl- and 3-methyl-histidine.

Incorporation of radioactivity from [^{14}C]dimethylnitrosamine into nucleic acids of liver slices

As mentioned above, incorporation of radioactivity from [^{14}C]dimethylnitrosamine into the nucleic acids as well as into the proteins of the slices appeared likely. The total nucleic acids extracted from the slices were chromatographed, after

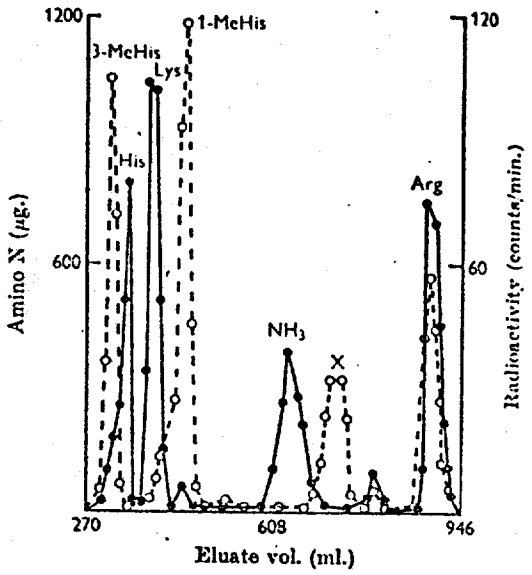

Fig. 2. Ion-exchange chromatography of an acid hydrolysate of proteins (250 mg.) prepared from rat-liver slices incubated *in vitro* with [^{14}C]dimethylnitrosamine. Dowex 50 (Na^+ form) column (1 cm. × 50 cm.) essentially according to Tallan, Stein & Moore (1954). ●, Amino N (μg.); ○, radioactivity (counts/min.). The radioactive material, X, was not identified.

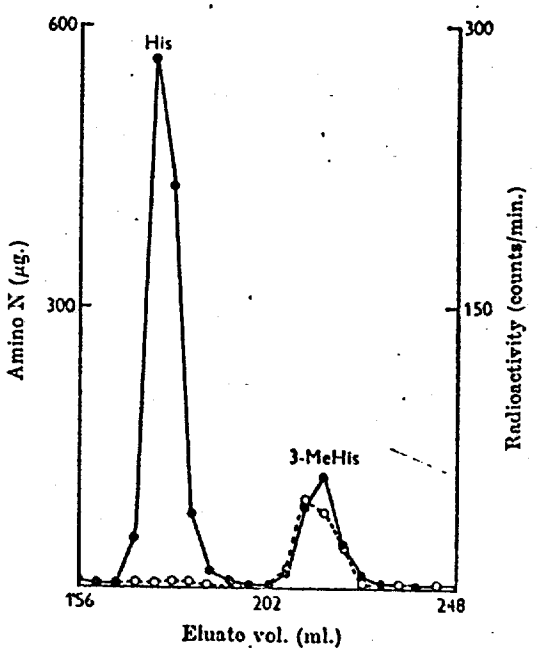

Fig. 3. Rechromatography of the 3-methylhistidine and overlapping histidine obtained previously (Fig. 2). Ion-exchange chromatography on a Dowex 50 (Na^+ form) column essentially according to Moore & Stein (1954). Symbols as in Fig. 2.

acid hydrolysis, on a cation-exchange column, and the major part of the radioactivity appeared in the effluent fraction between guanine and adenine, though there was no detectable ultraviolet-absorbing material corresponding to this fraction. In view of subsequent work (Magee & Farber, 1962), it is probable that this radioactive material was 7-methylguanine, present in amounts inadequate for spectroscopic detection. In later experiments the mixed nucleic acids were hydrolysed with alkali (Schmidt & Thannhauser, 1945), and the resulting RNA nucleotides were chromatographed by anion-exchange chromatography. A large peak of radioactivity appeared immediately before the guanylic isomers, merging into them to a greater or lesser extent in different experiments (Fig. 4). There was also a smaller peak of radioactivity corresponding exactly to that of the two isomers of adenylic acid. The radioactive material in the region of guanylic acid could be well separated from it by paper chromatography with the propan-2-ol–ammonia solvent system. It is probable that this material was a pyrimidine derivative arising by ring opening induced by alkaline hydrolysis of RNA methylated on the 7-position of some guanine moieties (Lawley & Wallick, 1957). On cation-exchange chromatography of the same alkaline hydrolysate of RNA, after further hydrolysis with N-hydrochloric acid to liberate the purine bases, the radioactive material appeared close to and shortly after the pyrimidine nucleotides. In no instance was enough material present to obtain ultraviolet-absorption spectra.

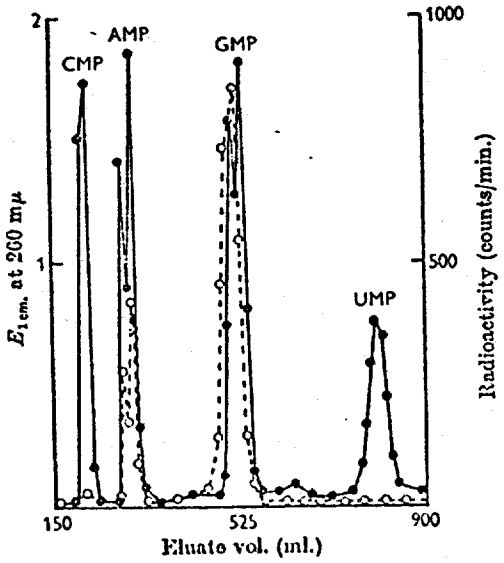

Fig. 4. Ion-exchange chromatography of an alkaline hydrolysate of RNA prepared from rat-liver slices incubated *in vitro* with [^{14}C]dimethylnitrosamine. Dowex 1 (formate form) column (1·5 cm. × 21 cm.) with gradient elution from 1 to 4 N-formic acid. ●, $E_{1cm.}$ at 260 mμ; ○, radioactivity (counts/min.).

DISCUSSION

The metabolic formation of formaldehyde from dimethylnitrosamine and its subsequent entry into the C_1 metabolic pool must be followed by labelling of all the cell constituents on the normal metabolic pathways of this compound. An attempt must therefore be made to distinguish between isotopic incorporation by this process and the formation of abnormal labelled tissue constituents which could not be derived from formaldehyde, since only the latter are likely to be related to the pathological action of dimethylnitrosamine.

The linear rate of incorporation of radioactivity from [^{14}C]dimethylnitrosamine into liver slices and the requirement for oxygen suggest that this process is enzymic. This probably involves the system present in the microsome + cell sap fraction of the liver which catalyses the decomposition of dimethylnitrosamine (Magee & Vandekar, 1958) and which has been recently shown to be a microsomal *N*-demethylase by Brouwers & Emmelot (1960). The absence of detectable radioactivity at zero incubation time in this experiment confirms that unchanged dimethylnitrosamine is very efficiently removed from the protein preparations by the washing procedures used, as would be expected from its solubility properties. It is possible, of course, that [^{14}C]dimethylnitrosamine might become more firmly adsorbed to the protein on incubation with the slices, but this is made unlikely by the failure to remove radioactivity by solution and reprecipitation of the protein in formic acid containing excess of non-radioactive carrier. This procedure has been shown by Gurnani, Kumta & Sahasrabudhe (1955) to make proteins more readily hydrolysed by acid, probably because the coiled form of the molecule is transformed into the extended form. Persistence of unchanged [^{14}C]-dimethylnitrosamine in any significant quantity is also ruled out by the absence of a radioactive peak in the chromatographic profile of the labelled protein (Fig. 1) in the expected region, i.e. that of dimethylamine. Solution in alkali, however, did remove a large amount of radioactivity from the protein. This method is widely used to remove [^{14}C]amino acids not bound by peptide bonds from protein preparations (Siekevitz, 1952), and the loss of radioactivity observed suggests that the alkali treatment splits off labelled groups chemically combined with the protein. This would be quite consistent with hydrolysis of methyl esters formed by methylation of protein carboxyl groups during incubation of the slices with dimethylnitrosamine.

It seems reasonable to conclude that the peak of radioactivity in the region of lysine and histidine (Fig. 1) is largely due to the presence of 1-methyl- and 3-methyl-histidine. Insufficient material was present to obtain a positive reaction with ninhydrin, but the close agreement between the chromatographic profiles of the added carrier compounds and the radioactivity (Figs. 2 and 3), including the small peak in the expected region for 1,3-dimethylhistidine, are all consistent with this. Also, since good chemical evidence for the presence of methylated nucleic acids is presented in the next paper (Magee & Farber, 1962), the intracellular formation of a methylating agent is strongly implied. The possibility that free histidine is methylated before incorporation into protein is unlikely. Although 1-methyl- and 3-methyl-L-histidine are excreted in the urine of man and other animals (Searle & Westall, 1951; Tallan *et al.* 1954) and 1-methylhistidine is known to occur in muscle as the dipeptide anserine (Behrens & du Vigneaud, 1939), methylhistidines have not been reported as constituents of protein (Greenstein & Winitz, 1961). Harms & Winnick (1954) treated rats with DL-1-methyl[1-^{14}C]histidine and found no significant labelling of tissue proteins, and Cowgill & Freeburg (1957) administered 1[^{14}C]-methyl-, 3[^{14}C]-methyl- and 1,3[$^{14}C_2$]-dimethyl-L-histidine to rats and found no detectable incorporation of these compounds into anserine or into other peptides or proteins of liver and other tissues. It seems highly probably, therefore, that the imidazole groups of some of the histidine residues in the protein molecules are methylated when rat-liver slices are incubated with dimethylnitrosamine. This, together with the probable methylation of the protein carboxyl groups, provides direct evidence in support of the methylation hypothesis of the mechanism of action of dimethylnitrosamine. The absence of methylated histidines from the proteins isolated from rat-liver slices incubated with [^{14}C]formaldehyde makes it unlikely that the observed methylation is mediated via this compound, although its metabolic formation from dimethylnitrosamine has been confirmed. Among the many reactions of formaldehyde with proteins *in vitro* (French & Edsall, 1945; Fraenkel-Conrat, 1951) methylation of histidine residues has not been reported.

The biological significance of the methylation of proteins in the liver is difficult to assess. Binding of carcinogens or their metabolites to tissue proteins has been investigated and discussed very extensively since the original observation by Miller & Miller (1947) of the combination of azo dyes with rat-liver proteins. Numerous correlations between protein binding and carcinogenesis have been established (Miller & Miller, 1953) and the enzyme deletion hypothesis, recently restated by Potter (1958), was formulated on the basis of combination between the carcinogen and cellular proteins as the initial reaction in chemical carcinogenesis. A similar protein–carcinogen interaction was postulated by Green (1954) in his immunological theory of cancer. Difficulties in acceptance of a primary protein change in carcinogenesis have been discussed by Anderson & Law (1960) and Magee (1962).

Since the cellular alteration in cancer must presumably be heritable, it seems more likely that the initial action of the carcinogen is on cellular nucleic acids, which are known to be capable of transmitting hereditary information, rather than on protein, which is not known to transmit such information. The possible relevance of methylation of nucleic acids to carcinogenesis by dimethylnitrosamine will be discussed in the following paper (Magee & Farber, 1962).

Methylation of proteins may be related to the acute hepatic injury induced by necrogenic doses of dimethylnitrosamine (Barnes & Magee, 1954). Although the extent of methylation of the total mixed proteins is very small indeed it must be remembered that certain specific protein molecules might be more extensively methylated. The imidazole groups of histidine residues in some enzyme proteins are known to be essential for activity (Barnard & Stein, 1958; Koshland, 1960). It is also established that minute quantities of an inhibitor combined irreversibly at the active centre can abolish enzymic activity, as in the phosphorylation of the serine hydroxyl group in esterases by organophosphorus compounds (Aldridge, 1956) and in the alkylation of protein sulphydryl groups by iodoacetate (Dixon, 1948). Such inactivation of an essential enzyme might play a part in the injury and subsequent death of liver parenchymal cells caused by dimethylnitrosamine. The possible role of reaction with sulphydryl groups in carcinogenesis by dimethylnitrosamine has been suggested by Emmelot & Benedetti (1961). Another possibility arises from the capacity of diazomethane to induce hypersensitive states (Fairhall, 1957), presumably following reaction with cellular proteins. Reiss & Tayeau (1959) have shown that methylation *in vitro* by diazomethane produces immunochemical changes in albumin, and similar changes have been shown to follow alkylation of proteins by sulphur mustard (Watkins & Wormall, 1952). In poisoning by dimethylnitrosamine, with local metabolic production of a methylating agent within the parenchymal cell, sufficient abnormal protein might accumulate to induce some form of immune reaction. The methylating agent would probably also methylate other cell constituents as well as protein and nucleic acid (Magee & Farber, 1962) and the cumulative effects of such reactions might well play a part in the cellular injury.

SUMMARY

1. The hepatotoxin and carcinogen dimethylnitrosamine, labelled with radioactive carbon, was incubated with rat-liver slices *in vitro*, and the fate of the label in some components of the acid-soluble protein and nucleic acid fractions of the tissue was studied.

2. Added carrier formaldehyde, isolated from the acid-soluble fraction as the dimedone derivative, was found to be radioactive; carrier serine, after periodate oxidation, also yielded a radioactive formaldehyde derivative.

3. Radioactivity was incorporated into total protein of liver slices and, probably, also into kidney protein. The incorporation process appeared to be enzymic with a requirement for oxygen. The radioactivity could not be removed from the protein by solution in formic acid but solution in dilute alkali led to considerable loss of radioactivity.

4. The radioactive liver protein was hydrolysed to free amino acids by hydrochloric acid, followed by ion-exchange column chromatography of the hydrolysate. Radioactivity appeared in the effluent in association with serine and methionine, and there was a peak of activity associated with lysine and histidine. An hydrolysate of liver-slice protein labelled by incubation with radioactive formaldehyde showed peaks of radioactivity associated with serine and methionine but none with the basic amino acids. An hydrolysate of non-radioactive protein with added radioactive dimethylnitrosamine showed virtually a single peak of radioactivity appearing between methionine and leucine which was probably dimethylamine.

5. Ion-exchange chromatography with better resolution of the basic amino acids showed that histidine and lysine were not labelled. Much of the radioactivity associated with them was resolved into two peaks which corresponded closely with carrier 1-methyl- and 3-methyl-histidine added to the protein hydrolysate before chromatography. Rechromatography of the 1-methyl- and 3-methylhistidine peaks gave very close agreement between the profiles of radioactivity and concentration of amino nitrogen.

6. Radioactivity was incorporated into the mixed nucleic acids of the liver slices. Ion-exchange chromatography of acid and alkaline hydrolysates of the labelled nucleic acids revealed most of the radioactivity in a single peak which did not correspond to any of the major nucleic acid components and was not identified. Most of the remaining radioactivity appeared to be incorporated into adenine.

7. These results are considered to be consistent with the hypothesis that dimethylnitrosamine, after enzymic oxidative *N*-demethylation, releases a methylating agent which methylates the imidazole group of some histidine residues and possibly also some free carboxyl groups of proteins. The carbon of the methyl group removed by oxidative demethylation appears to enter the general metabolic pool. The probable chemical alteration of tissue proteins by dimethylnitrosamine is discussed in relation to some current theories of tissue injury and carcinogenesis.

We would like to thank Dr D. F. Heath for the [^{14}C]-dimethylnitrosamine and Dr A. R. Mattocks for the methylated histidines. We are grateful to Dr R. Bridges for helpful discussion and to Mrs G. Beskow and Mr J. A. E. Jarvis for technical assistance.

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Toxic Liver Injury and Carcinogenesis

METHYLATION OF RAT-LIVER NUCLEIC ACIDS BY DIMETHYLNITROSAMINE *IN VIVO*

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In the preceding paper (Magee & Hultin, 1962) it was shown that methylation of proteins of rat-liver slices occurred during incubation of the slices with the hepatotoxin and carcinogen dimethylnitrosamine. This finding supports the hypothesis that dimethylnitrosamine undergoes enzymic oxidative *N*-demethylation in the liver with subsequent intracellular formation of a methylating agent, possibly diazomethane. Evidence for reaction with nucleic acids was also obtained. The reactions of nucleic acids and their components with alkylating agents have been studied very extensively *in vitro*

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The Possible Nitrosation of Amines in Smoked Chub

Studies were undertaken on the formation of dimethylnitrosamine (dmna) in aqueous model systems containing methyl amines and sodium nitrite. The reaction conditions (pH, reaction time, and temperature) were more severe than those occurring during the commercial processing of nitrite treated smoked chub. Polarographic analyses indicated that detectable amounts of dmna (1.4×10^{-5} mole) were not produced. It was concluded that in all probability dmna does not form in concentrations greater than 10 ppb during the smoking process.

Possible formation of the carcinogen dimethylnitrosamine (dmna) in food products has aroused wide concern (Lijinsky and Epstein, 1970; The Lancet, 1968). Accordingly, the issuance of a regulation by the U.S. Food and Drug Administration on the safe use of sodium nitrite in preserving smoked chub from the Great Lakes required evidence that dmna does not form via the nitrosation of methyl amines. A petition for a commercial process included 100 to 200 ppm of residual sodium nitrite in loin muscle and a minimum temperature of 160° F (71° C) for at least 30 min. A regulation for this process has been recently published (Federal Register, 1969).

In analyses of amines in chub, maximum concentrations of 25 ppm of trimethylamine (tma) and 107 ppm of trimethylamine oxide (tmao) have been documented (Grieg, 1970). A concentration of 2 ppm of dimethylamine (dma) has also been reported (Grieg, 1970). The pH values of smoked chub varied from 5.9 to 7.3 (unpublished data, Bureau of Commercial Fisheries, Ann Arbor, Mich., 1966), with the majority of the values in the range of 6.4 to 6.8 (Weckel and Wosje, 1966). It seemed probable from the studies of Sander *et al.* (1968), that the pH values were too high and the amine (tma, tmao, and dma) concentrations too low for the nitrosation reaction to take place. To test this hypothesis we studied in aqueous model systems the reaction under more severe conditions (pH, reaction time, temperature, and amine concentrations) than would occur during the commercial process.

Solutions (50 ml) were buffered at pH 6.4 and 5.8 with phosphate-citrate acid (Colowick and Kaplan, 1955). The concentrations of amines were varied from 200 to 2000 ppm (Table I). Reactions were conducted for 2.5 hr at 80 and 100° C in solutions containing 400 ppm of sodium nitrite (this concentration represents an estimate of the maximum sodium nitrite level reached during processing). Reaction products were analyzed for dmna by conventional DC polarography using a Heath EUW 401 operational amplifier, chopper stabilizer, and a three-electrode polarograph (Schlumberger Products Corp., St. Joseph, Mich.) and the electrolyte solutions and workup procedures described by Heath and Jarvis (1955). A fast scan system consisting of Chemtrix modules (Chemtrix Corp., Beaverton, Ore.) was also employed. The lower limit of detection was 1 μg (1.4×10^{-5} mole) of dmna.

Dmna was not detected in any reaction conducted at 80° C under the conditions shown in Table I. About 1 μg of dmna was detected at a tma concentration of 400 ppm at 100° C and a pH of 6.4. However, a slightly lower pH (5.8) resulted in the synthesis of 8.5 μg of dmna. These results suggest that a slight increase in the acidity of the chub may result in a significant increase in the nitrosation of the methyl amines (Sander *et al.*, 1968). Under all conditions shown in Table I,

Table I. Reactions of Amines with $NaNO_2$ in Aqueous Solutions

Amine mole × 10^{-4}	Concentration ppm	Mole Ratio amine:nitrite	pH	Dmna Formed 80° C	(μg) 100° C
Tma					
0.85	100	0.29	6.4	...	N.D.
0.85	100	0.29	5.8	N.D.[a]	...
1.7	200	0.58	6.4	N.D.	N.D.
1.7	200	0.58	5.8	N.D.	...
3.4	400	1.2	5.8	...	8.5
3.4	400	1.2	6.4	...	1.0
5.1	600	1.8	6.4	...	2.1
6.8	800	2.3	6.4	N.D.	2.6
8.5	1000	2.9	6.4	...	4.1
10.2	1200	3.5	6.4	N.D.	5.7
13.5	1600	4.7	6.4	N.D.	5.7
16.9	2000	5.8	6.4	N.D.	6.9
Tmao					
5.3	800	1.8	6.4	N.D.	N.D.
6.7	1000	2.3	5.8	...	N.D.
8.0	1200	2.8	6.4	N.D.	N.D.
10.6	1600	3.7	6.4	N.D.	N.D.
13.3	2000	4.6	6.4	N.D.	N.D.
Dma					
8.9	800	3.1	6.4	N.D.	N.D.
11.1	1000	3.8	5.8	...	N.D.
13.3	1200	4.6	6.4	N.D.	N.D.
17.7	1600	6.1	6.4	N.D.	N.D.
22.2	2000	7.6	6.4	N.D.	N.D.

Constants: 400 ppm (2.9 × 10^{-4} mole) $NaNO_2$; 2.5 hr reaction time.

[a] N.D.: not detected.

dmn and tmao did not react to yield detectable amounts of dmna.

The results of these studies, conducted under exaggerated reaction conditions, indicate that, in all probability, concentrations of dmna greater than 10 ppb would not result from the commercial processing of chub. It is recognized, however, that the aqueous model systems do not fully reflect the actual chemical environment in the smoked chub. These results are supportive of the data obtained by Howard *et al.* (1970) with smoked chub. These workers, employing glc techniques, were unable to find evidence for the nitrosation of amines under the prescribed processing conditions.

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Determination of Nitrate and Nitrite in Milk and Dry Milk Products [1]

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Abstract

A modified spectrophotometric method based on the selective diazotization-coupling reaction has been developed capable of detecting 1 ppm nitrite in dry milk products. Nitrate can be determined by the same reaction following quantitative reduction to nitrite with a cadmium column. Zinc hydroxide satisfactorily precipitated the milk fat and protein. Nitrate and nitrite levels have been determined in nonfat dry milk samples prepared on a single pilot plant dryer designed for either indirect (steam) heating or direct (gas) firing with natural gas. Slight increases in nitrate content attributable to gas firing were detected, whereas increases in nitrite were nil. A survey of 30 dry milk samples from 13 commercial plants revealed that products produced by direct-firing contained 1 to 3 ppm more nitrate-nitrogen than those produced by indirect heating designs.

Questions have been directed to us concerning the design of direct-firing spray dryers and the possibility that the nitrate and nitrite content of dry milk might be increased by contact with fuel gas combustion products. The combustion of fuels is the primary (and almost sole) mechanism responsible for the formation of nitric oxide (NO) and nitrogen dioxide (NO_2) in the atmosphere, and their emission concentration depends on: a) the temperature achieved during combustion, b) the efficiency with which combustion effluents are quenched, and c) the presence of excess air (or completeness of combustion) (5). Natural gas burners of the general design found in direct-firing spray dryers generate nitrogen oxide levels (calculated as NO_2) of about 10 to 100 ppm in the drying air (1). Moisture is required in the conversion of nitrogen dioxide gas to nitrate ion (18). Thus, if sufficient nitrate were absorbed (or trapped) by the drying milk mass, toxic levels possibly could be attained which might affect the health of persons, particularly infants, ingesting these dry milk products. Following these reports we decided to analyze a variety of dry milk samples for nitrate and nitrite, and in this regard to compare direct (gas) firing spray dryers with indirect (steam) heating designs.

Normal, fresh cow's milk contains less than 1 ppm nitrate (and nitrite) content (4, 10). However, most foods and water supplies contain levels higher than this (2, 4, 12). Serious and occasionally fatal cases of infantile nitrate poisoning have occurred following ingestion of well water containing excessive nitrate. Nitrite is equally dangerous in water supplies, but concentrations that occur naturally are of little public health significance. In recognition of the nitrate toxicity hazard, the U.S. Public Health Service has assigned an upper limit of 45 ppm in drinking water used for infant feeding (1). This value is equivalent to 10 ppm when nitrate is reported on a nitrogen basis. One must be cognizant of these two analytical conventions of reporting when interpreting literature results.

Most nitrate methods reported in the literature are arduous. In part, this is due to the colloidal nature of most foods as well as the interference of one or more food constituents (6). It was apparent that nitrite is more readily determined in foodstuffs than is nitrate (17).

The fact that most water supplies contain higher nitrate levels than milk forms the reasoning behind several early methods developed to indirectly detect adulteration of milk by added water. These procedures are at best microqualitative and lack specificity. Either diphenylamine or diphenylbenzidine usually is listed as the analytical reagent, but both are readily oxidized by agents other than nitrate (4). Sensitive spectrophotometric methods for determining nitrate in well water and waste water are now well established (2). These are based on the nitration of reagents such as 2,4-phenoldisulfonic acid, 2,4- or 3,4-xylenol, or brucine. Unfortunately, direct nitration procedures are not very reliable for analyzing complex food products because organic matter (especially carbohydrate), chloride, and

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nitrite interfere (6, 11, 15). For example, the tedious and laborious removal of all these interferences is incorporated into the nitro-xylenol steam distillation procedure adapted by Hanni (9) for milk and milk products.

In contrast, several suitable methods are available for determining nitrite in food (11, 17). These are based on the well-known, selective, diazotization-coupling reaction in acidic media to form an azo dye. Primary aromatic amines such as 4-aminobenzenesulfonamide, 4-aminobenzenesulfonic acid, and their analogs usually are specified as the diazotizable reagents, whereas the coupling reagents are either 1-naphthylamine or N,N-dimethyl-1-naphthylamine. Kamm et al. (12) found 1-naphthylamine diazotized rapidly and then coupled with excess amine to yield a chloroform–soluble diazo product identified as 4-(1-naphtholazo)-1-naphthylamine.

Another advantage of the diazotization-coupling methods is that nitrate-containing samples can be determined by the same reaction following quantitative reduction to nitrite. Nitrite is determined directly as well as nitrate plus nitrite after reduction; then nitrate is calculated by difference. Zinc dust is the reducing agent specified in several papers (14). Follett and Rateliff (6) claim nitrate reduction with zinc lacks precision, since results vary due to particle size and source of supply of the dust, so specify cadmium in their method for nitrate in cured meat. A modification of the Follett and Rateliff (6) method has been devised by Kamm et al. (12) which permits the accurate determination of both nitrate- and nitrite-nitrogen in baby foods at the 1 ppm level.

Both methods call for potassium aluminum sulfate as the fat-protein precipitating agent (6, 12). We encountered some difficulty in obtaining filtrates free of colloidal aluminum hydroxide, however, so alternate clarification techniques were investigated (16). These included such classical clarifying agents as copper hydroxide (19), cadmium hydroxide (3), and zinc hydroxide (7, 8). The most satisfactory precipitating agent for milk fat and protein removal was found to be zinc hydroxide. The method described here is a modified and simplified version developed from several literature sources and is suitable for routine determination of parts per million levels of nitrate and nitrite in milk and dry milk products.

Experimental Procedures

Milk and milk products. Milk obtained from the University dairy barn was used during the method development and other preliminary studies. Plain condensed skimmilk was obtained locally for the pilot plant dryer experiments. Commercial dry milk samples for the survey were obtained from several manufacturers.

Reagents

Buffer solution. Twenty milliliters of conc H_3PO_4 and 50 ml conc NH_4OH were added to about 500 ml distilled water. After diluting to one liter, the final pH was about 9.6.

1-Naphthylamine solution. One gram 1-naphthylamine[2] was dissolved in 200 ml 25% acetic acid solution. This reagent was prepared fresh daily.

Methanol-HCl solution. Seventeen milliliters conc HCl were added to two liters absolute methanol.[3]

Chloroform.[3]

Zinc hydroxide precipitating agent. A 12% sulfate ($ZnSO_4 \cdot 7\ H_2O$) solution and a 0.5 N NaOH solution were prepared. When 10 ml of each solution was diluted to a final volume of 200 ml in the procedure, the final pH was about 7.8.

Nitrate reduction column. Metallic cadmium preparation and column specifications are described in detail by Kamm et al. (12) and were followed without modification. The reducing efficiency was checked periodically by passing a known amount of nitrate through the column. A stock standard solution containing 100 μg NO_3-N/ml was prepared by weighing 0.6068 g $NaNO_3$ and making to one liter. Then a 1 μg/ml working standard solution was prepared for the actual recovery work.

Glassware. All glassware was rinsed carefully with HCl diluted 1:3, followed by distilled water to prevent nitrate contamination.

Preparation of the standard nitrite curve. A stock standard solution containing 100 μg NO_2-N/ml was prepared by dissolving 0.4925 g $NaNO_2$[3] in 5 ml of buffer solution and making to one liter with distilled water. From a 1 μg/ml working standard solution, 1, 2, 3, 5, and 10 ml aliquots were pipetted into a series of 250-ml Erlenmeyer flasks and made to about 100 ml with distilled water. Then 5 ml of buffer solution and 2 ml of 1-naphthylamine solution were

[2] Fisher Certified grade purchased from Fisher Scientific Company.

[3] Analytical reagent grade purchased from Mallinckrodt Chemical Works.

pipetted into each flask. After mixing, flasks were placed in the dark for 2 hr for color development at 20-25 C. Next, each solution was transferred to a 250-ml separatory funnel and the flask rinsed with 10 ml of $CHCl_3$. After shaking for about 1 min, most of the color had been transferred to the $CHCl_3$ layer, which was drawn off into a 50-ml volumetric flask. The extraction procedure was repeated three times with 10-ml volumes of $CHCl_3$. Finally, 5 ml of HCl-MeOH solution were added which changed the color from yellow-orange to purple. The flasks were made to mark with $CHCl_3$ and the colored solutions transferred to matched 19- by 150-mm colorimeter tubes. The absorbance was measured at 555 mμ against a reagent blank using a Coleman[4] Model 14 spectrophotometer.

Extraction of the milk sample. A 4-g sample of dry milk product (or 40 g of fluid milk or skimmilk) was weighed into a 200-ml volumetric flask[5] containing about 150 ml of distilled water (110 ml for fluid samples). The sample solution was mixed by swirling and warmed to 70 C in a water bath. Then 10 ml of $ZnSO_4$ solution and 10 ml of NaOH solution were added to each flask, the contents mixed, and held at 70 C for 10 min. After cooling to room temperature, flask was made to mark and mixed. The sample solution was decanted through Whatman no. 42 paper and a sufficient amount of filtrate collected in a 125-ml Erlenmeyer flask for subsequent analyses.

Determination of nitrite content. A 20-ml aliquot of sample filtrate was pipetted into a graduated 250-ml Erlenmeyer flask. The procedure listed under Preparation of the Standard Nitrite Curve was then followed, beginning "...and made to about 100 ml with distilled water."

Determination of nitrate plus nitrite content. A second 20-ml aliquot of sample filtrate was pipetted into a 30-ml beaker, 5 ml of buffer solution added, and quantitatively transferred into the reservoir of a cadmium reduction column (1). As the reservoir emptied, four successive 20-ml portions of distilled water were added and the entire eluate collected in a 250-ml Erlenmeyer flask. The procedure listed under Preparation of the Standard Nitrite Curve was then followed, beginning " . . . and 2 ml of 1-naphthylamine solution were pipetted into each flask."

[4] Coleman Instruments, Inc., Maywood, Illinois.

[5] Wide-mouth phosphoric acid flask, Cat. no. 5810, purchased from Corning Glass Works.

Calculations. The 20-ml sample aliquots represent 0.4 g of dry milk product. We found it convenient to construct direct reading standard curves by plotting absorbance vs. ppm NO_2-N or μg/g dry milk sample. Similar curves can be constructed also for fluid milk or skimmilk samples, except here, 20-ml aliquots are equivalent to 4 g of sample.

Results and Discussion

Follett and Ratcliff (6) caution that the nitrate-reduction and color-development steps in diazotization-coupling methods are very pH-dependent, so possible procedural effects introduced by the zinc hydroxide modification were investigated. Table 1 shows that zinc hydroxide performs well as a fat-protein precipitant and does not affect the pH limiting steps of the modified procedure.

Recently, Kamm et al. (13) subjected their naphthylazo-naphthylamine method (12) to a thorough statistical assessment, including a collaborative study, to evaluate such effects as type of food, nitrate-nitrogen levels, etc. on analytical precision and accuracy. They found virtually 100% reduction of nitrate to nitrite using their cadmium column design and also that the colored diazo product remained stable up to 4 hr in chloroform. Their statistical data indicate that the method is very precise and accurate. However, they did find the variation within a single laboratory was less than that for inter-laboratory comparison, but concluded this was due to nitrate interference in the distilled water used by several collaborators. We have run numerous trials to check the reproducibility and recovery of the modified procedure. We also found the precision of our own technicians to be excellent. The accuracy is illustrated in Table 2. Added nitrate and nitrite levels were essentially 100% recoverable, which substantiates the conclusions of Kamm et al. (12, 13).

Using our pilot plant spray dryer, a series of nonfat dry milk samples was prepared from

TABLE 1. Comparison of aluminum hydroxide and zinc hydroxide as milk clarification reagents.

Condition	Aluminum hydroxide	Zinc hydroxide
Fat removal	(Most)	(Complete)
Protein removal	TCA (−)	TCA (−)
Colloidal filtrates	Yes	No
pH after clarification	6.7	7.8
pH after buffer addition	9.6	9.5
pH after nitrate reduction	9.5	9.4
pH after acidification	4.3	4.3

TABLE 2. Accuracy of the modified method for determining nitrite- and nitrate-nitrogen in milk and milk products.

Sample[a]	Added		Recovered			
	Nitrate-nitrogen	Nitrite-nitrogen	Nitrate-nitrogen		Nitrite-nitrogen	
	(μg)		(μg)	(%)	(μg)	(%)
Reagent blank	5		4.8	96		
Reagent blank		5			4.9	98
5% Lactose	5		5.1	102		
Milk ultrafiltrate	5		4.9	98		
Isoelectric acid whey	5		4.9	98		
Isoelectric acid whey		5			4.6	92
Raw skimmilk	5		5.1	102		
Raw skimmilk		5			4.7	94
Nonfat dry milk	5		4.9	98		
Nonfat dry milk		5			4.9	98

[a] Control samples contained no NO_3-N or NO_2-N.

two different lots of low-heat plain condensed skimmilk, both by direct (gas) firing and indirect (steam) heating of the drying air. These results are listed in Table 3. Increases of about 2 to 3 μg nitrate-nitrogen g MSNF attributable to gas firing were detected, whereas increases in nitrite-nitrogen content were nil.

Thirty dry milk samples from 13 plants in Minnesota, Wisconsin, and Iowa were analyzed for nitrite- and nitrate-nitrogen content. A summary of these data is presented in Table 4. Nitrate-nitrogen levels in all but one of the samples produced by direct-firing were higher than those produced by indirect (steam) heating designs. Again, nitrite-nitrogen differences were relatively less than nitrate, except for those products produced by Gray-Jensen spray dryers. It is of interest that only two of the seven steam-dried samples contained no nitrate. This suggests that the 23 samples produced by gas firing might have contained some nitrate prior to dehydration. However, in no instance did samples produced by gas firing contain more than 3.8 ppm total nitrate-nitrogen, and 21 samples contained 3.0 ppm or less.

Most home and municipal water supplies contain parts per million levels of nitrate (2, 4, 12). Generally, dry milk products are reconstituted about 1:10 with water for beverage purposes. Thus, milk solids usually will contribute less nitrate to a recombined product than the water diluent. If nitrate-free water were used, it is obvious that a dry milk product would have to contain in excess of 100 ppm nitrate-nitrogen to prepare a beverage exceeding the 10 mg per liter limit established by the U.S. Public Health Service for water supplies used for infant feeding.

In all burning processes some atmospheric nitrogen is fixed with oxygen to yield equilibrium concentrations of nitric oxide and nitrogen dioxide (5). Therefore, it is not surprising that direct-firing spray dryers increase nitrate levels in dry milk products produced therein

TABLE 3. Comparison of direct versus indirect heating on nitrite- and nitrate-nitrogen content of nonfat dry milk samples prepared using a single pilot plant spray dryer.

Sample	Inlet air temp	Source of heat	Nitrite-nitrogen	Nitrate-nitrogen
	(C)		(μg/g MSNF)	
Supplier 1				
Skimmilk			0	0
Condensed skimmilk			0	0
NFDM 1	155	Steam	0	<0.5
NFDM 2	155	Natural gas	0	3.0
NFDM 3	215	Natural gas	<0.5	2.5
Supplier 2				
Skimmilk			0	<0.5
Condensed skimmilk			0	<0.5
NFDM 4	155	Steam	0	<0.5
NFDM 5	155	Natural gas	<0.5	1.9
NFDM 6	190	Natural gas	<0.5	2.5
NFDM 7	215	Natural gas	0.7	2.8

TABLE 4. Effect of dryer design on nitrite- and nitrate-nitrogen content of various dry milk products.

Dry milk product	Sample code	Type of spray dryer	Source of heat	Nitrite-nitrogen	Nitrate-nitrogen
				(ppm)	
Low heat nonfat dry milk	A	Coulter	Natural gas	0	1.2
	B	Coulter	Natural gas	<0.5	0.7
	C	Coulter	Natural gas	<0.5	1.7
	D	Coulter	Natural gas	0	2.5
	E	Coulter	Natural gas	0	3.0
	F	Coulter	Natural gas	<0.5	2.8
	G	Coulter	Natural gas	0.7	2.5
	H	Rogers	Natural gas	0	1.2
	I	Rogers	Natural gas	0	1.0
	J	Rogers	Natural gas	0	2.7
	K	Buflovak	Natural gas	1.0	2.2
	L	Buflovak	Natural gas	<0.5	1.7
	M	Gray-Jensen	Natural gas	2.0	0.8
	N	Gray-Jensen	Natural gas	1.5	1.5
	O	Coulter	Steam	0	<0.5
	P	Coulter	Steam	0	<0.5
	Q	Gray-Jensen	Steam	0	0.8
	R	Gray-Jensen	Steam	0	0
High-heat nonfat dry milk	A	Buflovak	Natural gas	0	3.0
	B	Gray-Jensen	Natural gas	<0.5	2.8
	C	Gray-Jensen	Natural gas	<0.5	2.5
	D	Gray-Jensen	Steam	0	0
	E	Gray-Jensen	Steam	0	0.8
Dry whole milk	A	Rogers	Natural gas	<0.5	3.8
	B	Rogers	Natural gas	0	2.8
	C	Coulter	Steam	0	<0.5
Buttermilk	A	Coulter	Propane	<0.5	3.2
	B	Gray-Jensen	Steam	<0.5	1.8
All dairy blend	A	Gray-Jensen	Natural gas	1.5	2.2
	B	Gray-Jensen	Natural gas	1.5	1.8

The mechanisms responsible for this increase are somewhat open to conjecture. Apparently, conditions (moisture, temperature, etc.) are suitable to convert some nitrogen dioxide to nitrate ions, which somehow become associated with the drying milk particles. It may be that some nitrogen dioxide becomes trapped in the void volumes of particles and subsequently reacts with bound water to form nitrate.

Direct gas fire for heating the air for spray dryers has almost completely replaced indirect heating with steam in this country because of reduced initial and operating costs. Many billions of pounds of dry milk products have been produced in direct gas-fired dryers. We are not aware of any reports of harm from the consumption of these products. Nevertheless, direct gas firing of spray dryers must be considered as another of the many possible sources of nitrate in foods.

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NITRATE METHEMOGLOBINEMIA

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MORE than forty years have passed since the first report of a fatal case of methemoglobinemia secondary to bismuth subnitrate ingestion.[1] Fifteen years ago Roe[2] reviewed the subject and reemphasized the hazards attending its use. Nevertheless, during the past four years 3 more cases have been reported.[3-5] A recent experience with a newborn infant has prompted us to review the general subject of nitrate methemoglobinemia, with emphasis on the causes, the types encountered, the mechanism involved and the rationale of present-day therapy.

Thirty cases of methemoglobinemia secondary to bismuth subnitrate, ammonium nitrate and nitrates in well water have been reported. The case reported below followed the ingestion of bismuth subnitrate on prescription.

We wish to point out that 4 of the 5 fatal cases of nitrate methemoglobinemia were in the group that followed bismuth subnitrate administration. Hence it is our opinion that still another warning to the medical profession regarding its use is indicated, particularly in view of the rapidly favorable outcome following the application of accepted therapeutic procedures.

CLASSIFICATION OF METHEMOGLOBINEMIAS

Methemoglobinemia may occur during the course of various diseases or may follow the ingestion of a variety of toxic agents. A slight modification of Ferrant's[6] classification is as follows:

Plasma methemoglobinemia. In this variety the methemoglobin is formed in the plasma after the liberation of hemoglobin from the red blood cells by hemolysis. This may occur in blackwater fever, eclampsia, paroxysmal hemoglobinuria, anaerobic sepsis and Winckel's disease of the newborn.

Cellular methemoglobinemia. In this variety hemoglobin, which contains iron in its ferrous form, is oxidized within the red blood cell by various agents to methemoglobin, which contains iron in its ferric form. This type is further subdivided into: idiopathic or enterogenous — according to van den Bergh,[7] nitrite is formed in the bowel from nitrate by nitrite-forming organisms, and absorption is encouraged by a damaged intestinal mucosa — and toxic, which comprises the largest and most important group of methemoglobinemias (the agents responsible include primarily the drugs containing nitro and amino groups, such as derivatives of aniline, certain sulfonamides, nitrates and nitrites[8-10]).

Our interest centers on the methemoglobinemias produced by nitrate ingestion.

REVIEW OF THE LITERATURE

According to Beck,[11] cases of bismuth subnitrate poisoning were reported as early as 1793, but impurities such as arsenic were thought to be responsible. Eusterman and Keith[12] cite Gamgee[13] as the first to demonstrate in 1886 that amyl nitrite could produce methemoglobinemia. In 1906 Bennecke and Hoffmann[1] reported the first fatal case of methemoglobinemia following bismuth subnitrate ingestion. Shortly afterward, Böhme[14] presented a report of a fatal case of methemoglobinemia following the administration of bismuth subnitrate by enema. He believed that intestinal putrefaction accelerated nitrite formation and rapid poisoning and stated that in fatal cases patients invariably suffered from intestinal disease. Thus, the danger of administration of bismuth subnitrate to infants, particularly when intestinal dysfunction existed, was established over forty years ago. Nowak and Gütig[15] showed that adults were not immune to nitrite poisoning after bismuth subnitrate. In reviewing the literature, Beck,[11] in 1909, made the first concise contribution on the general subject of poisoning due to nitrate, though limiting himself to bismuth subnitrate.

Years later, Eusterman and Keith[12] reported 2 nonfatal cases of methemoglobinemia in adults following the administration of ammonium nitrate as a diuretic. In both cases there was evidence of intestinal dysfunction. Tarr[16] collected 9 cases of methemoglobinemia following the ingestion of ammonium nitrate, of which he personally observed 4. In his cases renal impairment rather than intestinal dysfunction was the common denominator.

Roe,[2] in 1933, reported the fourth case of methemoglobinemia due to bismuth subnitrate ingestion and recommended the use of bismuth subcarbonate in its place for the treatment of diarrhea.

In the past few years a series of papers on methemoglobinemia due to the ingestion of well water of high nitrate content have appeared. Schwartz and Rector[17] reported a case of idiopathic methemoglobinemia in an infant fed a powdered milk formula and living in the country. Comley,[18] in describing 2 cases of this type, clarified the above

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syndrome and attributed the methemoglobinemia to the nitrates in well water. Additional cases were reported from Belgium by Ferrant,[6] again from country communities in the United States by Faucett and Miller,[19] Chapin[20] and Stafford[21] and from Canada by Medovy, Guest and Victor.[22]

Case Report

D. B., a 3-week-old infant with a birth weight of 4 lb., 9 oz., was discharged from the hospital on the 9th day on an Olac formula. At the age of 2 weeks he had attained a weight of 5 lb., 12 oz. Six days prior to admission diarrhea developed, and a change of formula was made. The diarrhea persisted, however, and bismuth subnitrate was administered in a compound prescription at the rate of 0.1 gm. (1½ gr.) every 3 or 4 hours. Medication was taken as ordered for 1 day but was withheld on the night prior to admission because of the patient's bluish color. On the following morning the color improved, and medication was resumed. That evening cyanosis became marked. The attending physician administered physiologic saline solution by hypodermoclysis and referred the patient to the hospital.

Physical examination revealed a grayish-blue cyanosis. The weight was 5 lb., 8 oz. The temperature was 98°F. Activity was normal, and there was no evidence of dehydration. The head and neck were essentially normal. The heart did not appear enlarged; its rate and rhythm were normal, and no murmurs were audible. The lungs were clear to percussion and ascultation. Except for phimosis the remainder of the examination was negative.

Urinalysis and a complete blood count were within normal limits. The blood was chocolate brown when the first specimen was drawn.

The admission diagnosis of methemoglobinemia was established after the initial withdrawal of blood for examination, though the etiology was not determined until the following day. Premature management was begun at once, and the infant was given a small blood transfusion and kept in an atmosphere of high oxygen concentration. Because respiratory difficulty was not marked, methylene blue therapy was withheld initially. This course was justified by the rapid improvement incident to the measures employed. Residual cyanosis was barely visible twelve hours after admission, and twenty-four hours after admission it had disappeared completely.

Present Concepts

Formation of Methemoglobin

Van den Bergh,[7] writing on enterogenous cyanosis, stated that nitrates were converted into nitrite within the intestinal tract by bacteria, the nitrite being absorbed through the injured intestinal wall and recovered in the blood stream. Years later ZoBell[23] showed that many organisms found in the intestinal tract could change nitrate into nitrite in vitro. It is now generally hypothesized that, after the introduction of nitrate into the intestinal tract, there is normally a conversion of a portion of the nitrate into nitrite and ammonia. After absorption in greater or lesser amount, nitrite may react with hemoglobin or may be excreted by the kidney. The reaction of nitrite with hemoglobin results in the formation of methemoglobin by oxidation, ferrous ion being converted to ferric ion. The process is reversible, but reconversion to hemoglobin is slow, requiring a minimum of eight or nine hours, according to Wendel,[24] and longer according to other authors. Inasmuch as anoxia is the basis of the symptomatology, the crux of the problem lies in the management of the patient during the first eight to twelve hours.

Before cyanosis due to methemoglobinemia occurs, a considerable amount of nitrite must be absorbed and retained in contact with hemoglobin. According to Wendel,[8] methemoglobin should make up at least 10 per cent of the total blood pigment before cyanosis becomes visible. Miller[4] quotes 3 gm. of methemoglobin per 100 cc. of blood as the critical figure for visible cyanosis.

Our review of the literature has uncovered many factors that play a part in the absorption of nitrite and its reaction with hemoglobin to form methemoglobin.

Chemical nature of the toxic agent. It is generally recognized that methemoglobinemia appears more quickly after nitrate ingestion than after sulfanilamide or plasmochin.[8]

Age of patient. Infants are more susceptible because of the greater frequency of gastrointestinal disturbances, the presence of a bacterial flora more favorable to nitrite production (Böhme,[14]) the existence of nitrate-splitting organisms high in the intestinal tract, which would facilitate the reduction of the nitrate to nitrites before the nitrate could be completely absorbed,[25] and possibly because of less renal efficiency in eliminating nitrites.

Mode of administration of nitrate. It has long been recognized that a more severe and prompt methemoglobinemia results from the rectal administration of nitrate (Böhme[14]).

Integrity of intestinal mucosa. Van den Bergh[7] implicated the injured intestinal mucosa as a factor many years ago.

Amount of nitrate administered. The appearance of cyanosis due to methemoglobinemia is generally dependent on the amount of nitrate ingested. This was recognized by Böhme[14] years ago, and substantiated by the cases of Barker and O'Hare[26] and Tarr.[16]

Renal status. Nitrite retention on the basis of renal insufficiency has been implicated by Tarr.[16]

Rapidity of passage through the intestinal tract. Incomplete reduction of nitrate to ammonia may be the result of rapid passage through the intestine with the intermediate nitrite present in greater quantity near the absorbing surface of the intestine.

pH of blood. Greenberg et al.[27] have shown that the conversion of hemoglobin to methemoglobin in the presence of nitrite occurs more rapidly in an acid medium. Furthermore, Marshall and Marshall[21] lessened the symptoms of nitrite poisoning experimentally by intravenous administration of sodium carbonate or bicarbonate.

Diagnosis of Methemoglobinemia

The diagnosis of methemoglobinemia with scientific certainty is made only by spectroscope or spectrograph. The average physician, however, will rely on the following points to establish a diagnosis

of nitrate methemoglobinemia: if no history of nitrate ingestion is obtainable, it is incumbent on the physician to ascertain whether or not well water of high nitrate content has been ingested; the cyanosis is described as an unusual grayish-brown color; the blood in methemoglobinemia is generally described as chocolate brown, brownish red or brownish black (hemolysis does not occur); blood transfusion frequently intensifies the cyanosis by enhancing the formation of additional methemoglobin; and chemical analysis of blood demonstrates nitrites.

Therapeutic Rationale

A certain percentage of patients with minimal cyanosis and symptoms will recover without therapy after withdrawal of the toxic agent. Wallace,[5] however, likens the methemoglobinemia following nitrate ingestion to the effects of rapid hemorrhage and that of enterogenous cyanosis and aniline poisoning to the effects of slow blood loss. The most urgent therapy is therefore required in cases of more advanced nitrate methemoglobinemia, and the critical period is the first twelve hours.

The treatment of choice in severe cases is the use of an agent that will hasten reconversion of methemoglobin to hemoglobin. Thionin and methylene blue are two such agents. Ascorbic acid and niacin possess the same property but act more slowly. Hence, they are more applicable to cases of methemoglobinemia due to aniline, sulfanilamide and plasmochin. Methylene blue catalyzes the physiologic reduction of methemoglobin by the body. According to Wendel,[8, 24] after administration in dilute solution, methylene blue is converted to the leuko or reduced form of dye by certain cellular enzymes in the red blood cell and other body cells. According to Hauschild,[29] who reported on the effectiveness of thionin and methylene blue (tetramethyl thionin hydrochloride) in the treatment of methemoglobinemia, a reversible oxidation-reduction system of thionin-leukothionin is produced initially. The hemoglobin-methemoglobin system is then shifted in favor of hemoglobin. Hauschild claimed that thionin administered intravenously was effective in ten minutes. Lederer[30] is quoted by Ferrant[6] as follows: "Thionin, a non-toxic, violet dye, not methemoglobin formative, has a reducing power five times that of methylene blue." Thus, the use of thionin in place of methylene blue bears investigation.

At present a rapid though brief action may be expected after the intravenous injection of methylene blue. The accepted dosage is 1 or 2 mg. per kilogram of body weight injected intravenously over a period of five minutes. An effect can be expected within thirty minutes. Injection of the dye under the skin and around the vein is painful and may produce necrosis. Furthermore, too rapid injection may produce hives and severe burning of the mouth. It must be emphasized again that the dye when used intravenously produces a rapid but brief action. Hence, it is important to supplement with oral therapy for prolonged action. Ten times the intravenous dose should be given orally at the same time and repeated once or twice to cover the critical period of anoxia. It has, moreover, been suggested that glucose in vitro transforms methemoglobin into oxyhemoglobin. We have also mentioned the favorable influence of an alkaline medium. Hence, the utilization of glucose and sixth-molar lactate solutions after the intravenous administration of methylene blue appears to be desirable.

Summary

The general subject of nitrate methemoglobinemia is reviewed.

A case of bismuth subnitrate methemoglobinemia recently encountered is presented.

The mechanisms involved in the formation of methemoglobin and in the evolution of rational therapy are discussed.

The increasing number of reports of methemoglobinemia due to nitrates in well water point to the growing importance of this variety.

It is re-emphasized that the use of bismuth subnitrate as a therapeutic agent is unnecessary, unwarranted and hazardous.

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STREPTOMYCIN IN THE TREATMENT OF SALMONELLA ENTERITIS IN INFANTS*

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THE susceptibility of various strains of salmonella in vitro to streptomycin has been adequately demonstrated by West et al.[1] However, the clinical efficacy of this antibiotic in salmonella enteritis has not been too extensively investigated, and relatively few cases have been reported thus far among children. Seligmann et al.[2] used streptomycin in 5 cases of enteritis due to *Salmonella typhimurium* in the newborn period and observed only a transitory sterilization of the bowel, and in all 5 cases the pathogenic organism reappeared in the stool, usually within forty-eight hours after the drug had been discontinued. A relatively small oral dose of streptomycin was used in Seligmann's series, ranging between 0.2 and 0.8 gm. a day for three or four days. In 1 case, combined oral and intramuscular administration was used and similarly failed to effect a permanent cure. Swiller and his associates,[3] on the other hand, reported a bacteriologic cure of an eight-month-old salmonella carrier when 1 gm. of streptomycin was given orally daily for twenty consecutive days. Pulaski and Amspacher[4] treated 3 cases of salmonella enteritis in infants, using a daily oral dose of 100 mg. per kilogram of body weight for four to seven days, and the stool cultures became negative and remained so during a three-week follow-up period. Except for these three reports, the literature on the treatment of salmonella enteritis in infancy with streptomycin has been limited.

During the past year, we have had the opportunity of treating 8 patients, ranging in age from three months to three years, with large doses of streptomycin for a prolonged interval. This report deals with the clinical and bacteriologic observations made in these cases.

TABLE 1. *Summary of 8 Cases of Salmonella Enteritis Treated with Streptomycin.*

Case No.	Age	Etiologic Organism	Streptomycin Sensitivity before Treatment	Streptomycin Sensitivity after Relapse	Dose of Streptomycin	
					Oral	Intramuscular
	mo.		unit/cc.	unit/cc.		
1	15	*S. manhattan*	5.0	5.0	0.4 gm. every 4 hr. for 7 days; 0.8 gm. every 4 hr. for 8 days.	None
2	3	*S. typhimurium*	5.0	5.0	0.4 gm. every 4 hr.	None
3	36	*S. oranienberg*	10.0	10.0	0.6 gm. every 3 hr.	0.1 gm. every 6 hr.
4	5	*S. typhimurium*	4.0	5.0	0.1 gm. every 4 hr.	None
5	4	*S. anatis*	12.5	—*	0.4 gm. every 4 hr.	0.1 gm. every 6 hr.
6	9	*S. meunchen*	10.0	7.5	0.4 gm. every 4 hr.	0.1 gm. every 6 hr.
7	1	*S. oranienberg*	10.0	12.5	0.4 gm. every 4 hr.	0.15 gm. every 8 hr.
8	8	*S. typhimurium*	6.0	7.5	0.6 gm. every 4 hr.	0.2 gm. every 8 hr.

*No relapse.

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Factors Affecting the Secretion of Staphylococcal Enterotoxin A

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The biosynthesis of enterotoxin A by replicating and nonreplicating cells was investigated. Unlike enterotoxin B, a secondary metabolite, enterotoxin A secretion resembled that of a primary metabolite by being secreted during the exponential phase of growth. The amount of toxin produced per unit of growth was not influenced by NaCl, $NaNO_2$, or $NaNO_3$. Several surfactants increased toxin secretion. Toxin secretion by nonreplicating cells was inhibited by chloramphenicol and 2,4-dinitrophenol but not by streptomycin or penicillin G. The optimal *p*H for enterotoxin A production was 6.5 to 7.0. The findings suggest a number of possible reasons for the higher incidence of food poisonings caused by enterotoxin A as compared to enterotoxin B.

In previous investigations (7, 8; Z. Markus, Bacteriol. Proc., p. 81, 1969), nonreplicating cells of *Staphylococcus aureus* were employed. This procedure separates the requirements for the secretion of enterotoxin B from those of growth and replication. As a result of these studies, a mechanism was postulated involving the presence of a toxin precursor (8). The assumption was based on the fact that cells resuspended in a nitrogen-free medium secreted a certain amount of toxin in the presence of chloramphenicol which was not detectable in cell extracts. The amount of toxin precursor present in the cell was dependent upon the rate of aeration and the age of the culture.

Although enterotoxin A has been implicated in a larger number of food poisoning cases than enterotoxin B (1), the conditions necessary for the secretion of enterotoxin A are not well defined. Enterotoxin A is produced in much smaller quantities than enterotoxin B, and there is more uniformity among strains in the amounts of toxin produced (5, 12). Donnelly et al. (2) have shown that the production of enterotoxin A in milk is associated with microbial growth, but the levels of enterotoxin were not quantitated.

This study was undertaken to compare the factors necessary for the secretion of enterotoxin A by replicating and nonreplicating cells with those previously found for enterotoxin B.

MATERIALS AND METHODS

Organism. *S. aureus* 100 and S-6 were obtained from M. Bergdoll, University of Wisconsin, Madison, Wis. Both strains possessed free coagulase and deoxyribonuclease and were bound coagulase-negative.

Media. As in the previous studies with enterotoxin B (7, 8), three media were employed, two utilizing N-Z amine as a source of nitrogen and energy (medium 1 and 2) and the third (medium 3A) lacking a nitrogen source but containing glucose. The latter two media were used in the studies involving toxin secretion by nonreplicating cells.

Medium 1, as described by Rosenswald et al. (13), was the growth medium and contains 4% N-Z amine type A (Sheffield Chemical, Norwich, N.Y.), 0.4% yeast extract (Difco), and 0.1% K_2HPO_4 in distilled water (*p*H 6.6 to 6.8).

Medium 2 contains 4% N-Z amine type A and 0.1% K_2HPO_4.

Medium 3A contains 0.5% glucose and 3.5% K_2HPO_4, the *p*H being adjusted to 7.0. The designation 3A was chosen to avoid confusion with medium 3 described previously (8), which has the same composition but a *p*H of 8.5.

Growth studies. Stock cultures of strain 100 were grown at 37 C for 16 to 18 hr on Trypticase Soy Agar slants (BBL) plus 0.5% yeast extract (Difco) and were stored at 4 C. A loopful of this culture was inoculated into a 500-ml shake flask, containing 100 ml of medium 1, and incubated at 37 C on a gyratory shaker (New Brunswick Scientific Co., Inc., New Brunswick, N.J., model V) for 18 hr. Repropagation was conducted in the same manner as above but with a 1% inoculum.

Experiments in fermentors. Toxin production was studied in 400 ml of medium 1 in a 1-liter vessel at 37 C and aerated at a rate of 2 liters/min; 100 μg of antifoam P-2000 (Dow-Corning, Midland, Mich.) per ml was used to avoid excessive foaming. The *p*H was automatically controlled at 6.9 by the addition of HCl.

Nonreplicating cells. In preliminary experiments, it was found that cells harvested after 3.5 hr of propaga-

ion at 37 C and resuspended in either medium 2 or 3 secreted over 85% of their toxin potential within 4 hr.

For this reason, the cells were propagated as described above and, unless otherwise noted, were harvested by centrifugation at 5,000 × *g* for 10 min, washed twice in 0.01 M phosphate buffer (*p*H 7.0), and adjusted in either medium 2 or 3A to a cell concentration of 5,000 to 6,000 Klett units (6×10^{10} to 8×10^{10} organisms/ml). A 10 ml amount of this suspension was agitated at 240 rev/min in a 100-ml flask at 37 C.

Assay of enterotoxin A. By using purified antigen and antiserum (provided by M. Bergdoll), the single diffusion technique of Weirether et al. (14) developed for enterotoxin B was found to be suitable for the assay of enterotoxin A. The response of the calibration curve for enterotoxin A was linear and sensitive to 0.4 μg/ml if the test was read within 24 hr. Longer incubation increased the sensitivity of the test.

The procedure for the assay of the supernatant was the same as that previously described for enterotoxin B (7, 8), care being taken to compensate for differences in ionic strength.

Chemicals. Aqueous stock solutions of chloramphenicol (Parke, Davis & Co., Detroit, Mich.), potassium penicillin G (Mann Research Laboratories, N.Y.), streptomycin sulfate (NBC, Cleveland, Ohio), and 2,4-dinitrophenol (Eastman, Rochester, N.Y.) were membrane sterilized and appropriately diluted in the final medium.

The surfactants Tweens 20, 40, 60, and 80, polyoxyethylene oleate, Span 60 and 80 (Atlas Chemical, Wilmington, Del.), sodium oleate (Baker Chemical, Phillipsburg, N.J.), Triton X-100 (Rohm and Haas, Philadelphia, Pa.), sucrose monopalmitate, and sucrose monostearate (Colonial Sugars, N.Y.) were autoclaved with medium 1.

Sodium chloride and sodium nitrate were added to medium 1 and sterilized by autoclaving. Sodium nitrite was sterilized by filtration and aseptically added to sterile growth medium 1.

RESULTS

The secretion of enterotoxin A in shake-flask cultures occurred mainly in the exponential phase of growth, although approximately 20% was secreted as the cells entered the early stationary phase of growth (Fig. 1). The cell density reached in these experiments was similar to that previously noted for the production of enterotoxin B by strain S-6 (8).

Growing strain 100 at a controlled *p*H of 6.9 increased the duration of exponential growth and, hence, the total cell crop (Fig. 2). Toxin production also increased during exponential growth, but the concentration produced per Klett unit remained fairly constant (3.4 to 4.6 μg of enterotoxin A per 1,000 Klett units).

Concentrations of NaCl of up to 10% did not essentially alter the ratio of enterotoxin A formation to growth (Fig. 3). Higher NaCl concentrations inhibited bacterial growth (also found for

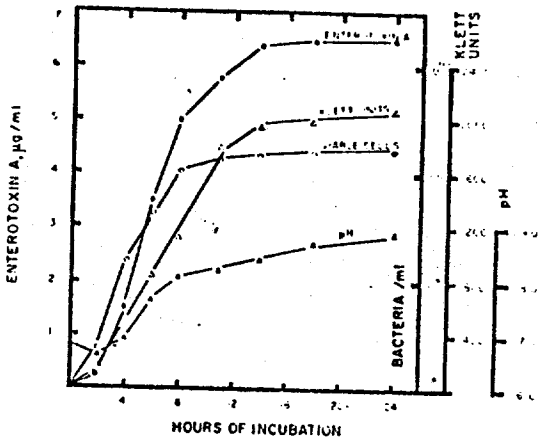

FIG. 1. *Enterotoxin A production by cells grown as shake-flask cultures.*

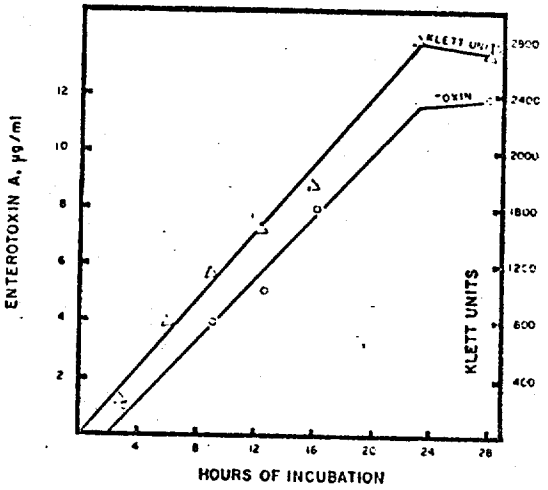

FIG. 2. *Enterotoxin A production by cells grown in a fermentor at a constant pH of 6.9.*

strain S-6); therefore, toxin concentration was determined after 48 hr of cell growth. As much as 200 μg of $NaNO_2$ per ml and 1,000 μg of $NaNO_3$ per ml did not affect either bacterial growth or toxin formation by strain 100. No synergistic effect between NaCl, $NaNO_2$ and $NaNO_3$ on bacterial growth or on enterotoxin A secretion was found. Strain 100 grown in medium 1 with 10% NaCl, 200 μg of $NaNO_2$ per ml and 1,000 μg of $NaNO_3$ per ml attained a cell concentration of 800 Klett units and produced 2 μg of enterotoxin A per ml.

Surfactants had a varied effect on growth and toxin formation (Table 1). Enterotoxin A secretion was increased by the presence of Tweens 40 and 60, whereas Tweens 20 and 80 had no effect. Span 60 behaved similarly to Tweens 20 and 80;

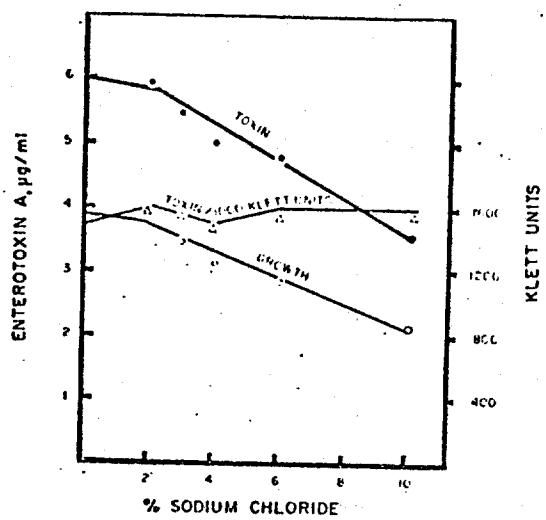

FIG. 3. *Effect of NaCl on growth and enterotoxin A secretion.*

TABLE 1. *Effect of surfactants on growth and toxin formation*

Surfactant[a]	Strain S-6		Strain 100	
	Growth[b]	Enterotoxin B	Growth	Enterotoxin A
		μg/ml		μg/ml
Control	1,450	150	1,500	6.5
Tween 20	1,200	70	1,400	6.8
Tween 40	1,200	150	1,550	8.5
Tween 60	1,600	290	1,550	9.0
Tween 80	1,250	17	1,400	6.5
Span 60	NG[c]	NG	1,500	5.5
Span 80	1,100	8	1,250	ND[d]
Polyoxyethylene oleate	1,200	ND	1,200	4
Sodium oleate	800	ND	300	ND
Triton X-100	180	ND	0	NG
Sucrose monostearate	NG	NG	800	2
Sucrose monopalmitate	550	ND	0	NG

[a] Final concentration used was 0.1% (v/v) in medium 1, except Triton X-100 which was at 0.05%.
[b] Determined from a 16 hr culture at 37 C in medium 1 with a Klett-Summerson colorimeter and a no. 66 filter; values are given in Klett units.
[c] No growth.
[d] Not detectable.

