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44

VI

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VII

THE EFFECT OF CURING SALTS ON BACTERIAL SPORES

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Canned cured meats are merchandized either as perishable or shelf stable products. In both categories the products are heat processed to an extent sufficient to destroy all vegetative forms of food poisoning bacteria. Even in shelf stable canned cured meats, however, the heat treatment given is not sufficient to eliminate all bacterial spores; the process given is necessarily far below that applied to other low acid foods. For example, canned vegetables are prone to spoilage if they are not given a heat process equivalent to about three minutes at 250° F.—expressed as $F_0 = 3$. Cured meats become totally inedible if subjected to that degree of processing. Indeed, severe organoleptic breakdown occurs well below $F_0 = 1$. Subculture of both shelf stable and perishable canned cured meats reveals small numbers of viable aerobic and anaerobic spores. Despite the relative ease with which these spores can be demonstrated in the finished product, the industry has enjoyed a long history of satisfactory commercial production.

One is concerned in canned cured meats, then, with a commercially sterile food, i.e., a product which has been heat processed to an extent sufficient to eliminate vegetative bacteria and has an environment which is unfavorable to the germination and outgrowth of surviving spores. Numerous shelf stable, commercially sterile, non-meat foods are to be found, viz. canned tomato juice and sauerkraut. In these products, the acidity prevents germination and outgrowth of spores. Perishable canned cured meats obviously owe their stability to the practice of merchandizing under refrigeration. With respect to shelf stable cured meats, the most obvious explanation for stability lies in the inhibitory effect of curing salts, NaCl, $NaNO_3$ and $NaNO_2$. As we shall see, however, the presence of curing salts in heat processed cured meats does not fully account for the stability of these products; rather, the presence of these agents in combination with heat shock to the contaminating spores is the key to the stability of canned cured meats.

THE TEST SYSTEM—SHELF STABLE CANNED CURED MEATS

Before attempting a more detailed discussion of the interplay of factors responsible for the stability of these products, it would seem profitable to define more precisely the test system (Table 1).

Many of the studies on the stability of canned cured meats have involved packs inoculated with large numbers of Cl. botulinum spores. Yet, a number of workers have reported that the raw material, red meat, consistently shows a very low order of contamination with putrefactive anaerobic spores (Harriman et al., 1948; Burke et al., 1950; Ayres and Adams, 1953; Brown, 1957; Silliker et al., 1958). Also of significance is the pH of meat (5.6 - 6.2) and its reducing capacity. These factors are of importance with respect to the anti-

TABLE 1

The Test System
Canned Cured Meat Products

Raw Material:	Uncooked beef and pork Indigenous putrefactive spore population less than one per gram.
Salt:	3-6% brine (no regulation)
$NaNO_3$:	0.0078 – 0.06% (no regulation)
$NaNO_2$:	Not greater than 200 p.p.m.
Processing:	1. Perishable – pasteurization 2. Shelf stable – F_0 less than 1.0.

bacterial activity of nitrite (Tarr, 1941; Henry et al., 1954).

As indicated, the brine content of cured meats varies over a rather broad range. The upper limit of salt in such products is, however, self limiting; products with greater than 3.5% salt (about 6% brine) are considered oversalty by the consumer.

No regulation limits the amount of nitrate that may be added to these products directly. Indirectly, however, the amount of nitrate that may be added is controlled by regulation of the final nitrite level in these products. This may not exceed 200 p.p.m. and, of course, includes nitrite that is formed through the bacterial reduction of added nitrate prior to processing.

As indicated previously, these products are given thermal processing in excess of pasteurization but not sufficient to render them sterile. The surviving organisms are anaerobic and aerobic spores.

SODIUM CHLORIDE

Tanner and Evans (1933) concluded that "salt is the most active preservative used in meat curing solutions." There seems little doubt that in high concentration sodium chloride is capable of preventing completely the germination and outgrowth of bacterial spores and that salt is necessary to the stability of canned cured meats. Just as certain is the fact that organoleptically acceptable levels of salt do not inhibit Cl. botulinum and other spore forming organisms. Selective data in support of this point are seen in Table 2. It will be noted that Tanner and Evans (1933) observed growth of Cl. botulinum in the presence of as much as 10.5% salt. Obviously, this is far above the salt content of merchandizable canned cured meats. Yesair et al. (1944) observed a difference between pasteurized spores and spores which were subjected to a heat treatment approximating F = 0.015. Pasteurized spores were not inhibited by 5% salt, whereas the more severely heated spores failed to grow in the presence of 3.5% salt. Stumbo et al. (1945) on the other hand observed growth of putrefactive anaerobic spores in meat which had received heat treatment in excess of F_0 = 1.0; Silliker et al. (1958) observed growth of surviving indigenous spores in meat containing 3.5% brine after processing to F_0 = 0.13. The work of Yesair et al. (1944) suggests that heat treatment slightly above pasteurization combined with an organoleptically acceptable salt level imposes dormancy on Cl. botulinum spores, but conflicting results of other workers warrant the conclusion that salt alone does not confer stability. Recent work with pasteurized Cl. botulinum spores in cured meats containing various amounts of salt has indicated that growth and toxin formation may be masked at high brine levels. At 7.1% brine 27 out of 201 apparently normal cans of comminuted beef were found to contain toxin (Greenberg et al., 1959). These findings suggest that high levels of salt impose a hazard, since the usual evidences of spoilage are masked.

TABLE 2

The Effect of Salt on the Growth of Putrefactive Anaerobes in the Absence of Other Curing Agents

Reference	Test System	Results
Tanner and Evans - 1933	Pasteurized spores in culture medium	Growth of Cl. botulinum in as high as 10.5% salt
Yesair, Bohrer, Cameron - 1944	Pasteurized spores in culture medium	Growth of Cl. botulinum in 5% salt but not in 10%
Yesair, Bohrer, Cameron - 1944	Spores treated at F_0 = 0.015* in culture medium	No growth of surviving Cl. botulinum spores in 3.5% salt
Stumbo, Gross, Vinton - 1945	Spores treated in meat given F_0 = 1.0 and more	Growth of putrefactive anaerobes at 5.5 - 6% brine*
Bulman and Ayres - 1952	Pasteurized spores in meat	Growth of putrefactive anaerobes at 7.5% brine*
Silliker, Greenberg, Schack - 1958	Spores treated in meat given F_0 = 0.13	Growth of putrefactive anaerobes at 3.5% brine
Greenberg, Silliker, Fatta - 1959	Pasteurized spores in meat	Botulism toxin formation in 7.1% brine

*Calculated from author's data.

SODIUM NITRATE

Jensen (1944) wrote: "In the field of microbiology of meats, there are few subjects over which there has been more controversy than on the effect of sodium nitrate on anaerobic bacteria in meats." Richardson (1907) attributed to sodium nitrate the ability to create aerobic conditions in an environment which is anaerobic in the bacteriologic sense. Jensen and Hess (1941) reported the results of experiments involving the stability of pasteurized meat cured with and without nitrate. Their conclusions were that nitrate in cured products is of benefit in preventing the growth of putrefactive anaerobic bacteria.

A critical examination of the literature fails to lend support to Richardson's hypothesis. The data summarized in Table 3 clearly indicate that putrefactive anaerobic bacteria are capable of growth in the presence of amounts of nitrate far in excess of that which is used in cured meats. It is of interest to note that Bulman and Ayres (1952) found that anaerobic spores had about the same tolerance for sodium nitrate as for sodium chloride. Yesair et al. (1944) reported inhibition of Cl. botulinum in culture media which were given a heat

Referenc	Test System	Results
Leifson - 1931	Pasteurized spores in culture media	Growth in 3% $Ca(NO_3)_2$
Tanner and Evans - 1933	Pasteurized spores in culture media	Toxin formation by Cl. botulinum at 4.4% $NaNO_3$
Jensen and Hess - 1941	Pasteurized meat	Growth of Cl. sporogenes in 0.25% $NaNO_3$ in presence of about 5.5% brine
Yesair, Bohrer, Cameron - 1944	Pasteurized spores in culture media	Growth of Cl. botulinum in presence of 0.25% $NaNO_3$
Yesair, Bohrer, Cameron - 1944	Spores receiving F_0 = 0.045 in culture media	Inhibition of Cl. botulinum by 0.1% $NaNO_3$
Stumbo, Gross, Vinton - 1945	Spores in meat receiving $F_0 > 1.0$	Growth of putrefactive anaerobes in presence of 0.18% $NaNO_3$
Bulman, Ayres - 1952	Pasteurized spores in meat	Growth of putrefactive anaerobes in presence of 3.5% $NaNO_3$
Silliker, Greenberg, Schack - 1958	Spores in meat receiving F_0 = 0.13	Growth of putrefactive anaerobes in presence of 0.0156% $NaNO_3$

process approximating F_0 = 0.045 in the presence of 0.1% sodium nitrate. Pasteurized spores grew in the presence of 0.25% sodium nitrate. Stumbo et al. (1945) and Silliker et al. (1948) failed to observe inhibition of anaerobic spore growth in meats containing nitrate, even though heat processes in excess of that reported by Yesair et al. (1944) were studied.

Jensen (1934) wrote "It is an easily proved fact that nitrate-nitrite-salt-sugar cure favors most phases of aerobic bacteria, whereas nitrite-salt-sugar cure always inhibits fermentation and aids putrefaction." Our own work confirms these conclusions in part (Silliker et al., 1958). Table 4 summarizes data from experiments in which comminuted meat with and without nitrate was subjected to heat processing slightly in excess of pasteurization. It will be noted that the addition of nitrate stimulated the appearance of non-putrid swells. The responsible organism in these cases was a member of the genus Bacillus. It is of interest to note, however, that in the absence of both nitrate and nitrite, non-putrid, Bacillus-induced swells occurred (note results with 5% brine and control variables). Further, in this series the only instance of putrefactive swelling was in the cure variable containing 3.5% brine together with nitrate.

In the Jensen paper (1934) it is stated that all the aerobic bacilli isolated from swollen cans of chopped cured ham were nitrate reducers. It would seem that a more logical explanation for the activity of nitrate, relative to organisms in the genus Bacillus, would lie in the position of nitrate as a hydrogen acceptor.

Canned Meat. F_0: 0.06 – 0.13

Cure	Total Cans Packed	Non-Putrid Swells	Putrid Swells	Sound Cans	Percentage of Pack Sound
5% Brine	12	4	0	8	67%
5% Brine + Nitrate*	12	11	0	1	9%
5% Brine + Nitrite†	12	0	0	12	100%
5% Brine + Nitrate + Nitrite	12	0	0	12	100%
3.5% Brine	12	6	0	6	50%
3.5% Brine + Nitrate	12	9	2	1	9%
3.5% Brine + Nitrite	12	0	0	12	100%
3.5% Brine + Nitrate + Nitrite	12	0	0	12	100%
0 Control	12	3	0	9	75%

*Sodium nitrate concentration: 0.0156%.

†Sodium nitrite concentration: 0.0078%.

Hence, with the addition of nitrate in an environment free of gaseous oxygen, the nitrate reducing Bacillus is able to carry out fermentation of sugar utilizing nitrate as a hydrogen acceptor. Support for this argument may be found in the work of Bulman and Ayres (1952) wherein concentrations from 0 to 4.5% nitrate were tested in ground pork inoculated with PA 3679. The product received a pasteurizing cook. Typical putrefactive spoilage occurred in tubes containing no nitrate and in those containing 3.5%. Certain tubes underwent a spoilage described as atypical. This was characterized by a change in color to gray-green, much gas production, no foul odor, and no extensive putrefaction. This spoilage did not occur in the absence of nitrate but was observed in the presence of 3.5, 4.0, and 4.5% sodium nitrate. The atypically spoiled meat contained large numbers of aerobic bacilli, and chemical analysis indicated the presence of 2000 p.p.m. of sodium nitrite. No nitrite was found in meat showing typical putrid spoilage nor in sound tubes.

It now seems apparent that nitrate is relatively inert with respect to putrefactive anaerobic bacteria but allows the development of nitrate reducing bacilli in environments free of gaseous oxygen. Thus, any cured meat product containing nitrate should be relatively less stable than the same product in the absence of nitrate, the degree of instability being determined by the amount of

contamination of the meat with organisms capable of utilizing nitrate as a hydrogen acceptor. The concept that nitrate produces an aerobic environment (Richardson 1903; Jensen, 1934) is true only to the extent that nitrate can for a particular group of organisms serve the same function as gaseous oxygen. The concept that nitrate prevents the development of anaerobic bacteria appears to be without foundation.

SODIUM NITRITE

Much of the published information on the antibacterial activity of nitrite is not applicable to an understanding of the stability of canned cured meat products, since much of the early work failed to take into account the profound effect of pH. Tarr (1941) showed that nitrites are particularly active against bacteria only at pH values below 7. Tarr's findings have been confirmed by Henry et al. (1954), Castellani and Niven (1955), and Eddy and Ingram (1956). In addition to pH, the ability of the test system to effect the reduction of nitrite to nitric oxide affects the activity of added nitrite (Henry et al. [1954]). Related to this is the reported greater toxicity of nitrite under anaerobic conditions (Castellani and Niven, 1955).

Table 5 summarizes representative data on the toxicity of nitrite against putrefactive anaerobic bacteria. It is apparent that at the levels of nitrite that are found in canned meats, this agent alone cannot confer stability.

TABLE 5

The Effect of Nitrite on the Growth of Putrefactive Anaerobes

Reference	Test System	Results
Tanner and Evans - 1934	Pasteurized spores in culture media. Alkaline	Growth of Cl. botulinum in presence of 0.39% $NaNO_2$
Jensen and Hess - 1941	Pasteurized meat	Growth of Cl. sporogenes in presence of 0.03% $NaNO_2$
Tarr - 1942	Spores and vegetative cells in culture media. pH 5.6	Inhibition of Cl. botulinum at 0.02% $NaNO_2$
Yesair, Bohrer, Cameron - 1944	Pasteurized spores in culture media. Alkaline	Growth of Cl. botulinum in presence of 0.02% $NaNO_2$
Yesair, Bohrer, Cameron - 1944	Spores receiving F_0 = 0.045 in culture media. Alkaline	Inhibition of Cl. botulinum in presence of 0.005% $NaNO_2$
Bulman and Ayres - 1952	Pasteurized spores in meat	Growth of putrefactive anaerobes in presence of 0.08% $NaNO_2$
Silliker, Greenberg,	Spores in meat receiv-	Growth of putrefactive

MIXED CURES

From the previous sections one is forced to conclude that no single curing ingredient at the concentration that it is used in canned meats is capable of preventing the germination and outgrowth of bacterial spores. We shall now consider the possibility that the effects of these agents are additive and that combinations of salt, nitrite, and nitrate can, under proper conditions, impose stability.

Tanner and Evans (1934) found that no combination of nitrate, nitrite, and salt (at the concentrations in which these agents occur in canned meats) is capable of inhibiting the growth of Clostridium botulinum and other putrefactive anaerobes. Yesair et al. (1944) reported that 0.0156% nitrite combined with 3.5% salt completely inhibited the growth of pasteurized Cl. botulinum spores in culture media. Nitrate at levels of 0.172 and 0.188% in combination with 3.5% salt did not inhibit growth. The addition of 0.172% nitrate to the curing system containing nitrite and salt resulted in delayed growth of Cl. botulinum. Bulman and Ayres (1952), working with pasteurized pork inoculated with PA 3679, found that no combination of curing agents would suppress indefinitely the germination and outgrowth of spores. The combined effects of the agents were, in general, greater than the individual effects. The active agents were sodium chloride and sodium nitrite. Sodium nitrate had little activity. These experiments are summarized in Table 6.

TABLE 6

Mixed Cures: The Additive Effect of Curing Salts

Pasteurized Meat - 50 P.A. Spores/g			
Cure			Result
3.5% NaCl	.125% NO_3^-	.015% NO_2^-	
X			Total spoilage - 2 weeks
	X		Total spoilage - 1 week
		X	Total spoilage - 4 weeks
X	X		Total spoilage - 2 weeks
X		X	Total spoilage - 12 weeks
X	X	X	Total spoilage - 19 weeks
	X	X	Total spoilage - 1 week

From Bulman and Ayres (1952).

It is apparent that whereas mixed cures have an advantage over cures involving the incorporation of a single curing salt, no combination of these agents is completely effective in the inhibition of spoilage. These findings involved spores which had received no more than a pasteurizing heat treatment. Meat products receiving such treatment are purveyed with the instruction "Perishable, Keep Under Refrigeration." Billions of pounds of such product have been sold without a single case of botulism. One would be naive to conclude that this excellent record can be attributed solely to refrigeration, since perishable products are frequently mistreated both on the retail and consumer level. Rather, it appears that this record can be traced, in part, to the low in-

cidence of putrefactive anaerobic spores combined with the tendency of such products to evince organoleptic degradation when they are mishandled.

There are, however, millions of pounds of shelf stable cured meats sold yearly. These products do not spoil even though they are held indefinitely at ambient temperatures. Such products do not receive heat treatment sufficient to eliminate contaminating spores. To what is the stability of these products to be attributed?

THE EFFECT OF HEAT INJURY COMBINED WITH CURING SALTS

A clue to the stability of shelf stable cured meats is found in the work of Yesair et al. (1944). Using pork infusion broth inoculated with Cl. botulinum spores, they tested the effects of curing salts after heat treatment at 185° F. for 1, 2, 3 and 4 hours. The inoculated organisms were recoverable from the tubes after these treatments, but the surviving spores did not grow in all instances. Tubes containing 3.5% salt and heated for one hour were subsequently negative for growth of Cl. botulinum after one year of incubation. Mixed cures were likewise stable, even though surviving organisms were demonstrated on sub-culture. These data suggest that heat treatment slightly above that necessary for pasteurization, combined with the presence of curing salts, imposes upon the spore an injury which renders it incapable of outgrowth in the presence of curing salts. Mr. G. A. Blodgett of our laboratories has computed the F_0 for the processes used in the study by Yesair et al. A treatment for 1 hour at 185° F., assuming no "come-up time," yields an F_0 of 0.0149.

Silliker et al (1958) studying uninoculated meat processed at F_0 values between 0.08 and 0.13 reported similar findings. Not all curing systems were found capable of producing a stable product. The results summarized in Table 4 show that either 3% or 5% brine combined with 0.0078% sodium nitrite produced a stable product. Neither brine alone nor brine combined with .0156% sodium nitrate was satisfactory. Nitrate increased the incidence of non-putrefactive spoilage over that observed in the absence of nitrate.

It was shown that the stability of these lightly processed cured products is dependent upon a low level of spore contamination. Intentional inoculation of such products with large numbers of putrefactive anaerobic spores resulted in prompt failure of the system. Table 7 shows the results obtained in cured meats receiving an F_0 of approximately 0.1 in the presence of 0.0078% sodium nitrite and 3.5% brine. Both products were inoculated with PA 3679, the first pack at a level of 15,000 per gram and the second, 2.0 per gram. The more heavily inoculated pack showed 66% putrid spoilage after 45 days of incubation. The lightly inoculated pack was completely sound after 90 days of incubation. At this time all cans were opened. There was no evidence of putrefaction, and bacteriological analysis showed that although 20% of the anaerobic spores survived the heat process, no growth of these organisms took place during subsequent incubation.

TABLE 7

Shelf Stability as a Function of Degree of Contamination

Cure*	Process	Inoculum†	Result
3.5% brine, 0.0078% $NaNO_2$	$F_0 = 0.13$	15,000/g	66% putrid spoilage, 45 days (100 cans)
3.5% brine, 0.0078% $NaNO_2$	$F_0 = 0.10$	2.0/g	No spoilage, 90 days (80 cans)

*Mixed with beef-pork emulsion.

†P.A. 3679 spores.

These results confirm those reported by Stumbo et al. (1945) and Vinton et al. (1947). In the earlier paper they reported results on meat tubes inoculated with putrefactive anaerobic spores in the presence of .09% sodium nitrate, .016% sodium nitrite, and 3.5% brine. The tubes received heat processing far above that reported in the studies of Yesair et al. (1944) and Silliker et al. (1958); yet no combination of curing ingredients inhibited the surviving spores. In the 1947 paper, however, uninoculated meat receiving F_0 values of 0.05, 0.2, 0.6 and 1.0 was stable. The authors concluded "That this work should be regarded as preliminary and possibly indicating a promising angle of attack upon the problems related to the thermal processing of canned meat. However, no definite or final conclusion should be drawn because this work should be checked and considerably extended before sufficient data are available to give final conclusions. It is also the opinion of the authors that no attempt should be made to interpret this work as indicating low thermal processes may be commercially safe or that inhibition by salt can be depended upon on a commercial scale to prevent spoilage. A vast amount of work yet remains to be done and to be checked by others on an industry-wide basis before such conclusions are warranted." What seemed to these workers a surprising result in 1947 has now become a fact well tested by commercial meat canners. This fact is that stable canned meats can be processed at levels such that putrefactive and aerobic spores remain in the finished product, provided that the level of contamination is no greater than obtains in commercial meat trimmings. The stability of the product is imposed by sub-lethal heat treatment combined with organoleptically acceptable levels of salt and concentrations of sodium nitrite which fall within legal limits.

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Effect of Individual Curing Ingredients on the Shelf Stability of Canned Comminuted Meats*

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(Manuscript received April 24, 1958)

SHELF STABILITY of canned cured meat products is a perplexing anomaly. Most of these items receive thermal processings far below those applied to other low acid products. Canned vegetables, such as peas, are prone to spoilage if they are not given a cook equivalent to about 3 minutes at 250° F.—expressed as $F_0 = 3$. Cured meat products become totally inedible if subjected to that degree of processing. Indeed, severe organoleptic degradation occurs well below $F_0 = 1$. Subculture of shelf stable cured meats reveals small numbers of viable aerobic and anaerobic spores. Despite the relative ease with which these spores can be demonstrated in finished product, the industry has enjoyed a long history of satisfactory commercial production.

The most obvious explanation for stability lies in the inhibitory effect of curing salts, NaCl, $NaNO_3$, and $NaNO_2$, which are an integral part of these products. However, a survey of the literature which has been built up over the last half century leads one to the conclusion that no reasonable concentration of a single curing salt or combination thereof will prevent completely the growth of *Clostridium botulinum* and other spore forming bacteria *(6, 7, 15, 16, 17, 18, 19, 20, 21)*.

It was our purpose to carry out studies to determine the factors responsible for the stability of lightly processed cured meats. Experimental procedures used were dictated by three primary considerations.

1. Virtually without exception, thermal processings used in the preparation of canned cured meats vary little among commercial producers. As has been indicated above, organoleptic requirements limit thermal processing to values well below spore lethality.

2. Salt concentrations are governed primarily by organoleptic considerations—levels above 3.5% in cured meats are often judged "over salty" by the consumer. Henceforth in this presentation, salt levels will be designated as "per cent brine"—i.e. the percentage of salt in the water phase of the meat emulsion. The bacteriostatic effect of salt in food products is generally believed to be inversely proportional to moisture level *(12)*. Analysis of commercial cured meat items showed a brine content ranging from 3–6%.

Nitrite and nitrate maxima are determined by Federal regulation *(11)*.

3. Ayers *et al. (1)* and Burke *et al. (5)* have demonstrated that a very low level of putrefactive anaerobic spores is present in commercial meat trimmings. Brown *(4)* and Harriman *et al. (11)* have also confirmed this finding; similar results were obtained in a year-long survey at Swift's Chicago Plant.

Studies on the stability of cured meats have often involved packs heavily inoculated with spores of *Cl. botulinum* and other putrefactive anaerobes showing especially high orders of heat resistance.

In view of the fact that previous investigations on the role of curing ingredients have in almost every case involved the use of heavily inoculated product as test material, combined with the observation that the conclusions reached on the basis of these studies is in contradiction to the observed history of the product, it seemed that a rational approach would involve product contaminated only with the indigenous flora. For the purpose of confirming conclusions reached on the bases of uninoculated packs, inoculated material was studied with close attention to the spore levels used. As will be indicated subsequently, the key to the interpretation of these results is, in fact, the level of meat contamination with spores.

MATERIALS AND METHODS

Meat emulsions composed of typical packing house beef and pork trimmings were stuffed in 300 x 308 cans and cooked in commercial retorts. Penetration data, obtained by means of geometrically centered copper constantan thermocouples, were calculated according to methods described by Ball *(2, 3)*.

All cans were incubated at 100° F. and observed each working day for evidence of spoilage. Routinely, spoiled cans were opened and examined organoleptically as well as bacteriologically to determine the nature of spoilage. Putrid swells were defined as cans in which there was any indication of putrefactive anaerobic development, as evidenced by organoleptic and bacteriological analysis of swollen cans.

Inocula used in the confirmatory experiments consisted of *Clostridium* species *P.A. 3679-20* grown in Anderson pork pea medium.

Determination of viable putrefactive anaerobic spores was accomplished by means of 3 tube most probable number analysis in liver infusion broth. Aerobic organisms were enumerated via direct plating on tryptone glucose extract agar.

RESULTS

Results obtained from pilot plant scale tests indicated that within organoleptically acceptable limits of salt and permissible levels of nitrate and nitrite, the key to the stability of lightly processed cured meats is nitrite. Table I summarizes the spoilage results on a pack which received an $F_0 = 0.08 - 0.13$ in 300 x 308 cans. It will be noted that salt (brine) effected no increase in stability of the product over the controls containing no added salt. This failure to increase stability as a result of increasing brine concentration within organoleptically acceptable levels is seen both in the presence and in the absence of nitrate. The nitrate at both levels of brine [illegible]

* Presented at the Eighteenth Annual Meeting of the Institute of Food Technologists held in Chicago, Illinois, May 27, 1958.

TABLE 1

Effect of individual curing ingredients on the shelf stability of canned meat. F_o: 0.08 – 0.13

Cure	Total cans packed	Non-putrid swells	Putrid swells	Sound cans	Percentage of pack sound
5% Brine	12	4	0	8	67%
5% Brine + Nitrate[1]	12	11	0	1	9%
5% Brine + Nitrite[2]	12	0	0	12	100%
5% Brine + Nitrate + Nitrite	12	0	0	12	100%
3.5% Brine	12	6	0	6	50%
3.5% Brine + Nitrate	12	9	2	1	9%
3.5% Brine + Nitrite	12	0	0	12	100%
3.5% Brine + Nitrate + Nitrite	12	0	0	12	100%
0 Control	12	3	0	9	75%

[1] Sodium nitrate concentration: 156 p.p.m.
[2] Sodium nitrite concentration: 78 p.p.m.

a stable product; on the contrary, presence of nitrate apparently enhanced spoilage in general, and as seen in the variable containing the lower level of brine, 2 of the 12 cans showed putrid spoilage, despite the presence of nitrate. Variables containing nitrite, either in the presence or absence of nitrate, were completely stable at both brine levels.

The effect of nitrite is a function of concentration. The experiments reported in Table 1 involved sodium nitrite added at a level of ⅛ ounce per hundred pounds of meat (78 p.p.m.). Data in Table 2 indicate that a level of 39 p.p.m. is not sufficient to prevent spoilage. Further, this pack gave indication of a stimulation effect of nitrate on spoilage organisms and the failure of this agent to inhibit the growth of putrefactive anaerobes. The data fail to reveal any useful function played by nitrate; nitrite must be present in adequate concentration to assure a shelf stable product. The nitrite and nitrate levels shown were those of the concentrations present in the meat emulsion prior to thermal processing. It will be recalled that nitrite is extremely reactive with protein materials and is likewise quite labile to heat; in contrast, nitrate is relatively inert and is not destroyed by heat. Hence, the residual nitrite in the processed meat is far lower than the added levels, whereas the nitrate concentrations in the finished product approximate those in the unprocessed emulsion. Usually, analysis of finished product to which 78 p.p.m. of sodium nitrite has been added before processing, will show between 10 and 20 p.p.m. residue after retorting.

It will be noted that a distinction has been made between putrid and non-putrid spoilage. This is based on the premise that any product allowing the development of putrefactive anaerobes is presumptively one which will allow growth and toxin development by *Cl. botulinum*. Table 3 summarizes data which indicate that sodium nitrite, at a level of 78 p.p.m., is an effective inhibitor of putrefactive anaerobes. The results shown represent a tabulation from a number of experiments similar to those cited in Table 1 and 2. Statistical analysis on the data yielded a Chi Square = 6.37, assuming one positive putrid ran in the series containing 78 p.p.m. of sodium nitrite. This indicates a probability of 98–99% that the apparent inhibition of putrefaction is not due to chance.

TABLE 3

Effect of 78 p.p.m. $NaNO_2$ on putrefactive spoilage in canned meat containing 3.5% brine

	With 78 p.p.m. $NaNO_2$/cwt.	Without 78 p.p.m. $NaNO_2$/cwt.
Cans packed	96	131
Putrid swells	0	13

Chi square: 6.37.
% Reliability: 98–99%.

These results are contingent upon a certain level of heat injury to the spores indigenous to this environment. Rapid spoilage occurs in product containing the same levels of curing ingredients but which has undergone a "pasteurizing" cook (155° F.). However, in a 235° F. retort, growth-restricting injury occurs somewhere below F_o = 0.10 in a 12 oz. container.

Stability is further dependent upon the contamination level of the raw product. Putrefactive anaerobic spore loads range up to a maximum of approximately 1 per gram. Apparently, fresh meat trimmings, rich in readily available organic materials and possessive of a very low O-R potential, tend to favor germination of indigenous P.A. spores. Artificial inoculation with large numbers of spores results in prompt failure of the system. As can be seen in Table 4, ⅔ of a pack containing 3.5% brine, 78 p.p.m. $NaNO_2$ inoculated with 15,000 P.A. spores per gram and retorted to an F_o = 0.13, had developed putrefactive spoilage within 45 days at 100° F. This is in sharp contrast to data reported in Tables 1 and 2 wherein the same curing and processing produced shelf stability when only indigenous spores contaminated the product. Table 4 also reveals that the presence of 156 p.p.m. sodium nitrate failed to change the rate of putrefactive spoilage.

It has previously been mentioned that small numbers of viable aerobic and anaerobic spores can be demonstrated in

TABLE 4

Putrefactive spoilage of cured meats inoculated with 15,000 P.A. spores per gram

F_o = 0.13		
Cure	45 days incubation at 100° F.	
	Total cans packed	Putrid swells
3.5% Brine + 78 p.p.m. $NaNO_2$	100	66
3.5% Brine + 78 p.p.m. $NaNO_2$ + 156 p.p.m. $NaNO_3$	100	64

TABLE 5

Survival of P.A. 3679 in luncheon meat containing 3.5% brine, 78 p.p.m. $NaNO_2$/cwt., processed to F_o = 0.10

	PA 3679
Added per gram	2.0
Indigenous P.A. spores	0.63
Total Viable P.A. spores/gm.	
Raw	2.63
Cooked	0.54
P.A. spore survival	20.5%
Cans incubated 100° F.	80
Cans sound: 90 days	80

TABLE 2

Effect of curing ingredients on shelf stability of canned meat containing 3.5% brine

Cure	F_o = 0.08				F_o = 0.13			
	Total cans in pack	Non putrid swells	Putrid swells	% of pack sound	Total cans in pack	Non-putrid swells	Putrid swells	% of pack sound
156 p.p.m. nitrate	8	7	0	13%	8	7	0	13%
78 p.p.m. NO_3, 39 p.p.m. NO_2	8	6	1	13%	8	5	1	25%
156 p.p.m. NO_3, 78 p.p.m. NO_2	8	2	0	75%	8	0	0	100%
78 p.p.m. NO_2	16	0	0	100%	16	0	0	100%
No nitrite or nitrate	32	25	6	3%	32	22	3	22%

shelf stable commercial product. This strongly indicates that the primary effect of nitrite is an inhibition of germination and/or outgrowth of heat injured spores. Recalling that Gross, Stumbo and Vinton *(8)* showed that an $F_s = 6$ was required to destroy completely a concentration of 5 *P.A. 3679* spores per gram in luncheon meat, we decided to confirm our results in packs inoculated with low concentrations of putrefactive anaerobic spores. An emulsion was prepared containing 78 p.p.m. sodium nitrite and 3.5% brine and processed to $F_s = 0.10$. The pack was inoculated with 2 *P.A. 3679* spores per gram. Analysis of the product before inoculation, after inoculation, and subsequent to retorting, showed that at least 20% of the P.A. spores survived processing. This pack gave no evidence of spoilage during a 90-day incubation period at 100° F. Bacteriological analysis revealed no growth of spores surviving processing. This suggested that the putrefactive anaerobic spores not destroyed by the mild heat treatment suffered injury sufficient to prevent their outgrowth during incubation.

DISCUSSION

The shelf stability of canned cured comminuted meats represents an area where a fortuitous combination of circumstances produces a protective result. In this type of product, a surprising degree of shelf stability is imposed by sub-lethal heat treatment combined with organoleptically acceptable levels of salt and concentrations of sodium nitrite which fall within legal limits. Although it is apparent from the experimental results that addition of 78 p.p.m. sodium nitrite to meat emulsions resulted in an antibacterial effect, the mechanism of nitrite action has not been established.

At the levels used, nitrate played no role in retarding putrid spoilage but actively stimulated aerobic spoilage. Our data confirm the findings of several investigators on this point *(15)*. Whether the observed effect of nitrate was due to an ability to overcome nitrite inhibition or involved an actual stimulation of spore outgrowth is not apparent from the data. However, whatever the mechanism, the net result of adding nitrate was an increase in product spoilage due to growth of organisms of the genus *Bacillus*.

Presence of brine, in concentrations up to 5%, does not guarantee stability. This would be predicted by the work of Tanner and Evans *(18)* and others *(6, 15, 16, 21)*. As Halvorson points out, "once a food is heavily contaminated it is no longer possible to check the bacteria by reasonable increases in the salt content" *(9)*.

The phenomenon of sub-lethal injury by mild thermal treatment is an important component of the stability system. Stimulation of spore germination by mild heat shock has long been standard practice in commercial fermentations *(13)*. However, processings of little more severity in a cured meat environment, effectively inhibit the germination and outgrowth of those organisms surviving the treatment. Injured spores, subcultured in a good nutrient medium, grow readily once they are removed from the product. The exact level of heat treatment required to prevent bacterial outgrowth in the cured meat environment has not been established in this work. As has been pointed out previously, pasteurization of product in the temperature range between 155–165° F. is not sufficient to impart shelf stability, whereas retorting procedures which impose barely measurable lethality values change the spores or their environment so that proliferation does not occur within the finished product.

We have presented data which we feel help explain the excellent commercial and public health record of canned cured comminuted meat products. A combination of 78 p.p.m. sodium nitrite, 3.5% brine and a very mild cook results in sufficient injury to the contaminating flora to prevent subsequent growth in the product. Use of sodium nitrate appears to be of questionable value.

The system breaks down when large concentrations of putrefactive anaerobic sports are introduced. Addition of artificially large inocula apparently creates a variable of its own which could be the primary basis for the failure of the bacteriologist to explain the shelf stability and excellent public health history of commercially canned cured meat products.

SUMMARY

Factors responsible for the shelf stability of canned comminuted cured meats were shown to be the combined effect of nitrite, salt, thermal injury and low indigenous spore load.

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Determination of Relative Value of Apples for Applesauce and Evaluation of Experimental Processing Apple Grades

(Manuscript received April 18, 1958)

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Apple processors have purchased apples for processing either on the basis of U. S. grades for apples for processing, or on their own grades, paying different prices for different grades and sizes and for different varieties. The amounts of the grade and size differentials have been a source of misunderstanding between processors and apple producers. Processors also have paid less for a given grade and size of apples that have been run over a grader ("table sorts") than for tree run fruit.

In the spring of 1956, the Virginia Apple Industry Advisory Committee of the Virginia State Horticultural Society, composed of both processor and grower members, requested the Virginia Agricultural Experiment Station to develop a method of calculating price differentials for apples of different qualities and sizes that would reflect the relative value of the fruit to the processors. They also requested an evaluation of the effect on grower returns for apples of different quality that a proposed new set of closer tolerance grades for apples for processing would have in comparison with present U. S. grades. The food technology section of the Horticulture Department already had work under way, in which time requirements in peeling and trimming apples of different grades and sizes and measuring the recovery of different apple fractions were being studied *(2, 3)*. These data were analyzed to determine their usefulness in solving the problem. Although some significant general relationships bearing on the problem were obtained, it was apparent that for determining price differentials applicable to the commercial processing industry some refinements of the experimental methods were desirable. Therefore, in the fall of 1956, the present study was initiated.

EXPERIMENTAL DATA

Data obtained at 4 applesauce processing plants in Virginia and Pennsylvania were included in this study. Experimental

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The Effect of Nitrate or Nitrite when Placed in the Rumens of Pregnant Dairy Cattle

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NONINFECTIOUS ABORTION has been prevalent among cattle grazing on unimproved lowland pastures in central Wisconsin during the past 50 years. Studies begun in 1954 and reported in part[3-5] revealed that nitrates or nitrites, or both, present in many weed species were involved in the lowland abortion syndrome.

The quantity of nitrate or nitrite necessary to cause abortion has not been previously reported. Earlier studies in Wyoming[1] showed that the lethal dosage of potassium nitrate (KNO_3) for cattle is 25 Gm. per 100 lb. of body weight. Studies reporting the death of cattle from nitrate poisoning in South Dakota[6] and Missouri[2] suggest that abortion without impending clinical signs may be a feature of excessive nitrate intake.

The feeding trials reported here were conducted between November, 1956, and August, 1957, to determine the dosage of nitrate or nitrite necessary to cause abortion in cattle and to verify the hypothesis that nitrates present in herbage eaten by them were the cause of the noncontagious abortions which occurred on weedy lowland pastures.

EXPERIMENTAL METHODS AND RESULTS

Four trials using pregnant dairy heifers or cows were conducted.

Trial 1.—Five pregnant heifers were given 20 Gm. of KNO_3 by capsule twice daily for three weeks. None aborted or manifested clinical reactions attributable to the administration of KNO_3. Methemoglobin levels were not significant. One heifer calved normally two days later and was therefore not used in the subsequent study.

Trial 2.—Four of the heifers employed in trial 1 were started on another trial approximately three weeks later. Two were given 10 Gm. of sodium nitrite ($NaNO_2$) twice daily and 2 were given 40 Gm. of KNO_3 twice daily for three weeks.

At the end of one week, the blood of the 2 animals given $NaNO_2$ contained 12.0 and 4.4 per cent, respectively, of the hemoglobin as methemoglobin. At the end of the second week, the methemoglobin in 1 cow had risen from 12.0 to 18.0. She had a normal calf two weeks after the termination of the experiment. However, there were lesions of the fetal membrane comparable to those previously described[4] in natural cases of lowland abortions. The animals given KNO_3 showed no signs of illness and their blood contained no measurable methemoglobin.

TABLE 1—Doses of KNO_3, Weight, and Gestation Stage of Heifers in Trial 4

Group	Animal No.	KNO_3 (Gm./day*)	Weight (lb.)**	Stage gestation (months)
A	1	70	600	3
	2	70	600	3½
	3	70	700	3
B	4	100	600	6
	5	100	675	6
	6	100	650	4
C	7	140	700	6
	8	140	700	5
	9	140	700	6

*Unless otherwise noted in text; **based on estimates by a local cattle dealer.

Trial 3.—Four lactating, mature, pregnant cows weighing 1,300 to 1,400 lb. were fed marsh (bluegrass) hay, corn silage, and grain for ten days prior to and during the administration of $NaNO_2$. Two were given 20 Gm. and 2 were given 10 Gm. of $NaNO_2$ twice daily for three weeks. None showed signs of illness and samples of their blood contained no appreciable amount of methemoglobin.

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TABLE 2—Methemoglobin and Nitrate Content of the Blood of Cows Which Died Following the Second Dose of 140 Gm. of KNO_3

Cow No.	Methemoglobin (%) of total blood	KNO_3 in blood (p.p.m.)
7	0.82	187.0
8	1.29	55.6
9	1.57	102.6

Trial 4.—The 9 pregnant heifers used were allotted to three groups of 3 animals each (table 1). Prior to and during the administration of KNO_3, they were kept in the barn and fed only bluegrass hay from the local marshes.

A plastic rumen fistula plug was installed in each heifer. The wounds were allowed to heal for 19 days prior to the administration of KNO_3.

The KNO_3 was given in dry form through a funnel inserted in the fistula plug at approximately 6:30 a.m. each day. Any material adhering to the funnel was washed into the rumen with water.

All 3 heifers of group C died approximately nine hours following the second administration of 140 Gm. of KNO_3. Necropsy revealed inflammation of the abomasum and small intestine in all 3 animals. The blood was dark purplish brown. The methemoglobin and nitrate content were determined 18 hours after death (table 2).

In group B, all 3 heifers given 100 Gm. of KNO_3 aborted. Heifer 5 aborted approximately eight hours after the third 100-Gm. dose of KNO_3 administered on Sunday, Monday, and Wednesday. Heifer 4 aborted after being given 100 Gm. of KNO_3 on Sunday, Monday, and Wednesday, and 90 Gm. on Friday. Rectal palpation revealed that the fetus in heifer 6 was dead 13 days after the initial treatment of 100 Gm. of KNO_3. Additional KNO_3 was administered for 23 days, but when abortion did not occur, stilbestrol was administered and the dead calf was aborted two days later.

The lesions in the fetal membranes of these 3 animals (group B) were identical with those found in animals which aborted on the lowland pastures.[4] The lesions of the fetuses were also similar to but not as extensive as those occurring in the fetuses aborted in the pastures. The perirenal hemorrhage was less marked and the pleura of the posterior lobes of the lungs was less thickened.

The percentage of methemoglobin varied considerably among the heifers at the same dosage level (table 3) and at various time intervals during the day (table 4).

Analyses of methemoglobin in the blood of experimental animals were made once prior to treatment and several times after

TABLE 3—Dosage Schedule of Heifers in Trial 4, Group B, and Methemoglobin Values (%) that Resulted

Date*	Heifer 4		Heifer 5		Heifer 6	
	Dose	Methemoglobin	Dose	Methemoglobin	Dose	Methemoglobin
7/12	0	0.08	0	0.12	0	0.04
7/14	100	.....	100	.....	100	.....
7/15	100	.....	100	.....	100	.....
7/17	100	7.72	100	5.53	100	0.39
7/18	0	2.00	0	0.12	90	0.39
7/19	90	8.62**	0	.....	100	2.51

*Blood samples taken at 11:30-11:45 a.m. on days given; **cyanotic—cyanosis first observed at 11:45 a.m. 250 ml. of 1 per cent methylene blue in 5 per cent glucose administered i.v. at 2 p.m.; at 4:30 p.m., appeared normal but aborted at 7:00 p.m.

TABLE 4—Percentage of Methemoglobin in Blood at Various Times on July 19

Heifer No.	Body weight (lb.)	KNO_3 (Gm. at 6:30 a.m.)	Methemoglobin (%) at: 8:30 a.m.	10:30 a.m.	11:45 a.m.	2:00 p.m.
1	600	70	0.35	1.14	0.24	0.43
2	600	70	0.31	2.51	1.69	0.71
3	700	70	0.74	0.74	0.55	0.27
4	600	90	0.86	6.23	8.62*	7.84
6	650	100	0.16	1.61	2.51	0.94

*Cyanotic—given methylene blue.

the administration of KNO_3 was begun. The blood of heifer 5 contained 5.53 per cent methemoglobin. Since her blood contained 12.49 per cent hemoglobin, 44.2 per cent (5.53 ÷ 12.49) had been converted to methemoglobin at the time of abortion (that afternoon). On the same day, 7.72 per cent of the blood of heifer 4 was methemoglobin. Her normal hemoglobin level was 11.75 per cent; therefore, 65.5 per cent had been converted to methemoglobin. However, she did not abort until two days later when the methemoglobin content of the blood was 8.62 per cent.

The blood of heifer 6 did not contain any appreciable amount of methemoglobin until the fifth day (table 3) following the first dose of 100 Gm. of KNO_3. On the succeeding days, her blood methemoglobin values were: 0.16, 3.72, 5.25, 2.74, 0.71, 6.98, 6.00, and 12.00 per cent. On July 28, with 12.0 per cent of the total blood as methemoglobin, methylene blue was administered since she was cyanotic. During the following 13 days, her methemoglobin values ranged from 7.06 to 9.43 per cent. All this time she retained a dead calf that was not aborted until she was given stilbestrol.

Heifers in group A (70-Gm. dosage) did not abort although 6.66 per cent of the total blood of heifer 2 became methemoglobin (table 5), a conversion of 54.5 per cent of her normal 12.2 per cent of hemoglobin. Furthermore, her calf at birth weighed less than 20 lb. Heifers 1 and 3 bore normal calves one and two months, respectively, after termination of KNO_3 dosing on August 30.

TABLE 5—Percentages of Methemoglobin in the Blood of Heifers in Trial 4, Group A

Date*	Hour samples taken	Methemoglobin (%) Heifer 1	Heifer 2**	Heifer 3
7/12	11:00 a.m.	0.12	0.04	0.04
			(First administration of 70 Gm. KNO_3 on 7/14)	
7/17	11:00	0.27	0.94	0.27
7/18	11:30	0.71	0.75	0.08
7/19	11:45	0.24	1.69	0.55
7/24	11:30	1.76	2.74	0.43
7/29	11:30	1.06	1.92	1.92
8/1	11:30	0.78	1.08	0.67
8/4	11:30	3.06	2.67	0.71
8/9	11:30	1.53	2.82	0.98
8/13	11:15	1.53	5.92	1.33
8/18	11:30	1.37	4.86	0.49
8/26	11:30	2.67.	6.66	1.76
8/22	11:30	2.67	6.63	0.71
8/30	11:30	2.08	4.61	1.45

*Methemoglobin was determined daily but all daily readings are not included because the values were similar to those given; variations in methemoglobin values apparent within and among cows are unexplainable; **No. 2 had a small (20 lb.) calf.

DISCUSSION

The results of the first three trials indicated that larger dosages of nitrate or nitrite or longer periods of feeding these chemicals might be necessary to produce abortion. In trial 2, the animals given $NaNO_2$ (usually considered ten times as toxic as KNO_3) showed an appreciable development of methemoglobin and fetal membrane lesions when they later calved; those given KNO_3 showed no appreciable development of methemoglobin.

Trial 4 was, therefore, initiated using 70, 100, and 140 Gm. of KNO_3 in heifers weighing approximately 700 lb. each. The 3 given 140 Gm. on two consecutive days died, presumably of anoxemia, before abortion could occur. They appeared healthy at noon and were found dead at 4:30 p.m. The level of KNO_3 was presumed safe according to the lethal dosage indicated by Bradley, *et al.*[1]

Investigations conducted in Missouri[2] indicate that a ration high in carbohydrates will offset the poisoning effect of nitrate. The animals in trials 1, 2, and 3 were fed good rations while those in trial 4 were on a poor ration, being fed only marsh-grown bluegrass hay.

The administration of 100-Gm. doses of KNO_3 for varying periods resulted in abortion with fetal membrane lesions identical to and fetal lesions comparable to those in naturally occurring marshland abortions. The pathogenesis of the disease may include anoxia through methemoglobin formation with resultant vascular changes in the placenta, interference with exchange

between the dam and fetus, fetal death, and ultimate abortion.

The 3 heifers given the low level of KNO_3 (70 Gm., or 10 Gm. per 100 lb. body wt.) did not abort, although the blood of 1 of them contained a substantial amount of methemoglobin. The rectal examinations made during the extended feeding period indicated that the fetus of heifer 2 was dead; however, it was born alive but was small and inactive. At nine months of age, it was the size of a 3-month-old calf.

The data on methemoglobin (tables 3 and 5) are difficult to assess. The percentages of methemoglobin fluctuate from day to day in the same animal and among animals given the same amount of KNO_3. This fluctuation is evident even at two-hour intervals on the same day (table 4). It is possible that other compounds of hemoglobin may be formed with nitrate or nitrite and that the methemoglobin level may not be the most reliable measure of nitrate poisoning. Perhaps a measurement of nitrate or nitrite, or of both, in the blood of the dam would permit a more reliable forecast of impending abortion than can be made by measuring methemoglobin.

Time of sampling the blood for methemoglobin determination is important; the highest readings were obtained four or five hours after administration of KNO_3. Many variables are to be considered in methemoglobin determinations, such as the time the blood sample is taken following ingestion of test material, time elapsed from sampling to determination, nutritional level of the ration, rumen flora, method of assay, gestation status, type of nitrogenous material fed, and other factors.

The data presented as the results of trial 4 verify the hypothesis that nitrates present in the herbage eaten were the cause of the noninfectious abortions which occurred on weedy lowland pastures.

SUMMARY

No abortions were produced in the first three trials when small doses of KNO_3 or $NaNO_2$ were given to cattle. Lesions found on the fetal membranes, however, indicated that abortions might have occurred at higher dosages.

In the fourth trial, 9 dairy heifers were given KNO_3 daily through a rumen fistula. The 3 given 140 Gm. daily died after the second dose; the 3 given 100 Gm. daily aborted after three, five, and 13 doses, respectively, and the 3 given 70 Gm. daily for approximately 47 days calved at the normal time but 1 calf was small (20 lb.) and weak.

The variation in levels of methemoglobin found among animals within groups and in individual animals on different days was so great that it was considered to have little diagnostic value for detecting nitrate intoxication.

Lesions on the fetuses and fetal membranes of the 3 heifers which aborted were comparable to those observed in naturally occurring marshland abortions.

Nitrates present in weeds are involved in the marshland abortion syndrome, although their specific mode of action remains undetermined.

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Position of the Foal During Gestation and Parturition

Observations on normal pregnant mares were made by rectal palpation of the uterus at various stages of gestation, by slaughter of 77 normal mares during parturition, and from 27 cases of serious dystocia. All but one were monocornual pregnancies with the fetus in a longitudinal position.

During the first six months of gestation, the fetus was often in the position of posterior presentation but this was completely reversed by the eighth month in 98 per cent of the cases. The exact type of presentation could not be predicted by rectal palpation even during the last week of pregnancy.—*Vet. Bull. (May, 1959): Item 1613.*

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NITRATE TOXICITY IN SHEEP*

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By K. B. SINCLAIR and D. I. H. JONES

The introduction of 50 g. of potassium nitrate into the rumen of sheep leads to the accumulation of nitrite which is accompanied by a methaemoglobinaemia. There was evidence of adaptation in the rumen of sheep fed without ill effect for 6 weeks on dried grass containing 0·5% of nitrate N.

It is concluded that poisoning is most likely to follow the sudden introduction of high-nitrate feeds.

Introduction

In 1939, Bradley *et al.*[1] showed that the syndrome of oat hay poisoning in cattle was similar to that caused by the ingestion of excessive quantities of nitrate and they concluded that it was the accumulation of nitrate in oat hay which made it toxic. Subsequently there have been several reports of fatalities following the ingestion of herbage containing high levels of nitrate, and recently attention has been given to less acute effects. There is evidence, for example, that the ingestion by cattle of feeds containing excessive amounts of nitrate may lead to abortion,[2] and adverse effects of vitamin-A metabolism[3] and thyroid function[4] have also been described. These aspects of the problem have been reviewed elsewhere.[5]

Methaemoglobinaemia is the most frequently reported symptom in nitrate poisoning, and Lewis[6] amongst others has shown this to be associated with the absorption of nitrite from the rumen. The nitrite arises from the reduction of nitrate by the rumen micro-organisms and is an intermediate in the formation of ammonia. Little is known of the factors involved in the accumulation and absorption of nitrite and it is possible that there are adverse effects other than the formation of methaemoglobin.

Our interest in the problem arose from earlier studies which revealed that under certain environmental conditions, grasses,[7] rape and kale[8] may accumulate levels of nitrate frequently in excess of those reported to be toxic to animals. For practical reasons sheep were used in our experiments although cattle are generally considered to be more susceptible to nitrate poisoning. Two experiments are described; in the first, potassium nitrate was administered intra-ruminally, and in the second, grass of high nitrate content was fed. Previous observations on the effect of nitrate on reproduction in sheep have been published elsewhere.[9]

Experimental

Samples of rumen contents were obtained from sheep fitted with rumen cannulae and the pH of the liquor determined immediately. A subsample was cleared as described by Lewis[6] and the solutions stored at −10° until required for the determination of nitrate,[10] nitrite[11] and ammonia.[12]

Samples of blood were collected from the jugular vein and estimations of packed cell volume, inorganic phosphorus, methaemoglobin and haemoglobin were carried out immediately. Calcium, magnesium, nitrate and nitrite were determined on samples of serum after storage at −20°. The analytical methods have been described elsewhere.[9]

Results

Experiment (1)

Effect of a single dose of potassium nitrate

Two Clun wethers each weighing approx. 60 kg. were kept in separate pens and fed *ad libitum* on frozen grass. Analysis of the grass fed to sheep No. 1 showed the soluble carbohydrate content to be 5% and *in vitro* digestibility 55%; that fed to sheep No. 2 contained 13% of soluble carbohydrate and the digestibility was 70%. After a preliminary period of a fortnight, 50 g. of potassium nitrate (25% aq. solution) was introduced into the rumen through the cannulae. This dose appeared to have no effect on the well-being of the sheep apart from

* Read before Agriculture Group, 18 February, 1964

inappetance on the day of dosing. Samples of rumen contents and blood were taken at intervals before and after dosing and the results of the analyses are shown in Figs. 1 and 2.

The rate of disappearance of nitrate was similar in both sheep, and only traces remained 16 h. after dosing. On the other hand, there were considerable differences in the levels and rate of disappearance of nitrite in the two sheep. In sheep No. 1, the nitrite rose to a peak of 47·5 mg. per l. after 3 h. and persisted for 20 h., but in sheep No. 2 the highest level was 17·5 mg. and after 15 h. nitrite could not be detected. In both sheep the appearance of nitrite was accompanied by the accumulation of ammonia which reached its maximum after 13 h. In contrast to nitrite, the highest levels of which occurred in sheep No. 1, the highest levels of ammonia occurred in sheep No. 2. It is possible that the higher ammonia and lower nitrite in the latter reflect a more complete reduction of nitrate to ammonia. Conversely, the lower ammonia in sheep No. 1 was consistent with the greater accumulation of nitrite.

The changes in methaemoglobin concentration in the blood of the two sheep are shown in Fig. 2 and it is evident that the higher level of rumen nitrite in sheep No. 1 was accompanied

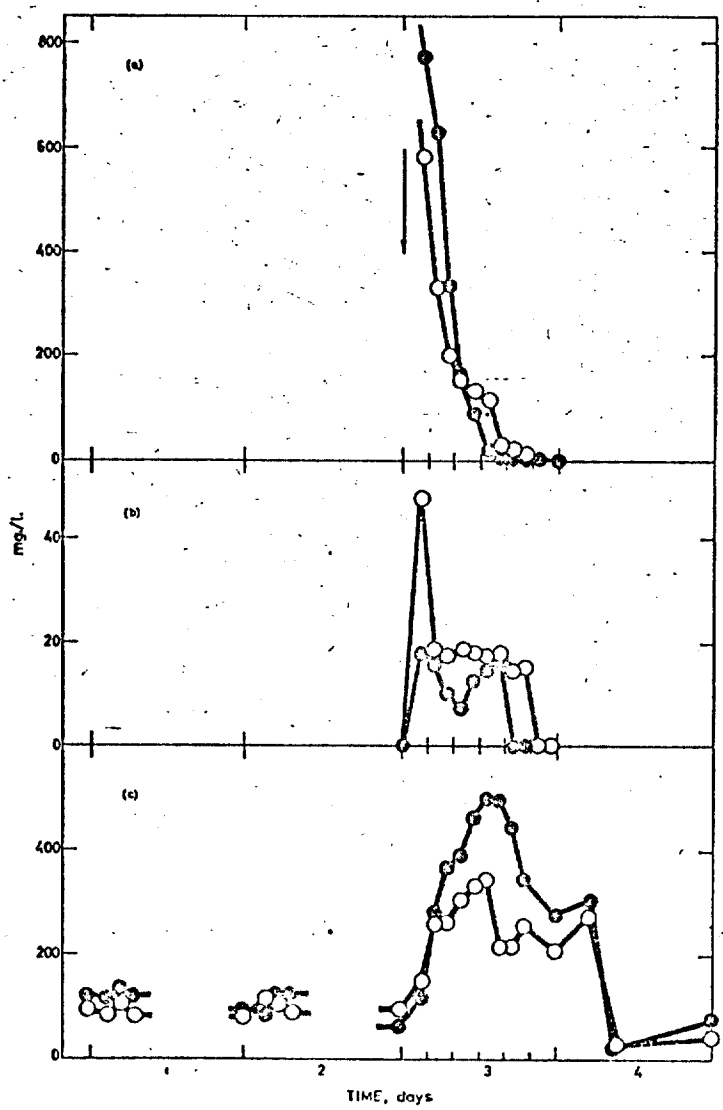

FIG. 1.—*Changes in composition of rumen liquor after dosage of sheep with potassium nitrate*

O sheep No. 1 ● sheep No 2

arrow indicates where 50 g. of KNO_3 were added

(*a*) nitrate-N (*b*) nitrite-N (*c*) ammonia-N

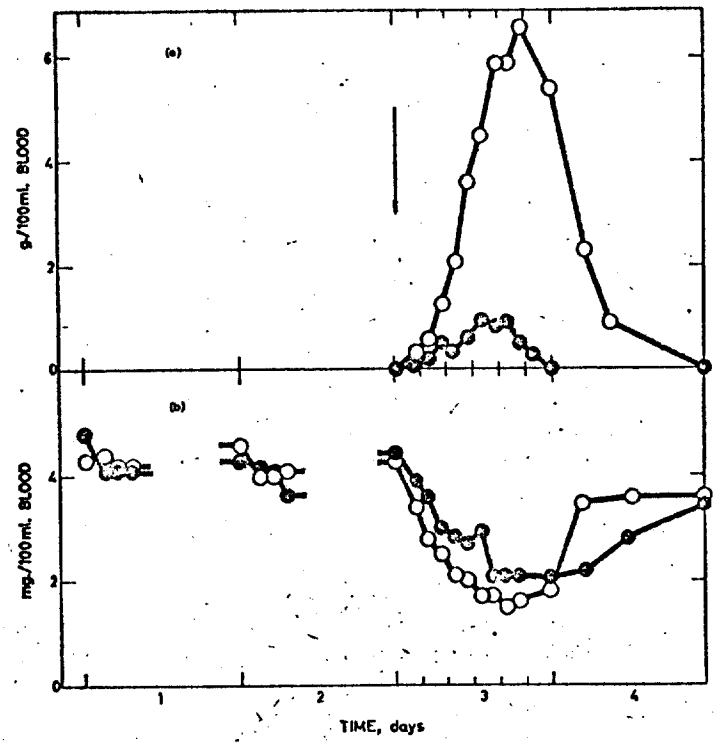

FIG. 2.—*Changes in blood composition after dosage of sheep with potassium nitrate*
(a) methaemoglobin (b) inorganic phosphorus
(legend as Fig. 1)

by a more marked methaemoglobinaemia. In this sheep methaemoglobin increased gradually to reach a peak value of 6·6 g. per 100 ml. 19 h. after dosage, while in sheep No. 2, the concentration did not exceed 1 g. per 100 ml. during the experiment. Comparison of Figs. 1 and 2 shows that the peak levels of methaemoglobin occurred just before the disappearance of nitrite from the rumen. In this experiment it was not possible to determine serum nitrate and nitrite but qualitative examination with diphenylamine showed close correlation with the occurrence of methaemoglobinaemia. Fig. 2 also shows a pronounced fall in the level of inorganic phosphorus in the blood of both sheep. The reason for this is not clear and requires further investigation. Determinations of serum calcium, serum magnesium, haemoglobin and packed cell volume showed that these were within normal limits. There was, however, a slight fall in packed cell volume and haemoglobin in sheep No. 1 shortly after dosing.