Span 80, although having a minor inhibitory effect on growth, completely inhibited enterotoxin A secretion. The remaining compounds inhibited growth, toxin formation, or both.

Cells harvested during various phases of growth had their toxin synthesizing abilities evaluated by the non-replicating technique (Table 2). Those cells obtained from the exponential phase (3.5 hr) produced high toxin levels, whereas cells harvested from the stationary phase of growth (16 hr) failed to produce significant amounts of toxin. The presence of N-Z amine in medium 2 doubled the concentration of toxin secreted but did not alter the incapability of cells harvested at 16 hr to synthesize toxin. The effect of *p*H was also important (Table 3). The maximal secretion of toxin occurred at *p*H 7.0, regardless of whether nitrogen was present.

The effect of several metabolic inhibitors on toxin secretion by nonreplicating cells is presented in Table 4. Chloramphenicol and 2,4-dinitrophenol prevented the secretion of enterotoxin A, but normal amounts of toxin were secreted by cells in the presence of streptomycin and penicillin G

TABLE 2. *Enterotoxin A production by nonreplicating cells harvested at different time intervals during the growth cycle*

Time of growth[a]	Enterotoxin A	
	Medium 2	Medium 3A
hr	μg/ml	μg/ml
3.5	3.5	1.9
6	2.1	1.0
16	ND[b]	ND

[a] At the sampling periods specified, the cells were collected, washed, and adjusted in medium 2 or 3A to a Klett reading of 5,000 to 6,000 units and incubated for 4 hr at 37 C as described in Materials and Methods.
[b] Not detectable.

TABLE 3. *Effect of pH on enterotoxin A production by nonreplicating cells*[a]

pH	Enterotoxin A	
	Medium 2[b]	Medium 3A[b]
	μg/ml	μg/ml
5.5	2.2	ND[c]
6.5	3.5	1.0
7.0	4.0	2.0
8.0	2.2	1.0
8.5	1.9	0.5

[a] Cells grown for 3.5 hr in medium 1 were harvested, washed, resuspended at the appropriate *p*H, and incubated at 37 C for 4 hr by the procedure described in Materials and Methods.
[b] Both media contained 0.2 M K_2HPO_4 and were adjusted initially to the appropriate *p*H with 0.5 M HCl or NaOH.
[c] Not detectable.

TABLE 4. *Effectiveness of various compounds to inhibit enterotoxin A production by nonreplicating cells*[a]

Inhibitor	Concn/ml	Enterotoxin A
		μg/ml
Control		3.0
Chloramphenicol	100 μg	ND[b]
2,4-Dinitrophenol	2 μmoles	ND
Streptomycin sulfate	0.007 μmoles	2.6
Penicillin G	100 units	2.9

[a] Harvested after 3.5 hr of growth, the standardized cell suspension was incubated for 4 hr at 37 C in medium 2 supplemented with the above compounds.

[b] Not detectable.

Cells propagated at 20 C attained the stationary phase of growth after 3 days. These cells were resuspended in medium 2 or 3A at 37 C by the nonreplicating technique. After an incubation period of 4 hr, 3.5 μg of enterotoxin A per ml was found in medium 2 and none in 3A. For cells grown at 37 C for 3.5 hr and resuspended at 20 C in medium 2 or 3A, only traces of enterotoxin A were subsequently found by the nonreplicating technique.

DISCUSSION

Enterotoxins A and B appear to be quite similar in their chemical structure and in their clinical symptoms of intoxication. Either toxin can be produced by nonreplicating cells in a nitrogen-free medium. This effect is increased by glucose, and air must be present. Toxin synthesis is also stimulated by the presence of protein hydrolysates. However, there are important differences in the biosynthesis of enterotoxins A and B.

It was previously established (7, 8) that enterotoxin B was produced by cells entering the stationary phase of growth and that, for a given medium, the secretion of enterotoxin B could be varied by altering cultural conditions. The optimal *p*H for the secretion of enterotoxin B in a nitrogen-free medium was 8.0 to 8.5 and 7.0 to 7.5 in the presence of protein hydrolysates. A certain amount of secretion occurred at 37 C with nonreplicating cells grown at 37 C in the presence of chloramphenicol. Cells propagated at 20 C and resuspended under nonreplicating conditions at 37 C demonstrated a lag of several hours before toxin secretion occurred.

In contrast, the secretion of enterotoxin A by strain 100 was found in the study to occur mainly during the exponential phase of growth, was directly related to cell numbers, and was influenced to a much lesser extent by cultural conditions. The optimal *p*H for the formation of enterotoxin A, in either the presence or absence of protein hydrolysates, was 6.5 to 7.0. Chloramphenicol completely inhibited toxin secretion by nonreplicating cells of strain 100. These results indicated differences in biosynthesis of enterotoxins A and B and the absence of any appreciable concentration of a partial or completely formed toxin precursor in strain 100.

In many cases, the effect of a given surfactant on toxin production by strains S-6 or 100 differed. However, these observations are not unique to this system because Tween 80, oleic acid, and other surfactants have been found by Friedman (3) to differ in their effect on two enterotoxin B producing strains. The value of surfactants for increasing enzyme yields was emphasized by Reese and Maguire (11), who assumed that these compounds affect cell permeability. An alternate explanation for their activity could be that they affect oxygen transfer in liquid media (4).

McLean et al. (6), using ATCC strain 14458, found that NaCl inhibited enterotoxin B secretion to a greater extent than growth. Our results with strain S-6 (*unpublished data*) confirm this observation. However, in the case of enterotoxin A, the amount of toxin secreted in the presence of NaCl was directly related to growth.

It was postulated (6, 8; Z. Markus, Bacteriol. Proc., p. 81, 1969) that enterotoxin B synthesis was characteristic of a secondary metabolite because its secretion was initiated during the late exponential and early stationary phase of growth. In contrast, it is apparent that enterotoxin A synthesis follows the pattern of a primary metabolite since it was synthesized continuously by cells during exponential growth. Cells of strain 100 harvested during the stationary phase did not produce significant amounts of enterotoxin A. However, the enterotoxins secreted by strains 100 and S-6 appear to satisfy the definition of Raynaud and Alouf (10) for true protein exotoxins. Neither strain secretes toxin during the late stationary phase of growth, and no intracellular toxin is detectable.

The incidence of food poisoning caused by enterotoxin A is higher than that caused by enterotoxin B (1). Our findings suggest a number of possible reasons for this observation. Being associated with growth, enterotoxin A is more likely to appear earlier than would enterotoxin B and at a *p*H range more commonly associated with food (*p*H 6 to 7). In certain situations, growth of strain S-6 occurred without enterotoxin B secretion. This is less likely in the case of enterotoxin A. Strain 100, an enterotoxin A producer, propagated at lower temperatures will begin to secrete toxin much sooner at 37 C than strain S-6 (8).

It is also conceivable that the higher incidence may be due to a greater frequency of enterotoxin A producing strains and to the fact that humans are more susceptible to this toxin than to enterotoxin B (9).

ACKNOWLEDGMENTS

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high dietary calcium appears feasible as a means of commercially reducing bone fragility.

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The Use of Turkey Poults and Chickens as Test Animals for Nitrate and Nitrite Toxicity

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INTRODUCTION

NITRATE and/or nitrite toxicity or poisoning has been the subject of extensive studies over the years. Numerous articles have appeared with information on reduction of nitrate to nitrite by micro-organisms and higher plants, Kurup and Vaidyanathan (1964). Conversion of nitrate to nitrite has, also, been reported in whole herbage, Jones and Griffith (1965). Perhaps the earliest recorded account of nitrate toxicity in animals was made by Mayo (1895) when he reported high mortality in range cattle fed corn stalks. Some forty years later Bradley *et al.* (1942) encountered similar problems when they fed oat hay to calves. It has now been established that these conditions only occur on the range when there is a large amount of nitrate present in the soil or when some other factors interfere with the normal processes of the plant.

Experimental results of recent years have produced evidence of retarded growth in cattle, Weichenthal *et al.* (1963) and Wallace *et al.* (1964), in sheep, Goodrich *et al.* (1964), and rats, Emerick and Olson (1962).

Although much of the work has been confined to the large animals, there have been several instances of this problem in poultry. One early report was made by Gallagher (1919) who was interested in avian toxicology. He fed three hens, weighing about four pounds each, doses of 9.7, 5, and 2 grams of KNO_3. The hen re-

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ceiving the larger dose died, but the other two survived. Guberlet (1922) investigated losses in a farm flock where 25 out of 225 affected had died. The birds he found had been fed KNO_3 in their feed instead of Epsom salts (magnesium sulphate). Conducting experiments of his own, he observed that a 3 gram dose of KNO_3 was toxic and 6 grams lethal. Riggs (1945) published a report on turkeys in which the birds developed nitrite poisoning when they were on range after a heavy rain ended a two-month drought.

Njaa *et al.* (1955) investigated the effect of sodium nitrite used to preserve herring meal fed to chickens. They found that the chicks could tolerate up to 0.2% nitrite in the feed. Beyond this level the chicks became sick and some died. The nitrite had no effect when vitamin A was added. Sell and Roberts (1963) reported that high levels of nitrite caused severe growth depression and high mortality in chicks. The same two researchers, Roberts and Sell (1963), determined that destruction of vitamin A in the presence of nitrite occurs in those areas of the digestive tract of sheep and chickens where the pH is 4 or less.

Another approach to this problem in poultry has been to supply the nitrate or nitrite in the drinking water of the birds. Bentley *et al.* (1965) reported that 300 p.p.m. nitrate added to the water supply of laying hens decreased early egg production. However, in the cooler months, addition of nitrate had no apparent effect; a change which would suggest that conversion of nitrate to nitrite is directly related to environmental temperature. Another explanation might be that less water was consumed in cool weather. Addition of iodine to a basal ration seems to have counteracted to some extent part of the deleterious effect of nitrate in the water. Kienholz *et al.* (1965) tested well water from four wells that had reportedly caused poor growth in turkeys. They found little effect of water source on bird performance up to four weeks of age. Continuing this experiment to 21 weeks using two treatments of distilled water and water from one well they found no effect on body weights, but a close relation in feed/gain ratio and water/feed consumption. A second trial, in which various levels of sodium nitrate were added to drinking water, yielded no difference in growth of poults at four weeks of age. Weights were improved at 16 weeks even though the nitrate was given only to four weeks.

Recently Adams *et al.* (1966) reported decreases in weight, feed consumption, water consumption, and high mortality in poults supplied with up to 200 p.p.m. nitrite-nitrogen in the drinking water. A significant decrease in liver vitamin A and β-carotene was also obtained at this level. Chicks in the same experiments fed different levels of nitrate showed no differences in feed or water consumption. All groups were given high levels of vitamin A.

The experiments reported here were undertaken because of field problems with turkeys during 1963 and 1964. During 1965 and 1966, the field problems were very minor; however, nitrate levels in the feedstuffs had also decreased to low levels.

PROCEDURE

Most of the studies reported here were conducted with day-old Broad Breasted Bronze (BBB) turkey poults of mixed sexes produced from a breeding flock maintained on the University experimental farm. The poults were wing-banded and weighed individually, then placed in an electrically heated starting battery. Each group consisted of 20 poults except for one experiment which had 24 per

group. The experiments lasted 3 to 4 weeks. The basal diet used is shown in Table 1. Feed and water were supplied *ad libitum*.

In the first three experiments (Tables 3, 4, and 5) certain vitamins, antibiotics, and minerals (1.59% Ca. in experiment 1) were used at different levels as additives to see whether they reduced or increased nitrite toxicity. In the other experiments, shown in Tables 6–11, KNO_3, KNO_2, and vitamin A were the only additives. In most instances in these experiments, the levels added were 1,000 p.p.m. NO_3^- and 400 p.p.m. NO_2^- except those shown in Tables 7–9 where they are used in combination and at varying levels. Table 6 shows the combined results of three experiments. In all these experiments, alfalfa meal was either deleted or used at a 1%, 2%, or 4% level depending on the total carotene content of the diet. Vitamin A acetate was added at levels of 20,000, 15,000, or 10,000 I.U. per kg. In the chick experiments, the basal diet was modified to bring it to a 20% protein level. Day-old chicks, also of mixed sexes from a NH♂×SCWL♀ mating, were used in these experiments. They were treated in the same manner as the poults,

TABLE 1.—*Basal diet*

	Gms./kg.
Ground corn	290
Soybean meal (44% protein)	577
Meat scrap (50% protein)	20
Fish meal (60% protein)	20
Alfalfa meal (220 I.U. vit. A/gm.)	20
Iodized salt	3
Calcium carbonate	20
Di-calcium phosphate	30
Choice white grease	15
Vitamin premix[1]	5
Manganese sulfate·H_2O	0.33

[1] Provided the following per kg. diet: Riboflavin, 2 mgs.; Ca pantothenate, 5 mgs.; menadione bisulfite, 0.5 mgs.; choline chloride, 0.5 gm.; vitamin B_{12}, 2 mcg.; vitamin D_3 (1,500 I.C.U./gm.), 0.33 gms.; sucrose used to make up 5 gms. per kg.

TABLE 2.—*Analysis of feed samples for nitrate during the years of 1964–1967*

Years	Corn	Soy	Alfalfa	Water
	p.p.m.	p.p.m.	p.p.m.	p.p.m.
1964	105	1,564	—	84
	74	984	—	31
	111	—	—	49
1965	235	251	—	—
1966	35	165	2,080	—
1967	187	167	—	90

prior to having been placed on experiment. All data collected from these experiments are shown in Tables 10 and 11. Table 10 is a combination of experiment 12 and 12a which lasted four weeks. Table 11 contains combined data from experiments 13 and 14 which lasted five weeks.

These experiments were conducted between 1964–1966. Nitrate determinations on the unsupplemented basal for both poults and chicks showed that it contained at various times levels of 1,177, 1,564, 472, and 154 p.p.m. nitrate. Nitrate levels of the feedstuffs were highest during 1964 and decreased during 1965 and 1966. The nitrate values of certain feedstuffs from 1964–1967 obtained in Wisconsin are shown in Table 2. These were determined using the procedure of Vogel (1948) for the 1964 material and the procedure of Bloomfield *et al.* (1965) for the other samples.

RESULTS AND DISCUSSION

The first experiment summarized in Table 3 gives some indication of the rate of growth and mortality of the poults during the three-week-experimental period. There were no measurable differences in growth or mortality among the treatments at the end of the first week. This was true for all experiments conducted. In this experiment, as in all the others, the toxicity symptoms (Fig. 1) caused by the nitrate, or nitrite, were characterized by poor growth, anorexia, incoordination and

FIG. 1. Nitrite toxic poults at about 3 weeks of age. Note emaciated condition and in coordination.

muscle tremors, rapid and labored breathing, frothing around the mouth, and high mortality. These symptoms were also observed in chickens by Guberlet (1922) and turkeys by Riggs (1945). Note that mortality was over 50% in some groups. Most of these symptoms were also observed in the field. There were also several severe cases of perosis among these groups. Frequently, the poults developed a "locked-hock" condition (Fig. 2). Addition of folic acid and furazolidone[1] in the first two experiments (Tables 3 and 4) did not appear to reduce or increase the toxicity.

[1] 3-(5 Nitrofurfurylideneamino)2-oxazolidinone.

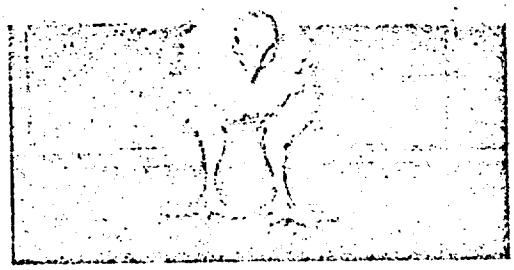

FIG. 2. Nitrite toxic poults showing "locked hock" condition.

TABLE 3.—*The effect of different additives on growth and mortality of poults when fed potassium nitrite*

Additions per kg.	WEEKS 1	2	3	
	gms.	gms.	gms.	% Mort.
1. Basal[1]+400 p.p.m. NO_2^-	129	221	264	56.0
2. as 1+1.8 gms. furazolidone	130	223	264	36.0
3. as 2+1 mg. folic acid	134	233	281	52.0
4. as 3+50 mgs. niacin	132	239	365	0
5. as 4+Vit. A (20,000 I.U.)	136	263	416	0
6. as 5+5 mgs. Vit. E	134	255	400	4.0
7. as 6+.9 gms. furazolidone	132	263	396	—
8. as 6 (No furazolidone)	132	252	392	4.0
9. as 2+100 p.p.m. NO_2^- in H_2O	127	222	247	32.0
10. as 6 (reduced Ca)	131	255	404	—

[1] Contained 1,177 p.p.m. NO_2^- on assay.

The groups receiving added vitamin A in these experiments grew normally in spite of the presence of the added nitrite in the diet, plus the nitrate present in the basal diets. The natural nitrate present in the basal diet was 1,177 p.p.m. for experiments in Table 3 and 1,192 p.p.m. for experiments shown in Table 4. Growth depression and mortality occurred when 400 p.p.m. NO_2^- was added even though the basal diet contained 2% alfalfa meal. The addition of niacin (Table 3—group 4) appeared to reduce toxicity of the high levels of nitrate and nitrite, but mortality was high even with niacin additions (Table 4—group 6). The addition of niacin to a diet containing 400 p.p.m. of added NO_2^-

TABLE 4.—*The effect of different additives on growth and mortality of poults when fed potassium nitrite*

	4-wk. gms.	% Mort.
1. Basal+folic acid (1 mg./kg.)[1]	401	.0
2. As 1+400 p.p.m. NO_2	300	40.0
3. As 2+1.8 gms. furazolidone	262	35.0
4. As 2+50 mg. niacin	338	5.0
5. As 2+niacin+Vit. A (20,000 I.U.)	395	15.0
6. As 3+niacin	302	40.0
7. As 6+Vit. A (20,000 I.U.)	444	10.0
8. As 3 (.9 gm. furazolidone)	421	35.0
9. As 5 (No added niacin)	477	5.0
10. As 5 (100 p.p.m. NO_2 in H_2O)	464	15.0

[1] Soybean meal contained 1,564 p.p.m. NO_3.
Corn contained 111 p.p.m. NO_3.
Basal diet contained 1,192 p.p.m. NO_3.

TABLE 5.—*The effect of different levels of potassium nitrite on growth of poults*

	Ave. wts.	% Ave. mort.	Liver vit. A units/gm.
1. Basal (2% Alfalfa+furazolidone+folic acid)[1] mg./kg.	426.6	12.5	3
2. +Vit. A (2,000 I.U.)	528.4	7.5	517
3. +100 p.p.m. NO_2	438.3	7.5	3
4. +200 p.p.m. NO_2	459.2	7.5	3
5. +400 p.p.m. NO_2	447.1	7.5	3
6. +800 p.p.m. NO_2	459.5	15.0	3
6A. +800 p.p.m. NO_2+Vit. A (20,000 I.U.)	521.6	15.0	396
7. +400 p.p.m. NO_2+50 mgs. niacin	442.6	2.5	—
8. +400 p.p.m. NO_2+Vit. A	494.0	5.0	421
9. +400 p.p.m. NO_2+Vit. A+niacin	520.8	5.0	—
10. +200 NO_2+Vit. A	488.4	7.5	—

[1] Contained 472 p.p.m. nitrate.

was not beneficial. Thus it is apparent, that while niacin gives protection under some conditions, it is not nearly as effective as vitamin A. In experiment 3 (Table 5), which lasted four weeks, all groups were given folic acid and furazolidone. The basal diet contained 472 p.p.m. nitrate. Different levels of NO_2^- were used, but the results showed no differences among groups either in weights or mortality. Because the level of nitrate was much lower in the basal diet, than in previous experiments, this is to be expected. However, the vitamin A assay on pooled liver samples from these poults showed that vitamin A storage was lowest in the groups receiving supplemented NO_2^- but no added vitamin A. This is in spite of 2% alfalfa meal in the basal diet.

Table 6 summarizes the next three experiments. In these experiments only certain groups were given alfalfa. The vitamin A supplement was reduced from 20,000 to 10,000 units per kg., and mixtures of KNO_3 and KNO_2 were utilized because the natural nitrate in the feed was reduced to 154 p.p.m. Groups receiving the basal diet without supplementary vitamin A exhibited generally poor growth and developed vitamin A deficiency after two weeks. The mixture of NO_3^- and NO_2^- without vitamin A supplementation caused poorer growth and high mortality. No real differences were apparent from the use of 2% or 4% alfalfa with mixtures of NO_3^- and NO_2^-. Assay on pooled liver samples from five groups again showed a high storage level in the vitamin A supplemented groups and extremely low values for the groups getting the NO_2^- and NO_3^-. The vitamin A storage from the unsupplemented groups was

TABLE 6.—*The effects of nitrate and nitrite on growth, liver, vitamin A, and mortality of turkey poults**

	3 wk. ave. wts.	Ave. mort.	Liver vit. A units/gm.	Ave. feed eff. (G/F)
1. Basal (Low A)[1]	295.7	16.1	9.5	.36
2. As 1+Vit. A (10,000 I.U.)	368.9	5.0	265.0	.55
3. As 1+1,000 p.p.m. NO_3+400 p.p.m. NO_2	276.3	20.2	5.0	.30
4. As 3+Vit. A	379.0	12.5	236.0	.42
5. As 3+2% Alfalfa	302.5	16.7	6.7	.36
6. As 3+4% Alfalfa	255.9	—	—	.46

* Three experiments.
[1] Contained 154 p.p.m. nitrate.

TABLE 7.—*The effects of various levels of potassium nitrate when added to a constant level of nitrite*

	Ave. Wts.	% Mort.	Ave. feed eff. (G/F)
1. Basal (Low A)[1]+400 p.p.m. NO_2^-	184	55.0	.07
2. As 1+250 NO_3^-	182	35.0	.18
3. As 1+500 NO_3^-	192	10.0	.26
4. As 1+750 NO_3^-	194	20.0	.29
5. As 1+1,000 NO_3^-	195	55.0	.09
6. As 5+Vit. A (15,000 I.U.)	353	0	.53

[1] No alfalfa calculated to contain 154 p.p.m. nitrate.

TABLE 8.—*The effects of various levels of potassium nitrite when added to a constant level of nitrate*

	Ave. wts.	% Mort.	Ave. feed eff. (G/F)
1. Basal (1% Alf.)[1]	307.2	0	.52
2. As 1+Vit. A (15,000 I.U.)	375.3	0	.56
3. As 1+1,000 NO_3^-	308.2	0	.49
4. As 3+100 p.p.m. NO_2^-	315.2	15.0	.41
5. As 3+200 NO_2^-	245.7	25.0	.37
6. As 2+400 p.p.m. NO_2^-	368.5	0	.56

[1] Calculated to contain 172 p.p.m. nitrate.

so low that a proper dilution was not possible. Thus, the actual levels of vitamin A are probably even lower than the indicated ones.

A different approach was employed for the next two experiments (Tables 7 and 8). Table 7 shows the results of one experiment in which a mixture of NO_3^- and NO_2^- was used. The NO_2^- level was fixed, but the levels of NO_3^- varied. The vitamin A was supplemented at 15,000 units per kg. No difference in weight between groups 1–5 was apparent, but mortality was very high. This was to be expected because the poults suffered from both vitamin A deficiency and NO_2^- toxicity. Varying the NO_3^- from 154 to 1,154 p.p.m. did not affect the weight gains or mortality. Because all weights were low, this suggests that the NO_2^- was the most serious component. Group 6 which received supplement vitamin A improved body weights and feed efficiency to normal levels.

The reverse method was utilized for the experiment shown in Table 8. A fixed level of NO_3^- was used along with varying levels of NO_2^-. Growth rates and feed efficiencies were better than in the previous experiment because of the lower levels of NO_2^-. However, mortality was high and weight gains reduced with 200 p.p.m. of NO_2^-. This indicates again the nitrite is more toxic than nitrate. The basal diet, although containing 1% alfalfa, was slightly deficient in vitamin A and additions of 1,000 p.p.m. nitrate only (group 3) did not decrease weight gain. Adding more than 100 p.p.m. NO_2^-, however, was enough to induce greater deficiency and toxicity. Again, supplementing with vitamin A, even with NO_2^- present, improved growth and feed efficiency to the normal range.

This experiment was repeated without added alfalfa in the diet, and the results are presented in Table 9. The groups that received the NO_2^- and NO_3^- gained less

TABLE 9. *The effect on turkey poults of different levels of nitrite when added to a constant level of nitrate*

	Ave. wts.	% Mort.	Ave. feed eff. (G/F)
1. Basal (Low A)+1,000 p.p.m. NO_3^-[1]	240.3	38.8	.24
2. As 1+100 p.p.m. NO_2^-	215.8	27.7	.41
3. As 1+200 p.p.m. NO_2^-	191.6	44.4	.15
4. As 1+400 p.p.m. NO_2^-	183.6	55.5	.27
5. As 1+600 p.p.m. NO_2^-	200.7	27.7	.25
6. As 1+400 p.p.m. NO_2^-+(Vit. A 15,000 I.U.)	318.5	27.7	.38
7. Basal+2% Alf.+1,000 NO_3^-+400 NO_2^-	217.1	44.4	.21
8. Basal (Low A)	204.4	55.5	.11
9. Basal 2% Alf.+Vit. A 15,000 I.U.	360.4	11.1	.50
10. As 1+Vit. A 15,000 I.U.	346.1	5.5	.53

[1] Contained 150 p.p.m. nitrate.

and had higher mortality than in the previous experiment. The mortality appeared to increase with the level of NO_2^- in the mixture up to 400 p.p.m. Deficiency symptoms were also very severe (group 8) as the vitamin A in this diet was lower than in the previous experiment. Two groups (7 and 9) received 2% alfalfa meal. One group was supplemented with a mixture of NO_3^- and NO_2^- and the other with vitamin A. The former group had a poorer growth rate and higher mortality which would indicate that the carotene from the alfalfa was not converted efficiently to vitamin A in the presence of NO_3. Growth and feed efficiency were normal in the latter group. Group 6 which received mixtures of the nitrites, nitrates, and vitamin A had mortality higher than normal. The effect of vitamin A can probably be determined by comparing groups 1 and 10. When vitamin A was present, 1,000 p.p.m. of nitrate had no effect on the poults.

The results of the chick experiments conducted for 4 and 5 week periods are recorded in Tables 10 and 11. In the first experiment, the basal diet was supplemented with .5% alfalfa meal. Examination of these data revealed no difference between treatments. The basal apparently contained a sufficient amount of vitamin A (compared groups 1 and 3) to prevent any effects from the NO_2^- and NO_3^-. When the alfalfa was left out of the basal in the next experiment (Table 11), the nitrate and nitrite did depress the growth significantly at 5 weeks. Adding vitamin

TABLE 10.—*The effect of nitrate and nitrite on chicks fed a low vitamin A diet*

Exp. 12 and 12a	Ave. wts.	% Mort.
1. Basal (.5% Alf.)[1]	306.9	0
2. As 1+400 p.p.m. NO_2^-+1,000 p.p.m. NO_3^-	306.9	0
3. As 1+Vit. A (10,000 I.U.)	317.1	0
4. As 3+NO_2^-+NO_3^-	327.0	4
5. Control diet	281.2	0

[1] Combination of duplicate groups—4 weeks experiment calculated to contain 161 p.p.m. nitrate.

TABLE 11.—*The effect of nitrate and nitrite on chicks fed a low vitamin A diet*

Exp. 13 and 14	Ave. wts.	% Mort.
1. Basal (no Alf.)[1]	431.1	0
2. As 1+400 p.p.m. NO_2^-+1,000 p.p.m. NO_3^-	329.5	9.0
3. As 1+Vit. A (10,000 I.U.)	450.8	0
4. As 2+Vit. A (10,000 I.U.)	467.5	10.0
5. Control diet	404.7	4.0

[1] Combination of duplicate groups—5 weeks experiment calculated to contain 150 p.p.m. nitrate.

A overcame the toxicity of the nitrate and nitrite.

All evidence compiled on avian species suggest that turkeys are more susceptible to nitrate toxicity than chicks, Krista *et al.* (1961) and Adams *et al.* (1966). Our experiments also support this theory. It probably results because the turkey has a higher requirement for vitamin A and because of faster growth. Whenever NO_2^- was used by itself, or in fairly high amounts in combination with NO_3^- (Tables 7 and 9), the toxicity symptoms manifested themselves soon after the first week of experiment and were very severe. Riggs (1945) has indicated that there is rapid absorption of nitrite from the digestive tract. In all instances, growth was poor. The results obtained with NO_3^- in fixed amounts and added NO_2^- (Tables 8 and 9) show that before symptoms developed, it must be present in relatively high amounts. Riggs (1945) demonstrated that the NO_3^- by itself was relatively nontoxic for turkeys. However, when the NO_3^- is reduced to NO_2^- in the digestive tract and then absorbed in the blood, it combines with hemoglobin to form methemoglobin. Feeding of NO_2^- by itself would therefore shorten this process. Sell and Roberts (1963) observed that 400 p.p.m. nitrite causes severe growth depression and high mortality in chicks.

It has also been shown experimentally that nitrate or nitrite reduces the conversion of carotene to vitamin A in other species (Garner *et al.*, 1958; O'Dell *et al.*, 1960). This may also be true for turkeys,

as it appears that they could not meet their vitamin A needs when they were fed alfalfa meal and corn in combination with the nitrates and nitrites. Liver storage in these poults was extremely low (Tables 5 and 6). Without the salts present, higher growth rates were obtained.

It is not clear what the role of vitamin A might be in eliminating toxicity to the nitrates and nitrites. It appears that these salts have little effect on the storage of vitamin A in the liver of ruminants (Weichenthal *et al.*, 1963; Smith *et al.*, 1962; Davison *et al.*, 1962; Cline *et al.*, 1962). Experimental results for avain species have shown somewhat different results. Adams *et al.* (1966) reported reduction of liver vitamin A and carotene of poults given 200 p.p.m. nitrite-nitrogen in water. Sell and Roberts (1963) observed a decrease in liver vitamin A of chicks fed vitamin A and KNO_2, but were able to alleviate this problem by injecting high levels of vitamin A equivalent to the levels fed. Njaa *et al.* (1955) reported that they were able to eliminate mortality in chickens fed herring meal with a high content of sodium nitrite by adding vitamin A. Our experimental results with poults have shown that growth and feed efficiency were not severely affected by nitrate and nitrite when adequate vitamin A is added. This is true even when the added vitamin A was as low as 10,000 units per kg.

The results of the chick experiments would indicate that chicks fed a high level of the nitrite with a high level of vitamin A up to 5 weeks had no problem of growth or feed efficiency. These results tend to agree with the work of Njaa *et al.* (1955).

SUMMARY

A series of 14 experiments were conducted with poults and chicks in which various levels of potassium nitrate and/or nitrite were added to the feed. The levels of nitrate found in the basal diet depended mainly upon the amount in the soybean meal and the alfalfa. Levels from 165 to 1,560 p.p.m. were found in soybean samples taken during different years.

Vitamin A was always able to overcome the toxicity of the nitrate or nitrite. When marginal levels of vitamin A were used, mortality was increased, growth was reduced, and rapid breathing developed when high levels of nitrate or nitrite were fed. The nitrite was more toxic than the nitrate. Vitamin E, folic acid, or furazolidone did not influence the toxicity of the nitrates or nitrites.

Chicks up to 5 weeks old are more tolerant to nitrate and nitrite toxicity than poults.

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Nucleic and Free Amino Acid Response in Avian Tissues During Tuberculosis Infection

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INTRODUCTION

SOLOTOROVSKY *et al.* (1949) pointed out that *M. tuberculosis*, avian strain, was an excellent disease model for the study of biochemical and nutritional interactions. Later studies by these workers on the effect of dietary vitamin A and fat on avian tuberculosis demonstrated that chicks survived longer when fed diets with lower fat content or adequate vitamin A (Solotorovsky *et al.*, 1961) and protein (Squibb *et al.*, 1965). Deficient or surfeit

Toxicity of Nitrate Nitrogen to Cattle

1363

Patric K. McIlwain, M.S., and I. A. Schipper, M.S., D.V.M.

ACUTE and chronic nitrate poisoning of livestock have been described frequently.[7,13,16,22,26] Increasing interest and reports of nitrate toxicity in livestock have been evident in the past several years.[2,4,17,28-30,32,33] Abortion, decreased milk production,[5] interference with conversion of carotene to vitamin A,[10] interference of growth,[31] and death have been suggested results of nitrate toxicity.[5]

The presence of nitrates in plants and their consumption by ruminants is a common occurrence.[6,11,12,26] Diets containing more than 0.5% postassium nitrate have been reported as toxic[2,5] although greater concentrations are not unusual in feeds and water consumed by livestock.[4,8] Reduction of nitrates to nitrites and nitrites to ammonia has been demonstrated in animals[15,16,19] and plants.[20,26] Suggested signs of intoxication have been interference with oxygen-carrying elements of the blood[28,29] and signs of vitamin A deficiency due to digestive irritation that results in decreased conversion of carotene.[1,25] The possibility of increased intoxication because of bacterial activity or avitaminosis A due to depressant activity on the thyroid gland has been briefly discussed.[3] It is known that microorganisms serve as a source of nitrate reduction.[14,27] The possible action of *Escherichia coli* as an adjuvant or "trigger mechanism" in nitrate intoxication cannot be ignored.[20] It has been suggested that the nitrate ion may serve as a thyroid depressant by interfering with iodine metabolism.[3,23] This action could account for some of the signs of toxicosis observed under field conditions and the variation in signs reported by investigators in different areas.[13,21] Because of a diversity of research approaches, the cause of toxicity and toxic levels of nitrates or nitrites remains controversial.[7,24,33]

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Materials and Methods

Fourteen 9- to 12-month-old male dairy calves were used for these investigations. The ration provided was a mixture of oats, barley, bran, and alfalfa hay, free choice. Bone meal and trace mineral salt were provided, free choice. The nitrate nitrogen was provided in the form of potassium nitrate to calves in all lots except lot 5, where the calves were given sodium nitrite. All fluid provided was water to which potassium nitrate or nitrite had been added.

The average water consumption per animal per day was approximately 10 to 15 gallons. One cup of molasses per 25 gallons of water was used to cover the undesirable taste of the nitrogenous additives and to assure water consumption.

Calves in lot 1 served as controls and were given *Esch. coli* in the water at 1 x 10^{12} per gallon on the 20th and 34th days of the investigation.

Calves in lot 2 were given 2.8 p.p.m. nitrate N in the drinking water for 42 days.

Calves in lot 3 were given 2.8 p.p.m. nitrate N (1 p.p.m. nitrate N = 7.22 p.p.m. KNO_3 equivalents or 0.0072% KNO_3) in the drinking water for 42

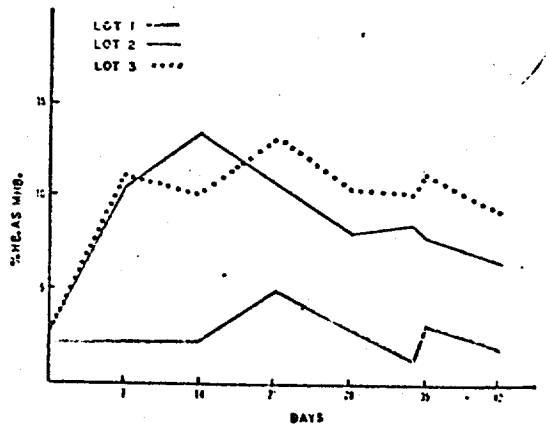

Fig. 1—Percentage of methemoglobin resulting from the administration of nitrate N alone in drinking water or in conjunction with Escherichia. Calves in lots 1 and 3 were given organisms on the 20th and 34th days. Those in lots 2 and 3 were given 2.8 p.p.m. of nitrate N for 42 days.

days with *Esch. coli* given in the same amount and schedule as lot 1. The strain of *Esch. coli* employed was isolated from well water suspected of causing nitrate poisoning and containing 86 p.p.m. nitrate N.

Calves in lot 4 were given a thyroid depressant daily at a per head dosage of 300 mg. for the first 10 weeks, 600 mg. from the 11th through the 14th week, and 1 Gm. thereafter until the termination of the investigation. The thyroid depressant was 1-methyl-2-mercapoimidazole* and was administered in the grain ration. The calves were given water containing 8.4 p.p.m. of nitrate N after 15 weeks and until the termination of the investigation.

Calves in lot 5 were given the same thyroid depressant at an identical rate as those in lot 4 and, in addition, were given 3.4 p.p.m. of nitrate N in the drinking water for the first 10 weeks of the investigation. The nitrate N concentration was increased to 6.8 p.p.m. from the 11th week through the 14th week and then increased to 8.4 p.p.m. until the termination of the investigation.

Calves in lots 6 and 7 were not given a thyroid depressant. Calves in lot 6 were given water containing nitrite N in a concentration of 2 p.p.m for 10 weeks, 3 p.p.m. for the 11th through the 14th week, and 4 p.p.m. for the final 6 weeks. Calves in lot 7 were given nitrate N in the same levels as those in lot 5.

Blood samples were collected from the jugular veins of the calves at weekly intervals. The methemoglobin concentrations were determined by the method of Evelyn and Malloy.[9] Determinations were made within 30 minutes after collection. Serum carotene and vitamin A concentrations were determined by the method of Kaser and Stekol.[13]

The objectives of this investigation were to determine toxic oral levels of nitrate or nitrite, the possibility of *Esch. coli* organisms serving as a "trigger mechanism" in the production of signs of nitrate intoxication, and the possibility of a thyroid depressant serving as an adjuvant to nitrate ions in the production of signs of intoxication.

Results

Methemoglobin levels for calves in lots 1 through 3 were determined (Fig. 1). The results indicate that the nitrate ion per se is relatively nontoxic at the levels administered. The methemoglobin content of the animals given 2.8 p.p.m. of nitrate N increased to a level of approximately 12% of the total hemoglobin within the first week of the trial. After this initial rise, the methemoglobin decreased throughout the remainder of the trial in the calves not given *Esch. coli*. The introduction of the *Esch. coli* organisms to calves in lots 1 and 3 produced an increase in the methemoglobin level at both the 19th and 24th days. The increase of methemoglobin in lots 1 and 3 upon the introduction of the *Esch. coli* organisms was not great; however, it might be considered significant because of

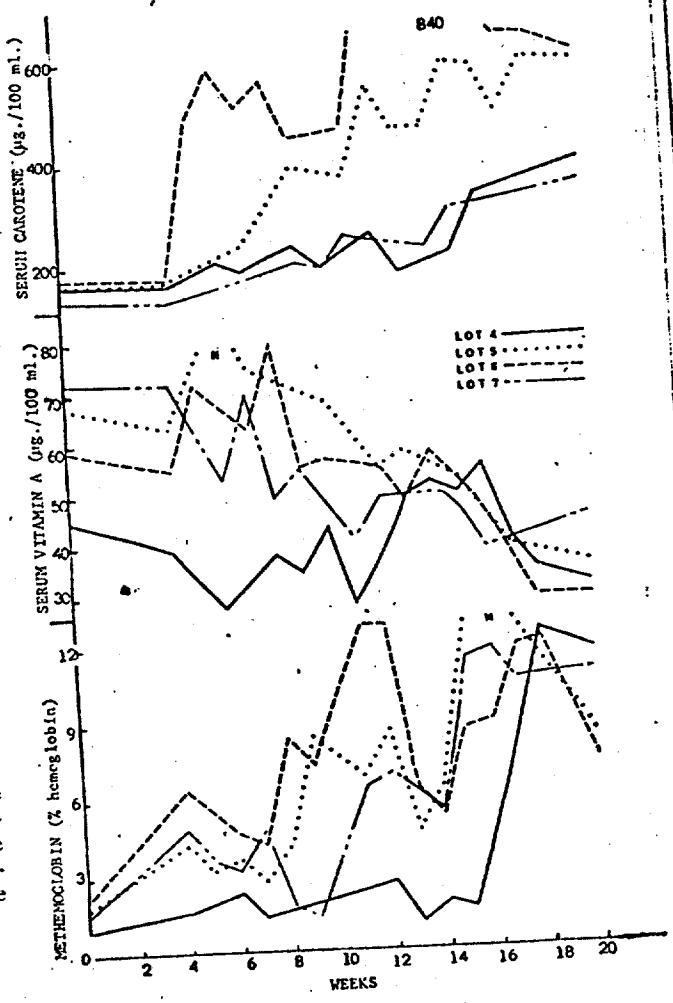

Fig. 2—Concentration of methemoglobin, serum vitamin A, and serum carotene resulting from administration of nitrate N. Calves in lot 4 were given 8.4 p.p.m. nitrate N from week 15 through 20. Thyroid depressant was given at a per head dosage of 300 mg. through the 9th week, 600 mg. through the 13th week, and 1 Gm. through the 20th week.
Calves in lot 5 were given 3.4 p.p.m. of nitrate N through the 9th week, 6.8 p.p.m. through the 13th week, and 8.4 p.p.m. through the 20th week. Thyroid depressant was administered at the same dosage as described for lot 4.
Calves in lot 6 were given 2.0 p.p.m. of nitrate N through the 9th week, 3.0 p.p.m. through the 13th week, and 4.0 p.p.m. through the 20th week. Calves in lot 7 were given nitrate N at the same dosage as those in lot 4.

*Taparole, Eli Lilly and Co., Indianapolis, Ind.

the relatively small number of organisms used.

In calves in lots 4 through 7 (Fig. 2), the methemoglobin levels increased in all animals given nitrate or nitrite. Sharp increases occurred after the administration of the higher amounts of nitrate. There was a methemoglobin decrease with a tendency to return to levels comparable to those observed at the initiation of the investigation in all calves following the primary sharp increase and continued consumption of each level of nitrate or nitrite. The calves given the thyroid depressant maintained a low methemoglobin concentration until given 8.4 p.p.m. of nitrate N at the 15th week of the investigation. A sharp increase of methemoglobin then occurred.

The serum carotene levels were determined (Fig. 2). The calves given nitrite and those given both nitrate and the thyroid depressant demonstrated a significant increase in circulating carotenes. The calves given nitrate only and those given the thyroid depressant only had much slower and less intense increases in serum carotenes.

The serum vitamin A levels in calves in lots 4, 5, and 6 decreased approximately 30 mg./100 ml. in the 20-week period (Fig. 2). The calves in lot 4 that were given only the thyroid depressant for 15 weeks had a decrease in serum vitamin A to levels near those at the beginning of the trial after they were given 8.4 p.p.m. of nitrate N. Serum vitamin A levels in calves in lot 3 decreased slightly from the initiation of the trial to the 11th week.

Discussion

The increase in the methemoglobin concentration in the blood of calves given nitrate or nitrite was expected. Nitrites convert the hemoglobin of the blood to methemoglobin which is unable to function as an oxygen carrier.

The increase in circulating serum carotene with an accompanying decrease in vitamin A in the calves given nitrates or nitrites is of interest. Apparently the conversion of carotene to vitamin A is inhibited. The actual mechanism of this inhibition is not established. Investigators have suggested that the intestine rather than the liver is the major site of the conversion of carotene to vitamin A.[1,35] The nitrite ion causes irritation to the mucous linings and underlying tissues of the gastrointestinal tract and may, in this manner, act as a blocking agent.

The increase of methemoglobin in calves in lots 1 and 3 upon the introduction of the *Esch. coli* organisms was not great; however, it might be significant because of the relatively small numbers of organisms used. Further investigations as to the possibility of *Esch. coli* acting on the nitrate ion in feed or in the rumen should be done. The function of the thyroid gland and its relation to conversion of carotene to vitamin A is still controversial.

An attempt was not made to determine the thyroid-depressing activity of 1-methylmercapoimidazole per se, only to evaluate the possible production of conventional signs of nitrate toxicosis. It is conceivable that this phenomenon may be responsible for some of the reported signs of vitamin A deficiency associated with nitrate intoxication.

Summary

Nitrate nitrogen was given to calves by adding it to drinking water in concentrations up to 8.4 p.p.m., either alone, or with *Escherichia coli*, or with a thyroid depressant.

Though no gross signs of toxicosis were observed under the procedures of this investigation, it is evident that nitrates or nitrites alone, or in conjunction with thyroid depressants or *Esch. coli* organisms, do interfere with normal carotene utilization. It is possible that if the trial had continued over a longer period, gross signs of toxicosis may have been initiated.

It would appear that concentrations of greater than 8.4 p.p.m. of nitrate nitrogen in water are necessary under field conditions to initiate signs of toxicosis. The possibility of unknown adjuvant factors initiating nitrate poisoning must be considered.

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Effects of Meat-curing Salts and Temperature on Production of Staphylococcal Enterotoxin B[1]

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We investigated the effect of time, temperature, and the presence of sodium chloride, nitrates, and nitrites in the medium on the growth and production of enterotoxin B by *Staphylococcus aureus*. Assays by the double gel-diffusion method showed that maximal enterotoxin B production occurs at the beginning of the stationary phase of growth. Lowering the temperature of incubation decreased the amount of toxin produced without affecting the total amount of growth. Increases in concentration of curing salts reduced toxin production more rapidly than cell growth. The relationship of these observations to food-poisoning outbreaks is briefly discussed.

Cured meats, particularly hams, have often been implicated in outbreaks of staphylococcal food poisoning (11), and staphylococci are known to be capable of growth in the presence of permissible and palatable levels of curing salts (1). In 1941, Segalove and Dack (18) showed (by the use of monkey-feeding tests) that enterotoxin production was apparently reduced when the incubation temperature was decreased, but quantitative studies of the effects of temperature, nutritional conditions, and other factors on toxin production, as distinct from total cell growth, have become possible only recently. Purification of enterotoxin B (17) has increased the availability of specific antiserum and thus has permitted a more critical examination of these factors. Genigeorgis and Sadler (9) revealed that enterotoxin B production in Brain Heart Infusion was detectable in media containing 10% NaCl at *p*H 6.9. In their study, a quantitative measurement of the NaCl effect was impossible because NaCl affected the zone width in the single gel-diffusion tube test that they employed. Occasionally, they also observed that growth was influenced only slightly when toxin production had been eliminated. Also, other investigators have illustrated that nutritional factors (13), some inorganic salts (8), and streptomycin and chloramphenicol (8, 16) can reduce toxin production with only a slight effect on growth. However, all of these investigators carried out their experiments at 37 C, whereas foods implicated in food poisoning are rarely maintained at this temperature. The combined effects of nitrates, nitrites, and sodium chloride on the quantitative production of enterotoxin have not previously been investigated, nor has the appearance of enterotoxin in a culture been correlated with the growth curve of the organisms. The data presented in this paper indicate that temperature and length of incubation, as well as presence of curing salts, influence the appearance of toxin independently of the total growth of the culture.

MATERIALS AND METHODS

Bacterial strains and culture media. We used *Staphylococcus aureus* ATCC 14458 (U.S. Food and Drug Administration no. 243) throughout this study. Although this strain was originally isolated from a case of enteritis, it has been designated the prototype strain for enterotoxin B production (6), and its enterotoxin has produced an emetic response in cats (3). Also, this strain is serologically identical to the purified enterotoxin B produced by strain S-6, which causes vomiting and diarrhea in monkeys when fed orally (17). In our experience, this organism decreased in enterotoxin production after repeated transfer. Therefore, when a culture failed to produce the expected titer in control flasks, a new culture was started from the stock. Stock cultures were maintained on porcelain beads (10) and working stock cultures on Veal Infusion Agar (Difco) slants.

Production of enterotoxin. Growth from a 21- to 24-hr slant incubated at 37 C was suspended in 0.85% (w/v) NaCl, and its turbidity was adjusted to a reading of 47.5 units on a Klett-Summerson colorimeter (no. 47 filter, at a wavelength of 370 to 510

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mμ). This suspension contained (by plate count) approximately 20 × 10^6 cells/ml and was used at the rate of 2% (v/v) as the inoculum for the various media. We employed Brain Heart Infusion (Fisher Scientific Co., Fairlawn, N.J.) for all studies on toxin production. Amounts of 100 ml in 1-liter Erlenmeyer flasks were incubated at appropriate temperatures in a reciprocating shaker bath at 90 rev/min with a 3-cm stroke (Aminco, Silver Spring, Md., model 4-8600) or at 74 rev/min with a 0.5 to 1.0-inch stroke (New Brunswick Scientific Co., New Brunswick, N.J., model 627). We obtained comparable results with the two shakers. Static broth cultures were incubated at 37 C in a Thelco (model 4) Incubator (Precision Scientific Co., Chicago, Ill.). NaCl and $NaNO_3$ in designated amounts were added, as required, to the media, the *p*H was adjusted to 7.0, and the flasks were sterilized by autoclaving at 121 C for 15 min. When nitrites were to be added, $NaNO_2$ solutions were sterilized by filtration and were added aseptically to the sterile media, adjusted to *p*H 7.0. All inorganic salts were reagent grade, and the concentrations described were in addition to any amount present in the medium. Except for experiments in which the effect of temperature on enterotoxin production and cell growth had been determined, all cultures were incubated at 37 C.

After incubation, samples from the cultures were diluted on a Klett-Summerson colorimeter, as necessary, to give accurate turbidity readings. The cultures then were centrifuged, and the concentration of enterotoxin in the supernatant liquid was measured.

Toxin assay. Enterotoxin B was determined by the double gel-diffusion test of Ouchterlony (14, 15) modified by Wadsworth (19), Crowle (7), and Casman (5). This procedure gives a roughly quantitative measure of toxin; a line visible with the naked eye forms in the agar when about 1 μg/ml of toxin meets an optimal concentration of antibody (4). The addition of one or two drops of an 8% (w/v) solution of $HgCl_2$ a few minutes before the test was read accentuated the line and increased the sensitivity (W. G. Roessler, *personal communication*). When we used $HgCl_2$ for this purpose, it was important that we make the agar employed on slides for diffusion in accordance with the following arbitrary procedure. Sodium barbital (0.8 g) and NaCl (0.85 g) were mixed in 100 ml of distilled water and adjusted with HCl to *p*H 7.4. We added 1 g of agar (Difco) and dissolved it by direct heating, with constant stirring. As soon as the agar had melted completely, the preparation was transferred in 25-ml portions into bottles and autoclaved at 121 C for 15 min. Slight deviations from this routine (probably resulting in different final *p*H value) produced agar in which a heavy white precipitate appeared immediately upon addition of $HgCl_2$. Experiments in which varying concentrations of NaCl (up to 10%, w/v) were added to known concentrations of enterotoxin indicated that NaCl would not interfere with the sensitivity of the test.

Antiserum and reference enterotoxin. Rabbit antiserum, produced by injecting purified enterotoxin B from strain S 6 and yielding a single line with purified toxin (17), was lyophilized and was stored at 2 to 4 C. Reconstituted serum also was stored at 2 to 4 C, and it was used for periods up to 1 month. Reference enterotoxin for routine assays was a crude preparation produced by *S. aureus* ATCC 14458. We grew the culture in Brain Heart Infusion under aerated conditions at 37 C for 24 hr; the supernatant fluid was dispensed in 0.1-ml portions into screw-cap vials and was then lyophilized and stored.

RESULTS

Enterotoxin stability under aerated conditions. Aerated cultures produced toxin more rapidly and in a larger amount than did static cultures (Fig. 1). To determine the stability of the enterotoxin in an aerated culture we divided the supernatant fluid from an 8-hr culture containing a high titer of enterotoxin into four portions and treated it as follows: (i) no further treatment, (ii) filtered through membrane filter (0.45-μ pore size), (iii) filtered through a Seitz filter (0.1-μ pore size), and (iv) added Merthiolate to make a final concentration of 0.01%. The samples were reincubated on the shaker at 37 C for 40 hr. In 18 hr, growth appeared in the untreated sample and it was not assayed because any toxin present could have resulted from new synthesis. In the other three samples, the toxin titer was unchanged during 40 hr on the shaker at 37 C.

Relationship of cell multiplication to enterotoxin production. Figure 2 shows that enterotoxin was first detectable after 5 hr of incubation—at about the end of the exponential growth phase and about the time the *p*H curve shifted. The enterotoxin titer increased steadily for 16 to 20 hr, although cell growth by 7 to 9 hr had reached the stationary phase.

Inorganic salts and toxin production. Concen-

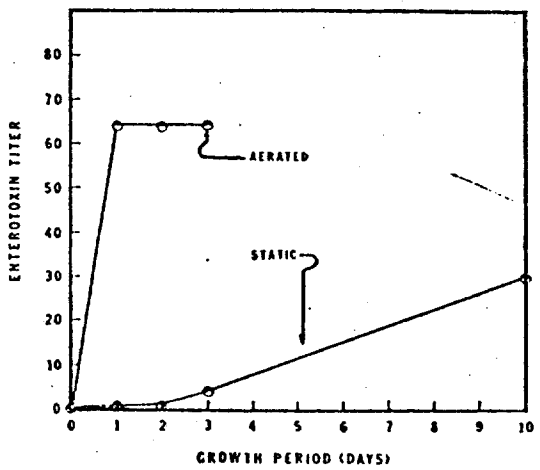

FIG. 1. *Comparison of aerated versus static incubation for enterotoxin production.*

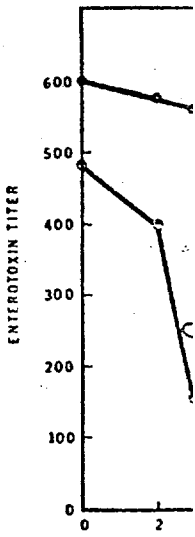

FIG. 2. ...

FIG. 3. *Effects ... and enterotoxin pr...*

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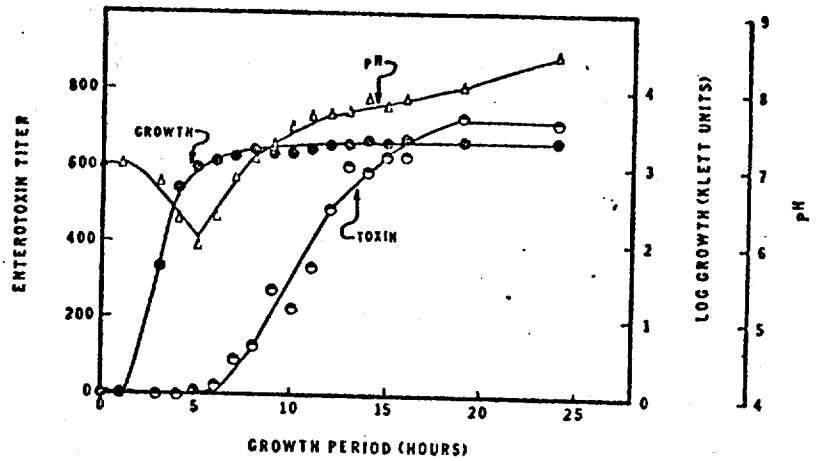

FIG. 2. *Enterotoxin production, growth, and pH changes in Staphylococcus aureus at 37 C.*

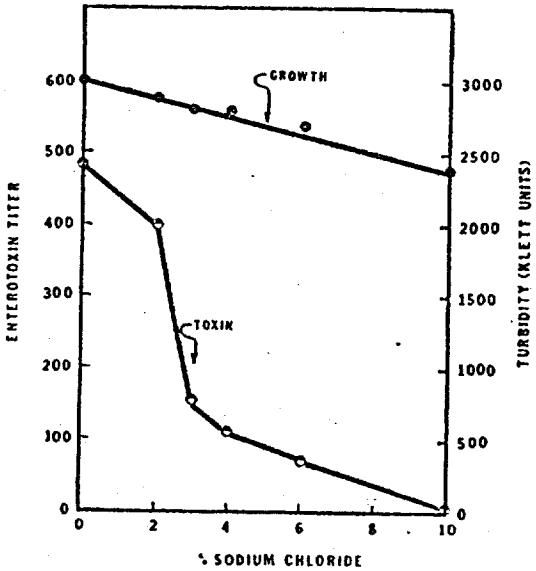

FIG. 3. *Effects of sodium chloride on total growth and enterotoxin production.*

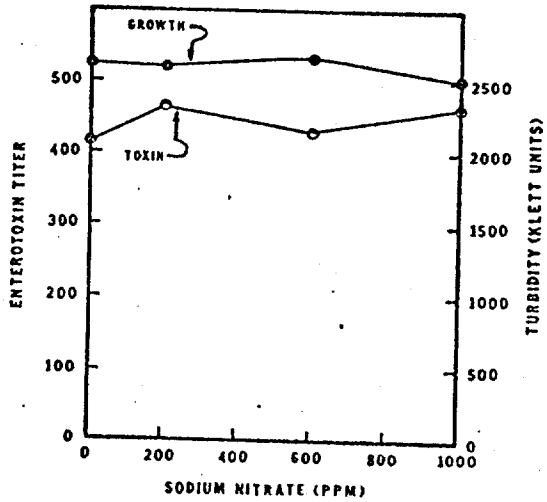

FIG. 4. *Effects of sodium nitrate on total growth and enterotoxin production.*

trations of NaCl up to 10% had a relatively slight effect on total growth, but they caused a definite decrease in toxin production above 3% NaCl (Fig. 3). Another medium that contained a casein hydrolysate as the nitrogen source was inoculated to determine whether the salt effect was related to the type of protein in the medium. Although enterotoxin levels were lower with the second medium, the rates of reduction in enterotoxin in relation to the NaCl concentration were comparable. Figures 4 and 5 suggest that neither $NaNO_3$ in concentrations up to 1,000 ppm nor $NaNO_2$ up to 200 ppm (maximum concentration permitted in cured meats) would affect either growth or enterotoxin production in broth. Table 1 indicates that combinations of the curing ingredients may have an effect at a lower salt concentration.

Effect of temperature. In an experiment in which frequent determinations were established during the first 85 hr of incubation at 20 C, we observed that the culture had reached the stationary phase between 55 and 60 hr, and toxin production had reached its maximal titer of 100 by 65 hr. The 37 C control had a titer of over 500. From another experiment (Table 2), we showed that with prolonged incubation at 16 C and 20 C, total growth approached that observed

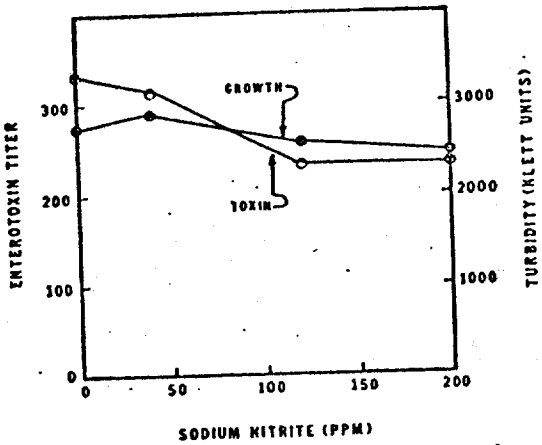

FIG. 5. *Effects of sodium nitrite on total growth and enterotoxin production.*

TABLE 1. *Effects of combination of curing ingredients on growth and enterotoxin production*

Medium	Klett reading[a]	Titer[a]
BHI control	2,650	400
NaCl, 2%	2,400	300
$NaNO_2$, 120 ppm	2,700	400
$NaNO_2$, 200 ppm	2,600	400
$NaNO_2$, 600 ppm	2,900	400
Combination A[b]	2,350	150
Combination B[c]	2,250	150

[a] Average of four trials.
[b] BHI broth, plus 2% NaCl, 120 ppm $NaNO_2$, and 200 ppm $NaNO_3$.
[c] Same as footnote *b* except that the amount for $NaNO_3$ was 600 ppm.

TABLE 2. *Effect of different incubation temperatures on growth and enterotoxin production by Staphylococcus aureus ATCC 14458*

Incubation time (hr)	Log of Klett reading			Enterotoxin titer		
	16 C	20 C	37 C	16 C	20 C	37 C
18	—[a]	—	3.4	—	—	150
112	3.0	3.0	3.2	8	20	340
170	2.9	2.8	3.3	10	25	260
207	2.7	—	—	8	—	—

[a] Determination not made.

at 37 C, but the production of enterotoxin remained low.

DISCUSSION

The ability of staphylococci to grow in the presence of curing salts (12) and at temperatures near 20 C to a cell density essentially equivalent to that of 37 C (18) has been known for many years. However, the data given in this paper emphasize that it is impossible to extrapolate the effect of these parameters on growth to a similar effect on enterotoxin B production. We recognized that the double gel-diffusion method for the detection of enterotoxin is only semiquantitative. Variations between experiments are commonly observed even though replicate determinations within a given experiment usually agree quite closely. Nevertheless, it is apparent that, in relatively low concentrations, curing salts will reduce toxin production. Whether the effect of combinations of curing salts on toxin production is more than an additive effect is not clear. Likewise, when we lowered the temperature of incubation of a culture, the amount of toxin produced decreased more rapidly than did its growth rate. Future studies will establish whether the temperature effect is on enterotoxin synthesis or on its release from the cell.

The absence of detectable enterotoxin until the end of the log phase and its accumulation during the stationary phase of growth further indicate that enterotoxin production is not inseparably bound to cell multiplication. This observation, plus the appearance of enterotoxin after the shift in *p*H and the association of enterotoxin with only a relatively few strains of *S. aureus*, suggest that it is a secondary metabolite, like many antibiotics (2).

Applicability of the data we obtained with this strain of *Staphylococcus* isolated from a nonfood source to known food-poisoning strains producing enterotoxin B might be questioned. However, the ability of the enterotoxin B from strain 243 to cause an emetic response in cats and its serological identity to the enterotoxin that produces the typical food-poisoning syndrome in monkeys implies that it is typical enterotoxin B. Obviously, other strains that produce the same chemical entity might respond differently to the environment. Nonetheless, the effects shown in this investigation could have served to limit food-poisoning outbreaks, even though large numbers of staphylococci were involved.

ACKNOWLEDGMENTS

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œsophagus in each case was normal in appearance. There was brownish discoloration at the anterior aspect of the pyloric end of the stomachs of the woman and child. On opening the stomachs the mucosa was found to be œdematous and injected, as was also the lining of the duodenum and upper ileum. The brain, kidneys, and other organs were rather congested.

The following observations were subsequently made. The dinner consisted of meat, potatoes, cabbage, Yorkshire pudding, rhubarb tart and custard. A salt cellar on the table contained an upper layer of a salt of a faint yellow colour and a lower layer of white salt similar in appearance to prepared table salt. A basin was found containing about 4 oz. of salt similar in appearance to that of the upper layer in the salt cellar. The contents of the basin and the upper layer in the salt cellar were found to be sodium nitrite of commercial purity containing 98 per cent. of the pure salt. The composition of the lower layer in the salt cellar corresponded to that of prepared table salt containing 98·2 per cent. of sodium chloride.

The following amounts of nitrites as sodium nitrite were found in organs, &c., examined.

	Sodium nitrite.
Man's stomach	4·275 grammes
Woman's stomach	1·284 "
Child's stomach (washed out before death)	0·005 "
Child's vomit	1·047 "

The unconsumed portions of the foodstuffs forming the meal contained the following percentages of nitrites as sodium nitrite.

	Sodium nitrite.
Yorkshire pudding	4·50 per cent.
Potatoes	0·75 "
Cabbage	6·50 "
Joint of meat (outer layer)	0·15 "
" (interior)	0·015 "
Rhubarb tart	Nil.
Custard	0·015 "

It was impossible to form a definite idea of the quantity of sodium nitrite taken by any of the three persons, since unfortunately neither the vomit of the man, nor of the woman, nor the contents of the child's stomach previous to washing out were obtainable for examination; but the quantity must have been appreciably in excess of the amounts subsequently recovered from the stomachs. No definite evidence could be obtained as to the origin of the sodium nitrite, but the man had access to the sodium nitrite plant of a factory at which he was employed.

My thanks are due to Mr. A. Scholes, of the Public Analysts' Laboratory, Middlesbrough, for the results of his chemical analysis of the various specimens submitted to him.

MEDICAL SOCIETIES

ROYAL SOCIETY OF MEDICINE

SECTION OF LARYNGOLOGY

A MEETING of this section took place on Nov. 6th, Mr. LIONEL COLLEDGE, the president, occupying the chair.

Tuberculosis of the Larynx Simulating Cancer

Dr. A. HAUTANT (Paris) submitted a contribution on unusual forms of tuberculosis of the larynx, and the manner in which they could simulate laryngeal cancer. He showed slides of cases of laryngeal epithelioma which were at first diagnosed as tuberculous disease of that part, but the biopsy showed them to be cancer. One case showed a unilateral lesion of the subglottic area, with the cords almost fixed, and an extension on to the surface of the anterior arytenoid. There were vegetations along the edge of the cord. Another slide showed a median subglottic cancer, with an expanding ventricle descending below the thyroid border. One case showed a pre-arytenoid lesion, with cord fixation, and X rays revealed fibrosis of the lungs. The biopsy left no doubt that it was epithelioma.

On the other hand, he showed slides of a case of tuberculous tumour of verrucose form on the ventricle, closely simulating in appearance carcinoma of the larynx. He urged that, whatever the appearance in the larynx and the apparent certainty of diagnosis, a careful pulmonary examination should always be made. Provocative X ray therapy was dangerous, and should be ventured on only with caution.

Dr. N. RH. BLEGVAD (Copenhagen) opening a discussion on the problem of

Early Laryngeal Tuberculosis

said it was not his purpose to enter into the differential diagnosis of tubercle of the larynx from cancer, syphilis, and lupus. Thirty years ago tuberculosis of the larynx was most widespread among men, but, as Sir StClair Thomson had pointed out, men and women were now affected about equally, probably because the modes of life of the two sexes were now more closely approximated. It was particularly important to make the diagnosis of laryngeal tuberculosis as early as possible, as the sooner the treatment was begun the greater were the chances of cure. In many text-books it was stated that people suffering from phthisis were especially susceptible to laryngeal tuberculosis, but Dr. Blegvad knew of no evidence in support of this, and it was contrary to his own experience. Most of the phthisical patients he had seen had told him they had never even been hoarse, and certainly they had not had acute laryngitis. In cases of laryngeal tubercle the disease at once manifested itself as such. Sir StClair Thomson had included among the symptoms which should give rise to suspicion of laryngeal tuberculosis, catarrh or congestion, limited to one part of the larynx, lasting for some time; thickness and congestion of one arytenoid or one ary-epiglottic fold; paresis of the muscles of abduction. There were six other changes which should arouse suspicion: (1) An isolated redness of one of the vocal cords. This, however, might mean cancer in an early stage, or a remnant of catarrh; but if it was seen in a phthisical patient it was safe to regard it as tuberculous. If the redness disappeared when silence and simple treatment were enjoined one could not tell whether it had been tuberculous or not. (2) Prolapse of the ventricle of Morgagni was an almost certain sign of laryngeal tubercle. This prolapse might be the only evident affection of the larynx. It was accompanied by redness and swelling along the border of the vocal cords. This prolapse largely disappeared on phonation. Often tubercle bacilli were recoverable from the ventricle of Morgagni. Further suspicious signs were (3) a swelling and redness of the posterior ends of the vocal cords, (4) swelling of the lower ends of the vocal cords, and (5) infiltration of the interarytenoid region. The last suspicious sign to which he drew attention was one he had only noted in the past year—namely, (6) the appearance of a little red cushion below the commissure on the anterior wall of the trachea. In these early examinations of the patient it was important, but often difficult, to induce the patient to breathe quite calmly. For the examina-

CLINICAL AND LABORATORY NOTES

PERMANENT STANDARDS FOR THE TURBIDOMETRIC ESTIMATION OF PROTEIN

By E. J. King, Ph.D. Toronto

READER IN PATHOLOGICAL CHEMISTRY IN THE UNIVERSITY OF LONDON AT BRITISH POSTGRADUATE MEDICAL SCHOOL; AND

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ASSISTANT IN PATHOLOGICAL CHEMISTRY AT THE SCHOOL

The determination of the amount of albumin in urine, C.S.F., and other body fluids is a matter of clinical, as well as laboratory interest. Of the several quantitative methods which have been proposed (Esbach,[1] Exton,[2] Van Slyke,[3] and others, the turbidometric procedure of Kingsbury and Clark[4] is probably the most convenient. This method can be applied to C.S.F. and other body fluids as well as to urine, and has been in use, with modifications in the apparatus required, in the laboratories of the British Postgraduate Medical School for some time. The procedure is very simple, and can be used with equal success by laboratory workers and general practitioners.

The principle of the method consists in comparing the turbidity in a set of permanent standards with that produced when a standard amount of sulphosalicylic acid is added to a measured amount of the albuminous fluid. The standards consist of a permanent suspension of formazin in gelatin. They have been standardised in this laboratory by reference to the turbidity produced with sulphosalicylic acid in a solution of crystalline horse-serum albumin, a diluted human serum whose protein content was established by Kjeldahl, and a solution of crystalline albumin isolated from the urine of an orthostatic albuminuric.

The Method.—Add 3 c.cm. of 3 per cent. sulphosalicylic acid to 1 c.cm. of urine, C.S.F., or other albuminous body fluid, in a small test tube, and compare the turbidity after 5 minutes with that in the standards. If the degree of turbidity is too great for comparison make a suitable dilution of the fluid with water, and repeat the test.

Preparation of the Standards.—The gelatin is clarified as follows: 65 grammes of pure gelatin are dissolved at about 90° C. in 500 c.cm. of water. The "white" of one egg, in approximately twice its volume of water, is vigorously stirred into the solution. The mixture is heated, with continual stirring, on a boiling water-bath for 1 hour. It is then filtered through a large coarse paper in a heated funnel. The clear slightly yellow filtrate is kept liquid at about 50° C. for the preparation of the standards described below.

The formazin is prepared as follows: 25 c.cm. of an aqueous solution (10 g. per 100 c.cm.) of hexamine (hexamethylene tetramine) are added to 25 c.cm. of a solution (1 g. per 100 c.cm. in water) of hydrazine sulphate. The mixture is stoppered, shaken, and left at room temperature for at least 15 hours. The resulting precipitate of formazin is carefully mixed by gentle shaking until it is evenly dispersed throughout the liquid.

14·5 c.cm. of the suspension are added to 100 c.cm. of gelatin together with 0·3 c.cm. of 40 per cent. formaldehyde to ensure permanent "setting." Such a gelatin suspension of formazin has been found to be equivalent to a concentration of serum albumin (precipitated with sulphosalicylic acid under the conditions described above) of 100 mg. per 100 c.cm. The gelatin suspension is now diluted with clarified gelatin (containing 0·3 c.cm. of 40 per cent. formaldehyde per 100 c.cm.) to give standards corresponding to other albumin concentrations. The following mixtures of gelatin and formazin-gelatin suspension are made in small tubes of uniform bore (7·5 by 1 cm.).

Tube.	C.cm. of gelatin.	C.cm. of gelatin-formazin.	Value in terms of albumin per 100 c.cm.
1	3·6	0·4	10
2	3·2	0·8	20
3	2·8	1·2	30
4	2·4	1·6	40
5	2·0	2·0	50
6	1·6	2·4	60
7	1·2	2·8	70
8	0·8	3·2	80
9	0·4	3·6	90
10	0	4·0	100

When cold, the tubes are stoppered with corks cut level with the top of the tube. The stoppered ends are then dipped in molten paraffin wax and allowed to cool. The standards may be checked against serum solutions standardised by nitrogen determinations. They should be mounted in a wooden rack painted black. Comparison is best made by viewing them against a strip of white cardboard with a transverse black line fastened to the rack.

Arrangements have now been made with Messrs. Gallenkamp and Co., London, to manufacture these standards. Sample sets have been checked against those prepared in this laboratory and against albumin solutions of known strength.

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FATAL POISONING BY SODIUM NITRITE

By T. A. C. McQuiston, M.B. Belf.

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As poisoning by sodium nitrite is rare, and as I have not been able to trace any record of fatal poisoning by it in this country, I have been prompted to contribute a few notes on three cases which recently came under my observation.

On the afternoon of Sunday, May 31st, 1936, I was called to see a family who had suddenly taken ill after the midday meal. The affected members were a man, aged 45, his wife, aged 43, and her daughter, aged 5. When I reached the house both the man and his wife were dead, and the child had been removed to hospital. I was told by neighbours who occupied adjacent rooms that the family had complained of abdominal pains immediately after the meal, that they had vomited, and their faces "went blue." External examination suggested that both adults had been dead about an hour (which would be within an hour after partaking of their meal). The lips were rather cyanosed; there were particles of cabbage and food about the mouth, but the buccal mucous membrane showed no signs of corrosion. The only vomited matter which was obtainable I was told was that of the child. When taken ill the mother had told her neighbour that another daughter, who was at Sunday school, was not to have her share of the meal which was being kept in the oven.

The child, I was informed, was moribund on arrival at hospital. She had a stomach lavage, and stimulants, but died about half an hour after admission.

I subsequently performed post-mortem examinations on all three victims. As might be expected from the sudden death the pathological changes were slight. The

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Kinetics of Dimethylamine Nitrosation in Relation to Nitrosamine Carcinogenesis[1,2]

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SUMMARY—The nitrosation of dimethylamine in buffered aqueous solutions was studied with the aid of the tritium-labeled amine. At pH 3.4, the rate of dimethylnitrosamine (DMN) formation was proportional to the dimethylamine concentration and to the square of nitrite concentration. The rate of reaction was maximal at about pH 3.4. The relative constancy of the appropriate rate constants under various conditions confirmed the third-order kinetics of the reaction, though discrepancies were apparent at pH 1–2. The rate constants were used to estimate the amount of DMN expected to be formed in the gastric contents after ingestion of food containing various concentrations of dimethylamine and nitrite and during storage of such food. For example, it was estimated that, if a man ate a 300-g meal containing 12 mg dimethylamine hydrochloride and 60 mg sodium nitrite, not more than about 3 μg DMN might be expected to be formed intragastrically.—J Nat Cancer Inst 44: 633–639, 1970.

DIALKYLNITROSAMINES ARE powerful carcinogens (*1*, *2*), which are readily produced by the reaction of dialkylamines with nitrous acid. Since both reactants occur in various foods, nitrosamines could be significant human carcinogens (*3*). As a preliminary to studies on the formation of nitrosamines in foods and biological systems, it was essential to examine the influence of various factors on this reaction under defined conditions. The formation of dimethylnitrosamine (DMN) is of particular interest, since much information has accumulated about its carcinogenic action (*1*, *2*), and DMN has been demonstrated to occur in toxic batches of fish meal preserved with nitrite (*4*), Central African alcoholic spirits (*5*), and an edible South African plant (*6*). Since nitrosation occurs most readily under acidic conditions, DMN and other nitrosamines could also be produced in the gastric contents during digestion (*3*, *7*, *8*).

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[2] Supported by a contract with the International Agency for Research in Cancer and Public Health Service contract PH 43-68-959 with the National Cancer Institute.

[3] I thank Professor D. Samuel, Weizmann Institute of Science, Professor J. H. Ridd, University College, London, and Professor W. Lijinsky, Dr. L. Wallcave, and Dr. G. Charnock, Eppley Institute, for useful discussions. I also thank Miss Shoshana Dabbi for excellent technical assistance.

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The nitrosation of dimethylamine (Me_2NH) was studied in 1929 by Taylor and Price (*9*), but the disappearance of dimethylamine was measured rather than the appearance of DMN, and the influence of *p*H was not examined. Sander *et al.* (*8*) recently studied the nitrosation of di-*n*-propylamine and more complex nitrosamines in solutions of varying *p*H and in gastric contents. However, the kinetics of the reaction were not investigated in detail, the yields of di-*n*-propylnitrosamine were very low, and the analytical method, which depended on the photochemical decomposition of nitrosamines to give 20–30% yields of nitrite, may be open to some question.

The present paper describes a reinvestigation of dimethylamine nitrosation, with the aid of tritiated dimethylamine labeled in the methyl groups, and gives estimates of DMN formation in the stomach and during food storage.

MATERIAL AND METHODS

The extent of [^{3}H]-Me_2NH nitrosation was determined by radioactive assay of [^{3}H]-DMN, extracted with ether from the acidified reaction mixture.

Anhydrous $Me_2NH \cdot HCl$ (from Eastman Organic Chemicals, Rochester, N.Y.; 0.5 g) was tritiated by the Wilzbach method at the Radiochemical Centre, Amersham, England. Labile tritium was removed by 4 successive additions of 0.1 N HCl (100 ml), each time distilling over all except 10–20 ml. The fourth distillate contained only 4–8% of the counts in the residual [^{3}H]-$Me_2NH \cdot HCl$ solution. The [^{3}H]-Me_2NH did not migrate on thin-layer chromatography (*10*) even after neutralization, as shown by scanning for radioactivity. The [^{3}H]-DMN was synthesized by treating the [^{3}H]-amine with HNO_2-acetic acid reagent at 50° for 1 hour and distilling most of the solution after adding excess KOH (*11*). The undistilled residue contained 2.9% of the counts in the distillate. Thin-layer chromatography of the [^{3}H]-DMN distillate revealed the presence of 8% [^{3}H]-Me_2NH, which could be converted completely into [^{3}H]-DMN by a second HNO_2 treatment. These observations serve to demonstrate the purity of the [^{3}H]-DMN and [^{3}H]-Me_2NH, which both showed specific activities of 15 μC/μmole—based on the radioactivity and optical density at 335 mμ of the [^{3}H]-DMN preparation (*11*). Other reagents (except ether) were of analytical grade and kept anhydrous.

Under the "standard conditions" used for the kinetic studies, 10 ml of a solution containing $Me_2NH \cdot HCl$ (0.02 M), $NaNO_2$ (0.10 M), NaCl (0.05 M), and potassium hydrogen phthalate (0.10 M) were prepared as follows: Appropriate stock solutions (freshly prepared in the case of $NaNO_2$) were mixed, and the *p*H was adjusted to 3.40 with H_2SO_4 using a Radiometer *p*H meter (model 25) fitted with a G202C glass electrode. The solution was diluted in test tubes to 9.75 ml, and 0.25 ml [^{3}H]-Me_2NH solution at *p*H 7 was added, containing 1.73×10^7 disintegrations per minute (dpm) and 0.45 μmoles [^{3}H]-Me_2NH, and 12 μmoles Cl^-. The solution was reacted at 25°C for 3 hours, 8 or 12 tubes usually being reacted in 2 or 3 successive batches on the same day. After reaction, 200 g/liter Na_2SO_4 (15 ml) and 5.0 N H_2SO_4 (5 ml) were added, and the mixture was extracted with ether (4×40 ml). (NaCl and HCl were not used here due to the possible involvement of nitrosyl chloride.) The volumes of the 2 phases were measured, and portions of combined ether phase (2×2.0 ml) and aqueous phase (2×0.5 ml, with 3 drops 50% aqueous triethanolamine per vial added) were assayed in 15 ml Bray's solution (*12*) using a Packard scintillation counter. Internal standards showed that the counting efficiency was 17.4% for the ether phase and 9.4% for the aqueous phase, with a consistent efficiency ratio of 1.85:1.

Thin-layer chromatography (*10*) of the ether extract of a standard reaction mixture (containing 10 times the usual level of radioactivity) showed that at least 90% of the label migrated as DMN. In blank experiments where $NaNO_2$ was omitted from the standard mixture, the 4 ether extractions contained 0.6 ± 0.1% (mean ± SD for 10 experiments) of the total dpm. When the standard mixture including $NaNO_2$ was extracted 4 times with ether as soon as possible after mixing, the combined ether extracts showed radioactivity similar to that in the blank experiments. When 8 solutions of [^{3}H]-DMN in standard mixtures (omitting [^{3}H]-Me_2NH) were each extracted 4 times with ether as soon as possible after mixing, the ether extracts contained 67 ± 2% of the total

dpm. (This recovery was corrected for the presence in the [^{3}H]-DMN solution of 8% [^{3}H]-Me_2NH, assumed not to be extracted.) Taken together with earlier studies on Me_2NH nitrosation (*18*), these results show that the radioactivity extracted by ether was essentially due to [^{3}H]-DMN. DMN decomposition, as measured by the ultraviolet absorption at 335 mμ, was undetectable when a DMN solution prepared under the standard conditions (but without $NaNO_2$) was allowed to stand for 3 hours at *p*H 3.4, and when the standard amounts of H_2SO_4 and Na_2SO_4 were added and the mixture was left for 1 hour at 25°.

For calculation of results, the total dpm in the ether phase were expressed as percent total dpm in both phases, and, after subtraction of 0.6% of the total dpm to allow for the blank, were multiplied by 100/67 = 1.49 to give percent conversion into [^{3}H]-DMN. All results represent the mean of at least 2 experiments.

RESULTS

Under the standard conditions described in "Materials and Methods," an aqueous solution containing $Me_2NH{\cdot}HCl$ (0.02 M), $NaNO_2$ (0.1 M), and NaCl (0.05 M), and buffered with phthalate at *p*H 3.4, was reacted at 25° for 3 hours. The yield of DMN in 13 such experiments was 15.8 ± 1.6% (mean ± SD), each value being the mean of duplicate runs. The difference between each duplicate and its mean was 0.6 ± 0.7% of the mean (12 experiments).

When the 0.05 M NaCl (added to imitate natural conditions) was omitted from the standard mixture, the yield of DMN rose by 8 and 9% in 2 duplicate experiments, possibly due to the increase in ionic activity. Taylor and Price (*9*) obtained a similar result. Reaction for 1, 2, 3, 5, and 8 hours under the standard conditions gave DMN yields of 6.2, 12.6, 16.7, 28.7, and 40.4%, respectively. At reaction temperatures of 15, 25, 35, and 45°, the yield of DMN under the standard conditions was 3.0, 15.8, 34.8, and 52.0%, respectively. The yield was not affected if the reaction was carried out in the dark. When the concentration of $Me_2NH{\cdot}HCl$ was varied, the percent yield of [^{3}H]-DMN under the standard conditions was nearly constant, so that the absolute amount of DMN produced was closely proportional to initial $Me_2NH{\cdot}HCl$ concentration. Thus at concentrations of 0.01, 0.02, 0.035, and 0.05 M $Me_2NH{\cdot}HCl$, the absolute yields were 16.8, 30.4, 48.6, and 71.5 μmoles DMN/10 ml incubation mixture, respectively.

Table 1 shows the effect of varying the stoichiometric concentration of $NaNO_2$ at a constant *p*H of 3.4. The low yields at low nitrite concentrations were not due to decomposition of the nitrite, since nitrite determinations by a standard diazo coupling method showed no significant decrease in nitrite concentration during incubation of 0.01, 0.02, or 0.10 M $NaNO_2$ for 3 hours under the standard conditions, apart from a slight decrease due to reaction with Me_2NH.

TABLE 1.—Values of $\bar{k}$ (equation [2]) for the nitrosation of dimethylamine at various concentrations of nitrite*

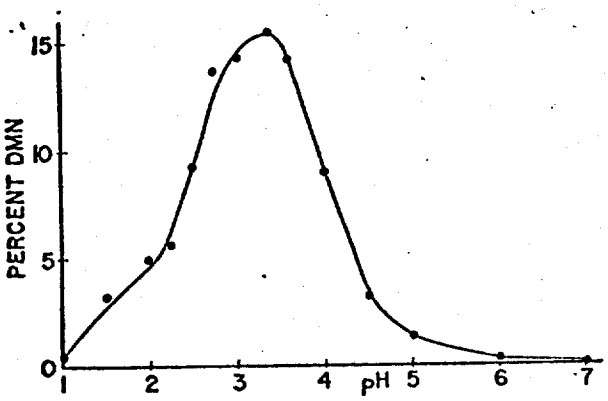
$NaNO_2$ (M)	Yield of DMN (%)	$10^3{\cdot}\bar{k}$
0.02	0.9	—
.025	1.7	16.5
.04	3.8	13.8
.05	5.5	12.8
.06	7.3	12.1
.075	12.4	13.6
.10	16.9	10.7
.125	21.8	9.1

*The results are for standard reaction mixtures containing $Me_2NH{\cdot}HCl$ (0.02 M) and various concentrations of $NaNO_2$. The mixtures were reacted at 25° and *p*H 3.4 for 3 hours. $\bar{k}$ is given in moles^{-2} min^{-1} liter^3.

The effect of varying the *p*H was studied using 0.1 M $NaNO_2$, 0.02 M $Me_2NH{\cdot}HCl$, and various buffer systems (text-fig. 1 and table 2). Highest yields of DMN were obtained at about *p*H 3.4, and the reaction rate dropped to low values above *p*H 5 and below *p*H 1.5. Yields at *p*H 2.75, 3.0, and 3.4 were similar with either citrate or phthalate buffers.

DISCUSSION

Taylor and Price (*9*) showed that the nitrosation of dimethylamine follows an equation whose modern form is equation [1]. Ridd (*14*) reviewed the formation of nitrosamines from secondary amines, together with the related reactions of diazotization and deamination, which proceed via the formation of unstable monoalkyl nitros-



TEXT-FIGURE 1.—Dependence of DMN formation on *p*H. Standard reaction mixtures containing $Me_2NH \cdot HCl$ (0.02 M), $NaNO_2$ (0.1 M), NaCl (0.05 M), and various buffers were reacted for 3 hours at 25°. All buffers were 0.1 M (final concentration), and the *p*H was adjusted with H_2SO_4 or NaOH. The buffers were sodium citrate (*p*H 2.0–3.0), potassium phthalate (*p*H 3.4–5.0), and sodium phosphate (*p*H 6.0–7.0). For *p*H 1.0–1.5, H_2SO_4 was used.

TABLE 2.—Values of $\bar{k}$ (equation [2]) and k (equation [1]) for dimethylamine nitrosation at various *p*H values*

pH	Yield of DMN (%)	$10^2\bar{k}$	$10^{-6}k$
1.5	3.3	1.88	32.2
2.0	5.0	2.88	16.4
2.5	9.3	6.24	13.3
3.0	14.3	8.94	9.5
3.4	15.5	9.66	8.8
4.0	9.0	5.34	7.7
4.5	3.1	1.76	6.1

*The results are taken from those shown in text-figure 1. $\bar{k}$ and k are given in $moles^{-2}\ min^{-1}\ liter^2$.

amines. In all cases, the reaction rate depends on the concentrations of *nonionized* amine and HNO_2. The chief nitrosating agent at *p*H>1 is nitrous anhydride (N_2O_3), which is formed *reversibly* from two molecules of HNO_2. The rate of reaction is proportional to N_2O_3 concentration and hence to the square of HNO_2 concentration, and we have the following equations, based on the concentrations of the nonionized species:

$$2HNO_2 \rightleftharpoons N_2O_3 + H_2O$$

$$Me_2NH + N_2O_3 \rightarrow Me_2N{\cdot}NO + HNO_2$$

$$\text{Rate} = k \times [Me_2NH] \times [HNO_2]^2 \qquad [1]^5$$

For most purposes it is more convenient to use the total concentrations of amine and nitrite rather than those of the nonionized species. At a constant *p*H, the proportions of total nitrite and total dimethylamine existing, respectively, as nonionized HNO_2 and Me_2NH are constant, and equation [2] based on the stoichiometric concentrations should apply. It is stressed that $\bar{k}$ (equation [2]) depends on *p*H, whereas k (equation [1]) is independent of *p*H.

$$\text{Rate} = \bar{k} \times [\text{total dimethylamine}] \times [\text{total nitrite}]^2 \qquad [2]$$

As expected from equation [2], the DMN yield at a constant *p*H of 3.4 was approximately proportional to dimethylamine concentration (*see* "Results") and also to [total nitrite]^2 at low nitrite concentrations (*see* table 1). For more precise evaluation of the results in table 1, it is necessary to test whether $\bar{k}$ (equation [2]) is independent of nitrite concentration. Therefore, values of $\bar{k}$ were calculated from the DMN yields and the stoichiometric concentrations of nitrite (A) and dimethylamine (B), using the appropriate integrated equation for reaction type A+B→products, with reaction rate proportional to $[A]^2 \times [B]$ [reference (*15*), p 21, equation [34]]. Table 1 shows that $\bar{k}$ was indeed fairly constant, at least between 0.01 and 0.1 M $NaNO_2$, although the yield of DMN varied nearly sixfold within this range.

The value of k (equation [1]) was calculated from $\bar{k}$ using equation [3], derived from the equivalence of equations [1] and [2].

$$k = \bar{k} \times \frac{[\text{total dimethylamine}]}{[Me_2NH]} \times \left(\frac{[\text{total nitrite}]}{[HNO_2]}\right)^2 \qquad [3]$$

The mean DMN yield of 15.8% under the standard conditions at *p*H 3.4 corresponded to a value for $\bar{k}$ of 9.9×10^{-2} $moles^{-2}\ min^{-1}\ liter^2$. At this *p*H, the fraction of total nitrite occurring as HNO_2 was 0.48, and that of total dimethylamine occurring as Me_2NH was 4.79×10^{-8} (based on pK_a values of 3.37 and 10.72, respectively). Using these fractions, k was calculated to be 8.94×10^6

[5] Brackets indicate molar concentration.

moles^{-2} min^{-1} liter^2, which does not differ very much from the figure of 2.4×10^7 moles^{-2} min^{-1} liter^2 derived by Ridd [(*14*), table 1)] from the data of Taylor and Price (*9*).

The *p*H curve (text-fig. 1) may be explained qualitatively as follows: For each decrease of 1 unit in the *p*H range 9–5, the concentration of nonionized Me_2NH decreases about tenfold and that of nonionized HNO_2 increases by a similar factor. As reaction rate is proportional to the square of $[HNO_2]$ and to $[Me_2NH]$, the rate increases about tenfold for each 1-unit drop in *p*H. Below the *p*K of HNO_2 at *p*H 3.36, nitrite becomes almost completely nonionized, so that the main effect of a further drop in *p*H is the continuing decrease in $[Me_2NH]$, causing a drop in the reaction rate.

Table 2 shows that, as the *p*H was varied, $\bar{k}$ also varied widely, and these $\bar{k}$ values may be used to calculate reaction rates at any *p*H (*see later*). However, fairly constant values were found for *k*, indicating that equation [1] was basically correct. The relatively high values of *k* at *p*H 1.5 and 2.0, and the subsequent gradual decrease in *k* with rising *p*H, may be due to the participation under acid conditions of other nitrosation mechanisms, *e.g.*, involving nitrosyl chloride or nitrous acidium ion (*14*). Alternatively, the different buffers used could have affected the results (*14*). Sander *et al.* (*8*) observed a fairly similar *p*H curve for the formation of di-*n*-propylnitrosamine.

Dimethylamine nitrosation thus mainly follows equation [1], at least at *p*H 3.4. In contrast, the rate of *N*-methylaniline nitrosation in 0.01 N $HClO_4$ is proportional only to $[HNO_2]^2$, since N_2O_3 formation is rate-limiting (though in 0.1 N $HClO_4$ the rate is also proportional to amine concentration) (*16*). This difference is attributed to the stronger basicity of dimethylamine (pK_b 3.28) compared with *N*-methylaniline (pK_b 9.15), which would make the reaction of N_2O_3 with dimethylamine rate-limiting at *p*H 3.4 because of the extremely low concentration of free amine.

Estimation of Dimethylnitrosamine Formation in Gastric Contents and During Food Storage

The methods used here may be useful for determining whether nitrite concentration, *p*H, and other conditions (excluding amine concentration) are suitable for DMN formation in food materials or gastric contents. Meanwhile, it is possible to use $\bar{k}$ (equation [2]) for rough estimates of DMN formation during digestion or storage of food containing dimethylamine and nitrite.

For example, consider a possible carcinogenicity experiment in which rats are fed a freshly prepared diet containing 1.0 g/kg each of $Me_2NH{\cdot}HCl$ and $NaNO_2$. We shall assume that 1) the food can be regarded as a homogeneous aqueous solution of 1 g/ml density, 2) the food remains in the glandular acid-secreting portion of the stomach for 1 hour at *p*H 3.4 and 37°, without dilution by gastric juice or drinking water, 3) $\bar{k}$ at 37° is twice that at 25°, *i.e.*, 0.198 moles^{-2} min^{-1} liter^2, and 4) a rat eats 15 g food/day. Substitution in equation [2] shows that 0.25% of the amine would be nitrosated, to give 2.3 mg DMN per kg food or 35 μg DMN/rat/day. This regimen would probably not be carcinogenic for rats, since the lowest DMN dose inducing significant numbers of hepatomas on continuous administration is 5 mg/kg food or about 75 μg/rat/day (*17*).

If the amine and nitrite concentrations are raised while remaining equal to each other, DMN formation should increase with the cube of reactant concentration. Thus if food containing 5 g/kg each of $Me_2NH{\cdot}HCl$ and $NaNO_2$ is administered under the conditions of the previous example, 9% of the amine should be nitrosated to give 400 mg DMN per kg food or 6 mg/rat/day. This dose of DMN would be highly carcinogenic, since 400 μg/rat/day induces a 100% incidence of liver tumors (*18–20*). However, the estimate may be too high, as the gastric contents may be diluted (a 50% dilution would reduce DMN formation eightfold) and the *p*H may not be in the 3.0–3.8 range of maximal nitrosation.

Druckrey *et al.* (*21*) found no liver tumors in rats fed for life with $NaNO_2$ in the drinking water and diethylamine hydrochloride in the food, at daily doses of 100 and 500 mg/kg body weight, corresponding perhaps to 2.0 g $NaNO_2$ per liter water and 10 g amine·HCl per kg food. Since the nitrite and amine were given in different vehicles and so would often reach the stomach at different times, possibly the concentrations used were too low. Also, Sander *et al.* (*8*) found no tumors in rats fed a diet containing 3 g/kg each of $NaNO_2$

and diethylamine, but the experiment had proceeded for only 6 months at the time of the report. Diethylamine (pK_b 3.02) should be slightly less readily nitrosated than dimethylamine (pK_b 3.28). However, the same workers did obtain esophageal tumors in rats fed a diet containing 5 g/kg each of $NaNO_2$ and *N*-methylbenzylamine (*8*). The formation of diethylnitrosamine was recently demonstrated after *in vitro* incubation of gastric contents with diethylamine and nitrite, and in the stomach contents of rabbits and cats after feeding the amine and nitrite (*22*).

Let us now consider the possibility of man being exposed to nitrosamines due to nitrosation in his gastric contents. The *p*H after an Ewald test meal of bread and water is about 1.4, but would presumably be higher during digestion of a large meal, which would stay in the stomach for several hours (*23*). A fish protein concentrate was found to contain 152 mg/kg of mixed amine hydrochlorides, of which about 25% was $Me_2NH{\cdot}HCl$ (estimated from the published gas chromatogram) (*24*). In the United States, the statutory limit of $NaNO_2$ added as preservative to fish and meat products is 200 ppm[6], *i.e.*, about 200 mg/liter (*25, 26*). Therefore, we shall take the example of food containing 40 mg/liter of $Me_2NH{\cdot}HCl$ and 200 mg/liter of $NaNO_2$ that is reacted in the stomach for 3 hours at *p*H 3.4 and 37°. Under these conditions, 11 μg/liter of DMN would be formed, or 3.3 μg per 300 g meal. This dose ingested daily may be too low to be significantly carcinogenic, though it should be stressed that even tiny incidences may assume importance in a large population.

Nitrosamines may also be formed during the storage of food. For example, consider the case of food containing 40 mg/liter of $Me_2NH{\cdot}HCl$ and 200 mg/liter of $NaNO_2$ (as in the previous example) that is stored for 180 days at 25° and *p*H 6. Under these conditions, $\bar{k}$ is estimated to be 8.2×10^{-4} moles^{-2} min^{-1} liter^2 (*i.e.*, 0.8% of the value at *p*H 3.4) and 65 μg/liter of DMN should be formed, without taking account of the possible decomposition of DMN during this long period. Far more DMN would, of course, be formed under more acidic conditions.

In conclusion, it should be possible theoretically to induce liver tumors in rats by feeding high doses of dimethylamine and nitrite, despite the negative results obtained by earlier workers (*8, 21*). Before deciding whether intragastric nitrosation is of importance in human carcinogenesis, a detailed study of nitrite and secondary amine concentration in foods is clearly necessary. It is important to find out whether nitrite concentrations ever exceed 200 ppm, *e.g.*, in brines used for pickling fish and meat. Nitrosamine formation should be suspected particularly in foods stored under mildly acidic conditions.

The formation of nitrosamines other than DMN could be a more serious problem than that of DMN itself, since the rate of nitrosation increases 1000-fold as the basicity of the amine decreases on proceeding from dimethylamine to aromatic amines such as *N*-methylaniline (*8*).

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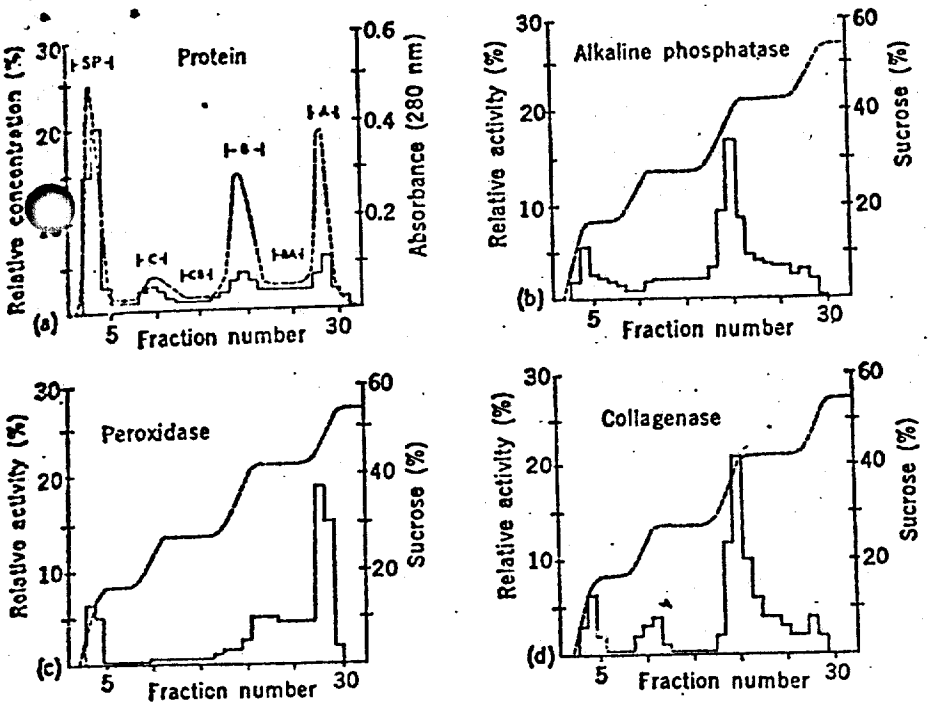

Fig. 1. Fractionation of leukocyte granules through a discontinuous sucrose gradient at 20,000 rev/min for 18 minutes. Absorbance was corrected for sucrose concentration. Protein was determined by the method of Lowry *et al.* (*11*). Peroxidase (c) was found at the heavy end of the gradient, and alkaline phosphatase (b) and collagenase (d) were consistently found together in the same region of the gradient. *SP*, Soluble phase; A, B, and C, regions of particle peaks; BA and CB, regions between peaks.

tion, which never exceeded 3 percent in reaction mixtures containing 0.5 μg of tr[illegible] per microgram of collagen. A number of detergent preparations designed to release latent enzyme activity were tested and found to interfere with the collagenase assay. Freezing and thawing repeated ten times was used, therefore, to release granule enzyme activity before all assay procedures. Assays were performed either on individual 20-ml fractions collected from the rotor or on granule pellets resulting from centrifugation of fractions pooled as shown in Fig. 1a.

Fractionation of rabbit PMN leukocyte granules yielded the distribution patterns in Fig. 1. Intact granules were demonstrated in areas C, B, and A by phase microscopy. A few particles were noted in area BA. Myeloperoxidase and alkaline phosphatase assumed modal distributions consistent with those reported elsewhere (*5*, *6*). Collagenase activity was consistently associated with the alkaline phosphatase–containing B granules (Fig. 1, area B). Assays of granule pellets from pooled fractions shown in Table 1 confirmed the strong co[illegible]ation between collagenase and alkaline phosphatase activity.

Granules in PMN leukocytes appear to possess many of the properties of lysosomes (*14*), and leukocyte granules are responsible for intracellular digestion of materials engulfed by these cells (*14*). Free extracellular granules have also been described in inflamed connective tissue (*15*), wound healing sites (*16*), tuberculin sensitvity reactions (*17*), and delayed hypersensitivity and arthus reactions (*18*). We report the presence of two alkaline hydrolases, alkaline phosphatase and collagenase, in a well-defined cytoplasmic body characteristic of lysosomes. The collagenase derived from PMN leukocyte granules of man manifests a broad *p*H optimum of 7 to 9.5 (*2*). In the case of polymorphonuclear leukocytes, therefore, particular lysosomal-type granules are associated with enzymatic degradation of constituents at an acid *p*H, and other types appear to be concerned with degradation of constituents at neutral to alkaline *p*H.

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Ascorbate-Nitrite Reaction: Possible Means of Blocking the Formation of Carcinogenic N-Nitroso Compounds

Abstract. *The formation of carcinogenic N-nitroso compounds by the chemical reaction between nitrous acid and oxytetracycline, morpholine, piperazine, N-methylaniline, methylurea, and (in some experiments) dimethylamine was blocked by ascorbic acid. The extent of blocking depended on the compound nitrosated and on the experimental conditions. Urea and ammonium sulfamate were less effective as blocking agents. The possibility of in vivo formation of carcinogenic N-nitroso compounds from drugs could be lessened by the combination of such drugs with the ascorbic acid.*

Most *N*-nitroso derivatives of secondary amines and amides are powerful carcinogens, which can act in several species (*1*). These derivatives might possibly be produced in the human stomach by the acid-catalyzed reaction between nitrite (*2*), which is present in some foods, and *N*-nitrosatable compounds,

which may be ingested as drugs, food additives, or natural constituents of food (*3*). As an experimental model for this hypothesis, tumors were induced in rats and mice by the long-term feeding of nitrite together with certain secondary amines (including the vermicide piperazine), alkylureas, and *N*-alkylcarbamates (*4*). The biological results are fairly well correlated with kinetic studies on the widely varying rates of the nitrosation reactions (*5*).

Since many drugs contain secondary amine, alkylurea, or *N*-alkylcarbamate groups, oral administration of these drugs might constitute a human health hazard, if nitrite-containing food were ingested at the same time. Examples of such drugs are piperazine, phenmetrazine, primaquine, pamaquine, physostigmine, synephrine, sulfanilylurea, and neohydrin. This possible hazard might be reduced by administering the drug together with a substance that preferentially reacts with and destroys any nitrite occurring in the stomach. We now present chemical data suggesting that ascorbic acid might be used for this purpose.

In addition to the classes of compound already mentioned, Lijinsky *et al.* (*6*) recently reported that certain drugs which contain tertiary amine or dialkylamide groups can be nitrosated to give dialkylnitrosamines. For example, reaction of the dimethylamino compound oxytetracycline with 0.30*M* nitrite at *p*H 4 and 37°C for 4 hours was reported to give a 65 percent yield of dimethylnitrosamine. We attempted to repeat this study on a commercial sample of oxytetracycline (Terramycin) and obtained negative results, but when pure oxytetracycline was used, we confirmed the published results. Our sample of Terramycin was compounded as a mixture containing 80 percent ascorbic acid, and when the same proportion of ascorbic acid was added to a sample of pure oxytetracycline and this was treated with nitrite, no nitrosation was observed. Further experiments were conducted in the presence and absence of ascorbate, and these confirmed that ascorbate almost completely blocked the nitrosation under conditions similar to those used by Lijinsky *et al.* (Table 1, experiments 1 and 2).

We then examined the effect of ascorbate on the nitrosation of five representative compounds whose nitrosation kinetics we have studied (*5*) (Table 1, experiments 2 to 21). Using a 2 : 1 ratio of ascorbate to nitrite, we found more than 98 percent blocking of nitrosation for morpholine and piperazine at all *p*H values tested. Blocking was only partial for *N*-methylaniline, but was fairly complete for methylurea. When the relatively slow nitrosation of dimethylamine was studied under conditions similar to those used for the other amines (reaction at 25°C with excess amine), blocking by ascorbate was fairly effective at *p*H 3 and 4, but at *p*H 2 there was little effect, and at *p*H 1, where the yield without ascorbate was only 0.042 percent, ascorbate increased the nitrosation to 0.076 percent (experiments 15 to 18). When dimethylamine was nitrosated under similar conditions to those used for the nitrosation of oxytetracycline, that is, prolonged reaction at 37°C with excess nitrite, the yield without ascorbate was much larger (2 to 82 percent) and more than 90 percent blocking was observed in the presence of ascorbate (experiments 19 to 21).

Dahn *et al.* (*7*) studied the kinetics of the reaction between nitrous acid and ascorbic acid, in which dehydroascorbic acid and nitric oxide are formed, probably via the nitrite ester of the 3-hydroxy group. In 0.1 to 0.5*N* perchloric acid the main nitrosating species is protonated nitrous acid ($H_2NO_2^+$), whereas in more dilute acid (*p*H 1.5 to 5.0) the nitrosating species is nitrous anhydride (N_2O_3). These are also the nitrosating species for second-

Table 1. Effect of ascorbate on the yield of *N*-nitroso compounds. A solution of oxytetracycline (250 mg), $NaNO_2$ (500 mg), and acetic acid (1 ml) in water (to 23 ml) was adjusted to the desired *p*H with HCl and kept for 22 hours at 37°C in a 100-ml flask fitted with a loosely stoppered condenser; it was then made alkaline with solid NaOH and extracted twice with 75 ml of CH_2Cl_2. The CH_2Cl_2 solution was extracted with 14 ml of 0.1*N* HCl and analyzed for dimethylnitrosamine by gas-liquid chromatography (*6*). For the experiments with secondary amines (*14*), 5 ml of a solution of $NaNO_2$ in 0.05*M* sodium citrate was acidified to the desired *p*H with perchloric acid and was added at 25°C to a solution of the amine plus sodium ascorbate in 15 ml of 0.05*M* citrate (previously adjusted to the same *p*H with perchloric acid). The 20-ml reaction mixture was kept in a stoppered 50-ml conical flask for 5 to 60 minutes at 25°C in the dark. The reaction was stopped by making it alkaline, and solid NaCl was added (in the case of some amines). The nitroso derivative was extracted with CH_2Cl_2 or ether, and its absorbance at 358 to 369 nm was measured. When the absorbance was less than 0.10, the extract was dried over Na_2SO_4, some of the solvent was removed by a rotary evaporator, and another absorbance reading was taken. Results were calculated from the molar absorptivity (ϵ) of the nitroso derivative and corrected for losses due to incomplete extraction. Each experiment was performed in duplicate with and without blocking agent. The full spectra of the extracts at 320 to 450 nm were identical to those of the nitroso derivative. When amine, nitrite, or ascorbate were separately run through the complete procedure, the extract showed no absorbance. When an acidic solution of nitrite plus ascorbate was made alkaline, it turned yellow and then red, but extracts showed no absorption above 320 nm. Methylnitrosourea formation at 25°C was determined by absorbance of the aqueous solution at 400 nm (ϵ, 79) (*5*).

Exp. No.	Base (m*M*)	Nitrite (m*M*)	Ascorbate (m*M*)	*p*H	Time (min)	Yield from amine or urea (%)		Blocking (%)
						Without ascorbate	With ascorbate	
				Oxytetracycline				
1	21.6	315	475	2.8	1320	44	0	100
2	21.6	315	475	3.8	1320	63	0.045	99.9
				Morpholine				
3	25	50	100	2	30	20	0.48	98
4	25	50	100	3	30	65	0	100
5	25	50	100	4	30	34	0	100
				Piperazine				
6	10	10	20	1	60	35	0.58	98
7	10	10	20	2	20	44	1.2	98
8	10	10	20	3	10	56	0.33	99
9	10	10	20	4	15	54	0.24	99
				N-Methylaniline				
10	2.5	2.5	5	3	5	68	27	60
11	2.5	2.5	5	4	10	16	8.8	45
				Methylurea				
12	5	10	20	1	10	85	6.9	92
13	5	10	20	2	10	58	7.6	87
14	20	20	40	3	30	27	0	100
				Dimethylamine				
15	500	50	100	1	60	0.042	0.076	−80
16	500	50	100	2	60	0.27	0.29	−7
17	500	50	100	3	60	0.76	0.20	74
18	500	50	100	4	60	0.92	0.03	97
19	20	300	500	2	1440	2.2	0.17	92
20	20	300	500	2.8	1380	20	1.7	91
21	20	300	500	3.8	1380	82	1.4	98

ary amines and alkylureas (*5*) [in the presence of bromide, iodide, and thiocyanate ions, the corresponding NOX species are active nitrosating species for *N*-nitrosation (*8*)]. Therefore, the block- of *N*-nitrosation by ascorbate was probably due to competition for available nitrite, or to be precise, for N_2O_3 and $H_2NO_2^+$. Ascorbate anion is 240 times more rapidly nitrosated than ascorbic acid (pK_a, 4.29), due presumably to the greater nucleophilic activity of the anion. At *p*H 3 to 5, where the proportion of anion is significant, reaction with nitrite is so rapid that the formation of N_2O_3 is rate-limiting. This explains why ascorbate is more effective as a blocking agent at *p*H 3 to 4 than at *p*H 1 to 2.

Ascorbate had no effect on decomposition of the *N*-nitroso derivatives of methylurea and the amines listed in Table 1, indicating that its action in reducing or eliminating the formation of *N*-nitroso compounds is solely a consequence of the reaction with nitrite.

Nitric oxide produced by the ascorbate-nitrite reaction may combine with atmospheric oxygen to form nitrogen dioxide, some of which would reenter solution and react with water to gen- e equimolar amounts of nitrous and c acids. Thus up to half of the nitric oxide could potentially be reconverted to nitrite. The presence of excess ascorbate in our experiments served to minimize the effect of such reconversions on the extent of *N*-nitrosation.

The nitrosation kinetics for the amines studied is in accord with the relative effectiveness of ascorbate in blocking the nitrosation of these compounds. For example, *N*-methylaniline, like ascorbate, is nitrosated so rapidly that N_2O_3 formation is rate-limiting (*9*), so that the amine competes rather successfully with ascorbate for N_2O_3, and blocking is only partial. Morpholine and piperazine are nitrosated less rapidly (*5*), N_2O_3 formation is not rate-limiting, and it is therefore not surprising that these nitrosations were effectively blocked by ascorbate. Dimethylamine, which is nitrosated much slower than the other amines tested (*5*), was not effectively blocked by ascorbate under those conditions in which relatively little nitrosamine was formed even in absence of ascorbate. When the re- ion conditions were adjusted to increase the yield of nitrosamine, the blocking action of ascorbate was more pronounced. The reason for this behavior is not clear. Blocking of methylurea nitrosation was perhaps stronger than might be expected at *p*H 1, where the $H_2NO_2^+$ mechanism is predominant and methylurea is rapidly attacked (*5*).

Table 2. Effect of the blocking agents urea and ammonium sulfamate on the yield of nitrosomorpholine and mononitrosopiperazine. The experimental procedure followed that described for Table 1, except that urea or ammonium sulfamate was substituted for ascorbate.

Base (m*M*)	Nitrite (m*M*)	Blocking agent (m*M*)	*p*H	Time (min)	Yield from amine (%): Without blocking agent	Yield from amine (%): With urea	Yield from amine (%): With ammonium sulfamate	Blocking (%): Urea	Blocking (%): Ammonium sulfamate
				Morpholine					
25	50	100	1	45	7.3	0.4	0	95	100
25	50	100	2	30	25	18	0	24	100
25	50	100	3	30	56	55	0.6	2	99
25	50	100	4	30	32		9.3		71
				Piperazine					
10	10	20	1	60	35	11	0	68	100
10	10	20	2	20	36	31	0	14	100
10	10	20	3	10	56	57	14	−2	75

The kinetics of oxytetracycline nitrosation have not yet been examined. Studies on other tertiary amines (*10*) suggest that nitrous acid first reacts with oxytetracycline to give dimethylamine, which is then nitrosated to give dimethylnitrosamine. Under similar conditions, ascorbate blocked the formation of dimethylnitrosamine more effectively from oxytetracycline (experiments 1 to 2) than from dimethylamine (experiments 19 to 21), suggesting that ascorbate reacts with nitrite sufficiently rapidly to prevent the formation of dimethylamine from oxytetracycline.

Since urea and ammonium sulfamate (*11*) also react rapidly with nitrite, the effect of these substances on the nitrosation of piperazine and morpholine was examined (Table 2). Urea was relatively ineffective, especially at higher *p*H. Ammonium sulfamate was even more effective than ascorbate at *p*H 1 and 2, but was relatively ineffective at *p*H 3 and 4. The *p*H of the fasted stomach can reach 0.9, but when a meal is ingested the *p*H of the food is lowered gradually over 1 to 2 hours until it reaches 1.5 to 2.0 (*12*). Hence ascorbate has the advantage over the other agents in that it acts efficiently at the higher *p*H values, and it could react with nitrite in the stomach before the *p*H is lowered to where extensive reaction with amines or ureas might occur.

Although under some conditions the nitrosation of dimethylamine was actually increased by ascorbate, the chemical results on the whole support the suggestion that drugs which can be *N*-nitrosated could be combined with ascorbate before they are administered. This would apply whenever nitrosation of the drug in vivo was likely to occur and combination with ascorbate would reduce this potential hazard without affecting the drug action. Furthermore, addition of ascorbate to certain foods containing nitrite or nitrosatable compounds might be worth considering. In fact, small amounts of ascorbate are often added to nitrite-preserved meat to increase the nitric oxide available for formation of the red nitrosomyoglobin color (*13*).

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14. For each amine, the amount of reagents added at the end of reaction, the volume of CH_2Cl_2 or ether, the wavelength (and the molar absorptivity) where absorbance was measured, the extraction efficiency, and the nitrosamine formed were as follows: morpholine, 2 ml of 5*N* NaOH, 2 × 10 ml CH_2Cl_2, 358 nm (106), 94 percent, nitrosomorpholine; piperazine, 4 ml of 5 NaOH and 2 g of NaCl, 4 × 15 ml of CH_2Cl_2, 358 nm (114), 86 percent, mononitrosopiperazine [kinetic studies (5) show that mono- and not dinitrosopiperazine is the main initial product]; *N*-methylaniline, 15 ml of saturated sodium tetraborate solution adjusted to pH 9, 3 × 10 ml of CH_2Cl_2, 369 nm (227), 100 percent, *N*-methyl-*N*-nitrosoaniline; dimethylamine (experiments 15 to 18), 5 ml of 5*N* NaOH and 2.5 g of NaCl, 4 × 35 ml of ether, 358 nm (119), 80 percent, dimethylnitrosamine; dimethylamine (experiments 19 to 21), temperature (37°C) and other reaction conditions as for oxytetracycline experiments, workup as for experiments 15 to 18.
15. We thank J. Sams for technical assistance, E. Conrad for running the gas chromatograms, and Pfizer Corporation, New York, for oxytetracycline. Supported by contract PH 43-68-959 from the National Cancer Institute and by grant BC-39 from the American Cancer Society.

14 February 1972; revised 18 April 1972

Decreased RNA Polymerase Activity in Mammalian Zinc Deficiency

Abstract. *The activity of DNA-dependent RNA polymerase has been measured in liver nuclei from suckling rats nursed by zinc-deficient dams, or by controls that were either pair-fed or given free access to the diet. In the zinc-deficient pups, the activity of the enzyme did not increase; it fell after the tenth day of life.*

Zinc is an essential nutrient that appears necessary for the synthesis of nucleic acids (*1–3*) and protein (*4*). The manner in which it influences these processes is unknown. Its requirement for the action of various metalloenzymes is well known (*5*), and its influence on the activity of mammalian (*1*) and *Escherichia coli* (*6*) DNA polymerase has been reported. While zinc may play a role in the maintenance of the conformation of nucleic acids (*7*), and zinc nutriture of the animal influences the sucrose density gradient profile of liver polysomes as well as the incorporation of uridine into those polysomes (*8*), it is far from clear how these latter events take place. Therefore, the influence of zinc deficiency on the activity of DNA-dependent RNA polymerase in nuclei of liver cells has been assessed in suckling rats.

Table 1. Effect of zinc deficiency on activity of liver nuclear DNA-dependent RNA polymerase of suckling rats. The activity of the enzyme rose very little from birth to day 10 of life and then decreased. Pair feeding did not suppress the activity of the enzyme.

Age (days)	Carbon-14 per milligram of deoxyribose (count min^{-1} mg^{-1} ml^{-1})		
	Zinc-deficient	Controls: Pair-fed	Controls: Free access to food
2	83.0*	81.5	78.6
4	85.9*		90.9
5		91.5	
6	85.0	87.0	94.8
7			103.5
8		98.9	105.0*
10	91.2	111.3*	
11			115.9*
12	80.4	114.4*	112.8
14	70.0	112.1	115.4†
15	64.8	111.1*	112.8*
16	60.4	107.8	107.4

* Average of two sets of pups. † Average of three sets of pups.

Feeding a biotin-enriched diet containing sprayed egg white (20 percent by weight) (*3*) to pregnant dams from the 18th day of gestation through the 16th day of the neonatal period produced zinc deficiency in their pups. Pair feeding of the zinc-supplemented dams (160 μg every other day, by intraperitoneal injection) also resulted in growth failure in their pups. The zinc supplementation was sufficient to maintain normal growth in pups nursed by dams given free access to an adequate diet (Fig. 1). Similar observations have been reported by Mutch and Hurley (*9*), who also found that the concentration of zinc in milk from zinc-deficient dams is decreased along with a decrease in total volume. In contrast, they found that milk from pair-fed dams has increased concentrations of zinc and protein while the volume is decreased. Growth retardation in pups nursed by pair-fed dams therefore reflects the fewer calories available to the pups as a consequence of maternal starvation.

The litters of the dams were sampled at intervals, the sampled pups were decapitated, and their livers were excised and pooled (3 to 11 pups per assay, with the usual number being 5 or 6). The effect of zinc deficiency, starvation, and free access to food on the activity of nuclear DNA-dependent RNA polymerase was measured. The method of Widnell and Tata (*10*) was used to prepare the nuclear suspensions. Enzyme activity was assayed as described by Weiss (*11*). The [^{14}C]adenosine triphosphate incorporated into RNA formed on the nuclear DNA was assayed by liquid scintillation counting and was reported in relation to the amount of deoxyribose (*12*) present. By duplicate assays, these techniques were found to have a high degree of reproducibility; in addition, the activity of the enzyme from pups of the same age and treatment was also similar.

Our studies indicate that starvation (pair feeding) did not inhibit the activity of the enzyme while zinc deprivation did (Table 1). In fact, the activity of the enzyme showed a steady decline in the pups deficient in zinc from day 10 of life ($P < .001$).

These observations are the first, of which we are aware, to show the requirement of zinc for the activity of nuclear DNA-dependent RNA polym-

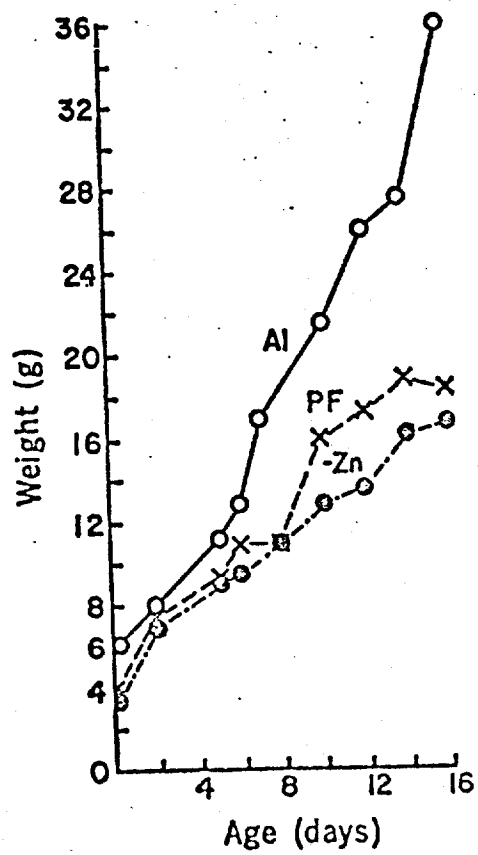


Fig. 1. Growth of suckling rats nursed by zinc deficient (*–Zn*) dams, pair-fed (*PF*) dams, and dams given free access to a normal diet (*Al*). Both zinc-deficient and pair-fed pups showed poor weight gain.

Schulung und soziale Stellung sichern mit dem Motto, non nobis solum, sed pro publico bono!

Zusammenfassung

Die Schnelligkeit des modernen Transportes, die Intensität des internationalen Fleischhandels und der wirtschaftliche oder auch politische Zusammenschluß von Staaten bringen die Gefahr der Einschleppung von Seuchen mit sich, wobei die Möglichkeit der Erkennung bedeutend vermindert wurde, zumal es sich bei den importierten Lebensmitteln oft um erhebliche Mengen handelt. Darüber hinaus sind die Verzehrsgewohnheiten andere geworden und die Massenverpflegung ist an die Stelle der Haushaltskost getreten. Lebensmittelvergiftungen können auf diese Weise erhebliche Folgen nach sich ziehen. Zur Abwendung dieser Gefahren ist die Verlegung der Untersuchung und Überwachung in das Ursprungsland die einzige sichere Möglichkeit. Dabei kommt der Fleischhygiene und dem tierärztlichen Überwachungsdienst auf der Basis einer engen internationalen Zusammenarbeit entscheidende Bedeutung zu und ist als Teil des Volksgesundheitsdienstes zu betrachten.

Seit dem Beginn der öffentlichen Fleischproduktion durch die Erlassung des preußischen Schlachthofgesetzes im Jahre 1868 und in der Folgezeit danach waren zwar noch viele Mängel in der Fleischhygiene nicht beseitigt, zumal entsprechende wissenschaftliche Erkenntnisse noch fehlten, aber im Zuge des modernen Schlachthofbaues und dem Bau moderner Anlagen in der Fleischindustrie, insbesondere nach dem zweiten Weltkriege, hat sich Wesentliches geändert.

Es wird heute auf Aufklärung und Zusammenarbeit mit allen Beteiligten Wert gelegt, um auch das Verständnis für die Lebensmittelhygiene weitesten Kreisen der Bevölkerung einschließlich der Konsumenten näherzubringen. Weiters bedarf es der Zusammenarbeit zwischen dem Veterinär- und Humanmediziner als gleichberechtigte Partner, wenn der Gesundheit der Menschen tatsächlich geholfen werden soll.

Das Niveau der Fleischhygiene hängt ganz wesentlich vom Stande der Hygiene in den Schlachtstätten, Kühlräumen, Fleischverarbeitungsbetrieben usw. ab. Es mußten nicht nur neue Arbeitsmethoden geschaffen werden, sondern auch die Kontrolle und die Fleischuntersuchung den Erfordernissen entsprechend geändert werden. Um eine Harmonisierung neuer gesetzlicher Bestimmungen bemühen sich seit 1955 die Weltvereinigung Tierärztlicher Lebensmittelhygieniker ebenso wie die FAO und WHO.

Summary

Ginsberg, A.: Problems associated with the Practical Application of New Scientific Knowledge in Meat Hygiene.

The rapidity of modern transport, the increased international meat trade and the economic and even political integration have greatly increased the risk of spreading infectious diseases with foodstuffs. At the same time the chances of safe and efficient control have considerably decreased, inasmuch as foods mostly are imported on a mass scale. Moreover, food habits are changing, meals in community have replaced the home-made meal. Outbreaks of food poisoning therefore may have serious consequences. To perform the control and surveillance of foods in the country of origin is the only safe means of avoiding these dangers. Meat hygiene and veterinary meat inspection which require close international collaboration play an essential role in this field and must be considered as part of the public health services.

Since the introduction of public supervision of meat production in 1868 when the Prussian law on abattoirs was enacted, and in the time afterwards many inadequate meat hygiene measures have still been in use, all the more since essential scientific knowledge was lacking. It was particularly in the post-war period that substantial progress has been made with newer developments in abattoir design and the construction of modern meat processing plants.

Actually particular attention is attached to the education of, and collabation with, all persons concerned, and attempts are made to make large groups of the populations, including the cosumer, familiar with the principles of food hygiene. Moreover the cooperation of medical and veterinary services should be encouraged as being essential for the safe protection of human health.

Standard of meat hygiene is largely dependent upon the hygienic standards of abattoirs, cooling facilities, processing plants, etc. It appears desirable that meat handling and processing techniques be improved and, in addition, official supervision and meat inspection should be modified according to practical requirements. Since 1955 the World Association of Food Hygienists and the FAO and WHO as well have taken useful steps to bring new legal regulations into a common line.

Anschrift des Verfassers: Dr. Dr. h. c. A. Ginsberg, New Zealand House, Haymarket, London SW 1.

Possible formation of toxic, particularly cancerogenic, substances in foods of animal origin ~~by~~ due to technological processing

Aus der Analytischen Abteilung der Deutschen Forschungsanstalt für Lebensmittelchemie und Fakultät für Landwirtschaft und Gartenbau der Technischen Hochschule München-Weihenstephan

Möglichkeiten der Bildung von toxischen, insbesondere kanzerogenen Stoffen in Lebensmitteln tierischer Herkunft durch technologische Verfahren[1])

Von K. Möhler

In letzter Zeit mehren sich Publikationen, bei denen die Grenze zwischen exakter Darstellung eigener oder fremder experimenteller Befunde und journalistischer

[1]) Gekürzte Fassung eines Vortrags bei der 4. Arbeitstagung der Sektion Lebensmittel tierischer Herkunft in der Österreichischen Gesellschaft der Tierärzte am 13. Mai 1970 in Wien.

Sensationsmache schwer zu finden ist. Bei Fleischerzeugnissen werden zwei Probleme in den Kreis solcher Betrachtungen einbezogen, einmal das Nitritproblem, erweitert auf die sogenannten Nitrosamine, und zum anderen die Räucherung. Die allgemeine toxische Situation bei Lebensmitteln tierischer Herkunft tritt demgegenüber in den Hintergrund, vor allem dann, wenn man sich als Chemiker auf das Fachgebiet Chemie und Technologie beschränkt. Damit entfallen die wichtigsten Quellen von möglichen Intoxikationen durch Mikroorganismen, z. B. Botulismus, Salmonellose, Aflatoxin-Vergiftung. Andere Quellen sind nur von geringer Bedeutung, doch soll der Vollständigkeit wegen ein kurzer Überblick gegeben werden.

Die Anwendung von Konservierungsstoffen war bei den Lebensmitteln tierischer Herkunft schon immer durch Verordnungen stark eingeschränkt und ist heute — zumindest für das Gebiet der Bundesrepublik Deutschland — fast bedeutungslos geworden. Selbst auf dem Fischsektor geht der Trend der Industrie nach dem Verbote des Hexamethylentetramins immer mehr zu den Vollkonserven, so daß aus der verbleibenden Anwendung von Konservierungsstoffen bei einigen kurzlebigen, meist rohen Erzeugnissen keine Gefahr mehr konstruiert werden kann. In welchem Umfang eine unzulässige Anwendung von Antibiotika eine Rolle spielt oder eine Gefahr werden kann, darüber dürften die in tiermedizinischen Instituten entwickelten Hemmstofftteste künftig einige Auskünfte bringen.

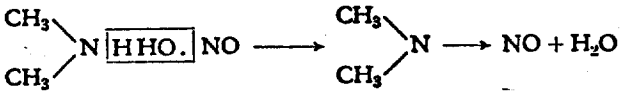
Nicht vernachlässigen darf man dagegen die Frage der Anwendung von Reinigungs- und Desinfektionsmitteln. Da dieses Thema auf dem Gebiet der Milchgewinnung und Verarbeitung sehr eingehend behandelt wird, kann man die dort gesammelten Erfahrungen bei der Fleischverarbeitung nutzbar machen. Auch bei regelmäßiger Anwendung von Reinigungsmitteln läßt sich gelegentlich ein Einsatz von stark wirkenden Desinfektionsmitteln nicht umgehen. Dieser Einsatz sollte aber dann massiv und kontrolliert erfolgen, um die Gefahr von toxischen Rückständen auszuschalten. Die Verwendung von Ozon zur Desodorisierung oder Entkeimung der Luft, z. B. in Kühlräumen, bringt nach bisherigen Forschungen keine Vorteile, ebenso ist die Verwendung von Ultraviolettstrahlen problematisch, zumal eine direkte Bestrahlung von Lebensmitteln in der Bundesrepublik ohnehin verboten ist.

Neuerdings hat man sich auch wieder Gedanken darüber gemacht, ob das Erhitzen, namentlich das Hocherhitzen von tierischen Lebensmitteln, neben einer Beeinflussung des Nährwertes auch Anlaß zur Bildung von toxischen Stoffen geben könnte. Es ist ziemlich sicher, daß mit der durch Erhitzen erzielten Bräunung von Fleisch keine Bildung von toxischen Stoffen eintritt. Ungeklärt ist dagegen die Frage, wie die Bräunungsreaktion in sterilisierter Säuglingsmilch zu bewerten ist. Da diese Milch unter Zusatz von Nährzuckern hergestellt wird, tritt beim Sterilisieren eine Maillard-Reaktion auf, die mit einer mehr oder weniger starken Braunfärbung einhergeht (15).

Die Behandlung der Zusatzstoffe bei der Fleischverarbeitung kann sich — abgesehen von Nitrit und Nitrat — auf einen kurzen Überblick beschränken. Bei der Anwendung von Kutterhilfsmitteln, z. B. von Polyphosphaten, von Hilfsmitteln zur Umrötung und Reifung, wie Askorbinsäure und Glukonodeltalakton, von Aromastoffen und Aromaverstärkern (Beispiel Glutamat und Ribonukleotide) sind keine toxischen oder kanzerogenen Wirkungen zu erwarten.

Eine kanzerogene Wirkung, abgegrenzt von den toxischen Stoffen, wird bei 2 Substanzgruppen für möglich gehalten, die im Rahmen der Zubereitung in Fleischerzeugnissen auftreten können, das sind die Nitrosamine und die Gruppe der kondensierten aromatischen Kohlenwasserstoffe, auch polyzyklische Kohlenwasserstoffe genannt.