This experiment confirms that nitrate is reduced in the rumen to nitrite and ammonia, the accumulation of nitrite being accompanied by methaemoglobinaemia. These findings suggest that the amount of nitrite which appears in the rumen may vary in animals on different diets, but, since only two animals were available for this investigation, the possibility of individual variation cannot be ignored. These results, however, which have been confirmed by other experiments, indicate that sheep can tolerate high doses of potassium nitrate with little ill effect.

Experiment (2)

Effect of feeding dried grass of high nitrate content

Three wethers weighing 60–70 kg. were housed in separate pens and fed on hay *ad libitum* for a preliminary period of about one month, after which the ration was changed to autumn-grown, artificially dried cocksfoot. The grass was harvested after a heavy application of nitrogen fertiliser, and chemical analysis showed it to contain 0·5% of nitrate nitrogen, 30% of crude protein and 2% of water-soluble carbohydrate.

Initially, the sheep were reluctant to eat the dried grass, but, after the first week, intake improved and it was readily consumed during the remainder of the experimental period. Samples of blood and rumen contents were obtained twice weekly during the last week of the preliminary period and for 6 weeks after the introduction of the dried grass. Determinations of pH, nitrate, nitrite and ammonia were carried out on the strained rumen liquor samples, and of nitrate, nitrite, methaemoglobin, haemoglobin, packed cell volume, inorganic phosphorus, calcium and magnesium on the blood. The most marked changes were in rumen pH and ammonia and the data for four sampling dates are given in Table I.

Table I

Effect of feeding high-nitrate grass on rumen pH and ammonia

(mean values for 3 sheep)

Feed	Rumen pH	Rumen ammonia (mg. N per litre)
Hay (preliminary period)	6·85	64
High-nitrate grass:		
after 1 week	7·15	258
after 3 weeks	7·07	257
after 6 weeks	7·30	266

The level of nitrate-N in the rumen liquor never exceeded 3 mg. per l., and only two isolated samples contained more than 0·1 mg. of nitrite-N per l. Furthermore, nitrate and nitrite were not detected in the blood and the absence of methaemoglobinaemia was not unexpected. Determinations of other blood constituents failed to show any marked deviation from normal levels. These results indicate that the reduction of feed nitrate was accomplished rapidly and completely, with little accumulation of nitrite.

Adaptation of the rumen micro-organisms to a high-nitrate diet could be an important factor in experiments involving prolonged ingestion of nitrate. It was decided, therefore, to examine the nitrate-reducing efficiency of the rumen liquor from two of the sheep used in this experiment. For this purpose the rumen liquor was diluted with phosphate–bicarbonate buffer, pH 6·9, and incubated at 38·5° under anaerobic conditions with added nitrate. Samples of rumen liquor from two other sheep fed *ad libitum* on nitrate-free hay were similarly incubated and the rate of disappearance of the nitrate compared. The results are in Table II.

It is apparent from Table II that the rumen liquor from the sheep fed on high-nitrate grass reduced the added nitrate more rapidly, indicating that adaptation to a high-nitrate diet can occur.

Table II

Changes in nitrate content during incubation of rumen liquor with added nitrate

Diet	Mean nitrate N, mg.			
	Initial	After 1 h.	After 2 h.	After 6 h.
High-nitrate grass	4·40	2·80	1·33	0·30
Nitrate-free hay	4·40	4·25	4·13	2·18

Discussion

These experiments show that considerable amounts of nitrate may be given to sheep without ill effect and emphasise the importance of the mode of administration. When sheep are dosed, the whole of the nitrate is introduced into the rumen at once, whereas when a high-nitrate diet is fed, the nitrate reaches the rumen in smaller quantities over a longer period of time. It is unlikely, therefore, that experiments which involve dosing with nitrate can determine the toxicity of nitrate incorporated in feed. Furthermore, it is evident that the composition of the diet plays an important part in the efficiency of nitrate reduction, and must be taken into consideration in determining toxicity.

These findings suggest that the rumen micro-organisms become adapted to a high-nitrate feed and enable the sheep to utilise considerable amounts of nitrate. If poisoning is to occur under practical farming conditions, it seems more likely to follow the sudden introduction of a readily consumed high-nitrate feed.

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The authors are indebted to their colleagues in the Grassland Agronomy Dept. for their co-operation in these studies.

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THE PROTEIN AMINO-ACID COMPOSITION AND NITROGEN DISTRIBUTION IN TWO TROPICAL GRASSES

By H. C. HODGSON*

Determinations have been made of the amino-acid composition of protein extracts from the dried green tissues of two perennial tropical grasses—*Brachiaria ruziziensis* and *Setaria sphacelata*, harvested at various growth stages. The relative contributions of the isolated protein N, soluble N, and residual or unextracted N to the total organic nitrogen have also been investigated. The results show general similarities of protein composition and pattern of nitrogen distribution throughout the development of the plants.

Introduction

The accurate evaluation of plant tissue proteins in terms of their amino-acid components has long been recognised as presenting formidable problems.[1] The principal difficulty is that of obtaining a quantitative extraction of protein uncontaminated by extraneous matter, for, in view of the partial destruction of certain amino-acids during mineral acid hydrolysis in the presence of carbohydrates, it is clearly desirable to isolate the protein in a substantially pure state. With existing methods of extraction, high yields are only obtainable at the expense of purity and vice versa. By use of juice expression techniques on fresh leaf material, yields of 50–60% of the total protein, with a nitrogen content of 12%, have been reported,[2] whereas, for less pure isolates, recoveries of the order of 90% are feasible by extraction with buffer solutions.[3] Where it is necessary to work on dried plant material, as in this present investigation, the yields are markedly lower due to denaturation and, although the products are reasonably

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Nitrite Toxicity in Sheep

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SUMMARY. Following the intra-ruminal administration of 5, 10 or 20 g. of potassium nitrite to 2 sheep, plasma nitrite rose to a peak within 2 hr. accompanied by progressive methaemoglobinaemia. A single dose of 20 g. proved lethal, but when this amount was incorporated in the daily feed of 2 other sheep, no ill-effects were observed.

In previous experiments (Sinclair and Jones, 1964a) it was found that sheep could ingest large amounts of nitrate in the feed without ill-effect, but when the nitrate was given in a single dose methaemoglobinaemia occurred. It has been shown by Lewis (1951), among others, that nitrate in the rumen is reduced to nitrite and that poisoning follows the absorption of nitrite. While it is clear that most of this reduction takes place in the rumen, Jones and ap Griffith (1964) have confirmed that some reduction of nitrate in the feed can occur before ingestion, due to the action of micro-organisms. In view of this, it seemed important to assess the toxicity of nitrite to sheep and the present paper describes a series of experiments involving the administration of potassium nitrite.

MATERIALS AND METHODS

Dosing Experiments

Two mature Clun wethers, fitted with rumen cannulae, and weighing about 50 kg. each, were placed in metabolism cages and fed *ad lib.* on pelleted S24 perennial ryegrass hay. Analysis of the hay showed the soluble carbohydrate content to be 11·8%, crude protein 9·6% and *in vitro* dry-matter digestibility 61·1%. All results are expressed on a dry-matter basis. Drinking-water was available as required. Three experiments were carried out using the same 2 sheep, each experiment being preceded by a period of 2 to 3 weeks during which preliminary samples of rumen liquor and blood were obtained. In the first experiment, 5 g. of potassium nitrite dissolved in 100 ml. deionised water was introduced as a single dose through the cannula into the rumen of each sheep. The dose was increased to 10 g. in the second experiment and to 20 g. in the third. Following each dose of potassium nitrite, serial samples were taken of blood and rumen liquor to measure the changes occurring in their compositions.

Feeding Experiment

Two other Clun wethers of similar weight to those used in the dosing experiments were placed in metabolism cages and fed a daily ration of 1,500 g. of the same pelleted ryegrass hay divided into 2 feeds. Drinking-water was available as required. After a preliminary period of one week, during which samples of blood were taken, the feed was changed to pelleted ryegrass hay containing 0·23% nitrite-N incorporated as a potassium nitrite solution during the pelleting process. The high nitrite pellets were fed for a period of 3 days, during which time samples of blood were taken at frequent intervals.

Analytical and Sampling Methods

These were as described by Sinclair and Jones (1964a, 1964b), except that serum magnesium was determined by atomic absorption spectroscopy and nitrite determinations were made on plasma instead of serum because laboratory tests revealed that only a very small proportion of nitrite added to blood could be recovered subsequently in the serum. Recoveries from plasma, on the other hand, were complete. To reduce sampling error nitrite was not determined in rumen liquor until 2 hr. after dosing.

RESULTS

Dosing Experiments

With the exception of inappetance, which followed the administration of nitrite on each occasion, there was no obvious effect on the well-being of the sheep until the highest level of dosing was reached. It can be seen from Tables I and II that the changes in the compositions of rumen liquor and blood showed similar patterns following the administration of

TABLE I

THE COMPOSITION OF RUMEN LIQUOR AND BLOOD FOLLOWING THE ADMINISTRATION OF 5 g. POTASSIUM NITRITE TO SHEEP

Sheep	Sample	Hours after dosing	Rumen liquor nitrite-N mg./litre	Plasma nitrite-N mg./100 ml.	Blood Haemoglobin g./100 ml.	Blood Methaemoglobin g./100 ml.	Blood Inorganic P. mg./100 ml.	Blood Packed cell vol. %
A	Preliminary	—	0	0	12·2	0	4·4	36
		—	0	0	10·4	0	4·3	32
	Experimental	0	0	0	12·9	0	5·0	35
		0·5	n.d	0·09	12·4	0·4	4·5	35·5
		1·0	n.d.	0·16	11·9	0·6	4·3	34·5
		2·0	6·2	0·20	n.d.	n.d.	n.d.	n.d.
		3·0	1·0	0·16	12·0	1·0	3·4	32
		4·5	0·1	0·03	11·8	0·7	4·3	33
		6·5	0·1	0·01	12·0	0·3	3·9	35
		9·5	0·1	0·01	12·0	0·6	3·7	34
		24·0	0	0	12·7	0·4	4·0	35
B	Preliminary	—	0	0	9·8	0	5·1	32
		—	0	0	10·0	0	5·0	29
	Experimental	0	0	0	11·2	0	5·2	32·5
		0·5	n.d.	0·25	10·0	0·7	4·8	29·5
		1·0	n.d.	0·27	10·8	1·2	4·9	31·0
		2·0	17·5	0·25	10·0	1·1	4·2	29·0
		3·0	13·4	0·18	n.d.	n.d.	n.d.	n.d.
		4·5	4·7	0·07	10·7	1·2	3·5	31
		6·5	0·1	0·02	10·2	0·4	3·5	31
		9·5	0	0·01	10·2	0·6	3·6	30·5
		24·0	0	0	10·3	0·2	4·5	31·5

n.d. = not determined.

either 5 g. or 10 g. of potassium nitrite. The smaller dose of nitrite disappeared from the rumen liquor within 5 hr., while traces of the 10 g. dose were still detectable after 13 to 15 hr. Nitrite appeared in the blood within 30 min. of dosing and reached peak levels in about 2 hr. In both experiments Sheep B showed the higher levels of plasma nitrite, but the time taken for the nitrite to disappear was similar in the 2 sheep. When the dose was increased from 5 g. to 10 g. the highest level of nitrite observed in Sheep A rose from 0·20 mg. to 0·27 mg./100 ml. only, while in Sheep B the level rose from 0·27 mg. to 0·53 mg./100 ml.

TABLE II

THE COMPOSITION OF RUMEN LIQUOR AND BLOOD FOLLOWING THE ADMINISTRATION OF 10 g. POTASSIUM NITRITE TO SHEEP

Sheep	Sample	Hours after dosing	Rumen liquor nitrite-N mg./litre	Blood: Plasma nitrite-N mg./100 ml.	Blood: Haemoglobin g./100 ml.	Blood: Methaemoglobin g./100 ml.	Blood: Inorganic P mg./100 ml.	Blood: Packed cell vol. %
A	Preliminary	—	0	0	12·2	0	4·0	34·5
		—	0	0	11·6	0	4·0	33·0
	Experimental	0·5	n.d.	0·22	11·6	0·2	4·0	33·0
		1·0	n.d.	0·26	11·0	1·8	4·1	32·5
		2·0	89·2	0·27	11·3	3·0	3·4	33·0
		3·0	86·8	0·22	12·2	3·9	3·0	35·0
		4·5	59·2	0·26	13·0	5·2	2·8	36·5
		6·5	34·0	0·17	13·6	4·5	2·5	37·0
		8·5	19·5	0·18	12·8	5·0	2·7	37·5
		10·5	12·3	0·17	12·4	4·9	3·3	36·5
		12·5	4·2	0·09	12·2	4·5	3·5	36·5
		14·5	0·4	0·05	14·0	3·6	4·1	39·0
		24·0	0·3	0·02	10·3	0·1	4·8	31·5
B	Preliminary	—	0	0	9·8	0	5·2	32·0
	Experimental	0	0	0	9·8	0	5·7	30·0
		0·5	n.d.	0·23	9·8	0·2	5·3	29·0
		1·0	n.d.	0·37	9·8	1·0	5·0	29·0
		2·0	88·8	0·52	10·5	3·3	4·0	31·0
		3·0	90·0	0·53	11·0	4·8	4·1	30·5
		4·0	85·2	0·43	11·1	6·3	3·4	33·0
		6·0	46·6	0·34	11·0	6·6	3·2	33·0
		8·0	18·8	0·22	12·0	6·3	3·1	34·0
		10·0	17·7	0·17	11·4	5·3	3·1	33·5
		12·0	12·5	0·18	11·0	4·0	3·2	33·0
		14·0	9·9	0·16	11·6	3·2	3·5	32·5
		24·0	3·3	0·05	9·5	0·1	5·1	29·0

n.d. = not determined.

This suggests that individual variation may occur in the susceptibility of sheep to nitrite. The appearance of nitrite in the blood was accompanied by methaemoglobinaemia which rose to a peak about 3 hr. after administration of 5 g. potassium nitrite and 6 hr. after the 10 g. dose. The pattern of the methaemoglobinaemia was similar in both sheep, although the higher level of plasma nitrite in Sheep B was reflected in a higher level of methaemoglobin. It is interesting to note that an increase in the dose of nitrite from 5 g. to 10 g. resulted in a disproportionate rise in methaemoglobin. Thus, for the lower dose, the maximum level of methaemoglobin recorded was about 1 g., while for the higher dose, it reached 7 g./100 ml. The rate of disappearance of the methaemoglobin appeared to be somewhat slower than that of the nitrite.

Apart from some fluctuation shortly after dosing with 5 g. potassium nitrite, there was

little change in either haemoglobin concentration or packed cell volume (PCV). The administration of 10 g., however, was followed by a pronounced rise in both haemoglobin concentration and PCV which persisted for more than 14 hr. These changes could be indicative of accelerated erythropoiesis stimulated by hypoxia.

In previous observations, a marked fall in the level of blood inorganic phosphorus followed the administration of potassium nitrate. Tables I and II show that the administration of either 5 g. or 10 g. of potassium nitrite led to a similar fall and a subsequent rise accompanied the disappearance of methaemoglobin. Serum magnesium values remained within normal limits throughout the experiments.

In our previous work, the administration of 50 g. of potassium nitrate was followed by a pronounced increase in rumen ammonia, but in the present experiments, the rumen ammonia concentration did not vary greatly after the administration of either 5 g. or 10 g. of potassium nitrite. Values for the pH of the rumen liquor were within normal limits, except that Sheep B showed very low values (minimum 4·85) some 6 hr. after administration of the 10 g. dose. The fall in rumen pH seemed to have no effect on Sheep B, although Holtenius (1957) found that a reduction of the pH to 5·7 resulted in a strong inhibition of the ability of the rumen micro-organisms to break down nitrite *in vitro*.

In the third experiment, the dose of potassium nitrite was increased to 20 g. and this proved lethal to both sheep. About 2·5 hr. after dosing, Sheep A became dull with accelerated respiration and heart-rate and with mud-coloured conjunctivae. Shortly afterwards the animal lay on its side and would not stand voluntarily. When a further sample of blood was obtained

TABLE III

THE COMPOSITION OF RUMEN LIQUOR AND BLOOD FOLLOWING THE ADMINISTRATION OF 20 g. POTASSIUM NITRITE

Sheep	Sample	Hours after dosing	Rumen liquor nitrite-N mg./litre	Blood: Plasma nitrite-N mg./100 ml.	Blood: Haemoglobin g./100 ml.	Blood: Methaemoglobin g./100 ml.	Blood: Inorganic P mg./100 ml.	Blood: Packed cell vol. %
A	Preliminary	—	0	0	10·2	0	4·3	29·0
		—	0	0	10·8	0	3·9	30·5
	Experimental	0	0	0	9·5	0	4·2	29·0
		0·5	n.d.	0·44	10·7	1	4·5	30·5
		1·0	n.d.	0·56	11·8	2·5	4·0	34·0
		2·0	182·0	0·67	13·3	6·3	3·1	38·5
		2·5	330·0	0·76	13·5	7·1	3·2	38·0
		3·0	296·0	0·61	12·8	8·2	3·4	36·0
				A died 3 hr. after dosing.				
B	Preliminary	—	0	0	10·8	0	4·7	31·5
		—	0	0	10·6	0	5·0	31·0
	Experimental	0	n.d.	0	10·1	0	6·2	29·5
		0·5	n.d.	0·66	10·4	1·6	5·7	31·0
		1·0	n.d.	0·89	9·6	3·4	5·5	28·6
		2·0	436·0	0·53	10·4	7·5	5·7	31·0
				B died 2 hr. after dosing.				

n.d. = not determined.

the disturbance involved seemed to exacerbate the condition and an expiratory grunt developed. Subsequently the sheep became comatose, there was excess salivation and the heartbeat became very rapid and feeble prior to death some 3 hr. after dosing. Table III shows that just prior to death the plasma nitrite level had reached 0·61 mg./100 ml. and methaemoglobin 8·2 g./100 ml. Similar symptoms were observed in Sheep B within 1·5 hr. of dosing and death occurred after 2 hr. when the plasma nitrite level was 0·53 mg./100 ml. and methaemoglobin 7·5 g./100 ml. (Table III). Retarded clotting-time was noted in samples of blood obtained from both sheep just prior to death.

The *post-mortem* findings were similar in the 2 sheep. There was intense congestion of intestine, liver and kidneys, with dark, unclotted blood. The portal vein was particularly evident and full of chocolate-coloured blood. There was a pinkish discolouration of the mucosae of the rumen and abomasum, and the mucosa of the latter stripped-off readily from the sub-mucosal tissue. A notable feature was the absence of food material adhering to the rumenal papillae so that the organ was emptied readily.

Feeding Experiment

The sheep readily consumed the pelleted ryegrass hay containing nitrite to give a daily intake of 3·45 g. of nitrite-N, that is, equivalent to about 20 g. of potassium nitrite. During the 3 days that the high-nitrite pellets were fed, there was no apparent effect on the well-being of the sheep and the levels of all the blood constituents determined remained within normal limits; nitrite was not detected. Unfortunately, insufficient ryegrass hay was available to prolong the investigation.

DISCUSSION

It is generally stated, for instance Garner (1961) and Radeleff (1964), that nitrite is considerably more toxic than nitrate. This is confirmed by the results of the present experiments which indicate that in sheep weighing about 50 kg. a single dose of between 10 g. and 20 g. of potassium nitrite is likely to be lethal: this is equivalent to 40 to 60 mg. of nitrite-N/kg. body-weight. The earlier work with potassium nitrate showed that 50 g. could be administered as a single dose without ill-effect to slightly heavier sheep, that is, equivalent to 120 mg. nitrate-N/kg. body-weight.

There is evidence, however, that composition of the diet may influence the toxicity of nitrate, and while this aspect was not studied in the present experiments, it seems likely that dietary factors may affect the toxicity of nitrite also. The mode of administration is of considerable importance too, for when administered as a single dose, 20 g. of potassium nitrite proved lethal, but when incorporated in the feed there were no ill-effects. The failure to detect nitrite in the blood of the sheep during the feeding experiment indicates a rapid breakdown of nitrite. On the other hand, in the dosing experiments when a quantity of nitrite was introduced into the rumen over a short time, the nitrite was absorbed rapidly and the peak blood levels were reached in about 2 hr. It is interesting to note that the levels of plasma nitrite found at the time of death were considerably higher than those reported by Setchell and Williams (1962).

ACKNOWLEDGMENTS

The authors are indebted to Mr. D. G. Miles for his co-operation in these studies.

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2026

Secondary Methemoglobinemia due to the Adulteration of Fish with Sodium Nitrite

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SECONDARY METHEMOGLOBINEMIA is a clinical entity that has been manifested in a wide variety of unusual circumstances (1–13). This rather dramatic syndrome has again appeared, the vehicle this time being fish adulterated with sodium nitrite in an attempt to mask or prevent spoilage.

CASE REPORTS

CASE 1

On March 24, 1959, at 7:46 PM, 2 brothers, 3 and 6 years of age, were brought to the emergency department of The Cooper Hospital, Camden. Patient 1, a 3-year-old boy, was dead on arrival. On examination, he appeared to be quite cyanotic, and there was no detectable evidence of life. When an intracardiac injection of epinephrine was done, the blood aspirated was found to be chocolate in color; the full significance of that observation was not appreciated at the time.

CASE 2

This was a 6-year-old brother of patient 1 who was brought into the emergency department in a semi-conscious, markedly cyanotic state. On examination, the patient was found to have a blood pressure of 95/50 mm Hg, pulse of 120/min, and respiratory rate of 32/min. The chest was clear, and there were no murmurs. Liver and spleen were not palpable, nor were there any other significant physical findings. Amphetamine sulfate, 10 mg, was given intravenously; the blood pressure rose to 115/70 mm Hg, although the cyanosis increased. An endotracheal tube was passed to assure adequate ventilation, and the patient was given 100% oxygen, without improvement in the degree of cyanosis.

In the absence of any apparent cardiac or respiratory basis for the cyanosis, it seemed that some acute toxic condition had rendered the blood incapable of carrying oxygen. The possibility of secondary methemoglobinemia appeared to be the most probable basis for this clinical picture (14). Methylene blue was given, therefore, as a therapeutic trial in the face of the obvious lethal potential of the acute toxic condition. Initially, 10 cc of a 1% solution, or 100 mg of methylene blue, were given intravenously. In approximately 10 minutes the patient's color began to improve; after 20 minutes, it showed definite improvement. A second ampule of methylene blue containing 10 cc was then given, due to some confusion concerning the number of mg contained in 10 cc of a 1% solution.

At approximately 10:35 PM, the patient was moved from the emergency department to the pediatric ward. The behavior of the child the first night was noted to be somewhat irritable, and the urine was recorded as being greenish-blue.

On March 25, 1959, the patient had 4 loose green stools, and the urine remained greenish-blue. Laboratory studies of the same day included a hemoglobin of 14 g/100 ml, hematocrit reading of 46%, total leukocyte count of 19,300/mm³, with 78% segmented neutrophiles, 16% lymphocytes, 5% monocytes, and 1% eosinophiles. The urine was reported as being green, alkaline, with a specific gravity of 1.023; it was negative for albumin and sugar, and was laden with mucus on microscopic examination. The patient was discharged on March 27, 1959, in good condition.

CASE 3

A third brother, age 4 years, was admitted, ambulatory, to the emergency department. As he passed into the treatment room, his color changed from dusky to that of deep cyanosis. By then, it was learned that an acute illness

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From The Cooper Hospital, Camden, New Jersey.

Requests for reprints should be addressed to Thomas L. Singley, III, M.D., 1207 Elm Avenue, Collingswood, New Jersey.

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had affected a family of 6, shortly after completion of their evening meal. It had resulted in nausea, vomiting, collapse, and cyanosis. The boy's stomach was then lavaged, as was that of patient 2; and he, too, was given 2 ampules containing 10 cc of 1% methylene blue intravenously.

Patient 3, however, did not appear to be responding quite as readily as patient 2, who was now sitting up and talking to his parents. Consequently, an exchange transfusion was performed on patient 3. This was continued until a total of 1,500 cc of blood had been exchanged. It was about midway through this procedure that the patient began to show satisfactory improvement in his level of consciousness, as well as recovery from cyanosis.

At 11:45 PM case 3 was moved to the pediatric ward in satisfactory condition. Nursing notes described the patient as being very upset and irritable, and crying for his mother through most of the night.

On March 25, 1959, the patient developed fever to 101 F, and on physical examination he showed some signs of hypervolemia, including hyperemia of mucous membranes and a palpable liver edge on inspiration. All urine collected was greenish-blue. Laboratory studies showed hemoglobin of 14 g/100 ml, hematocrit reading of 43%, and total leukocyte count of 25,950/mm³, with 60% segmented neutrophiles, 21% band forms (both showing toxic granulations), 13% lymphocytes, and 3% eosinophiles. The urine was reported as dark blue, alkaline, with specific gravity of 1.037; it was negative for albumin and sugar, and amorphous phosphates were observed on microscopic examination.

Investigation of Cases

The following information was obtained through the cooperation of the local police and agents of the Food and Drug Administration.

At approximately 5:05 PM on March 24, 1959, a mother of 4 children purchased 3 pieces of fillet of flounder, weighing a total of 2 pounds, at a local supermarket. The fish was prepared with bread crumbs, salt, and pepper. The 3-year-old boy, patient 1, being particularly fond of fish, ate his portion before the entire meal was prepared. His meal consisted of fish and milk alone, although he ate "nearly all" of one of the 3 pieces. At approximately 6:00 PM the boy left the table to visit his grandmother in the neighborhood. Within a few minutes, the grandmother called, informing the mother that the boy was very sleepy. This was attributed to a missed nap; however, very shortly another call told of his crying and vomiting. The mother reached the child at 6:35 PM and described him as "dark blue in color and appearing to be in shock."

Upon returning home with the critically ill child, she found that the 6-year-old boy, patient 2, had become nauseated after his meal, but was unable to vomit. The fourth child, a 6-year-old girl, and twin of patient 2, had eaten no fish, since she was waiting for her tomatoes to finish cooking, and she did not become ill. It was stated by the father that "the fish didn't taste quite right."

The family physician was called. Upon his arrival 15 minutes later, he found patient 1 lying on a chair with no detectable blood pressure, eyes rolled back, and absent reflexes. Patient 2 was on the couch in acute distress with abdominal cramps, cyanosis, and no perceptible blood pressure. Patient 3 was running around at this time, although he had been the last to come from playing, and the last to eat his supper. The mother had vomited, but otherwise appeared to be well; the presence of cyanosis in the father was questionable. Patients 1 and 2 were brought to the hospital by ambulance, oxygen being administered on the way.

Investigation was begun the following morning by agents of the Food and Drug Administration. Specimens of the family's supper had been obtained the night before by local police, who also obtained samples of similar fish from the supermarket at which the original purchase had been made (Table 1). More specimens were obtained by the federal authorities, and a detailed inspection of the home was made in an attempt to eliminate other agents capable of methemoglobin production (15).

TABLE 1. Specimens Examined for Nitrite Content

	Mg Nitrite/100 g Specimen
Specimen (Laboratory A)	
Fish from store	135
Liquid portion from sample 1	494
Fish from store	207
Fish from table at home	230
Fish from garbage can at home	388
Specimen (Laboratory B)	
Fish from store	611.1
Fish from store	592.8

Through the efforts of the federal and local authorities, it was ascertained by 3:30 PM on March 25, 1959, that approximately 1,800 pounds of flounder adulterated with sodium nitrite were in the Philadelphia-Camden area. A radio announcement at 5:00 PM cautioned that all recently purchased flounder should be discarded. The public reaction was inevitable: many people had their stomachs lavaged, and received emetics the following night in overcrowded emergency departments, probably unnecessarily. However, approximately 40 other cases of secondary methemoglobinemia were described by physicians throughout the area, although there were few laboratory data to support these reports. There were no other deaths.

AUTOPSY FINDINGS OF CASE 1

The autopsy was performed on March 25, 1959, and revealed a well-nourished child with an apparent age of 3 years. Marked cyanosis about the lips and nailbeds was noted. Extensive congestion of all organs was noted when the thoracic and abdominal cavities were opened. Samples of stomach contents and blood from the left atrium were sent to the State Toxicological Laboratories and were reported on April 2, 1959, as showing "stomach contents demonstrating a positive reaction for sodium nitrite" and "blood sample positive for methemoglobin."

COMMENT

If patient 1 ate 200 grams of a 300 gram piece of fish containing 230 mg of sodium nitrite per 100 g of fish, he consumed at least 460 milligrams of sodium nitrite. The patient's weight was 14 kilograms; therefore, he ingested 32.9 mg per kg of body weight. The toxic dose was studied by Stead, Kunkle, and Weiss, who found that a dose of 180 milligrams produced hypotension, pallor, sweating, nausea, dizziness, and loss of consciousness in the adult (16).

SUMMARY

Three cases of secondary methemoglobinemia resulting from the ingestion of fish adulterated with sodium nitrite are described clinically and supported by some laboratory data, although blood levels were not obtained. One death resulted, and the acute public health problem that ensued is discussed. Coordination between local and federal authorities resulted in a public warning less than 24 hours after the onset of the first case, and prevented the consumption of most of the 1,800 pounds of adulterated fish that was in the Philadelphia-Camden area. The 3-year-old patient who died consumed approximately 460 milligrams, or 32.9 mg per kg, of sodium nitrite.

SUMMARIO IN INTERLINGUA

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2036

THE EFFECT OF SODIUM NITRITE ON THE EMPTYING TIME OF THE NORMAL HUMAN STOMACH (*)

BY

CLARK K. SLEETH AND EDWARD J. VAN LIERE

(Received for publication 1-2-1940.)

That nitrites may exert a quieting influence on the gastro-intestinal tract has long been known. HIRSCHFELDER (1) observed intestinal movements of anmials with experimental plumbism, and noted that the spasms of the gut could be relaxed by administration of amyl nitrite or nitro-glycerine. HOLMES and DRESSER (2) found nitrites a valuable aid in differentiating spastic from organic lesions of the gastro-intestinal tract during fluoroscopy. BEAMS (3) confirmed their work, and added the information that the nitrite was most effective in the stomach and colon, and that pylorospasm and cardiospasm were only slightly affected by nitrite. The same worker later reported (4) that all pain of gastro-intestinal origin, if unaccompanied by an organic lesion could be relieved by nitrites, and that in 21 of 33 cases, pain was relieved even though some organic lesion was present. Of 200 patients fluoroscoped, only ten failed to show a decrease in peristalsis and a fall in tone of the stomach after nitrites. BEAMS and BARLOW (5) studied isolated segments of intestine, and concluded that the nitrite action was independent of nervous or vascular mechanisms.

No carefully controlled work could be found regarding the quantitative action of a therapeutic dose of any nitrite preparation on the emptying time of the normal human stomach. Consequently, the authors determined to make such a study.

(*) This research was made possible through a grant from the Therapeutic Research Committee of the Council on Pharmacy and Chemistry of the American Medical Association.

Methods

In seven healthy male medical students the normal emptying time of the stomach was determined fluoroscopically. The standard meal was the same as has been reported previously (6). Fifteen grams of Quaker Farina were added to 350 cc. of water and cooked to a volume of 200 cc. Fifty grams of barium sulphate were added after the meal had cooled. A number of control observations were made for each subject, and the average was used as the normal in each case.

The effect of sodium nitrite was then studied. One grain (0.065 Gm.) of sodium nitrite was given by mouth, with 100 cc. of water, 15 minutes before the meal was to be eaten. The emptying time of the stomach was then determined in the usual manner. At least 3 or 4 determinations were made on each subject, and the average figure was used. It had previously been determined that 100 cc. of water did not influence appreciably the normal gastric emptying time for the test meal used.

Results

The accompanying table shows the results obtained.

Table I

Effect of Sodium Nitrite on Emptying Time of Human Stomach

Subject Number	Normal Hrs.	After $NaNO_2$ Hrs.	Percent Delay	"p" (*)
1	1.65	2.75	65.7	
2	2.17	2.75	26.7	
3	2.17	2.75	26.7	
4	2.00	2.37	18.5	
5	2.37	2.75	16.0	
6	2.50	2.75	10.0	
7	2.33	2.37	1.7	
Average.....	2.17	2.64	23.6	< 0.01

(*) "p" refers to chance occurrence, calculated according to Fisher (7). A "p" of 0.05 is regarded significant.

Discussion

An average delay of 23.6 percent is quite significant, using the method outlined here. As may be seen in Table I, a statistical analysis shows that the probability of the results occurring by chance is less than 1 in 100. Although considerable individual variation has been noted by the authors when the effects of other substances on gastric emptying were studied (8 and 9), the variations noted with the nitrite are much more marked. This may, perhaps, be explained on the basis of individual differences in susceptibility to nitrites.

The data suggest that there may be a relationship between the normal emptying time and the amount of delay produced by the nitrite. That is, the shorter the normal emptying time, the greater the delay. Further work would be necessary to prove or disprove this, as no conclusions can be drawn from work on so small a number of subjects. It should also be mentioned that the three individuals in whom the greatest delay in gastric emptying was noted also presented the most severe complaints of headache and general malaise. The subject with the 65 percent delay complained most of all. Although he was not a migrainous subject, his symptoms simulated an attact of migraine. Migraine is known to be accompanied by delayed gastric emptying if vomiting does not occur, and often accompanies hypotensive states. Some degree of unusual sensitivity to nitrites may have been present in this case. In such cases, one cannot rule out the possibility that the nitrites may be exerting an indirect depressant influence on the stomach through a central action. The authors feel that an average delay of about 25 percent is likely to be encountered in any series of individuals chosen at random and subjected to the procedure outlined above.

Beams and Barlow (5) showed that nitrites depressed tone and motility of segments of intestine removed from the animal. It is also well established that nitrites lower blood pressure by a direct relaxing influence on the smooth muscle of the vessel walls. These observations suggest that the action of nitrites in delaying gastric emptying can be explained by a direct action on the smooth muscle of the stomach wall.

Summary

A standard test meal consisting chiefly of 15 grams of Quaker Farina and 50 grams of barium sulfate was fed to seven healthy young adults,

and an average gastric emptying time was established for each individual. Administration of a therapeutic dose of sodium nitrite, 1 grain (0.065 Gm.) produced an average delay in gastric emptying time of 23.6 percent. The results as shown by statistical analysis were significant; the probability of chance occurrence, was less than 1 in 100. There were rather marked individual variations; one of the subjects was virtually unaffected by the administration of sodium nitrite, whereas a delay in gastric emptying of 65 percent was caused in another of the subjects. In this individual the possibility of an added central depressant action cannot be ruled out. The direct depressant effect of nitrites upon the smooth muscle of the stomach, however, is doubtlessly the chief cause of the delayed gastric emptying time.

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TOXICOLOGY AND APPLIED PHARMACOLOGY 12, 179–185 (1968)

Reproduction in the Guinea Pig as Affected by Chronic Administration of Potassium Nitrate and Potassium Nitrite[1,2]

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Received October 9, 1967

Toxicosis produced by nitrates and related compounds is a problem in man and in domestic animals. The most prominent effect of nitrate and nitrite is the conversion of hemoglobin to methemoglobin. Nitrate must be reduced to nitrite before excessive methemoglobinemia occurs. Nitrites are therefore several times more toxic than nitrates. Pharmacologically, KNO_3 had been used as a diuretic, and much of this action is due to a direct irritant action on renal cells. The effects of nitrates and nitrites on reproduction have assumed considerable importance in recent years. Abortions in swine, sheep, and cattle have been attributed by Case (1957) to nitrate and nitrite poisoning. Experimental work emphasizing the effects of nitrates or nitrites on reproduction has been reported in cattle, sheep, swine, and rats. Simon *et al.* (1959) repeatedly administered potassium nitrate intraruminally to pregnant cows and thereby caused abortions. Davidson *et al.* (1963) produced one death and one abortion in a group of 40 cattle given varying levels of nitrates in feed. Winter and Hokanson (1964) reported that female cattle could tolerate quantities of nitrite sufficient to convert 40% to 50% of the total hemoglobin to methemoglobin with no apparent detrimental effects on maintenance of pregnancy. Eppson *et al.* (1960) and Setchell and Williams (1962) reported no apparent effects on reproduction in sheep. In swine, Tollett *et al.* (1960) reported that 5% levels of potassium nitrate in feed had no effect on the number of corpora lutea, the percentage of implantations, or the weights of ovaries, placenta, or thyroid gland. Abortions were not produced. Muhrer *et al.* (1956) reported that only 2 of 8 rats fed a diet containing 2% KNO_3 had normal litters. At necropsy, 4 had placental tissue indicating absorption or abortion.

In man, interest in this area has been on the problem of methemoglobinemia in infants due to nitrates or nitrites in drinking water. Typical of many reports of poisoning was a case described by Bucklin and Myint (1960). Schmitz (1961) postulated a relationship between high maternal methemoglobin levels and abortions in pregnant women.

There has been relatively little experimental work in laboratory animals such as the guinea pig, and basic information is lacking. The present experiments were attempts to determine the effects in guinea pigs of chronic administration of potassium nitrate and potassium nitrite in drinking water. The clinical, hematologic, and pathologic effects

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[2] Michigan Agricultural Experiment Station Journal Article No. 4282.
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were studied. This report emphasizes the reproductive aspects in the female guinea pigs used.

MATERIALS AND METHODS

Forty-six female guinea pigs were used. The chemical and concentration given, the number of guinea pigs per treatment, and the average number of days each group was given the chemical are shown (Table 1). Potassium nitrate was given in the water at concentrations of 300, 2500, 10,000, and 30,000 ppm. Concentrations of KNO_2 included 300, 1000, 2000, 3000, 4000, 5000, and 10,000 ppm. No more than 5 guinea pigs were in any one cage, and at least 1 was a male.

Food and water consumption was recorded. Guinea pigs were weighed weekly. A blood sample was obtained from a guinea pig of each group at approximately 2-week intervals and terminally from all guinea pigs killed. Hemoglobin and methemoglobin levels and differential and total leukocyte counts were determined by standard procedures. Serum nitrite levels were determined by the method of Diven *et al.* (1962). Levels of blood urea nitrogen were determined by utilizing an Auto-analyzer. Serum potassium levels were measured by flame photometry.

Those guinea pigs that died during the course of the experiment were necropsied as soon as possible after death. There was variability in the number of days the guinea pigs were given the chemicals since experiments were designed to determine pathologic effects of various lengths as well as levels of treatment. At necropsy tissue samples were collected from ovaries, uterus, cervix, thyroid, liver, spleen, kidneys, adrenals, skin, heart, and bone marrow. Tissue sections were fixed in 10% buffered formalin. Tissues were processed by routine paraffin technique and stained with hematoxylin and eosin.

RESULTS

Reproduction was not appreciably affected by KNO_3 at levels as high as 10,000 ppm in drinking water whereas, at 30,000 ppm, reproductive performance was poor (Table 2). One female (30,000 ppm) died and had 4 mummified fetuses *in utero*. Food and water consumption was essentially normal at all levels of treatment (Table 1) and weight gains were normal. There were no significant gross or microscopic lesions in the reproductive organs.

The most serious effects on reproduction were seen in females given 5000 or 10,000 ppm of KNO_2 in drinking water. No live births were recorded at these levels (Table 3). Reproductive disturbances were manifested in different ways. At 5000 ppm 6 fetuses were aborted, 3 were mummified and 1 was in the process of absorption at the time of necropsy (Table 3). One female (5000 ppm) died from apparent toxemia.

Histologic examination revealed placental, uterine, and cervical lesions especially in females in which the fetuses had died. Lesions were most extensive in females given 5000 ppm of KNO_2. Placental lesions were degenerative in nature and were characterized by necrotic foci with pycnosis, karyorrhexis, and calcification. Uterine and cervical lesions were typically inflammatory with accumulations of lymphocytes and neutrophils in the lumens, in the endometrium, and in the cervical epithelium.

Food and water consumption was maintained at or near normal at all levels of treatment (Table 1). The rate of weight gains was normal except for those guinea pigs

TABLE 1

AVERAGE FEED, WATER, KNO_3, AND KNO_2 CONSUMPTION BY GUINEA PIGS

Concentration of chemicals in water (ppm)	Number of females	Average number of days given chemical	Daily feed consumption (g/100 g body wt.)	Daily water consumption (ml/100 g body wt.)	Daily intake of chemical (mg/100 g body wt.)	Daily intake expressed as nitrate or nitrite nitrogen (mg/100 g body wt.)
Control						
0	4	191	8.9	32.5	0	0
KNO_3						
300	6	166	7.2	29.7	8.9	1.2
2,500	3	194	7.1	29.7	74.2	10.2
10,000	3	143	8.0	36.7	367.9	50.7
30,000	3	204	7.5	27.3	819.0	113.0
KNO_2						
300	3	240	8.1	36.4	10.9	1.8
1,000	3	176	6.6	27.2	27.2	4.5
2,000	3	165	12.1	47.1	94.2	15.4
3,000	5	123	9.1	37.1	111.2	18.2
4,000	6	100	8.7	29.0	119.2	19.2
5,000	4	135	8.4	29.8	149.0	24.4
10,000	3	118	6.1	35.2	352.0	57.7

TABLE 2

REPRODUCTIVE PERFORMANCE OF GUINEA PIGS GIVEN KNO_3 IN WATER

Conc. of KNO_3 in water (ppm)	Number of females	Average number of days given KNO_3	Litters produced	Live births	Relative[a] percent reproductive performance	Fetal deaths			Percent of fetal loss
						Stillborn	Aborted	Mummified or absorbed	
0 (control)	4	191	8	31	100	1	0	0	3
300	6	166	12	33	81	11	0	0	25
2,500	3	194	6	19	80	3	0	0	14
10,000	3	143	5	15	86	1	0	0	6
30,000	3	204	2	2	8	0	0	4	67

[a] Relative percentage was calculated on the assumption that the controls had 100% reproductive performance. The number of females in each group, the number of days the chemical was given, and the total live births were taken into consideration.

TABLE 3

REPRODUCTIVE PERFORMANCE OF GUINEA PIGS GIVEN KNO_2 IN WATER

Conc. of KNO_2 in water (ppm)	Number of females	Average number of days given chemical	Litters produced	Live births	Relative[a] percent reproductive performance	Fetal deaths			Percent of fetal loss
						Stillborn	Aborted	Mummified or absorbed	
0 (control)	4	191	8	31	100	1	0	0	3
300	3	240	8	31	106	3	0	0	9
1,000	3	176	6	17	79	3	0	0	15
2,000	3	165	4	12	60	1	0	0	8
3,000	5	123	8	20	80	4	0	0	17
4,000	6	100	4	10	41	2	0	2	29
5,000	4	135	4	0	0	0	6	4	100
10,000	3	118	1	0	0	0	2	0	100

[a] Relative percentage was calculated on the assumption that the controls had 100% reproductive performance. The number of females in each group, the number of days the chemical was given, and the total live births were taken into consideration.

given 10,000 ppm of KNO_2. At this level the rate of gain was about one-seventh normal. Weight losses took place after parturition in several females given higher levels of KNO_2. These losses were associated with the subacute endometritis and cervicitis.

Hemoglobin levels were slightly depressed at the higher levels of treatment with KNO_2. Recorded methemoglobin levels did not exceed 20% of the available hemoglobin, and the highest levels were in those guinea pigs given 10,000 ppm of KNO_2.

There were no significant alterations in serum nitrite, blood urea nitrogen, or serum potassium in any of the blood samples tested throughout the experiment.

DISCUSSION

Conception took place at all levels of treatment. Since males were treated similarly, their fertility was apparently not impaired. Reproductive disturbances in the female were dose related and particularly evident at 5000 and 10,000 ppm of KNO_2 and 30,000 ppm of KNO_3. Abortion, fetal absorption, fetal mummification, and maternal death were important manifestations.

Hypoxia of the fetus due to maternal methemoglobinemia is hypothesized as a cause of fetal death. In the present study, recorded methemoglobin levels did not exceed 20% of the available hemoglobin, but there were only limited numbers of blood samples taken. Fetal death could occur following a relatively short period of severe methemoglobinemia. One might also relate the placental and uterine lesions to fetal death but, in our opinion, most of these lesions developed after the fetuses had died.

The relatively normal reproductive performance by guinea pigs given levels of KNO_2 as high as 3000 ppm and KNO_3 as high as 10,000 ppm indicated considerable tolerance by the guinea pig to these chemicals.

SUMMARY

Guinea pigs were given KNO_3 in doses ranging from 300 to 30,000 ppm in the water and KNO_2 in amounts from 300 to 10,000 ppm for periods ranging from 100 to 240 days. Reproduction in the female was impaired by 30,000 ppm of KNO_3 with reproductive performance only 8% of that of the controls. Fetal losses were 100% in females given 5000 and 10,000 ppm of KNO_2. Reproduction was maintained at lower levels of treatment. Male fertility was apparently not impaired since conception took place at all levels of treatment. Food and water consumption and weight gains were normal except for a diminished rate of gain at 10,000 ppm of KNO_2. Uterine and cervical inflammatory lesions and degenerative placental lesions were present in females in which the fetuses had been aborted, mummified, or absorbed

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2046

Range-Finding Toxicity Data: List VII

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Acute toxicity and irritation data on about 200 compounds are presented, accumulated in a continuing program for screening potential commercial products.

DURING THE SEVEN years since the appearance of our last communication on the range-finding test,[1] many additional data have become available for presentation. Table I lists these data under names conforming to the nomenclature used in *Chemical Abstracts*. Table II lists only the oral LD_{50} values for a number of inorganic compounds. All materials included are either in commercial production or have been evaluated for commercial potential within the past few years.

As has been stated in earlier communications,[1-6] the range-finding test is relied upon only to allow predictions of the comparative hazards of handling new chemicals. Acute toxicity studies, no matter how precisely executed, yield no more than an indication of the degree of care necessary to protect exposed workmen or lead to an opinion that certain technically feasible applications of a chemical may or may not eventually be proved safe.

Our last paper[1] included data under the name of triisooctyl phosphine. The compound should have been entered as triisooctyl phosphite.

Experimental methods have remained unchanged. They are described in our 1962 publication.[1]

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TABLE I
Range-Finding Toxicity Data

Material Studied	Single Oral LD_{50} for Rats ml/Kg	Single Skin Penetration LD_{50} for Rabbits ml/Kg	Concentrated Vapor Inhalation by Rats Maximum for No Death	Inhalation of Metered Vapor Concentration by Rats: Concentration ppm	Time, hr.	Mortality	Irritation on Uncovered Rabbit Belly	Corneal Injury in Rabbits
Hydrocarbons								
2-(4-Cyclohexenyl)-bicyclo-(2.2.1)-hept-5-ene	4.29 (3.07 – 5.98)	3.54 (2.19 – 5.72)	8 hr.	–	–	–	5	2
Cyclopentene	2.14 (1.45 – 3.17)	1.59 (0.97 – 2.59)	3 m. §	16,000	4	4/6	5	5
5-Ethylidene-2-norbornene	2.83	9.17 (1.35 – 6.33)	–	4,000	4	5/6	5	2
p-Menth-3-ene	20.75 (15.36 –28.03)	5.66 (2.59 –12.3)	30 m.	2,000	4	2/6	5	1
2-Norbornene	11.3 (7.0 –18.3)*	>5	5 m.	–	–	–	1	7
1,7-Octadiene	19.7 (15.0 –25.8)	14.1 (4.66 –42.9)	15 m.	8,000	4	2/6	4	1
3a,4,7,7a-Tetrahydro-1H-indene	3.73 (2.68 – 5.21)	14.1 (8.75 –22.9)	4 hr.	–	–	–	3	2
Toluene	7.53 (6.73 – 8.43)*	14.1 (9.63 –20.77)	–	4,000	4	1/6	4	9
4-Vinyl-1-cyclohexene	3.08 (2.49 – 3.81)	20	15 m.	8,000	4	4/6	4	2
5-Vinyl-2-norbornene	5.19 (4.22 – 6.41)	15.9 (11.7 –21.5)	–	4,000	4	4/6	5	2
Hydroxyl Compounds								
p-tert. Butylphenol	3.25 (2.48 – 4.26)	2.52 (0.93 – 8.58)	8 hr.	–	–	–	6	9
Cyclohexenyl-4-phenol	2.83 *	3.18 (1.94 – 5.18)*	8 hr.	–	–	–	–	9
2-Cyclopenten-1-ol	0.47 (0.34 – 0.66)	0.18	1 hr.	500†	4	0/6	2	5
3,4-Dihydroxy-1,5-hexadiene	1.62 (1.24 – 2.13)	0.40 (0.29 – 0.54)	–	–	–	–	2	6
Eicosanol, mixed isomers	>64	>20	8 hr.	–	–	–	3	1
2-Ethylhexanol	2.46 (1.82 – 3.33)	2.38 (1.70 – 3.34)	8 hr.	–	–	–	3	5
Hydroxydihydrocyclopentadiene	3.25 (2.11 – 4.99)	3.15 (2.33 – 4.26)	8 hr.	–	–	–	5	9
3-Methyl-1-butanol	7.07 (4.82 –10.4)	3.97 (2.93 – 5.37)	8 hr.	–	–	–	2	8
Methyl-3-cyclohexenyl-methanol	1.41	0.62 (0.42 – 0.92)	8 hr.	–	–	–	3	8
Nonylphenol, mixed isomers	1.62 (1.06 – 2.50)	2.14 (1.52 – 2.99)	4 hr.	–	–	–	6	10
2-Norbornanemethanol	1.62 (1.24 – 2.13)*	0.80	8 hr.	–	–	–	3	9
Tetradecanol, mixed isomers	32.5 (29.1 –36.3)	7.13 (4.41 –11.52)	8 hr.	–	–	–	2	1
Ethers								
Bicyclo(2.2.1)hept-2,5-ylene bis allyl ether	3.73 (2.43 – 5.74)	5.95 (3.57 – 9.09)	8 hr.	–	–	–	3	1
2-Cyclopentyl ether	11.3	1.59 (1.03 – 2.59)	4 hr.	–	–	–	5	2
Ethyl propenyl ether	19.0 (13.4 –27.0)	>10	5 m.	8,000	4	0/6	2	2
Ethyl vinyl ether	8.16 (7.03 – 9.48)	>20	–	64,000	4	0/6	1	1
Methoxy triethylene glycol vinyl ether	11.3	10.0 (4.56 –21.9)	8 hr.	–	–	–	2	3
alpha-Methylbenzyl ether	9.80 (6.38 –15.06)*	–	8 hr.	–	–	–	4	1
2-Methoxypropene	1.87 (1.26 – 2.76)	>20	–	64,000	4	4/6	1	1
1,1,3,5-Tetramethoxyhexane	4.09 (3.52 – 4.76)*	–	8 hr.	–	–	–	2	2
Hydroxy Ethers								
1-Butoxyethoxy-2-propanol	4.00 (2.55 – 6.27)	2.83 (1.75 – 4.57)	8 hr.	–	–	–	3	5
3-Butoxy-1-propanol	5.95 (3.65 – 9.68)	1.59 (1.17 – 2.15)	8 hr.	–	–	–	2	7
Diethoxytetraethylene glycol	4.29 (2.90 – 6.34)	6.35 (3.89 –10.4)	8 hr.	–	–	–	2	2
3-Ethoxy-1-propanol	13.0 (8.46 –20.0)	2.83	8 hr.	–	–	–	2	5
2-Ethylbutoxypropanol, mixed isomers	3.56 (2.21 – 5.74)	6.0	8 hr.	–	–	–	2	9
2-Hexyloxy-2-ethoxyethyl ether	3.73 (2.68 – 5.21)	6.30 (4.66 – 8.52)	8 hr.	–	–	–	2	5
Isobutoxypropanol, mixed isomers	7.5	3.56 (1.95 – 6.52)	8 hr.	–	–	–	3	7

TABLE I
Range-Finding Toxicity Data (Continued)

Material Studied	Single Oral LD_{50} for Rats ml/Kg	Single Skin Penetration LD_{50} for Rabbits ml/Kg	Concentrated Vapor Inhalation by Rats Maximum for No Death	Inhalation of Metered Vapor Concentration by Rats: Concentration ppm	Time, hr.	Mortality	Irritation on Uncovered Rabbit Belly	Corneal Injury in Rabbits
2-Isopropoxyethanol	5.66 (4.11 - 7.79)	1.60 (1.08 - 2.37)	2 hr.	4,000	4	4/6	3	5
3-Methoxy-1-propanol	13.0 (9.90 -17.0)	5.66	8 hr.	-	-	-	1	5
2-Propoxyethanol	4.89 (4.16 - 5.73)*	0.96 (0.93 - 0.99)	-	-	-	-	2	8
Propoxypropanol, mixed isomers	3.25 (2.48 - 4.26)	4.00 (2.70 - 5.92)	8 hr.	-	-	-	2	7
Aldehydes, Ketones								
Cyclohexanone	1.62 (1.20 - 2.20)	1.00 (0.63 - 1.63)	30 m.	2,000†	4	1/6	1	5
5-Ethyl-3-nonen-2-one	8.12 (7.27 - 9.06)*	8.48 (5.04 -14.24)	8 hr.	-	-	-	2	1
Hexanal, mixed isomers	9.51 (5.84 -15.5)	>10	30 m.	-	-	-	2	5
2-Methylcyclohexanone	2.14 (1.48 - 3.10)	1.77	-	2,800	4	3/6	3	5
2-Methyl-1,2,3,6-tetrahydrobenzaldehyde	5.66	3.15 (2.33 - 4.26)	8 hr.	-	-	-	5	5
1-Tetralone	0.81 (0.62 - 1.07)	>20	8 hr.	-	-	-	4	3
Valeraldehyde	5.66	6	15 m.	4,000	4	3/6	2	5
Acids, Anhydrides, Lactones								
3-Hydroxybutyric acid lactone	17.2 (12.3 -23.9)	>20	8 hr.	-	-	-	5	5
b-Methyl-delta-valerolactone	12.9 (8.4 -19.9)	>10	-	-	-	-	1	4
Methyltetrahydrophthalic anhydride	2.14 (1.48 - 3.10)	1.41	8 hr.	-	-	-	1	9
Tri-isobutenylsuccinic anhydride	0.94 (0.61 - 1.44)*	0.89 (0.55 - 1.44)*	-	-	-	-	4	7
Valeric acid, mixed isomers	2.00 (0.90 - 4.44)	0.31 (0.06 - 1.50)	8 hr.	-	-	-	6	9
Esters, Acetals								
Acetic acid, 2-methyl-2,4-pentanediol diester	3.36 (2.06 - 5.48)	16.0 (3.33 -76.8)	8 hr.	-	-	-	3	2
Acetic acid, 2-methyl-2-propene-1,1-diol diester	0.44 (0.28 - 0.68)	0.044(0.024- 0.082)	1 hr.	62.5	4	5/6	5	9
Acetic acid, phenyl ester	1.63 (1.03 - 2.74)	8.00 (3.65 -17.5)	8 hr.	-	-	-	2	2
Acetic acid, propyl ester	9.8 (7.5 -12.9)	>20	30 m.	8,000	4	4/6	1	2
Acetic acid, triethylene glycol diester	22.6	8.0 (1.91 -33.5)	8 hr.	-	-	-	1	1
Acrylic acid, 2-butoxyethoxy ester	6.50 (4.72 - 8.95)	0.64 (0.39 - 1.04)	8 hr.	-	-	-	5	2
Acrylic acid, decyl ester, mixed isomers	12.3 (9.98 -15.3)	3.54 (1.17 -10.7)	8 hr.	-	-	-	5	1
Acrylic acid, hexyl ester	26.0 (18.9 -35.8)	5.66 (3.50 - 9.14)	4 hr.	-	-	-	5	1
Acrylic acid, 2-hydroxypropyl ester	1.23 (0.89 - 1.70)	0.16 (0.10 - 0.26)	8 hr.	-	-	-	5	7
Acrylic acid, 2-methoxyethoxy ester	0.81 (0.59 - 1.21)	0.25 (0.17 - 0.37)	15 m.	500	4	3/6	4	5
Acrylic acid, 2-norbornyl ester	5.66	0.88 (0.54 - 1.43)	8 hr.	-	-	-	5	2
Azelic acid, dihexyl ester	16.0 (10.2 -25.0)	>20	-	-	-	-	2	1
Benzoic acid, 2-ethylhexanediol diester	29.2 (15.8 -53.9)	20	8 hr.	-	-	-	2	1
Fumaric acid, ethyl 2,3-epoxypropyl ester	1.62 (1.06 - 2.50)	0.35 (0.22 - 0.57)	-	-	-	-	5	8
Fumaric acid, dimethyl ester	2.24 (1.15 - 4.37)*	1.25 (0.84 - 1.85)*	8 hr.	-	-	-	5	9
Malonic acid, diethyl ester	14.9 (10.7 -20.8)	>16	8 hr.	-	-	-	2	5
Methacrylic acid, allyl ester	0.43 (0.31 - 0.60)*	0.50 (0.37 - 0.68)	8 m.	250†	4	0/6	5	2
Methacrylic acid, butyl ester	22.6	11.3 (5.19 -24.7)	8 hr.	-	-	-	5	1
Phthalic acid, butyl decyl ester	20.8 (16.8 -25.7)	15.9 (11.7 -21.5)	8 hr.	-	-	-	2	1
Phthalic acid, decyl hexyl ester	49.4 (39.9 -61.0)	>20	8 hr.	-	-	-	2	1

(Continued on Next Page)

TABLE I
Range-Finding Toxicity Data (Continued)

[illegible]	2.2 [illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Malonic acid, diethyl ester	14.9 (10.7 -20.8)	>10		8 hr.	–	–	–	2	5
Acrylic acid, allyl ester	0.43 (0.31 - 0.60)*	0.5[illegible] (0.37 - 0.68)		8 m.	250†	4	0/6	5	2
Acrylic acid, butyl ester	22.6	11.[illegible] ([illegible].19 -24.7)		8 hr.	–	–	–	5	1
Phthalic acid, butyl decyl ester	20.8 (16.8 -25.7)	15.9 ([illegible]1.7 -21.5)		8 hr.	–	–	–	2	1
Phthalic acid, decyl hexyl ester	49.4 (39.9 -61.0)	>20		8 hr.	–	–	–	2	1

(Continued on Next Page)

TABLE I
Range-Finding Toxicity Data (Continued)