Ausgangspunkt der Nitrosamine sind sekundäre Amine und salpetrige Säure, die unter Wasserabspaltung miteinander reagieren, wie es am Beispiel des Dimethylnitrosamins dargestellt wird.



Dimethylamin + salpetrige Säure ⟶ Dimethylnitrosamin

Die Nitrosamine sind flüssige bis feste Substanzen; das Dimethylnitrosamin ist z. B. ein gelbes Öl, das in Wasser und Fettlösungsmitteln annähernd gleich gut löslich ist. Über den Gehalt an sekundären Aminen in Lebensmitteln ist noch relativ wenig bekannt; mit Sicherheit kommt Dimethylamin im Fisch vor. Soweit natürliche Gehalte unmittelbar in Frage kommen, werden sie sehr gering sein, anderseits ist aber eine Abspaltung aus verwandten Gruppierungen möglich, z. B. aus Proteinen sowie Lezithin, häufig unter Mitwirkung von Mikroorganismen. Die salpetrige Säure kann durch Hydrolyse aus Nitrit entstehen, das z. B. als Nitritpökelsalz zugesetzt wird oder aus Salpeter durch mikrobielle Reduktion gebildet wird. Bei der Pökelung entsteht bekanntlich Stickoxidmyoglobin und, soweit noch Blut vorhanden ist, auch Stickoxidhämoglobin. Das zugesetzte oder aus Nitrat entstandene Nitrit wird aber nur zum Teil zur Bildung des Stickoxidkomplexes verbraucht; ein Teil wird zu Nitrat oxydiert und ein weiterer Teil wird für vorerst noch unbekannte Reaktionen verbraucht. Im Modellversuch (11) werden z. B. von 15 mg $NaNO_2$ auf 100 g Fleisch 12% als NO an den Pökelfarbstoff gebunden, 17% zu Nitrat oxydiert, 17% unbekannt verbraucht und 54% als Restnitrit wiedergefunden. Der unbekannte Verbrauchsposten hat zu der

Annahme geführt, daß in Fleischerzeugnissen auch Nitrosamine entstehen könnten. Dazu muß gesagt werden, daß der pH-Wert von Fleischerzeugnissen, ausgenommen vielleicht stark gesäuerte Rohwürste, in der Regel um 6 liegt, wobei die Bildung von salpetriger Säure aus Nitrit noch sehr gering ist. Außerdem reagieren die sekundären Amine um so schwächer, je stärker basisch sie sind. Die Basizität nimmt mit steigender Kohlenstoffzahl der Alkylketten ab. Das Diphenylnitrosamin bildet sich daher besonders leicht, und zwar bereits bei pH 7 in merklicher Menge.

Die Entdeckung der kanzerogenen Wirkung (3) hängt eng mit der technischen Darstellung und Verwertung des Dimethylnitrosamins zusammen. Aus diesem Nitrosamin gewinnt man durch Hydrierung das unsymmetrische Dimethylhydrazin, das zusammen mit Stickstofftetroxid und Hydrazin als Raketentreibstoff in der Mondlandefähre des Apollo-Unternehmens dient (1). Lebererkrankungen bei Fabrikarbeitern konnten auf die Toxizität des Dimethylnitrosamins zurückgeführt werden, und *Magee* und *Barnes* (16) fanden später, daß das Nitrosamin in wesentlich geringerer Dosis bei Ratten Krebs hervorruft. Die „Nitrosamin-Forschung" hat seither einen erheblichen Umfang angenommen und zur Erforschung des Krebsproblems einen wichtigen Beitrag geleistet. Von den Eigenschaften oder Wirkungen seien nur kurz folgende genannt:

In der Regel ist die kanzerogene Wirkung um so stärker, je kürzer die Alkylkette ist. Dimethylnitrosamin ist am stärksten kanzerogen, Diphenylnitrosamin überhaupt nicht. Die kanzerogene Wirkung entfällt außerdem, wenn wenigstens ein Kohlenstoffatom, das an den Stickstoff gebunden ist, ein tertiäres C-Atom ist. Die Nitrosamine wirken organotrop, d. h. in Abhängigkeit von der Struktur der Alkylketten werden spezifisch bestimmte Organe im Körper befallen. Die Wirksamkeit ist bei oraler Aufnahme gegeben. Für den Wirkungsmechanismus gibt es eine sehr plausible, experimentell zum Teil gestützte Theorie. Durch enzymatische Hydroxylierung und Abspaltung einer Alkylkette als Carbonyl entsteht ein Diazoalkan, das eine Alkylgruppe in die RNS bzw. DNS einbauen kann. Die Nitrosamine zählen daher zur Gruppe der alkylierenden Stoffe. Durch Zufuhr von Dimethylnitrosamin entsteht z. B. aus Guanin das 7-Methylguanin. Durch die Alkylierung wird die genetische Reproduktion gestört; es kann zur Bildung eines Tumors und zur Entstehung von Krebs kommen (2).

Die ersten Angaben über Nitrosamine in Lebensmitteln (10) bewegten sich in der Größenordnung von 50 mg/kg Lebensmittel und verursachten einige Aufregung. Bald konnten jedoch die tatsächlichen Mengenverhältnisse genauer angegeben werden; sie liegen im Bereich von Mikrogramm/kg Lebensmittel (12). Die Isolierung erfolgt im wesentlichen nach dem organischen Trennungsgang für Neutralstoffe, erfordert aber einen hohen Einsatz an Substanz und Lösungsmitteln. Letztere müssen daher extrem rein sein. In der Schlußphase wird eine Kombination Gaschromatographie/Dünnschichtchromatographie durchgeführt. Da die Verluste bei der Aufarbeitung sehr hoch sind, muß eine Probe mit innerem Standard parallel aufbereitet werden. Der enorme Arbeits- und Kostenaufwand hat uns veranlaßt, die Untersuchung auf den Mechanismus der Bildung und des Abbaues von Nitrosaminen zu richten, um durch entsprechende Voraussagemöglichkeiten den Bereich der tatsächlich nötigen Lebensmitteluntersuchungen auf gezielte Proben zu beschränken.

Das Nitrosaminproblem ist mit den in Lebensmitteln vorkommenden Verbindungen noch nicht ausgeschöpft, da überschüssiges Nitrit im menschlichen Magen beim dort herrschenden niedrigen ph-Wert zur Bildung von salpetriger Säure Anlaß geben kann. Wenn gleichzeitig passende sekundäre Amine vorhanden sind, könnten sich Nitrosamine bilden. *Sander* in Tübingen (13) und *Sen* u. Mitarb. (14) in Kanada haben darüber Untersuchungen in vitro und mit Tieren angestellt. Während mit Magensaft in vitro in geringem oder mäßigem Umfang positive Resultate aus Nitrit und reinen Aminen zu verzeichnen waren, konnte z. B. die Bildung von Diäthylnitrosamin weder chemisch noch tierexperimentell nachgewiesen werden (13). Nach unseren Untersuchungen darf man das Problem nicht isoliert betrachten, sondern muß die anderen Lebensmittelbestandteile mit berücksichtigen, die durch kompetitive Hemmung die Bildung von Nitrosaminen sehr unwahrscheinlich machen.

Während die Nitrosamine, wie beschrieben, erst im Lebensmittel entstehen, werden die Vertreter der zweiten Gruppe, die polyzyklischen Kohlenwasserstoffe, stets von außen zugeführt. Ausgangspunkt ist bei Fleischerzeugnissen der Rauch. Wenn Holz, Kohle oder Torf verbrennen, entsteht neben den gasförmigen und im Aerosolzustand befindlichen flüssigen Stoffen Teer. Im Teer sind die schwer flüchtigen hochmolekularen Stoffe angereichert, von denen ein Teil kanzerogen wirkt. Für diese Wirkung gibt es eine Anzahl von Voraussetzungen (2), wie Kondensation von mindestens 3 und höchstens 7 Benzolringen, flacher Molekülbau ohne Substituenten, bestimmte Ringanordnungen. Am stärksten wirksam ist das 3,4-Benzpyren oder Benzo[a]pyren, wie es neuerdings genannt wird. Für die Bildung von polyzyklischen Kohlenwasserstoffen ist Voraussetzung, daß Stoffe vorliegen, aus denen sich solche Ringe bilden können, wie Proteine, Fette, Kohlenhydrate, Harz, Pech usw. Aus reinem Kohlenstoff, Koks und Holzkohle entstehen diese Ringsysteme nicht. Weiterhin ist die Bildung an gewisse Temperaturen gebunden, die etwa zwischen 500 und 900° C liegen. Im Torfrauch ist der Gehalt an polyzyklischen Kohlenwasserstoffen relativ hoch, er

wurde deshalb in der Bundesrepublik Deutschland als Räuchermaterial verboten.

Es gibt verschiedene Theorien zu der Frage, welche chemischen Eigenschaften der polyzyklischen Kohlenwasserstoffe die kanzerogene Wirkung auslösen (2). Sie sind aber alle umstritten und nicht annähernd so wahrscheinlich wie die Alkylierungstheorie bei den Nitrosaminen. Beachtenswert ist die Tatsache, daß nur etwa 20% der kanzerogenen Wirkung des Tabakteers durch die bisher bekannten, definierten polyzyklischen Kohlenwasserstoffe erklärt werden können (16).

Die Isolierung der polyzyklischen Kohlenwasserstoffe aus Lebensmitteln erfolgt in ähnlicher Weise wie bei den Nitrosaminen. Die endgültige Identifizierung gelingt durch Aufnahme des Ultraviolett- bzw. Fluoreszenzspektrums. Eine scharfe Differenzierung ist notwendig, da z. B. andere polyzyklische Kohlenwasserstoffe Benzpyren vortäuschen können (9). Die Gehalte, die in Lebensmitteln gefunden werden, liegen für die kanzerogenen polyzyklischen Kohlenwasserstoffe im Bereich von Mikrogramm/kg Lebensmittel (11). Im Ruß und Teer von Rauchkammern wurden zwar 800 bis 1400 µg/kg 3,4-Benzpyren gefunden, in stark geräuchertem Schinken dagegen nur 4 µg/kg (19). Vielleicht ist auch bei den polyzyklischen Kohlenwasserstoffen die Gefahr von anderer Seite viel größer, da Abgase von Kraftfahrzeugen, von Heizungen und Industrieanlagen usw. beachtliche Mengen von polyzyklischen Kohlenwasserstoffen erzeugen. Es ist daher nicht verwunderlich, daß Salat oder Gemüse, die in der Nachbarschaft von viel befahrenen Autobahnen gezogen werden, beachtliche Gehalte an Benzpyren aufwiesen (6, 7, 8). Auch bei der direkten Trocknung von Lebensmitteln mit den Verbrennungsgasen von Heizöl kann unter Umständen eine beachtliche Anreicherung mit 3,4-Benzpyren eintreten. Von den geräucherten Fleischerzeugnissen scheint nach bisherigen Untersuchungen die geringste Gefahr auszugehen.

Wenn man sich über eine mögliche kanzerogene Wirkung der in Lebensmitteln enthaltenen Nitrosamine und polyzyklischen Kohlenwasserstoffe beim Menschen Gedanken macht, muß man berücksichtigen, daß alle Erfahrungen über diese Wirkung ausschließlich im Tierversuch gemacht wurden. Dies hat zur Folge, daß über die Dosis, die beim Menschen Krebs auslösen könnte, nichts bekannt ist. Man kann sich daher nur der Statistik zuwenden und die besagt eindeutig, daß kein Zusammenhang zwischen vermehrtem Auftreten von Krebs und bestimmten Ernährungsformen besteht (5). Eine Ausnahme stellen unter Umständen die Bantu-Neger dar, bei denen sich die überhöhte Rate an Leberkrebs vielleicht durch einen relativ hohen Nitrosamingehalt einer Solanum-Art erklären läßt (4); oder die Isländer, die ihre Fische mit getrocknetem Schafsmist räuchern, womit der Benzpyrengehalt sehr stark erhöht wird. Es ist im Augenblick also völlig unmöglich, zu sagen, welcher Gehalt an 3,4-Benzpyren oder kanzerogenen Nitrosaminen in Lebensmitteln als bedenklich anzusehen ist. Vergleiche zwischen den Gehalten in Lebensmitteln und den etwa im Tabakrauch auftretenden Mengen solcher Stoffe sind als unwissenschaftlich in den Bereich der journalistischen Phantasterei zu verweisen, zumal bei diesem Beispiel die Applikationsweise völlig verschieden ist. Selbstverständlich muß das Bemühen aller an der Lebensmittelproduktion und -untersuchung Beteiligten dahin gehen, die Gehalte an diesen bedenklichen Stoffen so niedrig wie möglich zu halten. Anderseits dürften die bisherigen Herstellungsgewohnheiten und die gesetzlich geregelten Anwendungsformen von Nitrit und Rauch nach unserem derzeitigen Wissen keinen Anlaß zu Befürchtungen geben, daß die Gefahren größer wären als das allgemeine Risiko des täglichen Lebens.

Zusammenfassung

Verfasser bespricht vor allem die Nitrosamine und polyzyklischen Kohlenwasserstoffe, ihr Auftreten in Lebensmitteln und ihre kanzerogene Bedeutung. Er kommt zu dem Schluß, daß die bisherigen Herstellungsgewohnheiten und die gesetzlich geregelten Anwendungsformen von Nitrit und Rauch derzeit keinen Anlaß zu Befürchtungen geben.

Summary

Möhler, K.: Potential Toxic and in particular Carcinogenic Hazards from Food of Animal Origin as a Result of Technological Processing.

Attention is particularly attached to nitrosamines and polycyclic carbohydrates, their occurrence in foods, and possible carcinogenic action. It is concluded that usual processing techniques and usage of nitrite and smoke in permissible concentration actually are no question of public concern.

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Anschrift des Verfassers: Priv.-Doz. Dr. K. Möhler, Analytische Abteilung der Forschungsanstalt für Lebensmittelchemie, D-8050 München-Weihenstephan.

Human health hazards from the use of hormones and antibiotics in the fattening of livestock

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Gefahren für die menschliche Gesundheit durch Anwendung von Hormonen und Antibiotika in der Tiermast[1])

Von O. Prändl

Hormone und Antibiotika sind Stoffe, die nicht notwendigerweise im Futter vorhanden sein müssen. Soweit der Tierkörper Hormone zur Steuerung gewisser Lebensfunktionen benötigt, produziert er sie selbst. Von Antibiotika ist nicht bekannt, daß sie für den Tierkörper erforderlich sind, wenn man von der therapeutischen Anwendung absieht. In der Tierernährung werden heute aber Hormone und Antibiotika als Wirkstoffe bezeichnet und neben Vitamine, Mineralstoffe und dergleichen gestellt, also neben Substanzen, die für den Tierkörper als Nahrungsbestandteile essentiell sind. Es wäre daher schon aus systematischen Gründen erforderlich, die Wirkstoffe in essentielle und nichtessentielle zu unterteilen.

Hormone und Antibiotika bewirken eine Steigerung der Futterverwertung, des Wachstums, des Fleischansatzes und damit der Nutzleistung. Dies gilt jedoch nur unter gewissen Einschränkungen, da die Tierart, der Zeitpunkt und die Dauer der Verabreichung, bei Hormonen auch die Art des Hormons sowie andere Bedingungen von Bedeutung sind. Der Wirkungsmechanismus beider Stoffgruppen ist sehr unterschiedlich, auch wenn die Endergebnisse, nämlich die Masterfolge, eine gewisse Ähnlichkeit aufweisen. In hygienischer Hinsicht sind Hormone und Antibiotika ebenfalls getrennt zu behandeln, da sie in völlig verschiedener Weise die menschliche Gesundheit gefährden können.

Zunächst zu den Hormonen: Hormone bewirken nicht schlechthin einen Masteffekt. Lediglich die Östrogene, also die brunstauslösenden Sexualhormone, können unter bestimmten Bedingungen das Wachstum und die Gewichtszunahme fördern. Ihre Wirkung ist jedoch je nach Tierart und Alter der Tiere verschieden und hängt außerdem stark von der Dosis ab. In geringen Dosen wirken die Östrogene vorwiegend beim Rind anabol, das heißt, sie fördern Assimilationsvorgänge und damit das Wachstum und den Fleischansatz. Eine sinnfällige Beeinflussung des Geschlechtsverhaltens der Tiere tritt dabei nicht auf, es wird also gleichsam lediglich eine Nebenwirkung der Östrogene zur Hauptwirkung. In hohen Dosen wirken die Östrogene bei männlichen Tieren wachstumshemmend und fettbildend. Dabei kommt es zu einer hormonalen Kastration, indem die Funktionen der männlichen Gonaden ausgeschaltet werden. Beim Rind ist unter gewissen Bedingungen mit Dosen von 10 mg pro Tier und Tag ein Masteffekt zu erreichen. Voraussetzung dabei ist, daß es sich um Jungtiere, also um wachsende Tiere handelt. Die besten Masterfolge bei Östrogenanwendung wurden in der Jungochsenmast erzielt, während in der Jungbullenmast die Leistungssteigerung bescheidener ist. Auch bei Kälbern wird durch Östrogene die Mastleistung gesteigert. (3, 4, 5, 7, 8, 10, 11, 14, 18, 21, 23, 25).

Bei den in der Rindermast üblichen Östrogenmengen von 10 bis 20 mg pro Tier und Tag sind östrogen-wirksame Rückstände im allgemeinen dann nicht zu erwarten, wenn die Östrogenzufuhr 48 Stunden vor der Schlachtung eingestellt wird (3). Es können aber unterschwellige Mengen von Östrogenen oder solche vorliegen, die durch chemische Umsetzung ihrer östrogenen Wirkung beraubt sind. Die Östrogene werden im Körper unter Glucuronidbildung inaktiviert (19). An Hand radioaktiv markierter Östrogene konnten solche inaktivierten Rückstände sogar über einen längeren Zeitraum nach Einstellung der Östrogenzufuhr im Fleisch nachgewiesen werden (16). Über eine gesundheitsschädliche Wirkung solcher inaktivierter Rückstände, die allerdings nur noch in Spuren vorhanden sind, ist bisher nichts bekannt geworden.

Mit den Östrogenrückständen in Fleisch befassen sich zahlreiche Publikationen (3, 4. 6, 10, 11, 12, 14, 15, 16, 17, 18, 19, 20, 24, 26, 27, 29 u. a.).

Beim Geflügel ist die Hormonmast insofern anders geartet, als hierbei hohe Dosen an Östrogenen angewendet werden müssen (20 mg/kg Körpergewicht), wodurch die bereits erwähnte hormonale Kastration herbeigeführt wird, die wiederum eine Steigerung des Fettansatzes auslöst. Daneben soll auch eine echte Verbesserung der Fleischqualität erreicht werden (3, 8).

Allerdings werden beim Geflügel die Östrogene meist erst in der Endmast, und zwar als Depot durch Implantation angewendet. Untersuchungen haben ergeben, daß bei Hähnchen, die in der üblichen Weise 4 Wochen vor der Schlachtung einmalig 20 mg Diäthyloxystilben

[1]) Referat, gehalten bei der 4. Arbeitstagung der Sektion Lebensmittel tierischer Herkunft in der Österreichischen Gesellschaft der Tierärzte am 13. Mai 1970 in Wien.

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EVIDENCE OF FORMATION OF N-METHYL-N-NITROSOUREA IN RATS GIVEN N-METHYLUREA AND SODIUM NITRITE

by

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N[^{14}C]-methylurea and sodium nitrite were administered simultaneously to rats by stomach tube in order to demonstrate methylation of the nucleic acids due to the formation in the stomach of the corresponding nitrosamide, N[^{14}C]-methyl-N-nitrosourea. The ion-exchange chromatograms of the hydrolysates of stomach, liver and small intestine nucleic acids show a radioactive peak corresponding to the altered base 7-methylguanine in the rat receiving N[^{14}C]-methylurea and sodium nitrite. No evidence of 7-methylguanine was obtained when N[^{14}C]-methylurea was administered alone. These findings are interpreted as indicating that the highly potent carcinogen N-methyl-N-nitrosourea is formed in the stomach as a result of the nitrosation of the corresponding secondary amide and absorbed into the circulation of the rat. The possible consequences of the in vivo *formation of carcinogenic N-nitroso compounds in the aetiology of cancer are discussed.*

The N-nitroso compounds are very potent carcinogens, inducing tumours in a wide variety of species and a large number of organs (Magee and Barnes, 1967; Druckrey *et al.*, 1963*a*, 1967). The possibility that this group of carcinogens can be formed in the acid conditions of the mammalian stomach from ingested nitrites and secondary amines has been repeatedly suggested (Druckrey *et al.*, 1963*a*; Magee, 1968*a*; *Lancet*, 1968; Lijinsky and Epstein, 1970).

Secondary amines occur ubiquitously in nature in the protein metabolism of plants, animals and microorganisms (Franzen and Eysell, 1969). Nitrites are widely present in the environment mainly as food additives (Fassett, 1966) and nitrates can be reduced to nitrites by bacteria (Sander, 1967; Sinios and Wodsak, 1965). These observations emphasize the possibility that nitrites may represent a carcinogenic hazard. However Druckrey *et al.* (1963*b*) failed to induce tumours in rats after feeding diethylamine and sodium nitrite over the entire life-span of the animals. These negative results have been attributed to various factors such as the relatively high pH of rat stomach, the pK_b of diethylamine (pK_b = 3.02) and different vehicles for the administration of the two substances (see Mirvish, 1970). All these factors would limit the nitrosation of diethylamine, so that the amount of diethylnitrosamine formed is not high enough to give a detectable incidence of tumours in the number of animals studied.

The detection of small amounts of nitrosamines in intact animals is particularly difficult because of their rapid metabolism. Dimethylnitrosamine in doses of 50 mg/kg body weight is completely metabolized within 8-10 h in rats (Heath, 1962) and the nitrosamide, N-methyl-N-nitrosourea, is broken down at an even higher rate (Swann, 1968). Both compounds form an active alkylating intermediate which alkylates cellular components including nucleic acids and proteins; DNA and RNA are methylated predominantly on guanine to yield 7-methylguanine (Swann and Magee, 1968).

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The formation of small amounts of dimethylnitrosamine in the stomach of rats fed with dimethylamine and sodium nitrite was briefly reported by Magee (1968*a*), but the rapid metabolism of the nitrosamine interfered with attempts to demonstrate its presence in other organs. These difficulties were overcome by treating the rats orally with [^{14}C]-dimethylamine and sodium nitrite and demonstration of methylation of liver nucleic acids following nitrosation of the dimethylamine in the stomach (Magee, 1968, unpublished data).

Various reports have appeared in the literature on the formation of nitrosamines by incubation of several secondary amines with nitrite in the presence of gastric juice. Sander *et al.* (1967, 1968) studied the nitrosation of different secondary amines, including d-*n*-propylamine, diphenylamine, N-methylaniline and N-methylbenzylamine, in solutions of varying pH and in gastric contents, and demonstrated the formation of the corresponding nitrosamines. Sander and Seif (1969) reported bacterial reduction of nitrate to nitrite in human stomach; 31 human subjects were given sodium nitrite and diphenylamine intragastrically and the presence of diphenylnitrosamine was later demonstrated in the stomach contents. Similar findings were obtained by Sen *et al.* (1969) who reported the *in vitro* formation of diethylnitrosamine after incubation of diethylamine and sodium nitrite with gastric juices from rats, rabbits, cats, dogs and man. Human and rabbit juices (pH 1-2) produced more diethylnitrosamine than did rat gastric juice (pH 3-4). The nitrosation reaction was observed *in vivo* in cats and rabbits. Mirvish (1970) studied the kinetics of nitrosation of dimethylamine in buffered aqueous solutions using the tritium-labelled amine; the rate of dimethylnitrosamine formation, which is maximal at pH 3-4, was proportional to the dimethylamine concentration and to the square of the nitrite concentration.

Recently Sander and Burkle (1969) induced carcinomas of oesophagus and liver in rats after simultaneous feeding of N-methylbenzylamine and morpholine respectively and sodium nitrite; the same tumours developed after treatment with the corresponding nitrosamines, nitroso-methylbenzylamine and nitrosomorpholine.

The above observations are related to the detection of small amounts of relatively stable nitrosamines produced by nitrosation of secondary amines in the stomach. However, even greater problems are presented by the equally carcinogenic nitrosamides, because of their chemical instability even at a neutral pH (Druckrey *et al.*, 1967) and their rapid metabolism (Swann, 1968). Furthermore, extraction of these compounds from the tissues by acid protein precipitants prior to their polarographic determination cannot be employed because the process would allow a possible further nitrosation of the secondary amides. Since the nitrosamides are not volatile they cannot be distilled from the tissue homogenates as can the volatile nitrosamines. We therefore decided to avoid these difficulties by administering N[^{14}C]-methylurea and nitrite to rats and investigating the methylation of nucleic acids of various organs.

In the present experiments we report presumptive evidence of formation of N[^{14}C]-methyl-N-nitrosourea in the stomach from nitrosation of N[^{14}C]-methylurea, since the presence of labelled 7-methylguanine was detected in DNA and RNA of several organs. No evidence of 7-methylguanine was observed when N[^{14}C]-methylurea was administered alone.

These findings suggest the possibility that tumours would be induced in animals fed with methylurea and sodium nitrite. This possibility is made highly probable by the very recent report of Sander (1970), who described the induction of tumours of the nervous system and other organs in rats fed with N-N′-dimethylurea and sodium nitrite, and of Ivankovic and Preussmann (1970) who induced neurogenic tumours in offspring of mothers treated intragastrically with ethylurea and sodium nitrite.

MATERIAL AND METHODS

Female Wistar rats (200 g body wt), bred at the Courtauld Institute, were used. N-[^{14}C]-methylurea was obtained from New England Nuclear Corp. (Boston, Mass., USA) with a purity of over 98.5%. The rats were allowed free access to drinking water but they were not given food for 20 h before they were treated. One rat was used for each experiment.

Samples of N-[^{14}C]-methylurea (250 μCi, 8.2 mg/ml), 8 μl and 40 μl respectively, were mixed with 0.7 ml of 1.2% sodium nitrite solution (w/v) just before being administered by stomach tube to the rat. After 5 h the animals were killed by

bleeding from the abdominal
thesia with sodium pentobarbi
and small intestine were rem
liquid nitrogen.

Stomach nucleic acids (DN
extracted by the Schneider
except that the nucleic acids
initially precipitated at pH 7 w
$ZnSO_4.7H_2O$ (10%, w/v) - 1
avoid an acid milieu which
reaction between sodium nitr
urea during the extraction pro
and RNA of liver and small
pared from the same sample
fed Kirby method (Kidson
and Magee, 1968).

The nucleic acids were hydr
at 100° C for 1 h and ch
Dowex-50W (×12, H⁻ form) w
1N-4N HCl gradient, and the
lected in 5 ml fractions, except
(10 ml). Carrier 7-methyl-gua
don, England) was added to a
except for the stomach nucle
intestine RNA of the rat tre
methylurea alone, in order to
active fractions eluted betwee
adenine bases.

INCORPORATION
INTO NUCL

Compounds / Doses
Stomach
Nucleic acids (mixed DNA + RN RNA; total dpm)
% 7-methylguanine
Liver
DNA (dpm/mg)
% 7-methylguanine
RNA (dpm/mg)
% 7-methylguanine
Small intestine
DNA (dpm/mg)
% 7-methylguanine
RNA (dpm/mg)
% 7-methylguanine

bleeding from the abdominal aorta after anaesthesia with sodium pentobarbital. Stomach, liver and small intestine were removed and frozen in liquid nitrogen.

Stomach nucleic acids (DNA and RNA) were extracted by the Schneider (1945) procedure, except that the nucleic acids and proteins were initially precipitated at pH7 with 7 vol H_2O+1 vol $ZnSO_4.7H_2O$ (10%, w/v)+1 vol 0.5N NaOH to avoid an acid milieu which could allow further reaction between sodium nitrite and N-methylurea during the extraction procedure. Both DNA and RNA of liver and small intestine were prepared from the same sample of tissue by a modified Kirby method (Kidson *et al.*, 1963; Swann and Magee, 1968).

The nucleic acids were hydrolysed in 1 N HCl at 100° C for 1 h and chromatographed on Dowex-50W (×12,H^+ form) with an exponential 1N-4N HCl gradient, and the effluent was collected in 5 ml fractions, except for the liver DNA (10 ml). Carrier 7-methyl-guanine (Sigma, London, England) was added to all the hydrolysates, except for the stomach nucleic acids and small intestine RNA of the rat treated with N-[^{14}C]-methylurea alone, in order to identify the radioactive fractions eluted between the guanine and adenine bases.

After measurement of the E_{260}^{1cm}, the fractions were evaporated to dryness and the residues were taken up with 0.5 ml of hyamine hydroxide, (1.0M solution in methanol) (Koch-Light Labs. Colnbrook, Buckingham, England) and 10 ml scintillation solution (0.6% 2,5-diphenyloxazole in toluene). The radioactive measurements were made in a scintillation counter (Model No. 3320 Packard Instr. Co., La Grange, Ill., USA) by conventional methods.

RESULTS

In the rats treated with N [^{14}C]-methylurea alone incorporation of radioactivity into major bases, but not into 7-methylguanine, of nucleic acids and proteins of stomach, liver and small intestine was observed. This suggests that methylurea is probably metabolized by the organism, assuming that no impurities were present which could give rise to nucleic acid precursors. Following simultaneous administration of N[^{14}C]-methylurea and sodium nitrite, the incorporation into the nucleic acids and proteins was much higher than in the N-[^{14}C]-methylurea-treated rats and in addition we observed methylation of the nucleic acids of all the organs examined. Table I shows the incorporation of radioactivity

TABLE I

INCORPORATION OF RADIOACTIVITY FROM N[^{14}C]-METHYLUREA (N[^{14}C]-MU) INTO NUCLEIC ACIDS OF RAT ORGANS WITH OR WITHOUT $NaNO_2$

Compounds / Doses	N[^{14}C]-MU 380μg-11.6μCi	N[^{14}C]-MU + $NaNO_2$ 350μg-10.7μCi+8.4mg	N[^{14}C]-MU + $NaNO_2$ 65.6μg-2μCi+8.4mg
Stomach			
Nucleic acids (mixed DNA+RNA; RNA; total dpm)	3056	7100	1150
% 7-methylguanine	0	0.0015	0.0003
Liver			
DNA (dpm/mg)	9	53	
% 7-methylguanine	0	0.0013	
RNA (dpm/mg)	20	322	
% 7-methylguanine	0	0.0016	
Small intestine			
DNA (dpm/mg)	50	189	
% 7-methylguanine	0	0.0008	
RNA (dpm/mg)	67	123	
% 7-methylguanine	0	0.0016	

from N-[^{14}C]-methylurea into the nucleic acids of the various organs; the degree of methylation of the nucleic acids, which is expressed as the percentage of the guanine converted to 7-methylguanine, was calculated as previously described (Swann and Magee, 1968). Liver and small intestine nucleic acids (DNA and RNA) were not extracted in the group receiving the lowest dose of N-[^{14}C]-methylurea and sodium nitrite.

Figures 1 and 2 show the ion-exchange chromatograms of the hydrolysates of the nucleic acids of stomach and small intestine DNA. A peak corresponding to the altered base 7-methylguanine is present in the rats receiving N-[^{14}C]-methylurea and sodium nitrite. No evidence of 7-methylguanine was obtained when N-[^{14}C]-methylurea was administred without nitrite. Alkylation of nucleic acids of stomach as well as of other organs following a single oral administration of N[^{14}C]-methyl-N-nitrosourea to rats has been reported (Swann and Magee, 1968). The relationship between carcinogenic effect and alkylation of nucleic acids by N-methyl-N-nitrosourea, dimethylnitrosamine and other alkylating agents has been discussed by Swann and Magee (1968). The present results are interpreted as indicating that the highly potent carcinogen, N-methyl-N-nitrosourea, is formed in the stomach as a result of the nitrosation of the corresponding secondary amide and adsorbed into the circulation of the rat.

DISCUSSION

The alkylation of nucleic acids as an indication of the formation of N-nitroso compounds due to the nitrosation of secondary amines or amides appears a very useful tool for qualitative as well as quantitative evaluations, and furthermore it gives a dynamic view of a developing phenomenon. The results shown in Table 1 indicate that the

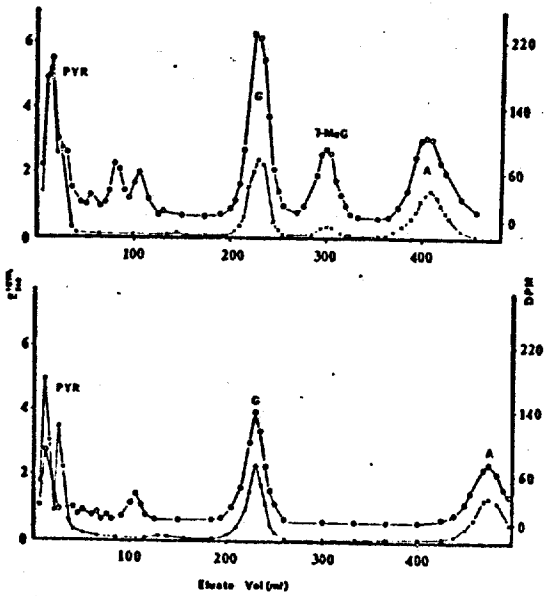

FIGURE 1

Ion-exchange chromatography of stomach nucleic acids of rats treated with N-[^{14}C]-methylurea + $NaNO_2$ (*top*) or with N-[^{14}C]-methylurea alone (*bottom*). Nucleic acids (DNA and RNA) were hydrolysed with 1N HCl for 1 h at 100°C and the hydrolysate was applied to a column of Dowex-50W (H^+ form) and eluted with a gradient of 1-4N HCl. The positions of bases eluted are indicated: PYR, pyrimidine nucleotides or nucleosides: G, guanine; A, adenine; and the position of the added marker base: 7-MeG, 7-methylguanine.

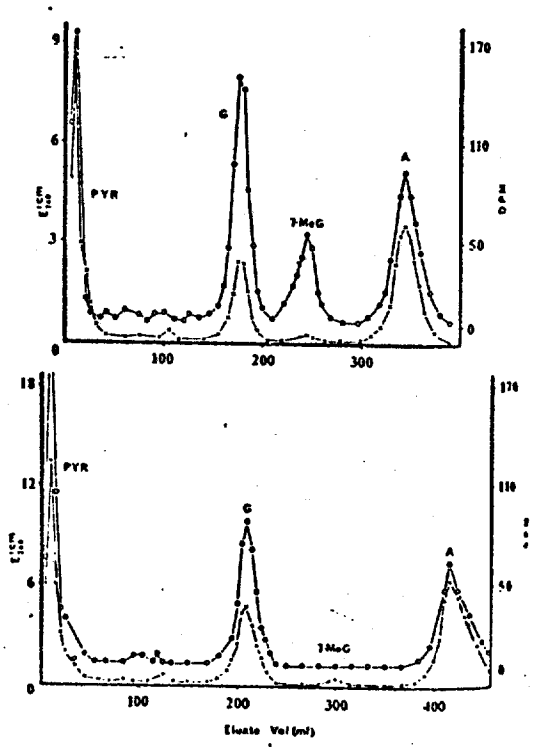

FIGURE 2

Ion-exchange chromatography of small intestine DNA of rats treated with N-[^{14}C]-methylurea + $NaNO_2$ (*top*) or with N-[^{14}C]-methylurea alone (*bottom*). DNA was hydrolysed and analysed as described in Figure 1.

percentages of 7-methylguanine formed in the stomach nucleic acids are directly proportional to the doses of N-[^{14}C]-methylurea administered. By comparing these values with the percentage of methylated guanine observed after N-[^{14}C]-methyl-N-nitrosourea treatment under similar experimental conditions we can determine the rate of nitrosation of the secondary amide. Further studies are under way to substantiate this quantitative relationship.

As mentioned before, secondary amines are widely distributed in nature; Asatoor and Kerr (1961) and Asatoor and Simenhoff (1965) reported the presence of various aliphatic and heterocyclic amines in human blood and urine and they found that the mean basal excretion of urinary dimethylamine in four human subjects amounted to 15.3 mg in 24 h. In many countries (Statutory Instrument 1962, No. 1532, HMSO, London; US Code of Federal Regulations, Title 21, Chapter 1, Sec. 121.1064; Canadian Food and Drug Act and Regulations, Table XI) the permissible concentrations of sodium nitrate and nitrite in a limited number of meat and fish products are 500 and 200 ppm respectively. However, much higher concentrations can be present in vegetables (Fassett, 1966). The average human daily intake of nitrite has been estimated by Sander (1967) as 1.5 mg $NaNO_2$; the daily intake of nitrates could be much higher than this. These data indicate that the formation of carcinogenic N-nitroso compounds in human stomach as a reaction between secondary amines and nitrite is a realistic possibility.

In this context the studies of Sato *et al.* (1959, 1961) appear particularly interesting. They investigated the relationship between gastric cancer mortality and the habit of taking salted food in various populations of Japan and Europe and suggested that salted food might be an important factor in the causation of gastric cancer.

N-methyl-N-nitrosourea induces tumours of stomach, kidney, small intestine and other organs after single or multiple oral or intravenous administrations (Druckrey *et al.*, 1961; Druckrey *et al.*, 1964; Druckrey *et al.*, 1965; Schreiber and Jänisch, 1967; Thomas *et al.*, 1967; Jänisch and Schreiber, 1967; Leaver *et al.*, 1969). Oral administration of the same compound caused the development of stomach and brain tumours in rabbits and adenocarcinomas of stomach and pancreas in guinea-pigs (Jänisch and Schreiber, 1967; Druckrey *et al.*, 1968). A single dose of dimethylnitrosamine induced kidney tumours in rats (Magee and Barnes, 1967) and liver tumours are reported in rats fed with 5 as well as 2 ppm of dimethylnitrosamine (Terracini *et al.*, 1967). A wide range of species including monkeys (Kelly *et al.*, 1966; O'Gara *et al.*, 1970) is known to be susceptible to the carcinogenic action of nitrosamines, and recent studies (Montesano and Magee, 1970) showing that fresh human liver slices can metabolize dimethylnitrosamine at a rate comparable to that of rat liver slices, suggest that man is sensitive to the carcinogenic action of dimethylnitrosamine and probably other nitrosamines. The possible role played by nitrosamines in the aetiology of human cancer is emphasized by reports of their presence in food for human consumption (Ender and Ceh, 1967; 1968; Sen *et al.*, 1969, 1970; Marquardt and Hedler, 1966; Hedler *et al.*, 1968; Du Plessis *et al.*, 1969; Keybets *et al.*, 1970) and their possible connection with some types of human cancer (McGlashan *et al.*, 1968; McGlashan, 1969).

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MISE EN ÉVIDENCE DE LA FORMATION DE N-MÉTHYL-N-NITROSOURÉE CHEZ LES RATS AYANT REÇU DE LA N-MÉTHYLURÉE ET DU NITRITE DE SODIUM

De la N[^{14}C]-méthylurée et du nitrite de sodium ont été administrés simultanément à des rats à l'aide d'une sonde stomacale en vue d'observer la méthylation des acides nucléiques due à la formation dans l'estomac du nitrosamide correspondant: N[^{14}C]-méthyl-N-nitrosourée. Les chromatogrammes d'échanges ioniques des hydrolysats des acides nucléiques de l'estomac, du foie et de l'intestin grêle révèlent un maximum radioactif qui correspond à la base 7-méthylguanine altérée chez le rat ayant

reçu de la N[^{14}C]-méthylurée et du nitrite de sodium. Aucune preuve de la présence de 7-méthylguanine n'a pu être obtenue lorsque les rats n'ont reçu que de la N[^{14}C]-méthylurée. Ces constatations semblent indiquer que le très puissant carcinogène N-méthyl-N-nitrosourée se forme dans l'estomac du rat en raison d'une nitrosation de l'amide secondaire correspondant et passe dans la circulation. Les auteurs étudient les conséquences possibles de la formation in vivo *de composés carcinogènes N-nitroso pour l'étiologie du cancer.*

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1958

EFFECT OF *PARA*-AMINO SALICYLIC ACID ON GROWTH OF MALE HOLSTEIN CALVES [1,2]

It has been suggested that *para*-amino salicylic acid (PASA) would stimulate the growth of calves when included in the diet *(2)*. A feeding trial has been conducted to test further this possibility.

Twelve male Holstein calves were divided into two groups at random. All calves had received colostrum for at least one day before being placed on trial. They were fed limited amounts of whole milk until ten days of age, along with milk replacement which was started at six days of age and continued through 42 days of age. Each calf received a total of 60 lb. of milk and 39.4 lb. of dry milk replacement. Calf starter was fed ad libitum until a maximum of 4 lb. was being consumed by each calf daily. No antibiotic was included either in the milk replacement or in the starter. The roughage used was second-cutting mixed alfalfa grass hay and was fed ad libitum. The diet fed the experimental group (Group I) was similar to that fed the control group (Group II), differing only in that it contained PASA at the rate of 90 mg/lb of milk replacement and 50 mg/lb of starter. The experimental animals consumed PASA at a rate of approximately 90 mg/100 lb. of body weight per day. Management and environment were the same for both groups.

Results of the trial (Table 1) show that Group II gained more in body weight, height at withers, and chest circumference than did Group I. The differences in favor of Group II were statistically significant at the 1% level of probability for body weight and at the 5% level for height at withers and chest circumference. Group II also required fewer pounds of TDN per pound of gain than did Group I, indicating a decrease in efficiency of utilization of TDN in the experimental group. These results suggest that PASA, when fed at the rate of approximately 90 mg/100 lb. body weight per day and without antibiotics, was not an effective growth stimulant for Holstein calves.

TABLE 1

Growth performances of calves fed para-*amino salicylic acid as compared to controls* [a]

	Group I (Fed PASA)			Group II (Controls)		
	Initial	6 wk.	12 wk.	Initial	6 wk.	12 wk.
Body weight *(lb.)*	101	136	191	104	144	214
Height at withers *(cm.)*	75.0	80.2	84.9	75.2	80.7	86.7
Chest circumference *(in.)*	30.8	33.9	37.6	31.3	34.7	39.0
TDN/lb gain			2.00			1.84

[a] Data represent means of six calves per group.

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[2] The *para*-amino salicylic acid used in this work was supplied by Chemo Puro Manufacturing Corp., Newark, N. J.

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1467

TOXICITY OF NITRATES AND NITRITES TO DAIRY CATTLE

During the past decade, the use of nitrogen fertilizers in the production of forage crops has become a wide-spread practice in many areas of the tropics. This practice has led to tremendous increases in yields of total digestible nutrients per acre. In tropical areas with 70-80 in. rainfall annually, the yields of dry forage per acre can be increased from 4-5 tons per year to 25-30 tons per year. This increase in over-all yield is accompanied by a threefold increase

the per cent crude protein, resulting in an 18- to 20-fold increase in total crude protein per acre. These yields are phenomenal when compared to forage yields obtained in the temperate zones.

The utilization of these high protein grasses has presented problems. These grasses have a relatively high nonprotein nitrogen fraction, including nitrates, which leads to undesirable effects unless certain specified feeding practices are adopted *(2, 5)*.

Recently, very severe losses from toxic agents of unknown origin occurred at two dairy farms in Puerto Rico. One dairyman lost 23 milk cows from his herd of 60; another lost 28 heifers. In both cases the losses occurred within 48 hr. after the first symptoms. These losses were not fully explained and, as both dairies were utilizing high-nitrogen forage, the possibility that nitrates were the causative agent was investigated. This investigation included four feeding trials. This report describes these trials and presents the results.

The grass, *Pennisetum purpureum,* used in these trials was produced on ¼-, ½-, and ¾-acre field plots. A high nitrate content was obtained by the application of 400 lb. per acre of a 10-10-10 fertilizer one week after cutting, followed 4 wk. later by an application of 800, 1,600, or 2,000 lb. of ammonium sulfate per acre. The grass was harvested and fed when 6 to 8 wk. old, depending on the nitrate concentration desired. Each individual ration was prepared daily by weighing both the fresh, finely chopped grass and the concentrate. All animals received the principal nutrients in excess of their requirements *(3)*. Feces and urine samples were collected manually. Nitrate concentrations in all samples were determined by the method of Swann and Adams *(4)*, and the methemoglobin levels by the procedure of Evelyn and Mallory *(1)*.

Trial 1. Two pregnant, nonlactating dairy cows, weighing about 950 lb. each, were placed in feeding pens and maintained on a chopped-grass ration that was free of nitrates. No supplements were given. An excess of fresh grass was available in the feed boxes at all times. After 2 wk., the nitrate-free grass was gradually replaced over a period of 1 wk. by a grass that contained 0.3 to 0.4% potassium nitrate (KNO_3) on a fresh weight basis (1.3 to 1.8% dry weight basis). The high-nitrate ration was then fed continuously for 60 days. The daily consumption of one cow varied from 115 to 175 lb., and averaged 135 lb.; that of the other from 125 to 210 lb., averaging 155 lb/day.

Weekly determinations of methemoglobin showed that the levels varied between 2 and 7%, and averaged 5%. Periodic inspections by a veterinarian revealed no symptoms of toxicity. One animal refused to eat on the 34th day but was normal again the next day. Thirty days after the feeding trial terminated one cow gave birth to a normal calf. The other cow gave birth to a normal calf 45 days after the trial ended.

Trial 2. A dairy herd of eight lactating and four nonlactating cows was switched from a nitrate-free pasture roughage to a high-nitrate, chopped roughage (1.8% potassium nitrate on a dry weight basis). The chopped roughage was fed from 12, 3 by 2.5 by 1.5 foot feeding boxes located in a large feeding pen. An excess of chopped grass was maintained in the feeding boxes at all times. Concentrate was fed at milking time in recommended quantities *(3)*. Feeding of the high-nitrate roughage continued 4 wk. The average consumption of roughage was 87 lb/cow/day over the 4-wk. period (336 cow-days).

Blood samples taken at 8 A.M. on the 22nd day of the trial contained normal amounts of methemoglobin, i.e., 3 to 5%. A veterinarian found the herd normal at the end of the trial. Milk production records revealed a slight decline not associated with the high nitrate content of the roughage.

Trial 3. Two yearling bulls, weighing approximately 400 lb. each, were placed on a balanced ration made up of a low-nitrogen roughage and a high-protein concentrate. After 1 wk., the animals were treated for parasites, and the low-nitrogen roughage was replaced with equal amounts of chopped grass which contained from 0.3 to 0.4% potassium nitrate (1.3 to 1.7% dry weight). This ration was fed for 2 wk. and the methemoglobin remained at normal levels. At the end of this 2-wk. period, the high-nitrate forage was replaced with a forage free of nitrates. Chemically pure potassium nitrate was then added to the forage until it contained 1.7% on a dry weight basis. This required 120 g. of potassium nitrate for the daily ration of each animal. After 2 wk., both animals appeared normal and continued to gain weight. The concentration of nitrates and nitrites in the feces and urine showed that less than 4% of the dietary nitrates was being excreted in the form of nitrates and nitrites. The concentration of potassium nitrate in the roughage was then raised from 1.7 to 3.0%. This required 210 g. of potassium nitrate for the daily ration of each animal. The animals were then maintained on this ration for 30 days, and together they consumed almost 30 lb. of potassium nitrate.

During this period, only 1% of the dietary nitrates was recovered unchanged in the feces, and approximately 3% passed unchanged in the urine. Methemoglobin levels ranged from 5 to 8%. At the end of the trial, the animals were examined by a veterinarian and appeared normal.

Trial 4. The two animals in Trial 3 were switched directly to Trial 4. The potassium nitrate (210 g.) in each daily ration was replaced by an equimolecular quantity of potassium nitrite (177 g.). After 1 wk., the methemoglobin levels were 3 and 7%. The quantity of potassium nitrite in each daily ration was raised to 200 g. Two weeks later the animals appeared normal to the examining veterinarian and the

trial was terminated. Methemoglobin levels were 2 and 3% at the end of the trial.

These trials led the authors to the conclusion that the severe losses mentioned previously were not caused by the nitrates in the forage being fed. This conclusion is based on these facts: *(1)* The losses occurred in herds that were obtaining 75% of their requirements from a forage that contained no more than 1.0% potassium nitrate on a dry weight basis. *(2)* Test animals showed no symptoms of nitrate poisoning and continued to gain weight while being fed rations that contained 3.0% potassium nitrate.

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OUR IN

NEW DEVELOPM
LACTIC CULT
BACTERI

Dairy Department,

Lactic cultures which do not produ uniformly every day present an importa lem to the dairy industry. Studies causes of slow acid production, or failure of a culture to produce acid, that bacteriophage often is responsible bacteriophage has been recognized as portant cause of culture failure, many mendations have been made to limit its into cultures and to decrease its activity. selection, sanitation programs, culture and propagation of mother cultures and ration of intermediate and bulk cultu relatively bacteriophage-free area, ha helpful in decreasing culture failures. H these methods have not entirely elimin bacteriophage problem.

A medium which would support good of lactic cultures, but which would not bacteriophage development would be of the dairy industry, provided that the such a medium would not constitute a tion. The addition to a medium such as some nontoxic substance that would bacteriophage development but permi growth of the culture organisms, wou have possibilities. In 1949, Shew *(5)* 1956, Reiter *(3)* reported that bacter active against the lactic streptococci req ium for maximum development. Pot Nelson *(2)* and Doull and Meanwell veloped media which were deficient in and which supported growth of lactic These media were used for fundamental on bacteriophage. Reiter *(4)* describe dium, based on milk, in which bacter did not multiply because of a lack of but in which certain lactic cultures gr ally. The medium was designate phage-resistant medium).

EXPERIMENTS WITH PRM

A supply of PRM was made avail experimental purposes by Fisons Milk Ltd., Loughborough, Leicestershire, The material was received in dry form

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[2] Presented at Research Conference Beltsville, Maryland, October 30, 1957. Eastern Utilization Research and Develop sion, USDA, Philadelphia, Pa.

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1494

EFFECT OF NA NITRITE IN LONG-TERM EXPERIMENTS. I.

(Účinky dusitanu sodného v dlouhodobém pokusu. - I)

Author: J. Musil

Source: Československá Hygiena - X - 10 - 1965, P. 589 - 597

In the worldwide and Czechoslovakian literature, there is an inportant disproportion between the studies devoted to the effect of Na nitrite on the blood pigment, and the investigations of the physiological and toxicological effects of this compound. The research workers of the Czechoslovakian Hygiene and Epidemiology Service have received an increasing number of requests to provide an answer to the problem of the reported toxicity of nitrates and of nitrites in potable waters. This complex of problems have gained importance because of the constant and relatively frequent occurrence of nitrate methemoglobinemias of the acute or latent type in babies. The problem to be solved is whether the long-term increase of methemoglobin (derivate with trivalent Fe) beyond the physical limit, which is observed in the regions with a higher occurence of nitrates in the drinking waters, must result in an important damage of the human organism. On these questions, the literature is scanty and fails to give a definite answer to the problem presented. The working group studying this problem at the Prague Hygiene Institute attracted the attention repeatedly to this fact in all its previous reports. The study conducted by Druckrey (Ref. 3) in recent time approaches the problem by means of laboratory tests conducted on rats; but in our opinion he uses relatively low rates for the animals. Riemann (Ref. 12) showed the effect of Na nitrite on the hypertrophy of the spleen and on the decrease of the thyroid gland in the mouse. Roberts and Sell (Ref. 13) investigated the destruction of vitamin A after incubation in the stomach content in vitro, and in vivo a decreased A vitamin content in the stomach content after a diet that contained Na nitrite. Other studies, discussed in previous reports, showed no efforts to evaluate the general toxicological values of nitrites and of their metabolical products.

This induced us to conduct an overall experiment in which the basic problems would be subjected to solution.

In this report, we present the first part of the results from tests in which we conducted a collective examination of the action of nitrites administered to laboratory rats. These laboratory tests must be the theoretical complement of an experimental work performed in order to resolves problems of prevention of babies's methemoglobinemias due to nitrates in food (Ref. 5, 6, 9, 14, 15).

Material and Methodology:

The tests were conducted on rats of both sexes, delivered to the collection of the Hygiene Institute. The animals were kept in breeding cages made of polystyrene with a metal lid. The Larsen diet, prepared each day, was used. Drinking was administered in glass containers with polystyrene suction tubes. The following indices were investigated: a. Food consumption, b. Liquid consumption c. Weight curves d. Hemoglobin level.

Table 1: a. Days of exposure b. Control group c. Males d. Females

In order to determine the values contained in Table 1, the animals were sacrificed at two time intervals: within 150 days after weaning, and after 200 days from weaning. The sacrifice was performed by cutting the carotid and by exsanguination. The extracted blood was used in determining the methemoglobin (d) by the Cruze method (Ref. 2), and was hematologically examined. The organs of the animals were extracted, weighed, fixed in formaldehyde, and prepared for histological evaluation.

The weight values of the organs were assessed for 100 g of the live weight of the animal tested. The weights of the myocardia were determined after cutting off the heart along the septum and by separately weighing the right and left alveoli of the septum.

Numbers of test animals in the groups:

The test involved 3 groups of experimental rats: the control group which was given drinking water* (* The water in the period mentionde exhibited a maximum concentration of NO_3^- of 10.5 mg/l liter, and of NO_2^- of 0.05 - 0.07 mg/l liter), the group which drank a solution of 10 mEquiv/l NO_2, and the group which drank a solution of 20 mEquiv/l NO_2 in concentration.

a The food consumption values obtained by weighing the remainders left by the tested animals in the cages show that these animals do not exhibit during the investigation period importance differences between the individual groups tested (Diagram 1). The average food consumptions were evaluated by the significance test of Student, and the values t for the collection of animals included in the test are much below the limit of significance. The mean values of the food remainders for the males did not involve a high dispersion. A statistically immaterial difference at the value of t = 0.517 existed between the group of control-females and the test group which drank the solution of 20 m Eqv $NaNO_2$.

b The curves concerning the consumption of the solutions administered to the test animals in the breeding cages exhibit in all the three groups a decreasing tendency which reflects the dependence of the ~~conversion~~ of the fluid consumed ~~into~~ on the increasing general body substances of the animals. In the first part of the test, we ascertained the highest consuption of the administered solutions in the groups . They amounted to 10 and 20 m Eqv NaO_2. In the females we did not detect a pronounced difference between the groups drinking the $NaNO_2$ solutions and the control group. For the groups, which received 10 and 20 mEqv $NaNO_2$ at the end of the test period, the consumption decreased below the mean value of the control groups. This fact was found to be true with the male and female rats. The differences in each group were not demonstrably important from the statistical standpoint. Such differences between the experimental male and female rates in the consumption values of the solutions administered were not significant (Diagram 2).

c The growth curves, based on standard weighing results of all the animals subjected to experimentation, exhibit a differend trend in the males and in the females (Diagram 3a and b). In the males at the start of the test, the controls were significantly heavier than the groups of animals which drank 10 and 20 m Eqv $NaNO_2$. After about 150 days of exposure, the weights leveled up, and at the end of the test, before the last sampling, the highest mean weight (404.0 g) was reached by the group which drank 10 mEqv $NaNO_2$, in contrast with the control group which exhibited the lowest average weidht of 359.0 g. The female rats, which drank the test solution during 200 days, had essentially the same initial weight. In the last phase of the test, there was an insignificant decrease in weight in the group which drank nitrite solutions. The highest mean weight was found in the control-female rats, that is 275.2 g. The lowest mean weight was found in the group which drank 10 mEqv $NaNO_2$ - 236.0. For the sake of completeness in the evaluation of the weight curve, it is necessary to obtain the weighing results of animals used during 320 days of exposure to $NaNO_4$ in testing the resistence of the myocardium to anoxia. In the male group there was no significant difference between the control group (392.0 g) and the test group (420.8 g), which drank 20 mEqv; on the other hand in the females an important difference was detected in the 2 groups which drank the nitrite solution, as compared with the control group; after 20 days the weight averages of the exposed groups were lower. Table 2 provides a survey of the average weight.

Table 2: Average weights of rats after a long-term exposure to $NaNO_2$

d The methemoglobin levels were expresed in per cent of the derivative with trivalent Fe from the total amount of blood pigment. The values determined show that after prolonged exposure of the test animals, the methemoglobin levels increase as compared with the control group.

In all the groups, which drank the $NaNO_2$ solution, higher methemoglobin levels were found. In the collections of animals with higher levels, in some samplings there were wide ranges of difference between the individual results (higher values.... in the Table). These results must be assessed as a possible effect

exerted on the methemoglobin level by the interval from the last drinking of the animals tested. The total results are reported in Table 3.

Table 3: Methemoglobin levels after prolonged exposure to $NaNO_2$

e The relative weight values of the organs were obtained by checking for 100 g of live weight before the animals were sacrificed. These values were determined immediately after exsanguination. They exhibit a first orientation evaluation of the reaction of the individual organs. The results reported in this article will be supplemented by an overall histological examination (Ref. 10).

Liver:

In evaluating the relative weights of the livers, the difference obtained for the average of the individual groups reached statistical significance only in females which drank 10 and 20 mEqv $NaNO_2$ during 200 days. In the males the difference in weight never exceed the limit values of the test used. The highest values of the average difference in the males were reached when 20 mEqv were administered during 200 days, but in this case the calculated value t = 2.00 is below the limit of significance. The results of weight evaluation for the livers of the experimental rats are presented in Table 4.

Table 4: Relative weight of the livers in rats during long-term exposure to $NaNO_2$.

Spleen:

The reaction of the spleen expressed in the indices of the relative weight of the organs of the test animals possesses again a different character in the males and in the females used in experimentation. Whereas in the males, during the entire test period, not even at higher doses of nitrites, no important difference existed in the averages of the individual group, the results obtained in the females tested exhibit an important difference of evaluation averages by the t-test after 150 days of exposure when 20 mEqv $NaNo_2$ were administered. An equal result was obtained in a 20 days exposure with the same dosage. The dose of 10 mEv $NaNO_2$

administered during 150 and 200 days results in an inportant and statistically significant increase of the spleen weight. The results are presented in Table 5.

Kidneys:

The kidneys of the animals during the weight evaluation of the individual groups differed considerably only before exposure for 200 days. The average difference, important in the females that drank 20 mEqv $NaNO_2$ during 150 days, was not statistically significant, when the calculated value t = 0.788 lay much below the limit value for the given group. Conspicuous changes were found in the females which drank 10 mEqv $NaNO_2$ during 200 days (statistically insignificant average difference), whereas in the group that drank 20 nEqv during 200 days, there was a statistically significant increase of the weight of the kidneys as compared with the controls, probably at the 0.01% level. In the males, which drank during 200 days 10 mEqv $NaNO_2$, no important weight difference was reported between the controls and the test group. In the group which drank 20 mEqv $NaNO_2$, there was an important difference at the 0.05% level. The results are presented in Table 6.

Table 6: Relative weight of the kidneys in rats during long-term exposure to $NaNO_2$

Myocardia:

The myocardia were weighed after cutting off the heart muscles into individual sections: left chamber, right chamber and septum. Separate weighing revealed that in all the myocardia of the animals exposed to $NaNO_2$ there was a uniform weight increase affecting uniformly all the heart sections.

Table 7: Relative weight of mycordia in rats during long-term exposure to $NaNO_2$.

The weight evaluation reported in Table 7 comprises only the data obtained in calculating the total weighing of all the heart sections for 100 g of live weight of tested animals. One calculated also the deviations of the myocardia in the tested animals from the standard developed for the weight assessment of the heart muscle for the Czechoslovakian breeds Poupa, Rakušan and Korecký (Ref. 11). It was found that the average deviations of the females tested after the administration of 20 mEqv $NaNO_2$ during 200 days amount to about

122 % of the calculated deviation from the standard for an animal of the same weight. The tested females, which received the same dose for the same period, exhibited a 129% deviation from the standard, although this value is deducted from the calculation of the standard values for the tested females, and the higher deviation thus is inaccurate from the standpoint of standard values. With respect to the fact that the standard is (? "stnoven") only for the tested females, in the final evaluation of the tested animals we did not use the deviations from the standard, but only the verification for 100 g of live weight of the tested animal.

The statistically important difference in the averages of the groups tested as compared with the control was found in the group which received 20 mEqv $NaNO_2$ during 150 and 200 days only in the males. Although the mean values of the tested females were nearly within the same range as the male averages, there was no statistical significance with respect to the wide dispersion of the values. The results are reported in Table 7.

Survey of the entire weight evaluation:

From the results mentioned it follows that in the test setup described in the article one find a different activity of the males and of the females subjected to experimentation.

As a conclusion of the weight evaluation, we offer a Table of the statistically important deviations of the average of the relative weight of the individual test groups with respect to the controls. The results of the statistical evaluation considered by the t-test are shown in Table 8.

Table 8: Survey of the va ues of the indices during long-term exposure of the rats to $NaNO_2$.

Discussion:

All the results reported are parts of an overall investigation of the reaction of laboratory rats to a long term administration of a Na nitrite solution.

This article contains the fundamental toxicological and physiological data necessary for a basic characterization of the compound examined. The conclusion offered by this evaluation will be supplemented in further articles by histological and hematological examinations.

On the basis of the information reported, it appears that the entire specific effect of Na nitrite is far higher with respect to the females than to the males. With the exception of the weight reaction of the myocardium, which was more pronounced in the males, all the other indices involve with greater probability a damage in the females.

If we analyze the individual indices from the standpoint of the distribution of the values, we find that in the males there is a higher percentage of animals that are insensitive to the toxic effect of the Na nitrite administered. A specific index of the effect of Na nitrite, as a methemoglobin-forming compound, is the direct determination of the level of the blood methemoglobin, a blood derivative with trivalent Fe, unable to transport O to the tissues. The fact that in the males it was possible to find animals with methemoglobin levels that did not differ from the values found in the controls or from the physiological values usually established in our tests and existing in the literature, can be explained in two ways: What may be involved is the influence of the interval elapsed from the last drinking of the animals, when the high methemoglobin leves was again reduced to the physiological limit, or the manifestation of a different reactivity of the redox system of the animal tested.

The investigation of the relative weight of the organs is a fundamental toxicological index providing information about the systems possible affected by the compound examined. The absence of a significant weight difference in the liver weights reported in the males cannot bear witness to the impossibility of an intervention in the hepatic functions. This view is definitely supported by the increase of the liver weight in the females; hence the analysis of the hepatic function must be regarded as a further step to the solution of the pathophysiological mechanism occurring during methemoglobinemias. In connection with the hypoxic states produced by the decrease of the overall blood pigment able to transfer O to the tissues, it was necessary to direct the attention also to the determination of

the spleen weight in the animals tested. In the males, under anoxic and hypoxic conditions, the spleen works as an important volume regulator of the entire circulating amount of blood. An increase in spleen weight in the females, as was found in the test, demonstrates that the involvement of the tissue of the spleen is not connected only with the mechanical function of the spleen, but has to do also with the function related to the blood formation dynamics in the experimental animal during its growth and puberty. The same result was ascertained in the relative weights of the kidneys. In the two test groups of males and females one found a statistically important increase of the kidney weight even after the last sampling after 200 days of exposure and during a higher administration of 20 mEqv $NaNO_2$. In our own tests we found that acute methemoglobinemia influences the eliminating function of the kidneys. In a chronic test it is possible to demonstrate, for the indices of the relative weight of organs, that a long-term contamination leading to methemoglovinemia can considerably damage the kidney tissue.

A overall discussion of the nonspecific organic reaction will be supplemented in a further article by a histological analysis, which will differentiate the damages of the individual structures.

The Na nitrite concentrations, used with the experimental animals were at the upper limit of concentrations possible under conditions of methemoglobinemia in babies. These concentrations had to be used in the experimental approach partly because we worked with relatively resistant animals and partly because it was necessary to gain an idea about the structure and about the extent of a possible nonspecific effect of the nitrite ion.

Conclusions:

1. For 121 rats of both sexes one conducted a long-term test in which the experimental animals received a concentration of 10 and 20 mEqv $NaNO_2$/liter of water. After 150 and 200 days, the animals were sacrificed by exsanguination, the methemoglobin level and the relative weights of the liver, spleen., kidney and myocardium were determined. During the test, the food and drink consumption and the growth curve were examined.

2. No important difference in the food and drink consumption and in the growth curves were found.

3. The methemoglobin levels in the tested females increased after 150 days of exposure with doses of 10 and 20 mEqv $NaNO_2$, and after 200 days with a dose of 20 mEqV. In the males the average methemoglobin values as compared with the controls were always higher, but not statistically significant.

4. The relative weight of the livers increased in the females which drank 10 to 20 mEqv $NaNO_2$ after 200 days; the relative weight of the spleen in the females increase with a dose of 20 mEqv $NaNO_2$ afrer 150 and 200 days of exposure; the relative weidht of the kidneys increased in the females with a dose of 20 mEqv $NaNO_2$ after 200 days. In the males there was an increase of the relative weight of the kidneys with a dose of 20 mEqv after 200 days and with a dose of 20 mEqv $NaNO_2$ after 150 and 200 days. There was an increase of the relative weight of the myocardium with a dose of 20 mEqv after 150 and 200 days.

5. The results will be used in resolving problems of latent nitrate methemoglobinemias found in regions with a high occurrence of nitrate in potable water.

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NOVINKY SZdN

Anestesiologie

Napsal doc. dr. H. Keszler a spolupracovníci

Ke spolehlivému a ekonomickému využití možností soudobé anesteziologie je nutná dobrá znalost těch úseků hraničních oborů, které se anesteziologie přímo týkají, ať již jsou to obory teoretické či klinické. Touto linií se důsledně řídí monografie; část obecná podává pečlivě vybrané stati z fyziky, chemie, fyziologie a patofyziologie vitálních funkcí a homeostázy vnitřního prostředí, zahrnuje farmakologii anestetik a pomocných látek v anesteziologii. Další kapitoly jsou věnovány vlastní anesteziologické problematice a pojednávají o předoperační přípravě a premedikaci, o celkovém i místním znecitlivění a o rušivých příhodách při všech způsobech anestézie a jejich léčení. Závěr obecné části tvoří kapitola o tlumení pooperačních bolestí. Odborná část řeší volbu anestézie, techniku provedení, předoperační přípravu a pooperační péči v závislosti na třech hlavních aspektech: na věku nemocného, na komplikujících onemocněních a na povaze operačního výkonu. Určeno především anesteziologům jako základní dílo pro přípravu ke specializaci i dalším odborníkům oborů operačních i neoperačních.

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ÚČINKY DUSITANU SODNÉHO V DLOUHODOBÉM POKUSU. — I.

Действие азотистокислого натрия в долговременном опыте. I.

Effect of Na Nitrite in Long-term Experiments. I.

J. Musil

Technická spolupráce V. Zahradniková, J. Zinkeová

Ústav hygieny, Praha

Выводы: В опытах на лабораторных крысах автор исследовал действие долговременного питья раствора азотистокислого натрия в концентрациях 10 и 20 мэкв на 1 литр воды. Значительных отклонений в потреблении пищи, питье и кривых роста доказано не было. Были доказаны статистически достоверные отклонения содержания метгемоглобина в крови подопытных животных у самок. У самок были установлены статистически достоверные отклонения относительного веса печени, селезенки и почек, у самцов отклонения относительного веса почки и миокарда.

Čs. Hyg. 10, 1965, 10: 589—597.

Summary: The author investigated in experiments on rats the effect of long-term drinking of a solution of Na nitrite in concentrations of 10 and 20 mEq./l water. No significant deviations in the food and fluid intake or grewth were found. Deviations were revealed in the methaemoglobin blood levels which were statistically significant in females. In the latter also statistically significant deviations in the weights of liver, spleen and kidneys were found and in the males deviations in the relative weight of the kidneys and the myocardium.

Čs. Hyg., 10, 1965, 10: 589—597.

Ve světovém i československém písemnictví existuje závažný nepoměr mezi pracemi věnovanými účinku dusitanu sodného na krevní barvivo a pracemi věnovanými fyziologickým a toxikologickým účinkům této látky. Pracovníci čs. hygienicko-epidemiologické služby jsou stále častěji nuceni zodpovídat otázky týkající se toxicity dusičnanů a dusitanů v pitných vodách. Tato problematika získala na závažnosti vzhledem ke stále relativně častému výskytu kojeneckých dusičnanových methemoglobinémií akutního i inaparentního typu. Je nutno řešit otázku, zda dlouhodobé zvýšení methemoglobinu (derivátu s trojmocným železem) nad fyziologickou mez, které je pozorováno v oblastech s vyšším výskytem dusičnanů v pitných vodách, může vést k závažnému poškození lidského organismu. Literatura v těchto otázkách je skrovná a nepodává na předloženou otázku jednoznačnou odpověď: na tuto skutečnost opakovaně upozorňovala ve svých předchozích sděleních pracovní skupina řešící tuto otázku na pražském Ústavu hygieny. Druckreyova práce[1] z poslední doby řeší otázku experimentem na laboratorním zvířeti (krysa), používá ovšem podle našich názorů pro krysy relativně nízké dávky. Riemann[12] prokázal vliv dusitanu sodného na hypertrofii sleziny a zmenšení štítné žlázy u myší. V práci Robertse a Sella[13] byla sledována destrukce vitaminu A po inkubaci v žaludečním obsahu in vitro a in vivo snížený obsah vitaminu A v žaludečním obsahu po dietě obsahující dusitan sodný. I další práce, o nichž se diskutovalo v předchozích sděleních, nejsou zaměřeny ke zhodnocení obecných toxikologických vlastností dusitanů a jejich metabolických produktů.

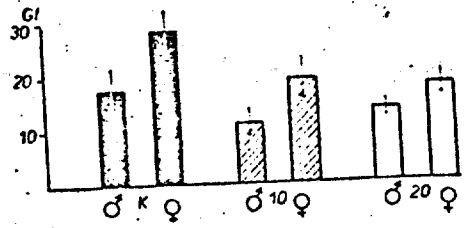
Rozhodli jsme se proto provést souborný pokus, ve kterém by byly řešeny základní otázky.

OF MEDICINE FOR PURPOSES OF STUDY OR RESEARCH IN LIEU OF LENDING THE ORIGINAL.

V tomto sdělení předkládáme první část výsledků z pokusů, ve kterých jsme souborně vyšetřili účinek podávaných dusitanů na laboratorní krysy. Tyto laboratorní pokusy mají být teoretickým doplněním experimentální i terénní práce zaměřené k řešení otázek prevence dusičnanových alimentárních methemoglobinémií kojenců[5 6 7 9 14 15].

Materiál a metodika

Pokusy byly provedeny na krysách obého pohlaví dodaných do zvěřince Ústavu hygieny Biovetcu. Zvířata byla chována v chovných klecích z polystyrénu s kovovým víkem, k výživě byla použita Larsenova dieta připravovaná pro každý den, pití bylo podáváno ve skleněných



Graf 1

nádobách s polystyrénovými savkami. V pokusu byly sledovány tyto ukazatele: a) spotřeba potravy, b) spotřeba pití, c) váhové křivky, d) hladina hemoglobinu.

Zvířata ke stanovení níže uvedených hodnot byla zabíjena ve dvou intervalech, do 150 dnů po odstavu a po 200 dnech od odstavu. Zabití se provádělo nástřihem karotid a exsanguinací,

Tabulka 1

Expozice dnů	Kontrolní skupina		10 mEkv		20 mEkv	
	samci	samice	samci	samice	samci	samice
150	10	10	8	9	10	10
200	10	10	10	9	12	13

odebraná krev byla zpracována ke stanovení methemoglobinu (d) metodou podle Cruze[3], hematologicky vyšetřena[8]. Ze zvířat byly vypitvány orgány, zváženy, fixovány ve formolu a připraveny k histologickému hodnocení[10].

e) Váhové hodnoty orgánů byly přepočteny na 100 g živé váhy pokusného zvířete. Váhy myokardů byly stanoveny po nástřihu srdce podél septa a odděleným zvážením pravé a levé komory a septa.

Počty pokusných zvířat ve skupinách:

V pokusu byly zařazeny tři skupiny pokusných krys: skupina kontrolní, která pila vodovodní vodou*), skupina, která pila roztok o koncentraci 10 mEkv/l NO_2, a skupina, která pila roztok o koncentraci 20 mEkv/l NO_2.

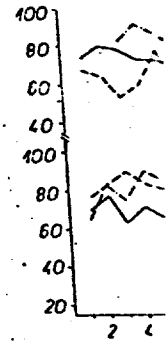
Výsledky

a) Hodnoty spotřeby potravy získané vážením zbytků zanechaných pokusnými zvířaty v klecích ukazují, že pokusná zvířata nevykazují ve sledovaném období významné rozdíly mezi jednotlivými pokusnými skupinami (graf 1). Průměrné spotřeby potravy byly hodnoceny testem významnosti podle Stu-

*) Použitá voda v uvedeném období vykazovala maximální koncentraci NO_3' — 10,5 mg/l litr a NO_2' — 0,05—0,07 mg/l litr.

denta a hodnoty t pro
hranicí významnosti P
významnějším rozptylu
byl mezi skupinou ko
20 mEkv $NaNO_2$.

b) Křivky spotře
klecí vykazují u všech



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tovat u samců i sa

Tab. 2

Skupina	Dny
Samci	
Samice	

ticky významné. T
spotřeby podaných
c) Růstové k
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U samců při zapo
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vyrovnaly a v zá
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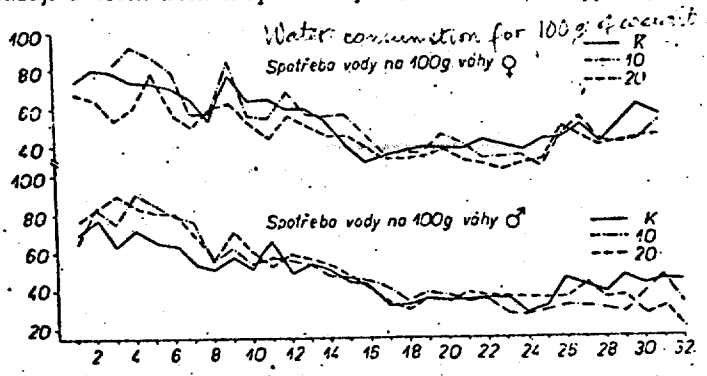
denta a hodnoty t pro soubory zvířat zařazených do pokusu leží hluboko pod hranicí významnosti. Průměrné hodnoty zbytků potravy u samců neležely ve významnějším rozptylu, statisticky nevýznamný rozdíl při hodnotě t = 0,517 byl mezi skupinou kontrolních samic a pokusnou skupinou, která pila roztok 20 mEkv $NaNO_2$.

b) Křivky spotřeby roztoků podaných pokusným zvířatům do chovných klecí vykazují u všech třech skupin klesající tendenci, která vyjadřuje závislost



Graf 2

přepočtu spotřeby tekutiny na stoupající celkové tělesné hmotě zvířat. V prvé části pokusu byla zjištěna vyšší spotřeba podaných roztoků u skupin, které pily 10 a 20 mEkv $NaNO_2$. U samic nebyl zjištěn významný rozdíl mezi skupinami pijícími roztoky $NaNO_2$ a skupinou kontrolní. Skupiny, které dostaly 10 a 20 mEkv $NaNO_2$ v závěru pokusného období snížily spotřebu pod hodnoty průměrných spotřeb skupin kontrolních. Tuto skutečnost bylo možno konstatovat u samců i samic. Rozdíly u žádné skupiny nebyly prokazatelně statis-

Tab. 2. Průměrné váhy krys po dlouhodobé expozici $NaNO_2$

Skupina	Dny expozice	Kontroly	10 mEkv	20 mEkv
Samci	200 320	359,0 392,0	404,0 nestan.	383,1 320,7
Samice	200 320	275,2 320,7	236,0 255,3	258,5 256,8

ticky významné. Také rozdíly mezi pokusnými samci a samicemi v hodnocení spotřeby podaných roztoků nebyly významné (graf 2).

c) Růstové křivky sestrojené z výsledků pravidelných vážení všech zvířat zařazených do pokusu mají odlišný průběh u samců a samic (graf 3a, b). U samců při započetí pokusu byla kontrolní zvířata nepatrně těžší než skupiny zvířat, které pily 10 a 20 mEkv $NaNO_2$, po cca 150 dnech expozice se váhy vyrovnaly a v závěru pokusu před posledním odběrem nejvyšší průměrné váhy (404,0 g) dosáhla skupina, která pila 10 mEkv $NaNO_2$, proti skupině kontrolní, která vykazovala nejmenší průměrnou váhu 359,0 g. Krysí samice, které pily

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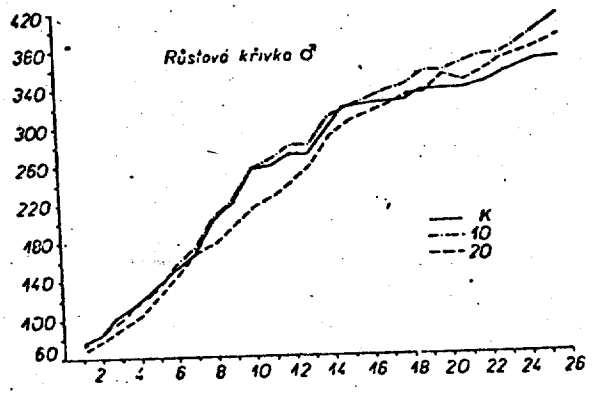
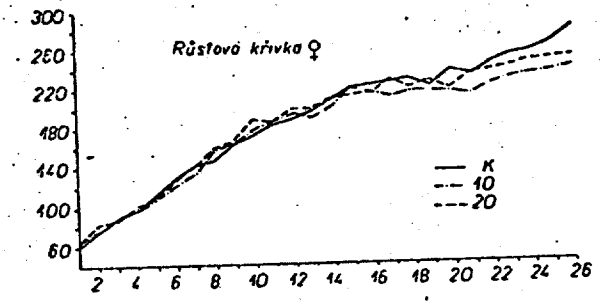
po 200 dnů pokusné roztoky, měly v podstatě totožné výchozí váhy, v poslední fázi pokusu se projevila nevýznamná snížení váhy u skupin, které pily roztoky dusitanů. Nejvyšší průměrnou váhu vykazovala u samic skupina kontrolní — 275,2 g, nejnižší průměrnou váhu skupina, která pila 10 mEkv $NaNO_2$ — 236,0. Pro úplnost hodnocení váhových křivek je nutno zařadit také výsledky



Graf 3a



Graf 3b

vážení pokusných zvířat použitých po 320 dnech expozice $NaNO_2$ k testování rezistence myokardu vůči anoxii. U skupiny samců nebyl významný rozdíl mezi skupinou kontrolní (392,0 g) a pokusnou (420,8 g), která pila 20 mEkv, naproti tomu u samic byl zjištěn významný rozdíl obou skupin, které pily roztok dusitanů, proti skupině kontrolní, přičemž stejně jako po 20 dnech byly váhové průměry exponovaných skupin nižší. Přehled průměrných vah uvádí tabulka 2.

d) Hladiny methemoglobinu byly vyjadřovány v procentu derivátu s trojmocným železem z celkového úhrnného množství krevního barviva. Stanovené hodnoty ukazují, že po dlouhodobé expozici pokusných zvířat se v krvi zvířat zvyšují hladiny methemoglobinu ve srovnání se skupinou kontrolní.

U všech skupin, které pily roztok $NaNO_2$, byly prokázány vyšší hladiny methemoglobinu. V souborech zvířat s vyššími hladinami se při některých odběrech projevovaly široké meze rozptylu mezi jednotlivými výsledky (v tabulce vyšší hodnoty SE). Tyto výsledky musí být posuzovány jako možné ovlivnění

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hladiny methemoglobinu intervalem od posledního napití pokusného zvířete. Souborné výsledky všech stanovení uvádí tabulka 3.

e) Relativní váhové hodnoty orgánů získané přepočtem na 100 g živé váhy zvířete před zabitím, které byly stanoveny ihned po exsanguinaci zvířete, vyjadřují první orientační hodnocení reakce jednotlivých orgánů. Výsledky uvedené v tomto sdělení budou doplněny souhrnným vyšetřením histologickým[10].

Játra

Při hodnocení relativních vah jater se prokázalo, že zjištěné rozdíly průměru jednotlivých skupin dosahují statistické významnosti pouze u samic, které pily po 200 dnů 10 a 20 mEkv $NaNO_2$. U samců váhové rozdíly nikdy nepřesahují limitní hodnoty použitého testu. Nejvyšší

Tab. 3. Hladiny methemoglobinu po dlouhodobé expozici $NaNO_2$

Skupina	Dny expozice	Kontroly	10 mEkv	20 mEkv
Samci	150	0,90 ± 0,090	2,83 ± 1,3 NS	1,71 ± 0,305 NS
	200	1,32 ± 0,397	2,09 ± 0,305 NS	4,42 ± 1,55 NS
Samice	150	1,07 ± 0,229	2,13 ± 0,271 0,01	5,34 ± 1,91 0,05
	200	1,09 ± 0,397	1,65 ± 0,250 NS	2,77 ± 0,432 0,05

* NS = nesignifikantní

hodnoty rozdílu průměru u samců bylo dosaženo při dávce 20 mEkv po 200 dnů, ale i v tomto případě leží vypočítaná hodnota t = 2,00 pod hranicí významnosti. Výsledky váhového hodnocení jater pokusných krys jsou obsaženy v tabulce 4.

Tab. 4. Relativní váhy jater u krys při dlouhodobé expozici $NaNO_2$

Skupina významnost	Dny expozice	Kontroly	10 mEkv	20 mEkv
Samci	150	3,36 ± 0,146	3,56 ± 0,180 NS	3,40 ± 0,176 NS
	200	3,11 ± 0,141	3,07 ± 0,011 NS	3,45 ± 0,090 NS
Samice	150	3,36 ± 0,072	3,99 ± 0,349 NS	3,81 ± 0,282 NS
	200	3,02 ± 0,164	3,56 ± 0,105 0,5	3,59 ± 0,112 0,01

Slezina

Reakce sleziny vyjádřená v ukazatelích relativní váhy orgánů pokusných zvířat má opět odlišný charakter u samců a samic použitých v pokusu. Zatímco u samců nebyla prokázána po celé pokusné období ani při vyšších dávkách dusitanů významnost rozdílů průměrů jednotlivých skupin, vykazují výsledky získané u pokusných samic vysoce významný rozdíl průměrů hodnocený t-testem po 150 dnech expozice při dávce 20 mEkv $NaNO_2$. Stejný výsledek byl pozorován u expozice po 200 dnech při stejné dávce. Dávka 10 mEkv $NaNO_2$ podávaná 150 a 200 dnů má za následek závazné zvýšení váhy sleziny, statisticky nevýznamné. Výsledky jsou obsaženy v tabulce 5.

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Ledvina

Ledviny zvířat z popisovaného pokusu se při váhovém hodnocení v jednotlivých skupinách lišily výrazně až do expozice 200 dnů. Rozdíl průměru zaznamenaný u samic, které pily 20 mEkv $NaNO_2$ po 150 dnech, nebyl statisticky významný, když vypočítaná hodnota t = 0,788 ležela hluboko pod limitní hodnotou pro daný soubor. Výrazné váhové změny byly u samic, které pily dávku 10 mEkv $NaNO_2$ po 200 dnů (rozdíl průměrů statisticky nevýznamný), zatímco u skupiny, která pila 20 mEkv po 200 dnů, se statisticky významně zvýšila váha ledvin

Tabulka 5

Skupina významnost	Dny expozice	Kontroly	10 mEkv	20 mEkv
Samci	150	0,353 ± 0,310	0,329 ± 0,035	0,334 ± 0,021
	t_s		NS	NS
	200	0,253 ± 0,020	0,264 ± 0,012	0,249 ± 0,053
	t_s		NS	NS
Samice	150	0,352 ± 0,011	0,487 ± 0,036	0,511 ± 0,040
	t_s		NS	0,01
	200	0,285 ± 0,014	0,331 ± 0,019	0,361 ± 0,023
	t_s		NS	0,05

proti kontrolám s pravděpodobností na 0,01% hladině. U samců, kteří pili po 200 dnů dávku 10 mEkv $NaNO_2$, nebyl zaznamenán významný váhový rozdíl mezi kontrolami a pokusnou skupinou; u skupiny, která pila dávku 20 mEkv $NaNO_2$ byl významný rozdíl na 0,05% hladině. Výsledky jsou obsaženy v tabulce 6.

Tab. 6. Relativní váhy ledvin u krys při dlouhodobé expozici $NaNO_2$

Skupina významnost	Dny expozice	Kontroly	10 mEkv	20 mEkv
Samci	150	0,706 ± 0,024	0,698 ± 0,027	0,718 ± 0,028
	t_s		NS	NS
	200	0,571 ± 0,024	0,601 ± 0,018	0,662 ± 0,020
	t_s		NS	0,05
Samice	150	0,650 ± 0,100	0,722 ± 0,021	0,735 ± 0,027
	t_s		NS	NS
	200	0,588 ± 0,017	0,640 ± 0,022	0,660 ± 0,017
	t_s		NS	0,01

Myokardy

Vážení myokardů bylo prováděno po nástřihu srdeční svaloviny na jednotlivé oddíly: levou komoru, pravou komoru a septum. Odděleným vážením bylo možno prokázat, že u všech myokardů zvířat exponovaných $NaNO_2$ šlo o zvětšení váhy rovnoměrné, postihující rovnoměrně všechny srdeční oddíly. Váhové hodnocení uvedené v tabulce 7 zahrnuje pouze údaje získané o přepočtu celkové váhy všech srdečních oddílů na 100 g živé váhy pokusného zvířete. V pokusu byly také hodnoceny úchylky myokardů pokusných zvířat od standardu vypracovaného pro váhové hodnocení srdeční svaloviny pro československé chovy Poupou, Rakušanem a Korcekým[11]. Bylo zjištěno, že průměrné odchylky u pokusných samců po dávce 20 mEkv $NaNO_2$ po dobu 200 dnů leží průměrně v hodnotě 122 % vypočtené úchylky od standardu pro zvíře stejné váhy. Pokusné samice, které dostávaly tutéž dávku po stejné období, vykazují úchylka 129 % od standardu, ovšem tento údaj je odečten z přepočtu standardních hodnot pro pokusné

samce a vyšší úchylka
že standard je stanoven
kusných zvířat odchyl
řete

Statisticky vyzna
prokázány u skupiny
průměrné hodnoty
mohla být prokázán
jsou uvedeny v tabulce

Celkový přehl
Z uvedených
to sdělení je nutné
zených do pokusu.
V závěru vá
významných odch
vzhledem ke kont
jsou uvedeny v ta

Všechny před
ratorních krys na
sdělení jsou obsa
nutné pro základ
toto hodnocení,
hematologické.
Z materiálu
tanu sodného
S výjimkou vá
než u samic,
škození samic je

Tab. 7. R

Skupina významnost	
Samci	
Samice	

rozboru jednotliv
se ve výsledcích
podávaného du
sodného, jako
methemoglobinu.
kyslík tkáním.
methemoglobinu

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amce a vyšší úchylka je proto z hlediska standardních hodnot nepřesná. Vzhledem k tomu, e standard je stnoven pouze pro pokusné samce, nepoužili jsme při závěrečném hodnocení pokusných zvířat odchylek od standardu, ale jen přepočet na 100 kg živé váhy pokusného zvíete

icky významné rozdíly průměrý pokusných skupin proti skupině kontrolní byly prokáz u skupiny která pila 20 mEkv $NaNO_2$ po 150 a 200 dnů pouze u samců. Přestože průměrné hodnoty pokusných samic ležely v téměř stejném rozsahu jako průměry samců, nenohla být prokázána statistická významnost vzhledem k širokému rozptylu hodnot. Výsledky sou uvedeny v tabulce 7.

Celkový přehled váhového hodnocení:

Z uvedených výsledků vyplývá, že v pokusném uspořádání popsaném v tomto sdělení je nutno konstatovat odlišnou aktivitu krysích samců a samic zařazených do pokusu.

V závěru váhového hodnocení předkládáme přehlednou tabulku statisticky významných odchylek průměru relativních vah jednotlivých pokusných skupin vzhledem ke kontrolám. Výsledky statistického hodnocení provedeného t-testem jsou uvedeny v tabulce 8.

Diskuse

Všechny předložené výsledky jsou částí souborného vyšetření reakce laboratorních krys na dlouhodobé podávání roztoku dusitanu sodného. V tomto sdělení jsou obsaženy základní toxikologické a fyziologické údaje, které jsou nutné pro základní charakteristiku zkoumané látky. Závěry, které poskytuje toto hodnocení, budou v dalších sděleních doplněny o vyšetření histologické a hematologické.

Z materiálu výše uvedeného je zřejmé, že úhrnný specifický účinek dusitanu sodného má výrazně vyšší účinek na pokusné samice než samce. S výjimkou váhové reakce myokardu, která byla u samců výraznější než u samic, ve všech ostatních ukazatelích bylo zjištěno, že o poškození samic je nutno uvažovat s vyšší pravděpodobností. Podrobíme-li

Tab. 7. Relativní váhy myokardů u krys při dlouhodobé expozici $NaNO_2$

Skupina významnost	Dny expozice	Kontroly	10 mEkv	20 mEkv
i	150	0,250 ± 0,037	0,256 ± 0,017	0,405 ± 0,023
	ts		NS	0,001
	200	0,239 ± 0,007	0,274 ± 0,015	0,291 ± 0,004
	ts		NS	0,01
Samice	150	0,229 ± 0,008	0,301 ± 0,025	0,318 ± 0,025
	ts		NS	NS
	200	0,297 ± 0,010	0,302 ± 0,10	0,321 ± 0,009
	ts		NS	NS

rozboru jednotlivé ukazatele z hlediska rozložení hodnot, zjistíme, že u samců se ve výsledcích objevuje vyšší procento zvířat, které byly vůči toxickému efektu podávaného dusitanu sodného necitlivé. Specifický ukazatel účinku dusitanu sodného, jako látky tvořící methemoglobin, je přímé stanovení hladiny krevního methemoglobinu, krevního derivátu s trojmocným železem, neschopného přenášet kyslík tkáním. Skutečnost, že u samců bylo možno najít zvířata s hladinami methemoglobinu nelišícími se ani od hodnot nalezených u kontrolních zvířat,

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ani od fyziologických hodnot obvykle v našich pokusech stanovovaných a uváděných v písemnictví, je možno vyložit dvojím způsobem: jednak může jít o vliv intervalu uplynulého od posledního napití zvířete, kdy zvýšená hladina methemoglobinu byla opět zredukována do fyziologického rozmezí anebo může jít o projev rozdílné reaktivity oxydoredukčního systému pokusného zvířete.

Sledování relativní váhy orgánů je základním toxikologickým ukazatelem, který poskytuje informaci o systémech, které mohou být zkoušenou látkou na-

Tab. 8. Přehled významnosti zjištěné u ukazatelů sledovaných při dlouhodobé expozici krys $NaNO_2$

Expozice	Samci				Samice			
	150		200		150		200	
	10	20	10	20	10	20	10	20
Methemoglobin	×	×	×	×	0,01	0,05	×	0,05
Játra	×	×	×	×	×	×	0,05	0,01
Slezina	×	×	×	×	×	0,01	×	0,05
Ledviny	×	×	×	0,05	×	×	×	0,01
Myokardy	×	0,01	×	0,01	×	×	×	×

padány. Nepřítomnost významného váhového rozdílu při sledování váhy jater v uvedených pokusech u samců nemůže svědčit pro nemožnost zásahu do jaterních funkcí. Tuto domněnku podepírá jistě nález zvýšení váhy jater u samic, a je proto nutno vidět v analýze jaterních funkcí další možný přístup při řešení patofyziologických mechanismů při methemoglobinémiích. V souvislosti s hypoxickými stavy vyvolanými snížením celkového krevního barviva schopného předávat tkáním kyslík bylo nutné zaměřit pozornost také na stanovení váhy sleziny u pokusných zvířat. U savců za anoxických a hypoxických stavů pracuje slezina jako významný objemový regulátor celkového obíhajícího množství krve. Zvýšení váhy sleziny u samic, které bylo v pokusech zjištěno, ukazuje, že postižení tkáně sleziny nesouvisí jen s mechanickou funkcí sleziny, ale že zasahuje i do funkcí souvisejících s dynamikou krvetvorby u pokusného zvířete v období dospívání i dospělosti. Totožný výsledek byl zjištěn u relativních vah ledvin. U obou pokusných skupin samců i samic bylo zachyceno statisticky významné zvýšení váhy ledviny až při posledním odběru po 200 dnech expozice a při vyšší dávce 20 mEkv $NaNO_2$. Ve vlastních pokusech jsme přinesli důkaz, že akutní methemoglobinémie ovlivňuje vylučovací funkce ledvin. V chronickém pokusu je možno na ukazateli relativní váhy orgánů prokázat, že dlouhodobá intoxikace vedoucí k methemoglobinémii může závažně poškozovat ledvinnou tkáň.

Souhrnná diskuse všech nespecifických orgánových reakcí bude v dalším sdělení doplněna rozborem histologickým, který bude diferencovat poškození jednotlivých struktur.

Použité koncentrace dusitanu sodného, které byly podány pokusným zvířatům v uvedených pokusech, leží na horní hranici koncentrací, které se mohou vyskytovat v podmínkách kojeneckých methemoglobinémií. Tyto koncentrace bylo nutno použít při experimentálním přístupu jednak proto, že jsme pracovali s relativně odolným zvířetem, a jednak proto, že bylo nutno získat představu o strukturách a rozsahu, ve kterém může dojít k nespecifickému účinku dusitanového iontu.

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Závěr

1. Na 121 krysách obého pohlaví byl proveden dlouhodobý pokus, ve kterém byly pokusným zvířatům podány k pití koncentrace 10 a 20 mEkv $NaNO_2$/ /1 litr vody. Po 150 a 200 dnech byla zvířata zabita exsanguinací, stanovena hladina methemoglobinu a relativní váhy jater, sleziny, ledviny a myokardu. V průběhu pokusu byla sledována spotřeba potravin, pití a růstová křivka.

2. V pokusu nebyly zjištěny významné rozdíly ve spotřebě potravy, pití a růstových křivkách.

3. Hladiny methemoglobinu u pokusných samic se zvýšily po 150 dnech expozice po dávkách 10 a 20 mEkv $NaNO_2$ a po 200 dnech u dávky 20 mEkv. U samců byly průměrné hodnoty methemoglobinu proti kontrolám vždy vyšší, ale nebyly statisticky významné.

4. Relativní váhy jater se zvyšují u samic, které pily 10 a 20 mEvk $NaNO_2$ po 200 dnů, relativní váhy sleziny u samic se zvyšují po dávce 20 mEkv $NaNO_2$ po 150 a 200 dnech expozice, relativní váhy ledvin se zvyšují u samic po dávce 20 mEkv $NaNO_2$ po 200 dnech. U samců bylo zjištěno zvýšení relativní váhy ledviny po dávce 20 mEkv po 200 dnech a po dávce 20 mEkv $NaNO_2$ po 150 a 200 dnech; zvýšení relativní váhy myokardu po dávce 20 mEkv po 150 i 200 dnech.

5. Výsledky budou použity pro řešení problematiky inaparentních dusičnanových methemoglobinémií prokázaných v oblastech s vyšším výskytem dusičnanů v pitné vodě.

Považuji za svou povinnost poděkovat inž. V. Pickovi, statistikovi ÚH, za vyhodnocení části výsledků.

Souhrn

Autor sleduje v pokusech na laboratorních krysách účinek dlouhodobého pití roztoku dusitanu sodného v koncentracích 10 a 20 mEkv/1 litr vody. Nebyly prokázány významné odchylky ve spotřebě potravy, pití a růstových křivkách. Byly zjištěny odchylky v hladinách methemoglobinu v krvi pokusných zvířat u samic statisticky významné. U samic byly zjištěny statisticky významné odchylky v relativní váze jater, sleziny a ledviny, u samců odchylky v relativní váze ledviny a myokardu.

LITERATURA

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EFFECT OF A CHRONIC SODIUM NITRITE INTOXICATION ON RATS

(Der Einfluß einer chronischen Natriumnitrit-Intoxikation auf Ratten)

Author: J. Musil

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The physiolo ical properties of methemoglobin unable to reversibly combine with O have frequently been studied in recent time (References 3, 11, 12, 14, 15, 24). Attention was turned also to the toxicology of the methemoglobin-forming substances (References 6, 7, 13, 16, 20, 22, 23). Nevertheless, the mechanisms enabling the mammalian organism to compensate the hypoxia, resulting during the prolonged intake of methemoglobin-forming substances because of the decrease of the functional hemoglobin, are unknown so far. Thus, for instance, the hypoxia of the elevation type is followed by an increase of the number of erythrocytes in the cardio-vascular system. In the case of an alveolar pO_2 of about 60 mm Hg, the O saturation of the blood amounts to about 90% (Ref. 1). With methemoglobin hypoxias, the pO_2 in the respiratory and alveolar air do not change; the O supply is normal. It is true that in this case lower hemoglobin amounts are available to the cardio-cascular system for the O transfer. During prolonged administration of methemoglobin-forming substances, the hypoxia possesses an undulating trend, since, in accordance with the absorption of the substance and of its methemoglobin formation, as well as with the enzymatic involution of the substance, variations in the O_2-contents of the blood develop. In this study we raise the question whether the mammalian organism compensates functionally a chemical formation of methemoglobin.

Procedure:

Wistar rats of the two sexes were used for the tests. They were kept in breeding cages of polystyrene with metal lids, and were fed a standard diet (LARSEN). Drinking water was contained in glass tanks with polystyrene suction devices. The animals were tested not before their 33rd day of life (after their ablactation).

The animals from the different litters were divided in individual test groups. The control group received water in which, during the entire test period, the nitrate concentration did not exceed 30 mg/l. Nitrites were not present. A test group receive water with 10 and a second with 20 m eq $NaNO_2$.

In the test, the basic biological parameter, such as animal weight, food and drinking water consumption, were studied (Ref. 17). Methemoglobin was determined by the method of CRUZ (Ref. 5) through difference spectrophotometry. The animals were sacrificed by opening the A. carotis, and the organs were weighed. The relative organ weight refer to the body weight of the animal before being killed. The heart was prepared according the FULTON and others (Ref. 9). For the purpose of hematological determination, the carotide blood was mixed with the residue of evaporation of the oxalate mixture. The number of red and white blood corpuscles was determined by the small flask method (Ref. 25) and the hemoglobin was ascertained by the cyanmethemoglobin method with the DRABKIN solution (Ref. 19). The hematocrite determination was carried out in the Prema equipment (Ref. 21). Further hematological indices, such the erythrocyte volume (V Ery), the hemoglobin content (I Hb) and the hemoglobin concentration in the erythrocyte (C Hb), were red according to the Czechoslovakian hematological Lineal Logarex.

Results

1. Methemoglobin Formation:

Table 1: Total hemoglobin concentration of the blood and methemoglobin content in the control and test groups

a. Days of exposure b. Group c. Males d. Number e. Significance

f. Females

K : control 10 = 10 m eq or 20 = m eq $NaNO_2$ in the drinking water; the mean va ues (M $\pm$ standard deviation (s) are indicated; NS - Nonsignificant.

Table 1 shows that the prolonged intake of nitrite-containing drinking water produces no change in the total hemoglobin concentration of the blood, but a high methemoglobin level. The methemoglobin values are indicated in per cent of the total amount of the red blood pigment. Because of the relatively high dispersion of the individual values, it is not possible to confirm statistically the differences as to the controls for all the test groups.

2. Relative Heart Weight:

Table 2: Relative heart weights in the control and test groups in g/100 of animal weight (Data as in Table 1).

In Table 2 the heart weights are expressed as relative weight per 100 g of weight of the living animal. From the results it is possible to conclude that the prolonged administration of Na nitrite to test rats causes a statistically significant increase of the heart weight in male rats. All the heart sections were uniformly involved in this.

3. Spleen Weight:

Table 3: Relative spleed weights in the control and test groups in g/100 g of animal weight (Indications as in Table 1).

With prolonged Na nitrite intake, only the females react with an increase of the spleen weight. In the males this weight decreases somewhat. In view of the dispersion of the results, this difference is insignificant. Table 3 provides a survey of these changes.

4. Hematological Parameters:

The erythrocyte number, as a fundamental value, was below the average values of the control animals in the test groups. In no case, however, was it possible to confirm the differences statistically. The hematocrit provides information about the volumes of the blood elements, preferably about those of the erythrocytes. In all the test groups (with exception of the 10 m eq/150 day group of the bucks (? males)) the hematocrit values increased. There was also a corresponding increase of the volume of the individual erythocytes (Compare Table 4).

Table 4: Hematological values in test and control animals (Indications as in Table 1).

The hemoglobin concentration of the blood does not differ in the individual groups (See Table 1) . A natural consequence of the nonsignificant decrease of the total number

of erythrocytes with the simultaneous increase of their volume, was, in the case of the parameters characterizing the single erythrocytes, the appearing of corresponding changes, especially an increase of the hemoglobin amount per erythrocyte with a simultaneous decrease of the concentration in the erythrocyte.

Table 5: Average Values of the hemoglobin content and of the hemoglobin concentration in the individual erythrocyte (Indications as in Table 1).

Discussion:

The action of Na nitrite on intact erythrocytes leads demonstrably neither to the formation of Heinz' corpuscles, nor to hemolysis (Ref. 4, 8, 10). No disturbance of the hem-globin combination occurs . In using the cyanmethemoglobin method to determine the total hemoglobin, no significant changes can be detected. The differences in the methemoglobin values are adequate answers of the organism of the test animals to the constant burden of a methemoglobin former. In males, however, the increase of the methemoglobin content in the t-test was not confirmed. This might be explained by the varying occurring in the individual animals in the interval between the last intake of the nitrite solution and the killing by bleeding, which causes an increased dispersion of the mean values. The lower scattering of the average values in the females might be explained hypothetically by species differences of the enzymatic reduction system in the erythrocytes. It will be practical to perform further tests regarding the sex specificity of the sensitivity to methemoglobin formers.

The oxidation of the hemoglobin follows a typical reaction curve (Ref. 15). From this results for our test conditions an intermittent increase of the methemoglobin values. The organism of the t st animals must compensate through adequate mechanisms the decrease of the effective O_2 capacity and guarantee the O saturation of the tissues within biologically unavoidable limits. Obviously in this case the main mechanism consists in the increase of the erythrocyte surface. Through this relative macrocytosis the emitting (supply) of the O combined with the residual functional hemoglobin is facilitated. The compensation mechanism through an increased heart performance is not much noticed in the hypoxias of the methemoglobin

type, even if the weight increase of the heart in the males of the 20 m eq group should not be neglected. However, the increase of the relative heart weights could also be induced by a decrease of the total body weight. Hence, one must place the heart weights in relation to the heart weight of equally heavy controls. In other tests and in other exposure times, no changes were found line those which occurred in the $NaNO_2$ tests. The increase of the heart muscle volume should be expressed in the females also where the differences in the methemoglobin levels were still more marked. These discrepancies can be explained only by further tests. In this case, special attention will have to be given to the reactivity of the heart muscle as to acute and chronic contamination (poisoning) by methemoglobin formers.

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Der Einfluß einer chronischen Natriumnitrit-Intoxikation auf Ratten

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(Eingegangen am 25. 10. 1965)

Zusammenfassung

1. Ein Gehalt von 10 bzw. 20 mÄq $NaNO_2$ im Trinkwasser bewirkt bei Ratten auch nach Expositionszeiten von 150 und 200 Tagen keine Veränderungen des Gesamthämoglobins; der prozentuale Methämoglobinspiegel ist erhöht.
2. Die Erniedrigung der Erythrozytenzahl ist statistisch nicht zu sichern, dagegen ist die Zunahme des Hämatokrits sowie des Hämoglobingehalts pro Erythrozyt signifikant.
3. Bei männlichen Versuchstieren steigt unter diesen Bedingungen das relative Herzgewicht (bezogen auf das Körpergewicht) signifikant an.
4. Bei weiblichen Ratten ist das relative Milzgewicht signifikant erhöht.

Die physiologischen Eigenschaften des MetHb[1], das nicht zur reversiblen Bindung von Sauerstoff befähigt ist, sind in der letzten Zeit häufig studiert worden [3], [11], [12], [14], [15], [24]. Auch der Toxikologie von MetHb-bildenden Stoffen wurde Aufmerksamkeit geschenkt [6], [7], [13], [16], [20], [22], [23]. Doch sind bisher die Mechanismen unbekannt, durch die der Säugetierorganismus die Hypoxie kompensiert, die bei langfristiger Verabreichung von MetHb-bildenden Stoffen infolge der Abnahme des funktionstüchtigen Hb entsteht. So folgt beispielsweise der Hypoxie des Höhentyps eine Erhöhung der Erythrozytenzahl im kardiovaskulären System. Bei einem alveolären pO_2 von annähernd 60 mm Hg beläuft sich die Sauerstoffsättigung des Blutes auf etwa 90% [1]. Bei Methämoglobinhypoxien ändern sich dagegen der pO_2 in der Atem- und der Alveolarluft nicht; die Sauerstoffzufuhr ist normal. Allerdings verfügt hier das kardiovaskuläre System über geringere Hb-Mengen für die Sauerstoffübertragung. Die Hypoxie besitzt bei der langfristigen Verabreichung von MetHb-bildenden Stoffen undulierenden Verlauf, da entsprechend der Aufnahme des Stoffes und seiner MetHb-Bildung sowie der fermentativen Rückbildung desselben Schwankungen der O_2-Kapazität des Blutes entstehen. In der vorliegenden Arbeit stellten wir uns die Frage, wie der Säugetierorganismus eine chemische MetHb-Bildung funktionell ausgleicht.

[1] Abkürzungen: Hb = Hämoglobin; MetHb = Methämoglobin

Methodik

Zu den Versuchen wurden Wistar-Ratten beiderlei Geschlechts verwendet. Sie wurden in Zuchtkäfigen aus Polystyrol mit Metalldeckel gehalten und mit einer Standarddiät (LARSEN) ernährt. Das Trinkwasser befand sich in Glasgefäßen mit Polystyrolsaugern. Die Tiere kamen an ihrem 33. Lebenstag (nach ihrer Abstillung) in den Versuch. Die Tiere aus den verschiedenen Würfen wurden in einzelne Versuchsgruppen eingeteilt. Die Kontrollgruppe erhielt Wasser, in dem während der ganzen Versuchsperiode die Nitratkonzentration 30 mg/l nicht überstieg, Nitrite waren nicht anwesend. Eine Versuchsgruppe bekam Wasser mit 10 und eine zweite mit 20 mÄq $NaNO_2$.

Im Versuch wurden die grundlegenden biologischen Parameter, wie Tiergewicht, Nahrungs- und Trinkwasserverbrauch, verfolgt [17]. MetHb wurde nach der Methode von CRUZ [5] durch Differenzspektrophotometrie bestimmt. Die Tiere wurden durch Entblutung aus der A. carotis getötet und die Organe gewogen. Die relativen Organgewichte beziehen sich auf das Körpergewicht des Tieres vor der Tötung. Das Herz wurde nach FULTON et al. [9] präpariert. Zur hämatologischen Bestimmung wurde das Karotisblut mit dem Abdampfrückstand der Oxalatmischung gemischt. Die Anzahl roter und weißer Blutkörperchen wurde durch die Kölbchenmethode [25] und Hämoglobin nach der Zyanmethämoglobinmethode mit der DRABKIN-Lösung [19] bestimmt. Die Hämatokritbestimmung erfolgte in der Apparatur Prema [21]. Weitere hämatologische Indizes, wie Erythrozytenvolumen (V Ery), Hb-Gehalt (I Hb) und Hb-Konzentration im Erythrozyten (K Hb) sind nach dem tschechoslowakischen hämatologischen Lineal Logarex abgelesen worden.

Ergebnisse

1. *Methämoglobinbildung*

Aus Tabelle 1 ist ersichtlich, daß die langfristige Verabreichung von nitrithaltigem Trinkwasser keine Veränderung der Gesamt-Hb-Konzentration des Blutes herbeiführt, aber andererseits einen erhöhten MetHb-Spiegel bedingt. Die MetHb-Werte sind in Prozent der Gesamtmenge des roten Blutfarbstoffes angegeben. Die Unterschiede zu den Kontrollen sind wegen der relativ großen Streuung der Einzelwerte nicht für alle Versuchsgruppen statistisch zu sichern.

2. *Relatives Herzgewicht*

Die Herzgewichte in der Tabelle 2 sind als relatives Gewicht pro 100 g Gewicht des lebenden Tieres ausgedrückt. Aus den Ergebnissen kann man schließen, daß das langfristige Verabreichen des Natriumnitrits an Versuchsratten eine statistisch signifikante Erhöhung des Herzgewichtes bei Männchen zur Folge hat. Daran waren alle Herzabschnitte gleichmäßig beteiligt.

3. *Milzgewicht*

Bei langfristiger Verabreichung von Natriumnitrit reagierten nur die Weibchen mit einer Erhöhung des Milzgewichtes. Bei Männchen senkten sie sich teilweise in geringem Maße. Im Hinblick auf die Streuung der Meßwerte ist diese Differenz jedoch insignifikant. Tabelle 3 gibt eine Übersicht über diese Veränderungen.

Tabelle 1

Gesamthämoglobinkonzentration des Blutes und Methämoglobingehalt bei Kontroll- und Versuchsgruppen

Exposition Tage	Gruppe	Männchen Anzahl	Männchen $M \pm s$	Männchen Signifikanz tP	Weibchen Anzahl	Weibchen $M \pm s$	Weibchen Signifikanz tP
		Hb-Konzentration in g/100 ml					
150	*K*	10	15,6 ± 0,65		10	15,2 ± 0,36	
	10	8	14,8 ± 0,83	*NS*	9	15,1 ± 0,43	*NS*
	20	10	15,9 ± 0,21	*NS*	10	15,6 ± 0,75	*NS*
200	*K*	10	15,7 ± 0,21		10	15,2 ± 0,20	
	10	10	15,6 ± 0,19	*NS*	9	15,0 ± 0,69	*NS*
	20	12	15,8 ± 0,30	*NS*	13	15,5 ± 0,36	*NS*
		MetHb-Gehalt in % des Gesamt-Hb					
150	*K*	10	0,90 ± 0,090		10	1,07 ± 0,305	
	10	8	2,82 ± 1,30	*NS*	9	2,13 ± 0,271	0,01
	20	10	1,71 ± 0,305	*NS*	10	5,34 ± 1,910	0,05
200	*K*	10	1,32 ± 0,397		10	1,09 ± 0,387	
	10	10	2,09 ± 0,305	*NS*	9	1,65 ± 0,250	*NS*
	20	12	4,42 ± 1,55	*NS*	13	2,77 ± 0,432	0,05

K = Kontrolle; 10 = 10 mÄq bzw. 20 = 20 mÄq $NaNO_2$ im Trinkwasser; angegeben sind die Mittelwerte (M) ± Standardabweichung (s); *NS* = Nicht signifikant

Tabelle 2

Relative Herzgewichte bei Kontroll- und Versuchsgruppen in g/100 Tiergewicht

(Angaben wie in Tabelle 1)

Exposition Tage	Gruppe	Männchen $M \pm s$	Männchen Signifikanz tP	Weibchen $M \pm s$	Weibchen Signifikanz tP
150	*K*	0,250 ± 0,037		0,299 ± 0,08	
	10	0,256 ± 0,017	*NS*	0,301 ± 0,025	*NS*
	20	0,405 ± 0,023	0,01	0,318 ± 0,025	*NS*
200	*K*	0,239 ± 0,07		0,297 ± 0,010	
	10	0,274 ± 0,015	*NS*	0,302 ± 0,10	*NS*
	20	0,291 ± 0,004	0,01	0,321 ± 0,09	*NS*

Tabelle 3
Relative Milzgewichte bei Kontroll- und Versuchsgruppen in g/100 g Tiergewicht
(Angaben wie in Tabelle 1)

Exposition Tage	Gruppe	Männchen		Weibchen	
		$M \pm s$	Signifikanz tP	$M \pm s$	Signifikanz tP
150	*K*	0,353 ± 0,310		0,352 ± 0,011	
	10	0,329 ± 0,035	*NS*	0,487 ± 0,036	*NS*
	20	0,334 ± 0,021	*NS*	0,511 ± 0,040	0,01
200	*K*	0,253 ± 0,020		0,285 ± 0,014	
	10	0,264 ± 0,012	*NS*	0,331 ± 0,019	*NS*
	20	0,249 ± 0,053	*NS*	0,361 ± 0,023	0,05

Tabelle 4
Hämatologische Größen bei Versuchs- und Kontrolltieren
(Angaben wie in Tabelle 1)

Exposition Tage	Gruppe	Männchen		Weibchen	
		$M \pm s$	Signifikanz tP	$M \pm s$	Signifikanz tP
		Erythrozytenzahl/mm³ · 10³			
150	*K*	6643 ± 307,89		5842 ± 341,0	
	10	6026 ± 284,7	*NS*	5700 ± 123,5	*NS*
	20	5908 ± 306,7	*NS*	5731 ± 258,5	*NS*
200	*K*	5650 ± 284,9		5259 ± 259,7	
	10	5303 ± 229,3	*NS*	5177 ± 209,5	*NS*
	20	4840 ± 247,7	*NS*	5174 ± 197,4	*NS*
		Hämatokrit ml/100 ml			
150	*K*	42,6 ± 2,5		39,8 ± 1,20	
	10	41,5 ± 1,74	*NS*	42,0 ± 1,48	*NS*
	20	45,1 ± 0,68	*NS*	42,1 ± 1,66	*NS*
200	*K*	41,4 ± 1,31		42,2 ± 0,64	
	10	45,1 ± 0,68	0,05	42,6 ± 2,31	*NS*
	20	48,8 ± 1,36	0,01	46,7 ± 1,13	0,01
		Erythrozytenvolumen μ^3			
150	*K*	65,0 ± 4,54		68,0 ± 4,47	
	10	72,0 ± 2,73	*NS*	72,81 ± 3,59	*NS*
	20	78,0 ± 4,61	0,05	73,7 ± 2,24	*NS*
200	*K*	75,2 ± 5,95		80,2 ± 1,80	
	10	89,5 ± 5,44	*NS*	81,4 ± 3,77	*NS*
	20	102,96 ± 4,91	0,01	91,9 ± 4,23	0,05

4. *Hämatologische Parameter*

Die Erythrozytenzahl als grundlegende Größe lag bei den Versuchsgruppen unter den Durchschnittswerten der Kontrolltiere. In keinem Falle ließen sich die Differenzen jedoch statistisch sichern. Der Hämatokrit gibt eine Information über die Volumina der Blutelemente, vorzugsweise der Erythrozyten. Bei allen Versuchsgruppen (mit Ausnahme der 10 mÄq/150 Tage-Gruppe der Böcke) stiegen die Hämatokrit-Werte an. Entsprechend ergab sich auch eine Erhöhung des Volumens der einzelnen Erythrozyten (vgl. Tab. 4).

Die Hb-Konzentration des Blutes unterschied sich bei den einzelnen Gruppen nicht (s. Tab. 1). Als natürliche Folge der zwar nicht signifikanten Herabsetzung der Gesamtanzahl der Erythrozyten bei gleichzeitiger Erhöhung ihres Volumens, ergaben sich bei den Parametern, die den Einzelerythrozyten charakterisieren, entsprechende Veränderungen, speziell eine Zunahme der Hb-Menge pro Erythrozyt bei gleichzeitiger Verminderung der Konzentration im Erythrozyten.

Tabelle 5

Durchschnittswerte des Hb-Gehaltes und der Hb-Konzentration im Einzelerythrozyten (Angaben wie in Tabelle 1)

Exposition Tage	Gruppe	Männchen		Weibchen	
		$M \pm s$	Signifikanz tP	$M \pm s$	Signifikanz tP
		Hb-Gehalt pg			
150	*K*	23,7 ± 1,25		26,4 ± 1,44	
	10	25,1 ± 0,59	*NS*	26,4 ± 1,04	*NS*
	20	27,8 ± 1,40	0,05	27,5 ± 1,21	*NS*
200	*K*	28,5 ± 1,45		28,9 ± 0,83	
	10	29,9 ± 1,39	*NS*	28,9 ± 0,86	*NS*
	20	32,8 ± 2,01	*NS*	30,4 ± 1,34	*NS*
		Hb-Konzentration g/100 ml			
150	*K*	38,8 ± 1,24		40,4 ± 0,90	
	10	41,0 ± 1,85	*NS*	38,4 ± 1,51	*NS*
	20	40,0 ± 1,72	*NS*	40,2 ± 1,26	*NS*
200	*K*	37,4 ± 1,66		36,1 ± 0,38	
	10	33,9 ± 0,82	*NS*	35,3 ± 0,97	*NS*
	20	32,6 ± 0,90	0,01	30,5 ± 0,98	0,01

Diskussion

Die Einwirkung des Natriumnitrits auf intakte Erythrozyten führt nachweisbar weder zur Bildung von Heinzschen Körperchen noch zur Hämolyse [4], [8], [10]. Es tritt auch keine Störung der Häm-Globin-Bindung ein.

Auch in den vorgelegten Ergebnissen zeigt sich, daß bei der Anwendung der Zyanmethämoglobinmethode zur Bestimmung des gesamten Hb keine signifikanten Veränderungen nachgewiesen werden können. Die Unterschiede in den MetHb-Werten sind adäquate Antworten des Organismus der Versuchstiere auf die ständige Belastung mit einem MetHb-Bildner. Bei Männchen war aber die Zunahme des MetHb-Gehaltes im *t*-Test nicht zu sichern. Dies kann man vielleicht damit erklären, daß bei den einzelnen Tieren das Intervall zwischen letztem Trinken der Nitritlösung und Entblutung variiert, wodurch sich die Streuung der Mittelwerte vergrößert. Die niedrigere Streuung der Durchschnittswerte bei Weibchen ließe sich hypothetisch über Speziesdifferenzen des fermentativen Reduktionssystems in den Erythrozyten deuten. Es wird zweckmäßig sein, weitere Versuche über die Geschlechtsspezifität der Empfindlichkeit gegenüber MetHb-Bildnern anzustellen.

Die Oxydation des Hb folgt einer typischen Reaktionskurve [15]. Daraus resultiert für unsere Versuchsbedingungen eine intermittierende Erhöhung der MetHb-Werte. Der Organismus der Versuchstiere muß durch adäquate Mechanismen die Abnahme der effektiven O_2-Kapazität kompensieren und die Sauerstoffsaturation der Gewebe in biologisch unumgänglichen Grenzen gewährleisten. Offensichtlich besteht dabei der Hauptmechanismus in der Vergrößerung der Erythrozytenoberfläche. Durch diese relative Makrozytose wird die Abgabe des am restlichen funktionstüchtigen Hb gebundenen Sauerstoffes erleichtert. Der Kompensationsmechanismus über eine verstärkte Herzleistung macht sich bei den Hypoxien des Methämoglobintyps wenig bemerkbar; auch wenn die Gewichtszunahme der Herzen bei den Männchen der 20 mÄq-Gruppe nicht übersehen werden sollte. Doch könnte die Erhöhung der relativen Herzgewichte auch durch eine Herabsetzung des gesamten Körpergewichtes bedingt sein. Man muß deshalb die Herzgewichte in Relation zum Herzgewicht gleichschwerer Kontrollen setzen. In anderen Versuchen und bei anderen Expositionszeiten konnten Veränderungen, wie sie in den $NaNO_2$-Versuchen auftraten, nicht festgestellt werden. Die Erhöhung des Herzmuskelvolumens müßte sich weiterhin auch bei den Weibchen äußern, wo die Unterschiede in den Methämoglobinspiegeln noch ausgeprägter waren. Diese Diskrepanzen lassen sich nur durch ergänzende Versuche aufklären. Dabei wird besonderes Augenmerk auf die Reaktivität des Herzmuskels gegenüber akuter und chronischer Intoxikation mit MetHb-Bildnern zu legen sein.

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Summary

J. MUSIL: The effect of a chronical sodium nitrite intoxication on rats

1. Drinking water containing 10 or 20 meq. of $NaNO_2$ respectively does not change the total hemoglobin of rats, even not after exposures of 150 and 200 days. The percentual methemoglobin level is enhanced.
2. The reduction of erythrocytes cannot be ensured statistically, whereas the increase of hematocrite and of the hemoglobin content per erythrocyte is significant.
3. The relative heart weight (related to the body weight) of male rats rises significantly under these conditions.
4. The relative spleen weight of female rats is significantly increased.

Резюме

Й. Музиль: Влияние хронической интоксикации нитритом натрия на крыс

1. Содержание в питьевой воде 10 или 20 мэкв $NaNO_2$ не вызывает изменения общего гемоглобина животных, даже не в течение 150 и 200 дней. Процентный уровень метгемоглобина повышен.
2. Уменьшение числа эритроцитов статистически недостоверно, однако повышение гематокрита и содержания гемоглобина на один эритроцит достоверно.
3. При этих условиях относительный вес сердца (отнесенный к весу тела) у самцев достоверно повышается.
4. У крыс-самок относительный вес селезенки достоверно повышен.

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EFFECTS OF $NaNO_2$ IN LONG-TERM EXPERIMENTS II

Authors: J. Musil and Z. Labounková

Source: Československá Hygiona - 12 - 2 - 1967, P. 82 - 88

In the previous article (Ref. 21) we summarized the results of a test in which laboratory rats of the Wistar strain were subjected during 200 days to the effect of a nitrate ion given in potable water in the breeding cages. The summary involved results obtained in examining the basic toxicological indices: weight curves, food and drink consumption, relative weight of the liver, spleen, kidneys and myocardium. Now we want to add the information regarding the results obtained during the hematological investigation of the animals tested.
The literature, providing the theoretical bases for the study of the hematological reaction to hypoxia of the methemoglobin type, is quite extensive already today. Although quite a number of authors studied the hypoxic and anoxic states in relation with the red and white blood picture (Ref. 20, 28, 6, 15, 26, 31, 32, 30, 3), and quite a number of other authors provided information about the results obtained by the hematological examination in different toxicological tests (Ref. 2) in which hypoxia occurred, no study was made of the connection between the chronic methemoglobinemias and the overall blood picture. Since in repeated and chronical methemoglobinemia, which were detected in babies in region with a rather high occurence of nitrates in drinking water, it is necessary to start from an experimental material, we investigated the animals by means of a chronic examination and with the help of basic hematological techniques.

Material and Procedure:

Rats of both sexes of the Wistar strain were used in the tests. They were kept in breeding cages made of polystyrene with a metal lid. A Larsen diet, prepared daily, was used. Drinking liquid was given in glass containers of polysterene. Three groups were used: a control group which drank pipe water, a group which drank a solution 10 m Eqv $NaNO_2$/ 1 liter of water in concentration, and a group which drank a solution of 20 mEqv $NaNO_2$/ 1 liter of water in concentration. The number of animals in the individual cages is given in Table 1.

Table 1: Number of animals in the experimental cages.

The evaluation of the food and drink consumption, of the weight curves and of the relative weights of the organs was reported in the previous article (Ref. 21). Methemoglobin was determined by the Cruze method (Ref. 7). For the hematological determination, the blood was sampled after subjecting the animals to an oxalate mixture evaporator. Only 1 pipette was used, by the same worker; likewise the reading of the blood elements in the Bürker chamber and the differentiation of the leukocytes (for 200 cells) without exception were performed by the same workers. The number of red and white blood corpuscles was determined by the current (? baničková") method and is mentioned for 1 mm^3 of blood. The cyanide method of Drabkin (Ref. 5, 18, 27) was used in examining the hemoglobin. The hematocrit was determined for standard conditions by the micromethod of Pravda with the halp of a Prem device (Ref. 24, 33, 35), centrifugation in 30 minutes, 3000 revolutions. The hemocrit value (%) was not corrected by the coefficient of blood plasma residue. Secondary parameters, such as the control hemoglobin content and the hemoglobin concentration in the erythrocyte (%), the erythrcyte volume (μ^3) and the color index were deduced with the help of the hematological rule (Logarex). The absolute differential values of the leukocyte count were not taken into consideration.

Results:

Overall results are contained in the Table of the hematological investigation. The test groups exhibit lower mean values in the erythrocyte count as compared with the controls. The difference was more marked in the males. The differences lay below the limit of statistical significance (Ery). In assessing the two indices characterizing the amount of blood pigment in the erythrocytes (per cent of hemoglobin - Hb and entire amount of hemoglobin - HbGIM) it was found that the test groups did not have very different average values as compared with the controls. The results obtained in determining the hematocrit values (Hk) and the basic information about the volume of formed blood elements, especially the erythrocytes, show that the effect of the nitrites applied in the test is seen in the important changes of the mean values. In the test animals the hematocrit value increases already after 150 days of application; in this test period, however, no statistical significance can be detected.

After 200 days of exposure, the mean hematocrit values differ in statistical significance in the group of test males which drank 10 mEqv $NaNO_2$ as compared with the control at the 0.05% level, and in the group of test males which drank 20 mEqv $NaNO_2$ at the 0.01% level. A smilar result was found in the females only in the group that drank 20 mEqv $NaNO_2$ (0.01 %). From the data measured we calculated also, for the sake of completeness, the values of the color index (Bi). The mean values in the males did not differ; in the females one observed an unimportant increase in the test groups as compared with the control groups. Quite different results were obtained in calculating the values characterizing the blood pigment content in the individual erythrocytes (OHb). In all the samplings, the groups which drank 10 and 20 mEqv $NaNO_2$ had a higher blood pigment content as compared with the control groups. This increase was systematic and corresponds to the increasing of doses of nitrite. Since the mean values in the test animals were connected with a wide dispersion, we did not calculated the values that were t-higher than the values of the Table. In calculating the volume of the individual erythrocytes (V Ery) we determined the differences in the average of the values obtained for the individual groups. The statistically important difference was recorded in the groups which drank 20 mEqv $NaNO_2$, in the males after 150 days at the 0.05% level, after 200 days at the 0.01 % level, in the females after 200 days at the 0.05% level.

In the analysis of the mean values of the blood pigment concentration (KHb) it is obvious that the effect of the administered nitrite ion in the index does not appear in 150 days. The mean values of the blood pigment concentration, however, differ markedly in the individual groups of the test animals after 200 days of experimentation. The mean values are lower than for the control group; in the males and in the females one finds a statistically important difference in the groups which drank 20 mEqv $NaNO_2$; but no significant difference was found in the groups which drank 10 mEqv $NaNO_2$. The decrease of the values is systematic. A lower dose of this index is reflected by a lesser effect than the one of a higher dose (Table 2).

In assessing the average amount of leukocytes, it is possible to conclude that after 200 days of exposure, the number of leukocytes in the test groups decreases; the difference in the t-test evaluation are not statistically

significant for the same dispersion of all the values measured. The mean amount of segmentary elements differed also after 200 days of exposure. In the two sexes there was a decrease of the measured values; in the females which drank 20 mEqv $NaNO_2$ the difference was important at the 0.05% level, whereas in the males at a high value of t = 1.961 there was no great statistically significant difference. The amount of T elements in the test groups, because of the absolutely unsystematic condition of the amounts founds, cannot be related in causale dependence on the effect determined in the test. The average number of eosinophil elements (E) cannot be evaluated as an index of the mechanisms connected with nitrite contamination. In the experimental groups there was a decrease of the mean values. These decreases, however, are not proportional to the doses administered, but to the difference in the test period. The highest deviations were noted in the females after 200 days of exposure. Because of the dispersion, it was not possible to detect a statistical significance. No difference in the averages of basophil elements (Ba) was observed. In the monocyte group (Mo) important differences were found in the males after 150 days of exposure, when there was a significant decrease of corpuscles, the difference being significant at the 0.01% level. Since after 200 days of exposure, no similar result was found in the females, this result must be considered as accidental. In the group the average number of lymphocytes (Ly) in the males and females after 200 days of exposure increased; in the males and females, which drank a higher dose, that is 20 mEqv $NaNO_2$, this increase was statistically significant when evaluation was performed by the t-test.

Discussion:

The nitrite ions are regarded as substances which exert a powerful oxidative effect on the iron of the heme component of hemoglobin . The oxidation of divalent Fe is as follows:

$$4\ NO_2 + 4\ Fe^{II}HbO_2 \text{----------} 4\ NO_3 = 4\ Fe^{III}HbOH + O_2 \quad (19)$$

It is usually thought that at low concentrations the nitrite oxidation does not cause any deterioration of the bond between globine and heme - with the histidine (?) group in the globin molecule. Only in some cases was it possible to prove the production of Heinz bodies in vivo (Ref. 11, 12, 22, 25). Tests in vivo,

closely comparable with the rates in the living organism as to the concentrations used, revealed the production of Heinz bodies only in blood corpuscles with a glucoso-6-phosphate dehydrogenase deficit or in the case of insufficient glucose in the incubation medium (Ref. 14). In the literature accessible, there is no study that might solve the dependence of the destruction of the erythocytes containing methemoglobin in the case of physiological amounts (Ref. 19, 22, 23) as well as of amounts exceeding the physiological limit. In connection with the fact that in chronical nitrite poisonings, a degradation of hemoglobin through Heinz bodies can only be assumed as an exception, it was necessary to examine not only the indices describing the rates between the individual elements of the blood, but also their size and their hemoglobin contents. The reaction of the blood picture observed in the tests described is the obvious response to prolonged hypoxia; in connection with the large amount of studies which exclude the effect of the redox system by the system of oxidized and reduced glutathione (Ref. 4, 8, 10), it is not possible even in our tests to assume intervention possibility in this system.

In the experimental animals, subjected to the tests previously referred, nitrite ion exposures were performed in two doses and in two periods; the toxic effect in each sampling increased in time and in dose. The difference in the blood picture values would then exist in addition to the probability significance and to the systematic gradation. In the experimental animals the erythrocyte values were always lower than in the controls. Since the differences were not important, it cannot be considered as proved that the chronical administration of ionite ions lowers the absolute erythrocyte counts, but the difference found requires attention in suitable further studies which should involve the examination of the other biological indices, like the length of life of the erythrocytes, the osmotic resistance, the alkaline resistance and the acid resistance. In other tests, the functional state of the active bone marrow should be determine. Considerable attention was given to the tests involving the determination of the hemoglobin content of the erythrocytes of the rats experimented. In all the indices, no important difference was found in the individual group averages; hence it must be assumed that the compensation of the decrease of the total amount of blood pigment during the formation of methemoglobin is not obtained through the formation of more blood pigment. In connection with the toxic action, the values of

the hematocrit changed to such extent that the groups tested always exhibited higher values as compared with the controls. The differences were systematic and involved the highest doses and the longest time periods. They were always statistically important at the 0.05% and 0.01% levels. In accordance with this fact, we obtained differences in the values calculated for the volume of the individual erythrocyte. As a function of the increase of the volume of the individual erythoryte, the concentration of hemoglobin in the individual erythrocyte decreased systematically also. Thus, it can be concluded that with an unimportant decrease of the number of red corpuscles and an important increase of the hematocrit values, the main mechanism compensating the decrease of the blood pigment capable of transferring O is extended to the entire surface area of the erythrocytes, hence to the area available to the organism for the saturation (? "syceni") and production of O. This provides basic information about the pathological mechanisms by which the entire organism reacts to a long-term administration of Na nitrite, a compound which forms methemoglobin.