Material Studied	Single Oral LD_{50} for Rats ml/Kg	Single Skin Penetration LD_{50} for Rabbits ml/Kg	Concentrated Vapor Inhalation by Rats Maximum for No Death	Inhalation of Metered Vapor Concentration by Rats: Concentration ppm	Time, hr.	Mortality	Irritation on Uncovered Rabbit Belly	Corneal Injury in Rabbits
Phthalic acid, decyl octyl ester	45.2 (28.0 -73.2)	>20	8 hr.	–	–	–	2	1
Propionaldehyde dierotyl acetal	2.14 (1.54 - 2.99)	3.54 (2.19 - 5.72)	30 m.	2,000	4	5/6	4	2
Succinic acid, di-2-[(2-ethylbutoxy)ethoxy] ester	3.3 *	7.11 (6.58 - 7.69)	–	–	–	–	3	2
Epoxy Compounds								
Bicyclononadiene diepoxide	2.14 (1.54 - 2.99)	1.77	8 hr.	–	–	–	3	5
Bis(3,4-epoxybutyl)ether	1.07 (0.77 - 1.50)	0.25 (0.17 - 0.37)	8 hr.	–	–	–	5	7
Bis(3,4-epoxy cyclohexylmethyl)adipate	4.39 (3.07 - 5.98)	>20	8 hr.	–	–	–	2	1
N,N-Bis(2,3-epoxypropyl)aniline	1.62 (1.18 - 2.24)	3.56 (1.68 - 7.57)	8 hr.	–	–	–	3	1
Butyl 2,3-epoxypropyl fumarate	0.71 (0.48 - 1.04)	1.26 (0.93 - 1.70)	8 hr.	–	–	–	5	8
t-Butylphenyl 2,3-epoxypropyl ether	3.73 (2.68 - 5.21)	5.66	8 hr.	–	–	–	5	2
1,2-Epoxy cyclohexane	1.09 (0.66 - 1.80)	0.63 (0.39 - 1.03)	30 m	2,000†	4	1/6	3	8
2,3-Epoxypropyl methacrylate	0.77 (0.57 - 1.04)	0.45 (0.21 - 0.95)	2 hr.	–	–	–	5	4
Propylene oxide	1.14 (1.05 - 1.23)	1.50 (1.07 - 21.0)	5 m. §	4,000	4	4/6	1	5
Heterocyclic Oxygen								
2-Aminomethyl-2,3-dihydro-4H-pyran	1.00 (0.64 - 1.57)*	0.18	4 hr.	–	–	–	6	9
2,5-Divinyltetrahydropyran	2.46 (1.79 - 3.39)	1.41 (0.65 - 3.08)	4 hr.	–	–	–	4	2
3,9-Divinyl-2,4,8,10-tetraoxaspiro (5,5) undecane	3.25 (2.48 - 4.26)*	7.92 (5.87 -10.73)*	8 hr.	–	–	–	2	2
2-Ethoxy-4-methyl-tetrahydropyran	5.16 (4.17 - 6.38)	> 5	4 hr.	–	–	–	2	3
Aliphatic and Alicyclic Amino Compounds								
1-Amino-3-aminomethyl-3,5,5-trimethyl cyclohexanol	1.30 (1.04 - 1.75)	–	8 hr.	–	–	–	7	9
2-Aminomethyltetrahydropyran	0.71 (0.44 - 1.13)	0.71 (0.44 - 1.14)	4 hr.	–	–	–	6	9
3-Aminopropoxy-2-ethoxy ethanol	6.50 (4.95 - 8.53)*	5.99 (4.27 - 8.42)	8 hr.	–	–	–	3	7
Amyl amine, mixed isomers	0.47 (0.33 - 0.65)*	1.12 (0.61 - 2.05)	30 m. §	2,000	4	4/6	6	9
Bis(2-dimethylaminoethoxy)ethane	2.83	1.2	–	–	–	–	7	8
Bis(2-dimethylaminoethyl) ether	1.23 (0.94 - 1.62)	0.28	8 hr.	–	–	–	6	9
Cyclohexylamine	0.71 (0.51 - 0.97)	0.32 (0.24 - 0.43)	2 hr.	4,000†	4	0/6	7	10
1,8-Diamino-p-menthane	0.77 (0.62 - 0.95)	0.63 (0.47 - 0.85)	4 hr.	–	–	–	7	9
2,4-Diamino-1-methylcyclohexane	1.41	0.50 (0.31 - 0.82)	8 hr.	–	–	–	6	9
Di(3-aminopropyl) ether of diethylene glycol	4.29 (3.07 - 5.98)	2.50 (1.14 - 5.48)	4 hr.	–	–	–	6	8
Di(3-aminopropoxy)ethane	2.83 (1.76 - 4.55)	1.25 (0.84 - 1.85)	8 hr.	–	–	–	6	8
N,N-Di(methyl-N-cyclohexylmethyl)amine	1.23 (0.94 - 1.62)*	0.21 (0.15 - 0.30)	8 hr.	–	–	–	7	9
N-(Methyl-N-cyclohexylmethyl)amine	1.41 *	0.63 (0.47 - 0.85)	4 hr.	–	–	–	6	9
N,N,N',N',N''-Pentamethyldiethylenetriamine	1.63 (1.24 - 2.13)	0.28	8 hr.	–	–	–	7	9
N,N,N',N'-Tetramethyl-1,3-butanediamine	0.93 (0.67 - 1.30)	0.40 (0.29 - 0.54)	8 hr.	–	–	–	6	9
N,N,N',N'-Tetramethyl-1,3-propanediamine	0.41 (0.31 - 0.53)	0.30 (0.21 - 0.42)	15 m.	1,000	4	2/6	5	9
Tetramethyl-2-tetrazene	1.78 (1.12 - 2.83)	—	4 hr.	–	–	–	2	4
Tripropylamine	0.096(0.071- 0.13)*	0.57 (0.26 - 1.23)	8 hr.	250	4	3/6	4	1
Aromatic Nitro and Amino Compounds								
3-Aminopyrene	1.07 (0.77 - 1.50)*	> 1	8 hr.	–	–	–	–	8
N-Methyl-1-naphthylamine	1.41 (1.03 - 1.95)		8 hr.	–	–	–	2	1

TABLE I
Range-Finding Toxicity Data (Continued)

Material Studied	Single Oral LD_{50} for Rats ml/Kg	Single Skin Penetration LD_{50} for Rabbits ml/Kg	Concentrated Vapor Inhalation by Rats Maximum for No Death	Inhalation of Metered Vapor Concentration by Rats: Concentration ppm	Time, hr.	Mortality	Irritation* on Uncovered Rabbit Belly	Corneal Injury in Rabbits
Amides								
N(2-Acetoxyethyl)-N-ethylacetamide	20.8 (16.8 -25.7)	>10	8 hr.	–	–	–	2	4
e-Caprolactam	2.14 (1.54 – 2.99)*	1.41	4 hr.	–	–	–	–	7
N-Ethyl-N-vinylacetamide	2.46 (1.88 – 3.23)	1.25 (0.84 - 1.85)	4 hr.	2,000	4	3/6	2	5
Propyl-N,N-diethylsuccinamate	12.93 (8.42 -19.87)	>20	8 hr.	–	–	–	4	5
Heterocyclic Compounds								
3,5-Dimethyl-1-(trichloromethylmercapto)-pyrazole	0.57 *	0.20 (0.15 - 0.27)*	4 hr.	–	–	–	6	10
Isopropylmorpholine	0.71	0.10 (0.07 - 0.15)	15 m.	500	4	5/6	6	7
Isopropylpiperazine	2.83	0.64 (0.47 - 0.86)	8 hr.	–	–	–	6	9
3,4-Lutidine	0.71 (0.40 – 1.24)	0.14	1 hr.	250†	4	0/6	6	8
N-Methyl-e-caprolactam	1.62 (1.18 – 2.24)	1.41	8 hr.	–	–	–	3	5
3-Methyl-2-oxazolidone	7.13 (3.32 –15.30)	10.8 (6.17 -16.5)	8 hr.	–	–	–	1	4
2-Methyl-5-vinylpyridine	1.30 (0.71 – 2.37)	0.80 (0.54 - 1.18)	4 hr.	4,000	8	4/6	5	8
1-(Trichloromethyl mercapto) pyrazole	0.35 *	0.063(0.047- 0.085)	–	200	1	6/6	7	9
1-(Trichloromethyl-mercapto)-4-methylpyrazole	1.04 (0.81 – 1.28)	0.079(0.059- 0.107)	2 m. §	–	–	–	7	8
Cyano Compounds								
Bis(2-isocyanatoethyl)carbonate	1.83 (1.21 – 2.79)	>12	8 hr.	–	–	–	3	9
Bis(2-isocyanatoethyl)-4-cyclohexene-1,2-dicarboxylate	10.4 (5.55 –19.4)	> 8	8 hr.	–	–	–	5	5
Bis(2-isocyanatoethyl)-5-norbornene-2,3-dicarboxylate	22.6 (13.4 –38.2)	16.1 (4.5 -57.2)	8 hr.	–	–	–	5	5
3-Butenenitrile	0.115(0.097- 0.138)*	1.41	5 m.	250†	4	0/6	2	3
N,N-Dibutyl-3-aminopropionitrile	3.25 (2.48 – 4.26)	5.04 (3.73 - 6.82)	8 hr.	–	–	–	2	2
1,6-Hexamethylene diisocyanate	0.71	0.57 (0.35 - 0.91)	8 hr.	–	–	–	8	9
Lactonitrile	0.087(0.060- 0.125)*	0.020(0.015- 0.027)	–	62.5†	4	0/6	2	–‡
3-Methoxypropionitrile	4.39 (3.07 – 5.98)*	>10	8 hr.	–	–	–	2	5
Methyl isocyanate	0.071(0.027- 0.18)*	0.22 (0.12 - 0.41)	–	31.25†	4	0/6	6	10
Tridecanitrile, mixed isomers	3.73 (2.68 – 5.21)	3.18 (1.43 - 7.06)	8 hr.	–	–	–	4	5
Halogen Compounds								
1-Bromooctane	4.49 (2.09 – 9.64)	8.00 (4.93 -13.0)	–	–	–	–	5	1
2-Chlorobutane	20.0 (12.8 -31.3)	20	5 m. §	8,000	4	3/6	1	2
Chloromethyl ether	0.21 (0.12 – 0.37)	0.28 (0.13 - 0.62)	–	8†	4	1/6	6	10
Dibromobicycloheptane, mixed isomers	0.21 (0.15 – 0.30)*	0.25 (0.19 - 0.34)	1 hr.	–	–	–	6	–
Dichlorobenzyl alcohol, mixed isomers	0.81 (0.50 – 1.31)	0.40 (0.24 - 0.65)	8 hr.	–	–	–	5	9
1,2-Dichloropropane(propylene dichloride)	1.9 (1.7 – 2.1)	8.75 (8.31 - 9.20)	10 m.	2,000	8	3/6	1	2
trans-2,3-Dichloro-1,4-dioxane	1.41	0.44 (0.27 - 0.71)	4 hr.	–	–	–	5	7
1,2-Dichloroethane(ethylene dichloride)	0.77 (0.67 – 0.89)*	3.89 (3.40 - 4.46)	–	–	–	–	2	3
Di(2-chloroethyl)acetal	0.31 (0.24 – 0.40)*	0.20 (0.14 - 0.30)	1 hr.	–	–	–	3	2
Fumaroyl chloride	0.81 (0.51 – 1.30)	1.41	–	500	4	3/6	6	10
1,1,1,3,3,3-Hexachloro-2,2-difluoropropane	0.54 (0.30 – 1.01)	4.53 (2.80 - 7.32)	–	–	–	–	3	2
m-Nitrobenzoyl chloride	2.46 (1.79 – 3.39)	0.79 (0.49 - 1.30)	8 hr.	–	–	–	5	9

(Continued on Next Page)

TABLE I
Range-Finding Toxicity Data (Continued)

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
trans-2,3-[illegible]-1,4-dioxane	1.41	0.44 (0.27 - 0.71)	4 hr.	–	–	–	–	2	3
1,2-D[illegible]hexane(ethylene dichloride)	0.77 (0.67 - 0.89)*	3.89 (3.40 - [illegible])	–	–	–	–	–	3	2
D[illegible](2-chlor[illegible]yl)acetal	0.31 (0.24 - 0.40)*	0.20 (0.14 - [illegible])	1 hr.	–	500	4	3/6	6	10
Fumaroyl chloride	0.81 (0.51 - 1.30)	1.41	–	–	–	–	–	3	2
1,1,1,3,3,3-Hexachloro-2,2-difluoropropane	0.54 (0.30 - 1.01)	4.53 (2.80 - 7.32)	–	–	–	–	–	5	9
m-Nitrobenzoyl chloride	2.46 (1.79 - 3.39)	0.79 (0.49 - 1.30)	8 hr.						

(Continued on Next Page)

TABLE I
Range-Finding Toxicity Data (Continued)

Material Studied	Single Oral LD_{50} for Rats ml/Kg	Single Skin Penetration LD_{50} for Rabbits ml/Kg	Concentrated Vapor Inhalation by Rats Maximum for No Death	Inhalation of Metered Vapor Concentration by Rats: Concentration ppm	Time, hr.	Mortality	Irritation on Uncovered Rabbit Belly	Corneal Injury in Rabbits
Phenyl chloroformate	1.41 (0.83 - 2.40)	3.97 (2.93 - 5.37)	15 m.	44	4	3/6	6	10
Propylene chlorohydrin	0.22 (0.19 - 0.27)	0.48 (0.34 - 0.67)	15 m.	500	4	1/6	1	8
Tetrachloroethane	0.20 (0.16 - 0.27)	3.99 (3.10 - 5.13)	–	1,000	4	3/6	6	–
1,1,2,3-Tetrachloropropene	0.35 *	0.40 (0.27 - 0.59)	30 m.	250	4	5/6	5	9
1,1,2-Trichloroethane	0.58 (0.47 - 0.71)	3.73 (3.30 - 4.21)	–	500	8	4/6	2	2
Trichloroethylene	4.92 (3.75 - 6.46)	>20	–	8,000	4	4/6	5	4
Phosphorous Compounds								
Bis(2-chloroethyl)phosphite	0.26 (0.19 - 0.35)*	0.141(0.065- 0.308)	8 hr.	–	–	–	2	3
Diethyl phosphite	5.19 (2.81 - 9.58)	2.02 (1.24 - 3.29)	4 hr.	–	–	–	1	5
Trimethyl phosphate	2.83 (1.75 - 4.57)	2.83	8 hr.	–	–	–	4	2
Tris(dipropylene glycol)phosphine	19.7 (15.0 -25.8)	>10	8 hr.	–	–	–	2	1
Tris(dipropylene glycol)phosphonate	29.9 (21.4 -41.6)	15.9 (11.7 -21.5)	8 hr.	–	–	–	3	1
Sulphur Compounds								
Bis(2-acetoxyethyl)sulfone	14.1	11.3	8 hr.	–	–	–	4	2
Bis(1,2-dichloroethyl)sulfone	0.25 (0.19 - 0.32)*	1.00 (0.40 - 2.51)	8 hr.	–	–	–	7	9
2,3-Epithiopropyl methoxy ether	2.14 (1.54 - 2.99)	> 5	1 hr.	–	–	–	3	2
n-Hexyl vinyl sulfone	0.57	0.84 (0.50 - 1.40)	–	–	–	–	7	5
Isopropyl sulfate	1.09 (0.64 - 1.87)	1.41 (0.88 - 2.29)	1 hr.	–	–	–	6	5
2(Methoxyethylthio)ethanol	13.00 (7.70 -21.93)	5.04 (3.09 - 8.23)	8 hr.	–	–	–	2	5
Methylvinyloxyethyl sulfide	3.25 (2.48 - 4.26)	> 8	–	3,200	8	4/6	2	2
Tetrahydrothiophene-1,1-dioxide (sulfolane)	1.54 (1.25 - 1.91)	3.18 (2.35 - 4.29)	8 hr.	–	–	–	2	4
2,2'-Thiodiethanol diacetate	9.25 (7.51 -12.9)	>16	8 hr.	–	–	–	3	1
Trichloromethyl allyl perthioxanthate	2.18 (1.39 - 3.42)*	0.6	–	–	–	–	6	1
Trichloromethyl methyl perthioxanthate	1.54 (0.89 - 2.68)*	1.3	–	–	–	–	6	2
Silicon Compounds								
Amyl trimethoxy silane	4.92 (3.75 - 6.46)	10.00 (4.56 -21.9)	4 hr.	–	–	–	4	1
2(3,4-Epoxycyclohexyl)ethyl trimethoxy silane	12.3 (9.98 -15.3)	6.30 (4.66 - 8.52)	8 hr.	–	–	–	3	1
3(2,3-Epoxypropoxy)propyl trimethoxy silane	22.6	3.97 (2.93 - 5.37)	8 hr.	–	–	–	3	2
2-Ethylbutyl silicate	19.7 (15.0 -25.8)	>10	8 hr.	–	–	–	3	1
2-Mercaptoethyl trimethoxy silane	2.46 (1.88 - 3.23)	–	2 hr.	–	–	–	3	2
3-Mercaptopropyl trimethoxy silane	2.83 (1.61 - 4.98)	5.66	–	–	–	–	3	2
Methyl trimethoxy silane	12.5 (9.98 -15.3)	>10	1 hr.	–	–	–	3	2
Trimethoxy silane	9.33 (6.69 -13.0)	6.30 (4.66 - 8.52)	15 m.	62.5	4	5/6	3	5
Vinyl trimethoxy silane	11.3	3.54 (2.03 - 6.16)	2 hr.	4,000	4	1/6	2	1

* as gm/kg in a suitable vehicle

§ inhalation time shown killed all six rats

† twice concentration shown killed all six rats

‡ caused death when introduced into the rabbit eye

TABLE II
Acute Oral Toxicity of Some Inorganic Compounds for Rats

Material*		Formula	Single Oral LD_{50} for Rats gm/Kg	Concentration Intubated gm/ml**	
Aluminum isopropylate	T	$Al[OCH(CH_3)CH_3]_3$	11.3 (7.0 -18.3)	0.200	A
Aluminum nitrate		$Al(NO_3)_3 . 9H_2O$	4.28 (3.86 - 4.76)	0.100	
Ammonium metavanadate	T	NH_4VO_3	0.16 (0.12 - 0.21)	0.0025	B
Ammonium paratungstate	T	$(NH_4O)_6W_7O_{15} . 6H_2O$	11.3	0.400	A
Ammonium persulfate		$(NH_4)_2S_2O_8$	0.82 (0.53 - 1.26)	0.200	
Borax		$Na_2B_4O_7 . 10H_2O$	5.66	0.010	
Boric acid		H_3BO_3	5.14 (4.75 - 5.58)	0.200	
Calcium acetate		$Ca(OOCCH_3)_2 . H_2O$	4.28 (3.86 - 4.76)	0.100	
Calcium acrylate	T	$Ca(OOCCH{=}CH_2)_2 . H_2O$	4.92 (3.75 - 6.46)	0.200	A
Calcium cyanide		$Ca(CN)_2$	0.039(0.030- 0.051)	0.001	
Calcium hydroxide		$Ca(OH)_2$	7.34 (4.83 -11.14)	0.100	
Calcium propionate	T	$Ca(OOCCH_2CH_3)_2 . H_2O$	5.16 (4.17 - 6.38)	0.100	
Chromium acetate	T	$Cr(OOCCH_3)_2 . H_2O$	11.26	0.200	
Chromium chloride (ous)	T	$CrCl_2$	1.87 (1.21 - 2.87)	0.050	
Chromium nitrate (ic)		$Cr(NO_3)_3 . 9H_2O$	3.25 (2.11 - 4.99)	0.200	
Cobalt oxide	T	CoO	1.70 (1.07 - 2.82)	0.100	A
Copper acetate		$Cu(OOCCH_3)_2 . H_2O$	0.71 (0.60 - 0.83)	0.010	
Copper nitrate		$Cu(NO_3)_2 . 3H_2O$	0.94 (0.61 - 1.43)	0.200	
Copper sulfate		$CuSO_4 . 5H_2O$	0.96 (0.71 - 1.30)	0.050	
Cuprous oxide	T	Cu_2O	0.47 (0.34 - 0.66)	0.100	A
Diammonium decaborane	T	$(NH_4)_2B_{10}H_{10}$	3.56 (1.85 - 6.86)	0.050	
Dicumene chromium	T	$(C_9H_{12})_2Cr$	0.81 (0.62 - 1.07)	0.100	M
Ferric nitrate		$Fe(NO_3)_3 . 9H_2O$	3.25 (2.56 - 4.26)	0.200	
Ferrous ammonium sulfate		$(NH_4)_2SO_4 . FeSO_4 . 6H_2O$	3.25 (2.48 - 4.26)	0.300	
Lithium carbonate		Li_2CO_3	0.71	0.200	
Magnesium chloride		$MgCl_2 . 6H_2O$	8.1 (7.3 - 9.1)	0.100	
Manganous acetate		$Mn(OOCCH_3)_2 . 4H_2O$	3.73 (2.68 - 5.21)	0.200	
Nickel nitrate		$Ni(NO_3)_2 . 6H_2O$	1.62 (1.06 - 2.50)	0.200	
Potassium acetate		$KOOCCH_3$	3.25 (2.48 - 4.26)	0.100	
Potassium carbonate		K_2CO_3	1.87 (1.34 - 2.60)	0.200	
Potassium hydroxide		KOH	1.23 (0.80 - 1.89)	0.100	
Potassium permanganate		$KMnO_4$	1.09 (0.70 - 1.71)	0.020	
Silver oxide	T	Ag_2O	2.82	0.200	A
Sodium arsenite		$NaAsO_2$	0.041(0.031- 0.053)	0.010	
Sodium cyanide		$NaCN$	0.015(0.011- 0.021)	0.0004	
Sodium fluoride		NaF	0.18 (0.12 - 0.26)	0.005	
Sodium nitrite		$NaNO_2$	0.18	0.010	
Sodium phosphate, diabasic		$Na_2HPO_4 . 7H_2O$	12.93 (9.85 -16.97)	0.200	
Sodium phosphate, tribasic		$Na_3PO_4 . 12H_2O$	7.40 (6.81 - 8.03)	0.200	
Sodium tripolyphosphate	T	$Na_5P_3O_{10}$	6.50 (4.03 -10.48)	0.200	
Sulfuric acid		H_2SO_4	2.14 (1.54 - 2.99)	0.250	
Vanadium dichloride	T	VCl_2	0.54 (0.38 - 0.75)	0.010	A
Vanadium oxytrichloride	T	$VOCl_3$	0.14 (0.09 - 0.22)	0.050	M
Vanadium tetrachloride	T	VCl_4	0.16	0.090	M
Vanadium trichloride	T	VCl_3	0.35 (0.22 - 0.57)	0.100	
Zinc acetate		$Zn(OOCCH_3)_2 . 2H_2O$	2.46 (1.60 - 3.78)	0.200	
Zinc naphthenate	T		4.92 (3.75 - 6.46)	0.200	L

* T Preceding formula designates technical grade, or development sample, otherwise reagent grade

** Intubation concentrations are in water except as noted by following symbols:

M In mineral oil

L In lard

A Suspended in 0.25% agar

B Suspended in 5% bentonite

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2148

Cellular Injury and Carcinogenesis

THE EFFECT OF A PROTEIN-FREE HIGH-CARBOHYDRATE DIET ON THE METABOLISM OF DIMETHYLNITROSAMINE IN THE RAT

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1. Rats fed on a protein-free high-carbohydrate diet for 7 days metabolized dimethylnitrosamine at only 55% the rate of rats fed on a commercial diet. 2. Dimethylnitrosamine was metabolized by liver slices from rats fed on the protein-free diet at less than half the rate attained by slices from rats fed on a commercial diet. But kidney slices from these rats metabolized dimethylnitrosamine at the same rate as kidney slices from rats on a commercial diet. 3. Methylation by dimethylnitrosamine (70mg/kg body wt.) of N-7 of guanine of the liver RNA and DNA of rats fed on a protein-free diet was only slightly higher than in rats fed on a normal diet given 27mg/kg body wt. In contrast, the methylation by dimethylnitrosamine of guanine in kidney nucleic acids of these rats was three times that in the rats fed on a normal diet. 4. In rats fed on a protein-free diet the incidence of kidney tumours produced by a single dose of dimethylnitrosamine is increased.

Rats fed on a protein-free diet for a short period became resistant to the lethal effects of dimethylnitrosamine. The median lethal dose is 45mg/kg body wt. in rats fed on a commercial pelleted diet (M.R.C. 41B) and 79mg/kg for rats fed on the protein-free diet (McLean & Witschi, 1966). During the feeding of a protein-free diet the activity of the enzyme system that metabolizes drugs, such as Pyramidon (aminopyrine), and foreign compounds, such as benzo[*a*]pyrene, falls to a low value in 4 days (McLean & McLean, 1966). In the present paper we report that a protein-free diet lowers the activity of the enzyme system metabolizing dimethylnitrosamine in the liver, but does not affect this enzyme system in the kidney. Because the rat is unable to excrete more than a small part of a dose of dimethylnitrosamine (Heath, 1962) the decrease in the amount metabolized in the liver leaves more to be metabolized in the kidney. A single dose of dimethylnitrosamine produces tumours of the kidney in all of the rats fed on this protein-free diet. This adds to the circumstantial evidence that the carcinogenic activity of dimethylnitrosamine is caused by some product of nitrosamine metabolism.

A preliminary report of this work has been published (Swann & McLean, 1968).

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MATERIALS AND METHODS

Animals. Porton-Wistar-derived albino rats from the Medical Research Council, Carshalton, Surrey, U.K., or Sprague–Dawley rats from the National Institutes of Health, Bethesda, Md., U.S.A., were maintained in cages with wire-grid bottoms and so denied access to sawdust and their own faeces as far as possible.

Diets. Rats were reared on an unlimited diet of M.R.C. 41B (Bruce & Parkes, 1956) or Wayne Lab-Blox (Allied Mills, Chicago, Ill., U.S.A.). The protein-free diet used was that described by McLean & McLean (1966). The bulk of this diet is carbohydrate (65% corn starch; 30% sucrose; 5% olive oil).

Rate of metabolism of dimethylnitrosamine by rats fed on diet 41B and a protein-free diet. The control rats for this experiment were fed on M.R.C. diet 41B up to and during the experiment. Twenty-five male rats (mean weight 180g) were given dimethylnitrosamine in 0.9% NaCl (50mg/kg body wt.) by intraperitoneal injection. The rats to be fed on the protein-free diet were reared on M.R.C. diet 41B until 8 days before the experiment. They were then transferred to the protein-free diet until and during the experiment. Twenty-five male rats, weighing an average of 170g at the start of the feeding period and an average of 140g on the day of injection, were each given dimethylnitrosamine in 0.9% NaCl (50mg/kg body wt.) by intraperitoneal injection.

Rats were anaesthetized in groups by intraperitoneal injection of sodium pentobarbitone (veterinary Nembutal; Abbott, Queenborough, Kent, U.K.) at various times after the injection and blood (3ml) was taken from the aorta. Dimethylnitrosamine concentration in the

blood samples was measured polarographically by using the variation of the method of Heath & Jarvis (1955) described by Heath (1962).

Rate of metabolism of dimethylnitrosamine by liver and kidney slices from rats on a normal diet and rats on a protein-free diet. Female rats weighing 170g, which had been reared on M.R.C. diet 41B, were fed on either M.R.C. diet 41B or a protein-free diet for 7 days before the experiment. At each occasion that the slice preparations were made, one rat fed on a normal diet and one rat fed on a protein-free diet were killed by a blow on the head. The livers and kidneys were placed in ice-cold Krebs-Ringer phosphate buffer (Umbreit, Burris & Stauffer, 1964) with the addition of glucose (2mg/ml). Slices (approx. 0.008in thick) from liver and kidney were cut, blotted and weighed. The slices (150–250mg total wt.) were equilibrated with O_2 in Warburg flasks each containing 2.8ml of the Krebs-Ringer buffer. Three separate samples were taken from each tissue. [^{14}C]Dimethylnitrosamine (40μg, 0.010μCi in 0.2ml of solution) was added from the side arm and the $^{14}CO_2$ produced taken up by NaOH (0.2ml; 10%, w/v) in the centre well. The slices were incubated for 2h. The NaOH was then removed from the well, the [^{14}C]carbonate precipitated as barium carbonate, and the radioactivity determined (Heath & Dutton, 1958; Swann, 1968).

Subsequently, Sprague–Dawley rats, maintained on Wayne Lab-Blox but otherwise treated exactly as described above, were used in an experiment analogous to that described above. Slices (about 500mg total wt.) cut from the livers of rats fed on the protein-free diet or Wayne Lab-Blox were incubated for 2h in flasks containing dimethylnitrosamine (150μg) in Krebs–Ringer phosphate buffer (5ml) with O_2 as the gaseous phase. The metabolism was stopped by addition of sulphosalicylic acid (5ml; 10%, w/v) and the amount of dimethylnitrosamine remaining determined by the method of Heath & Jarvis (1955).

Methylation in vivo *of guanine in liver and kidney RNA and DNA after a single dose of* [^{14}C]*dimethylnitrosamine was given to rats previously fed on a protein-free diet.* Seven male rats weighing 150–160g were fed on the protein-free diet for 7 days before the experiment. On the day of injection the mean weight of the rats was 125g. Each rat was given 70mg of dimethylnitrosamine/kg body wt. Water but no food was given to the rats between the injection of dimethylnitrosamine and their death. At 19h after the injection of dimethylnitrosamine the rats were anaesthetized with sodium pentobarbitone and blood was taken from the aorta. The dimethylnitrosamine concentration in the blood was measured by polarography (Heath, 1962). The liver and kidneys of the rats were removed and the amount of 7-methylguanine in RNA and DNA was measured (Swann & Magee, 1968).

RESULTS

The effect of feeding a protein-free diet on the rate of metabolism of dimethylnitrosamine (50mg/kg body wt.) was assessed by measuring the rate of disappearance of dimethylnitrosamine from the blood of the animals by using the method of Heath (1962). The rate of metabolism in the period in which the concentration fell to 20% of the initial value was calculated by linear regression analysis from the results shown in Fig. 1 (Snedecor, 1946). The results in terms of change of concentration were expressed in terms of mg metabolized/h per kg body wt. by extrapolating the regression line to find the concentration at zero time (68.7μg/ml for rats on normal diet, 67.0μg/ml for rats on a protein-free diet) and assuming that this corresponded to 50mg/kg body wt. The rate of metabolism in male rats fed on diet 41B was 4.9mg/h per kg and that for rats on the protein-free diet 2.7mg/h per kg, which may be compared with the results of Heath (1962), who found that female rats from the same colony fed on M.R.C. 41B diet metabolized 5.7mg/h per kg body wt.

The metabolism of dimethylnitrosamine takes place predominantly in the liver, and to a lesser extent in other tissues such as kidney and probably lung (Magee & Vandekar, 1958). To discover if the decrease in the rate of metabolism of dimethylnitrosamine in the living rat was the result of a change in the rate at which the liver metabolized dimethylnitrosamine, the rate of production of $^{14}CO_2$ from [^{14}C]dimethylnitrosamine by liver

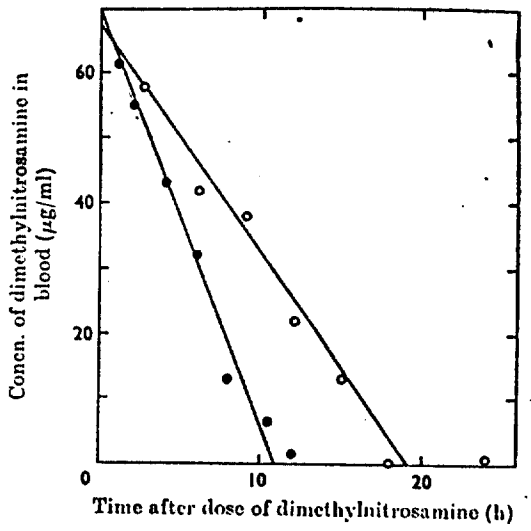

Fig. 1. Metabolism of dimethylnitrosamine in rats fed on normal and protein-free diets. The concentration of dimethylnitrosamine remaining in the blood of male rats fed on a normal (M.R.C. 41B) (●) or protein-free (○) diet at various times after a dose of dimethylnitrosamine (50mg/kg body wt.) was measured by polarography. The results at 1, 2, 8, 10½ and 12h for rats on a normal diet are each the mean of determinations on four rats; that for 4h on three rats; and that for 6h on two rats. Except for the result at 6h, which is the mean of determinations on four rats, each result for the rats on a protein-free diet is the mean of determinations on five rats.

slices *in vitro* was measured. The results (Table 1) show that liver slices from rats fed on a protein-free diet for 7 days before the experiment produced $^{14}CO_2$ from [^{14}C]dimethylnitrosamine at less than half the rate of slices from rats fed on M.R.C. diet 41B. By contrast, the slices of the kidneys of rats fed on a protein-free diet produced $^{14}CO_2$ from [^{14}C]dimethylnitrosamine at a rate not significantly different from that of kidney slices from rats fed on a commercial diet. Slices of liver from the rats fed on a protein-free diet respired much less than slices from the rats fed on M.R.C. diet 41B. This might suggest that the rate of metabolism of dimethylnitrosamine in these slices is not impaired but because of the change in respiratory rate the production of $^{14}CO_2$ does not accurately reflect the rate of metabolism. For this reason the experiment was repeated but this time the rate of disappearance of dimethylnitrosamine from the incubation medium and the slices was directly measured. Because of a change in laboratory, rats of a different breed, Sprague–Dawley instead of Porton–Wistar, were used, and Wayne Lab-Blox had to be used in place of M.R.C. diet 41B. Otherwise the experiment was essentially the same except that the disappearance of dimethylnitrosamine from the slices and medium during a 2h incubation with the slices was measured polarographically (Heath & Jarvis, 1955). In this experiment also liver slices from rats fed on a protein-free diet metabolized dimethylnitrosamine at only 55% of the rate obtained with slices from rats on a normal diet (Table 1).

This result would suggest that the effect of a protein-free diet on the rate of metabolism of dimethylnitrosamine in the living rat is the result of a change in the rate at which the liver can metabolize this compound, but that the rate at which the kidney metabolizes dimethylnitrosamine is unaltered. It is possible to make an estimate of the amount of metabolism of dimethylnitrosamine taking place in each tissue *in vivo* and to show that this difference between liver and kidney is not an artifact of the system *in vitro*. During the metabolism of dimethylnitrosamine a methylating agent is produced, which reacts with susceptible sites in the components of the organ in which the metabolism takes place. There is no evidence for migration of this active intermediate from the tissue in which it is produced to other tissues, and the amount of methylation of susceptible sites seems to parallel the rate of metabolism of the dimethylnitrosamine measured by other means. The amount of methylation at N-7 of guanine in rat liver and kidney nucleic acids produced by a dose of 70mg of dimethylnitrosamine/kg body wt. in rats that had been fed on a protein-free diet for 7 days previously was compared (Table 2) with the amount of methylation at N-7 of guanine produced in the nucleic acids of liver and kidneys of rats maintained on a normal diet (M.R.C. 41B) and given 27mg of dimethylnitrosamine/kg body wt. (Swann & Magee, 1968). The time-interval between the injection of the dimethylnitrosamine and the death of the animals is different in each case. Each group was allowed to live long enough to metabolize most, but not all, of the nitrosamine. Thus, the rats on the normal diet given 27mg of dimethylnitrosamine/kg body wt. were killed 5h after the dose. Those on the protein-free diet given 70mg of dimethylnitrosamine/kg body wt. were killed 19h after the dose. The mean proportion of dimethylnitrosamine not metabolized at this time was

Table 1. *Metabolism of dimethylnitrosamine by liver and kidney slices taken from rats fed on a normal or protein-free diet*

Slices were cut from the liver and kidneys of rats fed on either a normal or protein-free diet. For the measurement of respiration and [^{14}C]dimethylnitrosamine metabolism Porton–Wistar rats were used and in these experiments the normal diet was M.R.C. 41B. In the experiment in which dimethylnitrosamine metabolism was measured Sprague–Dawley rats were used and the normal diet was Wayne Lab-Blox. Experimental details and numbers of animals are given in the text. Results are given as means ±S.E.M. Estimates of statistical significance were by Student's '*t*' test (Snedecor, 1946): $*P = 0.001$; $\dagger P = 0.1$; $\ddagger P = 0.025$.

	Normal diet		Protein-free diet	
	Liver	Kidney	Liver	Kidney
Respiration (μl of O_2/h per g wet wt.)	756± 39	2628±60	322± 35	2585±123
(% of normal)			(50%)*	(98%)
[^{14}C]Dimethylnitrosamine metabolism (d.p.m. of CO_2/h per g of tissue)	3575±265	550±55	1347±161	423± 50
(% of normal)			(38%)*	(77%)†
Dimethylnitrosamine metabolism (μg metabolized/2h per g of tissue)	106± 15		58± 5.0	
(% of normal)			(55%)‡	

Table 2. *Methylation of N-7 of guanine in the nucleic acids of the living rat fed on a normal diet (M.R.C. 41B) or a protein-free diet*

Results for the protein-free diet are from the pooled livers and pooled kidneys of seven rats killed 19h after the dose of 70mg of dimethylnitrosamine/kg body wt. Results for the normal diet, taken from Swann & Magee (1968), are for the pooled livers and pooled kidneys of six rats killed 5h after a dose of 27mg of dimethylnitrosamine/kg body wt. The methods for preparation of nucleic acids and the determination of 7-methylguanine are given in the Materials and Methods section.

	Proportion of guanine residues methylated (% of total guanine)	
	Normal diet (M.R.C. 41B)	Protein-free diet
Liver RNA	1.16	1.4
Liver DNA	0.87	1.19
Kidney RNA	0.16	0.48
Kidney DNA	0.106	0.36

estimated by polarography to be approx. 6% of the dose, by use of the method of Heath (1962). Despite the much higher dose given to the rats on the protein-free diet methylation at N-7 of guanine in rat liver DNA and RNA was approximately the same as in the normally fed animals. In contrast, methylation of kidney nucleic acids was more than three times as great in the rats fed on a protein-free diet as in those fed on a normal diet.

DISCUSSION

The work reported above shows that the feeding f a protein-free diet, even for the short period of 7 days, decreases the rate of dimethylnitrosamine metabolism by the living rat to half the rate achieved by rats fed on a commercial diet (M.R.C. 41B). Experiments with liver and kidney slices *in vitro* show that the rate of metabolism of dimethylnitrosamine by liver slices from the Porton-Wistar or Sprague–Dawley rats fed on a protein-free diet was half that of liver slices obtained from rats on a commercial diet (M.R.C. 41B or Wayne Lab-Blox). The rate of metabolism of dimethylnitrosamine by kidney slices from Porton-Wistar rats fed on a protein-free diet was not significantly different from that of kidney slices from rats fed on a normal diet (M.R.C. 41B). This difference between the effect on liver and kidney found *in vitro* is probably a true representation of the changes occurring in the living rat, for it corresponds to the changes in the methylation of guanine found in these tissues. Although the dose of dimethylnitrosamine given to the rat fed on a protein-free diet is more than twice that given to the rat on the normal diet, the amount of methylation of rat liver DNA and RNA is scarcely increased. In contrast, the methylation of the guanine in the kidney nucleic acids of the rats given a protein-free diet before administration of 70mg of dimethylnitrosamine/kg is more than three times as great as the methylation achieved in the rats fed on a commercial diet and given 27mg of dimethylnitrosamine/kg.

The results of Heath (1962) show that the rate of dimethylnitrosamine metabolism is not dependent on the concentration of dimethylnitrosamine in the range of concentrations used in this experiment. Therefore, as the kidney of the rat fed on a protein-free diet is exposed to dimethylnitrosamine for nearly four times as long (19h instead of 5h) and metabolizes it at 77% the rate of the kidney of the rat on a commercial diet, it would be expected that if the result *in vitro* is correct the amount of methylation found in the kidney nucleic acids of the rat fed a protein-free diet should be $0.77 \times 19/5 = 2.93$ times as great as the amount of methylation of kidney nucleic acids found in the rat fed on a normal diet, i.e. the percentage methylation at N-7 of guanine in kidney DNA in the rat fed on a protein-free diet might be 0.31 and that in RNA 0.47. In the experiment *in vivo* the results were DNA 0.36, RNA 0.48. The experiments *in vitro* showed that liver slices from rats fed on a protein-free diet metabolized dimethylnitrosamine at only 38% of the rate in liver slices from rats fed on a normal diet. If this were a true representation of the change in the activity of the liver in the living rat, one would expect the methylation of guanine by dimethylnitrosamine in the liver nucleic acids of the rat fed on a protein-deficient diet to be $0.38 \times 19/5 = 1.44$ times as great as in the liver of the rat fed a normal diet, i.e. the percentage methylation at N-7 of guanine in liver RNA would be 1.67 and of DNA 1.25. The amount found in the experiment *in vivo* (RNA 1.4, DNA 1.19) was slightly less than predicted. The discrepancy is greater for the RNA than for the DNA, which would be compatible with the results of Craddock & Magee (1963) that 7-methylguanine is lost more rapidly from RNA than

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DNA. Craddock & Magee (1963) also showed that the loss of 7-methylguanine from the kidney was proportionately very much less than from the liver, which would account for the better correspondence between the predicted and actual results for the methylation of N-7 of guanine in the kidney than in the liver.

This short period of feeding on a protein-free diet increases the median lethal dose 1.7-fold and probably decreases the damage to the liver (McLean & Verschuuren, 1969). The toxicity of dimethylnitrosamine is the result of metabolism of the compound (Heath, 1962), but dimethylformamide, a competitive inhibitor of dimethylnitrosamine metabolism, although considerably more effective than the protein-free diet in decreasing the rate of metabolism of dimethylnitrosamine, had less effect in decreasing the toxicity of dimethylnitrosamine (Heath, 1962). The explanation for this is probably that the protein-free diet does not affect the metabolism of dimethylnitrosamine in all organs to an equal degree: the metabolism in the liver, which is the organ most badly damaged normally, is selectively inhibited. The metabolism in the kidneys, which normally show no overt damage, is not affected and they metabolize a greater proportion of the dose. A single dose of dimethylnitrosamine (60 mg/kg body wt.) produces kidney tumours in every rat on a protein-free diet (Swann & McLean, 1968; Hard & Butler, 1970*a*; McLean & Magee, 1970) but in only 35% of rats on a commercial M.R.C. 41B diet (Hard & Butler, 1970*a*). Studies of the relationship between chemical structure and carcinogenic activity have suggested that the carcinogenic activity of nitroso compounds is the result of the production of an active intermediate in their breakdown (Druckrey, Preussmann, Ivancovic & Schmähl, 1967). The results reported here are compatible with that view and it is probable that the increased incidence of tumours in the rats on a protein-free diet is the result of the relative increase of the amount of dimethylnitrosamine metabolized in the kidney.

The change in the activity of the enzyme system metabolizing dimethylnitrosamine in the livers of rats fed a protein-free diet is probably the result of lack of protein, rather than the high content of carbohydrate in the diet. The effect of a protein-free diet on the toxicity of carbon tetrachloride (McLean & McLean, 1966), which seems analogous to the effect on dimethylnitrosamine toxicity, is reversed by addition of only 10% casein to the diet, and thus protein would appear to play a more positive role than carbohydrate. Venkatesan, Arcos & Argus (1970) showed that the metabolism of dimethylnitrosamine *in vitro* by rat liver microsomal fraction is inhibited by feeding only glucose for 24h, but is increased by feeding cellulose for 24h or by 24h starvation. Feeding pure casein for 24h produced an even greater stimulation of activity.

In the experiments reported in the present paper the high content of carbohydrate in the diet suppresses the breakdown and utilization of protein from muscle, but starving the rat, either by feeding cellulose or by withholding food altogether, would lead to a mobilization of the animal's own protein and causes a high protein influx to the liver. Venkatesan *et al.* (1970) believe that their results show a direct inhibition of dimethylnitrosamine metabolism by carbohydrate, but the stimulation by starvation and the greater stimulation by a pure casein diet show that the inhibition of dimethylnitrosamine metabolism by a pure glucose diet could be caused by lack of protein rather than presence of carbohydrate.

The experiments reported in the present paper show the intimate relationship between the diet and the carcinogenic and toxic effects of dimethylnitrosamine. In this, they add to the already considerable literature on the effect of diet and enzyme inducers on the carcinogenic activity of chemicals. In addition, this experiment provides a means for the production of kidney tumours, resembling the Wilms tumours found in children, selectively in all the animals treated with a single dose of carcinogen. This procedure has been used in a microscopic study of the development of kidney tumours (Hard & Butler, 1970*a*, *b*) and promises to be a powerful tool in experimental cancer research.

We were very fortunate in having excellent assistance from Mr J. A. E. Jarvis. This work was begun at the Medical Research Council Toxicology Research Unit, Carshalton, Surrey. It was completed with support from the Cancer Research Campaign and while P.F.S. was an Astor Foundation Fellow.

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Nitrosamine-Induced Carcinogenesis

THE ALKYLATION OF N-7 OF GUANINE OF NUCLEIC ACIDS OF THE RAT BY DIETHYLNITROSAMINE, *N*-ETHYL-*N*-NITROSOUREA AND ETHYL METHANESULPHONATE

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1. The extent of ethylation of N-7 of guanine in the nucleic acids of rat tissue *in vivo* by diethylnitrosamine, *N*-ethyl-*N*-nitrosourea and ethyl methanesulphonate was measured. 2. All compounds produced measurable amounts of 7-ethylguanine. 3. A single dose of diethylnitrosamine or *N*-ethyl-*N*-nitrosourea produced tumours of the kidney in the rat. Three doses of ethyl methanesulphonate produced kidney tumours, but a single dose did not. 4. A single dose of diethylnitrosamine produced twice as much ethylation of N-7 of guanine in DNA of kidney as did *N*-ethyl-*N*-nitrosourea. A single dose of both compounds induced kidney tumours, although of a different histological type. 5. A single dose of ethyl methanesulphonate produced ten times as much ethylation of N-7 of guanine in kidney DNA as did *N*-ethyl-*N*-nitrosourea without producing tumours. 6. The relevance of these findings to the hypothesis that alkylation of a cellular component is the mechanism of induction of tumours by nitroso compounds is discussed.

The *N*-nitroso compounds are highly toxic and carcinogenic. The characteristic toxic lesion produced by the nitrosamines is centrilobular necrosis of the liver, but the damage produced by the nitrosamides is more widespread, with the most severe lesions in organs of rapid cellular proliferation such as the bone marrow, the intestinal epithelium and the lymphoid tissue (Magee & Barnes, 1967). This difference in behaviour of the two classes of nitroso compounds is possibly caused by a difference in their chemical properties: the chemically stable nitrosamines damage only organs such as the liver, which have enzyme systems capable of their breakdown, but the unstable nitrosamides damage cells in any organ that they penetrate (for review see Magee & Swann, 1969). The *N*-nitroso compounds include some of the most powerful known carcinogens, several being capable of producing tumours when given as a single dose in spite of their rapid elimination from the body. They are thus valuable agents for the study of mechanisms of the induction of cancer.

Administration of several *N*-nitroso compounds to animals results in different degrees of alkylation of bases in the nucleic acids of different organs, but the role, if any, of this alkylation in the production of tumours is not known. In a previous attempt to elucidate this role the amounts of methylation of the N-7 position of guanine produced in rat tissues *in vivo* by single doses of *N*-methyl-*N*-nitrosourea, dimethylnitrosamine, methyl methanesulphonate and dimethyl sulphate were compared and contrasted with the carcinogenic activity of the same dose of each compound (Swann & Magee, 1968). The experiments reported here extend this comparison to three ethylating agents, diethylnitrosamine, *N*-ethyl-*N*-nitrosourea and ethyl methanesulphonate.

Diethylnitrosamine is an acute hepatotoxin and is carcinogenic in many species of animals (Druckrey, Preussmann, Ivancovic & Schmähl, 1967). Prolonged feeding to rats at a high dosage in the diet produces liver tumours (Schmähl, Preussmann & Hamperl, 1960), but as the dose is decreased oesophageal tumours are produced (Druckrey, Schildbach, Schmähl, Preussmann & Ivancovic, 1963). In contrast, administration of diethylnitrosamine to hamsters commonly results in tumours of the lung and bronchi (Dontenwill & Mohr, 1961; Herrold, 1964). Druckrey, Steinhoff, Preussmann & Ivancovic (1963) showed that a single large dose of diethylnitrosamine produced kidney tumours in two of four rats of the BD strain. This result has been confirmed in a Wistar-derived stock (P. F. Swann & P. N. Magee, unpublished work). All the kidney tumours induced by diethylnitrosamine were derived from epithelial cells, with no tumours of the mesenchymal type. The major interest in the carcinogenic activity of *N*-ethyl-*N*-nitrosourea has centred on the induction of tumours of brain and

nerve in the offspring of rats treated around the fifteenth day of pregnancy (Ivancovic, Druckrey & Preussmann, 1966; Wechsler *et al.* 1969). Single doses administered to young rats produced mesenchymal tumours of the kidney as well as tumours of the nervous system (Druckrey, Schagen & Ivancovic, 1970). Ethyl methanesulphonate given in three large doses increased the incidence of pulmonary adenomas and induced epithelial-cell tumours of the kidney in mice (Alexander & Connell, 1963) and produced mesenchymal cell tumours of the kidney in rats (Swann & Magee, 1969).

Magee & Lee (1963) reported that administration of diethylnitrosamine to rats resulted in ethylation of N-7 of guanine of nucleic acids of the liver, but Krüger, Ballweg & Maier-Borst (1968) failed to find ethylation of N-7 of guanine when *N*-ethyl-*N*-nitrosourea was given to the same species. In the present paper it is shown that *N*-ethyl-*N*-nitrosourea does produce 7-ethylguanine in nucleic acids of rat tissue and the amount of ethylation produced by this and the two other carcinogenic compounds, diethylnitrosamine and ethyl methanesulphonate, are compared. A similar approach to that used in studies on methylating agents was adopted (Swann & Magee, 1968). The amount of alkylation was measured in rats given each compound in a dose similar to that used in the tests for carcinogenic activity, at a time when the alkylation was expected to have reached its highest value from knowledge of the rate of metabolism of the compound. The rate of metabolism of ethyl methanesulphonate (Roberts & Warwick, 1958) and of diethylnitrosamine (Heath, 1962) were already known. The rate of decomposition of ethyl methanesulphonate in the rat was approximately exponential, the half-life of a dose of 300mg/kg body wt. being about 6.5h. The rate of metabolism of diethylnitrosamine was linear through the greater part of its course, 4.3% of a dose of 204mg/kg body wt. being metabolized each hour.

In the work described in the present paper, the rate of decomposition of *N*-ethyl-*N*-nitrosourea *in vivo* was measured; *N*-[^{14}C]ethyl-*N*-nitrosourea, [^{14}C]diethylnitrosamine and [^{14}C]ethyl methanesulphonate were prepared; and the amount of ethylation of *N*-7 of guanine in nucleic acids of tissues of the rat that each produced *in vivo* was measured and compared with the carcinogenic activity of each compound. A preliminary report of this work has been given (Swann & Magee, 1970).

METHODS

Animals. Male or female Wistar-derived albino rats from the Courtauld Institute stock were maintained in cages with floors covered in wood-chips or sawdust. The rats were fed on Rowett Research Institute diet M. Syrian golden hamsters were obtained from the Laboratory Animals Centre, Carshalton, Surrey, U.K.

Chemicals. *N*-Ethyl-*N*-nitrosourea was obtained from Fluka A. G., Buchs, Switzerland. Ethyl methanesulphonate and diethylnitrosamine were obtained from Eastman-Kodak, Rochester, N.Y., U.S.A. Both were redistilled before use.

Radioactive chemicals. *N*-[2-^{14}C]Ethyl-*N*-nitrosourea (Sp. radioactivity 0.325mCi/mmol) was synthesized from [2-^{14}C]acetonitrile by reduction of the nitrile to [^{14}C]ethylamine with the complex of lithium aluminium hydride and aluminium chloride (Nystrom, 1955). *N*-[^{14}C]Ethylurea was formed by reaction of the [2-^{14}C]ethylamine and sodium isocyanate and nitrosated with nitrous acid (Cox & Warne, 1951; Jones & Skraba, 1953). The *N*-[2-^{14}C]ethyl-*N*-nitrosourea was extracted from the nitrosation mixture with diethyl ether and recovered by evaporation of the ether (yield by polarographic analysis, 34% of theoretical). [1-^{14}C]Ethyl methanesulphonate (sp. radioactivity 0.412mCi/mmol) was prepared by reaction of [1-^{14}C]ethyl iodide and silver methanesulphonate *in vacuo* without the use of solvent (Swann & Magee, 1968). [1-^{14}C]Diethylnitrosamine was prepared from [1-^{14}C]ethyl iodide by Dr D. F. Heath (Dutton & Heath, 1956) or from sodium [1-^{14}C]acetate through intermediate production of ethyl acetamide and diethylamine by Dr A. R. Mattocks (A. R. Mattocks, unpublished work). All radioactive precursors were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Measurement of radioactivity. All radioactivity measurements were made in a scintillation counter (Packard Instrument Co., LaGrange, Ill., U.S.A.) by conventional methods. Corrections from c.p.m. to d.p.m. were normally made by addition of standard [^{14}C]toluene (Packard Instrument Co.) but where the solutions were highly radioactive the method of pulse-height shift was used (Baillie, 1960). Two scintillation solutions were used: 0.6% 2,5-diphenyloxazole in toluene for samples that did not contain water, and 1% 2,5-diphenyloxazole, 0.05% 1,4-bis-(4-methyl-5-phenyloxazole-2-yl)benzene and 9% naphthalene dissolved in dioxan–cellosolve–toluene (3:3:1, by vol.) for aqueous samples (Bruno & Christian, 1961).

Conduct of animal experiments. (*a*) General procedure. The animals were allowed access to water but were not given food for 20h before being killed by bleeding from the abdominal aorta after anaesthesia with sodium pentobarbital (Vetinary Nembutal, Abbott, Queenborough, Kent, U.K.). The organs were removed and frozen in liquid N_2. In the experiment in which the rate of decomposition of *N*-ethyl-*N*-nitrosourea was measured the rats (150g) were allowed free access to food until immediately before the experiment. *N*-Ethyl-*N*-nitrosourea was dissolved in 0.01M-sodium citrate buffer, pH6, containing 8.5g of NaCl/l to give a final solution of 20mg/ml. This solution (1.5ml) was injected into the tail vein of each rat. After various time-intervals the rats were bled from the abdominal aorta and the amount of *N*-ethyl-*N*-nitrosourea in the blood was determined by polarography (Heath & Jarvis, 1955; Swann, 1968). Rats treated for a short period of time remained under sodium pentobarbital anaesthesia throughout, but those left for longer

periods were injected under light ether anaesthesia and anaesthetized again before being bled.

(*b*) Ethylation by diethylnitrosamine. A solution of [1-^{14}C]diethylnitrosamine in NaCl was prepared (40mg; 250μCi/ml of 0.9% NaCl) and 1.125ml was injected intraperitoneally into each of eight female rats (180g) (i.e. 250mg, 1.56mCi/kg of body wt.). The rats were treated as detailed in the 'general procedure' above and killed 24h later. Nucleic acids were prepared from liver, kidney, lung and small intestine. In other experiments rats were given 200mg (242μCi/kg of body wt.) or Syrian golden hamsters (70g) were given 166mg (330μCi/kg of body wt.).

(*c*) Ethylation by ethyl methanesulphonate. [1-^{14}C]-Ethyl methanesulphonate (283mg; 970μCi) was dissolved in 0.9% NaCl (10.5ml) and 1ml of this solution given by intraperitoneal injection to each of ten female rats (90-110g) (i.e. 270mg; 920μCi/kg of body wt.). These animals were starved for 17h after injection, then killed, and nucleic acids were prepared from the liver, kidney, small intestine, lung and brain.

(*d*) Ethylation by *N*-ethyl-*N*-nitrosourea. *N*-[2-^{14}C]-Ethyl-*N*-nitrosourea (229mg; 330μCi) was dissolved in 0.9% NaCl–0.01M-sodium citrate, pH6 (12ml) and 1.2ml was given to each of eight male rats (150g) that had been starved as in the other experiments (i.e. 150mg; 220μCi/kg of body wt.) by intravenous injection. These were killed 1.5–2h later and nucleic acids were prepared from liver, kidney, small intestine, large intestine, lung and brain.

Preparation of nucleic acids. Both RNA and DNA were prepared from the same sample of frozen tissue by the method of Kidson, Kirby & Ralph (1963) except that phenol–*m*-cresol–8-hydroxyquinoline–water (100:14:0.1:11, by wt.) was used in place of 90% (w/v) phenol. The DNA was freed of RNA by ribonuclease treatment and carbohydrates were removed from both RNA and DNA by the methoxyethanol–phosphate procedure (Kirby, 1956). The nucleic acids were finally precipitated by addition of an equal volume of cetyltrimethylammonium bromide (1%, w/v). The cetyltrimethylammonium salt of the nucleic acid was washed with water and converted into the sodium salt by treatment with 2% (w/v) sodium acetate in 70% (v/v) ethanol. The sodium salt of the nucleic acid was washed in ethanol, ethanol–diethyl ether (1:1, v/v), and diethyl ether and dried *in vacuo*. In one experiment where diethylnitrosamine was given, DNA, microsomal, soluble and nuclear RNA from the liver and kidneys of the rats were prepared by the method of Villa-Treviño, Shull & Farber (1966).

Determination of the proportion of guanine residues ethylated on N-7. The nucleic acids were hydrolysed in 1M-HCl by heating to 100°C for 1h. The hydrolysate was chromatographed on a Dowex 50W (X12; H^+ form) column (10cm × 1cm) by using an exponential gradient of 1–4M-HCl (Magee & Farber, 1962). In all cases a radioactive peak was eluted between guanine and adenine, in the same fractions as an authentic sample of 7-ethylguanine generously given to us by Dr P. D. Lawley. The amount of 7-ethylguanine was calculated from the amount of radioactivity in the peak by assuming that the specific radioactivity was the same as that of the ethyl group of the injected ethylating agent. The amount of guanine was calculated from the extinction of the guanine peak by taking ϵ_{260} in acid to be 8000.

RESULTS

Polarographic determination of the amount of *N*-ethyl-*N*-nitrosourea remaining in the blood of a rat given a dose of 200mg/kg of body wt. by intravenous injection showed that the concentration fell rapidly, with a half-life of about 5–6min (Fig. 1). Thus it appears slightly more stable *in vivo* than the methyl homologue, which seemed to have a half-life of approx. 1min (Swann, 1968).

A peak of radioactivity that eluted from the Dowex 50 column in a similar position to 7-ethylguanine was found in the acid hydrolysate of nucleic acids of tissues of rats given [1-^{14}C]ethyl methanesulphonate, [1-^{14}C]diethylnitrosamine or *N*-[2-^{14}C]ethyl-*N*-nitrosourea (Fig. 2). The amount of 7-ethylguanine is given in Table 1.

The first experiments with [1-^{14}C]diethylnitrosamine were done with a sample synthesized from [1-^{14}C]ethyl iodide by the method of Dutton & Heath (1956). Hamsters were used because it was hoped to discover why the respiratory tract of the hamster is so highly susceptible to the carcinogenic effects of diethylnitrosamine (Dontenwill, Mohr & Zagel, 1962). When an acid hydrolysate of nucleic acid from the livers of these animals was chromatographed a peak of radioactivity that eluted in a position similar to that of 7-methylguanine was found, as well as a peak corresponding to 7-ethylguanine (Fig. 3). By contrast, when a sample

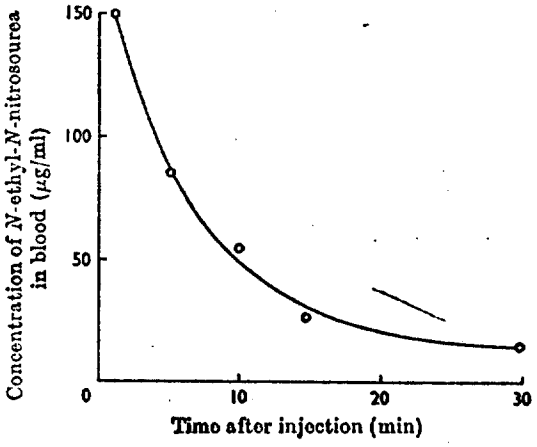

Fig. 1. Rate of disappearance of *N*-ethyl-*N*-nitrosourea from the blood of rats after an intravenous injection. Rats (each 150g) were each given 200mg/kg of body wt. The blood proteins were precipitated with sulphosalicylic acid and the amount of *N*-ethyl-*N*-nitrosourea in the supernatant was determined by polarography.

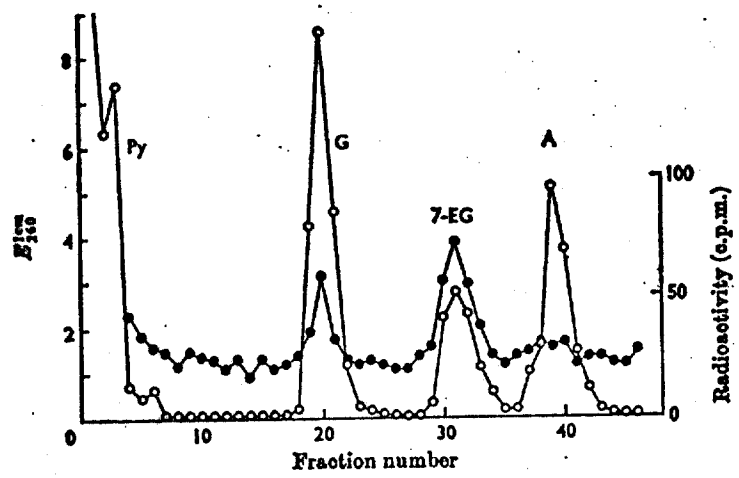

Fig. 2. Ion-exchange chromatography of an acid hydrolysate of lung RNA from rats given *N*-[2-^{14}C]ethyl-*N*-nitrosourea (150mg; 220μCi/kg). The isolated nucleic acid was hydrolysed in 1M-HCl at 100°C for 1h and chromatographed on a column (10cm×1cm) of Dowex 50 (X12; H^+ form) with exponential 1–4M-HCl gradient elution. Fractions (8.6ml) were collected ○, E_{260}; ●, radioactivity. Py, pyrimidine nucleotides; G, guanine; A, adenine; 7-EG, added authentic 7-ethylguanine.

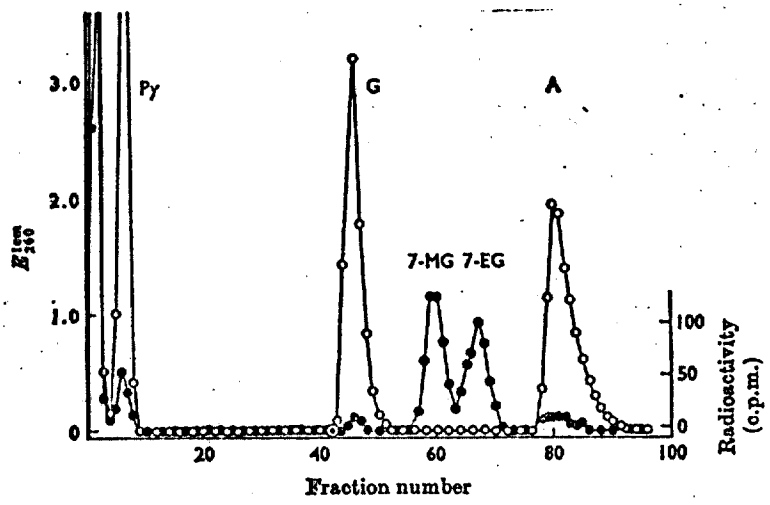

Fig. 3. Ion-exchange chromatography of an acid hydrolysate of liver RNA from hamsters given [1-^{14}C]-diethylnitrosamine synthesized from [1-^{14}C]ethyl iodide. The isolated nucleic acid was hydrolysed in 1M-HCl at 100°C for 1h and chromatographed on Dowex 50 (X12; H^+ form) with exponential 0.1–4M-HCl gradient elution. Fractions (10ml) were collected. ○, E_{260}; ● radioactivity. Py, pyrimidine nucleotides; G, guanine; A, adenine; 7-EG, 7-ethylguanine; 7-MG, 7-methylguanine. The 7-methylguanine co-chromatographed with a known specimen of 7-methylguanine.

of di[1-^{14}C]ethylnitrosamine synthesized from sodium [1-^{14}C]acetate was given to rats no 7-[^{14}C]methylguanine, but only 7-[^{14}C]ethylguanine, was detected in their nucleic acids. This perplexing result was at first thought to be caused by contamination of the [^{14}C]ethyl iodide. No detectable impurities were found in the first sample of diethylnitrosamine and the reason for the appearance of 7-methylguanine is still unknown.

As part of this investigation tests for carcinogenic activity were done with diethylnitrosamine given as a single dose (P. F. Swann & P. N. Magee, un-

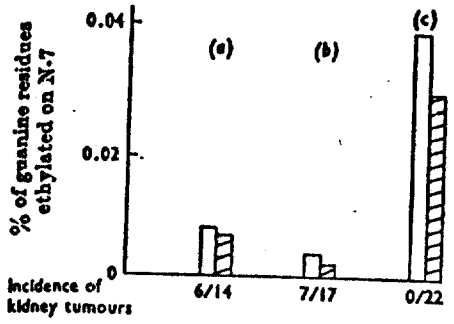

Fig. 4. Comparison between the amount of 7-ethylguanine produced in nucleic acids of rat kidney by diethylnitrosamine (*a*) (280 mg/kg), *N*-ethyl-*N*-nitrosourea (*b*) (150 mg/kg) and ethyl methanesulphonate (*c*) (270 mg/kg) and the carcinogenic activity of a similar dose of each compound. Although a single dose of ethyl methanesulphonate did not produce kidney tumours, three doses did. RNA, ▨; DNA □.

published work) and with ethyl methanesulphonate given as three separate doses (Swann & Magee, 1969). The results of these and previous investigations are given in Table 2.

DISCUSSION

In experiments with methylating agents (Swann & Magee, 1968) a single dose of methyl methanesulphonate produced greater methylation of N-7 of guanine in rat kidney DNA than that by dimethylnitrosamine, and roughly equivalent methylation of N-7 of guanine in rat kidney DNA to that by *N*-methyl-*N*-nitrosourea, but methyl methanesulphonate did not induce kidney tumours whereas both the nitroso compounds did. Methyl methanesulphonate was not entirely without carcinogenic activity, however, because it induced a significant number of tumours of the nervous system (Swann & Magee, 1969). This lack of correlation between the amount of alkylation of N-7 of guanine in nucleic acids by each compound and their carcinogenic activity cast some doubt on the importance of alkylation of N-7 of guanine for the initiation and development of cancer. The experiments reported here add to that doubt. It has been found that each of the compounds studied ethylates N-7 of guanine of nucleic acids *in vivo*. The amount of ethylation produced is less than the amount of methylation that the analogous methylating agent produced in the same organ. In kidney, diethylnitrosamine produces approximately one-tenth the alkylation of N-7 of guanine produced by dimethylnitrosamine; and *N*-ethyl-*N*-nitrosourea one-twentieth that produced by *N*-methyl-*N*-nitrosourea; and ethyl methanesulphonate one-

Table 1. *Ethylation of N-7 of guanine in nucleic acids of the rat by diethylnitrosamine, ethyl methanesulphonate and N-ethyl-N-nitrosourea*

The amount of 7-ethylguanine is expressed as a % of guanine. The method of calculation is given in the Methods section. Microsomal RNA (mic RNA), soluble RNA (S RNA) and nuclear RNA (Nuc RNA) were prepared by the method of Villa-Trevino, Shull, and Farber (1966); other nucleic acids were prepared as outlined in the Methods section.

Compounds	Dose (mg/kg) and route	Time after dose (h)	Content of 7-ethylguanine																	
			Liver				Kidney				Lung		Ileum		Brain	
			DNA	Mic RNA	S RNA	Nuc RNA	DNA	Mic RNA	S RNA	Nuc RNA	DNA	RNA	DNA	RNA	DNA	RNA
Diethylnitrosamine	250 (intraperitoneal)	24	0.034	0.025	0.06	0.015	0.008	0.0065	*	0.0078	0.007	0.0015	None detected		*	*
			0.031													
Ethyl methanesulphonate	200 (intraperitoneal)	24			0.033		0.007		0.0073						0.038	
N-Ethyl-*N*-nitrosourea	270 (intraperitoneal)	17	0.045		0.024		0.041		0.031		0.047	0.028	0.053	0.024	0.003	0.002
	152 (intravenous)	2	0.004		0.002		0.0034		0.002		0.0032	0.0015	*	0.002		

* Indicates that no measurement was made.

Table 2. *Carcinogenic activity in the rat of compounds producing 7-ethylguanine in nucleic acids in vivo*

This is not an exhaustive report on the carcinogenic activity of these compounds but shows those experiments where the dose used was similar to that used in the experiments reported here for determination of the amount of ethylation.

Compound	Dose and route	No. of rats	Tumours found	References
Diethylnitrosamine	280 mg/kg (intravenous)	4	Kidney (2)	Druckrey *et al.* (1964)
	280 mg/kg (intravenous)	14	Kidney* (6)	P. F. Swann & P. N. Magee (unpublished work)
				Alexandrov (1969)
N-Ethyl-*N*-nitrosourea	60–80 mg/kg (intravenous)	9 (pregnant)	Uterus (2), ovaries (1)	Druckrey *et al.* (1970)
	80 mg/kg (oral)	16 (10 days old)	Kidney† (9), nervous system (16)	Druckrey *et al.* (1970)
	80 mg/kg (oral)	17 (30 days old)	Kidney† (7), nervous system (16)	Swann & Magee (1969)
Ethyl methanesulphonate	350 mg/kg (intraperitoneal)	22	Brain (1)	Swann & Magee (1969)
	3 × 275 mg/kg (intraperitoneal)	24	Kidney† (12)	

* Epithelial cell tumours.
† Mesenchymal tumours.

fifth that produced by methyl methanesulphonate. These results imply that if alkylation of N-7 of guanine is responsible for the induction of tumours then ethylation is much more potent than methylation.

The correlation between the amount of ethylation of N-7 of guanine produced by these compounds *in vivo* and their carcinogenic activity can best be examined in the case of kidney, since they all can produce kidney tumours in the rat under suitable conditions. In comparing the incidence of tumours with the amount of ethylation it should be emphasized that the tumours that each compound induced were not of the same histological type. However, the proportion of mesenchymal to epithelial tumours may be influenced by the age (Murphy, Mirand, Johnston, Schmidt & Scott, 1966) or sex of the animals (Jasmin & Cha, 1969). Therefore it is impossible to decide whether the production of epithelial tumours with diethylnitrosamine and mesenchymal tumours with both ethyl methanesulphonate and *N*-ethyl-*N*-nitrosourea represents a real difference in the action of these chemicals, and the significance of the twofold difference between the amount of ethylation of N-7 of guanine by diethylnitrosamine and *N*-ethyl-*N*-nitrosourea (Fig. 4) is difficult to assess. However, a single dose of 270 mg of ethyl methanesulphonate/kg of body wt. produced five times more 7-ethylguanine in rat kidney DNA than the carcinogenic dose of diethylnitrosamine, and ten times more than a carcinogenic dose of *N*-ethyl-*N*-nitrosourea, yet a dose even larger than this (350 mg/kg body wt.) failed to induce kidney tumours. It is not clear why this single dose of ethyl methanesulphonate failed to induce kidney tumours in the rat when three separate injections of a smaller amount did (Swann & Magee, 1969).

The lack of quantitative correlation between the amount of 7-alkylguanine produced in the nucleic acids of the kidneys of rats by both these ethylating agents and by the methylating agents previously studied (Swann & Magee, 1968) might be taken to imply that the alkylation of N-7 of guanine plays no major role in the initiation of cancer by these compounds. This may be so, but it cannot be conclusively proved by evidence such as that reported here, for these measurements give no information about the distribution of alkylation between cell populations in the kidney, and it is possible that the distribution may vary with the various compounds; neither do the measurements describe the distribution of alkyl groups within a nucleic acid molecule. Singer & Fraenkel-Conrat (1969*a*,*b*) have suggested that an affinity by nitroso compounds for parts of the nucleic acid in suitable conformation, an affinity not shared by other alkylating agents, is the basis for the difference in the mutagenic action of the two

classes of compound. Similar considerations may apply to their carcinogenic activity. It is also possible that the different agents may have different effects on metabolic and immunological processes, which determine whether tumours do or do not follow the initial interactions with cellular components. Also, it must be emphasized that these experiments compare only the extent of alkylation of N-7 of guanine by each compound. This is the greatest product of the reaction of methylating agents *in vivo* and possibly also of ethylating agents but reaction at other sites in the nucleic acids such as the 0-6 position of guanine (Loveless, 1969) which may be the cause of mutation in DNA containing bacteriophage or the 3-position of cytosine which appears to cause miscoding *in vitro* (Ludlum, 1970*a*,*b*) may be of greater importance in the induction of cancer.

In these experiments it was decided to study alkylation rather than any other reaction of the nitroso compounds, and to study nucleic acids rather than other components of the cell. The carcinogenic activity of ethyl methanesulphonate, though not a proof, would seem sufficient justification for a belief that alkylation can be a sufficient initiator of the growth of tumours. The importance of alkylation of some cellular component other than nucleic acids cannot be excluded, but there is evidence from viral oncology (see Dulbecco, 1969) and from the work of Cleaver (1969) on xeroderma pigmentosum that nucleic acids do play a central role in carcinogenesis.

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2163

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MALIGNANT TUMORS INDUCED IN MICE FED WITH N-NITROSODIMETHYLAMINE

(Plates LXV~LXVII)

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Synopsis

A systemic experiment to induce malignant tumors in mice with N-nitrosodimethylamine is reported for the first time.

Twenty-seven ddN mice were fed a diet containing N-nitrosodimethylamine in a concentration of 50 p.p.m.. After 5 months of feeding, adenocarcinomas of the lung in 4 cases, multiple adenomas of the lung in 21 cases, an anaplastic carcinoma of the kidney, malignant hemangioendotheliomas of the liver in 2 cases, and a hemangioendothelioma of the spleen were observed.

INTRODUCTION

Barnes and Magee demonstrated that N-nitrosodimethylamine provoked acute liver necrosis in rats[3] and induced tumors in the liver after extended feeding.[3] Later they noticed that tumor can be produced in the lung and kidney also in that way.[13,14] This has been reconfirmed by Schmähl and Preussmann,[16] Zak, Halzner, Singer, and Popper,[20] and by Magee and Barnes.[14]

When the methyl groups were replaced by ethyl groups, the compound, N-nitrosodiethylamine, proved also to be carcinogenic to rat liver.[3,7,17] In Syrian hamsters, N-nitrosodiethylamine has induced squamous carcinoma of the trachea, lung, and ethmoidal cavity as later reported by Dontenwill *et al.*,[4,5] and by Herrold and Dunham.[8] In 1963, Argus and Hoch-Ligeti[1] observed the production of hepatic carcinoma in guinea pigs after oral administration of N-nitrosodiethylamine.

Application of this group of carcinogenic compounds to mice has not been reported except for a brief summary by Schmähl, Thomas, and König,[18] who reported that hemangiosarcoma and benign hepatoma were produced in the liver of DBA mice by feeding N-nitrosodiethylamine.

The present experiment was carried out by feeding an inbred strain of mice with N-nitrosodimethylamine for 5 months, in order to see whether the compound displays organotropic potency in mice or not, and if it has any specific organotropism in this species of animals.

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An inbred strain of mice, ddN, which has been widely in use in Japan for the past 10 years and supplied by the Central Laboratory for Experimental Animals, Tokyo, was selected for the experiment.

Materials and Methods

The experiment was started with 27 animals, 14 females and 13 males, when they were about 8 weeks old and weighed around 20 g on an average.

N-Nitrosodimethylamine (Eastman Organic Chemicals) was dissolved in warm peanut oil and added to the basal diet*[2] (CE-2-CLEA), adjusting the carcinogen content in the diet to 50 p.p.m.

The animals were fed with this diet for 5 months, after which they were kept on the basal diet only until the experiment was discountinued on the 203rd day and all the surviving animals were killed.

The total amount of N-nitrosodimethylamine taken in by a mouse was calculated from the total amount of the carcinogenic diet consumed per cage during 5 months.

When the animals died or were killed, thorough autopsies were performed and their organs were fixed in 4% Formalin solution. Paraffin sections were stained with Hematoxylin-Eosin for the histopathological examination.

Result

Five mice died within 50 experimental days due to pneumonia and without tumor. Twenty-two mice survived over 121 experimental days.

Findings in the Lung All mice, except one which died on the 121st day (#1), developed multiple pulmonary adenomas. Most of the tumors were 2 or 3 mm in diameter and were located in the periphery of the lungs. They presented papillary or tubular arrangements, mostly consisting of columnar or cuboidal cells (Photo 4).

Of those surviving over 166 days, adenocarcinoma was recorded in 4 cases, whose details are as follows:

Case 1 (#12): The animal died on the 166th day due to lung tumor. At autopsy, about 1 ml of hemorrhagic pleural fluid was noticed. The 4th lobe of the right lung was almost entirely occupied by a mass, measuring 6 mm in diameter, and the lower part of the left lung was also replaced by another large tumor nodule. No metastasis was observed. Histologically, cellular atypism, abundance of mitotic figures, irregularity of the cellular arrangement, and destruction of the preformed lung structures were apparent in comparison with the findings on adenomatous nodules (Photos 1 and 5).

*[2] Composition of basal diet: 49.2% of crude carbohydrate, 24% of crude protein, 10% of water, 6% of ash, 4.5% of crude cellulose, 3.5% of crude fat, 1.2% of calcium, 0.8% of phosphorus, 0.5% potassium, and 0.3% of sodium.

Table I. Findings of Tumor and Other Lesions in Mice fed N-Nitrosodimethylamine

Animal No.	Sex	Exptl. days	Body weight (g)	Liver weight (g)	Spleen weight (g)	Kidney weight (g) left	Kidney weight (g) right	Total amt. $(CH_3)_2N\text{-}NO$ (mg)	Histological Diagnosis: Lung	Liver	Spleen	Kidney
1	♀	121	20.0	1.8	0.2	0.3	0.3	14.5				
2	♂	129	24.4	3.2	0.4	0.3	0.3	18.8	Adenoma	A-type lesion*		
3	♂	129	27.0	1.9	0.2	0.3	0.3	18.8	Adenoma	Hemangioendothelioma, malignant		
4	♂	139	25.0	1.2	0.2	0.2	0.2	18.8	Adenoma	A-type lesion		
5	♀	134	24.7	1.5	0.4	0.3	0.3	16.4	Adenoma			
6	♂	140	27.3	2.3	0.5	0.3	0.3	20.0	Adenoma	A-type lesion	Diffuse cellular reticulosis	
7	♂	140	16.1	0.9	0.2	0.2	0.2	20.0	Adenoma	Hemangioendothelioma, malignant		
8	♂	145	28.7	1.3	0.2	0.2	0.2	21.0	Adenoma	B-type lesion*		
9	♀	159	24.5	1.2	0.1	0.3	0.2	20.0	Adenoma			
10	♀	166	29.7	2.2	0.6	0.37	0.35	20.0	Adenoma			
11	♀	166	28.0	1.6	0.45	0.3	0.25	20.0	Adenoma	B-type lesion		
12	♀	166	22.0	1.5	0.4	0.33	0.55	20.0	Adenocarcinoma	A- 〃	Hemangioendothelioma	
13	♂	170	20.0	1.3	0.75	0.15	0.25	23.0	Adenoma	A- 〃	Diffuse cellular reticulosis	
14	♀	174	22.8	1.3	0.25	0.2	0.15	20.0	Adenocarcinoma	B- 〃		
15	♀	181	18.0	2.4	0.45	0.4	0.35	20.0	Adenoma	A- 〃		
16	♀	181	20.0	1.3	0.5	0.35	0.35	20.0	Adenoma	A- 〃		
17	♂	189	15.5	1.1	0.2	0.15	0.2	20.0	Adenoma	B- 〃		
18	♀	190	19.0	1.0	0.25	0.3	0.3	20.0	Adenocarcinoma	A- 〃		Anaplastic carcinoma
19	♂	197	19.0	1.1	0.3	0.25	0.25	23.0	Adenocarcinoma			
20	♂	201	16.0	0.8	0.2	0.15	0.15	23.0	Adenoma			
21	♂	203	21.0	1.3	0.25	0.25	0.25	23.0	Adenoma	B-type lesion		
22	♀	203	21.0	0.9	0.5	0.27	0.27	20.0	Adenoma	A- 〃		

* See the section on Result.

Case 2 (♀14): The animal died on the 174th day. A white nodule measuring 7×6 mm was found in the upper part of the first right lung lobe, partly adhering to the pleura. It was histologically consistent with the adenocarcinoma in Case 1 and was invading the adjacent adenomatous nodules. The other part of the lung possessed multiple adenomas.

Case 3 (♀18): The mouse died on the 190th day due to lung tumors. At autopsy, the first right lung lobe was found occupied by a rather soft, partly hemorrhagic tumor mass measuring 6×8 mm. There was about 0.8 ml of hemorrhagic pleaural fluid. Histologically, the mass was composed mainly of unusually large papillary structures with large basophilic columnar cells and a portion of solid proliferation of the same type of cells. Destruction of the bronchial walls was notable. No metastasis was observed.

Case 4 (♀19): This mouse died on the 197th day. In the first lobe of the right lung, an ovoid tumor mass measuring 6×9 mm was found (Photo 2). Several pleural adhesion existed between the 1st and 2nd lobes. Histologically, this tumor was represented by fine papillary overgrowth not unlike that of benign adenoma. However, the pleura was involved and extensive destruction of the bronchial tree was observed (Photos 3, 7, and 8). No metastasis was found.

Findings in the Liver Two months after the initiation of the experiment, 10 out of 22 mice had hemorrhagic exudation in their peritoneal cavity. The amount of the exudate was more than 10 ml in 1 week.

Most of the mice showed severe but non-tumorous lesions of the liver. In 10 cases, there was extensive coagulative necrosis of the hepatic cells in association with dilatation of the sinusoids and occasionally focal hyperplasia of stellate cells (type-A lesion), while patchy disappearance of the hepatic cells has occured in 5, resulting in diffuse, occasionally localized, cystic dilatation of the sinusoids simulating ill-defined hemangiomatous structures (type-B lession). The sinusoid endothelia were swollen but no mitotic figures were observed.

In two cases, localized proliferation of bizarre-shaped endothelial elements was observed. The lesions appeared not unlike those seen in Kaposi's sarcoma of the human beings and in malignant hemangioendothelioma hitherto described in C3H mouse liver.

One mouse (♀2) had a solitary, blooded, reddish-purple, and somewhat cystic tumor nodule in the 6th liver lobe. The omentum adhered to a part of the hepatic tumor with indication of a small hemorrhage from the surface of the liver. At autopsy, 5 ml of hemorrhagic ascites fluid was obtained. Histologically, the tumor consisted of densely proliferating spindle cells with occasional arrangement forming irregular cleft-like lumina. It was largely consistent with malignant hemangioendothelioma (Phato 9). Another mouse (♀6) died on the 140th day. A whitish, nodular mass meas-

uring 5×5 mm was found in the 2nd lobe. This mouse had about 3 ml of peritoneal exudate at autopsy. Histological finding of this tumor was also consistent with malignant hemangioendothelioma.

Findings in the Spleen In most of the animals, the spleen was within a normal range of weight, grossly appeared rather anemic, and revealed no particular histological abnormalities. However, diffuse cellular reticulosis was observed in 2 mice (♂5 and ♂12).

A tumor, histologically diagnosed as hemangioendothelioma, was found in a limited area of the spleen of ♂11 which expired on the 166th day (Photo 10). It represented a miniature of hepatic lesions described above.

Findings in the Kidney One male mouse which died on the 189th day showed anaplastic carcinoma of the right kidney (Photo 11). This tumor appeared as a firm, hemorrhagic, irregular-shaped mass with no adhesion to adjacent organs. The renal structure had been completely destroyed by the cancer and massive hemorrhage had resulted.

Discussion

The carcinogenic potency of this group of chemicals with rather simple molecular structure has been explored by several groups of investigators. Rats have been most extensively used. It has been well established that N-nitrosodimethylamine can induce tumors of the lung, liver, and kidney in rats, and N-nitrosodiethylamine in the esophagus, trachea, lung, and paranasal sinuses in the same animal species. Due to relative simplicity of the structure the chemical is believed to be extremely suitable for the analysis of carcinogenic mechanism. Magee and his collaborator suggested methylation of nucleic acids as the probable cancer inducing step.[12,13]

In spite of popularity of mice in carcinogenic experiments, almost no authentic report has appeared in the literature in which this species of animals was employed for systematic investigation of this group of carcinogens. It might have been due to high toxicity or to negative result in inducing hepatic malignancy.

The present result revealed that the mice tolerate continuous feeding of N-nitrosodimethylamine and that several organs are susceptible to induction of malignant tumor. The lung was regularly affected and benign adenomas were induced as early as within 120 days. In 18% of the experimental animals, pulmonary tumors with histological malignancy were found, although no distant metastasis was observed and no transplantation study was performed.

It has been well known that certain strains of mice develop pulmonary adenoma spontaneously after the age of 12 months. The strain used in the present experiment was ddN which has been developed first in the Institute of Infectious Diseases, University of Tokyo, about 20 years ago, and for the last 10 years the strain has

been under maintenance and control of several animal laboratories. During the past 4 years, over 2,000 ddN mice were used for long-term experiments with various kinds of chemical carcinogens. Personally we have had no experience of seeing pulmonary tumors either grossly or microscopically among them. In 1962, Tsubura *et al.*[19] reported that incidence of lung tumor was none among his colony of dd_1, while 75% of the same strain developed spontaneous mammary carcinoma. This is rather in sharp contrast with the finding of Mori and Yasuno[15] who reported that, in their colony of dd mice between 15 and 18 months of age, microscopical isolated lung tumors were observed in 15%. Both the substrain dd and ddN have the same common ancestor with dd mice used by the last-named workers. In any case, our personal experience and scantly reported data indicate that in the particular strain of mice used for the present investigation, the chance for observing spontaneous pulmonary adenoma grossly is very low and that the tumors seen should represent the effect of the chemical ingested.

The distinction between adenoma and carcinoma of the lung in the experimental animals without evidence of distant metastasis may be ultimately subjective. In all 4 cases, the mice with adenocarcinoma in our series had hemorrhagic exudate in the pleural cavity and the tumor sometimes adhered to the parietal pleura. There were marked cellular atypism, irregularity in the histological patterns, frequent mitotic figures, and evidences of local destructive growth, especially of the bronchial walls and pleura. It is believed that nitrosodimethylamine is capable of producing pulmonary adenoma and carcinoma in ddN mice when fed for 5 months or over. There was also indication that the carcinoma has arisen from the focus of benign adenoma.

A transplantable malignant hemangioendothelioma of the liver in C3H mice has been reported by Edwards, Andervont, and Dalton,[6] and later by Lippincott, Edwards, Grady, and Stewart.[9] According to Edwards *et al.*, spontaneous hemangioendothelioma of the liver occurred occasionally in C3H mice and they have followed one by serial transplantation. So far as we are aware, there is no report of spontaneous hemangioendothelioma in ddN mice. In the present experiment, hemangioendothelioma was found in the liver in 2 cases.

It has been noticed that nitrosodimethylamine induces severe and diffuse hepatocellular damages leading to coagulation necrosis, followed by absorption of the hepatic cells. A marked dilatation of the sinusoids and, occassionally, focal proliferation of the stellate and endothelical cells occur as the consequence. Similar changes were also observed in limited cases in the spleen. This apparently compensatory hyperplasia of the endothelial cells may have a chance to develop malignant proliferation.

The production of kidney tumor in rats by feeding nitrosodimethylamine was reported by Magee and Barnes.[14] Only one case of anaplastic carcinoma was found

in the present experiment. However, no attempt was made to pursue histogenesis of this tumor.

We would like to thank Director Tomizo Yoshida. We also wish to acknowledge the generous gift of N-nitrosodimenthylamine from Dr. Takashi Sugimura, National Cancer Center Research Institute. Their thanks are also due to Miss Akiko Sakagami and Mr. Seijiro Sato for their technical assistance.

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Explanation of Plates LXV~LXVII

Photo 1. Posterior view of the lung of mouse # 12 on the 166th experimental day. Besides multiple small round nodules, larger ones with clover-like limits are seen in bilateral lower lobes, especially large on the right side.

Photo 2. Posterior view of the lung of mouse # 19 on the 197th experimental day. Besides multiple adenomatous nodules, an irregularly shaped large mass is seen in the right first lobe.

Photo 3. Frontal section of the lung in Photo 2. An ovoid mass is almost completely occupying the first lobe.

Photo 4. Representative section of multiple pulmonary adenoma. Case 22, on the 203rd experimental day. H-E. ×200.

Photo 5. Histology of the mouse shown in Photo 1. Note marked cellular atypism, frequent mitotic figures and irregular tissue pattern consistent with adenocarcinoma. H-E ×200.

Photo 6. Progressive involvement of the pleura in Case 2. Note also cellular atypism. Adenocarcinoma of the lung. H-E. ×100.

Photo 7. Destructive growth of adenocarcinoma in the mouse # 19. The peribronchial stroma is invaded. H-E. ×200.

Photo 8. Invasion of the interlobular connective tissue by an adenocarcinoma in the mouse # 19. H-E. ×200.

Photo 9. Macroscopic and microscopic pictures of the liver in mouse # 2. The 6th lobe is entirely occupied by a hemorrhagic mass. The tumor is histologically consistent with malignant hemangioendothelioma. H-E. ×100.

Photo 10. Histological picture of the spleen in mouse # 11 showing a localized hemangioendothelioma. H-E. ×200.

Photo 11. Histological picture of anaplastic carcinoma of the kidney in mouse # 17. H-E. ×200.

H-E: Hematoxylin-Eosin stain.

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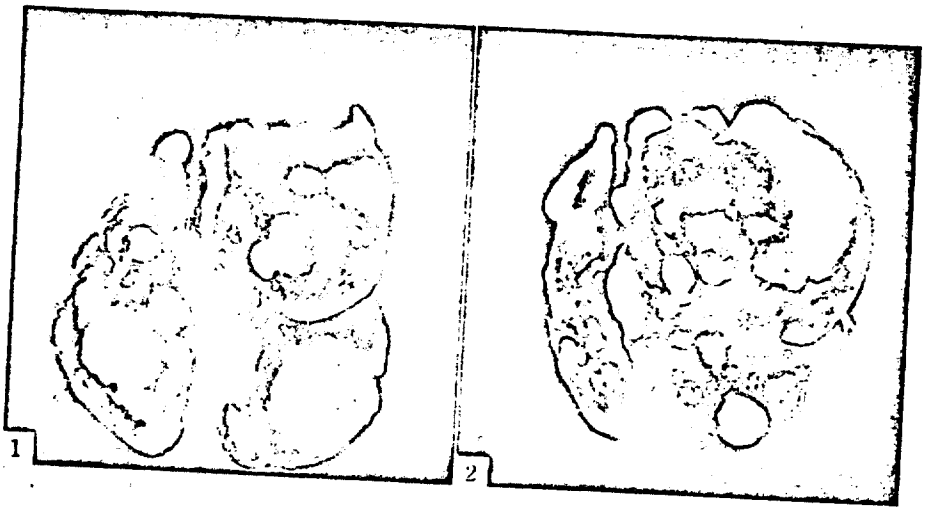

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Int. J. Cancer: 4, 373-383 (1969)

2166

SEQUENTIAL EFFECTS OF CHEMICALLY DIFFERENT CARCINOGENS, DIMETHYLNITROSAMINE AND 4-DIMETHYLAMINOAZOBENZENE, ON HEPATOCARCINOGENESIS IN RATS

by

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Induction of liver tumors was studied in rats given carcinogens as follows: (1) N-nitrosodimethylamine (DMN) alone; (2) 4-dimethylaminoazobenzene (DAB) alone; (3) DMN followed by DAB; and (4) DAB followed by DMN. The main findings were:

(1) DAB feeding for 0.62 to 2.5 months, followed by DMN feeding for 5 months, induced liver carcinomas more frequently than did DMN alone given for 5 to 10 months, or DAB alone given for 2.5 months.

(2) Likewise, DMN feeding for 0.62 or 1.25 months, followed by DAB feeding for 5 months, induced liver carcinomas more frequently than did DMN alone given for 5 to 10 months or DAB alone given for 2.5 months. Interestingly, when DMN was given for a longer time (2.5 months), followed by DAB for 5 months, 50% of the induced liver tumors were non-epithelial.

(3) Liver tumors were induced by sequential administration of the two carcinogens in doses that did not induce tumors when each carcinogen was given alone over a comparable time period.

(4) The latent period for liver tumor development was shorter in groups that received the two carcinogens sequentially than in those that received DAB or DMN alone for comparable time periods.

An important aspect of chemical carcinogenesis is the question whether or not the carcinogenic process initiated by one carcinogen can be brought to completion by another carcinogen of different chemical nature. Muta (1943) reported that tar or 3,4-benzpyrene painting, following the initial feeding with *o*-aminoazotoluene to the point where microscopic nodules were produced in the liver, enhanced the malignant transformation of these nodules. Similar phenomena were reported with 20-methylcholanthrene painting following a suitable period of 4-dimethylaminoazobenzene (DAB) (Odashima, 1962) and with diethylnitrosamine followed by DAB (Schmähl *et al.*, 1963*b*).

Dimethylnitrosamine (DMN) and its derivatives have been shown to induce liver tumors in various animal species since the discovery of their carcinogenicity (Magee and Barnes, 1956). The nitroso compounds have not only shown great versatility as carcinogenic agents, but also possess the following interesting characteristics: (1) a parallel between carcinogenic action and chemical structure has been demonstrated (Druckrey *et al.*, 1961, 1967); (2) if the administration period or

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daily dose is changed, there may be a change in the preferred target organs from which tumors arise (Magee and Barnes, 1962); and (3) DMN dissolved in peanut oil and given with the basal diet, or dissolved in drinking water of mice, induced non-epithelial tumors in the liver (Takayama and Oota, 1963, 1965; Takayama, 1968; Schmähl and Preusmann, 1959; Schmähl *et al.*, 1963*a*; Clapp and Craig, 1967; Clapp *et al.*, 1968). It was demonstrated in our preliminary experiments that in rats similar non-epithelial tumors were induced when DMN was administered in a given diet, but when it was replaced by DAB, typical hepatomas were induced.

In the present work, the effects of DAB and DMN on the two different targets in the liver were observed after sequential administration of these compounds to rats. The influence of initial treatment with each of these carcinogens was investigated in animals subsequently given insufficient doses of the other carcinogen.

This combination of carcinogens was chosen because each produces liver tumors when administered orally. The experiments were designed to permit observation of tumor yields when the two carcinogens were given to rats sequentially, maintaining the dose of each carcinogen at a level at which either one alone would fail to produce liver tumors. These sub-threshold doses were found empirically to be 100 μg/g of DMN in the diet for 5 months, and 600 μg/g of DAB in the diet for 2.5 months.

MATERIAL AND METHODS

Animals

The Donryu rats (155 males and 149 females) used in these experiments were obtained from the Nippon Rat Co. Ltd., Tokyo, Japan. All were 8 weeks old at the start of the experiment. They were housed in plastic cages in groups of three, and were given basal diet (CE-2) [1] and tap water *ad libitum* with the exceptions to be described.

[1] The basal diet CE-2 was composed of the following (in parts per 100): crude carbohydrate, 49.20; crude protein, 24.00; ash, 6.00; crude cellulose, 4.50; crude fat, 3.50; calcium, 1.20; phosphorus, 0.80; potassium, 0.50; sodium 0.30; and water, 10.00. The content of vitamins A, D_3, E, B_1, B_2, and B_6 was 10 and 2 IU, 10, 5.6, 10 and 4 μg/g diet, respectively.

DAB diet

4-Dimethylaminoazobenzene (Merck) was dissolved in peanut oil and added to the basal diet, adjusting carcinogen content in the final diet to 600 μg/g.

DMN diet

Dimethylnitrosamine (Eastman Organic Chemicals, Rochester, N.Y., USA) was dissolved in warm peanut oil and added to the basal diet, adjusting the carcinogen content in the final diet to 100 μg/g.

Peanut oil diet

Peanut oil (R. Nagai Co. Ltd., Tokyo, Japan) was added to the basal diet, adjusting the content to 1%.

Experimental groups and feeding periods

The rats were divided into 11 groups. The experimental groups and the treatments are summarized in Table I.

Group 1: Continuous DMN diet was given for 10 months. Group 2: Continuous DMN diet was given for 5 months. Group 3: Continuous DAB diet for 5 months. Group 4: Continuous DAB diet for 2.5 months. Group 5: After 2.5 months of DAB feeding, the DMN diet was given for the following 5 months. Group 6: After 1.25 months of DAB feeding, the DMN diet was given for the following 5 months. Group 7: After 18 days of DAB feeding, the DMN diet was given for the following 5 months. Group 8: After 2.5 months of DMN feeding, the DAB diet was given for the following 5 months. Group 9: After 1.25 months of DMN feeding, the DAB diet was given for the following 5 months. Group 10: After 18 days of DMN feeding, the DAB diet was given for the following 5 months. Group 11: Diet containing peanut oil was given continuously for 10 months. All groups were returned to basal diet until the end of the 22nd experimental month.

Total amounts of DMN and DAB ingested by each rat were calculated from the total amounts of the carcinogenic diets consumed per cage during 0.62, 1.25, 2.5, 5.0, and 10 months, respectively. The numbers of rats, numbers of liver tumors, and other data pertaining to rats consuming the diet containing each carcinogen are given in Table I.

Group	Carcinogen	P[illegible]
1	DMN	1[illegible]
2	DMN	5
3	DAB	5
4	DAB	2.5
5	DAB DMN	2.5 5
6	DAB DMN	1.2[illegible] 5
7	DAB DMN	18 5
8	DMN DAB	2.5 5
9	DMN DAB	1.2[illegible] 5
10	DMN DAB	18 5
11	Control	

[1] Reticulum cell sarcoma

All rats surviving [illegible] of the experiment we[illegible]

Thorough autop[illegible] animals as soon as t[illegible] killed, and their organ[illegible] solution. Paraffin [illegible] hematoxylin and eo[illegible] tion, and selected [illegible] periodic-acid-Schiff [illegible] Gomori's silver impr[illegible]

TABLE I

NUMBER OF ANIMALS, NUMBER AND TYPES OF LIVER TUMORS INDUCED, AND TOTAL DOSE OF DMN AND DAB

Group	Carcinogen	Period of treatment (months)	Initial no. of animals	Average intake of carcinogens (mg/rat)		No. of rats surviving over 32 weeks	Histological pattern of liver tumors			
				DMN	DAB		Typical hepatoma	Anaplastic hepatoma	Hemangio-endothelial sarcoma	Fibro-sarcoma
1	DMN	10	30 ♂17 ♀13	♂465 ♀268		20 ♂10 ♀10	2 ♂0 ♀2	1 ♂1 ♀0	4 ♂3 ♀1	2 ♂0 ♀2
2	DMN	5	27 ♂13 ♀14	♂288 ♀180		18 ♂ 9 ♀ 9				
3	DAB	5	28 ♂13 ♀15		♂1229 ♀1031	12 ♂ 5 ♀ 7	12 ♂5 12 ♀7			
4	DAB	2.5	29 ♂15 ♀14		♂ 583 ♀ 540	21 ♂11 ♀10	1 ♂0 ♀1			
5	DAB DMN	2.5 5	29 ♂15 ♀14	♂193 ♀153	♂ 650 ♀ 475	19 ♂10 ♀ 9	5 ♂2 ♀3	2 ♂1 ♀1		1 ♂1 ♀0
6	DAB DMN	1.25 5	27 ♂14 ♀13	♂175 ♀169	♂ 332 ♀ 280	22 ♂12 ♀10	6 ♂3 ♀3		1 ♂0 ♀1	
7	DAB DMN	18 days 5	28 ♂14 ♀14	♂211 ♀179	♂ 240 ♀ 200	19 ♂10 ♀ 9	4 ♂2 ♀2	1 ♂0 ♀1		2 ♂1 ♀1
8	DMN DAB	2.5 5	30 ♂15 ♀15	♂147 ♀ 83	♂1365 ♀ 951	20 ♂11 ♀ 9	2 ♂2 ♀0	2 ♂1 ♀1	3 ♂1 ♀2	1[1] ♂0 ♀1
9	DMN DAB	1.25 5	28 ♂15 ♀13	♂ 66 ♀ 50	♂1493 ♀ 987	20 ♂11 ♀ 9	9 ♂4 ♀5	2 ♂1 ♀1		
10	DMN DAB	18 days 5	29 ♂15 ♀14	♂ 31 ♀ 33	♂1170 ♀1154	19 ♂10 ♀ 9	9 ♂4 ♀5	1 ♂1 ♀0		
11	Control		19 ♂ 9 ♀10			16 ♂ 7 ♀ 9				

[1] Reticulum cell sarcoma of the liver.

All rats surviving at 22 months after the start of the experiment were killed at that time.

Thorough autopsies were performed on all animals as soon as they were found dead or were killed, and their organs were fixed in 4% formalin solution. Paraffin sections were stained with hematoxylin and eosin for histological examination, and selected material was stained by the periodic-acid-Schiff method, alcian-blue, and Gomori's silver impregnation.

RESULTS

The first liver tumor was found in a rat of Group 3, killed after 32 weeks. Therefore, all rats that survived longer than 32 weeks were regarded as cases on which frequencies of liver tumor development could be validly based.

Histological classifications of liver tumors induced by chemical carcinogens have been proposed by many investigators (Edward and White,

1941; Opie, 1944; Stewart and Snell, 1959), and have varied according to the emphasis of the study concerned. In this work, liver tumors were divided into four types on the basis of the following criteria:

Typical hepatomas

Cancer cells were arranged in cords and tubules, and were sometimes medullary or solid with thin bands of fibrous tissue between the clusters (Figs. 1-4). Three patterns were observed within this type. In the first, cancer clusters formed cord-like or tubular arrangements surrounded by endothelial cells which were connected directly to the blood sinuses (Fig. 2). In the second pattern, clusters of cancer cells formed a solid arrangement and were separated by thin bands of fibrous stroma carrying blood capillaries (Fig. 3). In the third type, the arrangement of cancer cells was tubular, follicular, or sometimes tubular with papillary infoldings (Fig. 4).

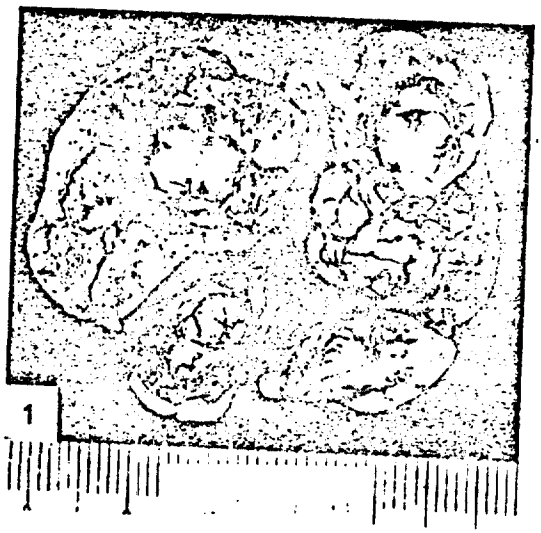

FIGURE 1

Posterior view of liver carcinoma in Group 3. The animal died during the 42nd week.

Anaplastic hepatomas

Cancer cells presented a polymorphous appearance, showed a variety of arrangements, and had thin connective tissue septa between the cells, or between small cell clusters (Figs. 5, 6).

Hemangioendothelial sarcomas of the liver

Tumors were represented by an ill-defined dark brownish swollen area, sometimes pedunculated into the peritoneal cavity (Fig. 7). These

FIGURE 2

Typical histological picture of the hepatoma (Type 1) shown in Figure 1. Cord-like arrangement of cancer cells. H. & E. ×250.

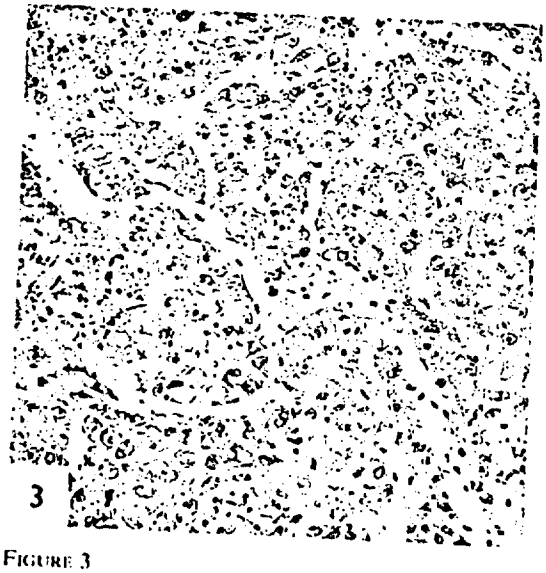

FIGURE 3

Typical hepatoma (Type 2) in Group 9 (animal died during the 50th week). Cancer cells arranged in cords and small solid aggregates. H. & E. ×250.

tumors were larger
vascular channels
vessels were lined
layers thick, often
extending into the
tumors consisted of
cells occasionally
cleft-like lumina (F

Fibrosarcoma of the

These tumors co
shaped cells of vary
directions. Mitotic
areas consisted of
11).

Group 1 (10 mon
died before the 32nd

FIGURE 4

Typical hepatoma (T
killed during the 70th
arrangement of cancer ce

between the 32nd and
tumor. In the remain
found between the 32n
average latent period
74 weeks.

Histological appear
group showed typic
anaplastic hepatoma

tumors were largely composed of blood-filled vascular channels varying in shape and size. The vessels were lined by tumor cells one or more layers thick, often forming papillary processes extending into the lumen. In some areas, the tumors consisted of densely proliferating spindle cells occasionally arranged to form irregular cleft-like lumina (Figs. 8, 9).

Fibrosarcoma of the liver

These tumors consisted of bundles of spindle-shaped cells of varying size, coursing in different directions. Mitotic figures were found, and some areas consisted of myxomatous tissue (Figs. 10, 11).

Group 1 (10 months on DMN diet): Ten rats died before the 32nd week and another 11 died between the 32nd and 87th weeks without liver tumor. In the remaining 9 rats, tumors were found between the 32nd and 86th weeks, and the average latent period to tumor recognition was 74 weeks.

Histological appearance of liver tumors in this group showed typical hepatoma in 2 females, anaplastic hepatoma in 1 male, hemangioendothelial sarcoma in 4, and fibrosarcoma in 2 females. In 2 rats, metastases from fibrosarcoma of the liver were detected in the lung and peritoneal cavity. One of the four hemangioendothelial sarcomas was accompanied by an adeno-

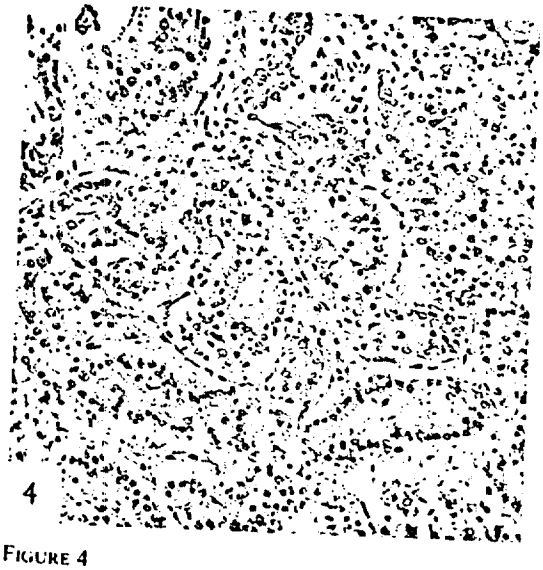

FIGURE 4

Typical hepatoma (Type 3) in Group 10, animal killed during the 70th week. Tubular or follicular arrangement of cancer cells. H. & E. ×250.

FIGURE 5

Anaplastic carcinoma in Group 5, animal killed during the 71st week. Cancer cells were pleomorphic (arrows) and surrounded by connective tissue stroma. H. & E. ×250.

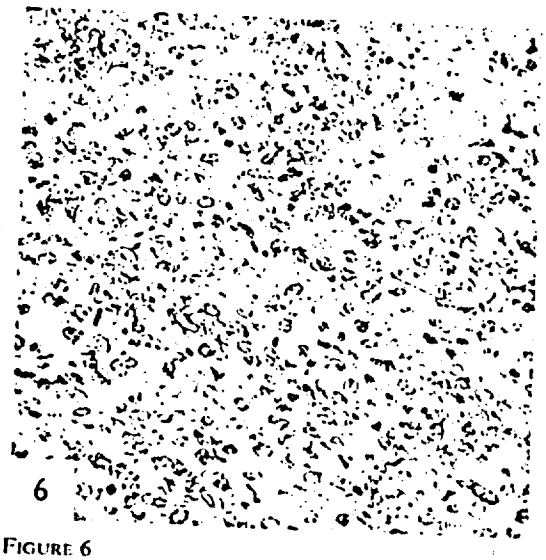

FIGURE 6

Anaplastic carcinoma in Group 5, rat died in the 65th week. Cancer cells present a moderately pleomorphic appearance. H. & E. ×250.

matous nodule in another lobe of the liver. Cyst formation was found in all the cases, one having all lobes covered with cysts. In two cases the liver weighed 50 g and contained multiloculated cysts with profusions of cells into the cyst cavities.

Group 2 (5 months on DMN diet): Nine rats died before the 32nd week, and 8 died between the 32nd and 86th weeks without tumor development. The remaining 10 rats were killed at the 87th week and no liver tumors were found.

Distinct cyst formation was found in all cases, three of which showed papillary proliferations of hyperchromatic cells within the cystic cavities.

Group 3 (5 months on DAB diet): Ten rats died before the 32nd week and another 6 died between the 32nd and 50th weeks without tumor. In the remaining 12, tumors were found between the 32nd and 73rd weeks, and the average latent period to tumor recognition was 67 weeks.

Histologically, all 12 cases were of the typical hepatoma type. Lung metastasis was detected in 2 out of the 12 rats. Development of anaplastic hepatoma, hemangioendothelial sarcoma, fibrosarcoma, and/or multiple cyst formation were not found in any animals in this group.

Group 4 (2.5 months on DAB diet): Eight rats died before the 32nd week and 11 died between the 32nd and 86th weeks. A tumor was found in only one female rat that died in the 81st week. The remaining 10 rats were killed in the 87th week; no liver tumors were detected.

8

FIGURE 8

Hemangioendothelial sarcoma of the liver shown in Figure 7. The tumor was composed of blood-filled vascular channels lined by tumor cells. Papillary processes extend into lumina. H. & E. ×200.

7

FIGURE 7

A large pedunculated hemangioendothelial sarcoma (arrow) of the liver in Group 1, animal died during the 80th week.

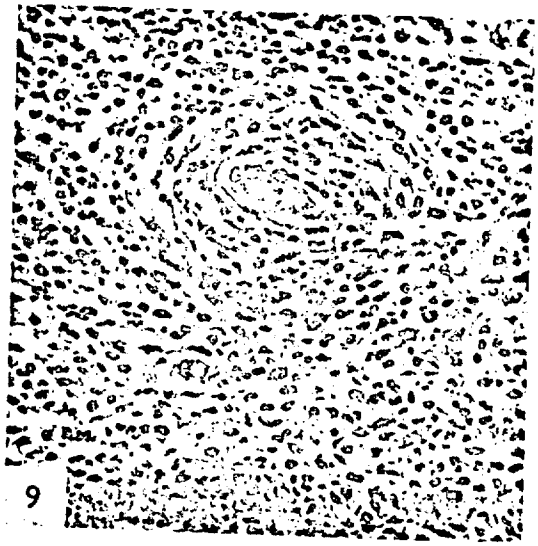

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FIGURE 9

Hemangioendothelial sarcoma of the liver in Group 8, animal died during the 65th week. Tumor consisted of dense proliferations of spindle cells. H. & E. ×250.

10

FIGURE 10

Gross appearanc
(arrow) in Group 1,
Tumor mass is seen
Scattered small cysts

In the rat that di
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Group 5 (2.5 mon
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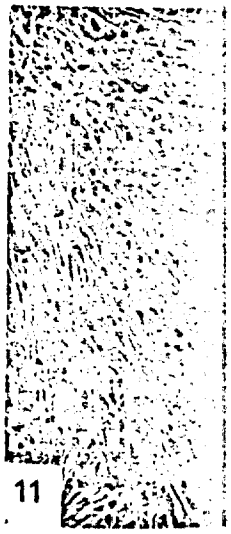

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FIGURE 11

Fibrosarcoma of
during the 53rd wee

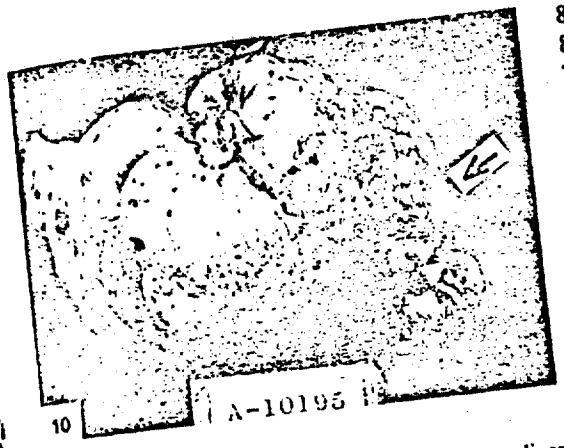

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FIGURE 10
Gross appearance of fibrosarcoma of the liver (arrow) in Group 1, animal died during the 80th week. Tumor mass is seen in the lower part of the left lobe. Scattered small cysts are seen.

In the rat that died in the 81st week, there was a solitary white nodule, 4×4×5 cm, in the right lobe of the liver. Histologically the tumor was composed entirely of typical hepatoma, and metastasis to the lung was present.

Group 5 (2.5 months on DAB feeding followed by 5 months on DMN diet): Ten rats died before the 32nd week and 11 died between the 32nd and 80th weeks without tumors. In all of the remaining 8 rats tumors were found between the 41st and 79th weeks, and the average latent period was 61 weeks.

The liver tumors in this group were classified as typical hepatomas in 5, anaplastic hepatomas in 2, and fibrosarcoma with metastasis to the lung in 1 rat.

Multiple small cyst formation was extensive in the livers in the majority of cases.

Group 6 (1.25 months' DAB feeding followed by 5 months on DMN diet): Five rats died before the 32nd week and another 15 died during the 32nd to 72nd week without tumor. In the remaining 7 rats, tumors developed between the 33rd and 83rd weeks, and the average latent period to tumor recognition was 68 weeks.

In 7 rats with liver tumors, 6 showed typical hepatoma and 1 had hemangioendothelial sarcoma.

Marked multiple cyst formation of the liver was also observed in all the cases.

Group 7 (0.6 month on DAB feeding followed by 5 months on DMN diet): Nine rats died before the 32nd week and another 12 died between the 32nd and 71st weeks without tumor. In the remaining 7 rats tumors were found between the 40th and 79th weeks, and average latent period to tumor recognition was 66 weeks.

Liver tumors in this group were classified as typical hepatoma in 4, anaplastic hepatoma in 1 and fibrosarcoma in 2 rats.

In non-tumorous areas of the liver, numerous cysts were observed in all cases.

Group 8 (2.5 months on DMN feeding followed by 5 months on DAB diet): Ten rats died before the 32nd week and 12 died between the 32nd and 86th weeks, without tumor. In the remaining 8 rats tumors developed between the 38th and 86th weeks, with an average latent period of 65 weeks.

Tumors were typical hepatoma in 2, anaplastic hepatoma in 2 and hemangioendothelial sarcoma in 3. The remaining one was diagnosed as reticulum cell sarcoma.

Multiple small cyst formation was observed in the livers of all rats.

Group 9 (1.25 months on DMN feeding followed by 5 months on DAB diet): Eight of

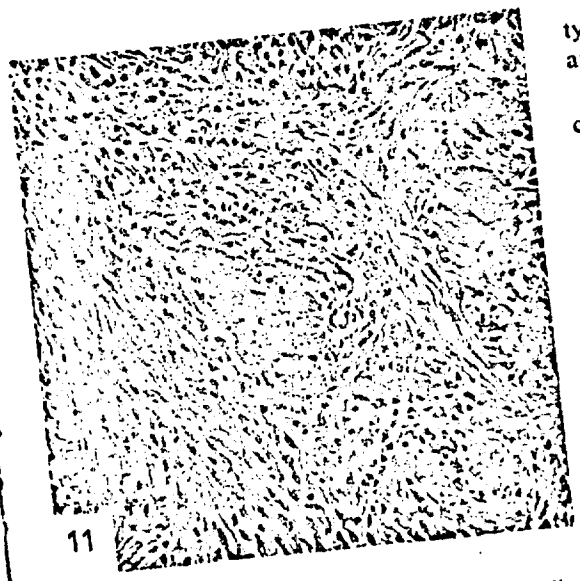

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FIGURE 11
Fibrosarcoma of the liver in Group 8, animal died [illegible] H. & E. ×250.

28 rats died before the 32nd week and 9 others died between the 32nd and 73rd weeks, without tumor. In the remaining 11 rats, tumors developed in the liver between the 55th and 85th weeks, the average latent period being 65 weeks.

Histological examination disclosed typical hepatomas in 9 and anaplastic hepatomas in 2 rats. There were no non-epithelial tumors in this group. However, extensive multiple cyst formation was observed in all rats.

Group 10 (0.62 month on DMN feeding followed by 5 months on DAB diet): Ten of 29 rats died before the 32nd week and another 10 died between the 32nd and 83rd weeks, without liver tumor. In the remaining 10, tumors developed in the liver between the 38th and 83rd weeks, for an average period of 60 weeks.

In one rat killed at the 73rd week, lymphoid leukemia involving liver, spleen, and bone marrow was found. Nine liver tumors were typical hepatomas, and one was an anaplastic hepatoma. Metastases were found in the lung in 3 cases, and in the peritoneal cavity in 1.