In the evaluation of the indices of the white blood components, an important difference was found in the amount of segmentary leukocytes in the animal subjected to the effect of a higher dose during 200 days. The entire values of the leukocyt s did not reveal a dependence on the compound administered. Other indices in the white blood picture did not differ in the groups of tested animals and of controls. In the tested animals, with a higher dose and a longer experimental period, one found a relative lymphocytosis that was statistically important. The changes in the values of the white blood picture can hardly be regarded as a manifestation of the direct toxic action of the oxidizing compound, as Na nitrite is.

The results obtained show that in the hematological evaluation of the test described there is no important difference between the sexes. The lower number of important differences in the hematological picture for the discrepancy from the nonspecific findings in the organisms, since the females were much more affected, can mean that the females possess less adaptability to toxic conditions.

The statistical significance for all the animals tested, without any difference as to sex, was tested by Engineer Picko, head of the statistical section of the Prague Hygiene Institute, who also confirmed the results as a whole.

English Summary at the beginning of the Czech text.

ÚČINKY DUSITANU SODNÉHO V DLOUHODOBÉM POKUSU. II.

Действие азотистокислого натрия в долговременном опыте. II.

Effects of $NaNO_2$ in Long-term Experiment. II.

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Technická spolupráce J. Zinkeová, V. Zahradníková, V. Šramotová

Выводы: На 121 крысе обоего пола исследовалась долговременная токсичность азотистокислого натрия, вещества, энергично образующего метгемоглобин. При гематологическом исследовании крови подопытных животных было установлено, что по истечении экспериментального периода 150 и 200 дней концентрация гемоглобина не изменяется и нет значительного различия в количестве эритроцитов у подопытных животных в сравнении с контрольными, но статистически достоверно повышается гематокрит и объем отдельных эритроцитов. На основании полученных результатов авторы высказывают гипотезу, что относительный макроцитоз является физиологическим механизмом, компенсирующим интермиттирующую гипоксию метгемоглобинового типа. При исследовании элементов белой крови было установлено, что статистически достоверно понижается у подопытных самок количество сегментированных лейкоцитов, а у самцов повышается число лимфоцитов.

Čs. Hyg., 12, 1967, 2: 82—88.

Summary: The long-term toxicity of $NaNO_2$ — a substance producing with a great energy methemoglobin — was tested in 121 rats of both female and male sex. The hematologic examining the blood of experimental animals has proved: 1. the Hb concentration does not change after an experimental period of 150 to 200 days, 2. the erythrocytes number does not differ from that of controls, 3. but the hematocrit and volume of individual erythrocytes are rising up significantly. Based on the results achieved an hypothesis is expressed: the relative macrocytosis represents a physiologic mechanism compensating the intermitent hypoxia of methemoglobin type. The following up the elements of white blood row demonstrated, that the quantity of segmented leukocytes is significantly decreasing, the number of lymphocytes is increasing by long-term action of $NaNO_2$.

Čs. Hyg., 12, 1967, 2: 82—88.

V předešlém sdělení[21] byly shrnuty výsledky pokusu, ve kterém byly laboratorní krysy kmene Wistar vystaveny po 200 dnů vlivu dusitanového iontu podávaného v pitné vodě do chovných klecí. V přehledu byly obsaženy výsledky dosažené při sledování základních toxikologických ukazatelů: váhových křivek, spotřeby potravy a pití, relativní váhy jater, sleziny, ledviny a myokardu. V tomto sdělení chceme podat zprávu o výsledcích získaných při hematologickém vyšetření pokusných zvířat. Literatura, ze které je možno získat teoretické podklady pro studium hematologické reakce na hypoxie methemoglobinového typu, je dnes už velmi obšírná. Přesto, že celá řada autorů studovala hypoxické a anoxické stavy ve vztahu k červenému a bílému krevnímu obrazu[20 28 6 15 26 31 32 30 34 3] a celá řada dalších autorů podala zprávu o výsledcích získaných he-

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matologickým vyšetřením při různých toxikologických pokusech[2], u nichž dochází k hypoxiím, nebyla zatím studována souvislost chronických methemoglobinémií s celkovým obrazem krevním. Vzhledem k tomu, že u opakovaných a chronických methemoglobinémií, které byly prokázány u kojenců v oblastech s vyšším výskytem dusičnanů v pitných vodách, je nutno vycházet též z experimentálního materiálu, vyšetřili jsme pokusná zvířata z chronického pokusu[21] také pomocí základních hematologických technik.

Materiál a metodika

Pokusy byly provedeny na krysách obého pohlaví kmene Wistar. Zvířata byla chována v chovných klecích z polystyrenu s kovovým víkem, k výživě byla použita Larsenova dieta připravovaná pro každý den, pití bylo podáváno ve skleněných nádobách s polystyrenovými savkami. V pokusu byly zařazeny tři skupiny krys: skupina kontrolní, která pila vodovodní vodu, skupina, která pila roztok o koncentraci 10 mEkv $NaNO_2$/1 litr vody a skupina, která pila roztok o koncentraci 20 mEkv $NaNO_2$/1 litr vody. Počet zvířat v jednotlivých skupinách je uveden v tabulce 1.

Tab. 1. Počty zvířat v pokusných skupinách

Expozice dnů	Kontrolní skupina		10 mEKv		20 mEkv	
	samci	samice	samci	samice	samci	samice
150	10	10	8	9	10	10
200	10	10	10	9	12	13

Hodnocení spotřeby potravy a pití, váhových křivek a relativních vah orgánů bylo provedeno v předešlém sdělení[21]. Methemoglobin byl stanovován metodou podle Cruze[7]. K hematologickým stanovením byla odebírána krev po podstřižení zvířete do odparku směsi oxalátu. Rozpipetováno bylo jednou a toutéž pipetou, týmž pracovníkem, rovněž počítání krevních elementů v Burkerově komůrce a diferenciace leukocytů (na 200 buněk) bez výjimky prováděli tíž pracovníci. Počet červených a bílých krvinek byl stanovován běžnou baničkovou metodou a je udán na 1 mm^3 krve. K vyšetření hemoglobinu byla použita cyanmethemoglobinová metoda Drabkinova[5,15,27]. Hematokrit byl vyšetřován za standardních podmínek mikrometodou podle Pravdy pomocí aparatury Prema[24,33,35], centrifugace 30 min., 3000 obrátek. Hodnota hematokritu (%) nebyla korigována koeficientem rezidua krevní plazmy. Vedlejší parametry jako střední obsah hemoglobinu a koncentrace hemoglobinu v erytrocytu (%), objem erytrocytu (μ^3) a barevný index byly odečteny pomocí hematologického pravítka (Logarex). Absolutní diferenciální hodnoty počtu leukocytů v přehledu neuvádíme.

Výsledky

Přehledné výsledky jsou obsaženy v souhrnné tabulce hematologického vyšetření. Pokusné skupiny vykazovaly nižší průměrné hodnoty počtu erytrocytů proti zvířatům kontrolním, rozdíl byl výraznější u samců, rozdíly ležely pod hranicí statistické významnosti (Ery). Při posuzování obou ukazatelů charakterizujících množství krevního barviva v erytrocytech (procento hemoglobinu-Hb a celkové množství hemoglobinu-HbGIM) bylo zjištěno, že pokusné skupiny neměly výrazně odlišné průměrné hodnoty proti kontrolám. Výsledky stanovení hematokritových hodnot (Hk), jako základní informace o objemu formovaných elementů krevních, tedy především erytrocytů, ukazují, že se účinek dusitanů aplikovaných v pokusu projevuje ve významných změnách průměrných hodnot. U pokusných zvířat se zvětšuje hematokritová hodnota již po 150 dnech aplikace, v tomto pokusném období však není možno prokázat statistickou významnost. Po 200 dnech expozice se průměrné hematokritové hodnoty liší statisticky vý-

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znamně u skupiny pokusných samců, kteří pili 10 mEkv $NaNO_2$ proti kontrolám na 0.05 % hladině a u skupiny pokusných samců, kteří pili 20 mEkv $NaNO_2$ na 0,01% hladině. Obdobný výsledek byl získán u samic pouze u skupiny, která pila 20 mEkv $NaNO_2$ (0,01 %). Ze změřených údajů byly také pro úplnost počítány hodnoty barvivového indexu (Bi). Průměrné hodnoty u samců v pokusu se nelišily, u samic byl pozorován nevýznamný vzestup u pokusných skupin v porovnání se skupinami kontrolními. Výrazně odlišné výsledky byly získány při výpočtu hodnot charakterizujících obsah krevního barviva v jednotlivých erytrocytech (OHb). Ve všech odběrech měly skupiny, které pily 10 a 20 mEkv $NaNO_2$ vyšší obsah krevního barviva ve srovnání se skupinami kontrolními. Tento vzestup byl systematický a odpovídal stoupajícím dávkám dusitanu. Vzhledem k tomu, že průměrné hodnoty u pokusných zvířat

Tab. 2. Samci

Skupina		K				
		ø	ø	ts	ø	ts
Ery	150	6643 ± 307,89	6026 ± 284,7	NS	5908 ± 366,75	NS
	200	5650 ± 284,90	5303 ± 229,30	NS	4840 ± 247,7	NS
Leuko	150	6390 ± 511,44	8588 ± 249,9	NS	5707 ± 315,0	NS
	200	5650 ± 752,32	5303 ± 753,76	NS	4838 ± 783,7	NS
Hb%	150	97,9 ± 4,03	93,2 ± 5,16	NS	99,7 ± 1,34	NS
	200	99,0 ± 1,35	−0 ± 1,182	NS	99,1 ± 1,87	NS
Hk	150	42,6 ± 2,5	41,5 ± 1,74	NS	45,1 ± 0,681	NS
	200	41,4 ± 1,318	45,1 ± 0,681	0,05	48,8 ± 1,362	0,01
HbGIM	150	15,6 ± 0,65	14,8 ± 0,836	NS	15,9 ± 0,2125	NS
	200	15,7 ± 0,2125	15,6 ± 0,1973	NS	15,8 ± 0,307	NS
Bi	150	0,74 ± 0,0378	0,78 ± 0,0225	NS	0,86 ± 0,0411	NS
	200	0,89 ± 0,0512	0,94 ± 0,0457	NS	1,04 ± 0,687	NS
OHb	150	23,7 ± 1,2574	25,1 ± 0,59	NS	27,8 ± 1,402	0,05
	200	28,5 ± 1,453	29,9 ± 1,39	NS	32,8 ± 2,013	NS
V Ery	150	65,0 ± 4,542	72,0 ± 2,739	NS	78,0 ± 4,61	0,05
	200	75,2 ± 5,954	89,55 ± 5,446	NS	102,96 ± 4,91	0,01
KHb	150	38,8 ± 1,245	41,0 ± 1,850	NS	40,0 ± 1,721	NS
	200	37,4 ± 1,666	33,9 ± 0,820	NS	32,6 ± 0,908	0,01
S	150	141, ± 1,586	15,5 ± 2,415	NS	17,0 ± 2,594	NS
	200	26,1 ± 4,23	23,0 ± 3,80	NS	16,9 ± 1,98	NS
T	150	1,0 ± 0,189	1,3 ± 0,351	NS	0,6 ± 0,164	NS
	200	0,5 ± 0,173	0,6 ± 0,219	NS	0,3 ± 0,109	NS
E	150	2,5 ± 0,781	2,3 ± 0,451	NS	2,3 ± 0,696	NS
	200	2,0 ± 0,599	2,4 ± 0,040	NS	1,7 ± 0,383	NS
Ba	150	0,15 ± 0,106	0,25 ± 0,189	NS	0,20 ± 0,122	NS
	200	0,35 ± 0,48	0,35 ± 0,195	NS	0,404 ± 0,046	NS
Mo	100	2,7 ± 0,109	2,6 ± 0,403	NS	1,0 ± 0,114	0,01
	200	0,3 ± 0,151	0,5 ± 0,229	NS	0,3 ± 0,182	NS
Ly	150	79,4 ± 2,89	77,9 ± 2,695	NS	78,6 ± 3,095	NS
	200	70,5 ± 4,29	75,0 ± 3,680	NS	80,5 ± 1,930	0,05
MHb	150	0,90 ± 0,090	2,83 ± 1,30	NS	1,71 ± 0,305	NS
	200	1,32 ± 0,397	2,09 ± 0,305	NS	4,42 ± 1,55	NS

Ery = počet erytrocytů, Leuko = počet leukocytů, Hb% = hemoglobin v procentech, Hk = hematokrit, HbGIM = hemoglobin v g%, Bi = barevný index, OHb = obsah hemoglobinu v červené krvince v gama-gama, V Ery = objem červené krvinky (μ³), KHb = koncentrace

byly spojeny s široký
noty tabulkové. Tak
byly zjištěny rozdily
tisticky významný ro
u samců po 150 dne
u samic po 200
hodnot koncentrace k
dusitanového iontu v
hodnoty koncentrace
pokusných zvířat po
skupině kontrolní, u
u skupin, které pily

Skupina		
Ery	150	584
	200	525
Leuko	150	433
	200	685
Hb%	150	95
	200	95
HK	150	39
	200	42
HbGIM	150	15
	200	15
Bi	150	0,8
	200	0,9
OHb	150	26
	200	18
V Ery	150	68
	200	80
KHb	150	40
	200	36
S	150	16
	200	26
T	150	1
	200	0
E	150	4
	200	2
Ba	150	0
	200	0,0
Mo	150	1
	200	0
Ly	150	77
	200	75
MHb	150	1,8
	200	1,0

hemoglobinu v erytro
Ba = počet bazofilů, M
na 200 buněk v %, MHb

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byly spojeny s širokým rozptylem, nebyly vypočítané hodnoty t- vyšší než hodnoty tabulkové. Také při propočtu objemu jednotlivého erytrocytu (V Ery) byly zjištěny rozdíly v průměru výsledných hodnot jednotlivých skupin. Statisticky významný rozdíl byl zaznamenán u skupin, které pily 20 mEkv $NaNO_2$, u samců po 150 dnech na 0,05% hladině, po 200 dnech na 0,01% hladině, u samic po 200 dnech na 0,05% hladině. Při rozboru průměrných hodnot koncentrace krevního barviva (KHb) je zřejmé, že se účinek podaného dusitanového iontu v tomto ukazateli neprojevuje v období 150 dnů. Průměrné hodnoty koncentrace krevního barviva se však výrazně liší u jednotlivých skupin pokusných zvířat po 200 dnech pokusu. Průměrné hodnoty jsou nižší proti skupině kontrolní, u samců i u samic byl zjištěn statisticky významný rozdíl u skupin, které pily 20 mEkv $NaNO_2$; ale nevýznamný rozdíl byl zjištěn

Female

Tab. 2. Samice

Skupina Group		K				
		ø	ø	t_s	ø	t_s
Ery	150	5842 ± 341,0	5700 ± 123,5	NS	5731 ± 258,5	NS
	200	5259 ± 259,7	5177 ± 209,5	NS	5174 ± 197,4	NS
Leuko	150	4355 ± 574,2	5657 ± 480,5	NS	8477 ± 1440,3	NS
	200	6833 ± 1019	6429 ± 889,0	NS	4530 ± 1086,5	NS
Hb%	150	95,2 ± 2,38	93,5 ± 2,836	NS	97,8 ± 6,03	NS
	200	95,3 ± 1,253	93,8 ± 4,41	NS	96,8 ± 2,307	NS
K	150	39,8 ± 1,202	42,0 ± 1,48	NS	42,1 ± 1,665	NS
	200	42,2 ± 0,645	42,6 ± 2,316	NS	46,7 ± 1,135	0,01
HbGIM	150	15,2 ± 0,368	15,1 ± 0,432	NS	15,6 ± 0,757	NS
	200	15,2 ± 0,201	15,0 ± 0,694	NS	15,5 ± 0,363	NS
Bi	150	0,83 ± 0,0453	0,83 ± 0,0338	NS	0,83 ± 0,01216	NS
	200	0,90 ± 0,0252	0,90 ± 0,0271	NS	0,95 ± 0,413	NS
OHb	150	26,4 ± 1,44	26,4 ± 1,046	NS	27,5 ± 1,215	NS
	200	18,9 ± 0,83	28,9 ± 0,864	NS	30,4 ± 1,340	NS
V Ery	150	68,0 ± 4,472	72,91 ± 3,591	NS	73,7 ± 2,242	NS
	200	80,2 ± 1,600	81,40 ± 3,770	NS	91,9 ± 4,235	0,05
KHb	150	40,4 ± 0,906	38,4 ± 1,517	NS	40,2 ± 1,262	NS
	200	36,1 ± 0,382	35,3 ± 0,975	NS	30,5 ± 0,982	0,01
S	150	16,6 ± 3,71	13,7 ± 3,29	NS	12,6 ± 2,762	NS
	200	20,3 ± 3,103	17,7 ± 3,23	NS	12,5 ± 1,710	0,05
T	150	1,2 ± 0,187	1,3 ± 0,374	NS	1,1 ± 0,245	NS
	200	0,4 ± 0,109	0,8 ± 0,118	NS	0,3 ± 0,118	NS
E	150	4,1 ± 0,442	3,7 ± 0,747	NS	3,0 ± 0,756	NS
	200	2,5 ± 0,223	2,0 ± 0,428	NS	1,6 ± 0,300	NS
Ba	150	0,1 ± 0,070	0,21 ± 0,089	NS	0,1 ± 0,070	NS
	200	0,05 ± 0,145	0,20 ± 0,122	NS	0,0	NS
Mo	150	1,6 ± 0,447	2,0 ± 0,586	NS	1,7 ± 0,442	NS
	200	0,8 ± 0,181	0,9 ± 0,216	NS	0,9 ± 0,170	NS
Ly	150	77,3 ± 3,980	80,9 ± 4,280	NS	80,6 ± 3,58	NS
	200	75,5 ± 2,700	78,3 ± 3,35	NS	84,2 ± 2,21	NS
MHb	150	1,07 ± 0,229	2,13 ± 0,271	0,01	5,34 ± 1,91	0,05
	200	1,09 ± 0,397	1,65 ± 0,250	NS	2,77 ± 0,432	0,05

hemoglobinu v erytrocytu v %, S = segmentů počet, T = počet tyčí, E = počet eozinofilů, Ba = počet bazofilů, Mo = počet monocytů, Ly = počet lymfocytů v diferenciálním rozpočtu na 200 buněk v %, MHb = procento methemoglobinu z celkového krevního barviva.

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u skupin, které pily 10 mEkv $NaNO_2$. Pokles hodnot je systematický, nižší dávka se v tomto ukazateli projevuje menším účinkem než dávka vyšší (tab. 2).

Při hodnocení průměrných množství leukocytů (L) lze dojít k závěru, že po 200 dnech expozice se u pokusných skupin snižuje počet leukocytů, rozdíly při hodnocení t-testem nejsou statisticky významné při stejném rozptylu všech naměřených hodnot. Průměrná množství segmentovaných elementů (S) se také lišila až po 200 dnech expozice, u obou pohlaví došlo k poklesu měřených hodnot, u samic, které pily dávku 20 mEkv $NaNO_2$, je rozdíl významný na 0,05% hladině, zatímco u samců i při vysoké hodnotě t = 1,961 není možno mluvit o prokázaném statisticky významném rozdílu. Stanovené množství tyčkových elementů (T) u pokusných skupin není možno vzhledem k naprosté nesystematičnosti zjištěných množství uvádět do příčinné závislosti na vlivech sledovaných v pokusu. Také průměrné počty eozinofilních elementů (E) se nedají hodnotit jako ukazatel mechanismů souvisejících s chronickou dusitanovou intoxikací. U pokusných skupin sice došlo k poklesu průměrných hodnot, tyto poklesy nejsou však úměrné podávaným dávkám a rozdílu v délce pokusného období. Nejzávažnější odchylky byly zjištěny u samic po 200 dnech expozice, vzhledem k rozptylu nebylo možno mluvit o statistické významnosti. Odlišnost průměrů nebyla pozorována ani u bazofilních elementů (Ba). Ve skupině monocytární (Mo) byly zjištěny významné rozdíly u samců po 150 dnech expozice, kdy došlo k významnému poklesu zjištěných tělísek, při čemž rozdíl byl významný na 0,01% hladině. Vzhledem k tomu, že po 200 dnech expozice u samic nebyl podobný výsledek zachycen, je nutno tento nález považovat za náhodný. Ve skupině lymfocytů (Ly) se u samců a samic po 200 dnech expozice zvýšil průměrný počet; u samců i u samic, které pily větší dávku, tj. 20 mEkv $NaNO_2$ byl při hodnocení t-testem tento vzestup statisticky významný.

Diskuse

Dusitanové ionty patří mezi látky, které mají mohutný oxidační účinek na železo hemové složky hemoglobinu. Oxidace dvojmocného železa probíhá podle reakčního schématu

$$4\ NO_2 + 4\ Fe^{III}HbO_2 \text{ ---------- } 4\ NO_3 - 4\ Fe^{III}HbOH + O_2 \quad (19)$$

Obecně je přijímána představa, že za nízkých koncentrací nezpůsobuje dusitanová oxidace porušení vazby globinu s hemem — s histidinovou skupinou v globinové molekule. Jen za některých okolností bylo prokázáno vytvoření Heinzových tělísek in vivo[11 12 22 25]. Pokusy in vitro těžko srovnatelné s poměry v živém organismu vzhledem k výšce použitých koncentrací prokázaly tvorbu Heinzových tělísek pouze u krvinek s deficitem glukózo-6-fosfát dehydrogenázy nebo při nedostatku glukózy v inkubačním médiu[14]. V dostupné literatuře zatím není studie, která by řešila závislost destrukce erytrocytů obsahujících methemoglobin jako při fyziologických množstvích[19 22 23], tak také při množstvích zvýšených nad fyziologickou mez. Vzhledem k tomu, že u chronických dusitanových intoxikací je možno jen výjimečně předpokládat degradaci hemoglobinu přes Heinzova tělíska, bylo nutno sledovat nejen ukazatele popisující vztahy mezi jednotlivými elementy krevními, ale také jejich velikost a zásobení hemoglobinem. Reakce krevního obrazu pozorovaná v popsaných pokusech je zřejmě odpovědí na prolongovanou hypoxii; vzhledem k velkému množství prací, které vyloučily ovlivnění oxidoredukčního systému přes systém oxidovaného a redukovaného glutathionu[4 8 10] není možno ani v našich pokusech předpokládat možnost zásahu v tomto systému.

U pokusných zv
reterovali, jsme pro
dvou obdobích, takže
tak i v dávce. Odli
pravděpodobnostní v
cytů byly stanoveny
trolních. Vzhledem k
za prokázané, že chr
erytrocytů, ale zjišt
dalších studiích, ve
jako je délka života
rezistence. V dalších
dřeně. Hlavní pozor
obsah hemoglobinu v
zjištěna významná o
předpokládat, že ko
tvorbě methemoglob
laci s toxickým pů
V korelaci s toxickým
skupiny vykazovaly v
žely systematicky a
statisticky významné
ním jsme obdrželi r
trocytu. V závislosti
maticky také koncen
umožňují vytvoření
nek a významném v
kompenzujícím úbyt
kové plochy povrchu
zvířat k dispozici pro
o patofyziologických
dobé podávání dusit

Při hodnocení u
liší množství segme
vyšší dávky po 200
na podávané látce.
ných i kontrolních
pokusném období zj
ke kterým došlo v ho
jako projev přímého

Z předložených
psaném pokusu není
odlišností v hemato
gánech, kdy byly sa
způsobivost samic t

Statistickou výz
pin podle pohlaví te
potvrdil v celém roz

Šesti skupinám
10 a 20 mEkv/1 ht

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U pokusných zvířat, která byla vyšetřena v pokusech, o nichž jsme výše referovali, jsme provedli expozici dusitanovými ionty dvěma dávkami a ve dvou obdobích, takže toxický vliv se při každém odběru stupňoval jak v čase, tak i v dávce. Odlišnost hodnot krevního obrazu by měla mít tedy i vedle pravděpodobnostní významnosti i systematickou gradaci. V hodnotách erytrocytů byly stanoveny u pokusných zvířat vždy hodnoty nižší než u zvířat kontrolních. Vzhledem k tomu, že rozdíly byly nevýznamné není možno považovat za prokázané, že chronické podávání dusitanových iontů snižuje absolutní počty erytrocytů, ale zjištěným rozdílům je nutno věnovat pozornost při případných dalších studiích, ve kterých by bylo vhodné sledovat další biologické ukazatele jako je délka života erytrocytů, osmotická rezistence a alkalirezistence a acidorezistence. V dalších pokusech by bylo nutné vyšetřit i funkční stav aktivní kostní dřeně. Hlavní pozornost byla v pokusech věnována výsledkům, které udávaly obsah hemoglobinu v erytrocytech pokusných krys. Ve všech ukazatelích nebyla zjištěna významná odlišnost jednotlivých skupinových průměrů a je tedy nutno předpokládat, že kompenzace úbytku celkového množství krevního barviva při tvorbě methemoglobinu neprobíhá výstavbou dalšího krevního barviva. V korelaci s toxickým působením se měnily hodnoty hematokritu pokusných zvířat. V korelaci s toxickým působením se měnily hodnoty hematokritu tak, že pokusné skupiny vykazovaly vždy vyšší hodnoty proti zvířatům kontrolním, rozdíly ležely systematicky a při nejvyšší dávce a v nejdelším časovém období byly vždy statisticky významné na 0,05% a 0,01% hladině. V souhlase s tímto stanovením jsme obdrželi rozdíly ve vypočtených hodnotách objemu jednotlivého erytrocytu. V závislosti na zvětšení objemu jednotlivého erytrocytu klesala systematicky také koncentrace hemoglobinu v jednotlivém erytrocytu. Tyto údaje umožňují vytvoření závěru, že při nevýznamném poklesu počtu červených krvinek a významném vzestupu hematokritových hodnot, je hlavním mechanismem kompenzujícím úbytek krevního barviva schopného přenášet kyslík zvětšení celkové plochy povrchu erytrocytů, tedy plochy, kterou má organismus pokusných zvířat k dispozici pro sycení a výdej kyslíku. Toto zjištění je základní informací o patofyziologických mechanismech, jakými reaguje savčí organismus na dlouhodobé podávání dusitanu sodného, látky tvořícího methemoglobin.

Při hodnocení ukazatelů bílé krevní složky bylo zjištěno, že se významně liší množství segmentovaných leukocytů u zvířat, které byla vystavena vlivu vyšší dávky po 200 dnů. Celkové hodnoty leukocytů neprokazovaly závislost na podávané látce. Jiné ukazatele v bílém obraze krevním se u skupin pokusných i kontrolních nelišily. U pokusných zvířat byla po vyšší dávce a delším pokusném období zjištěna relativní lymfocytóza statisticky významná. Změny, ke kterým došlo v hodnotách bílého obrazu krevního, mohou být stěží hodnoceny jako projev přímého toxického působení oxidační látky, jako je dusitan sodný.

Z předložených výsledků je zřejmé, že v hematologickém hodnocení v popsaném pokusu není závažný rozdíl mezi pohlavími. Menší počet významných odlišností v hematologickém obraze na rozdíl od nespecifických nálezů na orgánech, kdy byly samice mnohem vážněji postiženy[21], může znamenat nižší přizpůsobivost samic toxickým podmínkám.

Statistickou významnost pro všechna pokusná zvířata bez rozdělení do skupin podle pohlaví testoval ing. Picko, vedoucí statistického oddělení ÚH Praha a potvrdil v celém rozsahu uvedené výsledky.

Souhrn

Šesti skupinám krys kmene Wistar byl podáván dusitan sodný v roztoku 10 a 20 mEkv/l litr pitné vody. Při hematologickém vyšetření po 150 a 200

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dnech pokusu byly zjištěny tyto odchylky průměrných hodnot pokusných zvířat proti kontrolám: 1. snižuje se nevýznamně počet erytrocytů; 2. nemění se úhrnné množství hemoglobinu; 3. zvětšují se hodnoty hematokritu; 4. zvětšuje se objem erytrocytů; 5. snižuje se koncentrace hemoglobinu v jednotlivém erytrocytu; 6. snižuje se počet segmentovaných leukocytů; 7. zvyšuje se počet leukocytů; 8. nebyl zjištěn závažný rozdíl mezi pohlavími.

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jejich rozlišování. Použité chromatografické metody jsou na současné úrovni této techniky, byly voleny správně a se znalostí problematiky a jsou doplněny řadou praktických a cenných poznatků. Lze předpokládat, že je autoři doplní některými dalšími technikami chromatografie na tenké vrstvě silikagelu. Spektrofotometrické a polarografické metody jsou potom vhodným doplňkem pro posouzení identity a kvality barviva.

Příručka je psána přehledně a jednotlivé otázky srozumitelně vysvětlovány. Věcných chyb (jako na str. 34 — SO_3H skupina sulfhydrylová místo sulfonová) jsem mnoho nenašel. Jako diskutabilní se mi jeví skutečnost, že „experimentální část" je psána formou původní práce. Ukazuje tak sice názorně na přístup k řešení problému, nevýhodou je však, že pracovníci s nižším odborným vzděláním se budou obtížněji orientovat při používání těchto metod, majíce třeba jiná zařízení apod. Pro tento případ by bylo vhodnější použít formy doporučených postupů s poukazem na to, co je nutno dodržovat.

Závěrem mohu konstatovat, že příručka přináší mnoho cenných poznatků pro všechny pracovníky zabývající se hygienickou a analytickou problematikou barviv v potravinách a lze ji proto vřele doporučit.

J. Gasparič.

Výzk. ústav org. syntéz, Pardubice-Rybitví

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J. Gasparič,
, Pardubice-Rybitví

ČESKOSLOVENSKÁ HYGIENA — 12 — 2 - 1967

PŘEŽÍVÁNÍ KMENE PSEUDOMONAS AERUGINOSA V RŮZNÝCH DRUZÍCH PLEŤOVÝCH MLÉK

Pseudomonas aeruginosa в разных видах молока для лица

Surviving of Pseudomonas Aeruginosa Strain in Various Sorts of Complexion Milk

M. Žežulková

Ústav hygieny, Praha

Выводы: Исследовалась возможность выживаемости и возможного размножения штамма Pseudomonas aeruginosa в шести видах молока для лица. Результаты исследования показали, что только в одном виде молока для лица была возможна выживаемость и размножение этого штамма в течение не менее пяти недель, в то время, как в остальных видах молока для лица этот штамм не выживал дольше 48 часов. Результаты ясно показали важное значение постановки пробы размножения штамма Pseudomonas aeruginosa во всех новых косметических изделиях до внедрения их в производство.

Čs. Hyg., 12, 1967, 2 : 89—92.

Summary: The possibility of survival and potential reproductive multiplication of Pseudomonas aeruginosa in 6 sorts of complexion milk were submitted to analysis. The experimental results indicated, that only one sort of complexion milk permited the surviving and reproductive multiplication of this strain during the period of minimum 5 weeks, whereas in other sorts of complexion milk it did not survive longer that 48 hours. The results have shown the clear-cut importance to perform the multiplication test with the Pseudomonas aeruginosa strain in all cosmetic products before their introducing into the mass production.

Čs. Hyg., 12, 1967, 2 : 89—92.

V roce 1965 bylo v n. p. Kozmetika znehodnoceno několik šarží jednoho druhu pleťového mléka masivní kontaminací kmenem Pseudomonas aeruginosa. Mikrobiologické vyšetření použitých surovin bylo negativní. Vyšetřením vzorků z výrobní a plnicí linky byl zdroj znečištění lokalizován v jedné části potrubí plnicí linky. Na téže plnicí lince však byly ve stejném období plněny i jiné druhy pleťových mlék, u nichž nebyl v žádném případě kmen Pseudomonas aeruginosa prokázán. Na základě zjištěných skutečností bylo možno předpokládat, že příčinou přežívání a pomnožování kmene Pseudomonas aeruginosa v tomto pleťovém mléku je vlastní složení výrobku, který je zřejmě příznivým prostředím pro tento mikrobiální kmen. Abychom potvrdili tento předpoklad, provedli jsme laboratorní pokus s umělou kontaminací 6 druhů pleťových mlék tímto kmenem.

Materiál a metodika

Pokusy byly prováděny se šesti druhy pleťových mlék, vyráběnými v n. p. Kozmetika, závod Holešovice. Způsob přípravy všech použitých mlék byl zhruba stejný, suroviny nekontaminované, mléka obsahovala konzervační přípravek a byla plněna na téže plnicí lince. Všechna pleťová mléka byla před zahájením

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EFFECTS OF $NaNO_2$ IN LONG-TERM EXPERIMENTS. III

Authors: J. Musil, K. Nejedlý

Source: Československá Hygiena - 12 - 7 - 1967, P. 381 - 384

Introduction:

In the two preceding investigations (Ref. 7 and 8), a report was made about the results of a long-term test in which laboratory animals (rats) were subjected to the chronic action of Na nitrite, that is a compound producing methemoglobinemias. Histological examinations were conducted in order to confirm the toxicological and hematological findings as a whole. In the present investigation, we publish the histological findings obtained through the examination of preparations from the organisms of the animals tested.

In the extensive literature devoted to the problems of the physiology and toxicology of the substances that form methemoglobin, no investigation has been conducted about the histological effects on the individual organisms. If we induce experimental methemoglobinemias under the conditions studied, like hypoxia and anoxia, it is possible to find some works which investigated the histological changes from other standpoints. Hills and Spector (Ref. 5) investigated the changes of the water content in the brain after anoxia caused by the bandage (? tying - "podvaz") a carotis communis dextrā. Cerebral edema was found only in advanced anoxia. On the other hand, no change was found as compared with the controls in the nitial stages. The reaction of the liver tissue to anoxia was investigated biochemically by Reed (Ref. 14), who revealed an increase of the transaminase activity (SGOT and SGPT) without histological control. Coret (Ref. 3) studied the reactivity of the smooth muscle in hypoxia. However, he studied only the functional responses without any morphological control of the tissue. In experimental altitude hypoxia, the functional response was investigated in correlation with the morphological changes, by Poupa and others (Ref. 11, 12, 13) and also by Kerr (Ref. 6).

In our tests we tried to study the histologically demonstrable changes in organisms of laboratory rats (Wistar) subjected to hypoxia of the methemoglobin type.

Material and Procedure:

Histologically we investigated altogether 3 groups of rats of both sexes, of 10 animals each. The first experimental group received a solution of a concentration of 10 mEqv/l NO_2 during a period of 150 and 200 days after weaning. The other test group drank during this period a solution of a concentration of 20 mEqv/lNO_2, and the control group received only pipe water (maximum concentration NO_3^- - 10.5 mg and NO_2^- 0.06 mg/l liter). The animals were killed by exanguination by sectioning the carotide, in order to exclude the possible effect of narcotics on the tissue examined. The extracted organs (bone, heart, liver, spleen, kidney, suprarenal gland) were fixed with 4% neutral phormol and were treated by the current histological technique in paraffin sections, which were colored with hematoxylin-eosin and with Mallory phospho-W-hematoxylin, and some of them also by the Weiger-van Gieson method or with resorcin-fuchsin (Ref. 17). The changes found in the organisms were also evaluated in comparison with the preparations obtained from the organisms of the control group.

Results

Liver:

The entire and predominantly nonspecific response of the liver tissue to the long-term administration of Na nitrite can be summarized in the picture of a turbid swelling expressed altogether in all the preparations examined. The highest damage of the liver tissue was found isolated in the apical part of the lobe of a necrose. In some cases one found under the serosa a connected layer of altered cells with sparce protoplasma around the center. The cell plasma is coarse-grained. The blood (? "siny") and vessels are considerably widened and sometimes filled with coagulated blood plasma, which blends with the surrounding cells; it is nearly impossible to distinguish the lever supports (beams ?). The protobiliary spaces and the layers of the lymphoid tissue are

nearly without any reaction. Some parenchyma parts have a decreased color, other parts exhibit a complete shifting of oxyphilia to the basophilia side (Figure 1).

Spleen:

The parenchyma exhibits a marked reaction of the white pulp. This is confirmed by the surprizing widening of the cords of the lymphoid tissue. The lower basophilia of the lymphatic ganglia indicates that this organ is affected by a long-term noxa. In some cases one finds around the spleen ganglia a fairly massive ligament cover, and the ligament septa (trabeculae) had grown thick, which is directly connected with the increase of the weight of this organ. Close under the spleen case, one finds a layer of considerably changed cells with an indication of starting hypertrophia. The red marrow is full of blood (? "překrvena") like the spleen vessels. The spleen reticula contains much hemosiderin (Figure 2).

Kidneys:

Damage of the kidneys is reflected in a turbid swelling of the epithelium of the tubules; in their widening of the lumen the tubular liquid is coagulated. The glomerular convolutions are suffused with blood and some of them are degenerated. In some cases under the case (capsule ?) the parenchyma has become damaged by layers (layerwise) (incipient necrobiosis). In the tubular field (?) of some glomeruli, there is a fine round cell infiltration. This is accompanied by considerable blood suffusion of the cortex and marrow (? medulla) (Figure 3).

Suprarenal Gland:

Under the ligament envelope there is a narrow area of cells with coarse grained protoplasma and finely diffused vacuoles. The nuclei of the cells of this part are marked by much basophilia. The plasma of the remaining cortical layers is turbid because of albuminoid degeneration. The bluish (?) reticular layers are quire dilated and suffused with blood. The sinusoidal cavities of the medulla are considerably suffused (Figure 4).
(marrow ?)

Brain:

The superficial and deep vessels of the brain are for the most part suffused with blood. Below the pia mater of the anterior part of the brain and in the lateral brain chambers there are small hemorrhages. The lymphatic vessels are also delated. On the brain cells it has not been possible so far to detect any histological changes characterizing long term hypoxias. In this connection, however, isolated areas of swollen cells with vacuoles in the cytoplasma can be found in the deep cortical layer of the anterior brain part; there is also a change in the color of the superficial layers of the cortex of the anterior part of the brain. No edema was found. Sometimes the lack of sharpness of the cortical layers cannot yet be regarded as a sign of desintegration of the brain cells. There is, however, an obvious suffusion of the vessel tissue of the brain chambers, and their results. No changes in the cytoplasm are evident in the Purkyň cells (Figure 5, 6).

Heart:

An evident finding concerning the heart muscle of all the tested animals as compared with the controls is a marked dilatation of the coronary vessels and of the capillaries of the myocardium. On the other hand, the presence of some fine lymphocytic layers under the epicardium is only isolated. In many cases, in the interstices of the myocardium there are small scattered groups of erythrocytes. On the longitudinally sectioned muscular fibers there is in some cases some intermittent increase, and on the contrary a considerable decrease of oxyphilia. Sometimes on the myocardium there is an evident shifting of the coloration to the basophilia side, which can be regarded as an indication of incipient hypoxia of the heart muscle (Figures 7, 8).

Discussion:

The analysis of the results of histological examination show that in the study of the effects of the methemoglobin-forming compounds, it is impossible to observe a direct of indirect influence on the tissue of the individual organs of the exposed organism. Na nitrite is a compound which produced methemoglobin

stoichimetrically (relatively to Fe of the prosthetic hemoglobin group). An important property of the nitrite ion with respect to the appearing of methemoglobinemia is the reversible formation of derivatives with trivalent Fe without the development of hemoglobin products (Ref. 2, 4, 10, 16). The irreversible changes in the organs can also be the mere result of these factors: 1. Defective supply of O_2 to the tissues as a result of the conversion of hemoglobin into methemoglobin, during long-term exposure of Na nitrite; 2. the dilating effect of Na nitrite on the smooth muscles of the wall of the vessels; 3. the direct effect on the biochemical processes of the tissues.

In evaluating the finding for the individual organs it is possible to state that the resulting effect is a combination of all the factors mentioned. The finding about the brain agrees with the observations made in human pathological anatomy, which ascertains in disorders of the O supply to the brain tissue marked damages and even encephalomalacia (Ref. 1). On the other hand our results differ from those of Rose, Harrisse and Chena (Ref. 15), who gave rats during 108 days 60 - 109 mg $NaNO_2$/kg of live weight and did not find definite changes in the CNS tissue. The morphological changes of the liver tissue correspond to the picture of chronic hypoxia. The severest damages manifested by the necrose in the apical part of the lobe demonstrate that the liver is markedly connected with the detoxifying mechanisms of chronic nitrite exposure. In comparison with the morphological finding about the liver in the case of hypoxia-O insufficiency, if as a rule one observes first of all a centroakinetic (? "centroacinozní") necrose connected with dystrophy, one must regard our results as the indication of vasodilatory effects of the compound administered. The same conclusion can be drawn in the evaluation of the myocardium. Vasodilatation and tissue hyperemia in the case of some preparations examined are obviously found as the acute reaction to the last administration of Na nitrite mentioned. During a current microscopic examination, the literature confirms that in the case of hypoxia the vascularization of the tissue of the myocardium increases considerably (Ref. 13). Quite a histological finding was made about the spleen. The relation of this organ to the pathophysiology of methemoglobinemia has not been studied in details. From the nonspecific finding it is possible to conclude that a further analysis of the function of the spleen tissue during methemoglobinemia of the chronic type could have some different results about the individual types of the physiological

functions of this organ. The damage of the kidneys may mean the rejection of one of the main defensive means of the organism during the development of methemoglobinemia: the exclusion of the toxic ions in the briefest physiologically possible time interval. The reaction of the glomeruli shows that the kidneys are a further organ which markedly reacts to vasodilatation resulting from nitrite ion. From the experimental material presented, it is difficult to relate to a picture of the overall damage of the organ the histological finding about the suprarenal gland. Without an objective and functional investigation of the reactivity of the suprarenal gland it is impossible to critically evaluate the independent finding. The functional investigation of the suprarenal gland will be in the future one of the means to render possible a further solution of the problem of the pathology of methemoglobinemia. The histological finding proposed in this article will be a suitable contribution to such investigations.

Conclusion:

We summarized the results of histological investigations of laboratory rats which chronically drank a solution of Na nitrite. In the kidneys we found a picture of turbid swelling and even necrose in some lobes of the liver tissue. Ths spleen exhibited a state of massive hyperemia with hemosiderin deposits, a multiplication of the ligament and an increase of the white marrow reaction. The glomeruli of the kidney showed suffusion and dilatation; the entire renal tissues exhibited albuminoid degeneration of the plasma of the epithelium of the renal tubules. In the suprarenal gland one observed hyperemia of the cortex. The heart tissue presented isolated erythrocyte infiltration, dilation of the coronary vessels and a change of the oxyphilia of the muscular tissue. In the brain tissue there were hypermia and increased eosinophilia of the cortex of the front part.

Souhrn

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

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

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Došlo 10. 10. 1966.

L. B., Sofia, Výzkumný ústav hygieny

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ÚČINKY DUSITANU SODNÉHO V DLOUHODOBÉM POKUSU. — III.

Действие азотистокислого натрия в долговременном опыте. — III.

The Effects of Sodium Nitrite in Long-Term Experiment.-III.

J. Musil, K. Nejedlý

Technická spolupráce: R. Brühlová, O. Kokešová, V. Zahradníková, J. Zinkeová

Ústav hygieny, Praha, Státní ústav pro kontrolu léčiv, Praha

Выводы: Авторы оценивают гистологическую картину органов лабораторных крыс, которые пили в течение 150 и 200 дней раствор 10 и 20 мэкв азотистокислого натрия. Были установлены тяжелые изменения на органах подопытных крыс в сравнении с контрольными животными. Гистологическое исследование дополняет в общем токсикологическое и гематологическое исследования, опубликованные в предшествующих сообщениях.

Čs. Hyg., 12, 1967, 7 : 381—384.

Summary: The authors evaluate histological findings in organs of laboratory rats, who have drunk solutions containing 10 and 20 mEq of $NaNO_2$, respectively, for 150 and/or 200 days Serious changes in organs of the experimental animals in a comparison with the control ones were found. Histological examination completes the general-toxicology and haematological examinations, published in previous reports.

Čs. Hyg., 12, 1967, 7 : 381—384.

Úvod

V předcházejících dvou sděleních [7, 8] byla podána zpráva o výsledcích dlouhodobého pokusu, v němž byla laboratorní zvířata (krysy) vystavena chronickému působení dusitanu sodného, tj. látky vyvolávající methemoglobinémii. K potvrzení obecně toxikologického a hematologického vyšetření bylo provedeno vyšetření histologické. V tomto sdělení jsou uvedeny histologické nálezy získané vyšetřením preparátů z orgánů exponovaných zvířat.

V obsáhlé literatuře věnované problematice fyziologie a toxikologie látek tvořících methemoglobin nebyly zatím sledovány histologické účinky na jednotlivé orgány. Zařadíme-li experimentální methemoglobinémie mezi stavy studované jako hypoxie a anoxie, lze najít některé práce, které sledují histologicky prokazatelné změny z jiných zřetelů. Hills a Spector [5] sledovali změny obsahu vody v mozku po anoxii vyvolané podvazem a. carotis communis dextra. Cerebrální edém nacházeli pouze v pokročilé fázi anoxie, naproti tomu nezjistili změny proti kontrolním zvířatům v počátečních stadiích. Biochemicky sledoval reakci jaterní tkáně na anoxii Reed [14], který prokázal vzestup transaminázové aktivity (SGOT i SGPT) bez histologické kontroly. Coret [3] se zabýval studiem reaktivity hladkého svalu v hypoxii. Věnoval však pozornost jen funkční odpovědi bez morfologické kontroly tkáně. U experimentální výškové hypoxie vyšetřovali funkční odpověď v korelaci s morfologickými změnami Poupa et al. [11, 12, 13] a dále Kerr [6].

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V našich pokusech jsme se snažili zachytit histologicky prokazatelné změny na orgánech laboratorních krys (Wistar), vystavených hypoxii methemoglobinového typu.

Materiál a metodika

Histologicky byly vyšetřeny celkem tři skupiny krys obého pohlaví po 10 zvířatech. První pokusné skupině byl podáván roztok o koncentraci 10 mEkv/1NO'$_2$ po dobu 150 a 200 dní po odstavu. Druhá pokusná skupina pila po tutéž dobu roztok v koncentraci 20 mEkv/1NO'$_2$ a kontrolní skupina dostávala pouze vodovodní vodu (max. konc. NO'$_3$-10,5 mg a NO'$_2$-0,06 mg/1 litr). Zvířata byla usmrcena vykrvácením nástřihem karotid, aby byl vyloučen event. vliv narkotik na vyšetřované tkáně. Vyňaté orgány (mozek, srdce, játra, slezina, ledvina, nadledvina) byly fixovány 4 % neutrálním formolem a zpracovány běžnou histologickou technikou v parafinových řezech, které byly obarveny hematoxylinem-eozinem a Malloryho fosfowolframovým hematoxylinem, některé z nich též metodou Weigertovou—van Giesonovou nebo resorcinem-fuchsinem ([17]). Nalezené změny na orgánech byly vždy hodnoceny ve srovnání s preparáty zhotovenými z orgánů kontrolní skupiny.

Výsledky

Játra

Celkovou a převážně nespecifickou odpověď jaterní tkáně na dlouhodobé podávání dusitanu sodného je možno shrnout do obrazu kalného zduření vyjádřeného vesměs ve všech hodnocených preparátech. Jako vrcholné poškození jaterní tkáně byla zachycena ojediněle v apikální části laloku nekróza. V několika případech byla nalezena pod serózou souvislá vrstva alterovaných buněk s prořidlou protoplazmou kol jader. Buněčná plazma je vesměs hrubě zrnitá. Krevní siny a cévy jsou značně rozšířeny a mnohdy naplněny sraženou krevní plazmou, která splývá s okolními buňkami, takže nelze téměř rozeznat jaterní trámce. Portobiliární prostory a ložiska lymfoidní tkáně jsou téměř bez reakce. Některé partie parenchymu mají sníženou tinkci, jiné jeví dokonce posun oxyfilie na stranu bazofilie (obr. 1).

Slezina

Parenchym vykazuje výraznou reakci bílé pulpy. Nasvědčují tomu nápadně rozšířené provazce lymfoidní tkáně. Snížená bazofilie lymfatických uzlíků svědčí o zatížení tohoto orgánu déle trvající noxou. V několika případech byl nalezen kol slezinných uzlíků dosti masívní vazivový obal a ztluštělá vazivová septa (trabeculae), což je v přímé souvislosti se zvětšením váhy tohoto orgánu. Těsně pod slezinným pouzdrem nalézá se vrstva značně změněných buněk s náznakem počínající hypertrofie. Červená dřeň je silně překrvena a stejně tak i slezinné cévy. V retikulu sleziny nachází se množství hemosiderinu (obr. 2).

Ledvina

Poškození ledvin se projevuje kalným zduřením epitelu tubulů, v jejichž rozšířeném lumen je sražená tubulární tekutina. Glomerulární klíčky jsou silně překrveny a některé z nich degenerovány. V několika případech se pod pouzdrem ložiskovitě poškodil parenchym (počínající nekrobióza). Při cévním pólu některých glomerulů je drobná kulatobuněčná infiltrace. Společným nálezem je též značné překrvení kůry i dřeně (obr. 3).

Nadledvina

Pod vazivovým pouzdrem nalézá se úzká zóna buněk s hrubě zrnitou protoplazmou a drobnými, difúzně rozesetými vakuolami. Jádra buněk této partie jeví zvýšenou bazofilii. Plazma ostatních korových vrstev je zkalena vli-

vem albuminoidní
a překrveny. Také

Mozek

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předního mozku a
gie. Lymfatické cé
sud možno zajistit
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s vakuolami v cy
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nalezen v žádném
vrstev nemůže b
Evidentním nález
výsledky. Na Pu
(obr. 5, 6).

Srdce

Zcela eviden
kontrolním je zn
tomu přítomnost
kardem je pouze
přítomny roztro
vláknech jeví se
ná oxyfilie. Něk
nu bazofilie, co
(obr. 7, 8).

Rozbor vý
látek tvořících
tkáně jednotlivý
která tvoří met
piny hemoglob
vzniku methem
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Harrise a Cl
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vem albuminoidní degenerace. Siny retikulární vrstvy jsou značně dilatovány a překrveny. Také sinusoidní dutiny dřeně jsou silně překrveny (obr. 4).

Mozek

Povrchové i hluboké cévy mozku jsou většinou překrveny. Pod pia mater předního mozku a v postranních mozkových komorách jsou drobné hemorrhagie. Lymfatické cévy jsou rovněž dilatovány. Na mozkových buňkách není dosud možno zajistit histologické změny charakteristické pro dlouhotrvající hypoxii. V souvislosti s ní může však být ojedinělý okrsek zbubřelých buněk s vakuolami v cytoplazmě nalezený v hluboké korové vrstvě předního mozku a dále změna tinkce povrchových vrstev kůry předního mozku. Edém nebyl nalezen v žádném případě. V několika případech zjištěná neostrost korových vrstev nemůže být ještě pokládána za výraz dezintegrace mozkových buněk. Evidentním nálezem je však překrvení cévní pleteně mozkových komor a jejich výsledky. Na Purkyňových buňkách nejsou patrny žádné změny v cytoplazmě (obr. 5, 6).

Srdce

Zcela evidentním nálezem na srdečním svalu všech pokusných zvířat proti kontrolním je značná dilatace koronárních cév a kapilár myokardu. Naproti tomu přítomnost několika drobných lymfocytárních ložisek uložených pod epikardem je pouze ojedinělá. V mnoha případech jsou v intersticiu myokardu přítomny roztroušené skupinky erytrocytů. Na podélně prořízlých svalových vláknech jeví se v několika případech střídavě zvýšená a naopak značně snížená oxyfilie. Někdy je dokonce na myokardu patrný posun barvitelnosti na stranu bazofilie, což lze považovat za projev počínající hypoxie srdečního svalu (obr. 7, 8).

Diskuse

Rozbor výsledků histologického vyšetření ukazuje, že při studiu účinků látek tvořících methemoglobin není možno přehlížet přímý a nepřímý vliv na tkáně jednotlivých orgánů exponovaného organismu. Dusitan sodný je látka, která tvoří methemoglobin stechiometricky (vztaženo k železu prostetické skupiny hemoglobinu). Významnou vlastností dusitanového iontu ve vztahu ke vzniku methemoglobinémie je reverzibilní tvorba derivátů s trojmocným železem bez vzniku degradačních produktů hemoglobinu ([2 4 10 16]). Ireverzibilní změny na orgánech mohou tedy být jen výsledkem těchto faktorů: 1. poruchami dodávky O_2 tkáním jako následku přeměny hemoglobinu v methemoglobin, ke které dochází v průběhu dlouhodobé expozice dusitanu sodného, 2. dilatačního účinku dusitanu sodného na hladké svaly stěny cévní, 3. přímým účinkem na biochemické pochody tkání.

Při posuzování nálezů na jednotlivých orgánech je možno usuzovat, že výsledný účinek je projevem uplatnění všech uvedených činitelů. Nález na mozku je v souhlase s pozorováními humánní patologické anatomie, která zjišťuje při poruchách dodávky kyslíku mozkové tkáni výrazné poškození až encefalomalacie ([1]). Naproti tomu jsou naše výsledky odlišné od vyšetření Rose, Harrise a Chena ([15]), kteří podávali krysám po 108 dnů 60—109 mg $NaNO_2$/kg živé váhy a nenalezli prokazatelné změny na tkáni CNS. Morfologické změny jaterní tkáně odpovídají obrazu chronické hypoxie. Nejtěžší poškození manifestované nekrózou v apikální části laloku dokazuje, že játra jsou výrazně zapojena do detoxikačních mechanismů chronické dusitanové expozice. Ve srovnání s morfologickým nálezem jaterním při hypoxii-kyslíkové insuficienci, kdy je zpravidla pozorována především centroacinozní nekróza spojená s dystrofií, je nutno naše výsledky považovat za projev vasodilatačních účinků podávané

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látky. Stejný závěr je možno učinit i při hodnocení myokardu. Vasodilatace a hyperémie tkáně je u části vyšetřených preparátů nacházena zřejmě jako akutní reakce na poslední přijaté dávky dusitanu sodného. Při běžném mikroskopickém vyšetření bylo možno zjistit rozdíly ve vaskularizaci tkáně a porovnávat výsledky s údaji v literatuře, a tím potvrdit, že při hypoxii se výrazně zvyšuje vaskularizace tkáně myokardu ([13]). Výrazný histologický nález byl zjištěn na slezině. Vztah tohoto orgánu k patofyziologii methemoglobinémií nebyl zatím zevrubně studován. Z nespecifického nálezu je možno uzavřít, že by další analýza funkce slezinné tkáně při methemoglobinémiích chronického typu měla být diferencovaně zaměřena na jednotlivé typy fyziologických funkcí tohoto orgánu. Poškození ledvin může znamenat vyřazení jednoho z hlavních obranných mechanismů organismu při vzniku methemoglobinémie: vyloučení toxických iontů v nejkratším fyziologicky možném časovém intervalu. Reakce glomerulů ukazuje, že ledviny jsou dalším orgánem, který výrazně reaguje na vasodilatační působení dusitanového iontu. Z předloženého pokusného materiálu je těžko zařadit do obrazu celkového poškození organismu histologický nález na nadledvině. Bez cíleného funkčního vyšetření reaktivity nadledviny není možno samostatný nález posuzovat. Funkční vyšetření nadledviny bude v budoucnu jedním z prostředků, pomocí nichž bude možno dále řešit otázky patofyziologie methemoglobinémií. Předložený histologický nález v tomto sdělení bude vhodným korelátem těchto vyšetření.

Závěr

Shrnuty výsledky histologického vyšetření laboratorních krys, které chronicky pily roztok dusitanu sodného. Na játrech byl nalezen obraz kalného zduření a v některých lalocích jaterní tkáně až nekróza. Na slezině byl zjištěn stav masivní hyperémie s hemosiderinovými ložisky, zmnožení vaziva a zvýšená reakce bílé dřeně. Glomeruly ledviny jevily překrvení a dilataci, v celé ledvinné tkáni pozorovali albuminoidní degeneraci plazmy epitelu ledvinných tubulů. V nadledvině byla pozorována hyperémie kůry. Tkáň srdeční obsahovala ojediněle erytrocytární infiltrace, dilataci koronárních cév a změnu oxyfilie svalových vláken. V mozkové tkáni byla zjištěna hyperémie a zvýšená eozinofilie kůry předního mozku.

Souhrn

Autoři hodnotí histologický nález na orgánech laboratorních krys, které pily po 150 až 200 dnů roztok 10 a 20 mEkv $NaNO_2$. Zjistili závažné změny na orgánech pokusných krys ve srovnání proti zvířatům kontrolním. Histologické vyšetření doplňuje obecně toxikologické a hematologické vyšetření uveřejněné v předchozích sděleních.

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DEZINFEKCE SPOR V PITNÉ VODĚ CHLORNANEM VÁPENATÝM

Дезинфекция спор в питьевой воде хлорноватистокислым кальцием

Disinfection of Spores in Drinking Water by Calcium

J. Veger

Výzkumný ústav vodohospodářský, Praha-Podbaba

Выводы: В работе приведены результаты лабораторной проверки спороцидного действия хлорноватистокислого кальция на спорах B. cereus в питьевой воде. Установлены следующие результаты: коэффициент концентрации = 1,06; температурный коэффициент = 1,045; с повышением исходного pH воды, или с повышением количества содержащихся органических веществ спороцидный эффект значительно снижается; разный химический состав естественных вод может в разной степени изменять действие; лабораторно установленные эффективные концентрации, например, 10 мг/л при 30 мин. воздействия, следует считать основанием — при практической дезинфекции разных питьевых вод необходимо, вследствие неблагоприятного воздействия разных факторов, учитывать применение даже в 20 раз высшей концентрации.

Čs. Hyg., 12, 1967, 7 : 385—392.

Summary: The paper presents the results of laboratory investigation of sporocidal effect of calcium hypochlorite upon spores of Bacillus cereus in drinking water. The results were as follows: concentration coefficient = 1.06, temperature coefficient = 1.045; the sporocidal effect proved to decrease considerably in relation with increasing original pH of water or increasing amount of organic compounds; different chemical composition of natural water may change the effect in different degree; the effective concentration found, e. g. 10 mg/l in 30 min. exposure should be considered as a basic one in disinfection of different kind of drinking water in practice, the concentration used must be established even twenty times higher than the found one in view of possible unfavourable effects of different factors.

Čs. Hyg., 12, 1967, 7 : 385—392.

Dezinfekce spor v pitné vodě při běžné praktické dezinfekci nepřipadá v úvahu. Jiná situace nastává za mimořádných událostí, kdy je nutno počítat s event. přítomností spor ve vodě v důsledku činnosti protivníka při použití bojových biologických prostředků. Podle Fraňka [5] jako zdroj nákazy z pitné vody alimentární cestou připadá ze sporulátů v úvahu jedině B. anthracis, ale právě spory tohoto mikrobu zaujímají podle údajů zainteresovaných odborníků [1, 13, 18, 23] místo v čelní pozici prostředků, uvažovaných pro bakteriologickou válku. Je tedy třeba zabývat se také likvidací spor v pitné vodě a pro tento účel se ukázal být jako relativně silný sporocidní prostředek chlornan vápenatý.

Materiál a metodika

Dezinfekční agens: chlornan vápenatý s 62 % akt. Cl_2 (všechny v práci uvedené váhové hodnoty rozumí se aktivní složka preparátu na začátku pokusu). K pokusům byla používána množství 1—100 mg/l. Analytické stanovení bylo prováděno jodometricky [7].

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THE TOXICOLOGY OF NITRITES*

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INTRODUCTION

Cases of poisoning by nitrites have been extremely rare till very recently. The first fatal case of poisoning by nitrite in medical literature was reported in the year 1935[1] by S. Rajagopal Naidu and P. Venkatrao (Chemical Examiner's Report, Madras, for 1935 and *British Medical Journal*, 27th June, 1935, page 1300). Death of three members of one family in Middlesborough two hours after consuming sodium nitrite in mistake for common salt was reported in the *Analyst*, 1936.[2] This was the first fatal case of nitrite poisoning reported in England. Cases of poisoning by nitrite have subsequently been reported not only by the Chemical Examiner, Madras,[3] but also by the Chemical Examiner, Bengal[5] and by the Chemical Examiner, Bombay[4] in their annual reports.

Sodium nitrite and potassium nitrite are extensively used by weavers in South India for dyeing purposes. These substances can be easily purchased, without any restrictions. Hence cases of poisoning by nitrites have become increasingly frequent in recent years. As very little information was available on the toxicity of the nitrites, analytical studies of cases of nitrite poisoning received in the laboratory were undertaken. Also some experiments were conducted on dogs to correlate the results obtained with the analyses done on human viscera and to find out the toxicity of nitrites in man.

Thirty cases of poisoning by nitrites were studied during the years 1935 to 1940. Of these nineteen were suicides, nine homicides, one accidental poisoning and one in which the nature of death was not definitely established. In all these cases, the stomach and its contents, liver, kidney and intestines and their contents were sent for examination. Only in a few cases were urine and vomited matters sent. A careful and accurate determination of the poison was made in each of these cases with a view to studying the distribution of nitrites in the various organs. The tables appended give a record of analysis in fatal cases of poisoning by nitrites.

In some cases the medical officers reported that nitrite was taken as poison ; in some cases they indicated that some substance used for dyeing purposes was taken ; and in some other

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† The first case of fatal nitrite poisoning was reported by the Chemical Examiner to the Government of Bengal in his Annual Report for 1930.—Editor.

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cases where there was no indication as to the poison swallowed, they reported them as cases of sudden death. A routine examination for nitrites in the stomach and its contents is now made to eliminate the possibility of nitrite poisoning.

SYMPTOMS OF NITRITE POISONING

Symptoms furnished in the above cases of nitrite poisoning by the medical officers in their post-mortem reports were very meagre. The symptoms noted were vomiting, giddiness and unconsciousness with convulsions. Blueness of the lips and finger tips were also observed. In one case a man experienced "reeling sensation, derangement of brain, could not stand or gaze at lamp light, made incoherent statements and became unconscious sometime later."

POST-MORTEM FINDINGS IN NITRITE POISONING

Discharge of sanguinous fluid from nostrils and mouth was noted in some cases. The right side of the heart was full and contained red, dark-red or cherry-red blood. The left side of the heart was empty. In some cases there was some sanguinous fluid in the pleural and peritoneal cavities. Abnormally fluid blood was found in the large vessels. In many cases the lungs were congested and exuded dark-red blood on section. In some cases the lungs were normal. In a few cases the lungs were collapsed. The stomach was congested in many cases and in some cases was vascular in patches. Intestines were normal in many cases and in a few cases were vascular. The liver, kidneys and spleen were congested. The brain and its membranes were congested and the ventricles were full.

A TYPICAL CASE OF NITRITE POISONING

A typical case of nitrite poisoning is described below:—

A man was seen alive and sitting on a bench at a railway station at 3 P.M. Half an hour later he was found dead. Amongst his possessions was a small bottle containing some white powder. The post-mortem examination revealed small serous effusions into the pleura, pericardium and peritoneum, dark fluid blood in the heart and great vessels, intense congestion of the lungs, liver, spleen and kidneys, congestion of the stomach and intestines in portions and congestion of the membranes of the brain. Nitrites were detected in the visceral matters.

ESTIMATION OF NITRITES

The following is a brief note about the methods adopted in this laboratory for the estimation of nitrites in viscera and vomited matters:—

1. *Colorimetric Estimation.*—This method, that of Griess-Ilosyay, serves the estimation of very small quantities of nitrite (of the order of 0·01 down to 0·0005 parts per million). It depends on the formation of an intensely coloured azo-dye which imparts a red coloration to the water.

The following solutions are required:—

(*a*) 1 Gram of sulphanilic acid dissolved in a mixture of 10 ml. of glacial acetic acid and 50 ml. of water and the whole made up to 100 ml. with water.

(*b*) 0·5 Gram of alphanaphthylamine dissolved in a mixture of 10 ml. of glacial acetic acid and 50 ml. of water and the whole made up to 100 ml. with water.

The above solutions are prepared with nitrite-free water and kept separately and equal quantities are mixed immediately before use.

(*c*) Standard solution of sodium nitrite:—To 2·23 Grams of pure silver nitrite, in nitrite-free distilled water, is added excess of pure sodium chloride solution to precipitate the silver completely, made up to a litre, mixed well and allowed to settle. 100 ml. are diluted to 1 litre and then 10 ml. of this is diluted to a litre, using in each case nitrite-free water. Each ml. of the last solution is equivalent to 0·001 mgm. of sodium nitrite.

(*d*) Saturated mercuric chloride solution.

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Procedure.—The entire viscus is weighed and the whole liquid is measured. The viscus is finely cut into very small bits and sampled. An aliquot part of the sampled viscus and liquid is placed into a 400 ml. beaker, neutralised if acid in reaction, diluted with nitrite-free distilled water and heated to a temperature of 65°C. The contents of the beaker are mixed thoroughly by stirring with a glass rod taking care to break up all lumps into small bits, treated with sufficient quantity of saturated solution of mercuric chloride and made up to 500 ml. in a measuring flask. The contents are then filtered and the filtrate used for determination.