Even though DMN was fed to this group for only 0.62 month, marked multiple cyst formation in the liver was observed in all the cases (Fig. 12).

Group 11 (Peanut oil diet for 10 months): Three rats died before the 32nd week and another 8 died between the 32nd and 75th weeks without tumor. In the remaining 8 rats killed at the 82nd week, there was no neoplastic change in any organ. Histological examinations were made in all cases, and slight periportal fibrosis of the liver was observed in 2 cases.

FIGURE 12

A case of multiple cyst formation in the liver in Group 10 which received DMN diet for 0.62 month followed by 5 months of DAB feeding.

DISCUSSION

Two liver carcinogens, DMN and DAB, were administered to rats sequentially to determine their possible synergistic effect in combination with each other.

In rats fed DMN alone for 10 months, 66.7% of the liver tumors were non-epithelial, while in rats fed DAB alone for 5 months, 100% of the tumors induced were typical hepatomas. The histologic patterns observed in these two groups were used as references for analysis of tumors in rats given combinations of the two carcinogens. In rats given DAB for 2.5, 1.25, and 0.62 months respectively, followed by DMN for 5 months, 71-87% of the liver tumors that appeared were epithelial in type. These data suggest that the initial administration of DAB may determine the type of the tumor irrespective of the subsequent 5-month administration of DMN. While no effect of initial administration of DMN was demonstrated with respect to the resulting tumor type when it was given for only 1.25 or 0.62 months, 50% of the induced liver tumors were non-epithelial when DMN was given for 2.5 months, preceding 5 months of DAB.

It thus appeared that a prolonged primary exposure to DMN brought about a shift in the pattern of tumors induced.

Multiple cysts were observed diffusely distributed in all lobes of the livers of rats fed DMN. These cysts did not show evidence of becoming malignant, and were found in all groups that received DMN. They contained clear, sometimes yellow fluid. Numerically and histologically, there was no difference between cysts occurring after 0.62 month of DMN feeding and those occurring after 5 months of DMN feeding.

The relationship between total dosage and rate of tumor induction has been a matter of interest in many previous investigations concerned with carcinogenesis (Druckrey and Küpfmüller, 1948; Nakahara and Fukuoka, 1960; Nakahara, 1961). In the present work, liver tumors were induced by the additive or synergistic effects of two carcino-

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TREATMENT

Group	Carcinogen	Period of treatment (months)
1	DMN	10
2	DMN	5
3	DAB	5
4	DAB	2.5
5	DAB DMN	2.5 5
6	DAB DMN	1.25 5
7	DAB DMN	18 days 5
8	DMN DAB	2.5 5
9	DMN DAB	1.25 5
10	DMN DAB	18 days 5
11	Control	

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● Hemangioendothelial sarco
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gens administered consecutively, in doses of each which alone would not have induced liver tumors. (Table II).

It was supposed that DMN and DAB have different target cells, as mentioned above. Cells that were given some orientation by initial exposure to one carcinogen subsequently became completely cancerized by exposure to the other carcinogen, regardless of the order of the exposures.

The average latent period for liver tumor appearance was shorter when the two carcinogens were given consecutively, in either order, than when either chemical was given alone. This indicated that either DAB or DMN accelerated liver carcinogenesis in rats given limited doses, but doses that were sufficient to sensitize the liver tissue. Similar results were reported by Miyaji *et al.* (1959) with DAB followed by DMN feeding; by Odashima (1962), with DAB followed by 4-dimethylaminostilbene feeding; and by Schmähl *et al.* (1963*b*), with diethylnitrosamine followed by DAB. The present work therefore confirms previous observations, and suggests that the two carcinogens act either synergistically or through an additive effect of different modes of action, rather than through the summation of similar modes of action.

TABLE II

TREATMENT, LATENT PERIOD OF TUMOR FORMATION, AND TYPES OF TUMORS

Group	Carcinogen	Period of treatment (months)	Experimental period (30 40 50 60 70 80 90)	Incidence of induced epithelial and non-epithelial liver tumors (%)
1	DMN	10		33.3
				66.7
2	DMN	5		
3	DAB	5		100
4	DAB	2.5		100
5	DAB	2.5		87.5
	DMN	5		12.5
6	DAB	1.25		85.5
	DMN	5		14.4
7	DAB	18 days		71.4
	DMN	5		28.6
8	DMN	2.5		50
	DAB	5		50
9	DMN	1.25		100
	DAB	5		
10	DMN	18 days		100
	DAB	5		
11	Control			

o Typical and anaplastic hepatoma.
● Hemangioendothelial sarcoma and fibrosarcoma of the liver.
▲ Reticulum cell sarcoma of the liver.

ACKNOWLEDGEMENTS

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We are greatly indebted to the Director of this Institute, Dr. Tomizo Yoshida, for his helpful criticism throughout the study.

EFFETS DE L'ADMINISTRATION CONSÉCUTIVE DE DEUX CARCINOGÈNES (DIMÉTHYLNITROSAMINE ET DIMÉTHYLAMINOAZO-4 BENZÈNE) SUR L'HÉPATOCARCINOGÉNÈSE CHEZ LE RAT

On a étudié l'induction de tumeurs du foie chez des rats auxquels on avait administré des carcinogènes de la manière suivante: 1) N-nitrosodiméthylamine (DMN) seule; 2) diméthylaminoazo-4 benzène (DAB) seul; 3) DMN suivi de DAB; 4) DAB suivi de DMN. Principales constatations:

1) L'administration de DAB pendant 0.62 à 2.5 mois, suivie de DMN pendant 5 mois, a produit plus souvent des hépatocarcinomes que le DMN administré seul pendant 5 à 10 mois ou le DAB seul pendant 2.5 mois.

2) De même, l'administration de DMN pendant 0.62 ou 1.25 mois, suivie de DAB pendant 5 mois, a produit plus souvent des hépatocarcinomes que le DMN administré seul pendant 5 à 10 mois ou le DAB seul pendant 2.5 mois. Il est intéressant de noter que, quand le DMN était administré pendant plus longtemps (2.5 mois) et suivi de DAB pendant 5 mois, 50% des tumeurs hépatiques provoquées n'étaient pas épithéliales.

3) Les tumeurs ont été produites par l'administration consécutive des deux carcinogènes à des doses qui n'en provoquaient pas lorsqu'on administrait l'un des deux seulement pendant un temps comparable.

4) La période de latence était plus courte dans les groupes qui ont reçu les deux carcinogènes consécutivement que dans ceux qui ont reçu le DAB ou le DMN seuls pendant un temps comparable.

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Effect of Meat Curing Solutions on Anaerobic Bacteria.

III. Sodium Nitrite.

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By Fred W. Tanner and Florence L. Evans.

The nitrites of sodium and potassium have an important role in imparting a desired red color, to cured meats. Food control officials have rightfully regulated the composition of curing solutions to prevent the addition of unnecessarily large amounts of certain constituents which might injure the health of the consumer.

Nitrites have recently been used in meat curing solutions as color fixatives. When nitrates are used, it is assumed that they are reduced to nitrites. During late years, evidence has been accumulating to indicate that nitrites may also function to some extent as preservatives. This paper reports results which have been secured in a study of this question. Results with sodium chloride and sodium nitrate have already been published [Tanner and Evans (1, 2)].

Use of nitrite in place of nitrate in the curing of meat is of more recent development. This practice arose from an observation made by Polenski (3, 4) that nitrite was present in old nitrate curing solution. He believed that reduction of nitrate to nitrite was accomplished by bacterial activity. Nothwang (5) made the same observation, but added that reduction might be due to the meat tissue itself. In light of Bernheim and Dixon's work (6), Polenski's explanation is more probable. Bernheim and Dixon found that muscular tissue from a considerable number of animals including the ox and the pig was completely without power to reduce nitrate to nitrite. Lehmann (7) and Kisskalt (8), working separately, found that red color of cured meat was due to nitrite and not to nitrate. Lehmann boiled fresh meat with nitrite in a weak acid solution and secured a product having the red color of cured meat. When treated with sodium nitrate, this red color did not appear. Kisskalt found in addition, that, if meat were allowed to stand several days in contact with nitrate, a red color would result. Haldane (9) proved that the color of meat cured with nitrate and nitrite was due to nitric-oxide-hemoglobin which was formed by the action of nitrous oxide on hemoglobin. He also found that the permanent pink color of boiled cured meat was due to a water insoluble compound which proved to be nitric oxide-hemochromogen. Hoagland (10, 11) repeated Haldane's experiments and obtained the nitric oxide-hemoglobin as the red colored element of cured meat. He concluded that neither nitrates nor nitrites, as such, had any influence in preserving the color of fresh meat. The brown color of meat cured with excessive nitrate was thought to be due to the action of sodium

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nitrite on hemoglobin. An interesting observation was made by Klut (12) who noted that some waters would change the color of beef to a lighter or deeper red on boiling. Experiments showed that very small amounts of nitrite were sufficient to cause the change in color. Nitrate containing waters might give the color if they were allowed to remain in contact with zinc, for the metal acted as a reducing agent in this case and the nitrite so produced would cause the change in color.

After it had been shown that the active agent in color fixation was nitrite and not nitrate, efforts were made to use the former in curing solutions. Among the first to recommend it was Glage (13) who proposed the use of partially reduced nitrate prepared by heating dry saltpeter in a kettle. This procedure, of course, led to a product of unknown nitrate-nitrite composition which, when used in the curing of meat, gave somewhat irregular results. From this time on, however, commercial preserving salts containing nitrite made their appearance on the market. Auerback and Riess (14) found that meat cured with nitrite contained more of this salt than meat cured with nitrate. They found that meat exposed to the action of an ordinary curing solution containing five per cent potassium nitrate for several weeks, contained, at the most, a few milligrams of nitrite per 100 grams of meat. Amounts exceeding ten milligrams per 100 grams were found only in the surface portions. Curing solutions which contained a large amount of sodium nitrite caused it to penetrate uniformly into the meat. Ten to forty milligrams per 100 grams were found in surface samples. Auerback and Riess believed that amounts of sodium nitrite exceeding fifteen milligrams per 100 grams indicated that a nitrite cure had been used. Pollak (15) found that a nitrite cure was practical. The amount of nitrite in the finished product was even samller than usually found in meat cured with nitrate. Careful control was required in order to obtain good results. Experimental work on a commercial scale by Lewis, Vose, and Lowery (16) demonstrated that a nitrite cure, when properly carried out, yielded a product equal, or superior, to that resulting from a nitrate cure. The amount of sodium nitrite found to be most satisfactory was one tenth the amount of sodium nitrate usually used. The resulting product contained no more, and often less, nitrite than when the customary amount of saltpeter was used. These conclusions were, in general, concurred in by Kerr, Marsh, Schroeder, and Boyer (17).

The results of this investigation were confirmed by Riess, Meyer, and Müller (18) who compared the effects of curing solutions containing nitrite with those containing nitrate. They found that meat cured with nitrite never contained more than 10 mg. of sodium nitrite per 100 grams and frequently only a fraction of a milligram was found. They also pointed out that meat cured with two per cent saltpeter[1]) contained, in addition to the traces of sodium nitrite, 100 to 150 mg. of saltpeter. The possibility exists that this saltpeter may be transformed suddenly under uncontrolled conditions into nitrite. They recommended, as a result of their experiments, a salt mixture containing not more than 0.6 per cent sodium nitrite with sodium chloride. When this mixture was stored in dry rooms, very little destruction of nitrite occurred, but, when it was placed in wet rooms, appreciable loss took place in a short time. Riess, Meyer, and Müller found that the advantages of the use of 0.6% sodium nitrite curing mixtures are as follows: (1) a curing time of one half to one third that usually necessary with saltpeter; (2) thorough reddening of the meat and thorough salt penetration proceeded faster when this cure was used; (3) the nitrite content of the finished product was no larger and was frequently smaller than that receiving a nitrate cure; (4) the appearance, odor, flavor, and keeping quality of such products were equal to, or superior to, those cured with saltpeter.

[1]) Saltpeter according to Riess, Meyer, and Müller refers to a mixture of sodium and potassium nitrates.

The equivalence of sodium nitrate to sodium nitrite in meat curing has been stated to be 10 to 1 by Lewis and Moran (19); others have stated it to be 5 to 1 and lower. An exact equivalence which will hold under all conditions is probably not possible since the conditions which obtain in each separate experiment will greatly effect the results. Assuming that nitrites are somewhat germicidal, the number of nitrate-reducing bacteria influence the results. If nitrate reduction is slow, there will be less effective nitrite. At the Iowa Agricultural Experiment Station (20), nitrites proved effective in a concentration of from 1/10 to 1/20 that of nitrates generally used. A desirable color was not produced until the concentration of salt was at least 10 per cent. It is quite well established that constant equivalence values do not exist, for the amount of nitrite resulting from bacterial reduction of nitrate, would depend upon many different factors: type, numbers and activities of microorganisms, physical conditions which obtain in the curing solution, time of curing, etc.

Another factor of importance is that constant relationships do not seem to exist between the amount of ingredients in curing solutions and their incidence in cured meats. This seems to be borne out by data in publications of Kerr, Marsh, Schroeder, and Boyer (17) and others. This would seem to indicate, therefore, that it might be better to control amounts of nitrate and nitrite in the finished product than in the curing solution.

Saltpeter (sodium nitrate) has had the longest use in meat curing solutions. Since nitrates must be reduced to nitrites, packers desired to use nitrites as such to avoid the dangers which might result from inadequate conversion of nitrates to nitrites. Losses from uneven or inadequate fixation of color were not uncommon. Since many bacteria exist which may reduce nitrites, it is necessary to either add sufficient to fix color before it is used up or to have a constant source of supply (nitrates). The nitrate cure permitted this but the nitrite cure introduced the hazard of nitrite disappearance. Consequently, there may be much merit in the mixed cure in which both nitrates and nitrites are added. The nitrates may act as a reservoir for replacement of lost nitrite.

Regulation of the amounts of these salts used in meat curing solutions rests in the United States with the Bureau of Animal Industry and the bureau has rightfully been diligent to prohibit the use of unnecessarily large amounts. Too little is known, however, about the toxicity of nitrites in cured meat products.

The conditions caused by the World War made it necessary for the Secretary of the United States Department of Agriculture (21) to revise regulations to permit the immediate use of sodium nitrate (Chile saltpeter) in place of potassium nitrate for curing of meat in establishments where Federal inspection is maintained. This salt has been used since then in spite of the fact that many packers claim that better results are secured with

1922 (22) the United States Department of Agriculture to permit among other things saltpeter and nitrate

Amendment 4 to B. A. I. Order 211, revised, issued October 19, 1925 (23) permitted the use of saltpeter, nitrate of soda, and nitrite of soda. This was indeed a mixed cure and a distinct departure from former practice. The bureau stated that meats could be successfully cured with quantities of sodium nitrite that would not result in the presence of more than 200 parts per million in the finished meats. It was stated that in general, the correct quantity of nitrite is approximately one-tenth that of nitrate. The substitution of one pound of nitrite for each 10 pounds of nitrate was recommended. The basis for this ratio (10 to 1) is hard to determine.

After use of excessive amounts of sodium nitrate in pumping pickle was observed by the Bureau of Animal Industry in 1926 (24), a warning was issued that amounts of saltpeter or sodium nitrate over one per cent would be held excessive in pumping pickle.

Immediately after release of these rulings, the bureau began to record itself in favor of a nitrite cure as the approved cure for comminuted meats and sausage. It was suggested that the use of nitrate be eliminated when as much as one-quarter oz. of sodium nitrite is used per 100 lbs. of meat. This opinion was based on the observations that satisfactory cured meat could be made with such a cure.

Although many meat packers favored the nitrite cure, others believed that a mixed cure containing both nitrate and nitrite, was more satisfactory. It was suggested that, although nitrites made cured meats red, a little residual nitrate would be desirable to act as a constant source of nitrites. Accordingly, in the fall of 1931, the chief of the Bureau of Animal Industry issued a statement permitting ¼ oz. of nitrite, and 2¾ oz. of nitrate per 100 lbs. of meat in curing solutions. The arguments in favor of such mixed cures were several. The most important was that satisfactory cured meats had been prepared for years with a mixed cure and to change abruptly to the nitrite-cure would change the properties of the final products from those packing plants which had worked out a satisfactory procedure. Furthermore, it would be unnecessary to rely absolutely on the activities of nitrate reducing bacteria.

The practice in Germany was reviewed by Riess, Meyer, and Müller and need, therefore, not be reviewed here.

The amount of nitrite in cured meats naturally varies with the product and the method used for curing it. Moulton (26) has summarized the knowledge on this question. Lewis and Vose (27) reported from 42 to 53 parts per million of nitrite in a ham which had received a regular nitrate cure. Jones (28) reported from 72 to 960 parts per million of nitrite in American ham. In the published article the methods of sampling, analysis, and manipulation of the samples were not given. The amounts which would be secured would depend largely on the type of sample and its treatment after collection.

The ruling of the B. A. I. that the finished product should contain less than 200 parts per million is in many respects meaningless. Nothing was said as to how or when the samples were to be taken. It is apparent that a surface sample of a ham recently removed from a curing solution with 600 or 700 p.p.m. of nitrite would show nitrite content considerably in excess of 200 p.p.m. On the other hand, a sample from a deeply seated part in the ham would show a nitrite content well within the limits of 200 p.p.m. Unless the method of sampling is specified, a ruling such as the one under discussion, can but lead to confusion. A standard such as that just mentioned is meaningless unless standard procedures or methods are used in its enforcement. A surface sample would contain large amounts of nitrite while a sample from the center of the ham might contain very little.

Another very serious element for error is the technic used for preparation and handling of the sample. If the samples are not properly prepared and analyzed immediately, nitrate and nitrite data will be inaccurate. Bacteria may reduce nitrates and yield very high nitrite results, or, on the other hand, they may decompose the nitrites giving lower data than should be expected. Samples for the determination of nitrates must be analyzed promptly or stored under adequate refrigeration only for very short periods of time.

In some products such as spiced luncheon loaf in tin cans, the nitrite content decreases rapidly during storage. the rate of disappearance depending on the temperature of storage and probably the numbers and activity of the bacteria present. Ruyle and Tanner (29) in an examination of commercial canned meats did not find curing salts in sufficiently high concentration to prevent development of common anaerobes. After storage for several months the nitrite content in many cans is zero and almost so in the majority. Cooking, as carried out in the home, also destroys nitrite in cured meats. In experiments now in progress, it has been shown that boiling and frying frequently destroys all of the nitrites not even a trace showing on analysis even with products which have received a commercial nitrite or nitrate cure.

Nitrates are readily reduced to nitrites by many different chemical substances and microorganisms. In the preservation of meats with curing solutions containing nitrate only, it is well known that it must be reduced to nitrite before the color may be fixed.

Bacteria are known to be active in reducing nitrates and probably play the most important role in this respect in meat curing solutions containing nitrates. The subject has been studied mainly from the standpoint of agriculture and methods for determining the reduction with pure cultures. Maassen (30) studied the process with over 100 species of microorganisms in peptone broth containing 5% of potassium nitrate. Eighty-five of these species formed large amounts of nitrites. Maassen stated that the amount of potassium nitrate used hindered the growth of some of the bacteria. He also stated that some of them were hindered by the nitrite formed showing that it has a detrimental effect on bacteria. Results of investigations by many others have shown the importance of bacteria as nitrate-reducers.

It is possible that the meat itself may also reduce nitrates. Abelous and Gerard (31) reported results of an investigation which seemed to indicate the presence of an enzyme in horse flesh which reduced nitrates. Their conclusions have been criticized, however, on the basis that they did not work with sterile extracts and the reduction they attributed to the enzyme may have been brought about by microorganisms. Bernheim and Dixon obtained just the opposite results (6). Horovitz-Vlasova (32) has recently reported Bacillus halobicus, an obligate

ophilic organism which reduces nitrates to nitrites and then to free nitrogen. Zobell ( ) showed that many bacteria could reduce nitrates to nitrites. The latter, in turn, were also reduceable. Stickland (34) reported nitrate reduction by Escherichia coli.

Nitrites may also be decomposed by bacteria and some of the loss in nitrite must be attributed to microorganisms.

Pharmacology of Nitrites.

The chemical properties of sodium and potassium nitrite made the pharmacology of these salts of importance in meat preservation. Most of the evidence in literature, however, involves ingestion of far larger amounts of nitrite than are used in meat curing and certainly larger amounts than would be found in cooked cured meats as they would be prepared for the table. There is no evidence as far as can be found in the literature, to indicate that the amounts of nitrite ordinarily found in cured meats are harmful.

Since sodium nitrite and nitrate have been added to meat curing solutions, many investigators have suggested that these salts might have a detrimental effect on the health of human beings. Important among the reports which have been published are those by Grindley and MacNeal (35). Physiologically, nitrites are very active, and they reached the conclusion that their poisonous action in large doses was due to two factors. The first was the effect on the blood. They found that animals poisoned with nitrite had had a large part of the blood oxyhemoglobin changed to methemoglobin. The second factor was the direct effect on the tissues. The nature of this action was not definitely known, but Grindley and MacNeal believed that it was direct tissue asphyxia. Nitrites have been found to be toxic for all animals examined, both hemoglobin containing and non-hemoglobin containing. This includes insects, arachnidae, protozoa, infusoria and mammals. Many plants were also injured by nitrites.

Riess, Meyer, and Müller (18) gave, as an introduction to their paper, a brief history of the use of nitrite in meat curing in Germany. Since 1916 it had been forbidden in the industrial preparation of meat products. The dangerous properties of the salt were demonstrated by the doses regarded as permissable in medical practice. The German pharmacopoeia gave, as the largest single dose for an adult man, 0.3 gm. and as the largest daily dose 1.0 gm. The Swiss pharmacopoeia gave as the corresponding doses 0.1 gm. and 0.3 gm. Doses of 0.2 to 0.3 gms. can produce humming in the ears, vertigo, sensitivity to light, nausea, cyanosis, and irregularity in heart action. The literature was found to contain records of severe illness caused by 0.3 to 0.6 gms. It was considered in Germany that any material that affected the health of men in amounts of less than one gram should be used with the greatest care. However, they found by experiment that a carefully controlled curing process made possible the use of nitrite in the preparation of cured meats without the resulting product containing this substance in quantities sufficient to be detrimental to health. Riess, Meyer, and Müller's experimental work seemed to be [illegible]. They found that substitution of nitrate with nitrite decreased curing time by one-third. Addition of 0.6 per cent of nitrite to common salt sufficient when using 80 gms. of salt for 1000 gms. of meat.

The few data on the reaction of the salts of nitrous acid on bacteria, but one would suspect that a substance affecting so many other types of living things, might have some influence on such small organisms as bacteria. Lewis and Moran (19) found this to be the case. Their experiment included determinations of the effect of nitrite alone and also the effect of combinations of nitrite with sodium chloride. They found that 0.2 per cent of sodium nitrite, the lowest concentration used in their work, completely inhibited proteolysis in cultures of Clostridium putrefaciens. In another experiment using concentrations of 0.01 to 0.05 per cent, they observed no inhibition at any concentration below 0.05 per cent. Apparently, the effective concentration lies between 0.05 and 0.2% sodium nitrite. An experiment was also conducted with beef round covered with a solution containing nitrite, nitrate or chloride. The nitrite solutions contained 0.03, 0.06, 0.09, 0.12, and 0.18 per cent of sodium nitrite. After 9 days, growth and putrefaction were present in all the vessels containing nitrate and salt of concentrations ten times that of the nitrite. The nitrite-beef combinations showed no evidence of spoilage even after 21 days. This would seem to indicate that nitrite is very effective in inhibiting bacterial growth even in very low concentrations. The actual amount of effective nitrite must have been lower than the percentage concentrations appearing in the tables, for the amount of meat added was not considered in the calculations. The effect of nitrite, according to these data, must be different for Clostridium putrefaciens than for the ordinary meat flora. Whether inhibition of bacteria would be noticeable in the curing cellar is another matter.

The experiment on the effect of combinations of sodium nitrite and sodium chloride was performed with Clostridium sporogenes as the test organism. Proteolysis for the 48 hour period was inhibited at 0.05 per cent sodium nitrite in all the salt concentrations from one per cent to five per cent. However, for the seven to fourteen day period, proteolysis was observed in all nitrite concentrations from zero to 0.2 per cent (the highest sodium nitrite concentration used) in all the tubes containing zero to two per cent salt. In other words, after two weeks incubation, growth had appeared over the entire range in the tubes containing nitrite only. Proteolysis was checked in nitrite concentrations at and above 0.05 per cent in salt concentrations from three per cent to five per cent. In another similar experiment, these authors found that concentrations of 0.05 per cent and above prevented proteolysis in the absence of salt for the same incubation period. The difference between the effective concentration for these two experiments is the difference between 0.05 per cent and 0.2 per cent or 0.15 per cent.

Some attention has also been given to the formation of nitrites from nitrates in the intestines and the possible evil effects of the former. Barth (36) in 1879 studied the effect of Chile Saltpeter on animals which had accidentally ingested it. Symptoms of illness resulted including weakness, unconsciousness, muscle twitchings, etc. Barth attributed the symptoms to nitrites formed from the nitrates, an opinion which was not generally accepted by toxicologists. Binz and Gerlinger (37) confirmed Barth's work and believed that nitrite poisoning occurred. The experiments just reported leave the situation in an unsatisfactory state. Clear-cut conclusions cannot be reached on the toxicity of nitrites in foods. It is evident, also, that the amounts of nitrite used in the above experiments were considerable greater than would be found in cured meats.

[redacted] subjects were u[redacted] in these experiments. The results of this investigation indicated that [redacted]ssium nitrate as used in meat curing solutions showed no effect on the subjects. Mathews (38), who studied the pharmacology of nitrates and nitrites in this investigation, stated that the potassium in the potassium nitrate could be disregarded when the nitrate was taken in such small doses as those given in the experiments. The nitrate ion, in itself, was said to possess about the same physiological power as the chlorine ion. Sodium nitrate as such was said to have the same action on metabolism and nearly all tissues of the body as sodium chloride.

The situation for sodium nitrite is apparently different. Mathews quoted a number of cases of illness following ingestion of nitrites but the amounts were usually greatly in excess of those used in curing solutions. Data from injection experiments are not pertinent to this discussion.

Experimental.

These experiments were carried out to determine whether or not sodium nitrite in concentrations approximating those added to meat curing solutions had any effect on the putrefactive anaerobes, some of which are frequently involved in meat spoilage according to Klein (39) and McBryde (40). Special attention was given to Clostridium botulinum because of the dangerous properties of the toxin formed by this organism.

Twelve strains of spore formers were used as follows: Clostridium botulinum type A, cultures 1 and 2; Clostridium botulinum type B, cultures 5, 6, 7, 8, and 9; Clostridium putrificum, cultures 12 and 13; and Clostridium sporogenes, cultures 10, 11 and 14. These were grown in four different media to which various concentrations of sodium nitrite had been added.

The technic used needs no special discussion since it differed in no way from that used in experiments, results of which have been published recently in this journal. Special attention was given, however, to sterility of apparatus and media. All media were incubated before use and all apparatus after especially long heating was tested with media which had passed sterility tests. This is necessary in work involving the use of such microorganisms as thermophilic bacteria and spore-forming anaerobic bacteria.

Nitrites are destroyed by the action of meat tissues and heat. It was found that they decreased in media during storage. Determinations were made when the medium was inoculated. Nitrites added to media before sterilization were uniformly destroyed to a great extent.

Plain Broth: Sterile nutrient bouillon was inoculated by means of Pasteur pipettes with approximately ¼ cc. of an uniform, detoxified, spore suspension. This had been prepared from cultures growing in brain mash. These cultures were incubated at 37° C. for two weeks; then they were stored at room temperature for the remainder of the incubation period. Growth was detected by comparing the tubes with corresponding ones containing no nitrite and uninoculated tubes containing a small amount of sterile brain mash medium. Only small amounts of gas were produced by these organisms when grown in this medium. The most reliable criterion of growth seemed to be turbidity. This was transient and lasted from a day or two [redacted] culture by feeding a portion of it to a guinea pi[redacted]

Sodium nitrite was added to the medium in the following concen[redacted]tions: [redacted]149, 0.187, 0.2234, 0.239, 0.299, 0.337, 0.374, and 0.449%. After sterilization and the preliminary incubation, sodium nitrite determinations were made on representative tubes from each lot of the medium. The concentrations thus obtained were as follows: 0.1372, 0.1563, 0.2352, 0.2744, 0.3528, 0.392, 0.490, and 0.588%.

Results: Both growth and toxin formation showed considerable irregularity. Visible growth appeared to be inhibited in six out of seven of Clostridium botulinum cultures by the lowest concentration of sodium nitrite (0.1372%) and the two highest ones (0.490 and 0.588%). All of them grew in this medium containing 0.2352 and 0.2744%. Clostridium putrificum and Clostridium sporogenes cultures grew more evenly. The only concentration that showed appreciable inhibition for the latter organisms was the highest one. Of the entire group of cultures, only three grew in this concentration (0.588%).

Toxin formation, while quite irregular, yielded more understandable results. If the "skips" are disregarded, the lowest concentration which prevented toxic Clostridium botulinum cultures was 0.3528%, and the lowest one which inhibited all toxin formation was 0.490% of sodium nitrite. Toxin formation and growth did not necessarily show simultaneously in a culture. For instance, Clostridium botulinum failed to show growth, except in one instance, in 0.1372%, but all but one of these cultures formed toxin in this concentration. On the other hand, cultures 7, 8, and 9 grew in 0.3528%, but failed to form toxin when this amount of sodium nitrite was present. Occasional failure of growth to accompany the appearance of toxin in cultures may be explained by the fact that the former is not always easily determined visually.

Glucose Broth: Glucose broth was made up in lots containing 0.149, 0.187, 0.2243, 0.239, 0.299, 0.337, 0.374, and 0.449% of sodium nitrite (before sterilization). It was inoculated, as in the previous experiments, with approximately ¼ cc. of heated, uniform spore suspensions of the organisms. The incubation period was two weeks in the 37° C. incubator and several months at room temperature. Growth was detected by gas formation and turbidity of the medium. Considerable gas was formed in this medium. Turbidity, although transient, was also important evidence of development. A portion of each of the Clostridium botulinum cultures was fed to a guinea pig in order to detect presence of toxin.

The concentration of sodium nitrite was determined after sterilization and two weeks preliminary incubation. It was found that the amounts of sodium nitrite present at the time of inoculation were: 0.1372, 0.1764, 0.2352, 0.2744, 0.294, 0.392, 0.490, and 0.588%. These concentrations correspond to a range of 1,372 parts per million to 5,880 parts per million.

Results: Under the conditions of this experiment, the repressing effect of nitrite was extremely irregular. The tubes that showed growth were scattered over the entire range excepting the last concentration (0.588%). Fewer tubes among those inoculated with Clostridium botulinum showed growth than among those inoculated with Clostridium putrificum and Clostridium sporogenes. Only one culture of Clostridium botulinum showed growth in concentrations

...ce, but it is doubtful whether significance can ...ation. The highest concentration, 0.588%, pre- ... of all the organisms used.

...idium botulinum formed toxin in the lowest concen- ...nitrite in all cases, and two strains formed it in the next ...n (0.1764%). None of the tubes containing 0.2352 and 0.2744% ...e, but cultures 6 and 8 formed toxin in 0.294% and culture 5 in 0.392% of sodium nitrite. None of the cultures formed toxin ...d 0.588%, the highest concentrations used.

Meat Medium: Tubes of sterile egg-meat medium were with detoxified spore suspensions of the organisms. The same detoxified spore suspension (¼ cc.) was introduced into the bot- tubes by means of Pasteur pipettes. The inoculated cultures ...ted for two weeks at 37° C. and at room temperature for several ...ubes which had been inoculated with Clostridium botu- ...ere tested for toxicity by feeding portions of the contents to guinea

... nitrite was added to the medium in such amounts that the ...ns were: 0.149, 0.187, 0.2243, 0.239, 0.299, 0.337, 0.374, and ... weight of the C. P. salt. Colorimetric determinations of the so- ... were made at the time of inoculation, and the following amounts ... 0.0588, 0.0882, 0.1862, 0.1862, 0.1862, 0.2156, 0.3724, and 0.392%.

... Sodium nitrite failed to completely inhibit growth in any of ...rations used in egg-meat medium. The amount of growth was ...n in the corresponding controls in most cases as judged by the ...gas present in the respective tubes. Growth also appeared later ...s containing sodium nitrite than it did in those containing none

... was produced by the Clostridium botulinum cul- ...most irregular manner: Only three of the seven strains did not ...ps". Four of the seven strains were inhibited by concentrations ...2% (0.1862—0.392%). One of the remaining strains produced ...e highest concentration used, which was 0.392%.

Infusion: Tubes of sterile pork infusion medium were ...ith uniform suspension of detoxified spores. The same cultures ... in the other experiments. The inoculated tubes were incubated ... two weeks and were then held at room temperature for several ...owth was said to have occurred when gas was present, when the meat had taken place, and when a detectable odor was pre- formation in the tubes inoculated with Clostridium bo- ... was detected by feeding portions of the contents to guinea pigs. ...dium was prepared in lots containing 0.149, 0.187, 0.2243, 0.239, ..., 0.374, and 0.449% by weight of C. P. sodium nitrite. After ...and preliminary incubation, colorimetric determinations of the ...itrite were made. It was found that the concentrations present ...f inoculation were: 0.0588, 0.0784, 0.1372, 0.1470, 0.1666, 0.1764, 0.3136%.

... Complete inhibition of growth in any culture with any concen- ...dium nitrite used was not observed. In most cases, less gas ...n cultures containing the compound than in cultures without it, ...nd of the incubation period, growth was evident in all of them.

Toxin formation by Clostridium botulinum was affected to some ...nt by sodium nitrite, but so many irregularities were observed, that definite conclusions cannot be drawn as to the effective concentration of the compound in this medium. Two strains formed potent toxin in the highest concentration used (0.3136%). One strain (culture 6) produced toxin in pork infusion medium throughout the entire range of sodium nitrite concentrations tested.

Table 1. Strains showing growth.

Pork Infusion		Egg Meat Medium		Nutrient Bouillon		Glucose Bouillon	
% $NaNO_2$		% $NaNO_2$		% $NaNO_2$		% $NaNO_2$	
0	All strains	0	All strains	0	All strains	0	All strains
0.0588	,, ,,	0.0588	,, ,,	0.1372	6, 10, 11, 12, 13, 14	0.1372	6, 9, 10, 11, 12
0.0784	,, ,,	0.0882	,, ,,	0.1568	6, 7, 8, 10, 12, 13, 14	0.1764	2, 9, 14
0.1372	,, ,,	0.1862	,, ,,	0.2352	All strains	0.2352	2, 5, 6, 9, 10, 11, 12, 13, 14
0.1470	,, ,,	0.1862	1, 2, 5, 7, 8, 9, 10, 11, 12, 14	0.2744	,, ,,	0.2744	1. 5, 8, 9, 10, 11, 12, 13, 14
0.1666	,, ,,	0.1862	All strains	0.3528	2, 6, 7, 8, 9, 10, 12, 13, 14	0.2940	1, 2, 6, 9, 10, 11, 12, 13
0.1764	,, ,,	0.2156	,, ,,	0.3920	1, 2, 5, 6, 8, 9, 10, 11, 12, 13, 14	0.3920	9, 11, 12, 13, 14
0.2548	,, ,,	0.3724	,, ,,	0.4900	6, 10, 11, 12, 13, 14	0.4900	7, 9, 10
0.3136	,, ,,	0.3920	,, ,,	0.5880	6, 10, 14	0.5880	

Table 2. Strains of Clostridium botulinum producing toxin.

Pork Infusion		Egg Meat Medium		Nutrient Bouillon		Glucose Bouillon	
% $NaNO_2$		% $NaNO_2$		% $NaNO_2$		% $NaNO_2$	
0	1, 2, 5, 6, 7, 8, 9	0	1, 2, 5, 6, 7, 8, 9	0	1, 2, 5, 6, 7, 8, 9	0	1, 2, 5, 6, 7, 8, 9
0.0588	1, 6, 8	0.0588	1, 2, 5, 6, 7, 8	0.1372	1, 2, 6, 7, 8, 9	0.1372	1, 2, 5, 6, 7. 8, 9
0.0784	1, 2, 6, 7, 8	0.0882	1, 6, 7	0.1568	2, 5, 6, 7, 8, 9	0.1764	1, 7
0.1372	1, 6	0.1862	1, 5	0.2352	1, 5, 7, 8, 9	0.2352	
0.1470	2, 5, 6	0.1862	1, 2	0.2744	2, 5, 6, 7, 8, 9	0.2744	
0.1666	5, 6, 9	0.1862	5	0.3528	2, 6	0.2940	6, 8
0.1764	5, 6, 7	0.2156	2	0.3920	1, 2, 5, 6	0.3920	5
0.2548	6, 7	0.3724	1	0.4900		0.4900	
0.3136	6, 7	0.3920	1	0.5880		0.5880	

Discussion: The discrepancy between the concentrations of sodium nitrite added and found upon analysis presents a different problem than in the case of sodium chloride and sodium nitrate. In plain broth and glucose broth, analysis showed lowered nitrite contents for the two lowest concentrations. The third in the series showed approximate equality, although the figures obtained by analysis are slightly higher. In plain broth the higher percentages showed more sodium nitrite upon analysis than was

[illegible] This is also true of glucose broth except in the high [illegible] where the values are approximately the same. This might [illegible] follows: A small amount of sodium nitrite is destroyed by [illegible]. In the lower concentrations this is not entirely made up by [illegible], but in the higher concentrations the small quantity destroyed is not as important a factor as the comparatively large increase due to evaporation. When the substrate used was either egg-meat or pork-infusion media the concentration of sodium nitrite, found upon analysis, was always smaller than the amount added to begin with. The loss amounted to 0.1—0.15% over the entire range. It is attributed to the destruction of sodium nitrite by the meat protein in the medium which presumably acted as a reducing agent and the heat of sterilization. A comparison of the final sodium nitrite values obtained after adding the same amounts of this salt to the various media is given below:

Amount secured by Analysis after Sterilization.

Amount added %	Glucose Broth %	Plain Broth %	Pork Infusion %	Egg-Meat %
0.149	0.1372	0.1372	0.0588	0.0586
0.187	0.1764	0.1568	0.0784	0.0882
0.2243	0.2352	0.2352	0.1372	0.1862
0.239	0.2744	0.2744	0.1470	0.1862
0.299	0.2940	0.3528	0.1666	0.1862
0.337	0.3920	0.3920	0.1764	0.2156
0.374	0.4900	0.4900	0.2548	0.3724
0.449	0.5880	0.5880	0.3136	0.3920

The results of these four experiments tend to show that the amounts of sodium nitrite used cannot be relied upon to suppress either growth or toxin production by Clostridium botulinum, or to prevent the growth of Clostridium putrificum and Clostridium sporogenes. It is evident that the higher concentrations of sodium nitrite do not allow the formation of as many toxic cultures as the low concentrations. However, these data must be interpreted with caution, for each strain may have been harmless for guinea pigs when grown in some concentrations, and yet form a potent toxin in tubes containing higher concentrations of sodium nitrite. In the case of glucose or nutrient broth, it seems safe to conclude that 0.490 per cent and 0.588 per cent, in either of these media, completely represses toxin formation under the conditions of the experiment. As far as growth is concerned, inhibition of nine out of twelve cultures in plain broth, and of all the cultures in glucose broth by 0.588 per cent of sodium nitrite were the only definite evidence of a harmful action on the part of this salt. When it is considered that the amount of sodium nitrite in the tests where a meat medium was used ranged from about three times to about 19 times the maximum allowed in finished cured meats, it is evident that reliance cannot be placed upon this salt alone to prevent spoilage by such organisms as were used here, when other conditions are favorable for their growth.

Conclusions.

1. Concentrations of sodium nitrite from 0.1372 to 0.588% in plain broth cannot be relied upon to prevent growth of all strains of Clostridium botulinum, Clostridium putrificum, and Clostridium sporogenes.

2. A concentration of 0.588% of sodium nitrite in glucose broth inhibited twelve strains of putrefactive anaerobes, including: Clostridium botulinum types A and B, Clostridium putrificum, and Clostridium sporogenes.

3. Concentrations of 0.490 and 0.588% completely inhibited toxin formation by Clostridium botulinum types A and B in both plain broth and glucose broth.

4. Concentrations of sodium nitrite from 0.0588 to 0.3920% failed to inhibit Clostridium botulinum, Clostridium putrificum, and Clostridium sporogenes in either pork infusion of egg-meat medium under the conditions of the experiments.

5. Concentrations of 0.0588 to 0.3920% did not prevent toxin formation by all strains of Clostridium botulinum when grown in either egg-meat medium or in pork infusion.

6. The concentrations of sodium nitrite present in cured meat cannot be relied upon, in itself, to prevent spoilage or toxin formation by these organisms.

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Über den Einfluß der Temperatur und der Jahreszeit auf die biologische Abwasserreinigung.

[Aus dem Laboratorium der städtischen Kläranlagen in Leipzig.]

Von Karl Viehl, unter Mitarbeit von Bernhard Meißner.

Mit 3 Abbildungen im Text.

Die vorliegende Arbeit stellt einen Versuch dar, die Wirkung der Temperatur und der Jahreszeit auf die biologische Abwasserreinigung, insbesondere das Belebtschlammverfahren in chemischer und biologischer Hinsicht zahlenmäßig zu erfassen. Außerdem wurde versucht zu ermitteln, ob bei diesem Prozeß noch eine jahreszeitliche Schwankung vorliegt, die unabhängig von der Temperatur ist. Dies schien besonders in biologischer Hinsicht von Interesse, weil bei der Abwasserreinigung die Bedingungen — abgesehen von der Temperatur, die sich aber nur verhältnismäßig wenig ändert, und einer geringen jahreszeitlichen Schwankung in der Zusammensetzung des Abwassers — keinen durch den Wechsel der Jahreszeiten hervorgerufenen regelmäßigen Schwankungen unterliegen, vielmehr in dieser Hinsicht so konstant sind, wie dies selten in der Natur der Fall ist. Dabei

A New Method for the Decipherment of Charred Documents

In view of the widespread destruction by fire of documents of all kinds which is taking place at the present time, we think it desirable to put on record the preliminary results of some experiments in progress at this laboratory on the decipherment of charred documents. In spite of its topical interest, the subject is not a new one[1]; but we have already obtained results which we think, taking into consideration the simplicity and general applicability of the method, mark a distinct advance on anything hitherto recorded.

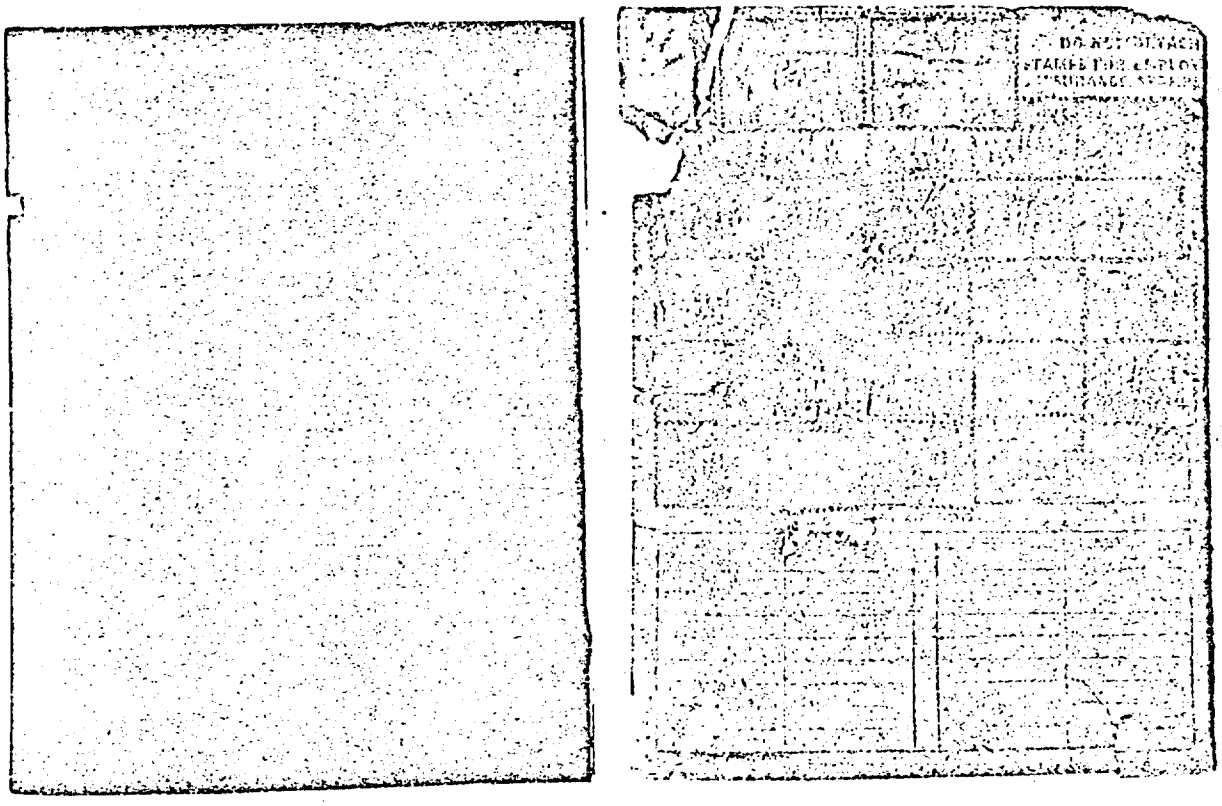
Briefly, the method consists in treating the document with chloral hydrate; this substance appears to exert an as yet unexplained 'clarifying' action on the burnt figures or letters. The chloral hydrate is applied in a 25 per cent alcoholic solution; after several applications, the document being dried at 60° C. between each, a mass of chloral hydrate crystals will form on the surface, and at this stage a similar solution containing 10 per cent glycerine is applied and the document dried as before. It may then be photographed. The most suitable type of plate is a contrasty non-colour sensitive one (for example, Ilford 'Thin Film Half-Tone').

The accompanying illustration shows the type of results obtained. The method is equally satisfactory for typescript and for printing, and with certain modifications has been found to restore writing. Furthermore, the reading matter is restored equally on both sides of the paper.

Certain distinct advantages which are possessed by this method are: (1) it appears to be applicable to any type of document; (2) it is fairly quick; (3) it has never yet in our hands failed to produce a readable result; (4) it requires no special apparatus other than a copying camera.

It has so far eluded us to find a satisfactory explanation in physical or chemical terms of this action by chloral hydrate; but it is hoped that this problem will also be solved. Such a solution ought to put the method on a rational basis, and open the way for further advances. The allied problem of rendering the burnt document less brittle and more easy to handle during treatment is also being attacked.

We hope to publish a more complete account of these experiments in due course.



PAGE OF AN UNEMPLOYMENT INSURANCE BOOK CHARRED DURING THE LONDON FIRE ON DECEMBER 29; BEFORE TREATMENT (LEFT) AND AFTER TREATMENT (RIGHT).

Our thanks are due to Dr. James Davidson, director of this laboratory, for permission to publish these results.

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[1] See, for example, Lucas, "Forensic Chemistry and Scientific Criminal Investigation", 3rd. edn., 142 (1935); Ainsworth Mitchell, "Inks", 3rd. edn., 173 (1924); Tyrrell, *J. Criminal Law and Criminology*, 30, 236 (1939). (The first two sources give a number of references to earlier publications on the subject.)

Bacteriostatic Action of Nitrates

ALTHOUGH nitrites have been used officially in meat curing processes for well over a decade in the United States[1], it is only comparatively recently that their use has been permitted in Great Britain[2]. Un-

doubtedly, the predominant reason for their inclusion in cured meats has been the fact that they occasion a desirable colour in the finished product[3]; but it appears that they may also play an important part in producing a typical "cured" flavour[2]. When consumed directly, quantities of sodium nitrite in excess of 0·2 gm. may produce marked toxic effects in man[4] so that their use in foods must be strictly controlled. For this reason pure food regulations both on the North American continent and in Great Britain forbid the inclusion of more than 0·02 per cent (200 parts per million) of sodium nitrite in cured meats.

Very little attention appears to have been paid to the fact that nitrites may, under certain conditions, play an important part in retarding bacterial development on, and thus delay the spoilage of, meats and related products. Lewis and Moran[5] observed that growth of the mixed bacterial flora of minced beef in experimental brines was retarded by 0·03–1·5 per cent of sodium nitrite. Their work, as well as that of Tanner and Evans[6], indicates that the growth of strict anaerobic bacteria of the genus Clostridium is inhibited only by relatively large amounts of nitrite. Other investigators[7,8] have observed that 0·4 per cent of sodium nitrite inhibits the growth of certain facultative anaerobes. Thus most of the available literature has indicated that it is only rather large concentrations of nitrite, usually greatly in excess of the 0·02 per cent permitted by law, which will hinder or prevent bacterial development.

About two years ago experiments were carried out at this Station in which it was shown that bacterial spoilage of fish flesh is strongly delayed by incorporation of small amounts of nitrite salts[9]. The reason for this was sought, and it was found that bacteria are only susceptible to low concentrations of nitrites at *p*H values below 7·0, and that the muscle of fish shortly after capture becomes sufficiently acid (for example, *p*H 6 to 6·6) for these salts to exert their bacteriostatic action[10]. Not all bacteria studied have proved equally susceptible to nitrite, and certain organisms do not appear to be inhibited. However, growth of species of the following bacterial genera at *p*H 5·7 or 6·0 has either been markedly inhibited, or in certain instances entirely prevented, by 0·02 per cent of sodium nitrite: Achromobacter, Flavobacterium, Pseudomonas, Micrococcus, Escherichia and Aerobacter (also one species of Torula)[11].

Attempts have been made to determine by what mechanism nitrites exert their inhibitory action. The possibility that they act by combining with respiratory haematin compounds suggested itself, especially in view of the fact that Ingram[12] has found that oxygen uptake in the case of the aerobic, cytochrome-containing organism, *Bacillus cereus*, is very strongly inhibited by traces of nitrite. However, it has been found that there is no apparent relationship between inhibition of growth by nitrite and its effect on respiration. Thus in certain instances growth of a given organism was strongly or completely inhibited at *p*H 6·0 by 0·02 per cent sodium nitrite, whereas under similar conditions its aerobic respiration was not significantly inhibited[11]. The possibility that salts of this type inactivate certain enzymes by combining with their amino groups as was originally suggested by Quastel and Wooldridge[13] must not be overlooked. Since it has recently been shown that certain organisms remain viable after exposure to 38·8 per cent sodium nitrite at *p*H 3·8 for ten minutes[14], it would appear rather unlikely that nitrites are able to act in a bactericidal capacity.

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β-Glucosaminase in Testes

DIFFUSING factor preparations from various sources cause a rapid decrease in the viscosity of certain mucins (for example, hyaluronic acid) and if contact is maintained for a longer period hydrolysis of the polysaccharide can be detected by an increase in the reducing power of the mixture[1]. Since the mucins in question are believed to be built up of N-acetylglucosamine and uronic acid residues it seemed likely that an enzyme capable of hydrolysing them would also hydrolyse other N-acetylglucosaminides. It has, in fact, been demonstrated (see accompanying table) that a concentrate of testicular diffusing factor[2] will hydrolyse N-acetyl-β-phenylglucosaminide much more readily than any other glycoside examined.

	Time of incubation at 37° C. (hr.)			
	1	2	6·5	24
	% hydrolysis (Bertrand)			
N-acetyl β-phenyl glucosaminide	4·6	9·3	18·7	24·4
β-phenyl glucoside	1·3	1·3	1·8	3·3
Salicin	0	0	1·5	1·8
Sucrose	1·7	1·7	3·3	2·7
β-menthyl glycuronide	0	0	0	0

The optimum *p*H for this β-glucosaminase (4·8) is close to that at which diffusing factor preparations exert their maximum effect on the viscosity of hyaluronic acid[3]. There are, however, certain facts which suggest that the two effects—decrease in viscosity and increase in reducing power—may not necessarily be due to the same enzyme. When mucins of the hyaluronic acid type are treated with *M*/5 HCl or *M*/5 NaOH at 25° C. a gradual and irreversible decrease in viscosity occurs. Glycosidic linkages are, as a rule, stable to alkali under these conditions, and it may well be that the viscous material contains linkages of some other type. Moreover, crude emulsin preparations which show β-glucosaminase activity do not affect the viscosity of hyaluronic acid.

A more detailed account of the β-glucosaminase activity of testicular extracts and its bearing on the problem of diffusing factors will be published elsewhere.

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The Action of Nitrites on Bacteria: Further Experiments

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ABSTRACT

Growth of the obligate anaerobes *Clostridium botulinum* and *C. sporogenes* is inhibited by 0.02 per cent sodium nitrite in fish digest broth at acid pH, inhibition not being accentuated by brief exposure of the cultures to air during the vigorous growth phase. Sodium nitrite inhibits growth of facultative anaerobes in nutrient broth under both strictly and semi-anaerobic conditions. Growth of facultative anaerobes is inhibited by sodium nitrite over a much wider pH range in nutrient broth than in fish digest broth or in a simple synthetic ammonium lactate medium. Sodium nitrite is capable of acting in either bactericidal or bacteriostatic capacity under different conditions, and inhibits growth of the human pathogens *Eberthella typhosa* and *Staphylococcus aureus*. The experiments described throw no light on the mechanism by which sodium nitrite, or its decomposition products, inhibit bacterial growth.

In previous publications (Tarr and Sunderland 1940, Tarr 1941) it was shown that sodium nitrite inhibited growth of bacteria in culture media and in fish muscle only when these subtrates possessed a somewhat acid reaction. The mechanism by which nitrite inhibited growth was not ascertained, but it was demonstrated that its action depended little, if at all, on its inhibitory effect on aerobic respiratory catalysts.

EXPERIMENTAL

Cultures

Certain of the organisms employed were those used in previous work (Tarr 1941), culture 4a being the strain of *Pseudomonas* formerly referred to as culture 4. The following organisms, obtained from the American Collection of Type Cultures, were also used: *Staphylococcus aureus* (no. 152), *Eberthella typhosa* (no. 167), *Clostridium botulinum* (no. 687) and *Clostridium sporogenes* (no. 459).

Media

FISH DIGEST BROTH

Defrosted halibut muscle was digested with Bacto trypsin following the procedure employed in preparing beef digest broth (Hartley 1922). The finished medium (pH 5.9) was stored under toluene. Prior to use portions of this medium

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were adjusted to desired pH values by addition of 1.0 normal HCl or NaOH. They were then heated in flowing steam, cooled, and filtered through a Seitz filter in order to remove bacterial cells and any insoluble precipitated protein material which might interfere with direct bacterial counts (*vide infra*). Immediately prior to use, 2-ml. portions of the filtered medium were sterilized in 12 by 150-mm. culture tubes. This medium, being naturally well buffered, required no added buffer solution.

BUFFERED NUTRIENT BROTH

Eight grams of Bacto dehydrated nutrient broth were dissolved in 1 litre of distilled water and adjusted to pH 5.2 by the addition of 3.5 ml. of 1.0 normal HCl, 1.5-ml. portions being dispensed into 12 by 150-mm. culture tubes. Prior to use, 0.5 ml. of sterile 0.2 molar Na_2HPO_4-KH_2PO_4 buffer (different pH values) was added to each tube of broth.

SYNTHETIC MEDIUM

This had the following composition: Lactic acid (Baker's A.R.) 5 ml., NaCl 1.0 g., trace of $MgSO_4$ and $FeCl_2$, strong (28 per cent) NH_4OH to adjust the solution to pH 5.0; 1.5-ml. portions were dispensed into 12 by 150-mm. culture tubes, sterilized, and adjusted to various pH values by addition of phosphate buffer as in the case of the nutrient broth medium.

For all media employed, the pH as reported in the various tables is that of the medium as diluted by addition of nitrite solution (or water) and the inoculum. It was found that with well-buffered media of this type the addition of 0.02 per cent of $NaNO_2$ did not occasion a significant change in pH.

TECHNIQUE OF INOCULATION AND INCUBATION

The facultative anaerobes were grown for 16 to 24 hours in a medium (pH 7.0) of the same type as that into which they were to be inoculated. The obligate anaerobes were grown for 24 hours in fish digest broth (pH 7.0) in cases where an inoculum consisting largely of vegetative cells was required, and for 7 days in the same medium in order to obtain cultures consisting mainly of endospores. Saline suspensions of the organisms for inoculation purposes were prepared by suitably diluting such cultures with sterile 0.01 per cent NaCl solution. Suspensions containing mainly endospores of the obligate anaerobes were heated at 80°C. for 10 minutes in order to destroy any vegetative cells which might be present. In the various experiments to be described individual tubes of medium were each inoculated with 0.5 ml. of a saline suspension of the organism being studied. Preparation and addition of $NaNO_2$ solutions to the various media, pH measurements, direct and viable bacterial counts and examination of inoculated cultures were carried out as described in previous publications (Tarr 1941, Tarr and Bailey 1939). Cultures of *C. botulinum*, *C. sporogenes*, *S. aureus* and *E. typhosa* were incubated at 37°C., the remainder being incubated at 25°C. Strictly anaerobic conditions were maintained by using McIntosh and Fildes' jars, employing the technique of removing some of the air by partial evacuation prior to letting in hydrogen and carrying out the combustion.

Inhibition in Fish Digest Broth

Obligate anaerobes

Experiment 1. Each tube of one set of fish digest broth medium was inoculated with approximately 17.7×10^6 *C. botulinum* organisms (largely vegetative cells or sporulating vegetative cells), the rate of growth being determined at intervals. The results of this experiment (table I) show that 0.02 per cent $NaNO_2$ markedly inhibited growth of this organism at pH values below 7.0, multiplication being entirely prevented at pH 4.6 to 5.6 even after 14 days.

Experiment 2. Two sets of fish digest broth were inoculated with *C. botulinum*, each tube receiving approximately 2.5×10^6 spores. One set was incubated anaerobically for 14 days, the other being similarly treated except that after the first day the medium was in each case exposed to air and shaken well in order to make bacterial counts, thus permitting access of air. The results (table II), show that 0.02 per cent $NaNO_2$ inhibited growth in both cases. It will be seen that, in the cultures exposed to air, growth did not occur below pH 6.1, while in the foregoing experiment under similar conditions a larger inoculum consisting mainly of vegetative cells caused growth even at pH 4.6. When the two sets are compared it will be seen that growth occurred at a much lower pH in both control and nitrite-treated cultures which were not exposed to air.

Experiment 3. Each tube of a set of fish digest broth was inoculated with approximately 19.6×10^6 *C. sporogenes* organisms (largely vegetative cells or sporulating vegetative cells), the rate of growth being determined at intervals

Table I. Growth of *C. botulinum* in fish digest broth of different pH with and without 0.02 per cent $NaNO_2$. (Inoculum 17.7×10^6 organisms, mainly vegetative cells; figures for growth are in millions per ml. by direct count).