An aliquot portion of the above filtrate is treated with 1 ml. of alphanaphthylamine and 1 ml. of sulphanilic acid and is nesslerised after 30 minutes by comparison with solutions of suitable standards similarly prepared.

[The method described above is exactly similar to the method described by Allen for the determination of nitrites in meat. The total volume occupied by the visceral matter and water is taken into consideration without any correction for the volume of visceral matter, as nitrites are uniformly distributed within the [illegible]re (*vide* Allen's Commercial Organic Analysis, [illegible] Edition, Vol. IX, footnote 1 on page 434).]

2. *Volumetric method.*—The protein-free filtrate obtained above is suitable for the titration method in cases in which the colour developed in the colorimetric method is too deep. The method used in such cases is to treat the solution with potassium iodide in the presence of acid in an atmosphere free from oxygen and to titrate the liberated iodine with sodium thiosulphate. In order to avoid the action of oxygen on nitric oxide it is essential to expel and displace all air by carbon dioxide.

Procedure.—A 500 ml. Florence flask is fitted with a two holed rubber stopper, one carrying a glass tube to a little above the bottom of the flask and the other the stem of an open tap cylindrical funnel of about 30 ml. capacity and with the tip of the stem just projecting below the stopper. Into the flask are put 50 to 100 ml. of the protein-free filtrate described above, 25 ml. of water, 10 ml. of 10 per cent potassium iodide and 1 ml. of amyl alcohol. The rubber stopper is fitted to the flask and a current of carbon dioxide is passed to replace the air in the flask. After the air inside has been replaced, 10 ml. of 5N oxygen-free sulphuric acid is run through the funnel into the flask and the liberated iodine titrated with N/20 sodium thiosulphate run through the funnel, using starch as indicator. (1 ml. N/20 sodium thiosulphate is equivalent to 3·45 mgms. of sodium nitrite).

BRIEF HISTORY OF THE THIRTY CASES

(*Vide* Table Nos. 1 to 6)

Case 1.—On 8-7-39 the deceased (Hindu, Devangulu) was brought to the dispensary in moribund condition and reported to have poisoned himself by taking sodium nitrite which is said to be available locally and used in the process of dyeing by the local professional men. It was reported that the deceased purchased the stuff and took it at 3 P.M. on 8-7-39 and developed symptoms of collapse very soon.

Cases 2 & 3.—It was suspected by the police that the deceased had been poisoned by sodium nitrite.

Cases 4 & 5.—Both cases ate food partly and both felt vomiting sensation, got up and vomited. Both became unconscious and died within two hours.

Cases 6 & 7.—Poison was mixed with food. Immediately after taking food, they felt giddy, vomited and died. It was suspected that nitrites used for dyeing clothes were used by the deceased.

Case 8.—The deceased said to have taken some food mixed with some poison on the night of 9-2-41 and developed signs of vomiting and giddiness and died on 10-2-41 at 6 A.M.

Case 9.—It was stated that the deceased committed suicide by taking Boora, a sort of chemical used in these parts for colour dyeing. It appears that she took the poison at about 10 or 11 P.M. in the night and expired at 1 A.M. the next day.

Case 10.—It was stated in the police report that the deceased died of poisoning. A female by name Pichamma in the village administered poison to the child, as a result of which he died.

Case 11.—Death due to poisoning—died suddenly.

Case 12.—The deceased was said to have committed suicide by taking poison, as his debtors demanded Rs. 325/- on 9-1-39.

Case 13.—Alleged to have taken poison on the night of 9-9-39.

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Case 14.—Sales (weaver). It appears he was involved in a criminal case and hence he put an end to himself by taking some poison. The exact nature of the poison is not known.

Case 15.—He was seen by some local people fainting and falling down at 11-30 P.M. and was found dead at 3 A.M.

Case 16.—The boy was brought to the hospital at about 3-30 P.M., cold, clammy and cyanosed. Two boys who accompanied him reported that the boy had taken some powder from the tin which was produced. The tin was found to contain potassium nitrite.

Case 17.—Sudden death.

Case 18.—The deceased (Sourastra, Hindu) appeared to have died suddenly at 10 A.M. in the morning, following a quarrel with her husband the previous day.

Case 19.—It was said that the deceased took some poison and died.

Case 20.—Death suspicious. The deceased fell down from the cot on a hot night and could not talk. She died a little later. Cause of death not known.

Case 21.—The deceased was said to have taken some white powder before his death. This powder was found by the side of his body and was sent in "packet No. 6". This was found to be potassium nitrite weighing 41 grams.

Case 22.—The deceased, Padma, salee by caste, died suddenly. The rumour was that she took some poison, some chemical to dye clothes.

Case 23.—The deceased and his two sons ate food at night and soon after all of them felt giddy, with tingling and numbness of the tongue, lips and mouth. Both the sons got better, while the old man went into deep coma and died. The exact nature of the poison is not known.

Case 24.—The deceased committed suicide by taking some poison on 6-12-35 and was brought to the hospital in an unconscious condition and died in five minutes. It was reported that he had convulsions. His finger tips were blue at that time.

Case 25.—It is alleged that the deceased took illicit arrack on 11-5-41 night, had motions and vomiting and died the same night.

Case 26.—Sali (weaver). There was a chit written by the deceased that he took poison, etc.

Case 27.—She was seen first before death, struggling with violent contractions and relaxations of limbs, but with no injuries on the body. The patient suffered for an hour or so. Hence poison was suspected. Further information regarding the suspected poison was that it was some white crystals just like sugar and magnesium sulphate used for dyeing purposes.

Case 28.—A case of suicide by taking some poison. Patient had vomited once. Her hands and face turned blue, had hurried respiration and died in an hour.

Case 29.—On 5-6-38 at about 3 P.M. a man was found sitting on a bench in Trichy Rown R.S., apparently intending to go to Srirangam. Half an hour later he was found dead near the bench where he was seated.

Case 30.—Salee by caste. She is suspected to have swallowed the powder, a sample of which was sent. The powder was found to be potassium nitrite.

DISTRIBUTION OF NITRITES IN THE VISCERA

In each of the thirty cases shown above nitrites were detected and on further examination no other poison was detected in them. The therapeutic dose of sodium nitrite is 30 to 60 mgms. In seven of the thirty cases, the stomach and its contents contained less than the therapeutic dose of sodium nitrite. These cases (1, 3, 4, 7, 8, 9 and 10) are shown in Tables 1 and 2. Of these the vomited matters

TABLE 1

RESULTS OF NITRITE ESTIMATIONS

Nitrite represented as Mgms. of sodium nitrite in the tissues sent.

Case No.	Death	Sex	Age (years)	Fatal period (Hours)	Stomach & its contents.	Liver	Kidney	Intestines & their contents	Urine	Vomit
1.	S	M	20	3½	52	...	0·032	0·032	0·032	294
2.	H	F	12	2½	1376	0·064	0·38	9	...	...
3.	H	M	32	2½	0·32	0·32	traces	0·064	...	...
4.	H	F	60	3	0·1	...	...	...	...	...
5.	H	F	50	2	851	...	...	...	...	...

Note: S = suicide; H = homicide; M = male; F = female.

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sent in cases 1, 8 and 10 contained 294, 60 and 102·4 mgms. of sodium nitrite respectively. In case 2 the stomach contained definitely large quantities of nitrite and in case 3 the stomach contained only 0·32 mgms. of sodium nitrite. Cases 2 and 3 should be taken together, as the history indicates that both the persons were poisoned by sodium nitrite simultaneously. Similarly cases 4 and 5 should be taken together since both persons developed symptoms of vomiting, purging and unconsciousness after eating the same food. Cases 6 and 7 are cases where a grand-father and his grand-child ate food poisoned with nitrite and died as a result of it. Hence there remains only one case (case 9) where the quantity of nitrite in visceral matters is less than even the therapeutic dose. As the history in this case indicates poisoning by a drug used for dyeing purposes and as no other poison was detected in the stomach contents, this has been included among the nitrite poisoning cases.

TABLE 2

RESULTS OF NITRITE ESTIMATIONS—(*contd.*)

Nitrite represented as Mgms. of sodium nitrite in the tissues sent.

Case No.	Death	Sex	Age (years)	Fatal period	Stomach & its contents.	Liver	Kidney	Intestines & their contents	Vomit
6.	H	M	54	sudden	1136	traces	11	6·4	...
7.	H	F	...	do.	12·8	0·25	0·16	0·64	...
8.	H	M	70	8 Hrs.	10·5	0·5	0·25	traces	60
9.	S	F	26	2-3 ,,	0·22	traces	traces	0·22	288
10.	H	M.C.	7	...	12	...	...	traces	102·4

Note : M = male; F = female; M.C. = male child; S = suicide; H = homicide; Hrs. = hours.

In the remaining twenty cases shown in Tables 3, 4, 5 and 6, the stomach and its contents contained nitrite which was much larger than the therapeutic dose of sodium nitrite, the maximum amount detected in the stomach and its contents being 16·96 Gms. and the minimum amount 66 mgms.

TABLE 3

RESULTS OF NITRITE ESTIMATIONS—(*contd.*)

Nitrite represented as Mgms. of sodium nitrite in the tissues sent.

Case No.	Death	Sex	Age (years)	Fatal period	Stomach & its contents.	Liver	Kidney	Intestines & their contents	Urine	Vomit
11.	S	F	25	sudden	922	traces	0·2	traces	...	...
12.	S	M	35	...	538	...	...	4	...	2522
13.	S	M	23	...	6816	225	...	305	...	13632
14.	S	M	35	...	2041	2	...	0·5	...	2509
15.	S	M	45	3 hrs.	2025	...	...	1·3	0·1	...
16.	A	M.C.	11	sudden	208	0·08	0·016	...	0·001	100

Note : S = suicide; H = homicide; M = male; F = female; M.C. = male child; Hrs. = hours.

Vomited matters were received in nine cases the largest amount of nitrite detected being 9·216 Gms. and the lowest amount 60 mgms.

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The intestines and their contents do not show large quantities. Only in case 23, 210 mgms. were detected and in the remaining cases only very small quantities were found.

cases the maximum quantity of sodium nitrite in the liver was 16 mgms., and the minimum quantity being traces or in some cases nothing at all.

TABLE 4

RESULTS OF NITRITE ESTIMATIONS—(*contd.*)

Nitrite represented as Mgms. of sodium nitrite in the tissues sent.

Case No.	Death	Sex	Age (years)	Fatal period	Stomach & its contents.	Liver	Kidney	Intestines & their contents	Urine	Vomit
17.	S	M	40	sudden	3558	traces	traces	0·4	...	...
18.	S	F	25	do.	16960	...	256	128	...	...
19.	S	M	25	do.	66	0·1	0·2	0·8	...	...
20.	S	F	25	do.	168	0·064	0·032	0·1	0·064	...
21.	S	M	22	...	256	...	...	...	0·0001	9216

Note: S=suicide; H=homicide; M=male; F=female; Hrs.=hours.

TABLE 5

RESULTS OF NITRITE ESTIMATIONS—(*contd.*)

Nitrite represented as Mgms. of sodium nitrite in the tissues sent.

Case No.	Death	Sex	Age (years)	Fatal period	Stomach & its contents.	Liver	Kidney	Intestines & their contents	Urine
22.	S	F	28	3 hrs.	2009	...	...	0·5	...
23.	...	M	70	...	176	...	...	...	...
24.	S	M	15	sudden	8504	...	...	...	...
25.	H	M	63	a few hrs.	122	0·64	0·128	0·8	0·0002
26.	S	M	45	...	16320	16	12·8	218	...

Note: S=suicide; H=homicide; M=male; F=female; Hrs.=hours.

TABLE 6

RESULTS OF NITRITE ESTIMATIONS—(*contd.*)

Nitrite represented as Mgms. of sodium nitrite in the tissues sent.

Case No.	Death	Sex	Age (years)	Fatal period	Stomach & its contents.	Liver	Kidney	Intestines & their contents	Urine
27.	S	F	20	1 hr.	689	...	...	...	0·03
28.	S	F	22	1 "	630	...	0·5	5	...
29.	S	M	25	½ "	637	...	...	125	3
30.	S	F	25	...	4225	...	...	0·1	...

Note: S=suicide; H=homicide; M=male; F=female; Hrs.=hours.

The livers show only very small quantities of nitrite and only in case 18, 256 mgms. of sodium nitrite were detected. In remaining

The detection of nitrites in the kidneys was also in minute quantities. The maximum quantity found in the kidney was 12·6 mgms.

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of sodium nitrite and the minimum quantity was either traces or nothing at all.

Only in eight cases were urine sent for examination, the maximum nitrite content being 3 mgms. and the minimum 0·0001 mgm. Only traces of urea were detectable in the urine.

EXPERIMENTS ON TOXICITY OF SODIUM NITRITE ON DOGS

Dog No. 1: Weight: 1.25 kilograms.

Dose: 200 mgms. of sodium nitrite in 5 ml. of water were administered by injection under the skin of the abdomen.

Symptoms: Started in three minutes with defecation, profuse loose motions, laboured breathing with expiration time increased, slight spasms in hind limbs, signs of respiratory failure, Cheyne-Stokes breathing, gasping and death 25 minutes after injection.

Post-mortem findings: Lungs collapsed; pleura contained serous fluid; heart engorged and full of dark fluid blood; liver highly congested; intestines pale, with blood vessels congested and prominent; stomach pale; bladder empty; kidneys congested. Spectral bands of methaemoglobin were demonstrated in the blood taken from the heart.

Dog No. 2: Weight: 2.7 kilograms.

Dose: 400 mgms. of sodium nitrite in 6 ml. of water were given by mouth.

Symptoms: Vomited three minutes after administration, respiratory difficulty, mild paralytic symptoms of the limbs, staggering, Cheyne-Stokes respiration, tremors in limbs, spasms, gasping and respiration stopped 45 minutes after the administration of the dose, but heart continued beating for about a minute and a half later.

Post-mortem findings: Stomach injected; intestines pale, with vessels red and prominent; liver very congested; kidneys congested; bladder empty; lungs collapsed; pericardium contained serous fluid; heart engorged with red fluid blood.

Analysis: Vomit contained about 32.4 mgms. of sodium nitrite and the stomach with its contents contained 1.6 mgms. of sodium nitrite. Liver, kidney and intestines contained traces of nitrites. Methaemoglobin spectral bands were demonstrated in the blood of the heart.

Dog No. 3: Weight: 2 kilograms.

Dose: 275 mgms. of sodium nitrite in 4 c.c. of water were administered by mouth.

Symptoms: Staggering and paralytic symptoms of the limbs started in about four minutes, fine tremors in the limbs, respiratory distress, Cheyne-Stokes respiration, spasms of the limbs, gasping, respiration stopped and heart stopped twenty-five minutes from time of administration.

Post-mortem findings: Stomach injected, mucosa slightly injected; intestines pale, with vessels red and prominent; liver very congested; kidneys congested; bladder empty; lungs collapsed; pericardium contained serous fluid; heart engorged, full of fluid blood.

Analysis: Stomach contained 26 mgms. of sodium nitrite. Liver, kidneys and intestines contained traces of nitrite. No vomit. Methaemoglobin spectral bands were demonstrated in the blood.

DISCUSSION

A regular feature of the above experimental dogs killed by nitrites, examined so far, was the presence of methaemoglobin in the blood taken from the heart after death. A common symptom in the experimental dogs was respiratory distress, probably the result of methaemoglobinaemia. Death was rapid, occuring in about half an hour after the administration of the poison. The experiments also revealed that when the poison was administered by the mouth, very little is left in the viscera, most of it having been absorbed or otherwise destroyed. In the Dog No. 2 referred to above which weighed 2·7 kilograms, 400 mgms. of sodium nitrite dissolved in about a quarter of an ounce of distilled water was given by mouth. About 32 mgms. of sodium nitrite were found in the vomit of the dog and only 1·6 mgms. of sodium nitrite in the viscera after death, the reminder having been absorbed or otherwise destroyed.

These observations appear to be in accord with the experience that in many cases of human poisoning from nitrites only small quantities of nitrites were detected in the viscera unless extremely large doses had been taken.

Fatal dose.—What is the smallest quantity of potassium nitrite or sodium nitrite capable of destroying life is a question that will be asked in every case of death by nitrite poisoning? In spite of the fair number of fatal cases studied above, the fatal dose of the poison is still without a satisfactory solution, since in none of

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these cases is there any mention of the quantity of nitrite administered. Recovery from nitrite poisoning is rare, except in one case where of two individuals who partook of the same poisoned food, one died and the other experienced giddiness, vomiting, and unconsciousness, but recovered. Analysis of liver, kidney and intestines revealed only very small quantities of nitrite, though the examination of the stomach and its contents and the vomited matters showed large quantities of nitrites. Sometimes the stomach contained small quantities and the vomited matters were not available. This may be due to the fact that nitrites are destroyed by the hydrochloric acid in the stomach and after absorption, are destroyed in the body by the aminobodies present, after a portion has been vomited out. Hence it is not possible to state definitely the lethal dose of nitrite from the amount of nitrite found in the body after death.

As poisonous symptoms observed in experimental dogs and those mentioned in the post-mortem reports of fatal cases of human poisoning by nitrites are similar, it is not unlikely that human beings are as susceptible as dogs to nitrites. From the experiments conducted on dogs the fatal dose to dogs works out to one milligram of nitrite ion per ten grams of the body weight of dog, there being no difference practically whether the drug was injected under the skin or administered by mouth. Calculating according to Mech's formula[7] ("Extra Pharmacopoeia" by Martindale, 21st Edition, Vol. 1, page 32) the fatal dose for human beings would probably be about 2 grams of sodium nitrite or 2·6 grams of potassium nitrite for a man weighing ten stones.

Fatal period.—The commencement of symptoms in animals is extremely rapid (3 minutes after the poison has been administered) irrespective of the fact whether the poison was administered by oral or by subcutaneous route. Similarly in human beings the commencement of symptoms is very rapid as mentioned in the history of some cases. Death occurs rapidly, the shortest period noted in post-mortem certificates being half an hour after ingestion of the poison and the longest period about eight hours. In the experimental dogs also death occured in half an hour after injection of the poison. Hence the fatal period for nitrite poisoning in man may be taken to be about half an hour to three hours on the average.

COMMENTS

The most direct proof that a poison has been administered or taken during life is the detection of the poison in matters vomited by the victim, in the urine or in the faeces during life time, and in the visceral matters after death. There is little doubt about the nature of the poison in cases where nitrites are detected in large quantities either in the vomited matter or in the stomach and its contents, as in cases 11 to 30 (*vide* Table Nos. 3 to 6). There was definite history that nitrite was taken in many of these cases and there was no evidence of the presence of any other poison. Hence it can safely be concluded that in these cases death was due to nitrite poisoning.

In cases where the quantity of nitrite detected in visceral matters is small, smaller than a therapeutic dose, the question may be asked whether or not death was due to nitrite poisoning. Further examination of the stomach and its contents in the cases revealed no other poison. Cases 1, 3, 4, 6, 8, 9 and 10 showed only small quantities of these, the vomitted matters sent in cases 1, 8 and 10 contained nitrite sufficient enough to prove that poisoning was a result of nitrites. The history of cases 2 and 3, 4 and 5 and of 6 and 7 are definitely sets of cases of simultaneous poisoning, the same poison having been partaken by two individuals at the same time. It is interesting to find that when poison was administered under similar circumstances to set of individuals in cases 2 and 3, the stomach and its contents in case 3, contained only 0·32 mgms. of sodium nitrite, whereas the stomach and its contents in case 2 contained 1376 mgms. of sodium nitrite.

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Similarly in the set of cases 6 and 7 nitrite was mixed with food and given to two individuals and the nitrite content of each of the two stomachs and their contents gave very different values, *i.e.*, 1136 mgms. of sodium nitrite in case 6 and 12·8 mgms. of sodium nitrite in case 7. Similarly in the set of cases 4 and 5 where two individuals took the same food, developed vomiting, purging, unconsciousness and died in two or three hours, analysis revealed that stomach and its contents in case 4 contained only 0·1 mgm. of sodium nitrite, while stomach and its contents in case 5 contained 851 mgms. of sodium nitrite.

Hence in the cases 3, 4 and 7 though analysis reveals only very small quantities of nitrite the cause of death must be due to nitrites and the nitrites were either vomited or eliminated from the system. Nitrites are very unstable in the body and they are easily decomposed by amino-bodies. The nitrite content of the liver, kidney, intestines and urine was found to be very low, probably due to nitrites being destroyed in the human tissues leaving only a trace of poison in them.

The animal experiments showed that healthy [illegible]s died in half an hour after administration of [illegible] nitrite, only very small quantities being detected in the stomach and its contents and traces in the liver, kidney and intestines and yet death was due to nitrite poisoning. These facts confirm the findings in the human cases that small quantities of nitrites detected in the viscera do not contra-indicate death by nitrite poisoning.

The commencement of symptoms in animals is very rapid, setting in, in three or four minutes after the administration of the poison, irrespective of the fact whether the poison was given orally or under the skin. Vomiting is the first symptom, as it is the first line of defence of the organism against the poison. A second line of defence appears to be spasm of the pylorus as is evidenced by the comparatively insignificant quantities in the intestines, in spite of the very considerable quantities found in the stomach and its contents. This fact has been noticed in cases of other poisons also. The absorption of the poison into the system gives rise to methaemoglobinaemia and the train of symptoms that follow, namely, respiratory distress, giddiness and unconsciousness, are probably due to methaemoglobinaemia and cardio-vascular collapse in addition to direct action on the brain. As a consequence of this, death occurs rapidly, the fatal period observed in dogs being half an hour and the fatal period in man being about half an hour to three hours.

A constant feature of nitrite poisoning in dogs was that spectral bands corresponding to methaemoglobin were seen in the blood. Since blood was not received in any of the human cases, this feature could not be recorded.

SUMMARY

1. Both nitrites of potassium and sodium are poisonous to human beings.

2. The lethal dose for man, as worked out from the lethal dose on dogs, is about 2 gms. of sodium nitrite or about 2·6 gms. of potassium nitrite for a man weighing ten stones.

3. Death is rapid and symptoms resemble those of cyanide poisoning.

4. Death results in half an hour to three hours in human beings on the average.

5. Most of the poison is detected in the stomach contents and vomited matters and only minute quantities are detectable in the liver, kidney, intestines and urine.

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Alkylierend wirkende Verbindungen

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Embryotoxic and Teratogenic Properties of Some Nitrosamines

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Embryotoxische und teratogene Eigenschaften einiger Nitrosamine

Aus der Literatur ist ersichtlich, daß viele tumorerzeugende Substanzen gleichzeitig stark teratogen wirken (Übersicht bei *DiPaolo* und *Kotin* 1966).

In unseren Versuchen wurden die embryotoxischen und teratogenen Eigenschaften einiger N-Nitroso-Verbindungen untersucht, die der Gruppe der symmetrischen Dialkylnitrosamine, Nitrosoaniline sowie der Alkylnitrosoharnstoff-Derivate angehören und die bei ausgewachsenen Tieren Tumoren verschiedener Lokalisationen hervorrufen.

Die Versuche wurden an Hühnerembryonen (400) und vor allem an weißen Ratten (kein Inzuchtstamm) zu genau feststellbaren Zeiten der Schwangerschaft durchgeführt. Bei den Ratten wurden die Befunde am 17. bis 21. Tag der Tragezeit erhoben. Insgesamt wurden 507 trächtige Ratten und rund 3400 ihrer Foeten untersucht, die mit den angeführten Verbindungen in unterschiedlicher Dosierung behandelt worden waren.

Symmetrische Dialkylnitrosamine

Dimethyl-, Diäthyl- und Dibutylnitrosamine wurden in verschiedenen Entwicklungsstadien der Embryogenese untersucht. Alle diese Substanzen, mit denen die trächtigen Tiere oral, intraperitoneal und intravenös behandelt wurden, erhöhten die Mortalität der Embryos. Die größte Empfindlichkeit gegenüber den embryotoxischen Einflüssen war bei den

Analysis of the data available from the literature shows that many blastomogens are at the same time strong teratogens (see the review of *DiPaolo* and *Kotin* 1966).

In our experiments embryotoxic and teratogenic properties of some nitroso-compounds, representatives of symmetrical dialkylnitrosamines, nitrosoanilines and alkylnitrosoureas, which can produce tumours of different localizations in adult animals, were studied.

Experiments were carried out in chicken embryos (400) and, mainly, in white, non-inbred rats with exactly fixed date of pregnancy. Results in rats were recorded on the 17th to 21st days of gestation. The total of 507 pregnant rats and about 3400 of their fetuses were examined after treatment with different doses of the above compounds.

Symmetrical dialkylnitrosamines

Dimethyl-, diethyl-, and dibutylnitrosamines were tested at different stages of embryogenesis. All the substances increased the mortality of the embryos, being administered orally, intraperitoneally or intravenously to the pregnant animals. Peaks in the sensitivity to embryotoxic effect of the above substances were found on the 3rd, 10th and 12th days of the intrauterine development in experiments with a single-dose treatment.

Experiments with treatment lasting a

Versuchen mit einmaliger Applikation der genannten Substanzen am dritten, zehnten und zwölften Tage der intrauterinen Entwicklung festzustellen.
In Versuchen, bei denen die Behandlung eine Woche lang oder über die ganze Schwangerschaft fortgesetzt wurde, waren in der letzten Woche der Tragezeit keine tödlich auslaufenden toxischen Wirkungen festzustellen.
Obwohl die applizierten Dosierungen nur wenig unter der LD 50 lagen, zeigte sich in keinen der Versuche eine teratogene Wirkung.
Nach Behandlung der Ratten mit DMNA über die ganze Zeit oder während des letzten Drittels der Schwangerschaft fanden sich Tumoren der Nieren bei 5 von 82 geworfenen Tieren. Von diesen leben noch 67 Tiere ohne Tumoren. Daraus kann geschlossen werden, daß bei den Nachkommen auch dann Tumoren erzeugt werden können, wenn die applizierte Verbindung bei derselben Tierart nicht zu kongenitalen Mißbildungen führt.

Nitrosoaniline

Bei den Versuchen mit Methyl- und Äthyl-Nitrosoanilinen zeigten sich deutliche embryotoxische Wirkungen mit eindeutigen Beziehungen zum embryonalen Entwicklungsstadium. Die Empfindlichkeit gegenüber den tödlichen Wirkungen der Versuchssubstanzen zeigte ähnliche Unterschiede wie bei der Behandlung mit DMNA. Bei Embryonen, die Einzeldosierungen von MNA (140 mg/kg) und ÄNA (180 mg/kg) überlebten, zeigten sich außer den embryotoxischen Wirkungen am zweiten, neunten und dreizehnten Tag der embryonalen Entwicklung verschiedene Mißbildungen wie Mikrophthalmie, Exencephalie und Syndaktylie.

Derivate von Alkylnitrosoharnstoff

Die Applikation von Methylnitrosoharnstoff (i. p., 10 mg/kg) wirkte bei Ratten am dritten und neunten Tage des embryonalen Lebens durch Schädigung der Em-

week or a whole pregnancy, revealed the absence of lethal effect during the last week of gestation.
In spite of the fact that all the doses applied were only a little lower than LD 50, no teratogenic effect was observed in any of the experiments.
Treatment of rats with DMNA during the whole pregnancy or only its last third resulted in 5 cases of renal tumour development out of 82 descendants. 67 of them are still alive without tumours. This suggests that tumorigenic effect on the progeny can be observed even if the substance does not induce congenital malformations in the same species.

Nitrosoanilines

Marked stage-specific embryotoxic effect was found in experiments with methyl- and ethyl-nitrosoanilines. Changes in sensitivity of the rat embryos to the lethal effect were similar to those observed with DMNA. In addition to embryotoxic effect, different malformations (microphthalmia, exencephalia, syndactyly) were found in fetuses which survived single injections of MNA (140 mg/kg) and ENA (180 mg/kg) on the 2nd, 9th and 13th days of embryogenesis.

Alkylnitrosoureas

Methylnitrosourea (i. p., 10 mg/kg) exerted strong lethal effect on the 3rd and 9th days of prenatal life in rats due to the damage of the embryoblast or allantoic tract. A distinct stage-specific effect was observed also in teratogenesis induced by MNU. Injections of MNU on the 9th day after conception resulted in development of malformations of the brain and spinal cord (exencephalia, bifid spine), of the maxilla (palatognathous fetuses) and of the eyes (anophthalmia). Treatment on the 13th day of embryogenesis induced reduction of the long bones, deformation of the ribs, general retardation of growth, ectrodactylia, polydactylia, syndactily, phocomely,

bryoblasten oder der Allantoisanlage in sehr vielen Fällen letal. Eine deutliche Abhängigkeit der teratogenen Wirkung vom Entwicklungsstadium war auch bei Behandlung mit MNU* zu beobachten. Wenn eine Verbindung am neunten Tage nach der Konzeption injiziert wurde, kam es zu Mißbildungen des Gehirns und des Rückenmarks (Exencephalie, Spina bifida), des Oberkiefers (Palatognathose) und der Augen (Anophthalmie). Eine Behandlung am 13. Tag bewirkte eine Verkürzung der langen Knochen, Deformitäten der Rippen, eine allgemeine Wachstumsverzögerung, Ektrodaktylie, Polydaktylie, Syndaktylie, Phokomelie, Mikrognathie, Dislokationen des Hüftgelenks etc. Bei Ratten, die nach dem 13. Tag der Embryonalentwicklung mit MNU behandelt wurden, fanden sich Mikrocephalien und Erweiterungen der Seitenventrikel.

Versuche mit Dimethyl-, Äthyl- und Propylnitrosoharnstoff führten zu ähnlichen Ergebnissen wie die Versuche mit MNU. Äthyl- und Propylnitrosoharnstoff zeigten bei Applikation am neunten Tag, Methyl- und Dimethylnitrosoharnstoff bei Applikation am 13. Tag die stärksten teratogenen Wirkungen. Bei Dosierungen, die in bezug auf ihre letalen Wirkungen vergleichbar waren, zeigten die genannten Verbindungen stärkere teratogene Wirksamkeit als die anderen Derivate des Nitrosoharnstoffs.

Unsere Untersuchungen der embryotoxischen und teratogenen Wirkung verschiedener Nitrosamine führte zu Ergebnissen, die ähnliche Einflüsse auf die Embryogenese erkennen lassen, wie sie von vielen alkylierenden Verbindungen bekannt sind.

Wenn DMNA, DÄNA und Äthylnitrosoharnstoff (ÄNU) in der letzten Zeit der Embryogenese appliziert werden, können sie zur Tumorbildung im späteren Leben der Tiere führen. Aber nur eine der genannten Verbindungen (ÄNU) wirkt teratogen.

Bei Tieren, die in den späten Abschnitten

* Methylnitrosoharnstoff

dislocation of the hip joint, micrognathia, etc. In rats exposed to MNU after the 13th day of embryogenesis microcephaly and dilatation of the lateral ventricles were found.

Experiments with dimethyl-, ethyl-, and propylnitrosourea gave results similar to those with MNU. The highest teratogenicity was brought about by the application of ethyl- and propylnitrosoureas on the 9th day and on the 13th day by methyl- and dimethylnitrosourea. The substance named showed, in doses which were comparable in their lethal effect, stronger teratogenicity than other derivatives of nitrosoureas.

Data obtained in experiments testing different nitrosamines on embryotoxic and teratogenic activity show that the effects of these substances resemble in many respects the influence on embryogenesis of different alkylating agents.

It is found that DMNA, DENA and ENU can induce tumours in the postnatal life of experimental animals when applied during the late stages of embryogenesis, but only one of them (ENU) is teratogenic.

Blastomogenic effect was observed in animals subjected to the substances at the late stage of prenatal life, whereas teratogenic action was observed when the substances were applied not later than 15th day of embryogenesis. Metabolic immaturity of embryonal tissue during the first two weeks of prenatal life is probably responsible for the absence of teratogenic effect of the substances, which require transformation to some proximate agents possessing biological activity (tumorigenic effect at late stages of embryogenesis and teratogenic at early ones).

Absence of teratogenic activity in a compound of the group of nitrosamines is not sufficient to conclude without further studies that this compound will not induce tumours in the offspring when applied during pregnancy.

des pränatalen Lebens einer Behandlung mit diesen Substanzen ausgesetzt wurden, fanden sich tumorerzeugende Wirkungen; wurde die Behandlung dagegen vor dem 15. Tag der embryonalen Entwicklung durchgeführt, so führte dies zu Mißbildungen. Wahrscheinlich liegt es an einer noch fehlenden Ausbildung bestimmter Stoffwechselvorgänge, zu denen das embryonale Gewebe in den ersten zwei Wochen noch nicht fähig ist, daß diese Substanzen dann noch nicht teratogen wirken. Offenbar bedarf es im Gewebe der Umsetzung in bestimmte „Wirkformen" für die biologische Aktivität (tumorerzeugend in den späten und teratogen in den frühen Abschnitten der embryonalen Entwicklung).

Wenn bei einer Verbindung aus der Gruppe der Nitrosamine keine teratogene Wirkung beobachtet wird, so genügt dies ohne weitere Untersuchungen noch nicht, um zu folgern, daß diese Verbindung im späten Leben der Tiere keine Tumoren hervorruft, wenn sie während der embryonalen Entwicklung appliziert wurde.

Diskussion

Druckrey: Die Arbeiten von *Napalkov* und unsere Untersuchungen geben ein Beispiel für Cancerogene, die von der Mutter pränatal auf den Embryo übergehen und bei Kindern zur Tumorbildung führen könnten.
Nach *Laqueur* ist beim Cycasin auch mit solcher Wirkung zu rechnen. Wenn Muttertiere diese Substanz erhalten, zeigen sich bei den geworfenen Tieren neurogene Tumoren.
Dies ist auch bei *Inhalation* von solchen Substanzen durch die Muttertiere möglich.

Magee: Angesichts dieser Befunde muß das Vorkommen von Nitrosaminen im Zigarettenrauch – selbst in geringsten Spuren – außerordentlich ernst genommen werden. Wie gering die wirksamen Dosen sein können, zeigen die Versuche von *Druckrey* und die von *Brune* geschilderten Ergebnisse.

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Discussion

Druckrey: Napalkov's work and our research work have disclosed cancerogenic substances which are passed from the pregnant mother to the embryo and which could cause the development of tumours in children.
The same effect, according to *Laqueur*, is also to be expected in the case of cycasin. When this substance has been applied to pregnant animals, they have given birth to offspring with neurogenic tumours.
This is also possible when the pregnant animals have been made to *inhale* these substances.

Magee: The presence of nitrosamines in cigarette smoke, even the slightest traces of such, has to be taken seriously in view of these findings. *Druckrey's* experiments and the results illustrated by *Brune* show us how small the dosages can be and still be effective.

Comparison of FID and AFID responses to volatile components in the equilibrium vapor over fish that had reached an advanced stage of spoilage demonstrated the advantage of the selective nitrogen detector. Although some volatile nonnitrogenous spoilage compounds masked DMA in the FID analysis, both DMA and TMA were easily quantitatively determined by the more specific alkali flame detector.

The procedures described above are more rapid and, in the case of on-column trapping, more sensitive than methods (Dyer, 1945; Dyer and Mounsey, 1945; Nonaka *et al.*, 1967) presently used for the quantitative analysis of TMA and DMA in fish.

Although columns, containing Graphon and TEP (2%), were used exclusively throughout this investigation, comparable separations and resolution of MA, DMA, and TMA were obtained using Graphon coated with 0.5% TEP (3.7-m × 3-mm o.d.). Contrary to results obtained by Di Corcia *et al.* (1970; Figure 1C), TMA and DMA could not be separated using Graphon and 5% TEP.

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Determination of Nitrosodimethylamine in the Low Parts Per Billion

John E. Newell* and Harold R. Sisken

Nitrosodimethylamine is measured in apples and milk in the low ppb range by vacuum distillation, concentration, gas chromatography, and microcoulometric nitrogen determination. The concentration of the NDMA is achieved by percolation of the aqueous distillate through polymer beads. The method is sensitive to 3 ppb and the recovery is 70% or better.

About 20 ppb nitrosamines have been detected in tobacco smoke and food by Kroeller (1967) in Germany using thin-layer and gas chromatography. Mayerhofer and Mochler (1967) and Sen *et al.* (1969) used these techniques to determine nitrosodimethylamine (NDMA) and nitrosodiethylamine in food. Rhoades and Johnson (1970) selectively pyrolysed *N*-nitrosamines to ammonia at 400–600° C. Howard *et al.* (1970) extracted NDMA from smoked fish and detected it by a gas chromatograph equipped with a modified thermionic detector. Fazio *et al.* (1971), also working with fish, determined NDMA by gas chromatography and confirmed its identity with a mass spectrometer. Lakata (1967) suggested the use of a Porapak column for the gas chromatographic resolution of NDMA from other volatile components of foods.

This paper presents an analytical method sensitive to 3 ppb of NDMA in raw apples, cooked apples, and in milk. The method should be applicable to the determination of NDMA in other foods as well, and also to the determination of other volatile nitrosamines. The use of Porapak to accumulate a few parts per billion of organic compounds from large volumes of water could also find applicability in water pollution analysis and urinalysis.

The method consists of five operations. (1) The NDMA and other volatile components are removed from the nonvolatile fraction of the sample by a vacuum distillation by a technique which minimizes loss by hydrolysis and volatility. (2) The NDMA and other organic materials in the distillate are removed from the water by percolation through a column of polymer beads. (3) The NDMA is removed from the polymer beads by heat and carried into a gas chromatographic column. (4) Programmed temperature gas chromatography separates the NDMA from other components. (5) The NDMA is catalytically reduced to ammonia, which is microcoulometrically titrated and continuously recorded. The area of a peak at a specific retention time established the amount of NDMA.

MATERIALS AND EQUIPMENT

The equipment for this method is shown in Figures 1, 2, 3. The rotary evaporator had a Teflon dri... 5001, Calif. Laboratory Equipment Co... In Figure 3, valve g-1 denotes a toggle va...

Uniroyal Chemical, Division of Uniroyal, Incorporated, Naugatuck, Connecticut 06770.

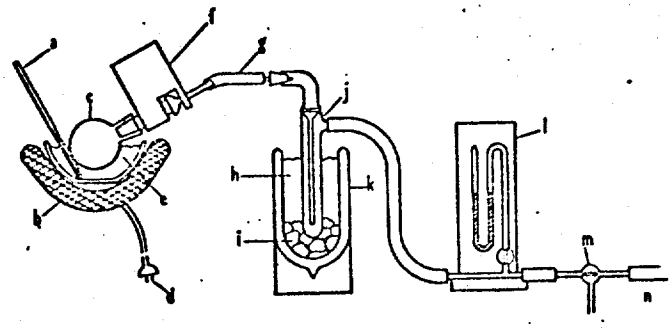

Figure 1. Vacuum distillation apparatus. Key: a. Thermometer; b. Heating mantle; c. 309-ml round-bottomed flask; d. To variable transformer; e. Evaporating dish containing water; f. Rotary evaporator; g. Teflon tubing; h. Acetone; i. Dry Ice; j. Vacuum trap; k. Dewar flask; l. Manometer; m. Three-way stopcock; n. To vacuum pump

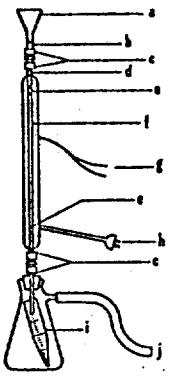

Figure 2. Scavenging assembly. Key: a. Funnel; b. Teflon connection; c. Swagelok fittings; d. Quartz tube; e. Glass wool; f. Porapak Q; g. Thermocouple leads; h. Heater plug-to variable transformer; i. centrifuge tube; j. To mild vacuum

and g-2 and g-3 denote valves having bellows instead of packing or O-rings, designed for leak-tight, moderately high temperature service. Valves g-2 and g-3 and the interconnecting T-joint and tubing were wound with asbestos-covered, No. 23 gauge, Nichrome wire and then covered with a wrapping of asbestos tape. By means of a variable transformer, the valve section was maintained at 50–60°C to eliminate condensation in this zone.

The scavenging column was a 19-in. length of 0.24 in. o.d., 0.16 in. i.d. quartz tube inserted in a 17-in. length of $^3/_8$-in. o.d., $^1/_4$-in. i.d. stainless steel tube wound with asbestos-covered 23 gauge Nichrome wire. A thermocouple was included between the Nichrome winding and the stainless steel tube. The winding was covered with several thicknesses of asbestos tape until the wrapped section was about 1 in. in diameter. The quartz tube had Swagelok fittings on the ends and the middle 11 in. was filled with 100/120 mesh Porapak Q held in place with quartz wool plugs.

The gas chromatograph was a Perkin-Elmer 881 instrument with the flame detector by-passed. The analytical column was a 6-ft × $^1/_8$-in. o.d. stainless steel column filled with 80/100 mesh Porapak Q-S.

Newly packed columns and scavenger tubes were preconditioned by heating to 175°C overnight with a low helium flow.

The analytical column in the chromatograph was connected to a quartz reducing tube in a furnace. The quartz reducing tube, micro reduction furnace, and titration cell enclosure have been described previously by Sisken and Newell (1971).

The detector was a Dohrmann C-200-A microcoulometer with a T-400 titration cell. The microcoulometer was operated with a Range Ohms setting of 100 or 200.

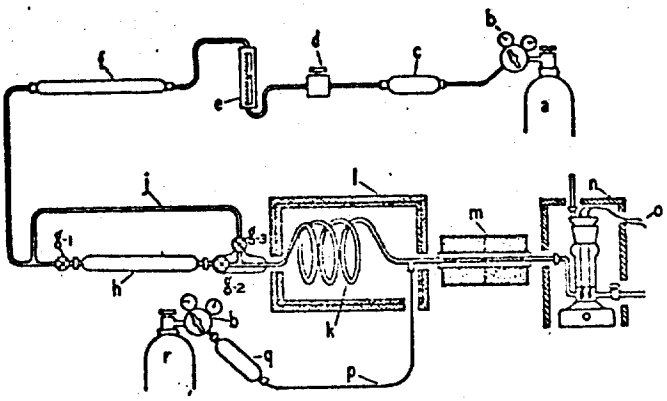

Figure 3. Gas chromatograph–microcoulometric titration assembly. Key: a. Helium; b. Pressure regulator; c. Molecular sieve drier; d. Constant flow regulator; e. Flow meter; f. Porapak Q precolumn; g-1. Valve 1; g-2. Valve 2; g-3. Valve 3; h. 19-in. Porapak Q scavenging column 0.24 o.d. with Swagelok fittings; j. Bypass; k. 6-ft × $^1/_8$-in. Porapak Q-S; l. Programmed temperature oven; m. Two-zone furnace; n. Titration cell; o. Electrical connections to microcoulometer; p. 0.010-in. i.d. S.S. capillary tubing; q. Molecular sieve moistener; r. Ultrapure hydrogen

The carrier gas was "High Purity Helium" (J. T. Baker Co.). Before entry into the chromatograph, the carrier gas passed through a cartridge containing Molecular Sieve 5A and a 23-in. × $^1/_2$-in. o.d. stainless steel tube filled with Porapak Q. This pre-column was conditioned at 150°C with a slow stream of helium passing through it for 16 hr, then cooled to room temperature. When in service, the helium flowed through the precolumn in the opposite direction to its flow during conditioning. Its flow rate was 20 ml/min when the analytical column was at 225°C. The hydrogen was "Ultra High Purity" (Matheson Co.) and its flow rate was 52 ml/min. Both flow rates were measured at the vent stack of the titration cell.

To maintain maximum sensitivity of the analytical column to nitrosodimethylamine, the system was treated to a relatively massive amount (1 μg in 1 μl of water) of NDMA (CAUTION: Carcinogen) at the beginning of each day's work. This was accomplished with a tube similar to the scavenger tube but containing no Porapak Q. The evaporation at 225°C, collection on the analytical column at 80°C, and temperature programming were as described below.

PROCEDURE

A 10-g portion of apple homogenate was weighed into a 300-ml round-bottomed flask containing 2 g of potassium carbonate and 2 ml of water, or 10 g of milk were introduced into the flask which contained 2 g of potassium carbonate. After connection to the rotary evaporator, the flask was partially immersed in a 10–15°C water bath which was a large evaporating dish supported in a heating mantle (Figure 1). The vacuum trap was chilled in a Dry Ice–acetone bath for several minutes, the vacuum line connected, the rotary evaporator tuned on, and vacuum slowly applied until an ultimate pressure of 1 mm was reached in 5 min. The variable transformer connected to the heating mantle was turned to a setting which raised the temperature to 50°C (40°C for milk) in 15 min. During the vacuum distillation, the vacuum was maintained at 1 mm or less.

The temperature of the bath was kept steady and the distillation continued for 30 min for apples. The apple pulp became almost dry. For milk, the distillation was discontinued when about 2 ml remained. Then air was slowly admitted to the system, and the cold trap was removed and warmed to room temperature. The distillate was rendered mildly alkaline (pH 8–9, with pH indicating paper) by adding 2 drops of 2% aqueous K_2CO_3.

Table I. Analytical Results

Untreated	Apples Raw	Apples Cooked	Milk, Raw
ppb NDMA found[a]			
High	1.3	1.7	2.7
Low	0.0	1.0	1.3
Avg	0.7	1.4	1.9
95% confidence limits	1.2		1.2
Number of analyses	12	4	11
Fortified (10 ppb)			
ppb NDMA recovered[b]			
High	12.4	7.1	9.3
Low	8.0	7.1	6.7
Avg	10.2	7.1	7.8
Avg % recovery		71	78
95% confidence limits	3.0		2.3
Number of analyses	12	2	7

[a] Small interference peak, calculated as NDMA. [b] Corrected for untreated sample.

Valves g-1 and g-2 of the apparatus (Figure 3) were closed, valve g-3 was opened, and the scavenger tube was removed from the apparatus. It was connected by a Swagelok fitting to a vacuum flask (Figure 2) and a small funnel was connected to the upper end with Teflon tubing. Using mild vacuum, the distillate was passed through the Porapak Q beads in the quartz tube, followed by 2 ml of water rinsing. The filtration was very slow, taking 30 or more minutes. Then clean air was sucked through the scavenger tube for 3 min.

The tube was removed from the filter flask and the uppermost end was connected to the assembly at valve g-1 (Figure 3), leaving the other end venting to the atmosphere. Valve g-1 was opened to permit helium to flow through the tube. The temperature of the stainless steel sleeve was slowly raised (in 8–10 min) to 100°C to expel water. A cold watch glass or metal sheet was held against the exhaust end and fogging showed the emission of water vapor. The open end of the tube was occasionally dried by insertion of cotton-tipped swabs. After a few minutes at 100°C, the fogging stopped and the heater was turned off and the helium was permitted to flow for another minute. Valve g-1 was closed and the tube was disconnected, reversed, and then reconnected, gas tight, at both ends so that the end to which the funnel had been attached was near valve g-2.

The programmed temperature oven, which had been maintained at 225°C to this point, was now cooled to 80°C and allowed to stabilize. With minimum loss of time, valves g-1 and g-2 were opened, valve g-3 was closed, and the scavenger tube variable transformer was turned on. The temperature of the scavenger tube was raised to 225 ± 5°C quickly and the variable transformer turned back to a setting that would hold this temperature. After 14 min, the recorder was started and the analytical column programmed from 80 to 225°C at 6°C/min. The upper temperature was held for 30 min thereafter. Nitrosodimethylamine recorded as a peak on the chart at a retention time of 26 ± 1 min. At 10 min into the programmed temperature run, the heater on the scavenger tube was turned off and the helium allowed to flow through it as it cooled.

The areas of peaks from the sample and from nitrosodimethylamine standards were found by tracing them on a piece of tracing paper. The peaks were then cut out and weighed. From these areas, the weight of the sample and Range-Ohms setting of the microcoulometer, the parts per billion of NDMA was calculated.

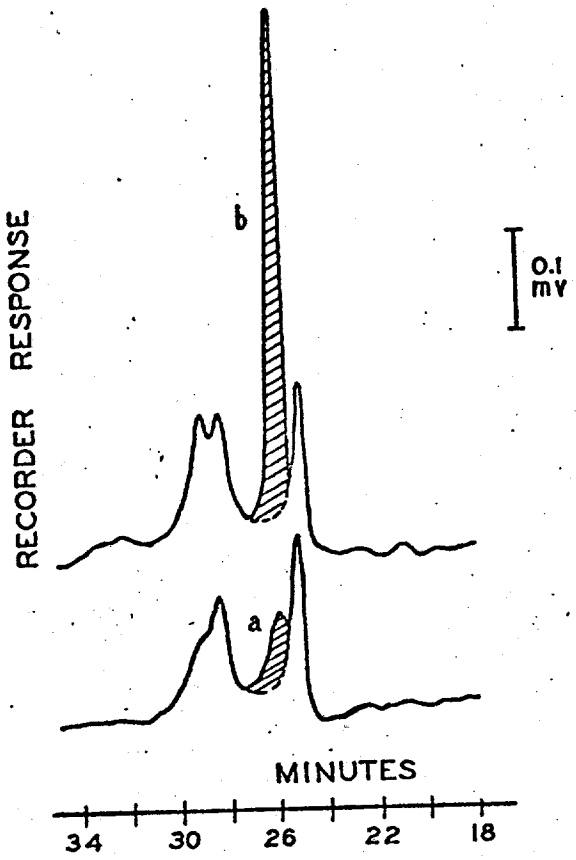

Figure 4. Chromatograms of untreated and NDMA-fortified cooked apples. Chromatogram b has been displaced upward for clarity

RESULTS

The samples of apples came from seven widely separated areas in the United States. Small positive blank results in untreated samples were due to a small peak of the same retention time as NDMA, caused by a naturally occurring nitrogenous component. The milk samples were from six cows. The chromatogram from untreated milk samples also showed a naturally occurring interference. However, no implication is intended that apples or milk contain nitrosodimethylamine. To find the efficiency of recovery, samples of cooked apples and milk were fortified with 10 ppb of NDMA prior to vacuum distillation. The data for raw apples were obtained as a part of an earlier project in which the efficiency of recovery was not measured. Several samples fortified with 10 ppb were analyzed and the average response of the equipment was set at 10. Therefore, the data for raw apples should be evaluated only as an indication of precision. The results of analyses are given in Table I. Typical chromatograms from untreated cooked apples, and cooked apples to which had been added 10 ppb NDMA, are shown in Figure 4.

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The Depletion of Added Sodium Nitrite in Ham

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Abstract

The concentration of added sodium nitrite in meat is progressively lowered during curing, cooking, and storage. The rate of this depletion appears to be exponentially related both to pH and temperature. For ham meat the rate doubles for approximately every 22°F (12°C) increase in temperature or for every 0.86 pH units decrease over the range studied. Thus the "half life", i.e. the time required for the nitrite to be depleted to ½ of its original value (with pH kept constant at 6.2), ranged from 513 hours at 40°F (4.4°C to only 1.6 hours at 220°F (104.4°C) and from 4.9 hours at pH 5.6 to 12 hours at pH 6.7 when temperature was held constant at 170°F (76.7°C). This relationship appears to be independent of initial nitrite concentration over the range of conditions tested and can be expressed mathematically by the equation "$\log_{10}$ (½ life) = 0.65 — 0.025 (Temperature °C) + 0.35 (pH). This relationship did not apply at room temperature. In this case the rate of nitrite depletion was unexpectedly rapid unless the product was first sterilized, indicating that bacterial consumption was probably a factor.

Résumé

La concentration en nitrite de sodium ajouté à la viande diminue progressivement au cours de l'affinage, de la cuisson et de l'entreposage. La vitesse de cette diminution semble être en relation exponentielle avec le pH et la température. Dans le cas du jambon, la vitesse double pour chaque diminution du pH de 0.86 dans la zone étudiée.

Ainsi la demi-vie de nitrite, c'est-à-dire le temps nécessaire pour une diminution à la moitié de sa valeur originale (au pH constant de 6.2) varie de 513 heures à 40°F (4.4°C) à seulement 1.6 heures à 220°F (104.4°C) et de 4.9 heures au pH 5.6 à 12 heures au pH 6.7 lorsque la température est maintenue à 170°F (76.7°C). Cette relation semble indépendante de la concentration initiale en nitrite dans la zone des conditions étudiées. Elle peut être exprimée mathématiquement par l'équation.

"$\log_{10}$ (½vie) = 0.65 — 0.025 température °C + 0.35 (pH)

Cette relation ne s'applique pas à la température ambiante. Dans ce dernier cas la diminution du nitrite est d'une rapidité imprévisible à moins que le produit soit d'abord stirilisé. Ceci indique que l'utilisation bactérienne est probablement en cause.

Introduction

It has long been known that the amount of nitrite actually required for cured flavour and colour development is very small. Normal tasting hams, for instance, have been produced by adding only 1/10 the normal amount of nitrite, about 15 ppm, (Barnett *et al*, 1965). More recently, work on the relationship between nitrite content and toxinogenesis by C. botulinum (Pivnick *et al*, 1967), has indicated a need to examine more fully the loss of nitrite that occurs during cooking and storage of cured meat products. Industry experience shows that canned hams contain only a fraction as much nitrite after several months storage as was present directly after canning. There is a rapid reduction of nitrite during retorting and then a further slower depletion on storage.

Other investigators have noted a similar depletion with time (Rose and Peterson, 1953; Morris, 1952; Kueck *et al*, 1966). Rose and Peterson (1953), noted that there was a progressive destruction of nitrite (which was independent of bacterial contamination) which he attributed to be due to the reducing systems of fresh pork. The pH of the product influenced the depletion rate.

Fresh pork extracts in the temperature range of 60-120°F (15.6 to 48.9°C) were used. Morris (1952) noted a correlation of nitrite content with production of gaseous pressure, during canning, (due presumably to oxides of nitrogen). Similar observations were made at Canada Packers Laboratories.

Walters & Taylor (1963) used differential absorption manometry to show that a gas having the solubility characteristics of nitric oxide was produced when pork muscle was incubated at 37°C (98.6°F) with nitrite at a concentration of 2500 ppm. Gas was passed through water, alkali, then alkaline sulfite, using a Warburg apparatus. The gas passed by alkali but trapped by alkaline sulfite was presumably NO. This was later confirmed by mass spectrometry.

Kueck (1966) in a patent on a process for canned meat reported data showing the rate of depletion of nitrite in processed canned meat held at various temperatures. It was claimed that canned meat heated for a period at low temperature (160-180°F; 71-82°C) then flash heated for a short period (20 minutes) at 230°F (110°C) followed by a 40 day storage period at 45°F (7°C) would be shelf stable. The step heating was intended to reduce consumption of nitrite and thus enhance shelf stability. Half lives calculated from his data are about 460 hours at 45°F (9°C), 170 hours at 75°F (23.9°C) and 96 houres at 100°F (37.8°C).

The experimental work reported in the literature has (with the exception of Kueck) been under very specific conditions, sometimes very different from industry operating and storage conditions. Rose, for instance, worked on aqueous pork extracts at 60-120°F (15.6 to 48.9°C) while Walters & Taylor used abnormally high levels of nitrite (2500 ppm). We felt therefore that a general investigation into the area of nitrite depletion was warranted. The importance of nitrite levels has been emphasized in papers by Silliker *et al* (1958) and by Pivnick et al (1967) in which they demonstrated that nitrite has an inhibitory effect on bacterial growth and toxinogenesis after processing.

Experimental

Fresh ham meat at several different pH values was ground through a ¼" plate, then chopped in a silent cutter together with 20% of its weight of salt brine containing 100 g NaCl/litre and various amounts of phosphate and nitrite.

The pH value of the meat containing the curing ingredients was determined. The product was then

vacuum sealed in small cans (211 x 103) (to allow rapid heat transfer) and held in an oil bath at various temperatures. Samples were selected at intervals and analysed for nitrite, using the α-napthylamine sulphanilic acid method (A.O.A.C., 1965)

The data obtained was plotted on semi-log graph paper with the nitrite concentration on the log scale and time on the linear scale.

A regression analyses was then made using data from the straight line portion of the curves, to obtain a half-life for each pH temperature combination.

A further multiple regression analyses of the temperature, and pH value and log (½ life) was then made. The data was weighted in favour of those half-life values which were based on more observations by submitting the data $(n-1)^{1/2}$ times, where n = number of nitrite determinations on which the half-life was based.

Table 1 lists all of the data obtained. The bracketed values indicate the point at which the data begins to depart from a straight line relationship and these were not included in the regression analysis.

Not all of the data has been plotted on graph paper since 47 experiments were run. Instead only enough data was plotted to illustrate the relationships obtained.

Results and Discussion

Figure 1 shows the rate of temperature increase at the geometric centre of the can when held at 170°F (76.7°C). The time required to reach a temperature of 169°F (76°C) was in the order of 45-50 minutes.

Figure 2 shows the effect of temperature on the rate of depletion of nitrite in samples with 0.53% sodium tripolyphosphate and 40 - 50 ppm nitrite added. Ham meat with a pH value of 5.9 was used (pH = 6.2 after pickle addition). Nitrite concentration has been plotted on the log-scale and time on the linear scale of semi-log graph paper. A straight line fits most of the data plotted in this manner, thus indicating a reaction in which the rate is proportional to the concentration of the reactant. The slopes of the curves have been defined by specifying their half-lives. For instance, at 190°F (87.8°C) a half-life of 4 hours was found for the ham being examined. If this ham originally contained 200 ppm sodium nitrite and was held at 190°F (87.8°C), it would contain 100 ppm after 4 hours, 50 ppm after 8 hours, etc.

As the level of nitrite decreases, the rate of depletion decreases until at low levels almost no change occurs. Note that for some samples there is a divergence from the straight line relationship as the concentration becomes low, indicating that other factors besides temperature and concentration contribute to the rate of depletion. This becomes very evident in the data presented in Figure 7, 8, and 9 where the product was held for extended periods.

At room temperature, the rate of depletion becomes unexpectedly rapid and is probably due to bacterial consumption of nitrite. The rapidly increasingly slope of the curve would be consistent with an increasing consumption of nitrite as the bacterial population multiplied. This theory is also supported by the fact

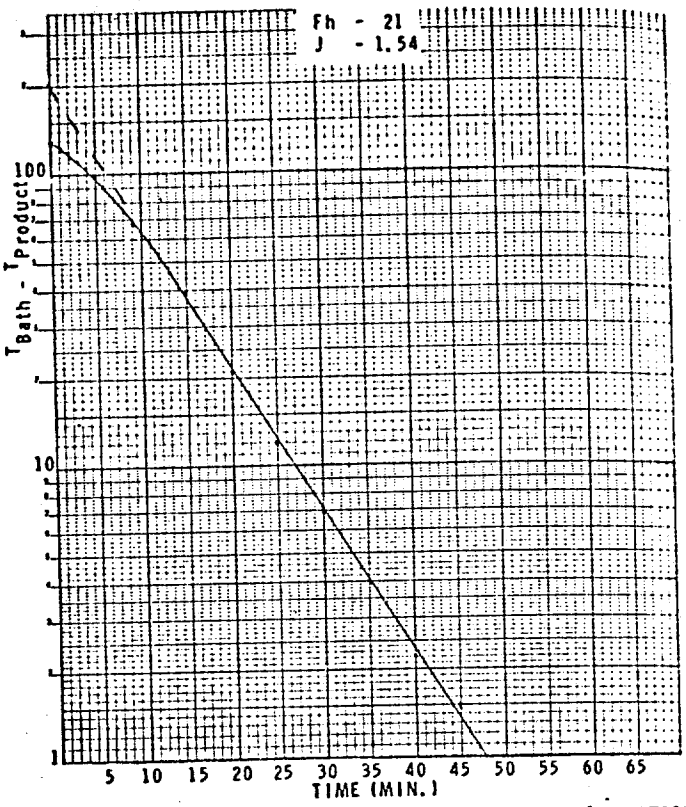

Fig. 1. Heating of emulsion in 211 x 108 cans in oil at 170°F (76.7°C).

that if the nitrite-containing meat is first sterilized to an Fo value of 3 and then held at room temperature, a normal curve of about the expected slope is obtained. This is shown in Figures 2 and 3. The lower original level of nitrite here was due to a reduction in the nitrite level during the sterilizing step. A comparison of the slope of the curves for the sterilized samples and the unsterilized samples indicates that the depletion rate is not greatly altered by sterilization.

Figure 4 shows the effect of different levels of

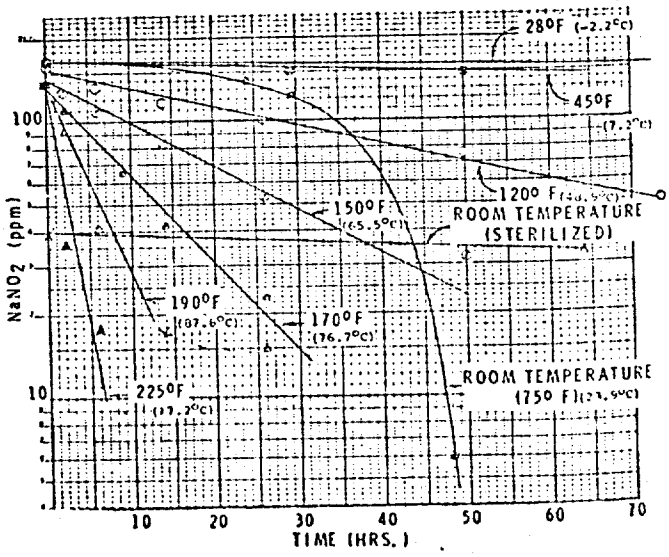

Fig. 2. Rate of depletion of $NaNO_2$ in chopped ham at various temperatures.