Initial pH of medium	Growth after the following incubation periods							
	1 day		2 days		7 days		14 days	
	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$
3.60	0	0	0	0	0	0	0	0
4.10	0	0	0	0	0	0	0	0
4.60	0	0	0	0	+	0	+	0
5.10	0	0	0	0	+	0	+	0
5.60	0	0	0	0	+	0	+	0
6.10	0	0	257	0	+	+	+	+
6.60	80	4	306	0	+	+	+	+
7.05	360	5.5	*155	180	+	+	+	+
7.50	296	330	*117	*119	+	+	+	+
7.80	224	219	* 36	* 60	+	+	+	+

*Decrease due to autolysis of vegetable cells; mainly spores remaining.
0 = No visible growth or increase in original inoculum.
+ = Positive growth by visual inspection.

TABLE II. Growth of *C. botulinum* in fish digest broth with and without 0.02 per cent $NaNO_2$ (Inoculum 2.5×10^6 spores; figures for growth are in millions per ml. by direct count).

Initial pH of medium	Medium shaken in presence of air in making direct counts on first day of experiment				Completely anaerobic conditions throughout experiment	
	Growth observed after the following incubation periods				Growth observed after incubating for 14 days	
	1 day		14 days			
	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$
3.60	0	0	0	0	0	0
4.10	0	0	0	0	+	0
4.60	0	0	0	0	0	+
5.10	0	0	0	0	+	0
5.60	0	0	0	0	+	+
6.10	3	0	+	0	+	+
6.60	125	0	+	0	+	+
7.05	225	0	+	+	+	+
7.50	230	226	+	+	+	+
7.80	219	202	+	+	+	+

and the organisms thus being exposed to air. The results (table III) show that nitrite inhibited growth of this organism, the general effect being similar to that obtained in experiment 1 with *C. botulinum*.

Experiment 4. Two sets of fish digest broth were inoculated with approximately 5.2×10^6 spores of *C. sporogenes* and treated as in experiment 2. The results (table IV) were very similar to those obtained in the case of *C. botulinum*. They show that large inocula consisting chiefly of vegetative cells (compare with table III) caused growth at lower pH values than did small inocula of spores, and that growth occurred at much lower pH values in cultures not exposed to air than in those exposed thereto. Nitrite inhibited growth over about the same range of pH in cultures exposed, or not exposed, to air.

It will be noticed that in certain of the experiments with anaerobes somewhat irregular results were obtained in the case of cultures not exposed to air during growth (see tables II and IV). Thus growth in one instance occurred at pH 4.6 but not at either pH 4.1 or at pH values between 5.1 and 6.1 The reason for this is not clear, but it is not altogether unexpected, for it is known that obligate anaerobes are rather fastidious with respect to their nutrient requirements, even small amounts of CO_2 being required for growth (Knight 1941). Minor differences in CO_2 content of the medium might thus account for the observed irregularity.

FACULTATIVE ANAEROBES

Experiment 5. One set of fish digest broth was inoculated with each of three

TABLE III. Growth of *C. sporogenes* in fish digest broth of different pH with and without 0.02 per cent $NaNO_2$ (Inoculum 19.6×10^5 organisms, mainly vegetative cells; figures for growth are in millions per ml. by direct count).

Initial pH of medium	Growth after the following incubation periods							
	1 day		2 days		7 days		14 days	
	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$
3.60	0	0	0	0	0	0	0	0
4.10	0	0	0	0	0	0	0	0
4.60	0	0	0	0	0	0	0	0
5.10	0	0	0	0	0	0	+	0
5.60	0	0	189	0	+	0	+	0
6.10	0	0	209	0	+	0	+	0
6.60	0	0	288	0	+	0	+	0
7.05	0	0	307	0	+	0	+	+
7.50	20	7	198	152	+	+	+	+
7.80	40	0	173	203	+	+	+	+

TABLE IV. Growth of *C. sporogenes* in fish digest broth of different pH with and without 0.02 per cent $NaNO_2$ (Inoculum 5.2×10^6 spores; figures for growth are in millions per ml. by direct count)

Initial pH of medium	Medium shaken in presence of air in making direct counts on first day of experiment				Completely anaerobic conditions throughout experiment	
	Growth observed after the following incubation periods				Growth observed after incubating for 14 days	
	1 day		14 days			
	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$
3.60	0	0	0	0	0	0
4.10	0	0	0	0	+	0
4.60	0	0	0	0	+	+
5.10	0	0	0	0	+	0
5.60	5	0	0	0	+	0
6.10	25	0	+	0	+	0
6.60	77	0	+	0	+	+
7.05	227	0	+	0	+	+
7.50	239	3	+	+	+	+
7.80	233	136	+	+	+	+

different organisms as follows: (1) *S. aureus*, 1.6×10^6 organisms per tube of medium. (2) *Culture 6* (*Flavobacterium* sp.), 4.8×10^6 organisms per tube of medium. (3) *Culture 9* (*Achromobacter* sp.), 1.6×10^6 organisms per tube of

medium. The tubes were incubated as usual, bacterial counts being made at intervals. The results (tables V, VI, and VII) show that $NaNO_2$ retarded growth of all three organisms in medium of acid pH values. However, the retarding or inhibiting effect of the nitrite was much less marked, and did not extend over nearly such a wide range of pH as was observed when the same or different organisms were cultivated in ordinary nutrient broth (compare tables VIII to XIII).

TABLE V. Growth of *S. aureus* in fish digest broth of different pH with and without 0.02 per cent $NaNO_2$ (Inoculum 1.6×10^6 organisms; figures for growth are in millions per ml. by direct count).

Initial pH of medium	Growth after					
	6 hours		1 day		14 days	
	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$
3.60	0	0	0	0	0	0
4.10	0	0	0	0	0	0
4.60	0	0	10.5	0	+	0
5.10	0	0	66	0	+	0
5.60	3.8	0	225	67	+	+
6.10	39	20	374	340	+	+
6.60	51	45	400	413	+	+
7.05	54	49	394	402	+	+
7.50	49	51	302	292	+	+
7.80	56	54	253	259	+	+

TABLE VI. Growth of culture 6 (*Flavobacterium* sp.) in fish digest broth of different pH with and without 0.02 per cent $NaNO_2$ (Inoculum 4.8×10^6 organisms; figures for growth are in millions per ml. by direct count).

Initial pH of medium	Growth after					
	1 day		2 days		14 days	
	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$
3.60	0	0	0	0	0	0
4.10	0	0	0	0	0	0
4.60	0	0	0	0	0	0
5.10	0	0	0	0	0	0
5.60	9	0	40	0	+	0
6.10	34	0	75	0	+	0
6.60	43	32	72	68	+	+
7.05	42	31	80	83	+	+
7.50	44	28	83	85	+	+
7.80	31	32	82	81	+	+

TABLE VII. Growth of culture 9 (*Achromobacter* sp.) in fish digest broth of different pH with and without 0.02 per cent $NaNO_2$ (Inoculum 1.6×10^6 organisms; figures for growth are in millions per ml. by direct count).

Initial pH of medium	Growth after							
	6 hours		1 day		2 days		14 days	
	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$
3.60	0	0	0	0	0	0	0	0
4.10	0	0	0	0	0	0	0	0
4.60	0	0	0	0	0	0	0	0
5.10	0	0	0	0	0	0	0	0
5.60	0	0	0	0	316	0	+	0
6.10	1.8	0	208	0	604	150	+	+
6.60	9.8	5.2	337	332	+	+	+	+
7.05	7.9	8.5	360	358	+	+	+	+
7.50	5.6	8.4	385	379	+	+	+	+
7.80	7.7	8.1	350	358	+	+	+	+

INHIBITION IN NUTRIENT BROTH AND IN SYNTHETIC MEDIUM

Only certain of the organisms available were able to grow in the simple synthetic medium employed, the few strains of *Micrococcus* studied showing no evidence of growth. The cultures used were transferred twice in the synthetic medium prior to diluting them with saline solution and using them to inoculate the media.

Experiment 6. One set each of synthetic medium and of nutrient broth was inoculated separately with different organisms as follows: *Culture 9* (*Achromobacter* sp.), 3.6×10^6 organisms per tube of medium; *Culture 17* (*Achromobacter* sp.), 7.8×10^6 organisms per tube of medium; *Culture 4a* (*Pseudomonas* sp.), 2.7×10^6 organisms per tube of medium.

The cultures were incubated and determinations of the rate of growth were made as usual. The results (tables VIII, IX and X) show that growth of all three organisms was inhibited by $NaNO_2$ both in nutrient broth and in the synthetic medium. However, in every case growth occurred at a much more acid reaction, and nitrite inhibited over a much wider pH range, in nutrient broth than in the synthetic medium. Somewhat unexpected results were obtained with culture 4a when cultivated in the synthetic medium, for it was found that only at pH 5.80 did nitrite inhibit growth permanently. At pH values between 6.18 and 6.85 nitrite actually accelerated growth. The reason for this finding is not clear, but it is suggested that it may have been due to the fact that the nitrite acted as an additional hydrogen acceptor at certain pH values, thereby favouring growth. It is of interest in this connection to note that Meiklejohn (1940) showed that certain species of *Pseudomonas* carried out denitrification under aerobic as well as under anaerobic conditions, and that they reduced nitrate to nitrite and further to gaseous nitrogen.

TABLE VIII. Growth of culture 9 (*Achromobacter* sp.) in nutrient broth and in synthetic medium at different pH and with and without 0.02 per cent $NaNO_2$. (Inoculum 3.6×10^6 organisms; figures for growth are in millions per ml. by direct count).

Nutrient broth							Synthetic medium						
Initial pH of medium	Growth after						Initial pH of medium	Growth after					
	18 hours		2 days		14 days			18 hours		2 days		14 days	
	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$		Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$
5.12	0	0	41	0	+	0	4.44	0	0	0	0	0	0
5.42	4	0	190	0	+	0	4.48	0	0	0	0	0	0
5.58	39	0	388	0	+	0	4.54	0	0	0	0	0	0
5.88	126	0	368	0	+	0	4.60	0	0	0	0	0	0
6.10	46	4	372	35	+	+	4.85	0	0	0	0	0	0
6.30	152	60	390	380	+	+	5.20	2	0	0	0	+	0
6.50	150	106	381	360	+	+	5.80	89	0	188	0	+	0
6.72	154	145	369	378	+	+	6.18	89	3.3	251	44	+	+
6.88	144	150	377	160	+	+	6.42	98	39	305	194	+	+
7.09	140	142	359	388	+	+	6.70	95	85	300	301	+	+
7.30	137	139	398	402	+	+	6.85	91	84	296	292	+	+

TABLE IX. Growth of culture 4a (*Pseudomonas* sp.) in nutrient broth and in synthetic medium at different pH and with and without 0.02 per cent $NaNO_2$. (Inoculum 2.7×10^6 organisms; figures for growth are in millions per ml. by direct count).

Nutrient broth							Synthetic medium						
	Growth after							Growth after					
Initial pH of medium	18 hours		2 days		14 days		Initial pH of medium	18 hours		2 days		14 days	
	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$		Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$
5.12	261	0	780	0	+	0	4.44	0	0	0	0	0	0
5.42	285	0	762	0	+	0	4.48	0	0	0	0	0	0
5.58	295	0	730	0	+	+	4.54	0	0	0	0	0	0
5.88	262	17	770	246	+	+	4.60	0	0	0	0	0	0
6.10	277	85	830	530	+	+	4.85	0	0	0	0	0	0
6.30	267	223	798	690	+	+	5.20	0	0	0	0	0	0
6.50	299	306	805	746	+	+	5.80	0	0	37	0	+	0
6.72	315	285	810	808	+	+	6.18	0	8	160	37	+	+
6.88	287	265	806	812	+	+	6.42	2	29	67	98	+	+
7.09	302	266	820	765	+	+	6.70	2	37	82	265	+	+
7.30	318	292	790	770	+	+	6.85	0	54	46	351	+	+

TABLE X. Growth of culture 17 (*Achromobacter* sp.) in nutrient broth and in synthetic medium at different pH and with and without 0.02 per cent $NaNO_2$. (Inoculum 7.8×10^6 organisms; figures for growth are in millions per ml. by direct count).

Nutrient broth					Synthetic medium				
Initial pH of medium	Growth after				Initial pH of medium	Growth after			
	1 day		14 days			1 day		14 days	
	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$		Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$
5.12	57	0	+	0	4.44	0	0	0	0
5.42	67	0	+	0	4.48	0	0	0	0
5.58	72	0	+	0	4.54	0	0	0	0
5.88	78	0	+	0	4.60	0	0	0	0
6.10	80	0	+	+	4.85	0	0	0	0
6.30	95	32	+	+	5.20	4	0	+	0
6.50	103	82	+	+	5.80	61	0	+	0
6.72	101	100	+	+	6.18	66	0	+	+
6.88	111	103	+	+	6.42	73	32	+	+
7.09	106	99	+	+	6.70	96	47	+	+
7.30	92	75	+	+	6.85	89	75	+	+

INHIBITION UNDER STRICTLY AND SEMI-ANAEROBIC CONDITIONS

Experiment 7. Each tube in two sets of nutrient broth medium was inoculated with approximately 4.5×10^6 cells of *culture 6* (*Flavobacterium* sp.), one set being incubated under strictly anaerobic conditions, and the other under the usual semi-anaerobic conditions. A similar experiment was made using *culture 17* (*Achromobacter* sp.). The results (tables XI and XII) show that nitrite inhibited growth under both anaerobic and semi-anaerobic conditions, the only noticeable difference being that growth both in presence and absence of nitrite was never nearly as great under anaerobic as under semi-anaerobic conditions.

EFFECT OF SMALL INOCULA

Experiment 8. Each of the following four organisms was inoculated into a set of nutrient broth, individual tubes receiving approximately the number of bacteria given: (1) *Culture 9* (*Achromobacter* sp.), 120 organisms per tube. (2) *Culture 17* (*Achromobacter* sp.), 160 organisms per tube. (3) *Culture 4a* (*Pseudomonas* sp.), 550 organisms per tube. (4) *E. typhosa*, 190 organisms per tube. When the results of this experiment (table XIII) are compared with those given in tables VIII, IX and X for very much larger inocula of cultures 9, 17 and 4a, the following differences will be observed. In the case of cultures 9 and 17 nitrite inhibited growth completely at pH 5.88 or lower when large inocula were employed, whereas with the small inocula as used above growth was totally inhibited at pH 6.1 or lower. With culture 4a no difference was noticed when either large or small inocula were used. In the case of *E. typhosa* nitrite severely inhibited growth with the small inoculum employed.

TABLE XI. Growth of culture 6 (*Flavobacterium* sp.) in nutrient broth of different pH under anaerobic and semi-anaerobic conditions in the presence and absence of 0.02 per cent $NaNO_2$ (Inoculum 4.5×10^6 organisms; figures for growth are in millions per ml. by direct count).

Initial pH of medium	Anaerobic growth				Semi-anaerobic growth			
	1 day		14 days		1 day		14 days	
	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$
5.12	0	0	0	0	5	0	+	0
5.42	3.5	0	1.7	0	23	0	+	0
5.58	6.3	0	12.5	0	33	0	+	0
5.88	7.0	0	13.0	0	37	0	+	0
6.10	7.2	0	10.8	0	41	0	+	0
6.30	9.6	0	15.5	0	49	0	+	0
6.50	10.5	2	11.0	0	51	0	+	0
6.72	11.5	11.9	16.0	15.0	69	32	+	0
6.88	12.1	12.4	12.8	13.5	64	50	+	+
7.09	13.3	13.4	13.0	11.0	73	69	+	+
7.30	12.0	10.0	12.1	11.5	70	58	+	+

TABLE XII. Growth of culture 17 (*Achromobacter* sp.) in nutrient broth of different pH under anaerobic and semi-anaerobic conditions in the presence and absence of 0.02 per cent $NaNO_2$. Inoculum 7.8×10^6 organisms; figures for growth are in millions per ml. by direct count).

Initial pH of medium	Anaerobic growth				Semi-anaerobic growth			
	1 day		14 days		1 day		14 days	
	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$
5.12	23	0	+	0	57	0	+	0
5.42	41	0	+	0	67	0	+	0
5.58	60	0	+	0	72	0	+	0
5.88	71	0	+	0	78	0	+	0
6.10	95	0	+	+	80	0	+	+
6.30	99	0	+	+	95	32	+	+
6.50	114	9	+	+	103	82	+	+
6.72	115	15	+	+	101	100	+	+
6.88	109	30	+	+	111	103	+	+
7.09	105	98	+	+	106	99	+	+
7.30	92	90	+	+	92	75	+	+

TABLE XIII. Growth of small inocula of cultures 9, 17, 4a and *E. typhosa* in nutrient broth of different pH with and without 0.02 per cent $NaNO_2$. (Inocula: culture 9, 120; culture 17, 160; culture 4a, 550; and *E. typhosa*, 190 organisms; figures for growth are in millions per ml. by direct count).

Initial pH of medium	Culture 9		Culture 17		Culture 4a		*E. typhosa*			
	14 days		14 days		14 days		1 day		14 days	
	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$	Control	0.02% $NaNO_2$
5.12	0	0	+	0	+	0	+	0	+	0
5.42	+	0	+	0	+	0	+	0	+	0
5.58	+	0	+	0	+	+	+	0	+	0
5.88	+	0	+	0	+	+	+	0	+	0
6.10	+	0	+	0	+	+	+	0	+	+
6.30	+	+	+	+	+	+	+	+	+	+
6.50	+	+	+	+	+	+	+	+	+	+
6.72	+	+	+	+	+	+	+	+	+	+
6.88	+	+	+	+	+	+	+	+	+	+
7.09	+	+	+	+	+	+	+	+	+	+
7.30	+	+	+	+	+	+	+	+	+	+

BACTERICIDAL ACTION

In the strict sense of the terms a bactericidal substance is one which renders an organism incapable of further growth, while a bacteriostatic substance hinders growth but does not render the organism incapable of further growth. It is obvious that it is possible for a given compound to be at the same time bactericidal and bacteriostatic, depending on the concentration employed, sensitivity of different bacterial cells, and various other conditions. The foregoing experiments have shown that sodium nitrite in 0.02 per cent concentration exerts a pronounced bacteriostatic action, and that it inhibits growth entirely in this concentration under certain conditions. The following experiments show that sodium nitrite is capable of pronounced bactericidal activity.

Experiment 9. Petri dishes containing Bacto nutrient agar 1.5 times its normal strength were inoculated with culture 9 and incubated for 2 days at 25°C. The resulting growth was suspended in a large volume of 0.01 M sterile phosphate buffer (pH 7.2) and centrifuged rapidly under aseptic conditions, thus concentrating the bacterial cells. The supernatant liquid was discarded and the organisms suspended uniformly in similar buffer solution. The suspension, which contained 203×10^5 organisms per ml. (viable count), was used immediately subsequent to preparation as follows.

Five ml. of bacterial suspension were placed in each of ten sterile, cotton-plugged, 50 ml., round-bottom centrifuge tubes. Each of six of these tubes received 5 ml. of 1 molar KH_2PO_4 solution (pH 4.45), the remainder receiving 5 ml. of 1 molar phosphate buffer (pH 7.3). To certain of the tubes 5 ml. of 3 per cent sterile $NaNO_2$ were added, water being added to the others (see table

XIV). After mixing the contents of the tubes gently they were incubated at 22°C., examinations being made at intervals. In this way the organisms were exposed to 1 per cent $NaNO_2$ for varying periods of time under both acid and faintly alkaline conditions.

The approximate number of organisms surviving the treatment was determined as follows. The tubes were centrifuged rapidly for 15 minutes in order to concentrate most of the bacterial cells, the faintly opalescent supernatant liquid being discarded after determining its pH. The precipitated organisms were resuspended in 15 ml. of sterile 0.01 M phosphate buffer (pH 7.2), mixed thoroughly, and viable bacterial counts made. It will be noticed (table XIV) that the pH of the solutions in the acid range varied between pH 5.55 and 5.80, and in the alkaline range between pH 7.20 and 7.50. Two factors appear to contribute to this slight variation, namely a decrease in pH due to the addition of $NaNO_2$, and an increase in alkali resulting from incubation of the organisms in the absence of nitrite. It will be seen that 1 per cent $NaNO_2$ caused a very rapid fall in the numbers of viable bacteria in suspensions maintained at acid pH value. On the other hand above pH 7 it exhibited only a very slight bactericidal action.

Experiment 10. This experiment was carried out similarly to the foregoing one except that the bactericidal activity of 10 per cent and 0.02 per cent $NaNO_2$ was tested. The suspension of cells of culture 9 used contained approximately 229×10^6 organisms per ml. (viable count). In the case of bacteria exposed to

TABLE XIV. Influence of pH on bactericidal action of sodium nitrite using culture 9 (*Achromobacter* sp.) as test organism.

$NaNO_2$ concentration %	Period of exposure	pH of exposure (supernatant liquid after centrifuging)	Viable bacteria in colonies per ml.
0	30 min.	5.55	$9{,}300 \times 10^6$
0	24 hr.	5.80	$5{,}670 \times 10^6$
1	30 min.	5.60	$1{,}620 \times 10^6$
1	2 hr.	5.60	137×10^6
1	6 hr.	5.60	4.1×10^6
1	24 hr.	5.60	0
0	30 min.	7.40	$12{,}300 \times 10^6$
0	24 hr.	7.50	$9{,}200 \times 10^6$
1	30 min.	7.20	$6{,}500 \times 10^6$
1	24 hr.	7.20	$3{,}500 \times 10^6$

10 per cent $NaNO_2$ the suspension was centrifuged a second time after suspending in phosphate buffer before preparing the final suspension, in order to remove the rather large amount of nitrite present in the first suspension. Bactericidal action was studied only in acid medium since the foregoing experiment showed that $NaNO_2$ (1 per cent) merely exerted minor bactericidal activity above pH 7. In order to ascertain whether osmotic or other effects due to high salt concentration were causing inhibition parallel tests were conducted with 10 per cent NaCl.

TABLE XV. Bactericidal action of 0.02 per cent and 10 per cent $NaNO_2$ using culture 9 (*Achromobacter* sp.) as test organism.

$NaNO_2$ concentration %	Period of exposure	pH of exposure (supernatant liquid after centrifuging)	Viable bacteria in colonies per ml.
0	30 min.	5.55	$6{,}000 \times 10^6$
0	24 hr.	5.75	$6{,}600 \times 10^6$
0.02	6 hr.	5.60	$4{,}700 \times 10^6$
0.02	24 hr.	5.60	$3{,}900 \times 10^6$
10	30 min.	5.40	3,270
10	1 hr.	5.40	0
10	6 hr.	5.40	0

The results (table XV) show that exposure of the bacterial cells to 10 per cent $NaNO_2$ caused a very rapid decrease in the number of viable bacterial cells. Ten per cent NaCl exerted a negligible germicidal activity in comparison. In 0.02 per cent concentration $NaNO_2$ caused only a very slight decrease in number of viable organisms.

Experiment 11. An experiment similar to the above was carried out with culture 4, a species of *Micrococcus*, the growth of which had not been inhibited appreciably by 0.02 per cent $NaNO_2$ in previous experiments (Tarr 1941). The suspension employed contained approximately 165×10^8 organisms per ml. (viable count). The results (table XVI) show that both 1 and 10 per cent $NaNO_2$ exerted a markedly destructive effect upon the cells of this culture.

Experiment 12. In view of the fact that Bittenbender, *et al.* (1940) had reported that *S. aureus* survived exposure to 38.8 per cent $NaNO_2$ for 10 minutes this organism was also studied. The technique employed was similar to that outlined in previous experiments, the suspension of *S. aureus* used having approximately 667×10^7 organisms per ml. (viable count). The results (table XVII) show that in the case of this organism 10 per cent $NaNO_2$ exerted a very marked bactericidal action.

TABLE XVI. Bactericidal action of 1 per cent and 10 per cent $NaNO_2$ using culture 4a (*Pseudomonas* sp.) as test organism.

$NaNO_2$ concentration %	Period of exposure	pH of exposure (supernatant liquid after centrifuging)	Viable bacteria in colonies per ml.
0	30 min.	5.55	$3{,}500 \times 10^6$
0	24 hr.	5.75	$2{,}200 \times 10^6$
1	30 min.	5.55	$3{,}500 \times 10^6$
1	6 hr.	5.55	1.2×10^6
1	24 hr.	5.55	0
10	30 min.	5.35	3,570

TABLE XVII. Bactericidal action of 10 per cent $NaNO_2$ using *S. aureus* as test organism

$NaNO_2$ concentration %	Period of exposure	pH of exposure (supernatant liquid after centrifuging)	Viable bacteria in colonies per ml.
0	30 min.	5.55	$1,990 \times 10^6$
0	6 hr.	5.55	$2,370 \times 10^6$
10	30 min.	5.40	28,700
10	6 hr.	5.40	0

DISCUSSION

The present experiments have shown that in acid medium 0.02 per cent of sodium nitrite inhibits growth of two species of obligate anaerobes of the genus *Clostridium*, and also of certain facultative anaerobes when cultivated under strictly anaerobic or the usual semi-anaerobic conditions. Previous investigators (Lewis and Moran 1928, Tanner and Evans 1934) found that growth of obligate sporogenous anaerobes was inhibited by sodium nitrite only when concentrations normally greatly in excess of 0.02 per cent were used. Since these workers did not state the pH of the media which they employed, their failure to record bacteriostatic activity with small concentrations of nitrite may well have been due to the fact that these nutrient substrates possessed a neutral or faintly alkaline reaction.

The fact that growth of these obligate sporogenous anaerobes is inhibited by nitrite is of interest. The theory has been advanced that inhibition of growth of such organisms during meat-curing processes is due to hydrogen peroxide, a substance which is extremely toxic for *Clostridia* even in extremely small amounts, and which may accumulate as a result of inhibition of catalase by the hydroxylamine formed as an intermediate during the bacterial reduction of nitrate to nitrite (Jensen and Hess 1941). While this effect may well account for some of the inhibition of growth of such organisms during curing, it would appear that, since meat curing is usually carried out in somewhat acid medium, nitrites may also retard multiplication of *Clostridia* under such conditions.

It was found that the pH range over which nitrite inhibited growth of facultative anaerobes varied considerably with the substrate employed. In general the range was greatest when nutrient broth was used, and considerably less when using fish digest broth and synthetic medium. The reason for this was not ascertained. The fact that nitrite inhibited bacterial growth in a simple ammonium lactate medium would make it seem highly unlikely that this compound effects inhibition by combining with and inactivating some essential growth factor which might be present in a more complicated medium, or by inhibiting proteolytic or other enzymes which are presumably not utilized in a synthetic medium. There is little doubt that nitrite acts directly on the bacterial cell, but whether its action is specific or general is not clear.

It was found that sodium nitrite in fairly high concentration (1 and 10 per cent) exerted marked bactericidal action in acid medium, but not above pH 7. Bittenbender *et al.* (1940) reported that *S. aureus* was not killed by exposure to

38.8 per cent sodium nitrite for 10 minutes at pH values between 3 and 8. Since these investigators recorded only positive or negative growth after such treatment, and not the proportion of organisms rendered incapable of further multiplication, it would seem probable that in their experiments some, but not all, of the bacteria were inactivated. The present work has shown that *S. aureus* organisms are readily inactivated by 10 per cent sodium nitrite, depending on the length of time they are exposed.

In 0.02 per cent concentration sodium nitrite exerted only a very trivial bactericidal action. It would appear that such factors as concentration, pH, length of exposure and sensitivity of bacterial cells all contribute in determining whether nitrite exerts a bacteriostatic or bactericidal action.

Nitrite inhibited growth of the obligate anaerobes to about the same extent in cultures which were exposed to air during the early growth stages as in those not so exposed. Cultures of anaerobes not exposed to air grew at lower pH values than these exposed to it, and this is probably explained by the fact that vegetative cells of *Clostridia* are readily killed by exposure to oxygen (Knight 1941).

In concluding it is of interest to note that Meiklejohn (1940) found that growth of certain species of denitrifying *Pseudomonas* isolated from soil was strongly inhibited by nitrite in neutral or faintly acid solution, but not in alkaline medium, and that the degree of inhibition depended on the concentration of nitrite used.

SUMMARY

Growth of the obligate anaerobes *Clostridium botulinum* and *C. sporogenes* in fish digest broth was inhibited by 0.02 per cent sodium nitrite at acid pH. When vegetative cells of these organisms were exposed to air during the maximum growth phase multiplication did not take place at such a low pH as in cultures not so exposed. The degree of inhibition by sodium nitrite was not appreciably affected by such treatment.

Growth of certain species of facultative anaerobes was inhibited over a much wider pH range in ordinary nutrient broth than in fish digest broth or synthetic medium.

In nutrient broth nitrite inhibited growth of certain facultative anaerobes when these were cultivated under either strictly anaerobic or semi-anaerobic conditions.

The size of inoculum employed exerted only a minor influence on the final inhibition of growth of certain facultative anaerobes by sodium nitrite in nutrient broth.

Growth of the human pathogens *Eberthella typhosa* and *Staphylococcus aureus* in nutrient broth was inhibited by sodium nitrite in acid medium.

Sodium nitrite can act in either a bactericidal or bacteriostatic capacity, depending on such conditions as concentration employed, pH, length of exposure and sensitivity of bacterial cells, but its mode of action has not as yet been ascertained.

Bittenbender
Ind. E
Hartley, P.
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Jensen, L. B.
Knight, B. C.
Lewis, W. L.
Meikeljohn, J.
Tanner, F. W.
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Action of Nitrates and Nitrites on Bacteria

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ABSTRACT

The amount of $NaNO_2$ formed by reduction of $NaNO_3$ by growing heterotrophic fish spoilage bacteria depended largely on the pH of the culture medium used. Thus $NaNO_2$ formation was strongly inhibited in media controlled at acid pH, but amounts as high as 6,950 p.p.m. were formed when the pH was permitted to rise unchecked. $NaNO_3$, in presence or absence of 200 p.p.m. of $NaNO_2$, had either a very trivial effect, or no effect, on the growth of fish spoilage bacteria in broth medium or fish muscle, and it was either not, or but feebly, reduced in fish muscle under the experimental conditions.

In previous papers (Tarr 1941, 1942) the bacteriostatic and bactericidal action of sodium nitrite was studied in detail with special reference to the effect of pH. Sodium nitrate is generally conceded to have practically no direct bacteriostatic action, other than that which might be due to osmotic effects when large concentrations of the salt are used (Holm and Sherman 1921). However, nitrates may influence bacterial growth in two different ways. They may be activated by bacterial *nitratase* and act as hydrogen acceptors in place of molecular oxygen, thereby rendering anaerobic growth possible, or at least facilitating it (Quastel, Stephenson, and Whetham 1925). On the other hand they have been shown to inhibit growth of, and toxin formation in, certain *Clostridia* (Tanner and Evans 1933). Nitrates inhibit growth of *Clostridia* in meat-curing processes. This is believed to be due to an indirect effect in which the hydroxylamine formed during bacterial reduction of nitrates inactivates catalase, thereby permitting an accumulation of hydrogen peroxide which is extremely toxic for *Clostridia* even in small concentrations (Jensen 1942); hydroxylamine has been shown to be formed during the reduction of nitrates by certain facultative anaerobes (Lindsey and Rhines 1932), and is probably similarly formed by certain *Clostridia* (Woods 1938). It has been shown that nitrites inhibit the growth of certain species of *Clostridia* in acid medium (Tarr 1942), but whether this finding applies to the nitrites formed by bacterial reduction of nitrates in meat curing processes is apparently not known.

The present work was undertaken in order to determine (1) the optimum conditions for production of nitrites from nitrates by fish spoilage bacteria, and

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TABLE I. Amount of $NaNO_2$ formed from $NaNO_3$ in Bacto nutrient broth at an initial pH of 7.0 in duplicate experiments (A and B).

Incubation period at 25°C. (days)	$NaNO_2$ (p.p.m.)				pH				Bacterial counts (× 10^6 per ml.)			
	Culture 7		Culture 23		Culture 7		Culture 23		*Culture 7		Culture 23	
	A	B	A	B	A	B	A	B	A	B	A	B
0	0.2	0.2	0.2	0.2	7.00	7.00	7.00	7.00	..	..	...	...
3	1500	1800	750	790	6.80	6.80	7.00	7.00	25	26	440	415
11	3050	2850	2650	2700	7.85	7.85	7.90	7.85	51	52	580	550
17	3800	3600	3250	3450	8.30	8.20	8.35	8.30	76	82	**	**
27	3900	3850	4050	3950	8.50	8.40	8.75	8.70	81	85	**	**
39	4100	4250	4000	3900	8.55	8.60	8.90	8.80	79	95	**	**
45	3650	4250	4050	3850	8.55	8.60	8.85	8.80	78	89	**	**

*This organism forms clumps and the counts given represent clumps or single organisms.
**Cells autolyzing and accurate count impossible.

th 0.5 ml. of a young broth culture of organisms 7 and 23 (*Achromobacter* sp.), ocula containing 1.4 × 10^6 cells (mostly clumps) and 85 × 10^6 cells respec- ly. The inoculated media were incubated at 25° C., and were examined at ted intervals as described in experiment 1. In the case of each organism the H of the medium in both duplicates of one of the two pairs of broth cultures s kept at about 6.0 by occasional careful addition of sterile 1.0 N HCl, the H in the remaining pair being permitted to rise unchecked. After the $NaNO_2$ ntent of the various cultures had risen to between 200 and 300 p.p.m., 10 ml. toluene was added to one of each pair, the flasks being then stoppered and aken well.

The results of this experiment are given in tables II and III. It was found t in all instances toluene treatment promptly stopped bacterial increase and n suppressed $NaNO_2$ formation, there being some increase of the latter during day following such treatment but none thereafter. In the cultures in which ttempt was made to hinder the normal increase in pH the $NaNO_2$ gradually ased until it reached 6,950 p.p.m. after 65 days with production of a pH of in culture 23, and 1,800 p.p.m. after 76 days with production of a pH of in culture 7. On the other hand nitrite formation was markedly suppressed n the normal increase in pH was checked either by addition of HCl or by l formed from glucose. Thus in culture 7 the pH of the glucose medium fell to pH 5.7, and the $NaNO_2$ content did not rise above 285 p.p.m.; in culture 23 the pH fell to 5.65 in this medium and the $NaNO_2$ content did rise above 420 p.p.m. When the pH of the ordinary buffered broth medium not allowed to rise above 6.00 due to occasional addition of HCl, the $NaNO_2$ t did not rise above 880 p.p.m. in culture 7 and 780 p.p.m. in culture 23. acterial counts were complicated by the fact that the organisms in culture 7 ed clumps throughout the experiment, while in culture 23 clumps of curious oid involution forms appeared after about two weeks. Autolysis also

(2) the effect of nitrates and mixtures of nitrates and nitrites on growth of such organisms in laboratory media at different pH values and in fish muscle itself.

EXPERIMENTAL

Reduction of Nitrates at Different pH Values

Though the reduction of nitrates to nitrites is one of the principal tests used in classifying bacteria, little appears to be known regarding the amount of nitrite which is produced under different conditions. Stickland (1931), when studying the *nitratase* enzyme of *B. coli*, found that washed suspensions of this organism produced in one instance 2,480 p.p.m. of potassium nitrite from 2,520 p.p.m. of the corresponding nitrate. The following experiments show that comparatively large amounts of nitrite can be formed from nitrate by growing fish spoilage bacteria under certain conditions. No attempt has been made to determine how much nitrite can be formed from the corresponding nitrate by washed suspensions of such organisms. The cultures employed were those used in previous experiments (Tarr 1939).

EXPERIMENT 1.

Culture 7 (*Micrococcus* sp.) and culture 23 (*Achromobacter* sp.), the growth of both of which had been shown in previous experiments to be strongly inhibited by 0.02 per cent sodium nitrite, were cultivated 24 hours on Bacto nutrient agar. A small amount of the growth from each culture was transferred to each of two 500-ml. conical flasks containing 250 ml. of Bacto nutrient broth plus 1 per cent chemically pure $NaNO_3$ (pH 7.0). The flasks were incubated at 25° C. and at stated intervals small portions were removed aseptically and the following three determinations were made: (1) pH to the nearest 0.05 unit using a Beckman pH meter; (2) direct bacterial counts using a Petroff-Hausser counting chamber and (3) amount of $NaNO_2$ using an Evelyn photo-electric colorimeter and a No. 520 Rubicon filter (White 1939). The comparatively low bacterial counts obtained with culture 7 were due to the fact that this organism formed clumps in liquid medium, and that these were recorded as a single unit.

The results (table I) showed that in nutrient broth at an initial pH of 7.0 the two cultures produced rather similar amounts of $NaNO_2$ from 10,000 p.p.m. of $NaNO_3$, maximum production (4,050 and 4,250 p.p.m.) being reached after 27 to 39 days' incubation. The production of $NaNO_2$ was accompanied by a pronounced increase in bacterial content up to the eleventh or seventeenth day and by a marked increase in pH.

EXPERIMENT 2.

Bacto nutrient broth containing 1 per cent $NaNO_3$ and 0.05 M KH_2PO_4 was prepared and adjusted to pH 5.95 with 1.0 N NaOH. Analytical reagent grade glucose was added in 0.1 per cent concentration to a portion of this medium. The resulting media were dispersed in 250-ml. portions into 500-ml. conical flasks, which were then autoclaved, the pH rising to 6.00.

Four flasks of the plain broth and two of the glucose broth were inoculated

TABLE I. Amount
initial

Incubation period at 25°C. (days)	$NaNO_2$	
	Culture 7	
	A	B
0	0.2	0.2
3	1500	1800
11	3050	2850
17	3800	3600
27	3900	3850
39	4100	4250
45	3650	4250

*This organism forms clumps
**Cells autolyzing and accurat

with 0.5 ml. of a young
the inocula containing 1
tively. The inoculated
stated intervals as descr
all of the medium in b
was kept at about 6.0 b
pH in the remaining pa
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of toluene was added t
shaken well.

The results of this c
that in all instances tol
was suppressed $NaNO_2$
the day following such t
no attempt was made to
increased until it reache
5.55 in culture 23, and
6.50 in culture 7. On th
when the normal increas
acid formed from gluco
was fell to pH 5.7, an
while in culture 23 the pH
not rise above 420 p.p.m.
was not allowed to rise a
content did not rise abo
The bacterial counts were
formed clumps throughou
coid involution form

TABLE I. Amount of $NaNO_2$ formed from $NaNO_3$ in Bacto nutrient broth at an initial pH of 7.0 in duplicate experiments (A and B).

Incubation period at 25°C. (days)	$NaNO_2$ (p.p.m.)				pH				Bacterial counts (× 10^6 per ml.)			
	Culture 7		Culture 23		Culture 7		Culture 23		*Culture 7		Culture 23	
	A	B	A	B	A	B	A	B	A	B	A	B
0	0.2	0.2	0.2	0.2	7.00	7.00	7.00	7.00	..	..	...	...
3	1500	1800	750	790	6.80	6.80	7.00	7.00	25	26	440	415
11	3050	2850	2650	2700	7.85	7.85	7.90	7.85	51	52	580	550
17	3800	3600	3250	3450	8.30	8.20	8.35	8.30	76	82	**	**
27	3900	3850	4050	3950	8.50	8.40	8.75	8.70	81	85	**	**
39	4100	4250	4000	3900	8.55	8.60	8.90	8.80	79	95	**	**
45	3650	4250	4050	3850	8.55	8.60	8.85	8.80	78	89	**	**

*This organism forms clumps and the counts given represent clumps or single organisms.
**Cells autolyzing and accurate count impossible.

with 0.5 ml. of a young broth culture of organisms 7 and 23 (*Achromobacter* sp.), the inocula containing 1.4 × 10^6 cells (mostly clumps) and 85 × 10^6 cells respectively. The inoculated media were incubated at 25° C., and were examined at stated intervals as described in experiment 1. In the case of each organism the pH of the medium in both duplicates of one of the two pairs of broth cultures was kept at about 6.0 by occasional careful addition of sterile 1.0 N HCl, the pH in the remaining pair being permitted to rise unchecked. After the $NaNO_2$ content of the various cultures had risen to between 200 and 300 p.p.m., 10 ml. of toluene was added to one of each pair, the flasks being then stoppered and shaken well.

The results of this experiment are given in tables II and III. It was found that in all instances toluene treatment promptly stopped bacterial increase and soon suppressed $NaNO_2$ formation, there being some increase of the latter during the day following such treatment but none thereafter. In the cultures in which no attempt was made to hinder the normal increase in pH the $NaNO_2$ gradually increased until it reached 6,950 p.p.m. after 65 days with production of a pH of 8.55 in culture 23, and 1,800 p.p.m. after 76 days with production of a pH of 6.50 in culture 7. On the other hand nitrite formation was markedly suppressed when the normal increase in pH was checked either by addition of HCl or by acid formed from glucose. Thus in culture 7 the pH of the glucose medium soon fell to pH 5.7, and the $NaNO_2$ content did not rise above 285 p.p.m.; while in culture 23 the pH fell to 5.65 in this medium and the $NaNO_2$ content did not rise above 420 p.p.m. When the pH of the ordinary buffered broth medium was not allowed to rise above 6.00 due to occasional addition of HCl, the $NaNO_2$ content did not rise above 880 p.p.m. in culture 7 and 780 p.p.m. in culture 23. The bacterial counts were complicated by the fact that the organisms in culture 7 formed clumps throughout the experiment, while in culture 23 clumps of curious coccoid involution forms appeared after about two weeks. Autolysis also

TABLE II. Amount of $NaNO_2$ formed from $NaNO_3$ in duplicate experiments (A and B) with culture 7 (*Micrococcus* sp.) in Bacto nutrient broth containing 1 per cent $NaNO_3$ (initial pH 6.00) with pH adjusted, pH not adjusted and with 0.1 per cent glucose. Inoculum 1.4×10^6 cells or clumps of cells per flask.

Incubation period at 25°C. (days)	$NaNO_2$ (p.p m.)						pH						Bacterial counts ($\times 10^6$ per ml.)					
	pH not adjusted		pH adjusted		0.1% glucose		pH not adjusted		pH adjusted		0.1% glucose		pH not adjusted		pH adjusted		0.1% glucose	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
0	0.2	0.2	0.2	0.2	0.2	0.2	6.00	6.00	6.00	6.00	6.00	6.00	..	..	..	..	..	..
2	..	..	..	..	*235	215	..	..	..	..	*5.70	5.70	..	..	..	..	*0.60	0.55
3	250*	230	*210	260	255	285	*6.00	6.00	*6.00	6.00	5.70	5.70	*0.55	0.65	*0.45	0.60	0.65	0.60
5	240	465	225	420	250	285	6.00	6.05	6.00	6.00	5.70	5.70	0.55	1.00	0.45	1.30	0.55	0.50
7	240	560	220	700	240	280	6.00	6.10	6.00	6.00	5.70	5.70	0.55	3.90	0.45	4.1	0.60	0.50
10	..	1500	..	860	..	285	..	6.25	..	6.20	..	5.70	..	63	..	30	..	5.2
12	..	1490	..	880	..	285	..	6.30	..	6.00	..	5.70	..	174	..	32	..	7.0
16‡‡	..	1500	..	830	..	275	..	6.30	..	6.00	..	5.70	..	180	..	18	..	9.0
20	..	1550	..	..	..	260	6.00	6.30	6.00	6.00	5.70	5.70	0.88	185	0.7	12	0.80	0.7
35	..	1400	..	..	..	..	..	6.35	..	..	..	..	..	250	..	..	..	..
42	..	1400	..	..	..	..	..	6.40	..	..	..	..	..	328	..	..	..	..
55	..	1750	..	..	..	..	..	6.50	..	..	..	..	..	..	..	..	..	..
64	..	1750	..	..	..	..	..	6.50	..	..	..	..	..	..	..	..	..	..
76	..	1800	..	..	..	..	..	6.50	..	..	..	..	..	‡243	..	..	..	..
82	..	1400	..	..	..	..	..	6.50	..	..	..	..	..	..	..	..	..	..

*Toluene added.
‡Autolyzing cells made an accurate count impossible.
‡‡All except "pH not adjusted, B" contained no viable bacteria in 1 ml. of medium.

TABLE III. Amount of $NaNO_2$ formed from $NaNO_3$ in duplicate experiments (A and B) with culture 23 (*Achromobacter* sp.) in Bacto nutrient broth containing 1 per cent $NaNO_3$ (initial pH 6.00) with pH adjusted, pH not adjusted and with 0.1 per cent glucose. Inoculum 85×10^6 cells per flask.

Incubation period at 25°C. (days)	$NaNO_2$ (p.p.m.)						pH						Bacterial counts ($\times 10^6$ per ml.)					
	pH not adjusted		pH adjusted		0.1% glucose		pH not adjusted		pH adjusted		0.1% glucose		pH not adjusted		pH adjusted		0.1% glucose	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
0	0.2	0.2	0.2	0.2	0.2	0.2	6.00	6.00	6.00	6.00	6.00	6.00	..	..	..	..	..	..
1	95	90	105	110	90	80	6.05	6.05	6.00	6.00	5.70	5.70	72	78	68	71	142	194
2*	285	280	280	300	265	280	6.05	6.05	6.00	6.00	5.65	5.65	87	83	78	89	139	191
3	295	500	310	530	320	420	6.05	6.10	6.00	6.05	5.65	5.65	93	88	73	86	142	187
4	290	610	310	560	310	405	6.05	6.15	6.00	6.05	5.65	5.65	88	84	73	93	138	181
6	285	735	320	780	310	400	6.05	6.20	6.00	6.00	5.65	5.65	91	88	79	84	134	184
8	280	720	310	770	310	400	6.05	6.20	6.00	6.00	5.65	5.65	..	..	..	..	..	..
10	280	720	315	755	310	410	6.05	6.20	6.00	6.00	5.65	5.65	87	83	74	85	139	179
13‡‡	..	1140	..	..	..	..	..	6.30	..	..	..	..	..	‡	..	..	..	..
17	280	1950	..	..	..	..	6.05	6.50	..	..	..	..	82	..	..	..	..	..
20	285	2500	..	..	..	..	6.05	6.60	..	..	..	..	..	..	..	..	..	..
27	260	3100	..	..	..	..	6.05	6.75	..	..	..	..	..	..	..	..	..	..
36	215	3450	..	..	..	..	6.05	7.15	..	..	..	..	..	..	..	..	..	..
43	215	4850	..	..	..	..	6.05	7.65	..	..	..	..	..	..	..	..	..	..
56	..	5750	..	..	..	..	..	8.40	..	..	..	..	..	..	..	..	..	..
65	..	6950	..	..	..	..	..	8.55	..	..	..	..	..	..	..	..	..	..
77	..	6750	..	..	..	..	..	8.60	..	..	..	..	..	..	..	..	..	..
83	170	5900	..	..	..	..	6.05	8.60	..	..	..	..	..	..	..	..	..	..

*Toluene added to all "A" cultures.
‡Cell clumping and autolyzing.
‡‡All except "pH not adjusted, B" contained no viable bacteria.

contributed to the difficult
tendency for $NaNO_2$ incre
cultures, but the above m
relation impossible. There
bacterial content and conc
broth cultures had more f
corresponding plain broth
produced was quite toxic a
glucose-containing and pH-
viable bacteria after 13 to 1
in which the pH was not c
commencement of the exper

EXPERIMENT 3

An experiment closely s
and, in place of Bacto nutri
having an initial pH of 5.90.
out, no toluene being added.
agreed fairly closely, but w
experiment. Thus in the g
transient, the organism evid
pH. After 70 days the pH
though not so markedly as in
in which the pH was control
rose above 375 p.p.m.; while i
to pH 8.25 or 8.3, from 3,800

TABLE IV. Amount of $NaNO_2$ for
culture 23 (*Achromobacter* sp.
pH 5.90) with pH adjusted,
36×10^6 cells per flask.

Incubation period at 25°C. (days)	$NaNO_2$ (p.p.m.)				
	pH not adjusted		pH adjusted		0.1 gluc
	A	B	A	B	A
4	180	155	54	130	33
5	265	245	130	145	150
7	485	440	285	275	265
10	700	695	375	370	255
18	800	790	305	315	365
26	1300	1300	315	335	740
33	2150	2550	275	290	1650
45	3350	3500	280	300	2100
54	3200	3700	270	290	2750
64	3650	4450	270	285	3200
70	3800	4200	265	280	2850

*Autolyzing cells and formation of

contributed to the difficulty of making accurate counts. There was a general tendency for $NaNO_2$ increase to be accompanied by bacterial increase in both cultures, but the above mentioned factors made any close comparison of this relation impossible. There was not necessarily any direct relation between bacterial content and concentration of $NaNO_2$ formed, for in culture 23 glucose broth cultures had more bacteria but considerably less $NaNO_2$ than had the corresponding plain broth cultures up to the tenth day. That the $NaNO_2$ produced was quite toxic at acid pH values was suggested by the fact that the glucose-containing and pH-adjusted cultures of both organisms contained no viable bacteria after 13 to 16 days' incubation. On the other hand the cultures in which the pH was not controlled contained viable organisms 105 days after commencement of the experiment.

EXPERIMENT 3

An experiment closely similar to the above was carried out using culture 23 and, in place of Bacto nutrient broth, a fish digest broth medium (Tarr 1942) having an initial pH of 5.90. In this experiment duplicates were carried throughout, no toluene being added. The results (table IV) showed that the duplicates agreed fairly closely, but were somewhat different from those of the foregoing experiment. Thus in the glucose medium the initial fall in pH was purely transient, the organism evidently resisting the amount of $NaNO_2$ formed at this pH. After 70 days the pH averaged 8.2 and the $NaNO_2$ greatly increased, though not so markedly as in the digest containing no glucose. In the cultures in which the pH was controlled by addition of HCl, the $NaNO_2$ content never rose above 375 p.p.m.; while in those in which it was permitted to rise unchecked to pH 8.25 or 8.3, from 3,800 to 4,450 p.p.m. of $NaNO_2$ were formed.

TABLE IV. Amount of $NaNO_2$ formed from $NaNO_3$ in duplicate experiments (A and B) with culture 23 (*Achromobacter* sp.) in fish digest broth containing 1 per cent $NaNO_3$ (initial pH 5.90) with pH adjusted, pH not adjusted and with 0.1 per cent glucose. Inoculum 36 × 10^6 cells per flask.

Incubation period at 25°C. (days)	$NaNO_2$ (p.p.m.)						pH						Bacterial counts (× 10^6 per ml.)					
	pH not adjusted		pH adjusted		0.1% glucose		pH not adjusted		pH adjusted		0.1% glucose		pH not adjusted		pH adjusted		0.1% glucose	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
4	180	155	54	130	33	32	6.00	6.00	5.95	5.95	5.90	5.95	21	19	19	20	28	31
5	265	245	130	145	150	155	6.10	6.10	5.95	5.95	5.80	5.80	20	23	24	20	28	30
7	485	440	285	275	265	270	6.20	6.20	6.00	6.00	5.85	5.85	19	30	22	32	32	44
10	700	695	375	370	255	255	6.50	6.50	6.00	6.00	6.00	6.00	35	36	32	32	47	51
18	800	790	305	315	365	380	6.60	6.60	6.00	6.00	6.30	6.30	88	83	34	31	50	50
26	1300	1300	315	335	740	725	6.90	6.90	6.10	6.10	6.50	6.50	145	138	40	44	84	74
33	2150	2550	275	290	1650	1250	7.40	7.30	6.00	5.95	7.00	6.75	940	812	44	43	792	660
45	3350	3500	280	300	2100	2150	8.05	7.90	6.00	6.00	7.85	7.40	*	*	*	*	*	*
54	3200	3700	270	290	2750	2500	8.30	8.15	6.00	6.00	8.15	7.90						
64	3650	4450	270	285	3200	3600	8.30	8.25	6.00	6.00	8.25	8.10						
70	3800	4200	265	280	2850	2800	8.30	8.25	6.00	6.00	8.25	8.15						

*Autolyzing cells and formation of clumps of coccoid forms made further accurate counts impossible.

Influence on Growth in Culture Media

In order to ascertain whether nitrate itself inhibited growth, or whether the inhibition, if any, depended on prior reduction of nitrate to nitrite, half the

Table V. Effect of $NaNO_3$, $NaNO_2$, and a mixture of these salts on the growth (—means "none ... to "much") of certain fish spoilage

*Culture	Inoculum **(× 10^6)	Initial pH of medium†	Amount of growth: Control 1 day	Control 2 days	Control 14 days	1% $NaNO_3$ 1 day	1% $NaNO_3$ 2 days	1% $NaNO_3$ 14 days	0.5% $NaNO_3$ 1 day	0.5% $NaNO_3$ 2 days	0.5% $NaNO_3$ 2 days	0.5% $NaNO_3$ 14 days	0.02% $NaNO_2$ 1 day	0.02% $NaNO_2$ 2 days	0.02% $NaNO_2$ 14 days	An 1% 0.0 1 day
7		5.95	−	+	+++	+	++	++	+	+	+	++	−	−	++	−
Micrococcus	1.6	6.35	+	++	+++	+	++	++	++	++	++	+++	+	+	++	+
+		7.20	++	++	+++	++	++	+++	++	++	++	+++	++	++	+++	++
18		5.95	−	−	−	−	+	−	+	+	+	−	−	−	−	−
Micrococcus	1.3	6.35	+	+	−	−	+	−	+	+	+	−	−	−	−	−
+		7.20	+	+	+	−	+	−	+	+	+	−	+	+	−	+
22		5.95	+++	+++	+++	++	++	+++	++	++	++	+++	+	++	+++	+
Achromobacter	6.8	6.35	++	+++	+++	++	+++	+++	++	++	+++	+++	++	+++	+++	++
+		7.20	++	+++	+++	+++	+++	+++	+++	++	+++	+++	++	+++	+++	+++
23		5.95	++	++	+++	++	++	+	++	++	++	+	−	−	−	−
Achromobacter	4.5	6.35	++	++	+++	++	++	++	++	++	++	++	+	++	+++	−
+		7.20	++	++	+++	++	+++	+++	++	++	+++	+++	++	++	+++	++
9		5.95	++	++	+++	++	++	+++	++	++	++	+++	−	−	−	−
Achromobacter	1.9	6.35	++	++	+++	++	++	+++	++	++	++	+++	−	−	−	−
−		7.20	++	+++	+++	++	++	+++	++	++	+++	+++	++	+++	+++	++
19		5.95	++	+++	+++	++	+++	+++	++	++	+++	+++	−	−	−	−
Achromobacter	6.1	6.35	++	+++	+++	++	+++	+++	++	++	+++	+++	+	++	+++	+
−		7.20	++	+++	+++	++	+++	+++	++	++	+++	+++	++	+++	+++	++
10		5.95	+	+	−	+	+	−	+	+	+	−	−	−	−	−
Micrococcus	1.8	6.35	+	+	−	+	+	−	+	+	+	−	+	+	−	−
−		7.20	++	++	+++	++	++	+++	++	++	++	+++	++	++	+++	++
24		5.95	−	−	++	−	−	++	−	−	−	++	−	−	−	−
Micrococcus	4.7	6.35	+	++	+++	+	++	+++	+	++	++	+++	+	++	+++	−
−		7.20	++	+++	+++	++	+++	+++	++	++	+++	+++	++	+++	+++	++ +

*In this column + indicates that a culture reduced nitrate to nitrite strongly, − that it did ... nitrite.
**All the *Micrococcus* formed clumps in broth and the counts given represent clumps plus single ...
†These pH values were approximately 0.15 of a unit lower in media containing 1% $NaNO_3$, ... media containing 0.5% $NaNO_3$.

cultures chosen were ones which reduced nitrate to nitrite strongly, while the remainder did not.

Sterile 16 × 150-ml. tub
broth; 0.5 ml. of one of thr
1 ml. of a sterile solution of

0.5 ml. of an inoculum prepare
culture of the organism under i

Sterile 16 × 150-ml. tubes received 2 ml. of double strength Bacto nutrient broth; 0.5 ml. of one of three 0.2 M phosphate buffers (pH 4.1, 6.0, or 7.5); 1 ml. of a sterile solution of $NaNO_3$, $NaNO_2$, a mixture of these, or water, and

to "much") of certain fish spoilage bacteria in Bacto nutrient broth of different pH values.

Amount of growth																	
NaNO₃			0.02% $NaNO_2$			1% $NaNO_3$ + 0.02% $NaNO_2$			0.5% $NaNO_3$ + 0.02% $NaNO_2$			0.1% $NaNO_3$ + 0.02% $NaNO_2$		
2 days	14 days		1 day	2 days	14 days	1 day	2 days	14 days	1 day	2 days	14 days	1 day	2 days	14 days
+	++		−	−	++	−	−	−	−	−	+	−	−	++
++	+++		+	+	++	+	++	++	+	+	++	+	+	++
++	+++		++	++	+++	++	++	+++	++	++	+++	++	++	+++
+	−		−	−	−	−	−	−	−	−	−	−	−	−
+	−		−	−	−	−	−	−	−	−	−	−	−	−
+	−		+	+	−	+	++	+++	−	−	+++	−	+	−
++	+++		+	++	+++	+	+	++	+	+	++	+	+	+++
++	+++		++	+++	+++	++	++	+++	++	++	+++	++	++	+++
++	+++		++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
+	+		−	−	−	−	−	−	−	−	−	−	−	−
+	++		+	++	+++	−	+	++	−	+	+	+	+	+
++	+++		++	++	+++	++	++	+++	++	++	+++	++	++	+++
+	+++		−	−	−	−	−	−	−	−	−	−	−	−
+	+++		−	−	−	−	−	+++	−	+	+++	−	−	+++
++	+++		++	+++	+++	++	++	+++	++	+++	+++	++	+++	+++
++	+++		−	−	−	−	−	−	−	−	−	−	−	−
++	+++		+	++	+++	+	++	+++	+	++	+++	+	++	+++
++	+++		++	+++	+++	++	+++	+++	++	+++	+++	++	+++	+++
−	−		−	−	−	−	−	−	−	−	−	−	−	−
+	−		+	+	−	−	+	−	+	+	−	−	+	−
+	+++		++	++	+++	++	++	+++	++	++	+++	++	++	+++
−	++		−	−	−	−	−	−	−	−	−	−	−	−
+	+++		+	++	+++	−	−	+++	−	++	+++	+	++	+++
+	+++		++	+++	+++	++	+++	+++	++	+++	+++	++	+++	+++

nitrite.

in media containing 0.5% $NaNO_3$.

0.5 ml. of an inoculum prepared by suspending a portion of a 24-hour broth culture of the organism under investigation in sterile 0.1 per cent NaCl. In

ited growth, or whether the nitrate to nitrite, half the

se salts on the growth (− means "no

Amount of growth				
1% $NaNO_3$			0.5%	
1 day	2 days	14 days	1 day	2 days
+	++	++	+	+
+	++	++	++	+
++	++	+++	++	+
−	+	−	+	+
−	+	−	+	+
−	+	−	+	+
++	++	+++	++	+
++	+++	+++	++	+
+++	+++	+++	+++	+
+	++	+	++	+
	++	++	++	+
+	+++	+++	++	+
++	++	+++	++	+
++	++	+++	++	+
++	++	+++	++	+
++	+++	+++	++	+
++	+++	+++	++	+
++	+++	+++	++	+
+	+	−	+	+
+	+	−	+	+
++	++	+++	++	+
−	−	++	−	−
+	++	+++	+	+
++	+++	+++	++	+

to nitrite strongly, − that it did
ints given represent clumps plus single
er in media containing 1% $NaNO_3$, a

nitrite strongly, while the

this manner a number of media of different pH values and containing nitrate, nitrite, or nitrate plus nitrite in varying proportions were prepared (table V). It was found that 1 per cent $NaNO_3$ lowered the initial pH of the broth by approximately 0.15 of a pH unit, while 0.5 per cent lowered it approximately 0.1 of a pH unit. The inoculated tubes were incubated at 25°C. and examined for visible growth at stated intervals.