TABLE 1

(a) Depletion of nitrite at 0°F (—17.8°C)

	pH of Meat and Curing Ingredients			
Time (Days)	6.3	6.3	7.0	7.0
0	350	280	360	410
14	118	86	244	280
42	42	40	186	238
73	(43)	(32)	172	204
102	(31)	(22)	110	150
Half-Life (Days)	14.3	15.9	68.9	80.8
Half-Life (Hours)	342.7	381.4	1653	1938

(b) Depletion of nitrite at 28°F (—2.2°C)

	pH of Meat and Curing Ingredients				
Time (Days)	6.2	6.3	6.3	7.0	7.0
0	182	280	350	410	360
7	148				
14	122	180	180	280	270
22	100				
43	44	68	50	156	160
73			20	86	91
102			10		44
120		10	(10)	50	
Half-Life (Days)	21.1	25.2	19.8	39.5	34.4
Half-Life (Hours)	506.1	603.8	474.9	948.1	825.3

(c) Depletion if nitrite at 40°F (4.4°C)

	pH = 6.2	
Time (Hours)	#1	#2
0	342	88
72	280	82
192	266	
Half-Life (Hours)	555.0	706.7

(d) Depletion of nitrite at 45°F (7.2°C)

	pH of Meat and Curing Ingredients				
Time (Hours)	5.9	6.0	6.2	6.2	6.6
0	365	112	178	160	
24	300				
45			166		
48				150	
72		97			173
112	280				
120			139		
144	240				
168			137		
240		86	(134)		160
480		67			147
910		47			116
Half-Life (Hours)	298.2	685.6	410.1	515.5	1750.3

(e) Depletion of nitrite at 75°F (23.9°C)

	pH of Meat and Curing Ingredients		
Time (Hours)	6.0	6.2	6.6
0	112	40	165
48	87		162
64		34	
72	73		154
88		32	
144	61		143
160		24	
192	53		141
3[illegible]	33		126
[illegible]			88
Half-Life (Hours)	186.5	217.1	553.7

(f) Depletion of nitrite at 120°F (48.9°C)

	pH of Meat and Curing Ingredients	
Time (Hours)	6.2	6.2
0	160	40
6	131	
14	117	
24		26
26	102	
50	76	
64		16
79	48	
88		(14)
Half-Life (Hours)	48.7	49.2

(g) Depletion of nitrite at 150°F (65.5°C)

	pH of Meat and Pickle Ingredients		
(Hours)	6.0	6.2	6.2
0	160	150	40
2	135	126	
4	126		
6	117	110	
10	104		
14	89	82	
24			14
26		53	
48			8
50		(33)	
Half-Life (Hours)	17.9	18.1	20.7

(i) Depletion of nitrite at 190°F (87.8°C)

	pH of Meat and Pickle = 6.2	
Time (Hours)	#1	#2
0	160	140
2	112	93
4	90	—
6	63	41
10	43	—
14	28	17
26		(15)
Half-Life (Hours)	5.7	4.7

(h) Depletion of nitrite at 170°F (76.7°C)

Time (Hr)	pH of Meat and Pickle Ingredients										
	5.6	5.6	5.9	6.2	6.2	6.2	6.2	6.2	6.7	6.7	6.9
0	100	365	152	160	140	342	88	166	390	340	226
1	—	—	—	—	—	—	—	—	—	—	218
2	60	220	—	146	110	272	85	—	270	284	—
3	—	—	109	—	—	—	—	154	—	—	208
4	40	150	—	117	—	240	76	—	230	250	—
6	30	130	78	100	66	184	69	110	200	206	190
7	—	110	—	—	—	—	—	—	195	—	—
8	(30)	—	—	—	—	136	52	—	—	190	—
10	—	100	—	78	—	—	—	—	168	—	—
12	—	—	55	—	—	116	47	83	—	—	170
14	—	55	—	60	42	—	—	—	—	—	—
16	—	—	—	—	—	92	28	—	125	—	—
24	—	—	20	—	—	55	17	36	—	—	113
26	—	(49)	—	—	23	—	—	—	75	—	—
28	—	—	—	—	—	43	12	—	—	—	—
30	—	(21)	—	—	—	—	—	—	(74)	—	—
31	—	(27)	—	—	—	—	—	—	(72)	—	—
36	—	—	—	—	—	26	9	—	—	—	—
40	—	—	—	—	—	17	—	—	—	—	—
48	—	—	(18)	—	—	—	—	(24)	—	—	60
50	—	—	—	—	6	—	—	—	—	—	—
72	—	—	(18)	—	—	—	—	(24)	—	—	(39)
144	—	—	(17)	—	—	—	—	(21)	—	—	(29)
192	—	—	(12)	—	—	—	—	(16)	—	—	(24)
Half-Life (Hr)	3.4	5.7	8.5	9.7	11.5	9.9	10.0	10.7	12.1	9.3	25.0

(j) Depletion of nitrite at 212°F (100°C)

	pH of Meat and Pickle Ingredients				
Time (Hours)	5.9	6.0	6.6	6.7	6.8
0	365	183	196	390	340
0.25	—	131	180	—	—
0.5	—	113	155	—	—
1.0	—	66	137	—	—
2.0	140	20	84	196	280
2.5	—	19	73	—	—
4	40	—	—	—	205
6	—	—	—	58	145
7	—	—	—	53	—
8	(30)	—	—	—	130
11	—	—	—	(25)	—
15	—	—	—	(17)	—
24	—	—	—	—	25
Half-Life (Hours)	1.25	.72	1.71	2.37	6.4

(k) Depletion of nitrite at 225°F (107.2°C)

	pH of Meat and Pickle Ingredients	
Time (Hours)	5.9	6.2
0	300	140
1.5	40	—
2	—	37
6	—	18
Half-Life (Hours)	0.52	2.2

(l) Depletion of nitrite at 170°F (76.7°C)

	The Effect of Phosphate Addition % Sodium Tripolyphosphate		
Time (Hours)	0	0.27	0.53
0	224	224	224
1	206	206	206
3	182	192	192
6	130	146	150
8	77	79	98
16	40	42	57
25	(24)	(25)	(40)
72	(11)	(14)	(20)
120	(10)	(12)	(22)
Half-Life (Hours)	6.1	6.3	7.8

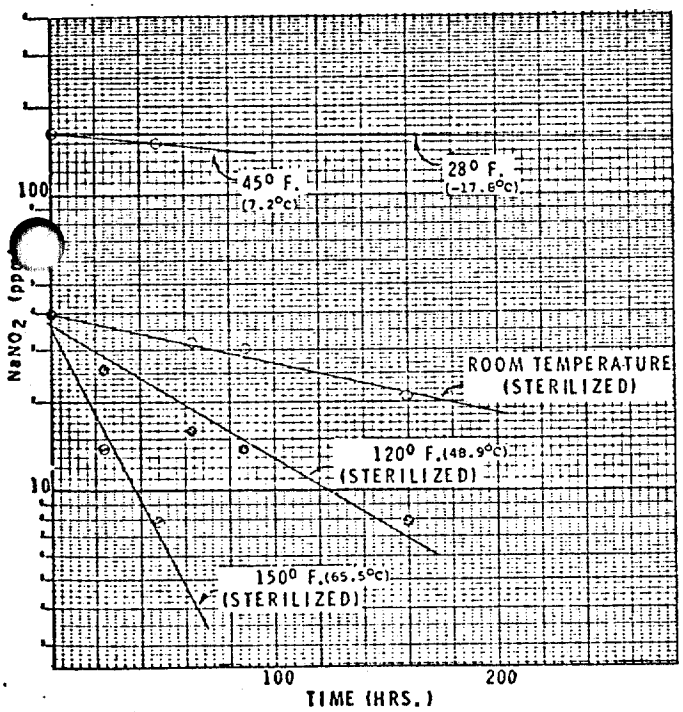

Fig. 3. Rate of depletion of $NaNO_2$ in chopped ham at various temperatures.

nitrite added to the same meat. The lines showing nitrite concentration with respect to time were essentially parallel over the range of 88-340 ppm added when the meat was held at 170°F (76.7°C).

Figure 5 shows the effect of the pH of the meat on depletion rate when held at 170°F (76.7°C) and 212°F (100°C). Ham meat with pH values of 5.5 and 6.4 was mixed with 20% of its weight of curing pickle in this [illegible]. This raised the pH to 5.9 and 6.7 respectively. [illegible] depletion rate is much greater at the lower pH levels.

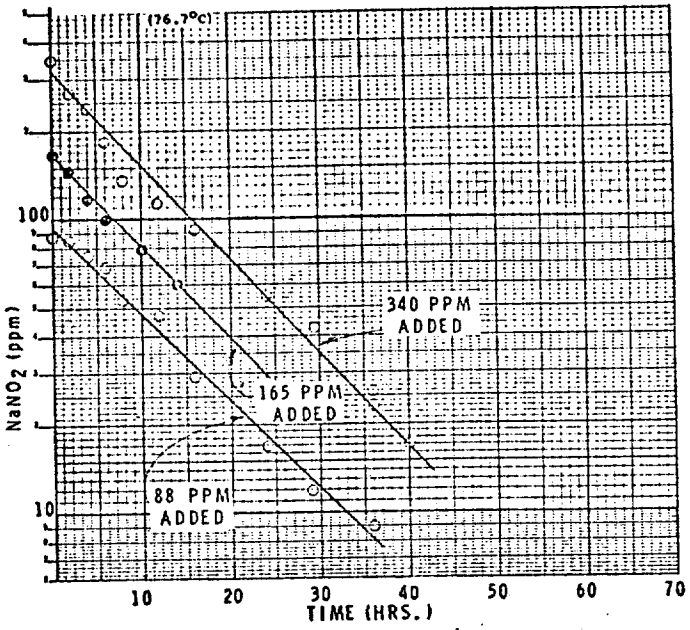

Fig. 4. Rate of depletion of $NaNO_2$ at 170°F when added at various levels.

Figures 6 and 7 show similar effects due to pH. In this case, ham meat with pH values of 5.5 and 7.0 was used. After mixing with pickle, the pH values were 6.3 and 7.0. Storage was at 28°F and 0°F.

Figure 8 gives data of a further experiment in which ham meat with a pH value of 6.0 was adjusted with HCl or NaOH to give pH values of 5.5, 6.0 and 6.9. 20% pickle was then added giving pH values of 5.9, 6.2 and 6.9, respectively before storing at 170°F (76.7°C. A similar pattern of pH effect is evident.

Figure 9 shows the relatively slight effect due to level of sodium tripolyphosphate in the meat. The effect is probably due to its influence on the pH.

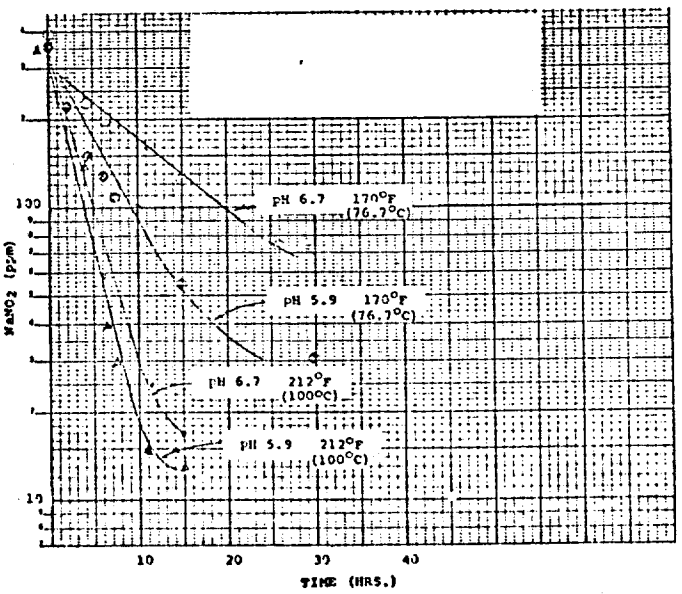

Fig. 5. Effect of pH on $NaNO_2$ depletion at 170°F (76.7°C) and 212°F (100°C).

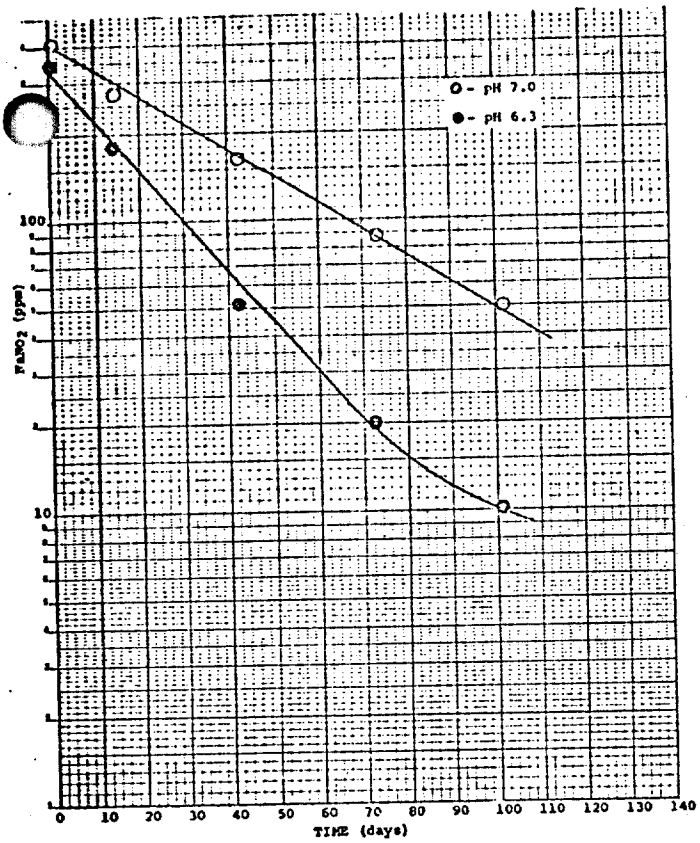

Fig. 6. Effect of pH on rate of depletion at 28°F (—2.2°C).

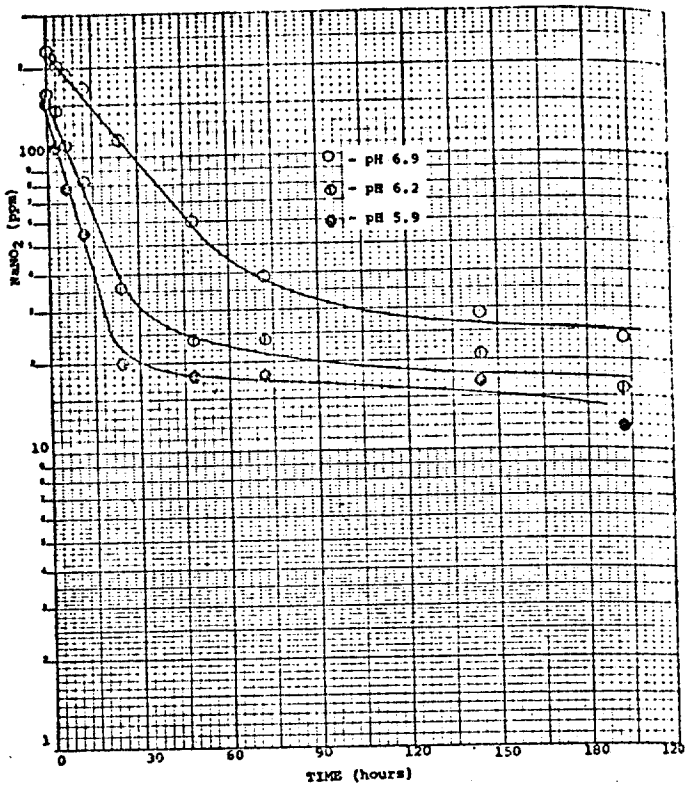

Fig. 8. Effect of pH on rate of depletion at 170°F (76.7°C).

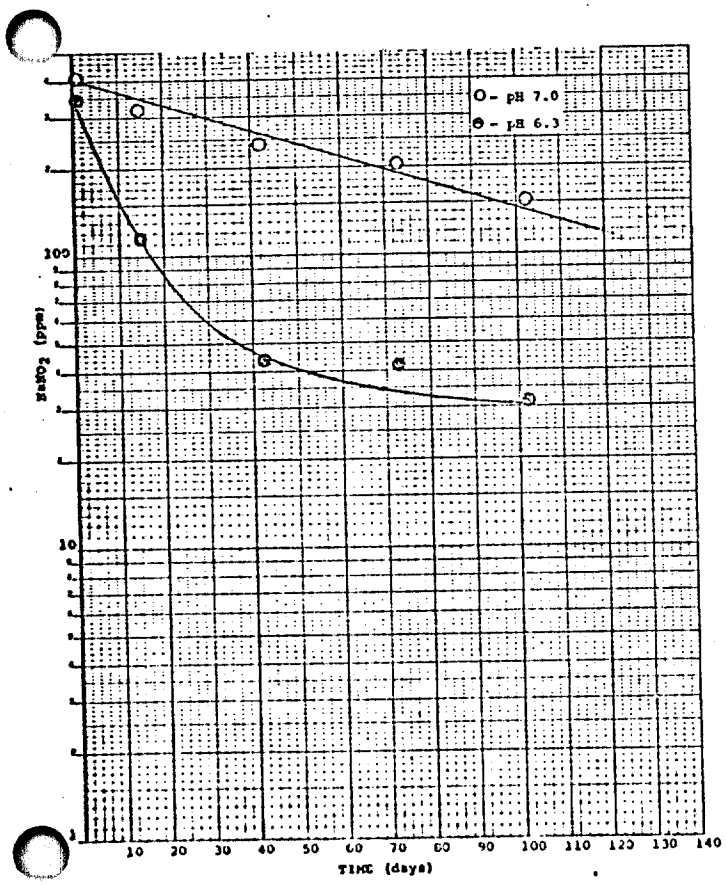

Fig. 7. Effect of pH on rate of depletion at 0°F (—17.8°C).

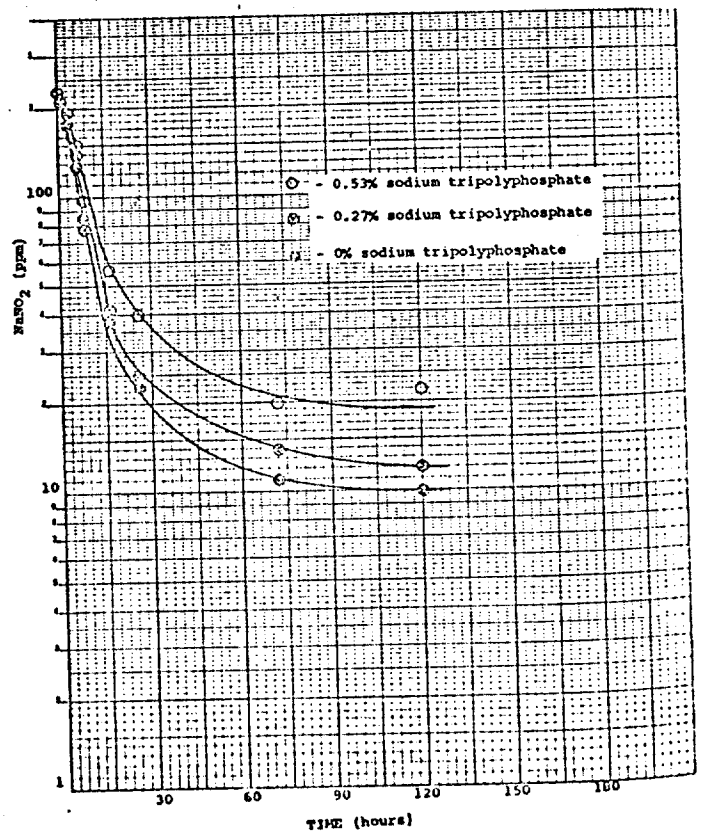

Fig. 9. Effect of sodium tripolyphosphate on rate of depletion at 170°F (76.7°C).

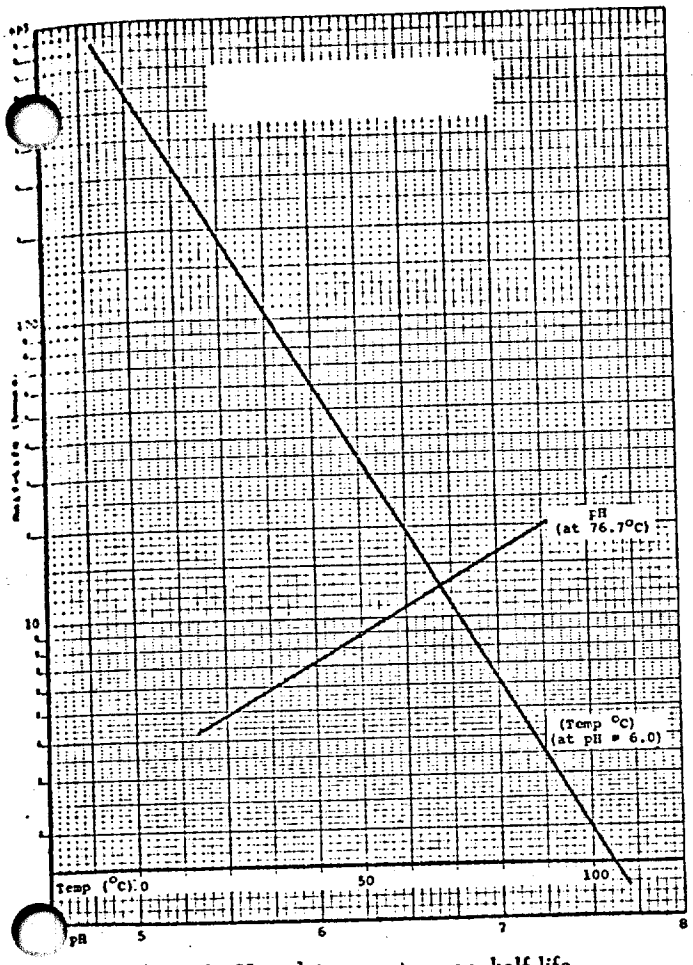

Fig. 10. Effect of pH and temperature on half-life.

A regression analysis of the data shows that depletion of nitrite can be expressed by the equations:

(a) $\log NaNO_2$ concentration) $= a + bt$

where $a = \log$ (initial concentration)

$b =$ constant depending on pH and temperature

$t =$ time

(b) $\log_{10}$ (½ life) $= 0.65 - 0.025$ (Temp °C) $+ 0.35$ (pH)

If the half-life is first corrected for temperature, i.e., $\log_{10}$ (½ life + 0.025 (Temp °C) is plotted against pH a correlation coefficient of 0.56 is obtained. Figure 10 illustrates this relationship.

If the half-life is similarly corrected for pH a correlation coefficient of 0.98 is obtained for temperature.

Figure 10 also illustrates this relationship.

Figure 11 shows the relationship between half-life, pH and temperature. Half-life has been plotted on the vertical axis on a log scale, temperature (°C) on the right axis (linear scale) and pH on the left axis (linear scale) of isometric paper.

The data falls in the plane bounded by the heavy lines shown and defined by the equation $\log_{10}$ (½ life $= 0.65 - 0.025$ (°C) $+ 0.35$ (pH)

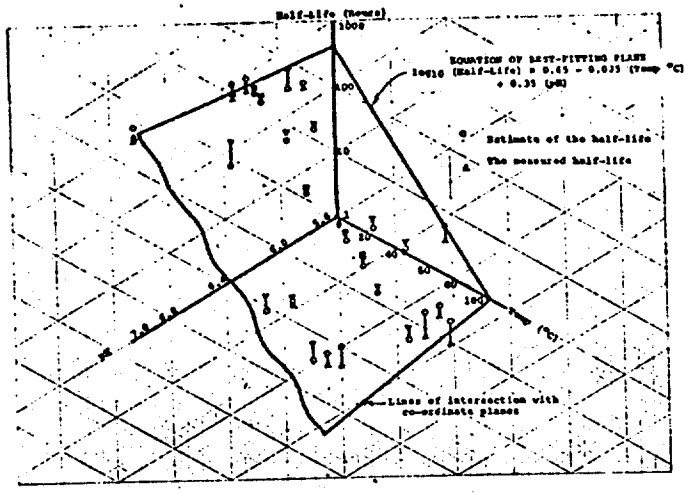

Fig. 11. Relationship between half-life, pH and temperature.

Summary

The rate of nitrite depletion is proportional to its concentration. It is exponentially related to both temperature and pH and doubles for every 22°F (12.2°C) increase in temperature or 0.86 units decrease in pH. The rate is not affected by heat denaturation of the meat. The relationship does not apply at room temperature unless the product is first heat treated.

Acknowledgement

The assistance of D. van Binnendyk who made the statistical analysis of the data and others who contributed to the accumulation of this data is gratefully acknowledged.

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Attachment Question 40 page [illegible] Company [illegible]

The Depletion of Added Sodium Nitrite in Ham

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1564 SUBSTANCE No. 6343 (SOD. NITRATE).

Abstract

The concentration of added sodium nitrite in meat is progressively lowered during curing, cooking, and storage. The rate of this depletion appears to be exponentially related both to pH and temperature. For ham meat the rate doubles for approximately every 22°F (12°C) increase in temperature or for every 0.86 pH units decrease over the range studied. Thus the "half life", i.e. the time required for the nitrite to be depleted to ½ of its original value (with pH kept constant at 6.2), ranged from 513 hours at 40°F (4.4°C to only 1.6 hours at 220°F (104.4°C) and from 4.9 hours at pH 5.6 to 12 hours at pH 6.7 when temperature was held constant at 170°F (76.7°C). This relationship appears to be independent of initial nitrite concentration over the range of conditions tested and can be expressed mathematically by the equation "$\log_{10}$ (½ life) = 0.65 — 0.025 (Temperature °C) + 0.35 (pH). This relationship did not apply at room temperature. In this case the rate of nitrite depletion was unexpectedly rapid unless the product was first sterilized, indicating that bacterial consumption was probably a factor.

Résumé

La concentration en nitrite de sodium ajouté à la viande diminue progressivement au cours de l'affinage, de la cuisson et de l'entreposage. La vitesse de cette diminution semble être en relation exponentielle avec le pH et la température. Dans le cas du jambon, la vitesse double pour chaque diminution du pH de 0.86 dans la zone étudiée.

Ainsi la demi-vie de nitrite, c'est-à-dire le temps nécessaire pour une diminution à la moitié de sa valeur originale (au pH constant de 6.2) varie de 513 heures à 40°F (4.4°C) à seulement 1.6 heures à 220°F (104.4°C) et de 4.9 heures au pH 5.6 à 12 heures au pH 6.7 lorsque la température est maintenue à 170°F (76.7°C). Cette relation semble indépendante de la concentration initiale en nitrite dans la zone des conditions étudiées. Elle peut être exprimée mathématiquement par l'équation.

"$\log_{10}$ (½vie) = 0.65 — 0.025 température °C + 0.35 (pH)

Cette relation ne s'applique pas à la température ambiante. Dans ce dernier cas la diminution du nitrite est d'une rapidité imprévisible à moins que le produit soit d'abord stirilisé. Ceci indique que l'utilisation bactérienne est probablement en cause.

Introduction

It has long been known that the amount of nitrite actually required for cured [illegible] and colour development [illegible]. Normal [illegible] for instance, have been produced by adding only [illegible] the normal amount of nitrite, about 15 ppm. (Barnett *et al*, 1965). More recently, work on the relationship between nitrite content and toxinogenesis by C. [illegible] (Pivnick *et al*, 1967), has indicated a need to examine more fully the loss of nitrite that occurs during cooking and storage of cured meat products. Industry experience shows that canned hams contain only a fraction as much nitrite after several months storage as was present directly after canning. There is a rapid reduction of nitrite during retorting and then a further slower depletion on storage.

Other investigators have noted a similar depletion with time (Rose and Peterson, 1953; Morris, 1952; Kueck *et al*, 1966). Rose and Peterson (1953), noted that there was a progressive destruction of nitrite (which was independent of bacterial contamination) which he attributed to be due to the reducing systems of fresh pork. The pH of the product influenced the depletion rate.

Fresh pork extracts in the temperature range of 60-120°F (15.6 to 48.9°C) were used. Morris (1952) noted a correlation of nitrite content with production of gaseous pressure, during canning, (due presumably to oxides of nitrogen). Similar observations were made at Canada Packers Laboratories.

Walters & Taylor (1963) used differential absorption manometry to show that a gas having the solubility characteristics of nitric oxide was produced when pork muscle was incubated at 37°C (98.6°F) with nitrite at a concentration of 2500 ppm. Gas was passed through water, alkali, then alkaline sulfite, using a Warburg apparatus. The gas passed by alkali but trapped by alkaline sulfite was presumably NO. This was later confirmed by mass spectrometry.

Kueck (1966) in a patent on a process for canned meat reported data showing the rate of depletion of nitrite in processed canned meat held at various temperatures. It was claimed that canned meat heated for a period at low temperature (160-180°F; 71-82°C) then flash heated for a short period (20 minutes) at 230°F (110°C) followed by a 40 day storage period at 45°F (7°C) would be shelf stable. The step heating was intended to reduce consumption of nitrite and thus enhance shelf stability. Half lives calculated from his data are about 460 hours at 45°F (9°C), 170 hours at 75°F (23.9°C) and 96 houres at 100°F (37.8°C).

The experimental work reported in the literature has (with the exception of Kueck) been under very specific conditions, sometimes very different from industry operating and storage conditions. Rose, for instance, worked on aqueous pork extracts at 60-120°F (15.6 to 48.9°C) while Walters & Taylor used abnormally high levels of nitrite (2500 ppm). We felt therefore that a general investigation into the area of nitrite depletion was warranted. The importance of nitrite levels has been emphasized in papers by Silliker *et al* (1958) and by Pivnick et al (1967) in which they demonstrated that nitrite has an inhibitory effect on bacterial growth and toxinogenesis after processing.

Experimental

Fresh ham meat at several different pH values was ground through a ¼" plate, then chopped in a silent cutter together with 20% of its weight of salt brine containing 100 g NaCl/litre and various amounts of phosphate and nitrite.

The pH value of the meat containing the curing ingredients was determined. The product was then

vacuum sealed in small cans (211 x 103) (to allow rapid heat transfer) and held in an oil bath at various temperatures. Samples were selected at intervals and analysed for nitrite, using the α-napthylamine sulphanilic acid method (A.O.A.C., 1965)

The data obtained was plotted on semi-log graph paper with the nitrite concentration on the log scale and time on the linear scale.

A regression analyses was then made using data from the straight line portion of the curves, to obtain a half life for each pH temperature combination.

A further multiple regression analyses of the temperature, and pH value and log (½ life) was then made. The data was weighted in favour of those half-life values which were based on more observations by submitting the data $(n-1)^{1/2}$ times, where n = number of nitrite determinations on which the half-life was based.

Table 1 lists all of the data obtained. The bracketed values indicate the point at which the data begins to depart from a straight line relationship and these were not included in the regression analysis.

Not all of the data has been plotted on graph paper since 47 experiments were run. Instead only enough data was plotted to illustrate the relationships obtained.

Results and Discussion

Figure 1 shows the rate of temperature increase at the geometric centre of the can when held at 170°F (76.7°C). The time required to reach a temperature of 169°F (76°C) was in the order of 45-50 minutes.

Figure 2 shows the effect of temperature on the rate of depletion of nitrite in samples with 0.53% sodium tripolyphosphate and 40 - 50 ppm nitrite added. Ham meat with a pH value of 5.9 was used (pH = 6.2 after pickle addition). Nitrite concentration has been plotted on the log scale and time on the linear scale of semi-log graph paper. A straight line fits most of the data plotted in this manner, thus indicating a reaction in which the rate is proportional to the concentration of the reactant. The slopes of the curves have been defined by specifying their half-lives. For instance, at 190°F (87.8°C) a half-life of 4 hours was found for the ham being examined. If this ham originally contained 200 ppm sodium nitrite and was held at 190°F (87.8°C), it would contain 100 ppm after 4 hours, 50 ppm after 8 hours, etc.

As the level of nitrite decreases, the rate of depletion decreases until at low levels almost no change occurs. Note that for some samples there is a divergence from the straight line relationship as the concentration becomes low, indicating that other factors besides temperature and concentration contribute to the rate of depletion. This becomes very evident in the data presented in Figure 7, 8, and 9 where the product was held for extended periods.

At room temperature, the rate of depletion becomes unexpectedly rapid and is probably due to bacterial consumption of nitrite. The rapidly increasingly slope of the curve would be consistent with an increasing consumption of nitrite as the bacterial population multiplied. This theory is also supported by the fact

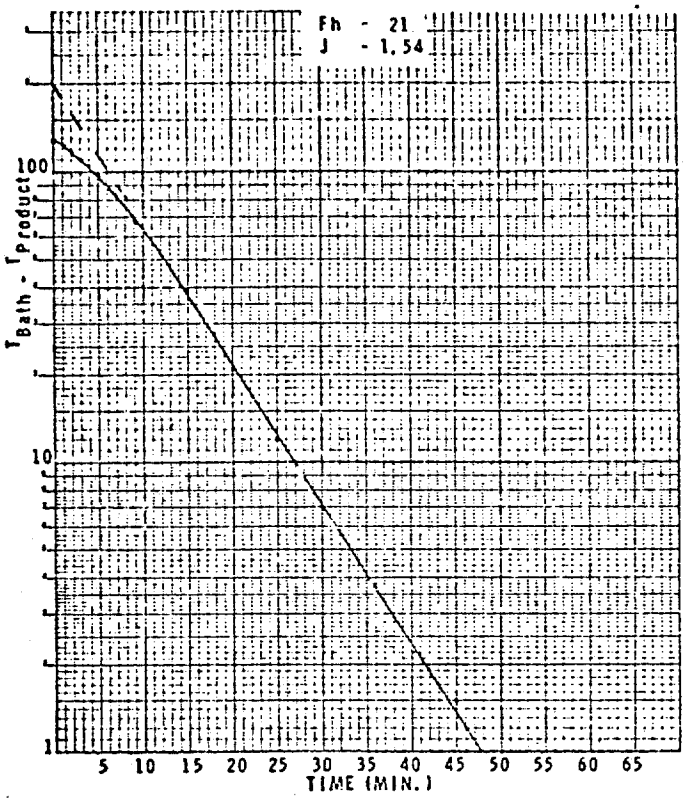

Fig. 1. Heating of emulsion in 211 x 108 cans in oil at 170°F (76.7°C).

that if the nitrite-containing meat is first sterilized to an Fo value of 3 and then held at room temperature, a normal curve of about the expected slope is obtained. This is shown in Figures 2 and 3. The lower original level of nitrite here was due to a reduction in the nitrite level during the sterilizing step. A comparison of the slope of the curves for the sterilized samples and the unsterilized samples indicates that the depletion rate is not greatly altered by sterilization.

Figure 4 shows the effect of different levels of

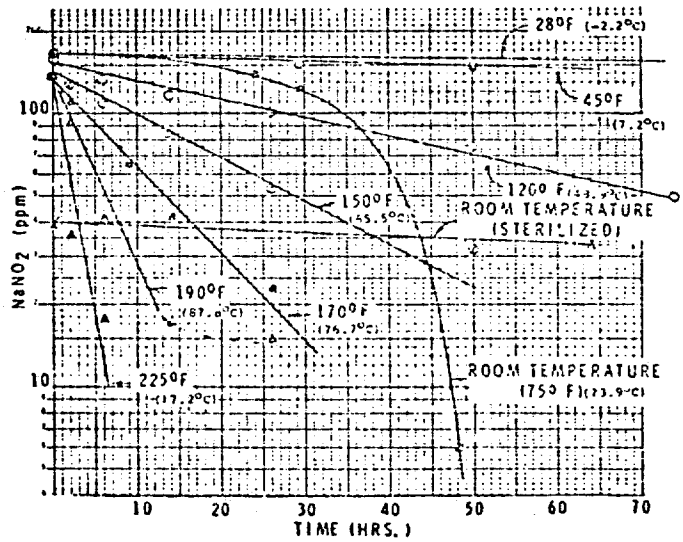

Fig. 2. Rate of depletion of $NaNO_2$ in chopped ham at various temperatures.

TABLE 1

(a) Depletion of nitrite at 0°F (—17.8°C)

	pH of Meat and Curing Ingredients			
Time (Days)	6.3	6.3	7.0	7.0
0	350	280	360	410
14	118	86	244	280
42	42	40	186	238
73	(43)	(32)	172	204
102	(31)	(22)	110	150
Half-Life (Days)	14.3	15.9	68.9	80.8
Half-Life (Hours)	342.7	381.4	1653	1938

(c) Depletion if nitrite at 40°F (4.4°C)

	pH = 6.2	
Time (Hours)	#1	#2
0	342	88
72	280	82
192	266	
Half-Life (Hours)	555.0	706.7

(b) Depletion of nitrite at 28°F (—2.2°C)

	pH of Meat and Curing Ingredients				
Time (Days)	6.2	6.3	6.3	7.0	7.0
0	182	280	350	410	360
7	148				
14	122	180	180	280	270
22	100				
43	44	68	50	156	160
73			20	86	91
102			10		44
120		10	(10)	50	
Half-Life (Days)	21.1	25.2	19.8	39.5	34.4
Half-Life (Hours)	506.1	603.8	474.9	948.1	825.3

(d) Depletion of nitrite at 45°F (7.2°C)

	pH of Meat and Curing Ingredients				
Time (Hours)	5.9	6.0	6.2	6.2	6.6
0	365	112	178	160	
24	300				
45			166		
48				150	
72		97			173
112	280				
120			139		
144	240				
168			137		
240		86	(134)		160
480		67			147
910		47			116
Half-Life (Hours)	298.2	685.6	410.1	515.5	1750.3

(e) Depletion of nitrite at 75°F (23.9°C)

	pH of Meat and Curing Ingredients		
Time (Hours)	6.0	6.2	6.6
0	112	40	165
48	87		162
64		34	
72	73		154
88		32	
144	61		143
160		24	
192	53		141
312	33		126
480			88
Half-Life (Hours)	186.5	217.1	553.7

(f) Depletion of nitrite at 120°F (48.9°C)

	pH of Meat and Curing Ingredients	
Time (Hours)	6.2	6.2
0	160	40
6	131	
14	117	
24		26
26	102	
50	76	
64		16
79	48	
88		(14)
Half-Life (Hours)	48.7	49.2

(g) Depletion of nitrite at 150°F (65.5°C)

Time (Hours)	pH of Meat and Pickle Ingredients 6.0	6.2	6.2
0	160	150	40
2	135	126	
4	126		
6	117	110	
10	104		
14	89	82	
24			14
26		53	
48			8
50		(33)	
Half-Life (Hours)	17.9	18.1	20.7

(i) Depletion of nitrite at 190°F (87.8°C)

Time (Hours)	pH of Meat and Pickle — 6.2 #1	#2
0	160	140
2	112	93
4	90	—
6	63	41
10	43	—
14	28	17
26		(15)
Half-Life (Hours)	5.7	4.7

(h) Depletion of nitrite at 170°F (76.7°C)

Time (Hr)	pH of Meat and Pickle Ingredients										
	5.6	5.9	5.9	6.2	6.2	6.2	6.2	6.2	6.7	6.7	6.9
0	100	365	152	160	140	342	88	166	390	340	226
1	—	—	—	—	—	—	—	—	—	—	218
2	60	220	—	146	110	272	85	—	270	284	—
3	—	—	109	—	—	—	—	154	—	—	208
4	40	150	—	117	—	240	76	—	230	250	—
6	30	130	78	100	66	184	69	110	200	206	190
7	—	110	—	—	—	—	—	—	195	—	—
8	(30)	—	—	—	—	136	52	—	—	190	—
10	—	100	—	78	—	—	—	—	168	—	—
12	—	—	55	—	—	116	47	83	—	—	170
14	—	55	—	60	42	—	—	—	—	—	—
16	—	—	—	—	—	92	28	—	125	—	—
24	—	—	20	—	—	55	17	36	—	—	113
26	—	(49)	—	—	23	—	—	—	75	—	—
28	—	—	—	—	—	43	12	—	—	—	—
30	—	(21)	—	—	—	—	—	—	(74)	—	—
31	—	(27)	—	—	—	—	—	—	(72)	—	—
36	—	—	—	—	—	26	9	—	—	—	—
40	—	—	—	—	—	17	—	—	—	—	—
48	—	—	(18)	—	—	—	—	(24)	—	—	60
50	—	—	—	—	6	—	—	—	—	—	—
72	—	—	(18)	—	—	—	—	(24)	—	—	(39)
144	—	—	(17)	—	—	—	—	(21)	—	—	(29)
192	—	—	(12)	—	—	—	—	(16)	—	—	(24)
Half-Life (Hr)	3.4	5.7	8.5	9.7	11.5	9.9	10.0	10.7	12.1	9.3	25.0

(j) Depletion of nitrite at 212°F (100°C)

Time (Hours)	pH of Meat and Pickle Ingredients 5.9	6.0	6.6	6.7	6.8
0	365	183	196	390	340
0.25	—	131	180	—	—
0.5	—	113	155	—	—
1.0	—	66	137	—	—
2.0	140	20	84	196	280
2.5	—	19	73	—	—
4	40	—	—	—	205
6	—	—	—	58	145
7	—	—	—	53	—
8	(30)	—	—	—	130
11	—	—	—	(25)	—
15	—	—	—	(17)	—
24	—	—	—	—	25
Half-Life (Hours)	1.25	.72	1.71	2.37	6.4

(k) Depletion of nitrite at 225°F (107.2°C)

Time (Hours)	pH of Meat and Pickle Ingredients 5.9	6.2
0	300	140
1.5	40	—
2	—	37
6	—	18
Half-Life (Hours)	0.52	2.2

(l) Depletion of nitrite at 170°F (76.7°C)

Time (Hours)	The Effect of Phosphate Addition % Sodium Tripolyphosphate 0	0.27	0.53
0	224	224	224
1	206	206	206
3	182	192	192
6	130	146	150
8	77	79	98
16	40	42	57
25	(24)	(25)	(40)
72	(11)	(14)	(20)
120	(10)	(12)	(22)
Half-Life (Hours)	6.1	6.3	7.8

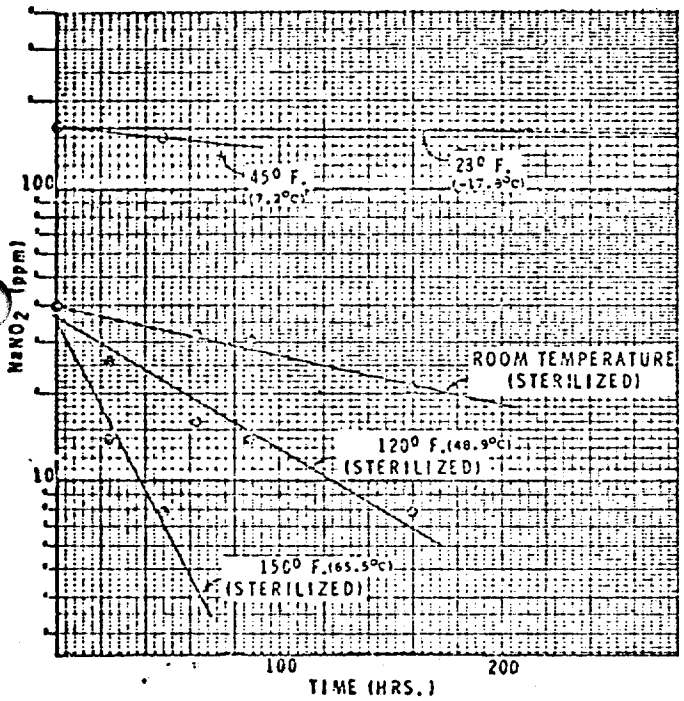

Fig. 3. Rate of depletion of $NaNO_2$ in chopped ham at various temperatures.

nitrite added to the same meat. The lines showing nitrite concentration with respect to time were essentially parallel over the range of 88-340 ppm added when the meat was held at 170°F (76.7°C).

Figure 5 shows the effect of the pH of the meat on depletion rate when held at 170°F (76.7°C) and 212°F (100°C). Ham meat with pH values of 5.5 and 6.4 was mixed with 20% of its weight of curing pickle in this study. This raised the pH to 5.9 and 6.7 respectively. The depletion rate is much greater at the lower pH levels.

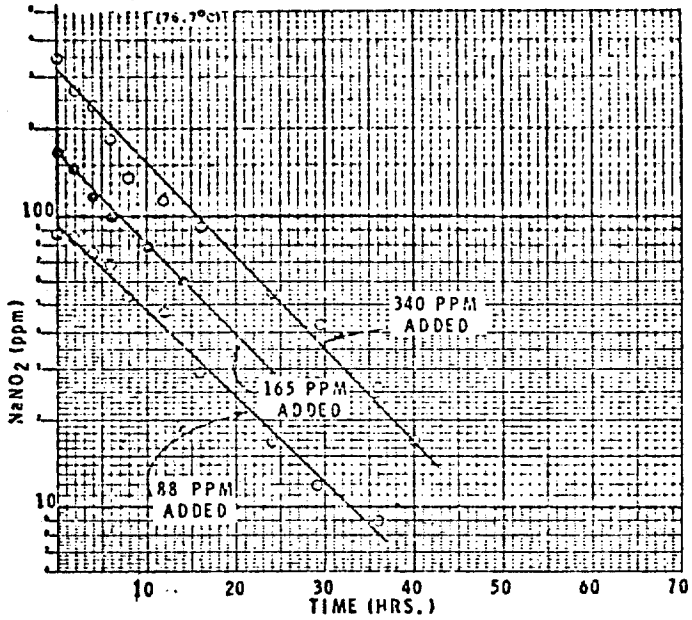

Fig. 4. Rate of depletion of $NaNO_2$ at 170°F when added at various levels.

Figures 6 and 7 show similar effects due to pH. In this case, ham meat with pH values of 5.5 and 7.0 was used. After mixing with pickle, the pH values were 6.3 and 7.0. Storage was at 28°F and 0°F.

Figure 8 gives data of a further experiment in which ham meat with a pH value of 6.0 was adjusted with HCl or NaOH to give pH values of 5.5, 6.0 and 6.9. 20% pickle was then added giving pH values of 5.9, 6.2 and 6.9, respectively before storing at 170°F (76.7°C. A similar pattern of pH effect is evident.

Figure 9 shows the relatively slight effect due to level of sodium tripolyphosphate in the meat. The effect is probably due to its influence on the pH.

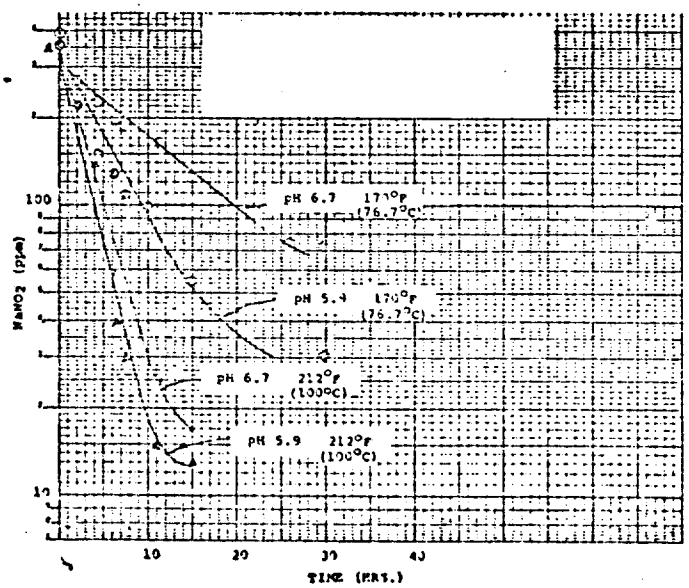

Fig. 5. Effect of pH on $NaNO_2$ depletion at 170°F (76.7°C) and 212°F (100°C).

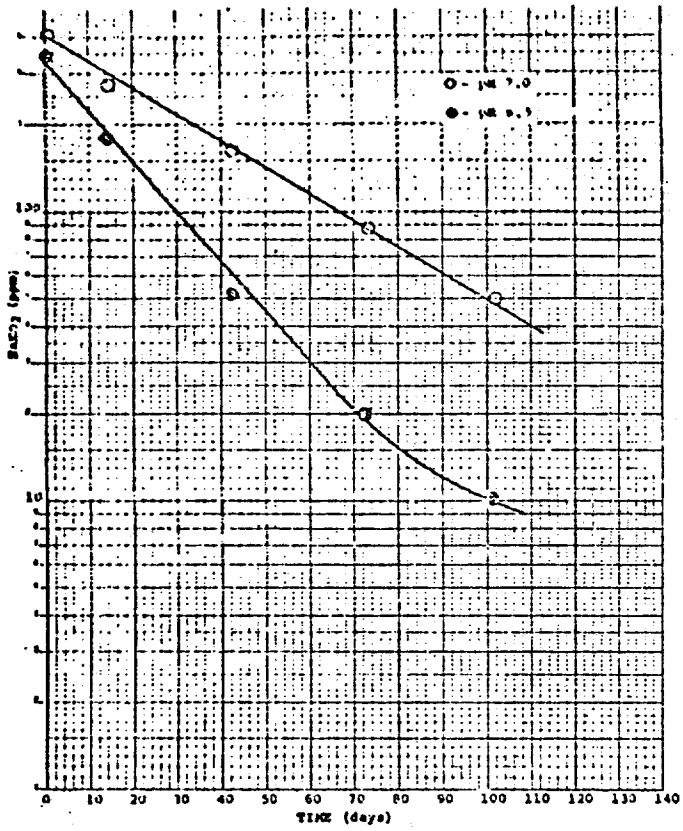

Fig. 6. Effect of pH on rate of depletion at 28°F (—2.2°C).

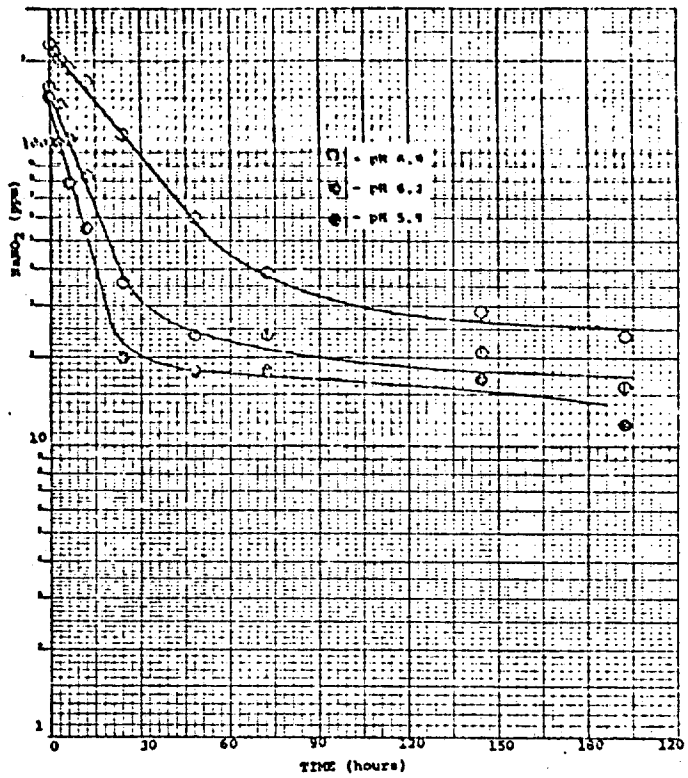

Fig. 8. Effect of pH on rate of depletion at 170°F (76.7°C).

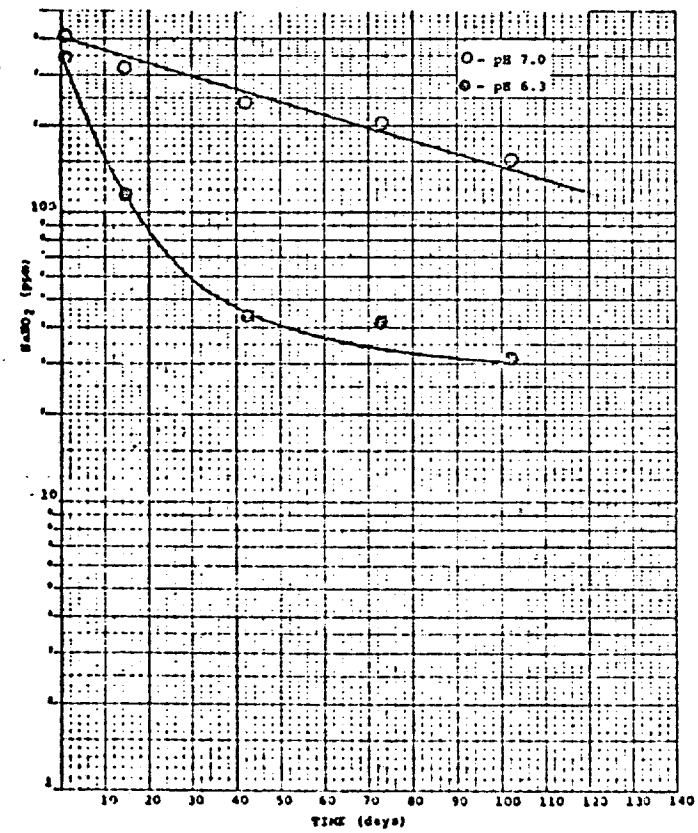

Fig. 7. Effect of pH on rate of depletion at 0°F (—17.8°C).

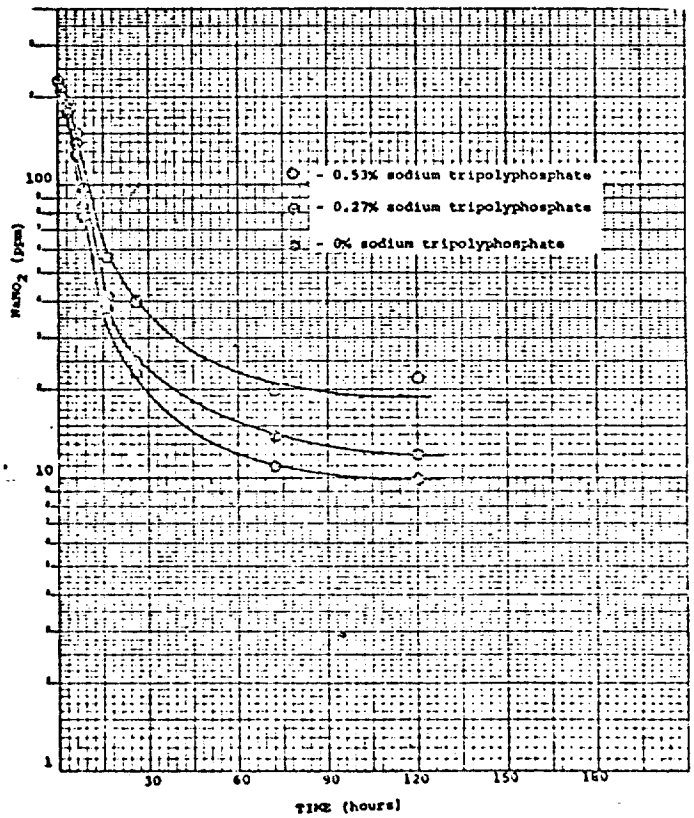

Fig. 9. Effect of sodium tripolyphosphate on rate of depletion at 170°F (76.7°C).

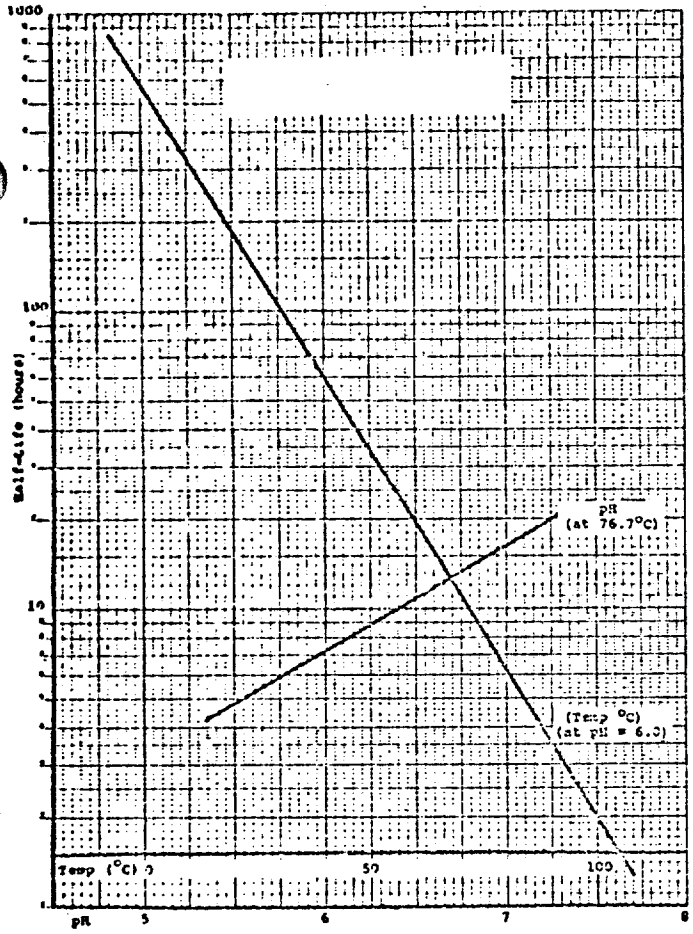

Fig. 10. Effect of pH and temperature on half-life.

A regression analysis of the data shows that depletion of nitrite can be expressed by the equations:

(a) log $NaNO_2$ concentration) = a + bt

where a = log (initial concentration)

b = constant depending on pH and temperature

t = time

(b) $\log_{10}$ (½ life) = 0.65 − 0.025 (Temp °C) + 0.35 (pH)

If the half-life is first corrected for temperature, i.e., $\log_{10}$ (½ life + 0.025 (Temp °C) is plotted against pH a correlation coefficient of 0.56 is obtained. Figure 10 illustrates this relationship.

If the half-life is similarly corrected for pH a correlation coefficient of 0.98 is obtained for temperature.

Figure 10 also illustrates this relationship.

Figure 11 shows the relationship between half-life, pH and temperature. Half-life has been plotted on the vertical axis on a log scale, temperature (°C) on the right axis (linear scale) and pH on the left axis (linear scale) of isometric paper.

The data falls in the plane bounded by the heavy lines shown and defined by the equation $\log_{10}$ (½ life = 0.65 − 0.025 (°C) + 0.35 (pH)

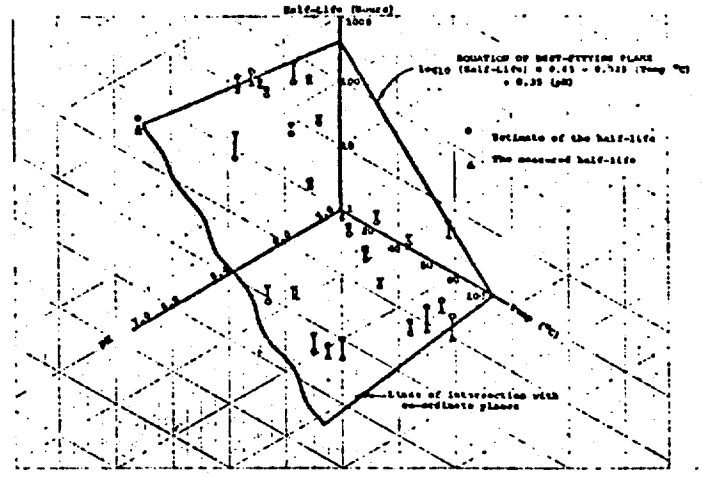

Fig. 11. Relationship between half-life, pH and temperature.

Summary

The rate of nitrite depletion is proportional to its concentration. It is exponentially related to both temperature and pH and doubles for every 22°F (12.2°C) increase in temperature or 0.86 units decrease in pH. The rate is not affected by heat denaturation of the meat. The relationship does not apply at room temperature unless the product is first heat treated.

Acknowledgement

The assistance of D. van Binnendyk who made the statistical analysis of the data and others who contributed to the accumulation of this data is gratefully acknowledged.

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THE ACUTE TOXICITY OF GLYCERYL TRINITRATE AND SODIUM NITRITE IN RABBITS[1]

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There are but few observations in the literature on the toxic action of glyceryl trinitrate or sodium nitrite in lethal or sublethal doses, and apparently no attempt at a quantitative determination of the minimum lethal dose has been made for either of these drugs. In view of the known differences in the action of glyceryl trinitrate and sodium nitrite in therapeutic doses, a comparison of the action of these drugs in toxic doses is of interest.

Merck (1) reviews the literature on the acute toxicity of glyceryl trinitrate. Most of the workers noted the occurrence of convulsions in mammals poisoned with the drug, and tetanic convulsions were also noted in the frog. Brunton and Tait (2) state that when the cord of the frog is transected, convulsions do not occur in the hind quarters. There was invariably a formation of methemoglobin. Kastle and Elvove (3) performed a few inconclusive experiments on the lethal dosage of sodium nitrite in rabbits. They administered the drug subcutaneously. Haldane, Makgill, and Mavrogordato (4) gave lethal doses of both sodium nitrite and glyceryl trinitrate subcutaneously to mice. In the rabbit they found that the symptoms coincided with the amount of methemoglobin produced. They could protect mice against lethal doses of sodium nitrite by placing them in an atmosphere of oxygen at several atmospheres pressure; in such an atmosphere the animals would survive even though 90 per cent of the hemoglobin was converted into methemoglobin. High oxygen

[1] This report is part of an extended study of the pharmacology of the nitrates and nitrites undertaken in the pharmacology laboratory of the University of Wisconsin under the direction of Professor A. S. Loevenhart (1878-1929).

pressure did not protect against lethal doses of glyceryl trinitrate in mice. Binz (5) found the M.L.D. of sodium nitrite for the average rabbit to be about 180 mgm. when given subcutaneously, and described the toxic symptoms which occur after oral and subcutaneous administration. Convulsions following sodium nitrite administration have not been described.

METHOD

Healthy adult rabbits weighing approximately 2 kgm. were used, and the drugs were administered intravenously. The glyceryl trinitrate was given in the form of a 10 per cent solution of the drug in alcohol diluted in each case to 5 cc. with physiological salt solution. The average injection contained, therefore, 10 per cent of alcohol, and was in the form of an emulsion. The time required for injection varied from five to fifteen seconds with an average of ten seconds. The sodium nitrite was dissolved in 20 per cent alcohol so that the amount of alcohol injected would approximate that used in the glyceryl trinitrate administration. The concentration of sodium nitrite ranged from 1.2 to 5 per cent. The time required for injection varied from ten to seventeen seconds. The rapid changes which follow these injections required that the heart and respiratory rates be taken almost continuously, though these observations were necessarily interrupted on the occurrence of convulsions. The animals which survived the injections were kept for a minimum time of ten days. In no instance did any animal which survived the first twelve hours, die during the following days of observation.

Of the forty-two rabbits employed in this investigation, twenty-three were given glyceryl trinitrate and nineteen sodium nitrite (tables 1 and 2).

The symptoms of glyceryl trinitrate poisoning after the intravenous injection of lethal or barely sublethal doses are as follows:

1. Almost immediately after cessation of the injection a respiratory stimulation appeared. This was almost invariably the first effect noted.

2. A slowing of the heart rate closely followed the respiratory stimulation.

3. Convulsions appeared within fifteen to twenty seconds after the injection. The convulsions were both tonic (opisthotonus) and clonic. They were commonly preceded by a brief interval of muscular twitching after which the animal went into severe

TABLE 1

Summary of a study of the toxicity of glyceryl trinitrate in rabbits injected intravenously

DOSE OF GLYCERYL TRINITRATE PER KILOGRAM BODY WEIGHT	NUMBER OF ANIMALS	LIVED	DIED	PER CENT OF DEATHS
mgm.				
70	2	0	2	100
60	2	0	2	100
50	7	2	5	72
45	6	3	3	50
40	6	5	1	17

Taking the minimum lethal dose as that dose at which 50 per cent of the animals die, the M.L.D. is 45 mgm. glyceryl trinitrate per kilogram body weight.

TABLE 2

Summary of a study of the toxicity of sodium nitrite in rabbits injected intravenously

DOSE $NaNO_2$ PER KILOGRAM BODY WEIGHT	NUMBER OF ANIMALS	LIVED	DIED	PER CENT OF DEATHS
mgm.				
150	1	0	1	100
120	1	0	1	100
104	1	0	1	100
100	3	0	3	100
90	4	1	3	75
80	9	7	2	22
70	2	2	0	0
60	1	1	0	0
24	1	1	0	0

M.L.D. of sodium nitrite is between 80 and 90 mgm. per kilogram body weight.

tonic convulsions of a strychnine-like type. These tonic convulsions were followed by the clonic type.

4. An increase in heart rate and decrease in respiratory rate was noted in the intervals between convulsive attacks. The decrease

in respiratory rate was progressive up to the time of death. The increase in heart rate reached its maximum shortly after the convulsions ceased and from that time on the heart rate decreased until death supervened. Respiration usually ceased before the heart stopped beating.

5. Other effects noted were (*a*) a primary constriction of the pupil followed by dilatation, (*b*) expulsion of urine and feces, (*c*) an increased flow of saliva and tears.

All but two of the animals died within twenty minutes after the injection. If an animal survived five hours, recovery was assured.

After poisoning with lethal or barely sublethal doses of sodium nitrite the following events occurred:

1. Immediately following the injection the skin and mucous membranes assumed a brownish color. Salivation occurred in some cases. In males erection of the penis invariably occurred. Urination and defecation took place in from ten to thirty minutes after the injection.

2. Approximately twenty minutes after the injection, a paralysis gradually set in, appearing first in the hind limbs. In the animals which received a fatal dose, the paralysis progressed to an extreme stage and passed over into coma and death. Paralysis was never observed in any animal which survived the dose of sodium nitrite.

3. In five of the twelve rabbits that died, a mild type of clonic convulsion followed the paralysis. The clonic convulsions were usually preceded and followed by a fine muscle tremor. Death always occurred shortly after the onset of the convulsions.

4. The respiratory rate increased immediately after the injections, and the heart rate increased soon thereafter. This condition was temporary, since both respiratory and heart rates soon showed a depression. Immediately preceding the convulsions the heart rate was immediately slow, being in the neighborhood of eighty to one hundred per minute. Following the convulsions respiration ceased whereas the heart rate increased. There then occurred a few terminal respiratory gasps with the heart be-

coming slow in action and finally ceasing to beat about one minute after the last gasp.

Death from sodium nitrite usually occurs within one and a quarter hours after the injection.

DISCUSSION

It is apparent that sodium nitrite and glyceryl trinitrate act very similarly in sublethal doses, just as are their therapeutic actions similar. The differences which occur are those of acuteness and duration of actions. We find the lethal dose of glyceryl trinitrate to be 45 mgm. per kilogram and that of sodium nitrite to be between 80 and 90 mgm. per kilogram. This difference in lethal dosage indicates that we must look elsewhere than to the nitrite ion for an explanation of the more toxic effects of glyceryl trinitrate. In an alkaline hydrolysis in vitro, glyceryl trinitrate yields two-thirds of its nitrogen content in the form of nitrite. Assuming that the same type of hydrolysis takes place in the body we may calculate that 27.3 mgm. of sodium nitrite would furnish the same number of nitrite ions as 45 mgm. of glyceryl trinitrate. Thus the lethal dose of sodium nitrite is approximately three times as large as would be expected if both compounds act only by the liberation of nitrite ions.

The symptoms of poisoning by these two drugs resemble remarkably the symptoms of death by asphyxia whether produced by strangulation or hydrogen cyanide. We have observed no symptom as the result of poisoning with these two substances which is not also present in asphyxia. Both glyceryl trinitrate and sodium nitrite produce evidences of primary stimulation of the central nervous system followed by depression, with the heart beat outlasting respiration. In death from sodium nitrite the convulsions are very mild or absent, which simulates a gradually induced asphyxia; whereas the convulsions following glyceryl trinitrate are very similar to those of sudden asphyxia (as from HCN).

Sodium nitrite and glyceryl trinitrate produce asphyxia either by the fall in blood pressure which follows their administration, or by the formation of methemoglobin. The fact that Haldane

and his co-workers were able to protect their mice against lethal doses of sodium nitrite by keeping them in chambers of compressed oxygen is further evidence in support of the asphyxiation hypothesis. It is probable that other factors than asphyxia must enter in the causation of death by these substances, but the evidence would seem to show that asphyxia is at least one of the more important causes.

SUMMARY

1. The lethal dose of glyceryl trinitrate by intravenous injection in adult rabbits is 45 mgm. per kilogram.

2. The lethal dose of sodium nitrite similarly determined is between 80 and 90 mgm. per kilogram.

3. It is noted that on the basis of available nitrite ion the lethal dose of sodium nitrite is about three times as high as that of glyceryl trinitrate; explanations for this fact are offered.

4. The symptoms of lethal poisoning by sodium nitrite and glyceryl trinitrate are discussed in detail, and their resemblance to asphyxial death is pointed out.

5. It is suggested that asphyxia is one of the major factors in death from lethal doses of these drugs.

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