It was found (table V) that the effect of $NaNO_3$ alone was both slight and inconsistent, and did not appear to be influenced by whether a given culture did or did not reduce nitrate to nitrite. Thus at acid pH it suppressed growth slightly in the case of culture 23, but under similar conditions favoured the growth of culture 18. In the other cultures nitrate had little or no effect on growth. As in previous work (Tarr 1941, 1942) 0.02 per cent sodium nitrite alone exerted a more or less pronounced inhibition of growth of all cultures studied at acid pH. In the case of culture 9 a mixture of $NaNO_3$ and $NaNO_2$ appeared to be less effective in inhibiting growth than was $NaNO_2$ alone, while in culture 24 both 1 and 0.5 per cent $NaNO_3$ stimulated the bacteriostatic action of the $NaNO_2$ slightly. Poor growth and rapid autolysis occurred in most of the media inoculated with culture 23, the same holding true in the case of culture 10 in media of acid pH.

Influence on Growth in Fish Muscle

Strictly fresh fish were filleted under clean conditions, the flesh was passed through a domestic mincer, and, after mixing thoroughly, 95-g. portions were transferred to sterile 250-ml. beakers covered with an inverted petri dish. The desired quantity of $NaNO_2$, $NaNO_3$ or NaCl was added in a 5-ml. portion of a sterile aqueous solution, and the treated flesh stored at 0.5 or 10°C. Direct bacterial counts (Tarr 1943) and determinations of the amount of $NaNO_2$ present were made at stated intervals. The results of three different experiments using lingcod (*Ophiodon elongatus*) and spring salmon (*Oncorhynchus tschawytscha*) are recorded in tables VI, VII and VIII.

It will be seen that while $NaNO_2$ inhibited growth in all cases, the effect of $NaNO_3$ was either negative, or feeble and inconsistent. Thus the results given

Table VI. Effect of $NaNO_3$ and $NaNO_2$ on rate of bacterial increase in lingcod flesh stored at 0.5°C.

Treatment	Bacteria (no. per g. of flesh) after			$NaNO_2$ (p.p.m.) after		
	4 days	8 days	11 days	4 days	8 days	11 days
Control	420,000	420×10^6	$1,400 \times 10^6$	0	0	0
0.02% $NaNO_2$	130,000	3.8×10^6	$2,500 \times 10^6$	206	206	216
0.02% $NaNO_2$ + 0.1% $NaNO_3$	190,000	2.5×10^6	$1,500 \times 10^6$	214	212	208
0.02% $NaNO_2$ + 0.5% $NaNO_3$	340,000	4.2×10^6	$1,100 \times 10^6$	202	206	200
0.02% $NaNO_2$ + 1.0% $NaNO_3$	210,000	2.3×10^6	440×10^6	220	212	204

Table VII. Effect of $NaNO_2$ [illegible] salm[illegible]

Treatment	[illegible]
Control	7
0.02% $NaNO_2$	
0.02% $NaNO_2$ + 0.1% $NaNO_3$	
0.02% $NaNO_2$ + 0.5% $NaNO_3$	
0.02% $NaNO_2$ + 1.0% $NaNO_3$	

Table VIII. Effect of $NaNO$ [illegible] lingcod f[illegible]

Treatment
Control
0.02% $NaNO_2$
0.02% $NaNO_2$ + 1% NaN[illegible]
0.02% $NaNO_2$ + 1% NaCl

in table VI indicated that 1 per [illegible] effect of $NaNO_2$, while those [illegible] ization of the inhibitory effect [illegible] 1 per cent NaCl as with $NaNO$[illegible] to $NaNO_2$ in either of two expe[illegible] stored at 10°C. the $NaNO_3$ was [illegible] that nitrate has no important [illegible] bacteria, and that it is but slow[illegible]

The amount of $NaNO_2$ f[illegible] medium depended on the pH [illegible] either by the acid formed fro[illegible] nitrite formation was strongly [illegible] viable bacteria. On the othe[illegible] checked the medium became [illegible] 1,800 to 6,950 p.p.m. of NaN[illegible] different conditions, the cultu[illegible] immediately stopped bacterial [illegible] $NaNO_2$ was noticed.

The growth of both nitra[illegible]

TABLE VII. Effect of $NaNO_3$ and $NaNO_2$ on rate of bacterial increase in spring salmon flesh stored at 0.5°C.

Treatment	Bacteria (no. per g.) after		$NaNO_2$ (p.p.m.) after	
	8 days	15 days	8 days	15 days
Control	74 × 10^6	15,000 × 10^6	0	0
0.02% $NaNO_2$	270,000	30 × 10^6	198	205
0.02% $NaNO_2$ + 0.1% $NaNO_3$	530,000	30 × 10^6	190	195
0.02% $NaNO_2$ + 0.5% $NaNO_3$	110,000	10 × 10^6	198	198
0.02% $NaNO_2$ + 1.0% $NaNO_3$	420,000	16 × 10^6	220	210

TABLE VIII. Effect of $NaNO_3$, $NaNO_2$ and NaCl on rate of bacterial increase in lingcod flesh stored at 10°C. for 3 days.

Treatment	Direct counts (× 10^6 per ml.)	$NaNO_2$ (p.p.m.)
Control	6,400	0
0.02% $NaNO_2$	200	188
0.02% $NaNO_2$ + 1% $NaNO_3$	280	448
0.02% $NaNO_2$ + 1% NaCl	280	168

in table VI indicated that 1 per cent $NaNO_3$ slightly improved the bacteriostatic effect of $NaNO_2$, while those given in table VII showed a very slight neutralization of the inhibitory effect of $NaNO_2$, but that this was just as marked with 1 per cent NaCl as with $NaNO_3$. At 0.5°C., $NaNO_3$ was not appreciably reduced to $NaNO_2$ in either of two experiments, though in the one in which fish flesh was stored at 10°C. the $NaNO_3$ was slightly reduced. The results obtained indicated that nitrate has no important bacteriostatic properties as regards fish spoilage bacteria, and that it is but slowly reduced even in stale fish flesh.

SUMMARY

The amount of $NaNO_2$ formed by bacterial reduction of $NaNO_3$ in broth medium depended on the pH of the medium. When the pH was kept near 6 either by the acid formed from added glucose, or by addition of sterile HCl, nitrite formation was strongly suppressed, and the medium soon contained no viable bacteria. On the other hand, when the pH was permitted to rise unchecked the medium became quite alkaline, and, with the two cultures studied, 1,800 to 6,950 p.p.m. of $NaNO_2$ was formed from 10,000 p.p.m. of $NaNO_3$ under different conditions, the cultures remaining viable for over 3 months. Toluene immediately stopped bacterial increase, and shortly after its addition no increase in $NaNO_2$ was noticed.

The growth of both nitrate-reducing and non-nitrate-reducing fish spoilage

bacteria was retarded by 0.02 per cent $NaNO_2$ in acid medium; but $NaNO_3$ in from 0.1 to 1.0 per cent concentration had either no effect, or a relatively trivial and variable effect on their growth either in the presence or absence of $NaNO_2$.

In from 0.1 to 1.0 per cent concentration, $NaNO_3$ had either no effect, or a relatively trivial and variable effect on the bacteriostatic action of 0.02 per cent $NaNO_2$ in fish muscle. In the experiments reported $NaNO_3$ was either not, or only slightly reduced to nitrite in spoiling fish muscle.

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I wish to thank Miss F. M. Lew for her technical assistance in the course of this work.

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BY ELIZA
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(*Received*

None of the recognized Micro
unidentified Micrococci from fish do

The present paper is a p
trimethylamine oxide. The
present work were identical w
which dealt with trimethylan
and Baird 1943).

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Identified Micrococci and
Type Culture collection throu
species from the four genera of
atories. These donations are
fish were isolated in this labora

I. GENUS *Micrococcus*

The following species and
been studied.

Micrococcus catarrhali
" *butyricum*
" *roseus*
" *cinnebaren*
" *freudenreic*
" *luteus*
" *flavus*
" *aurantiacu*
" *varians*
" *conglomerat*
" *perflavus*
Micrococcus ureae
" *candicans*

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The Effect of Sodium Nitrite on Experimental Animals

By H. L. A. Tarr and N. M. Carter
Pacific Fisheries Experimental Station

(*Received for publication October 28, 1942*)

ABSTRACT

Incorporation of sodium nitrite in the diet of cats and white rats on the basis of an average sized man consuming 1 lb. (454 g.) of fish containing 0.2 per cent (908 mg.) of this salt daily for six days each week does not appear to affect their growth rate nor the development (weight) of their thyroid, heart, lungs, spleen, liver, kidneys or adrenals. The fecundity of white rats as judged by their ability to breed and raise normal litters is apparently not affected thereby. The lethal dose of sodium nitrite by oral route is about 1.1 to 2.0 g./kg. for healthy male rats, 0.46 to 1.2 g./kg. for healthy female rats and 0.073 g./kg. for cats (one animal). The lethal dose by subcutaneous route is about 0.19 to 0.20 g./kg. for healthy male rats and 0.057 to 0.13 g./kg. for healthy female rats.

INTRODUCTION

Recent work has shown that nitrites inhibit bacterial spoilage of fish (Tarr and Sunderland 1940) and that under certain conditions they are also able to exert a pronounced bacteriostatic and bactericidal action (Tarr 1941a and b, 1942). The present regulations in Canada (Food and Drugs Act, Ottawa, 1939) permit the incorporation of sodium nitrite in amounts not exceeding 0.02 per cent in *cured* meats (including fish, shellfish, etc.). In view of these facts and the knowledge that nitrite salts are regularly consumed by those who partake of cured meats, it was thought advisable to ascertain what effect continued oral administration of sodium nitrite in sub-lethal amounts might have on experimental animals, with special consideration of its possible cumulative toxic effect. A few experiments were also undertaken to estimate the lethal dose.

It is well known that nitrites, both organic and inorganic, act as vasodilators and anti-spasmodics, their action in small doses being to dilate the capillaries and thus to reduce the blood pressure. Both sodium nitrite and amyl nitrite have been used for years in the treatment of certain heart conditions (Dixon 1921). According to Reiss, Meyer and Müller (1928) the German Pharmacopeia lists 1 g. of sodium nitrite as the maximum permissible daily dose and the maximum single dose is stipulated as 0.01 to 0.08 g. Excessive doses of nitrites have a narcotic effect on the nervous system, produce cold shivers, dizziness, vertigo, singing in the ears, suffocating feeling, vomiting, cyanosis, methaemoglobinaemia and possibly death through a process analogous to asphyxiation.

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It has been stated that at least 0.2 g. must be consumed orally in one dose to produce the mildest symptoms in adults, slightly less than this amount being tolerated safely (Reiss *et al.* 1928).

Cases of nitrite poisoning resulting from the consumption of cured meats do not appear to be of very frequent occurrence. Thus Behre (1939) stated that, "Trotzdem Pökelfleisch sowohl das in salpeter- und zuckerhaltiger als auch in das in nitrithaltiger Pökellake hergestellte Fleisch, in ungeheuer grosser Menge genossen wird, ist bisher in Deutschland kein Fall bekanntgeworden in dem Vergiftung durch den Genuss solchen Fleisches mit Sicherheit festgestellt worden ware." He described a few rather mild and non-fatal cases of nitrite poisoning in a German family due to consumption of "pickled ribs". Several cases of fatal poisoning due to consumption of sodium nitrite in apparent mistake for ordinary table salt have been recorded (Scholes 1936, Hunziter-Kramer 1936, Padberg and Martin 1939).

EXPERIMENTAL

Effect of Continued Oral Doses

In the following two experiments growing animals were fed daily except Sunday for some months a diet incorporating weighed amounts of a flesh supplement containing 0.2 per cent of sodium nitrite (ten times the legal concentration). Control animals were fed a corresponding diet with nitrite-free supplement. The amounts of supplement fed were adjusted periodically to be proportional to the maximum weights of fish considered likely to be consumed *per diem* by a normal human being at corresponding stages of growth (table I).

Table I. Amount of sodium nitrite administered orally to experimental animals with changing body weight.

Man (hypothetical case)		White rats		Cats	
Weight (g.)	Wt. $NaNO_2$ ingested (g.)	Weight (g.)	Wt. $NaNO_2$ ingested (g.)*	Weight (g.)	Wt. $NaNO_2$ ingested (g.)*
13,600	0.226	30	0.005	790	0.0226
31,800	0.454	70	0.010	1930	0.0454
49,900	0.680	110	0.015	2940	0.0680
68,000	0.908	150	0.020	**4080	0.0908

*The amount of sodium nitrite fed was not increased until animals attained the next highest weight. Thus rats which weighed between 30 and 70 g. were fed 0.005 g. of $NaNO_2$.

**None of the cats studied attained this weight prior to the conclusion of the experiment.

The nitrite-containing supplement for albino rats was prepared from freshly-opened canned pink salmon which, after thorough mixing with 0.2 per cent of

sodium nitrite, was stored in sealed ¼-lb. cans held at −20°C. The nitrite content of the stored material was found not to alter appreciably during the period of the experiments. The nitrite-containing supplement for cats was prepared by thoroughly mixing the appropriate amount of a 20 or 40 per cent solution of sodium nitrite with 15 to 20 g. of finely divided flesh (not necessarily fish) immediately before feeding.

EXPERIMENT 1 (WHITE RATS)

Five brother and four sister pairs were used (three pairs from each of three litters, A, B and C). Initial weights varied from 29 to 66 g. (males) and from 26 to 60 g. (females). One of each pair was fed nitrite-containing fish, the other (control) fish without nitrite. They were segregated in six groups, each of three animals (two males and one female for each of litters A and B, and two females and one male for litter C). The feeding experiments lasted 127 to 168 days in different instances.

Except as undernoted, the animals were given free access to water and an unlimited quantity of a stock diet mixed in the following proportions: ground (whole) wheat 3000 g., ground "Purina" fox chow 1000 g., sodium chloride 50 g., dried brewer's yeast 100 g., dogfish liver oil 100 g., kelp meal 50 g., and dried "Trumilk" 750 g. In addition they received daily small amounts of one or more of the following: raw or cooked beef liver, raw carrot, apple, orange, or green vegetable.

The supplemental fish ration was controlled as follows. Every day all food and water was removed from the cages for a period varying from 2 to 5 hours. Each animal was then transferred to a separate cage, the weighed amount of fish was introduced on a metal dish, and the animal was watched until the entire ration was consumed, usually voraciously. The animal was then returned to its cage and to its regular diet.

The animals matured, mated, and the females raised one or more litters. They were at first weighed bi-weekly, later once a week, to the nearest gram. Females were isolated shortly before littering and were returned to the males subsequent to weaning of the young. Some first litters died through lack of adequate development of the mothers' mammary glands (figure 1). One second litter (of a control) was lost through this cause. The young were almost all healthy and of average weight, there being no perceptible difference between nitrite-fed animals and controls in this respect

The sole symptom attributable to nitrite feeding was shown by three litter rats which, during the first 10 days of the experiment, exhibited slight transient respiratory distress, lasting 30 minutes or less.

Growth curves (shown for each pair in figure 1) indicated no effect of nitrite on growth-rate. Fecundity was about the same, 53 young from nitrite-fed mothers, as against 58 from controls. The offspring from the nitrite-fed animals appeared more resistant, only 30 per cent dying before weaning, as against 52 per cent of the young of the controls, but in view of the smallness of the number of animals this difference is probably not significant.

At the end of the feeding experiment the rats were killed by chloroform, and

frozen at −20°C. Prior to dissection they were only partially thawed, so that the liver and heart remained filled with blood. Thyroid, heart, lungs, liver, spleen, kidneys and adrenals were dissected out and weighed (Cameron 1925).

The extremes and mean organ weights for each group of animals are shown in table II, and exhibit no essential difference between experimental animals and controls. The figures are in general of the same order as those reported by Cameron and Sedziak (1921) and by Cameron (1925) though, owing to the blood content, those for hearts are uniformly higher.

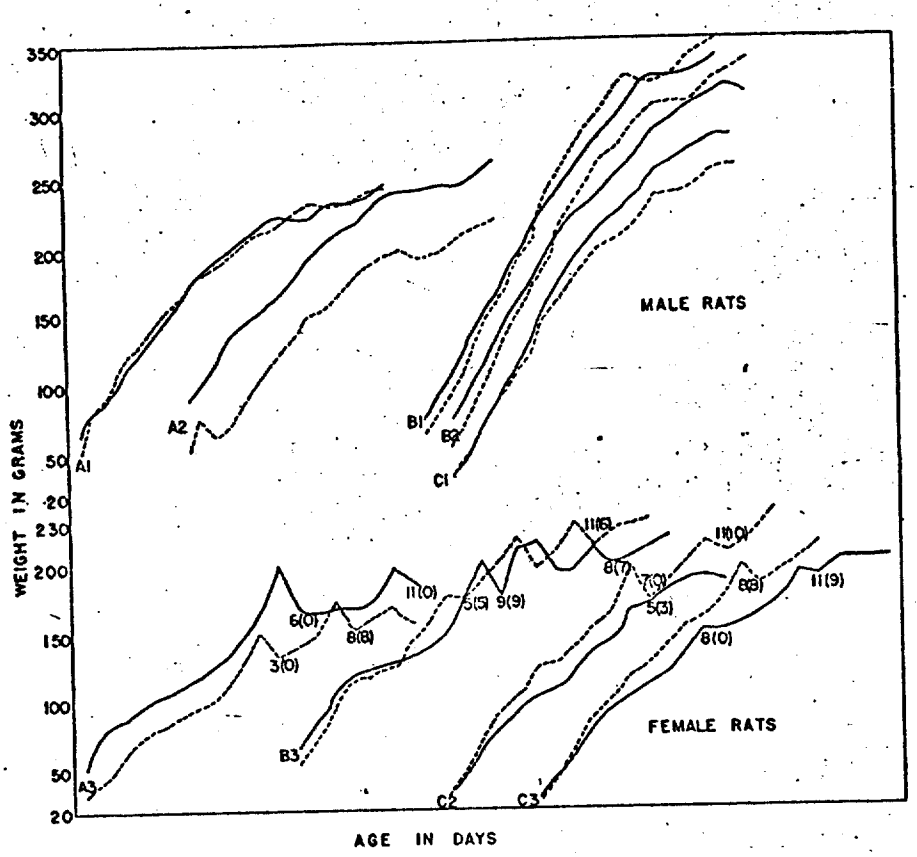

FIGURE 1. Growth curves of white rats fed diets with (broken lines) and without (continuous lines) added sodium nitrite (experiment 1). The figures along the female growth curves represent the number of young in litters, the figures in parentheses showing the number of young successfully reared and weaned in the litter.

Hence the regular feeding of sodium nitrite in the described amounts over a long period does not appear to affect the health, rate of growth, size of body organs, fecundity, and health of offspring of the white rats. It may be noted that a typical animal in this experiment received in 121 days 0.167 g. of sodium nitrite, an amount almost 50 per cent greater than the maximum non-lethal single oral dose indicated for the rat in experiment 3.

TABLE II. Comparison of organ weights of nitrite-fed and control rats.

Data obtained	4 females (nitrite-fed)	4 females (controls)	5 males (nitrite-fed)	5 males (controls)
Total body weight (g.)				
Initial	27–56	26–60	29–66	31–63
Final				
Extremes	186–229	183–217	218–350	243–335
Mean	210	204	280	285
Organ weight (%):				
Kidneys				
Extremes	1.00–1.09	0.98–1.11	0.81–0.95	0.82–1.05
Mean	1.05	1.07	0.90	0.94
Liver				
Extremes	7.3–8.8	6.5–7.3	4.0–5.1	4.1–5.0
Mean	7.7	6.6	4.7	4.6
Heart				
Extremes	0.84–1.06	0.76–1.05	0.63–1.01	0.64–0.79
Mean	0.97	0.87	0.70	0.73
Spleen				
Extremes	0.18–0.23	0.16–0.38	0.16–0.27	0.18–0.26
Mean	0.21	0.28	0.22	0.22
Thyroid				
Extremes	0.0058–0.0081	0.0054–0.0078	0.0039–0.0064	0.0052–0.0069
Mean	0.0072	0.0069	0.0053	0.0059
Lungs				
Extremes	0.79–1.20	0.77–0.93	0.75–1.00	0.74–1.04
Mean	0.94	0.82	0.81	0.83
Adrenals				
Extremes	0.025–0.032	0.027–0.028	0.0086–0.013	0.0091–0.013
Mean	0.028	0.028	0.011	0.011

EXPERIMENT 2 (CATS)

Five kittens from three different litters were used, two being retained as controls and the others being fed sodium nitrite in their diet (table III). They were kept in a large wire enclosure in a room at uncontrolled temperature (3 to 15°C.). Their diet consisted of a liberal quantity of milk ("Trumilk") and one of the following foods which were rarely fed for more than two days successively: cooked beef liver or heart, cooked fish (several varieties), "Pet" canned animal food kindly donated by the B.C. Packers Ltd., and a small quantity of mixed fish liver oils as source of vitamins A and D. The animals were normally fed in the morning and in the afternoon, no food being given for from 12 to 16 hours prior to isolating and feeding individual animals their nitrite-containing supplement.

Six days after the commencement of the experiment the animals were treated for worms, a single capsule containing 0.2 ml. of tetrachloroethylene (Parke Davis) being administered orally to each animal. This treatment, when coupled with the fact that during the first few days of the experiment the rather large

food requirement of the animals was not fully met, undoubtedly accounts for the fact that most of them either failed to gain weight or lost a little during the first week. No unusual external symptoms were observed in cats which received the nitrite-containing supplement.

The cats were normally weighed each week, and after 15 weeks were chloroformed, frozen, partially defrosted and their organs dissected out and weighed as in experiment 1. The organ weights of a 7- to 8-months-old female cat from a different litter which was killed by a single large dose of sodium nitrite (see experiment 4) have been included in this work.

The results (figure 2 and table III), although based on a very small number of experimental animals, strongly indicated that the regular feeding of sodium nitrite in the described amounts resulted in no important differences in either the

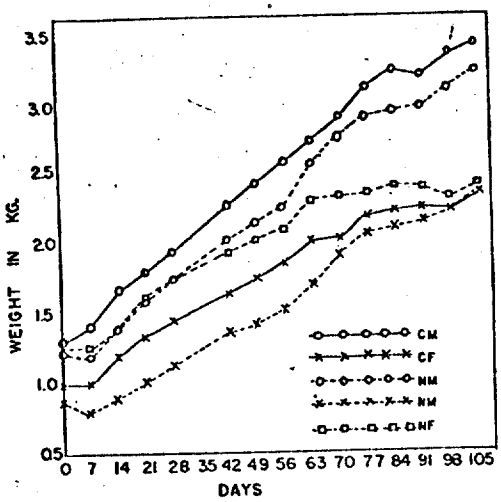

FIGURE 2. Growth curves of cats fed diets with (broken lines) and without (continuous lines) added sodium nitrite (experiment 2).

TABLE III. Comparison of organ weights of nitrite-fed and control cats.

Initial wt. (g.)	Final wt. (g.)	Body weight (%)						
		Kidneys	Liver	Heart	Spleen	Thyroid	Lungs	Adrenals
1300 CM	3400	1.01	4.5	0.87	0.23	0.0061	1.3	0.012
1010 CF	2310	0.88	4.5	0.84	0.18	0.0068	1.2	0.012
1230 NM	3200	0.79	4.6	0.75	0.14	0.0055	1.0	0.010
855 NM	2370	1.2	5.1	0.93	0.20	0.0089	1.5	0.015
1270 NF	2410	0.89	5.2	0.86	0.15	0.0071	0.85	0.009
.... F*	2550	1.1	4.4	0.80	0.20	0.0067	0.91	0.016

*This female was killed by a lethal dose of $NaNO_2$ and had not received the same treatment as the other animals.

C = control, N = nitrite-fed, M = male, F = female. Second, third and fourth animals were from the same litter.

growth rate or organ weights of the nitrite-fed cats as compared with the controls. The third cat in table III ingested approximately 4.1 g. of sodium nitrite during the experiment without apparent ill effect.

Lethal Dose

Rappeport-Lewey (1936) found that the minimum lethal dose of sodium nitrite for rabbits was 0.0876 grams per kilogram of body weight, the definite lethal dose being about 0.12 g. per kg. Sheep are sometimes poisoned by the nitrite which arises when the nitrate of Australian mint weed is reduced enzymically (Williams and Hines 1939). Oral ingestion of 0.08 g. of sodium nitrite per kilogram produces anoxaemia in sheep, and this is rapidly corrected by administration of methylene blue solution (Scott 1941). The lethal dose of sodium nitrite for man is probably not known, but in susceptible individuals 0.0043 g. per kg. of body weight may produce marked symptoms of poisoning (Reiss *et al.* 1928). Reference has already been made to fatal cases of nitrite poisoning in adults occurring as a result of seasoning food with sodium nitrite in mistake for salt.

Experiment 3 (White Rats)

Oral administration. Preliminary tests indicated that the rather large amount of sodium nitrite required to kill a rat could not readily be incorporated in its food, so solutions (10, 20 or 40 per cent by volume) were injected well down the oesophagus of the animals, using a small hypodermic equipped with a glass cannula. With care it was found that little of the liquid thus introduced was lost, though with small rats it was necessary to introduce amounts in excess of 0.1 ml. subdivided into successive doses at brief intervals. The treated animals were kept under observation and those which survived were usually retained for further tests.

The results (table IV) showed that for apparently healthy vigorous male rats the minimum lethal dose of sodium nitrite by oral route was 1.1 grams per kilogram of body weight, the maximum surviving dose being 2 g. per kg. It can therefore be assumed that the lethal dose will normally lie between these limits. For female rats the corresponding limits were 0.46 and 1.2 g. per kg. That the toxic effect of sodium nitrite in this experiment was not due to the introduction of rather large quantities of hypertonic salt solution is shown by the fact that oral introduction of sodium *nitrate* in amounts as great as 4 g. per kg. was in no case fatal. The results showed that old feeble rats were much more susceptible to sodium nitrite than were young vigorous rats.

Subcutaneous injection. A small patch of hair over the biceps femoris muscle of the hind leg was removed and the skin washed with tincture of phenyl mercuric nitrate (Birkhaugh 1933). Solutions of sodium nitrite (1, 10 or 20 per cent by volume and sterilized by Seitz filter) were injected subcutaneously into a number of rats of different sex and age, using a small hypodermic and a no. 25 needle (table V). Male rats recovered after injection of from 0.03 to 0.19 g. of sodium nitrite per kilogram, but died when from 0.20 to 0.48 g. per kg. were administered; the lethal dose therefore appeared to lie between 0.19 and 0.20 g. per kg. Females appeared to be more sensitive than males. They recovered when from

TABLE IV. Lethal dose of sodium nitrite for white rats by oral route.

Weight (g.) and sex	Solution administered		Amount of $NaNO_2$ or $NaNO_3$ administered (mg.)	Amount of $NaNO_2$ or $NaNO_3$ introduced (g. per kg. of body wt.)	Result
	Amount (ml.)	Concentration (%)			
	$NaNO_2$	$NaNO_2$			
230 M	0.20	40	80	0.35	Survived
*142 M	0.20	"	60	0.42	"
86 F	0.10	"	40	0.46	Died in 3 hr. 37 min.
40 F	0.05	"	20	0.50	Survived
216 M	0.30	"	120	0.55	"
71 M	0.20	20	40	0.56	"
34 F	0.05	40	20	0.58	"
207 M	0.30	"	120	0.58	"
*133 M	0.20	"	80	0.60	"
+ 32 F	0.10	20	20	0.62	"
** 65 F	0.20	"	40	0.62	"
44 M	0.15	"	30	0.68	"
140 F	0.30	40	120	0.86	Died in 29 min.
42 F	0.20	20	40	0.95	Survived
+ 36 M	0.20	"	40	1.1	"
76 M	0.20	40	80	1.1	Died in 28 min.
34 F	0.10	"	40	1.2	Died in 40 min.
34 M	0.10	"	80	1.2	Survived
** 66 F	0.40	20	80	1.2	"
* 98 M	0.30	40	120	1.2	Died in 1 hr. 4 min.
30 M	0.10	"	40	1.3	Survived
+ 30 M	0.10	"	40	1.3	Died in 10 min.
47 M	0.30	20	60	1.3	Died in 2 hr. 20 min.
81 M	0.30	40	120	1.5	Died in 22 min.
35 F	0.15	"	60	1.7	Died in 12 min.
45 F	0.20	"	80	1.8	Died in 13 min.
+ 40 M	0.20	"	80	2.0	Survived
40 F	0.40	20	80	2.0	Died in 26 min.
+ 38 M	0.20	40	80	2.1	Died in 3 hr.
40 M	0.50	20	100	2.5	Died in 2 to 3 min.
29 F	0.20	40	80	2.8	Died in 11 min.
** 64 M	0.50	"	200	3.1	Died in 8 min.
22 M	0.30	"	120	4.0	Died in 8 min.
** 38 M	0.40	"	160	4.2	Died in 10 min.
‡222 F	0.20	20	40	0.18	Died in 3 hr. 30 min.
‡248 M	0.40	"	80	0.32	Died in 1 hr. 30 min.
‡229 F	0.30	40	120	0.52	Died in 1 hr. 45 min.
	$NaNO_3$	$NaNO_3$			
92 F	0.30	40	120	1.3	Survived
32 F	0.20	"	80	2.5	"
40 F	0.30	"	120	3.0	"
44 F	0.40	"	160	3.6	"
29 F	0.30	"	120	4.0	"

*Animals which had recovered from a sub-lethal dose of sodium nitrite administered at least 7 days before, and which were apparently healthy.

**Animals which had recovered from a sub-lethal dose of sodium nitrite administered at least 7 days before, were apparently healthy and had been starved from 16 hours prior to administering the above nitrite solution.

+ Starved for 5 hours prior to administration of nitrite solution.

‡ Old feeble rats.

TABLE V. Lethal dose of sodium nitrite for white rats by subcutaneous route.

Weight of rat (g.) and sex	Solution injected		Amount of $NaNO_2$ or $NaNO_3$ injected (mg.)	Amount of $NaNO_2$ or $NaNO_3$ introduced (g. per kg. body wt.)	Result
	Amount (ml.)	Concentration (%)			
	$NaNO_2$	$NaNO_2$			
73 F	0.10	1	1	0.014	Survived
26 M	0.08	1	0.08	0.034	"
138 F	0.025	20	5	0.036	"
112 F	0.05	10	5	0.045	"
*106 F	0.03	20	6	0.057	"
70 M	0.05	10	5	0.072	"
26 M	0.02	"	2	0.077	"
105 M	0.1	"	10	0.095	"
* 94 M	0.05	20	10	0.11	"
33 M	0.02	"	4	0.12	"
61 F	0.04	"	8	0.13	Died in 1 hr. 25 min.
29 M	0.04	10	4	0.14	Survived
62 F	0.05	20	10	0.16	Died in 43 min.
27 M	0.05	10	5	0.19	Survived
*101 M	0.10	20	20	0.20	Died in 34 min.
33 M	0.04	"	8	0.24	Died in 1 hr. 12 min.
83 M	0.2	10	20	0.24	Died in 17 min.
104 F	0.15	20	30	0.29	Died in 24 min.
33 M	0.08	"	16	0.48	Died in 27 min.
	$NaNO_3$	$NaNO_3$			
123 M	0.20	20	40	0.33	Survived
30 M	0.08	"	16	0.53	"
61 M	0.25	"	50	0.82	"

*Animals which had recovered from a sub-lethal dose of sodium nitrite administered at least 7 days before and which were apparently healthy.

0.014 to 0.057 g. per kg. were injected, and died after administration of from 0.13 to 0.29 g. per kg., the lethal dose therefore lying between 0.057 and 0.13 g. per kg. Thus a much larger dose of nitrite was required to kill a rat when introduced orally than when injected subcutaneously. Subcutaneous injection of from 0.33 to 0.82 g. of sodium *nitrate* per kilogram did not cause any important reaction in rats.

EXPERIMENT 4 (CAT)

An apparently normal healthy female cat maintained on the usual mixed diet was fed at intervals increasing amounts of sodium nitrite incorporated in approximately 30 g. of finely minced cooked liver or fish, after starving for some hours as in experiment 2. The results were as follows:

Day	Weight (kg.)	Mg. of $NaNO_2$ fed	Reaction
0	3.34	80	None observed
2		160	" "
4		180	Vomited 80 minutes after feeding; suffered severe anoxaemia and was unable to stand. Vomited a second time about 20 minutes later. Four hours after the nitrite was administered the animal had apparently recovered completely.
18	3:28	240	Vomited about 1½ hours after feeding, suffered severe anoxaemia and cyanosis and died about 2 hours after ingesting the nitrite.

This experiment indicates that about 0.073 g. of sodium nitrite per kilogram will kill a cat when introduced orally with its food, assuming that previous administration of the salt in sub-lethal amounts did not in any way influence the final reaction of the animal.

Another female cat weighing 2.55 kg. and about 7 to 8 months old was fed 400 mg. of sodium nitrite in approximately 40 to 50 g. of chopped liver. The animal vomited about 10 minutes after feeding, suffered from severe anoxaemia and cyanosis, and died about 1 hour after the nitrite was ingested.

DISCUSSION

These experiments indicate that the growth rate and weight of certain internal organs of cats and white rats, and the fertility of the latter, are not appreciably affected by continued oral administration of sub-lethal quantities of sodium nitrite.

Brooks (1937) showed that *in vitro* in the absence of oxygen and the presence of a reducing agent, haemoglobin was converted quantitatively into nitrosohaemoglobin, the reaction proceeding much more rapidly at pH 5.15 to 6.63 than at pH 7.16 to 7.75. In the absence of a reducing agent one molecule of sodium nitrite combined with two equivalents of reduced haemoglobin to give one equivalent each of nitrosohaemoglobin and methaemoglobin. According to Brooks it is probable that in the presence of oxygen oxyhaemoglobin reacts with sodium nitrite to form nitrosohaemoglobin, methaemoglobin and possibly other compounds. The usual product of the *in vivo* interaction of sodium nitrite and haemoglobin is apparently methaemoglobin (Rappeport-Lewey 1936, Padberg and Martin 1939, Cox and Wendel 1942), though Hunziter-Kramer (1936) stated that in some fatal human cases of nitrite poisoning, the blood contained no methaemoglobin but was bright red in colour and may therefore have contained some nitrosohaemoglobin.

Recent experiments by Cox and Wendel (1942) support the contention that continued oral administration of nitrite is probably not harmful. They found that prolonged methaemoglobinaemia in a dog which received intravenously 30 mg. of sodium nitrite per kilogram of body weight at intervals of 5 to 6 hours (7 injections over a 36-hour period) had no apparent effect on the ability of the

natural reducing systems of the body to reduce the methaemoglobin so formed. In this experiment 52 to 65 per cent of the total haemoglobin was maintained in the form of methaemoglobin over the 36-hour period, and they calculated that the total blood pigment must have undergone reduction and oxidation at least four times. The average rate at which methaemoglobin was reduced *in vivo* to functionally active haemoglobin in dogs was 11.3 ± 2.0 per cent of the total blood pigment per hour. The injection of methylene blue accelerated the reduction. Experiments with rabbits and rats have shown that the methaemoglobin formed *in vivo* slowly disappears (Gelinsky 1940).

It appears that *in vivo* nitrite itself is probably partly oxidized to nitrate or further broken down, though some may be excreted unchanged (Sollmann 1922, Dixon 1921).

Hale (1910) believed that protein digestion was impaired in the presence of small amounts of nitrite, and more recently Sleeth and van Liere (1941) have stated that nitrite delays the emptying time of the human stomach. Further experimental data are needed to confirm or disprove these statements.

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Use of Vacuum Distillation and Gas Chromatography–Mass Spectrometry for Determination of Low Levels of Volatile Nitrosamines in Meat Products

2204

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A method is described for the identification and determination of volatile nitrosamines in meat products based on vacuum distillation linked with gas chromatography–mass spectrometry. Lower limits of detection for the technique range from 25 ppb for *N*-nitrosodimethylamine (DMN) to 65 ppb for *N*-nitrosodipentylamine. The technique has been used to screen seven types of meat products for the presence of volatile nitrosamines, and all results were negative. The paper also discusses techniques by which certain individual nitrosamines, including DMN, can be detected at levels of <10 ppb.

Since Magee and Barnes (1956, 1967) published their work on the carcinogenic properties of nitrosamines, numerous authors claim to have identified nitrosamines in a wide range of foodstuffs. These included smoked fish (Eder and Ceh, 1968), raw and cured meats (Mohler and Mayrohfer, 1968), flour (Kroller, 1967), milk (Hedler and Marquardt, 1968), and spirits (McGlashan *et al.*, 1968). In particular, efforts have been made to find them in cured meats where, theoretically, naturally occurring secondary amines might react with nitrite to form nitrosamines.

A wide range of analytical techniques was used (Fishbein and Falk, 1969) including nitrite release (Daiber and Preussmann, 1964), polarography (Heath and Jarvis, 1955; Lydersen and Nagy, 1967), tlc (Preussmann *et al.*, 1964) and glc (Serfontein and Hurter, 1966). Many of these methods are open to criticism and several recent studies (Heyns and Koch, 1970; Foreman *et al.*, 1970; Howard *et al.*, 1970; Henriksen, 1970) indicate that many, if not all, of these methods may lack the necessary specificity and/or sensitivity to give reliable results. It follows that if the methods themselves are suspect, the results thus obtained are also suspect. These doubts and criticisms are disturbing, in view of the significance which has been placed on the presence of small amounts of nitrosamines in foodstuffs (Druckrey *et al.*, 1963).

Early in our studies we examined samples of luncheon meat for their volatile nitrosamine content, using a steam distillation clean-up by ion-exchange chromatography and determination by gas chromatography with flame ionization detection. The type of chromatogram obtained is shown in Figure 1. From retention times we could presume the presence of significant amounts of several nitrosamines, but when this extract was examined by gas chromatography–mass spectrometry (gc–ms), we found that the peaks which might be ascribed to nitrosamines on the basis of retention times alone were, in fact, due to a range of compounds such as terpineol, benzaldehyde, and nonanol, and that no nitrogen-containing compounds were present.

To evaluate the many claims for nitrosamines in foodstuffs, we developed a determinative technique using gc–ms and applied it to the determination of volatile nitrosamines in meat products.

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EXPERIMENTAL

IMPORTANT SAFETY NOTE: Nitrosamines are highly carcinogenic compounds and all experimental work should be done in a well ventilated area. Safety gloves should be worn whenever nitrosamines are being handled.

Apparatus. A Pye 104 gas chromatograph was used, fitted with a 5.5-m × 4-mm i.d. glass column packed with 10% Carbowax 20M on acid washed, silanized Celite (100–120 mesh) and temperature programmed from 100° to 200° C at a rate of 2° C per min. Helium, at a flow rate of 40 ml per min, was used as the carrier gas in the gc–ms determinations. In all other gas chromatography work, nitrogen was used. Injections were made directly on to the column.

The column effluent was split approximately 10:1, the larger amount passing to an A.E.I. MS 902 mass spectrometer by means of a fritted glass separator (Watson and Biemann, 1965), and remainder to a flame ionization detector.

Fragment ions of composition NO^+ were monitored by using the peak matching facilities of the mass spectrometer, the mass scale being calibrated by reference to the background O_2^+ ion. The NO^+ ion current was recorded with a Brüel and Kjaer Level Recorder (Type 2305).

Compounds studied were the *N*-nitroso derivatives of dimethylamine (DMN), *N*-methylethylamine (MEN), diethylamine (DEN), *N*-methylisopropylamine (Mi-PN), *N*-methylisobutylamine (Mi-BN), *N*-methylbutylamine (Mn-BN), diisopropylamine (Di-PN), dipropylamine (Dn-PN), *N*-ethylbutylamine (En-BN), diisobutylamine (Di-BN), dibutylamine (Dn-BN), *N*-methyloctylamine (MON), diisopentylamine (Di-Pent N), dipentylamine (Dn-Pent N), pyrrolidine (NNPyrr), and piperidine (NNPip).

Analytical Procedure. Weigh 250 g of mixed sample into the container of a macerator, add 50 g of sodium chloride, 10 g of potassium carbonate, and 250 ml of water, and macerate the mixture for 10 min. Transfer the resultant suspension to a 2-l. round-bottomed flask, washing out the macerator with a minimum of water. Immerse the flask in a water bath at <10° C and connect it as part of a vacuum still, passing ice water through the condenser and cooling the 500-ml receiver in an ice bath. Apply the maximum vacuum of a rotary oil pump and heat the water bath to 65° C, ensuring that the whole surface of the distillation flask is kept at this temperature. Maintain these conditions until no more distillate is produced (3–5 hr). Release the vacuum and wash the condenser and adaptors with water, adding the washings to the distillate. Note the volume of distillate (≤250 ml). Extract the distillate and washings with two 250-ml portions

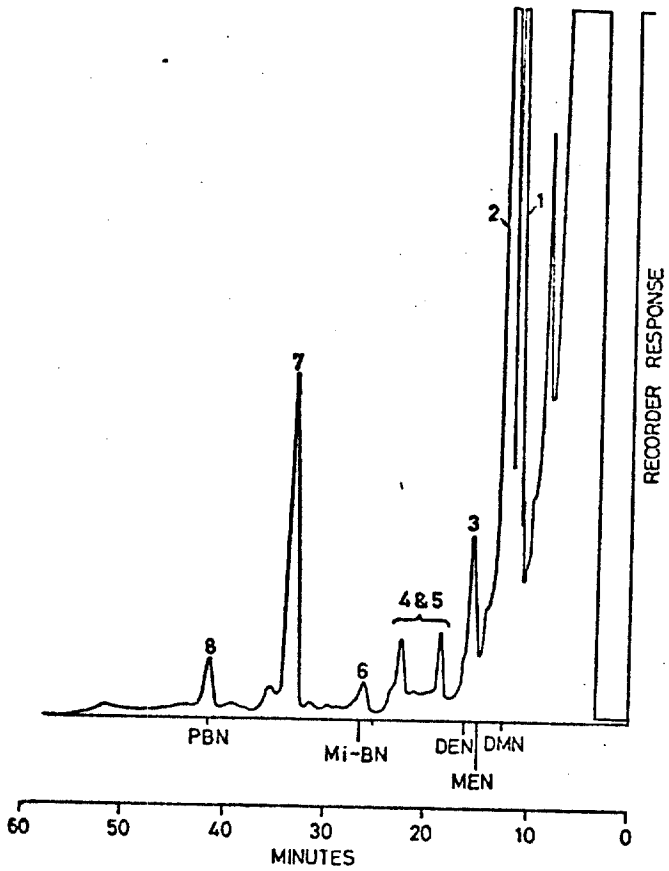

Figure 1. Gas chromatography of pork luncheon meat extract prepared by steam distillation and ion exchange

Instrument: Pye 104 F.I.D. Column: 5 ft 10% Carbowax 20M on acid washed HMDS treated Celite. Temperature: Isothermal at 170° C. Carrier gas: Nitrogen. Inlet pressure: 28 psi. Flow rate: Approximately 36 ml/min. Peak 1, acetoin; 2, hydroxy acetone; 3, nonanol; 4 and 5, terpene $C_{10}H_{18}O$; 6, benzaldehyde; 7, sesquiterpene and furfuryl alcohol; 8, α-terpineol. The corresponding retention times of nitrosamines are indicated

of dichloromethane, running the dichloromethane extracts into a Kuderna-Danish evaporator.

Immerse the lower part of the evaporator in a water bath held at 50° C, and evaporate the dichloromethane, making sure that the level of solution in the evaporator is always above the water level in the bath.

When the volume of the extract is approximately 5 ml, remove the bulb from the collection tube, fit a Quickfit cone to the tube, and continue evaporating until the volume is reduced to 1 ml. Add 0.4 ml of redistilled hexane and continue the concentration until the volume is 0.1 ml.

Examine 10 μl of this concentrated extract by high-resolution gc-ms with the mass spectrometer set to detect the NO^+ ion mass (29.99799) at a resolution of 15,000.

Note those peaks in the chromatogram which give a positive NO^+ response. If a positive result is obtained, inject an additional 10-μl sample and record the low resolution mass spectrum of the corresponding peaks in the conventional way.

High-resolution mass spectrometry monitoring for NO^+ ions will detect a minimum of 0.1-0.2 μg of individual nitrosamines. An identifiable low-resolution mass spectrum can be obtained from 0.05 μg of a single pure nitrosamine injected on the column, but in the presence of other species distilled from meats by the above technique, a minimum of 0.5 μg is required to give a sufficiently intense spectrum for positive identification. For a 10-μl injection this corresponds to a level of 20 ppb of individual nitrosamines in the original sample, assuming 100% recovery through the distillation and extraction procedures.

But tests made on the nitrosamines listed above using the procedures resulted in recoveries which ranged from 92% for DEN and MEN to 31% for Dn-Pent N (Table I). Therefore the 20 ppb detection limit must be increased to 25 ppb for DEN and MEN and 65 ppb for Dn-Pent N.

Table I. The Percentage Recovery of Nitrosamines at the 10-μg Level from an Aqueous Salt Solution and from a 250-g Sample of Ham Using Steam Distillation and Vacuum Degassing Without Cleanup

Nitrosamine	From aqueous salt soln: Steam distillation	From aqueous salt soln: Vacuum degassing	From 250-g ham: Steam distillation	From 250-g ham: Vacuum degassing
DMN	86	88	*a*	*a*
MEN	92	82	46	92
DEN	93	87	88	93
Mi-PN	93			87
Mi-BN	97			*a*
Mn-BN	93	89	80	85
Di-PN	96	89	67	89
Dn-PN	96		64	86
En-BN	91	90	70	86
Di-BN	88		53	78
Dn-BN	91	84	44	
MON	80	54	22	33
Di-Pent N	98			47
Dn-Pent N	46	57	31	31
NNPyrr	21	79	41	44
NNPip	76	90	33	73

[a] Interference from naturally occurring materials. All results represent the mean value for at least ten determinations.

Table II. Percentage Recoveries of 10 μg Nitrosamine through Various Stages of the Proposed Method

Nitrosamine	Carbon no.	Ion exchange	% Recovery: To 1.0 ml in dichloromethane	% Recovery: To 0.1 ml in hexane
DMN	2	94	93	60
MEN	3	97	89	89
DEN	4	94	97	81
Mi-PN	4	89	101	
Mi-BN	5	92	105	
Mn-BN	5	91	95	91
Di-PN	6	95	90	85
Dn-PN	6	84	96	84
En-BN	6	84	91	91
Di-BN	8	79	97	87
Dn-BN	8	72	95	86
MON	9	25	98	98
Di-Pent N	10	63	110	
Dn-Pent N	10	63	110	
Dn-Pent N	10	20	102	102
NNPyrr	5	68	98	92
NNPip	6	91	90	82

All values represent the mean value of at least six determinations.

RESULTS AND DISCUSSION

Development of the Method. CLEANUP OF DISTILLATES The three main column cleanup techniques which are quoted in the literature for nitrosamine extracts are based on alumina (Sen *et al.*, 1970), ion exchange (Sen *et al.*, 1969) or acid Celite (Howard *et al.*, 1970). Alumina has only been used as an extra cleanup step after ion exchange and the sulfuric acid-Celite technique proposed by Howard is only suitable for DMN and MEN.

We tried a mixed Amberlite IR 120/IRA 407 column as a cleanup step. As the recoveries (Table II) of 10-μg quantities

of individual nitrosamines show, appreciable losses occur for dialkyl nitrosamines with a carbon number greater than 8 as well as for NNPyrr. Since our aim was to develop a screening method for as many volatile nitrosamines as possible, we decided to develop a technique which did not include a cleanup stage.

THE USE OF STEAM DISTILLATION. Steam distillation can be used in place of vacuum distillation and is quicker, but the recoveries of added nitrosamines are generally much lower, as shown in Table I, than those from vacuum distillation. These lower recoveries mean lower sensitivity.

RECOVERIES OF NITROSAMINES THROUGH DISTILLATION AND PARTITION CONCENTRATION STEPS. When aqueous solutions of nitrosamines are extracted once with an equal volume of dichloromethane, more than 95% is found in the organic phase except for DMN, where only 77% is transferred to the organic phase. However, by partitioning with two portions of dichloromethane, less than 5% of the DMN is left in the aqueous phase.

Losses of nitrosamines during concentration from 500 ml of dichloromethane solution to 1 ml, transfer to hexane, and concentration to 0.1 ml are given in Table II. The only significant loss is that for DMN in the final concentration step.

Recoveries through the whole process as far as concentration to 1 ml are given in Table I. For recoveries from meat we used samples of cooked ham as our base material. We found that the background due to this product on the chromatographic scans was sufficiently low for an accurate assessment of recoveries to be made for all the nitrosamines except DMN and Mi-BN.

When recoveries of the higher nitrosamines from ham slurries and from an aqueous solution of NaCl and K_2CO_3 are compared, it can be seen that recoveries are much lower from the former than from the latter. It is not known whether these higher nitrosamines are decomposed or whether they are merely not extractable using the techniques employed.

THE USE OF GC-MS AS THE DETECTION STAGE. If each member of a class of compounds gives rise to an ion in its mass spectrum which is unique to that class, then by setting the mass spectrometer to monitor for the unique ion only, the mass spectrometer becomes a specific detector for that class of compound. Previous uses of the fixed mass technique in combination with gc-ms have been mainly confined to low-resolution mass spectrometry (Henneberg and Schomburg, 1971).

Each of the standard nitrosamines available for examination contained an ion of *m/e* 30 in its mass spectrum and high-resolution mass spectrometry established that this ion was composed of the species NO^+ and CH_4N^+. We decided to use the NO^+ ion as the fixed mass in preference to the CH_4N^+ ion, as the latter is a prominent ion in the spectra of many amino compounds (Budzikiewicz *et al.*, 1967), whereas NO^+ ions arise only from compounds containing NO or NO_2 groups.

The more probable combinations of C, H, O, and N of mass 30 and the resolution required to separate each combination from NO are given in Table III. A resolution of 15,000 is sufficient to separate the species NO^+ from all others, with the exception of $C^{18}O^+$. As we were aiming to identify nitrosamines at the lowest possible level, it was decided that, although a resolution of 25,000 is attainable with the MS 902 mass spectrometer, the resultant loss of sensitivity is too great for this particular application. Therefore the extracts prepared by the procedures already described were examined for the presence of NO^+ ions at a resolution of 15,000.

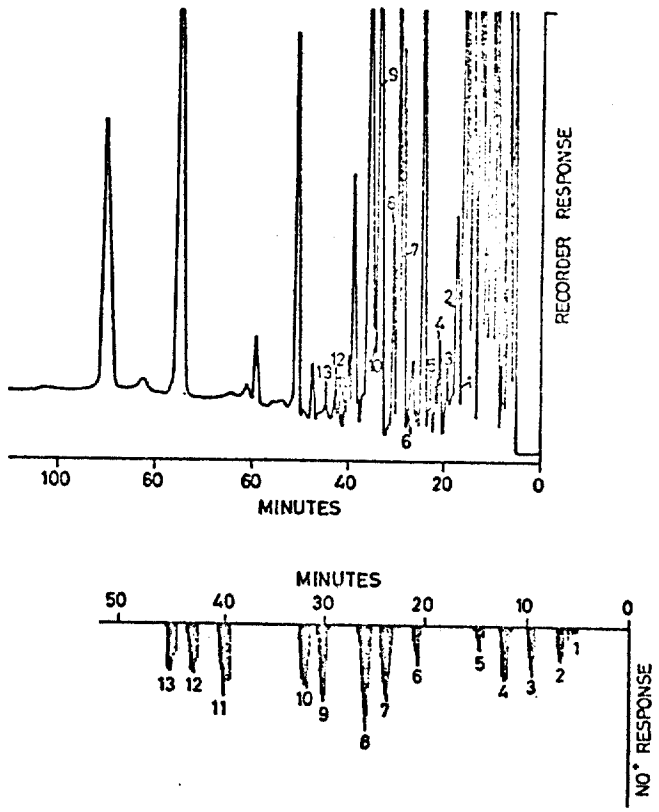

Figure 2. Chromatogram and corresponding NO^+ detector trace from pork luncheon meat spiked with nitrosamine. Peak numbers as in Table IV

Table III. Possible Elemental Compositions of Mass 30 and Resolution Required to Separate from NO^+

Composition	Exact mass	Approximate resolution to separate NO^+
NO	29.9980	
$C^{18}O$	29.9992	25,000
^{15}N	30.0002	13,600
^{13}CHO	30.0061	3,700
CH_2O	30.0106	2,400
H_2N_2	30.0208	1,300
$CH_3{}^{15}N$	30.0236	1,200
$^{13}CH_3N$	30.0309	900
CH_4N	30.0344	800
$^{13}CCH_5$	30.0425	700
C_2H_6	30.0469	600

The sensitivity of the gc-ms combination was determined in the following way. Standard nitrosamines were added at the 5-μg level to the dichloromethane extract prepared from a sample of pork luncheon meat after reduction to 1-ml volume. The mixture was then concentrated to a volume of 0.1 ml in hexane. Figure 2 shows the chromatogram and corresponding NO^+ ion record for this sample. Low-resolution gc-ms of the numbered peaks resulted in the identification of all the added nitrosamines as shown in Table IV. The success of the method is demonstrated by the location and identification of the nitrosamines which were not resolved from major components of the mixture. Although Mi-BN and Dn-PN were not resolved under the gas chromatographic conditions used, both were identified as components of the mixture. Similarly, Di-BN and En-BN were not resolved but were successfully identified. Peaks 1, 5, 6, and 9, which also gave a positive response, were found

Table IV. Compounds Identified in Spiked Pork Luncheon Meat Extract

Peak	Compound
1	Monoterpene hydrocarbon, hydroxyacetone
2	DMN
3	MEN, monoterpene hydrocarbon
4	DEN
5	Silicone, two oxygenated compounds
6	Oxygenated monoterpene
7	Dn-PN, Mn-BN, sesquiterpene hydrocarbon
8	Di-BN, En-BN, oxygenated monoterpene
9	Oxygenated monoterpene
10	Pn-BN, sesquiterpene hydrocarbon
11	Dn-BN
12	NNPip
13	NNPyrr

not to contain nitrosamines. As the compounds responsible for these peaks are oxygenated and do not contain nitrogen, their response is attributed to $C^{16}O^+$ and not to NO^+ ions. The peak due to hydroxyacetone is particularly interesting. In the presence of an excess of this compound it is very difficult to identify trace amounts of DMN by low-resolution mass spectrometry alone since their retention times are almost the same, their molecular weights are the same, and their fragmentation patterns are similar.

Certain specific nitrosamines can be detected at a sensitivity of 10 ppb or better by monitoring for ions other than NO^+. If it is suspected that, *e.g.*, DMN is present in the extract, the mass spectrometer under high-resolution conditions is set to record only the mass corresponding to the molecular ion of DMN. As this is approximately five times more abundant than NO^+ ion, a fivefold gain in sensitivity is achieved. Similarly, a gain in sensitivity will be realized for any nitrosamine which has a more abundant molecular ion than NO^+ ion. The greatest gains are given by DMN, DEN, MEN, Mi-PN, NNPyrr, and NNPip.

Monitoring the molecular ion has the additional advantage that if a positive result is found it need not be confirmed by recording a low-resolution mass spectrum, as it is exceedingly unlikely that any other compound of the same retention time as the nitrosamine will contain a fragment ion in its mass spectrum which corresponds to the chemical composition of the nitrosamine molecular ion.

It is not necessary to perform a separate run for every nitrosamine suspected to be present, provided that there is sufficient time between the elution of the nitrosamines from the gas chromatograph to allow the mass spectrometer to be readjusted to detect the next molecular ion.

All low-resolution mass spectral data obtained during this study have been submitted to the Mass Spectrometry Data Centre, A.W.R.E., Aldermaston, Berks, U.K.

Application to Meat Samples. The NO^+ ion monitoring technique described above has been used to examine a number of commercial meat products for the presence of volatile nitrosamines at the levels described in the method.

Products examined were canned pork luncheon meat, Danish back bacon, and injection-cured and slice-cured English bacon, all of which were cured with nitrate and nitrite. Other products examined were nitrite-cured cooked ham, fresh pork, and fresh beef.

In all cases we failed to detect the presence of any nitrosamine at the levels claimed for this method (*i.e.*, 25-65 ppb) depending on the nitrosamine.

These results are clearly at variance with a number of published claims to have identified nitrosamines in meat products such as raw beef (0.25 ppm of DMN) (Mohler and Mayrohfer, 1968).

Although this technique will not allow the analyst to prove the complete absence of nitrosamines in meat products, it does give a means of proving or disproving claims of the presence of volatile nitrosamines in the ppm rather than ppb range.

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pathological changes in soft tissue or bony structures. Pneumoperitoneum (fig. 4) revealed the right side of the pelvis to be normal. The left side showed a nodular mass of tissue indicating tumor growing in the lymph nodes and causing obstruction to the lymphatics of the left leg. As a result of this information, further intense roentgen therapy was given, and the swelling promptly disappeared.

These are only two cases from the series that I have been privileged to examine, but they indicate the importance of the information that this procedure will produce.

SUMMARY

In summary, I feel that pneumoperitoneum offers a method of determining the size, shape and position of pelvic structures so that accurate evaluation of clinical information can be made. This is an accurate method of determining intrauterine or ectopic pregnancy without interrupting the pregnancy. This is a method for evaluating progress and determining treatment of patients with malignant disease in the pelvic structures.

Pneumoperitoneum should be more widely used as a diagnostic aid in the leading centers of this country.

ABSTRACT OF DISCUSSION

DR. JOHN O'SULLIVAN, Melbourne, Australia: How much air does Dr. Maxfield inject?

DR. FRANK J. RIGOS, Tacoma, Wash.: What position does he use for the roentgen examination?

DR. J. MAURICE ROBINSON, San Francisco, Cal.: I should like to ask whether he uses carbon dioxide instead of air.

DR. J. R. MAXFIELD JR., Dallas, Texas: I apparently left out [illegible] than I should have on the subject of technic. These technics have been described in several papers. The amount of carbon dioxide (and that is the gas that should be used in all of these, because one can eliminate the possibility of danger by using carbon dioxide) is approximately 2,000 cc. This does not cause undue discomfort to the patient. A patient who has marked ascites, in which there has been the previous removal of fluid, will tolerate 4,000 or 5,000 cc. without difficulty. The positions that I use routinely for pelvic structure study are the right lateral decubitus and the left lateral decubitus, with the head down at a Trendelenburg position of 15 degrees. The patient is then turned to the prone position, with the table inclined to 45 degrees and the beam inclined downward through the pelvic strait, so that the patient's pelvis is up, the head is down and the beam goes through at an angle to outline pelvic structures. In this position the structures seem to have the gas around them for the best contrast. Dr. Irving Stein, in his numerous writings, has described the technic in which a knee-chest position is used. A special table has been made so that the patient can actually be in a knee-chest position in which the pneumogram of the pelvis can be taken. However, I feel that one should encourage the use of pneumoperitoneum in the general radiological laboratories. It can be done on the standard tables, and excellent films can be obtained without great difficulty to patient or radiologist. The paper has numerous bibliographical references that will describe the use of pneumoperitoneum in detail. The apparatus to induce it is quite simple and is also described in previous papers.

Infectious Mononucleosis.—There is no specific therapeutic agent for infectious mononucleosis. Sulfonamides and penicillin may benefit a complicating streptococcus throat infection. Convalescent infectious mononucleosis and scarlet fever sera have given dramatic results in some seriously ill patients. Human plasma and gamma globulin have occasionally appeared to be beneficial. Aureomycin and Chloromycetin seem effective in some cases when started early in the disease. Hepatitis is extremely common, and when severe should be treated exactly like cases of infectious hepatitis.—Robert E. Kaufman, M.D., Treatment of Infectious Mononucleosis, *American Practitioner*, April, 1951.

CLINICAL NOTES

METHEMOGLOBINEMIC CYANOSIS

REPORT OF AN EPIDEMIC DUE TO CORNING EXTRACT SUBSTITUTED FOR MAPLE SYRUP

2206

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Sudden onset of cyanosis is an alarming event that usually suggests a cardiovascular or pulmonary catastrophe. A rarer cause is acute toxic methemoglobinemia. We recently had an opportunity to study an "epidemic" of the latter condition involving at least seven patients. All had eaten wheatcakes and "maple syrup" at a diner. This report briefly summarizes the case histories and describes the series of events leading to discovery of the offending agent. A similar epidemic of methemoglobinemia due to accidental nitrite ingestion was reported by Greenberg, Birnkrant and Schiftner,[1] who reviewed the literature up to 1945. At that time, they called attention to the serious nature of the poisoning and mentioned the high fatality rate. At least 19 deaths due to sodium nitrite have been reported. Since Greenberg's article appeared, the therapeutic use of methylene blue for cyanosis has been established beyond doubt. Although five of our seven patients in the present series were not particularly ill and recovered spontaneously, the remaining two patients appeared moribund on hospital admission. In these two patients, methylene blue was employed intravenously and appeared to be lifesaving.

REPORT OF CASES

CASE 1.—C. C., a 37 year old male taxi driver, was admitted to the ward service of the Syracuse Memorial Hospital at 9:15 a. m. on May 15, 1950, because of severe pounding of the heart and increasing bluish discoloration of the skin of one hour's duration.

At 7:30 a. m. on this day, the patient had pancakes and syrup at a diner in Syracuse. He thought the butter tasted rancid. At 8:15 a. m. he became nauseated and vomited small amounts of food. Shortly thereafter, dizziness, palpitation and cyanosis developed. At 6:15 on the morning of admission, he had taken one tablet of a commercial headache preparation and for 48 hours before entry he had been taking acetylsalicylic acid. He also stated that in the course of his occupation he had to wear dark trousers because the dye from the seat covers of the taxi was apt to stain his trousers and his skin.

Physical examination on admission showed the patient to be conscious but markedly cyanotic and dyspneic. His blood pressure was 120/70, temperature 99.8 F., pulse rate 140 and respiration rate 22. Except for the cyanosis and a few rales over the left lower lung field, the entire examination was

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Dr. Jaime Paris assisted in the clinical management of the first patient. Mr. Richard Wilson and Mr. Melvin Rossman, of the class of 1951, aided in collecting the samples of food which permitted the identification of the toxic substance. The following physicians permitted us to report their patients: Dr. S. Schwartzbart, case 2; Dr. S. Schwartzberg, case 3; Drs. W. Saile and J. G. F. Hiss, case 5.

1. Greenberg, M.; Birnkrant, W. B., and Schiftner, J. J.: Outbreak of Sodium Nitrite Poisoning, Am. J. Pub. Health 35: 1217-1220 (Nov.) 1945.

noncontributory. His heart and lungs showed no abnormality on physical examination.

Laboratory Data.—On admission, the patient's hematocrit reading was 49 per cent, and his leukocyte count was 11,500, with 67 per cent polymorphonuclear cells, 1 per cent monocytes, 1 per cent eosinophils and 31 per cent lymphocytes. Results of urinalysis were normal. The nonprotein nitrogen concentration was 42 mg. per 100 cc. and the fasting blood sugar level was 110 mg. per 100 cc. On the day after admission his leukocyte count was 14,000 with 70 per cent polymorphonuclear cells.

Hospital Course.—On admission the patient was placed in an oxygen tent, but despite this his cyanosis increased. At 10:15 a. m. he became irrational; his blood presure fell to 70/50. He then fainted and had minor generalized convulsions. After he was placed in the Trendelenberg position, his rationality returned, and he complained of tingling of his hands. This process was reversed by raising his back, and then he would faint. Blood was drawn at this time, and it appeared grossly to be of dark chocolate color. Oxygen bubbled through it did not change its color, and a clinical diagnosis of methemoglobinemia was made. At this time, 100 mg. of methylene blue was administered intravenously. Immediately before administration of the methylene blue, blood was drawn for a methemoglobin determination according to the method of Evelyn and Malloy.[2] Methemoglobin was estimated to be present in the amount of 75 per cent of the total pigment. Twenty minutes after the administration of the methylene blue the tingling had disappeared from the extremities and the man became rational. Fifty minutes after the administration of methylene blue the methemoglobin concentration was 60 per cent. Cyanosis was definitely less. The blood pressure was 100/70. Three hours after the administration of methylene blue, cyanosis could not be detected clinically but the methemoglobin concentration was reported as 18 per cent. Another 100 mg. of methylene blue was injected intravenously at this time, and thereafter all signs and symptoms improved. He was discharged from the hospital on May 19. An electrocardiogram taken when the patient's methemoglobin concentration was 75 per cent showed sinus tachycardia with slight RS-T segment depression in leads V_4, V_5 and V_6. Two days later the electrocardiogram was entirely within normal limits.

The etiological factors considered in this case on the day of admission were acetanilid, aniline dye and sodium nitrite or nitrate adulteration of the food. The overwhelming nature of the methemoglobinemia and the rapidity of its onset following the morning meal suggested that the offending toxin was most likely a nitrite and that the patient had ingested it with his breakfast. However, no steps were taken to identify the toxic agent until after the admission of the second patient.

CASE 2.—H. R., a 54 year old telephone lineman, was admitted to the Crouse-Irving Hospital on May 16, 1950, at 11:00 a. m., complaining of a faint feeling and dyspnea of three hours' duration. This man had been in excellent health until the morning of admission, when, after a breakfast of pancakes and syrup at 8:00 a. m. at the same diner as the first patient, he suddenly became nauseated and felt "numb" and had generalized tingling. He then became weak and dyspneic, staggered and then fainted. A physician was called who found him extremely cyanotic, dyspneic and disoriented and referred him to the hospital.

Physical examination showed the patient to be comatose with marked generalized cyanosis. His temperature was 99.8 F., pulse rate 150, respiration rate 40 and blood pressure 90/50. Aside from a small bruise on the vertex of his head, the remainder of the physical examination was entirely noncontributory.

Laboratory Data.—On admission his hemoglobin was 13 Gm., erythrocyte count 4,000,000, leukocyte count 50,000 with 90 per cent polymorphonuclear cells and 10 per cent lymphocytes. Nonprotein nitrogen concentration was 44 mg. per 100 cc., fasting blood sugar 254 mg. per 100 cc. (intravenous feeding was in progress at the time). Urinalysis revealed no abnormality. A lumbar puncture showed the spinal fluid to be clear with no cells and with a protein content of 27 mg. per 100 cc. The dynamics were normal.

Hospital Course.—The blood of this patient was also noted to be chocolate brown and did not change when it was equilibrated with air. A clinical diagnosis of methemoglobinemia was made, and he was given 100 mg. of methylene blue intravenously. Blood drawn before the admission of methylene blue showed 78 per cent concentration of methemoglobin. Twenty minutes after the administration of methylene blue the patient was able to speak coherently and at this time gave the above history. In 30 minutes the cyanosis had markedly lessened. His blood pressure rose to 120/80, and two hours after the first injection of methylene blue his methemoglobin was 18 per cent. He was given another 100 mg. of methylene blue and he continued to improve. He was discharged on May 18. This case was complicated at first by the suggestion that the patient's condition might have been due to a head injury he had sustained in the course of his work on the day before admission.

After the discovery that the only etiological factor in common between the first and the second patient was the wheatcakes, butter and syrup which each had eaten at the same restaurant, samples of these materials were collected and investigated by the department of pharmacology. Three cubic centimeters of an aqueous extract of the wheatcakes, syrup and butter was fed to each of three adult male rats by stomach tube. All the animals died in about 35 minutes with quantitative conversion of their hemoglobin to methemoglobin. A qualitative test of this extract was strongly positive for nitrite ion. The department of health was then notified, and samples of all materials which might possibly have been involved in the meal were sent to the department of pharmacology for acute toxicity studies. The pancake batter and the butter were nontoxic, but what was thought to be maple syrup produced methemoglobinemia in rats. It was then learned that the diner prepared its maple syrup by adding maple flavoring extract to sugar syrup. However, when each of these ingredients was tested separately in animals neither produced methemoglobinemia. On further questioning of the restaurant proprietor, it became apparent that a gallon jug of corning extract, used in corning beef and freshening hamburgers, had been substituted inadvertently for a gallon jug of maple syrup. The corning extract contained large amounts of sodium nitrite and some sugar and appeared grossly like the maple syrup and bore a remarkable resemblance to it in taste.[3]

All hospitals in Syracuse were then notified, and physicians were contacted with regard to any patients with unexplained cyanosis who had appeared within the previous week. In retrospect, the following five patients were given diagnoses of probable methemoglobinemia. None of them had laboratory confirmation of this diagnosis.

CASE 3.—L. B., a 52 year old laborer, had pancakes and syrup on May 11, 1950, at 8:00 a. m. at the diner mentioned above. About one hour later, he appeared dizzy and faint and his fellow workers noticed a bluish tint to his skin and

2. Evelyn, K. A., and Malloy, H. T.: Micro-Determination of Oxyhemoglobin, Methemoglobin and Sulfhemoglobin in a Single Sample of Blood, J. Biol. Chem. 126: 655-662 (Dec.) 1938.

3. This corning extract is known as Afral and contains 8 per cent nitrites, 4 per cent nitrates and sucrose.

that day but left his job during the morning, went home, drank some milk and went to sleep. At 2:30 p. m. he awoke feeling better and walked about. He felt slightly weak for about a week but did not return to work for 12 days. Eleven days after this incident, he received a physical examination which revealed no abnormality.

CASE 4.—M. S., a 38 year old male newspaper employee, was admitted to St. Joseph's Hospital in Syracuse at 4:00 a. m. on May 15, 1950. At 2:00 a. m. on the same morning he had pancakes and syrup at the same diner. He noticed that the syrup tasted salty. One hour later he began to feel weak and his fellow employees noticed his bluish color. At the hospital he was placed in an oxygen tent, with slight improvement, but no other symptoms developed nor were there any positive physical findings. By noon his color was normal and he felt completely well except for some headache. He was discharged at 2:00 p. m.

CASE 5.—R. B., a 46 year old white male cabinetmaker, on May 13, 1950, at 9:00 a. m. had pancakes and syrup at the previously mentioned diner. He noticed that the syrup tasted salty. At 10:00 a. m. he began to notice pain in his chest over the region of the heart and epigastric and arm pain, accompanied with palpitation and dyspnea. He was seen by a physician at 1:00 p. m., who noticed extreme cyanosis, dyspnea, auricular fibrillation and a blood pressure of 100/60, although it had previously been 130/80. The patient improved gradually; auricular fibrillation stopped in 24 to 36 hours; cyanosis cleared in 3 to 4 days, and the blood pressure returned to its former level. On May 16, he had some abdominal pain and diarrhea which cleared within 24 hours. An electrocardiogram taken eight days after the acute episode, was normal. The provisional diagnosis on May 13 was coronary occlusion, but the diagnosis in retrospect was methemoglobinemia with coronary insufficiency due to anoxia.

CASE 6.—H. H., a 35 year old male newspaper employee, had pancakes and syrup on May 13, 1950, at 7:15 p. m., at the above diner. One hour later he felt nauseated and vomited a small amount of food. He then noticed that his nails were extremely dark. Because of his unsteadiness on his feet, he took a cab home. On arrival home, his wife noticed that he was markedly cyanotic and dyspneic. He went to bed and at 4:00 a. m. felt distinctly better, and his cyanosis had lessened. At 9:00 a. m. he was completely well. He did not see a physician professionally, but reported the episode to a friend who was a physician.

CASE 7.—P. T., a 27 year old newspaper employee, had pancakes and syrup with the preceding patient (case 6). He noticed that the pancakes and syrup tasted peculiar and did not finish them. He took two bromo-seltzer* tablets and immediately felt sick but did not vomit. On returning to work, his colleagues noticed that he was extremely cyanotic. He noticed only a peculiar taste in his mouth and continued to work for the next 2½ hours; then he went home. The following morning he felt well but retained a peculiar taste in his mouth. He did not consult a physician but reported this incident to the patient mentioned in case 6, who included it in his report to his physician friend.

COMMENT

The clinical diagnosis of methemoglobinemia in case 1 was suggested by (1) the history of sudden onset of cyanosis after eating, (2) the presence of intense cyanosis without gross evidence of cardiac or pulmonary disease, (3) the symptoms and signs of severe anoxia while in an oxygen atmosphere and (4) the peculiar chocolate brown appearance of the blood, with no change in color after oxygen had been bubbled through the sample. In retrospect, typical nitrite hypotensive reactions were observed in cases 1 and 2. The effects of anoxia on the coronary circulation were evi- the symptoms of coronary insufficiency in case 5. Presumably, the sickest patients had the greatest nitrite intake; indeed, the first patient even finished the syrup remaining after the wheatcakes had been eaten.

The physiological and pharmacologic mechanisms involved in the appearance and disappearance of methemoglobinemia have been thoroughly reviewed by Finch,[4] whose article presents an excellent discussion. Our patients all had secondary methemoglobinemia, due to accidental nitrite ingestion. Once absorbed, the nitrites oxidized hemoglobin to methemoglobin at a rate faster than the reverse reaction could be carried out by the hypothetical normal cellular mechanisms. There was no evidence that any of these patients suffered a congenital lack of these normal cellular mechanisms (primary methemoglobinemia), and neither ascorbic acid nor glutathione, useful in this congenital type of deficiency[5] was given to these patients. The treatment of secondary methemoglobinemia is based on the observation that methylene blue enormously potentiates the action of the normal red blood cell enzymatic system that reduces formed methemoglobin at a rapid rate.[6] This rate is faster than that at which hemoglobin can be oxidized by most of the known methemoglobin formers.

SUMMARY

As a result of the clinical diagnosis of methemoglobinemia in two patients, the responsible toxin was identified and traced to "maple syrup" served in a public restaurant. Corning extract containing nitrite and sucrose had been inadvertently substituted for the syrup. In retrospect, the diagnosis of methemoglobinemia was made for five additional patients. Methylene blue administered intravenously to the two sickest patients produced dramatic, apparently life saving results.

It is suggested that in view of this episode, suitable steps be taken by public health authorities to identify properly such corning extracts and to call attention to their toxic effects if consumed in quantity.

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Preparing Children for Pain.—Virtually all children have a great deal of anxiety about being in hospitals, particularly about the so-called minor operations so common in childhood. Although hospital and operative procedures leave their greatest scars in neurotic children, they also leave some psychic scars in normal children. We have noticed that these are more traumatic to the child when he is not told the truth about the procedure and when it is not discussed with him before it is done. We would like to condemn the common practice of telling a child, "This won't hurt," and then sticking him with a needle, putting an ether mask over his face, etc. It is less traumatic to tell a child that it is going to hurt, but you will try to get it over with quickly and with no more hurting than absolutely necessary, and that he will feel better afterward.—F. G. Ebaugh, M.D., and J. J. O'Hearne, M.D., Psychiatry, in Medicine of the Year: Philadelphia, J. B. Lippincott Company, 1951, p. 71.

HEPATIC PATHOLOGY IN RATS ON LOW DIETARY LEVELS OF DIMETHYLNITROSAMINE

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DIMETHYLNITROSAMINE (DMN) is a very powerful liver carcinogen for the rat (Magee and Barnes, 1956) and will produce malignant tumours at other sites and in other species (Magee and Barnes, 1967).

Because of the possible importance of certain nitrosamines as aetiological agents for cancer in the human environment (Magee and Barnes, 1967) it was important that an attempt should be made to try to obtain experimental data on the basis of which it might be possible to calculate a non-effective dose of this compound. This also required an evaluation of non-neoplastic lesions appearing in animals exposed to the carcinogenic nitrosamine and their relation to tumour development. The experiments reported here were carried out in this laboratory since 1957 and dietary concentrations of DMN ranging between 2 and 50 p.p.m. were used. No non-neoplastic lesions were found to be specifically linked with the appearance of tumours. The findings are discussed in relation to the problem of constructing the dose-response relationship of a powerful carcinogen which in this case is probably produced within the animal body from the compound which is added to the diet.

MATERIALS AND METHODS

Male and female white rats (Porton strain) 4–6 weeks old were housed 6 to a cage. All rats were given Diet 41B (Bruce and Parkes, 1957) in powder form. Food intake was measured throughout the experiments and the rats were weighed twice weekly. All sick animals and any animal found to be losing weight were killed with coal-gas.

DMN (British Drug Houses, Ltd.) was added to the powdered diet as a solution in arachis oil. The solutions in oil were so made up that oil was added at the rate of 10 ml. to each kilo of powdered diet and mixed in a Hobart food mixer. The controls had oil alone added to their diet except for one group of 6 male and 6 female included from another experiment which were fed pellets. At intervals during the earlier experiments the level of DMN in the diets was checked by the method of Heath and Jarvis (1955). The amount of DMN was always within 20% of the expected concentration and it did not fall off within the periods (1–2 weeks) during which the batches of prepared diets were used.

Pathology

Autopsies were carried out shortly after death and the tissues fixed in either Helly's solution or formol saline. Several pieces of the liver were removed and in

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most experiments both kidneys together with any other tissue appearing abnormal at autopsy. The skull was opened and pituitary tumours and evidence of aural sepsis sought.

Tissues were fixed, cleared and imbedded in the usual way. Sections were cut at 7 μ and stained by haematoxylin and eosin.

RESULTS

Details of the animals used in the various experiments are given in Table I. Survivors at 104 weeks were usually killed; a few animals were left alive for up to

TABLE I.—*Summary of the Experimental Animals Used in Various Experiments in Which DMN was Given in the Diet*

Conc. of DMN in the diet (p.p.m.)	Sex	No. of animals: At start	No. of animals: After 60 weeks	Rats with liver tumours
0	M	12	11	0
	F	29	25	0
2	M	19	13	1
	F	18	13	0
5	M	6	5	1
	F	62	55	4
* 5	F	15	14	3
10	F	5	5	2
20	F	23	13	15
50	F	12	0	10

* These rats received 5 p.p.m. in their diet for 52 weeks and then received a normal diet.

120 weeks. Deaths before 60 weeks in untreated rats and those on the lower doses of DMN were due to intercurrent disease but on the higher doses (20 and 50 p.p.m.) were often due to liver tumours.

In one experiment animals received 5 p.p.m. DMN for only 52 weeks and were then returned to a normal diet and compared with those fed DMN at this level continuously. Six rats were killed at 52 weeks for examination of the liver. Because DMN can produce kidney tumours in rats under some conditions (Magee and Barnes, 1962) special attention was paid to the kidneys. No tumours visible to the naked eye were found at autopsy but a search was made for histological evidence of early lesions or other damage.

A complete autopsy was carried out on all but 2 of the rats but in a further 5 no histological material was prepared because of the advanced decomposition at the time of autopsy.

Pathological Changes

Liver

A special search was made for the cytological changes described in an earlier paper (Magee and Barnes, 1956). Large hepatic parenchymal cells with prominent nuclei were found in the livers of rats receiving 50 and 20 p.p.m. DMN both in the non-tumorous parts of livers with tumours and in those livers without tumours. However, at lower concentrations of DMN the numbers of large cells which were seen occasionally in non-neoplastic livers of animals dying late in the experiment did not differ significantly from the numbers seen in untreated rats. There are a number of changes which have been described as being frequently

associated with tumour formation in the liver of animals exposed to chemical carcinogens (Stewart and Snell, 1957). Of these changes areas of adenofibrosis were not found but pseudotubules and glandular lesions were seen during the early stages of exposure to the highest concentrations of DMN (Fig. 1). Slightly enlarged portal spaces with gland-like structures but without distortion of the liver architecture were seen in both treated and untreated animals (Fig. 2). The two changes which were obviously related to exposure to DMN but not clearly related to each other were tumours and cysts similar to those described by Stewart and Snell (1957). In Table II these findings are summarised for rats killed or

TABLE II.—*The Incidence of Tumours and Cyst Agglomerates in the Livers of Rats Receiving DMN in Their Diet*

DMN p.p.m.	Sex	Experiment weeks	Animals total	Tumours	Tumours and cysts	Cysts	Nil
0	F	0–60	4	—	—	—	4
		61–120	25	—	—	—	25
	M	0–60	1	—	—	—	1
		61–120	11	—	—	—	11
2	F	0–60	5	—	—	—	5
		61–120	13	—	—	—	13
	M	0–60	6	—	—	—	6
		61–120	13	1	—	—	12
5	F	0–60	8	—	—	—	8
		61–120	69	*5	2	14	48
	M	0–60	1	—	—	—	1
		61–120	5	*1	—	—	4
10	F	0–60	0	—	—	—	—
		61–120	5	**1	1	2	1
20	F	0–60	10	3	2	1	4
		61–120	13	4	*6	1	2
50	F	0–60	12	4	6	1	1

* Including 1 sarcoma.
** One carcinoma with glandular features.

dying in the first 60 weeks and the second 60 weeks of the various experiments. Cyst agglomerates were often recognisable to the naked eye and measured up to 2 cm. in diameter. They were multilocular and might replace extensive portions of the liver parenchyma. The contents of the larger ones were gelatinous. The cysts were lined with flat or cuboidal epithelial cells and might contain a pale eosinophilic PAS negative material. Septa between the cysts were thin but some papillary formation might be seen. Remnants of liver cords were often seen at the periphery and there was no capsule (Fig. 3 and 4). No nuclear irregularities were observed. Small isolated subcapsular cysts (Fig. 5) may be found in both experimental and in untreated rats and are considered to be distinct from the agglomerates. While the livers of rats on 20 and 50 p.p.m. DMN were grossly distorted with multiple cysts and tumours, these usually occurred singly in rats on lower doses. When cysts and tumours occurred together in the same liver the lesions were quite distinct. Of the 88 rats that received 5 or 10 p.p.m. DMN 19 had cyst agglomerates but only 3 had liver tumours as well; on the other hand of the 9 rats with tumours only 3 also had cystic agglomerates. Thus these lesions, while associated with an exposure to DMN, seem to develop independently.

Hyperplastic nodules (Fig. 6) were seen in 9 rats and in 7 there were also frank liver tumours making the differential diagnosis difficult but not of particular importance. Frank hepatomas were seen in 36 rats and the incidence was clearly dose-dependent in spite of the fact that animals on the lower doses lived longer. Of the tumours 33 were carcinomas of the trabecular or anaplastic type. Haemorrhage and areas of necrosis were common and metastases were found in the lungs of 11 rats. In 3 rats the tumours were spindle cell sarcomas with haemorrhage and necrosis but no metastases were found.

Kidney

No renal tumours were seen. The characteristic senile changes (Magee, 1959) were noted in rats which survived for more than a year. In 42 rats enlarged tubules similar to those described by others (Allen, Fisher and Adams, 1964; Foley *et al.*, 1964, and Terracini, Palestro, Gigliardi and Montesano, 1967) were observed. These were lined by large epithelial cells with pale uniformly staining cytoplasm and round nuclei. Small papillae were sometimes seen (Fig. 7). These lesions appeared irrespective of the sex or treatment of the rats and were common in the older rats, although not always associated with the senile changes.

Lungs

In addition to the inflammatory changes and associated bronchiectasis and liver tumour metastases, a benign primary tumour was found in 4 rats on 2 or 5 p.p.m. DMN. In one a papilloma obstructed a bronchus of a female which also had a breast tumour. In 3 rats there were small adenomas replacing lung parenchyma with the formation of irregular cavities lined with cuboidal cells with large nuclei. Although they had no capsule there were no histological signs of malignancy. The tumours were quite distinct from the hyperplasia seen associated with chronic inflammation.

Other tumours

The incidence of other tumours is given in Table III. There is no obvious relationship between the site and frequency and the dose of DMN which the animals had received.

EXPLANATION OF PLATES

FIG. 1.—Rat (F). DMN 20 p.p.m. for 50 weeks. Liver: enlarged and confluent portal spaces with gland formation. H. & E. × 125.

FIG. 2.—Rat (F) control killed 115th week. Small cluster of glandular cavities lined by cuboidal cells in a portal space. H. & E. × 125.

FIG. 3.—Rat (F). DMN 5 p.p.m. for 110 weeks. Liver: circumscribed cystic lesion. Cysts are empty, irregularly shaped and intermingled with liver parenchyma. H. & E. × 50.

FIG. 4.—Rat (F). DMN 5 p.p.m. for 111 weeks. Liver: cyst agglomerate; cysts lined by flat epithelium are separated by thin fibrous septa. No atypical cells. H. & E. × 140.

FIG. 5.—Rat (F). DMN 5 p.p.m. for 104 weeks. Liver: isolated cyst lined by flat epithelial cells. This was the only change found in the liver. H. & E. × 140.

FIG. 6.—Rat (F). DMN 20 p.p.m. for 61 weeks. Liver: well defined nodule composed of sheets of liver cells with no topographical relations to vascular structures or portal spaces. No atypical cellular features or mitoses. This animal had no liver tumours. H. & E. × 55.

FIG. 7.—Rat (F). DMN 2 p.p.m. for 95 weeks. Kidney: hyperplastic tubule lined by tall cells with pale cytoplasm. Some nuclei are piled up. P.A.S. × 140.

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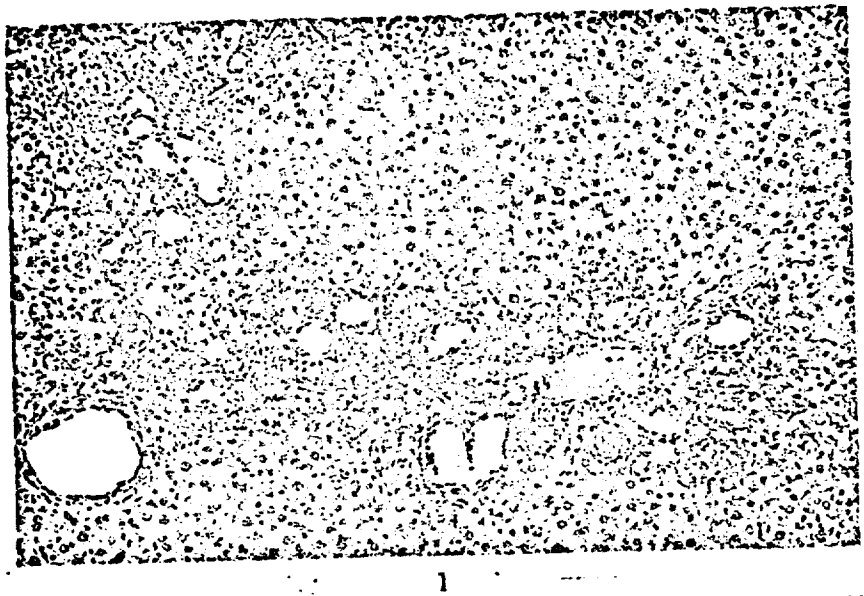

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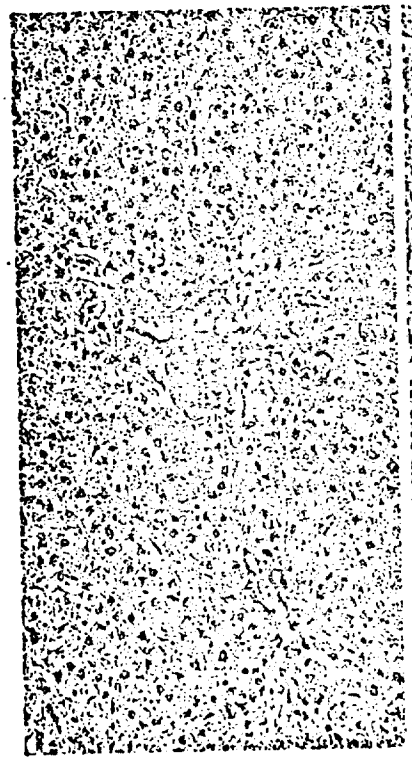

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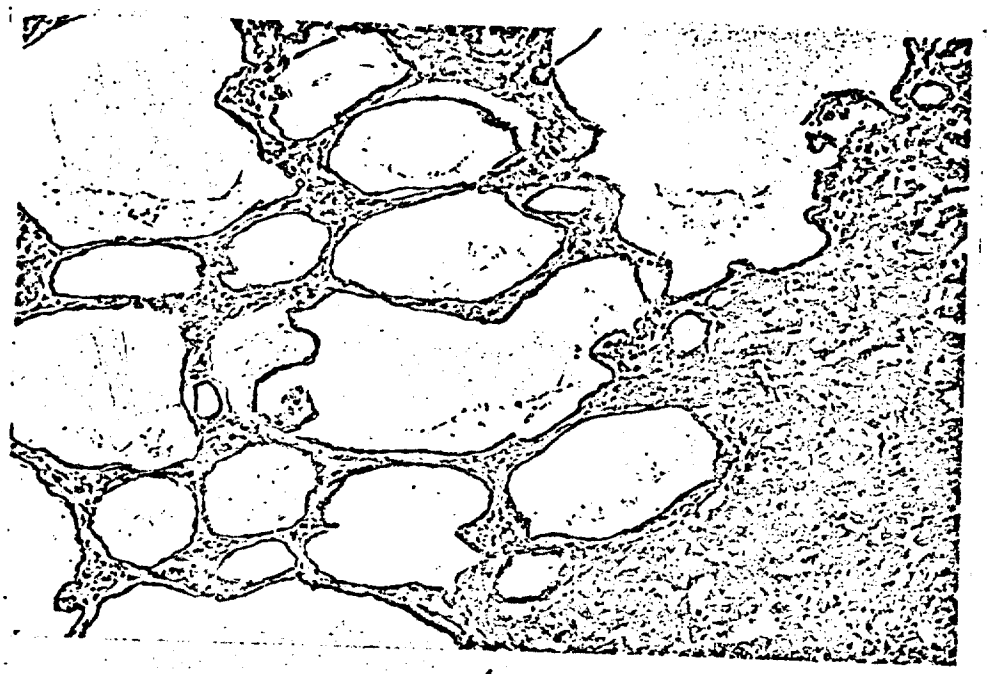
Terracini, Magee and Barnes.



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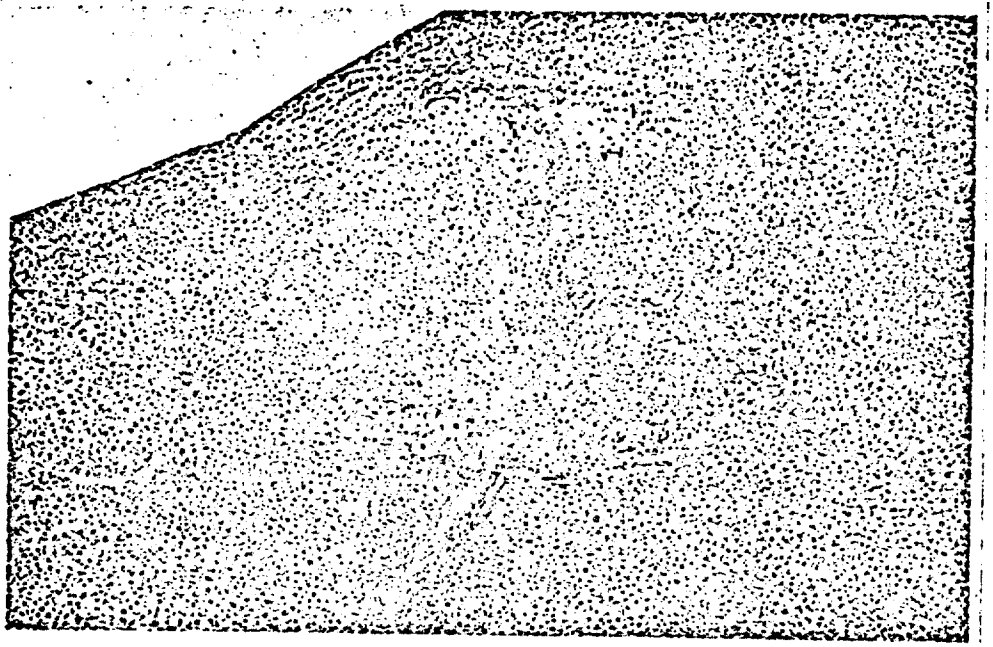

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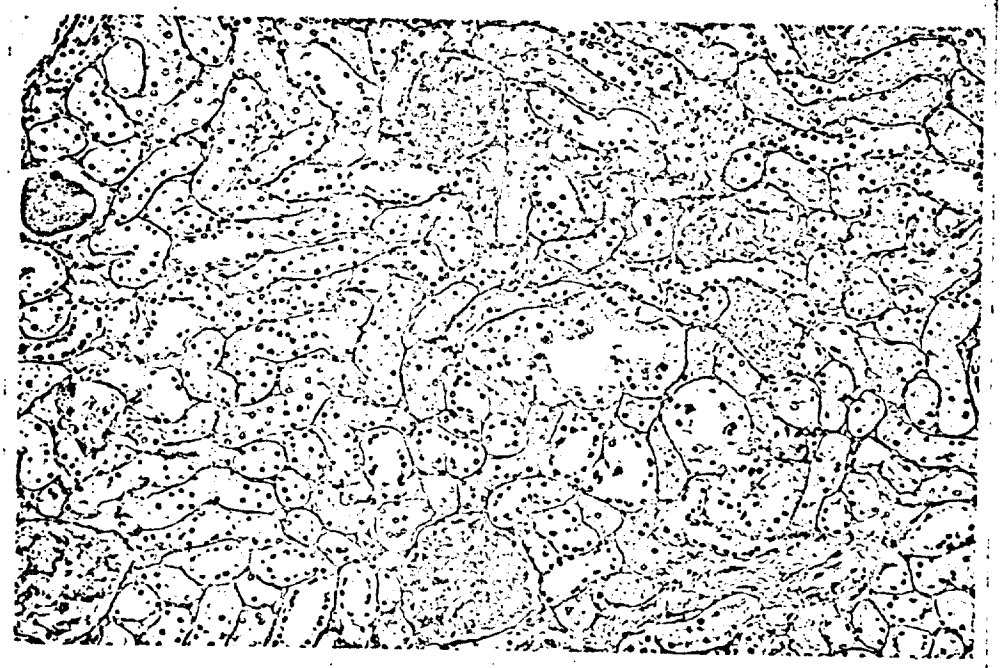

7

Terracini, Magee and Barnes.

TABLE III.—*Kidney Lesions and Incidence of Other Tumours in Rats Receiving DMN*

DMN p.p.m.	Sex	No.	*Renal tubule hyperplasia	Lung tumours	Mammary tumours	Lymphoma	Other tumours
0	M	12	2/11	0	0	1	1 subcutaneous tumour (no histology).
0	F	29	6/26	0	3	0	1 uterine sarcoma. 1 pyloric adenocarcinoma.
2	M	19	4/18	1	0	1	1 carcinoma of mouth.
2	F	18	7/12	2	3	0	1 adenocarcinoma of ovary.
5	M	6	3/5	0	0	0	—
5	F	77	17/72	1	5	3	1 carcinoma of mouth. 1 intestinal sarcoma. 2 uterine adenocarcinomas. 1 pyloric adenocarcinoma. 1 peritoneal sarcoma. 1 ovarian tumour.
10	F	5	0/5	0	0	0	1 carcinoma of mouth.
20	F	23	2/20	0	0	0	1 peritoneal sarcoma.
50	F	12	0/9	0	0	0	—

* Number of animals showing the lesion from among those from which kidney sections were available.

DISCUSSION

The exposure of rats to low doses of DMN throughout their life span was undertaken in order to try to establish a sequence of pathological changes that might run parallel with the change in tumour incidence. Since the whole liver of a rat exposed to high doses of DMN undergoes a plethora of histological and cytological changes in association with the development of tumours, it was hoped that there might be some widespread non-cancerous changes which would provide evidence of possible impending malignant transformation. The comprehensive study of the liver changes in the rats exposed to low doses of DMN revealed no such indication. While the incidence of tumours fell rapidly when the dose was decreased from 50 to 5 p.p.m., some malignant tumours were seen at the latter dose and even at 2 p.p.m. The only other liver changes consistently found were cyst agglomerates; these were somewhat more frequent than hepatomas in rats on 5 p.p.m. DMN and were not seen at all in those on 2 p.p.m. As indicated above, these cyst agglomerates appeared to arise quite independently from the hepatomas. It was not possible to detect cytological changes or minor deviation from normality in hepatic architecture that made it possible to distinguish rats on a diet containing 2 or 5 p.p.m. DMN that would produce a low incidence of tumours, and rats on a normal diet of which none in our laboratory has developed hepatomas.

It is very important to try to establish some idea of the dose-response relationships to the exposure of animals to carcinogens. However, in most laboratories it is impractical to expose very large numbers of animals to a carcinogen at doses that may be expected to produce only a very low incidence (say 1 : 1000) of tumours. Such an incidence would of course represent an appalling hazard to human populations. Druckrey (1952) has already expressed the view that all doses of carcinogens are purely additive but attempts to establish the truth of this at low dose levels of a carcinogen are frustrated by the limited life span of the experimental animals. The use of specific pathogen-free rats and mice with a longer life span will provide more opportunities for doing such studies. However, the simple concept of the additive effect of repeated doses is not applicable to every situation. The incidence of kidney tumours in rats given DMN illustrates such an exception.

Among rats surviving a single intraperitoneal LD_{50} of DMN (6 mg.) 20% developed kidney tumours while a much higher incidence was seen in rats receiving a diet containing 200 p.p.m. DMN for 1 week (21 mg.) or 100 p.p.m. for 4 weeks (42 mg.) (Magee and Barnes, 1962). Furthermore, Jasmin and Riopelle (1964) found 100% incidence in rats given 1·5 mg. DMN daily for 6 days only (9 mg.). In the experiments described here no kidney tumours were found in rats receiving 5 p.p.m. DMN for up to 104 weeks (54 mg.). (The total dose of DMN given in parentheses is calculated on the basis of a daily intake of 15 g. of diet.) Thus the total dose of carcinogen ingested is not always the only factor in producing tumours. It is probable that the crucial factor is the amount of the carcinogen which reaches the site of action as has been accepted for other drug effects (Brodie, Cosmides and Rall, 1965). In the case of DMN which almost certainly has to be metabolised before it becomes carcinogenically active it has long been known that the metabolism of a second dose of DMN is slowed when it follows closely after the first dose (Magee, 1956). Under these circumstances it is easy to visualise an effective dose reaching the kidneys.

The main object of these long-term studies on the effect of low doses of carcinogens was to try to provide a basis from which to extrapolate a no-effect dose. The present work indicates that in the case of the liver and the active nitrosamines, morphological changes cannot provide a basis for recognising effects before the appearance of tumours. Any hope of establishing a no-effect dose of a carcinogen must rest upon finding out more about the biochemical changes that must precede the development of neoplasia with the hope of being able to establish a dose below which such biochemical disturbances do not take place.

SUMMARY

1. The pathological changes in rats kept for a lifetime on diets containing 5 and 2 p.p.m. dimethylnitrosamine (DMN) are described.

2. While the incidence of liver tumours falls rapidly when the dietary concentration of DMN is reduced from 50 to 5 p.p.m. a single rat on 2 p.p.m. developed a liver tumour so that a no-effect level has not been established.

3. There were no pathological changes in the liver other than tumours which provided a more sensitive index of exposure to DMN.

4. No kidney tumours were seen in any rats and the dose response relationship for DMN and tumours of the kidney is discussed.

We wish to thank Mr. C. R. Kennedy for his constant attention to the animals and their diets during these experiments; Mr. R. Legg for the photographs and Mr. J. A. E. Jarvis for making the determination of DMN in the diets.

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2242

Carcinogenesis Study with Dimethylnitrosamine Administered Orally to Adult and Subcutaneously to Newborn BALB/c Mice*

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SUMMARY

Dimethylnitrosamine was tested for carcinogenic action by oral administration as a 0.001 per cent solution in the drinking water for 141 days to adult and by doses of 1.5, 15, 30, and 75 μg. in 0.05 ml. of physiologic saline in a single subcutaneous injection to newborn BALB/c inbred mice. The treatment resulted in the induction of lung adenomas, hemangiomas, and hemangiosarcomas mainly in the liver, adenomas of kidney, hepatomas, and liver cell carcinomas. Since benign and malignant liver cell tumors were found only in animals treated at birth, the influence of age on oncogenic response is discussed.

The toxicity of dimethylnitrosamine (DMN) in man was recognized in the past (13, 14) but received relatively little attention. Acute toxicity tests in rats showed that DMN is highly toxic (LD_{50} about 30 mg/kg of body weight), all the animals dying with severe centrilobular hemorrhagic necrosis of the liver (2). Similar results were obtained in the rabbit (16). The compound was later found to be carcinogenic in rats, inducing tumors of the liver (1, 23, 31), kidney (1, 24, 25, 39), and lung (1, 39). The carcinogenicity of many other nitroso compounds has also been demonstrated in rodents, and systematic investigation of possible correlations between chemical structure and carcinogenic activity are now in progress (7–11).

Following the suggestion that DMN must be metabolized before becoming tumorigenic, it was shown that enzymic demethylation of the compound is followed by methylation of cell constituents, presumably as a result of the intracellular formation of a methylating agent (3, 20, 26, 27).

The present experiment was undertaken primarily to study the possible carcinogenic effect of DMN on another species, the mouse. In view of the particular susceptibility of newborn rodents to the action of carcinogenic chemicals (5, 12, 18, 19, 21, 22, 28–30, 33, 35, 37, 38), the action of DMN in newly born mice was also investigated.

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† On leave of absence from the Toxicology Research Unit, Medical Research Council Laboratories, Carshalton, Surrey, England (present address).

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MATERIALS AND METHODS

BALB/c inbred mice, originally obtained through the courtesy of Dr. Kurt Stern, University of Illinois, Chicago, and since 1961 bred in our laboratory by brother-to-sister mating, were used. Each litter was housed with its mother until weaned, then separated according to sex. All mice were housed in plastic cages with sterilized granular cellulose bedding in groups of ten, and were given Rockland diet in pellets and tap water *ad libitum* with the exception to be described.

The carcinogen used was dimethylnitrosamine (N-nitrosodimethylamine, Eastman Organic Chemicals, Rochester, New York), purified by distillation (b.p. 150° C.). The experimental and control groups and the treatments are briefly described as follows.

Group 1.—DMN was dissolved in the drinking water as a 0.001 per cent solution and was given continuously for 141 days to 46 females and 41 males which were 5–10 weeks old at the beginning of the experiment. The solution was prepared twice a week, and the total water consumption was measured at the same intervals during the first 10 weeks of treatment. The average daily water consumption per animal was 3.9 ml. in the females and 4.2 ml. in the males.

Group 2.—One litter consisting of seven siblings was treated by S. C. injection in the dorsal region on the day of birth with a tuberculin syringe with a 30-gauge needle. Each received 1.5 μg. of DMN (1 mg/kg of body weight, average body weight being 1.5 gm.) in 0.05 ml. of physiological saline.

Group 3.—Three litters consisting of 22 siblings were treated in the same way as Group 2 with 15 μg. of DMN (10 mg/kg of body weight).

Group 4.—Four litters consisting of 28 siblings were

TABLE 1

Survival Rate and Distribution Pattern of Tumors in DMN-treated and Control BALB/c Mice

Group no. and treatment	No. of mice injected at birth	Effective no. of animals	Survivors at weeks of age										Animals with:														
			10	20	30	40	50	60	70	80	90	100	Lung adenomas			Hemangiomas			Hemangiosarcomas			Hepatomas		Liver cell carcinoma		Other neoplasms latent period (in weeks)
													No.	%	Latent period*	No.	%	Latent period*	No.	%	Latent period*	No.	Latent period*	No.	Latent period*	
Group 1. 0.001% DMN in drinking water for 141 days	—	46♀	44	40	15	1	—	—	—	—	—	—	41	89.1	26 (15–40)	15†	34.7	28 (21–36)	12‡	26.0	27 (23–36)	—	—	—	—	§
		41♂	40	37	22	—	—	—	—	—	—	—	35	85.3	31 (21–39)	9#	—	29 (21–35)	12¶	29.2	32 (25–38)	—	—	—	—	‖
Group 2. 1.5 μg. DMN by S.C. injection at birth	7	3♀	3	3	3	3	2	2	2	1	—	—	—	—	—	—	—	—	1**	—	83	—	—	—	—	—
		4♂	4	4	4	4	4	4	1	1	—	—	—	—	—	—	—	—	—	—	—	1	83	—	—	—
Group 3. 15 μg. DMN by S.C. injection at birth	22	10♀	10	10	10	8	7	6	—	—	—	—	1	—	56	5**	—	49 (36–68)	—	—	—	2	59 (50–68)	6	61 (56–68)	—
		12♂	12	12	7	2	1	—	—	—	—	—	1	—	24	1**	—	36	—	—	—	1	44	1	58	—
Group 4. 30 μg. DMN by S.C. injection at birth	28	5♀	5	5	3	2	2	2	—	—	—	—	2	—	27 (24–30)	2**	—	42 (24–61)	1**	—	62	—	—	2	61–62	††
		10♂	10	10	10	3	—	—	—	—	—	—	4	—	39 (37–44)	3**	—	42 (38–49)	1**	—	44	2	43 (38–49)	1	38	—
Group 5. 75 μg. DMN by S.C. injection at birth	27	1♀	1	1	1	1	1	—	—	—	—	—	1	—	57	1**	—	57	—	—	—	—	—	1	57	—
Group 6. Untreated controls	—	51♀	49	48	48	48	45	29	11	6	1	—	1	—	64	—	—	—	—	—	—	—	—	—	—	‡‡
		49♂	46	45	42	37	34	27	16	4	2	—	3	—	78 (68–90)	—	—	—	—	—	—	—	—	—	—	§§

* In weeks.

† Nine in livers; one in liver, ovary, and spleen; one in liver and pararenal tissue; one in liver and ovary; one in liver and lymph node; one in pararenal tissue; one in ovary and peripheral nerve.

‡ Five in livers; one in liver and spleen; one in liver, spleen, ovary, and lung; one in ovary; one in uterus; one in salivary gland; one in pancreas; one in pararenal tissue.

§ One adenoma of kidney (40).

Eight in livers; one in pararenal tissue.

¶ Nine in livers; two in liver and lymph node; one in liver and pancreas.

‖ One squamous-cell carcinoma of skin (30); one papillomatosis of gall bladder (38); one adenoma of kidney (34).

** In livers.

†† One malignant lymphoma, stem cell type (30).

‡‡ One skin papilloma (79).

§§ One malignant lymphoma, lymphocytic type (48).

treated in the same way as Group 2 with 30 μg. of DMN (20 mg/kg of body weight).

Group 5.—Four litters consisting of 27 siblings were treated in the same way as Group 2 with 75 μg. of DMN (50 mg/kg of body weight).

Group 6.—As a control, 51 females and 49 males were kept untreated.

The number of treated and control animals and the number of survivors at weaning (5 weeks) are shown in Table 1. The experimental and control animals were carefully checked and weighed at weekly intervals, and the gross pathologic changes were recorded. The mice were allowed to die without interference or were killed with ether when found in poor condition. A complete necropsy was performed on all animals except three males in the control group, which were lost through cannibalism. All organs were examined macroscopically and were fixed in 10 per cent buffered formalin. Several lung lobes from each mouse were studied histologically as well as those tissues which showed gross pathologic changes, with the use of a routine hematoxylin-eosin stain and the addition of special methods when necessary.

RESULTS

The survival rates at weaning in animals given injections at birth is recorded in Table 1. A considerable number of siblings died in the group treated by injection with 30 μg.; the solitary survivor in the 75-μg. group indicates a close lethal dose effect. The survival rates after weaning are also recorded in Table 1. It can be seen that with the exception of the group receiving 1.5 μg., the treatment markedly reduced the survival in the various groups, including the animals treated at adult age.

The number, incidence, and latent period of the tumors are summarized in Table 1. In Group 1, in which the mice were treated with DMN in the drinking water, a high incidence of lung adenomas and a considerable number of hemangiomas and hemangiosarcomas, mainly in the liver, were found. Summarizing the results of Groups 2, 3, 4, and 5, in which the mice were treated at birth with different doses of DMN, it appears that in addition to the induction of lung adenomas, hemangiomas, and hemangiosarcomas (often in the liver), which exhibited various incidences, a significant number of benign and malignant liver cell tumors were obtained.

The microscopic examination of lung tumors revealed the typical appearance of benign adenomas (Fig. 1), and in a few instances their morphology suggested malignancy since they invaded blood vessels and bronchii or showed many mitoses. It is notable also that of the 224 mice observed, 64 (35 in the control group) developed nodular, usually multiple, lung necroses.

At necropsy several animals had internal hemorrhage and the pleural and peritoneal cavities were filled with blood. The macroscopic appearance of the lesions in the liver was as follows: all lobes showed irregularity in size and shape and were often distorted and shrunken (Fig. 2). The livers were often nodular, and the majority contained varying numbers of hemorrhagic cysts. Microscopically, in the early stages there were hyperemia and dilation of the liver sinusoids, often accompanied by severe centrilobular necroses (Fig. 3). Later on, blood lakes were also seen in several animals. Our criterion for the diagnosis of hemangioma was the characterization of many layers of proliferating endothelial cells (Fig. 6). To be considered a hemangiosarcoma, a lesion had to be composed of highly anaplastic cells forming vascular clef and invading the surrounding tissue endovascularl (Fig. 7), occasionally giving metastases to various organ (Fig. 8). In animals treated at birth, we frequently saw hyperplastic nodules in the liver (Fig. 4). The task of distinguishing between hyperplastic nodules and benign hepatomas was difficult, and our diagnosis was based primarily on size, ability to compress surrounding parenchyma, and presence or absence of portal tracts and central veins in the nodule (Fig. 5). Two main types of liver cell carcinomas were seen. One was composed of a densely populated mass of atypical cells without any trabecular structure (Figs. 9 and 10); the other type exhibited neoplastic cells in a cordlike arrangement, separated by large, dilated vascular spaces (Figs. 11 and 12). These malignant tumors were morphologically similar to those previously reported induced in the Syrian golden hamster with carbon tetrachloride (4).

In addition, a small number of other types of tumor appeared in the treated and control animals and, therefore, with the possible exception of the two adenomas of kidney, cannot be attributed to the treatment.

DISCUSSION

The present experiment demonstrates that oral and subcutaneous administration of DMN to adult and newborn mice induces lung adenomas and also hemangiomas and hemangiosarcomas, mainly in the liver. In addition, a considerable number of benign and malignant liver cell tumors were found only in mice treated at birth. Since not a single hepatoma or liver cell carcinoma was seen in the mice treated at an adult age, and because no tumor was found at the site of administration of DMN in either age group, the neoplastic response appears to be qualitatively influenced by the age of the hosts.

This finding is somewhat similar to our previous results, in which the subcutaneous injection of 7,12-dimethylbenz(a)anthracene (DMBA) in adult Swiss mice induced mainly local sarcomas, while the same treatment in newborns gave rise to malignant lymphomas and lung adenomas, but only a few subcutaneous sarcomas (37). The suggested explanation of this result was that either the carcinogen had been retained subcutaneously in the adults and produced local tumors, or the target tissue susceptibility at certain age levels had been responsible for the different response.

The present results, of course, differ from our previous findings with DMBA in Swiss mice. The differences might be due to various reasons. In this instance, DMN was given subcutaneously to newborns and orally to adult mice; in prior studies the carcinogen was administered to both age groups only subcutaneously. Furthermore, DMN is a water-soluble substance, whereas DMBA was injected in an oily solution. In addition, it is notable that no tumor was found at the application site of DMN in either age group.

More recently our attention was directed toward the enzymatic and metabolic studies in this field. The activities of certain liver enzymes in newborn mice were shown to be 5 times smaller than in adults (15). Moreover, it was demonstrated in our laboratory that DMBA persists for a longer period in newborns than in adult mice (6). In accord with this finding, the catabolic rate of urethan was reported considerably higher in adult mice than in younger and newborn animals (17). The results obtained in these different experiments do not explain, of course, the qualitative differences in tumor type observed in adult and newborn mice in the present investigation; however, they do point to the direction in which further studies could be performed.

In relation to incidence and distribution of tumors induced by DMN in different organs of various species, it seems to us of particular importance to correlate these findings with the results obtained in the metabolic studies of radioactive DMN. It was shown that when rats, mice, and hamsters were treated with tritium- and carbon-14-labeled DMN, most of the radioactivity was present as 7-methylguanine in the liver and kidney of the rat, in the liver of the hamster, and in the liver and lung of the mouse (20). Since the induction of tumors by DMN was observed mainly in these particular organs of the various species (23, 24, 34, 36), it is possible that a link exists between the structural changes of RNA and tumor induction.

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wish to thank Mrs. Irene Boreisha for valuable technical assistance, and Mrs. Helen Cummins for histological preparations.

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Fig. 1.—Lung. Adenoma. 26-week-old female mouse treated orally with DMN; killed 20 weeks after the beginning of the treatment. Hematoxylin and eosin, × 160.

Fig. 2.—Liver. Carcinoma. 56-week-old female mouse treated with 15 μg. of DMN at birth, × 12.

Fig. 3.—Liver. Severe hemorrhagic necrosis. 11-week-old male mouse treated orally with DMN; died 7 days after beginning of the treatment. Hematoxylin and eosin, × 95.

Fig. 4.—Liver. Regenerative nodules. 50-week-old female mouse treated with 15 μg. of DMN at birth. Hematoxylin and eosin, × 80.

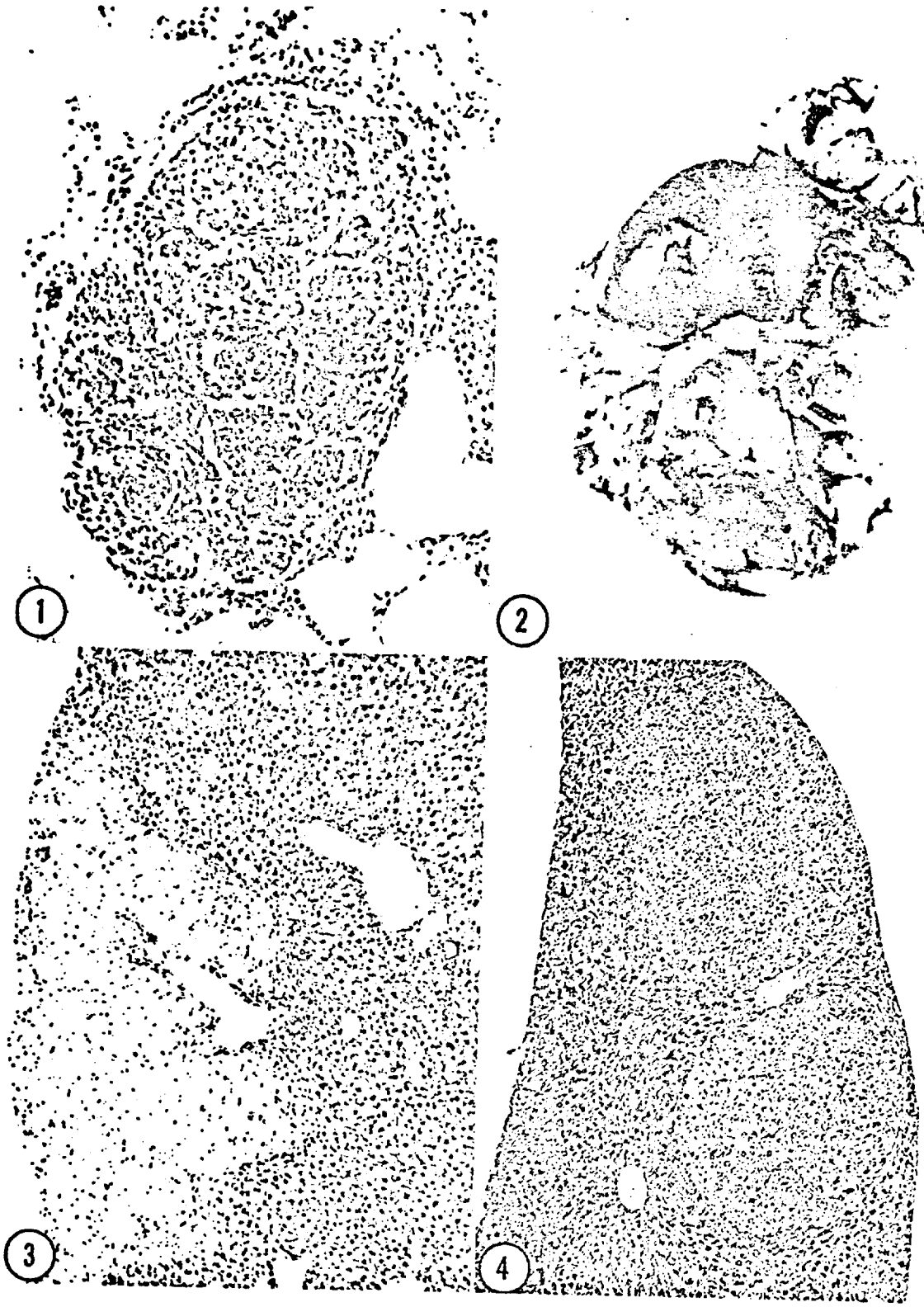
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FIG. 5.—Liver. Hepatoma. Note the absence of portal tracts and central veins in the lower part of the picture. 38-week-old male mouse treated with 30 μg. of DMN at birth. Hematoxylin and eosin, × 180.

FIG. 6.—Liver. Hemangioma, showing several layers of proliferating endothelial cells. 28-week-old female mouse treated orally with DMN; killed 20 weeks after the beginning of the treatment. Hematoxylin and eosin, × 95.

FIG. 7.—Spleen. Hemangiosarcoma, exhibiting vascular growth and invasion of the spleen. 30-week-old female mouse treated orally with DMN; killed 22 weeks after the beginning of the treatment. Hematoxylin and eosin, × 110.

FIG. 8.—Lung. Metastasis from the spleen. Same as Figure 7. × 120.

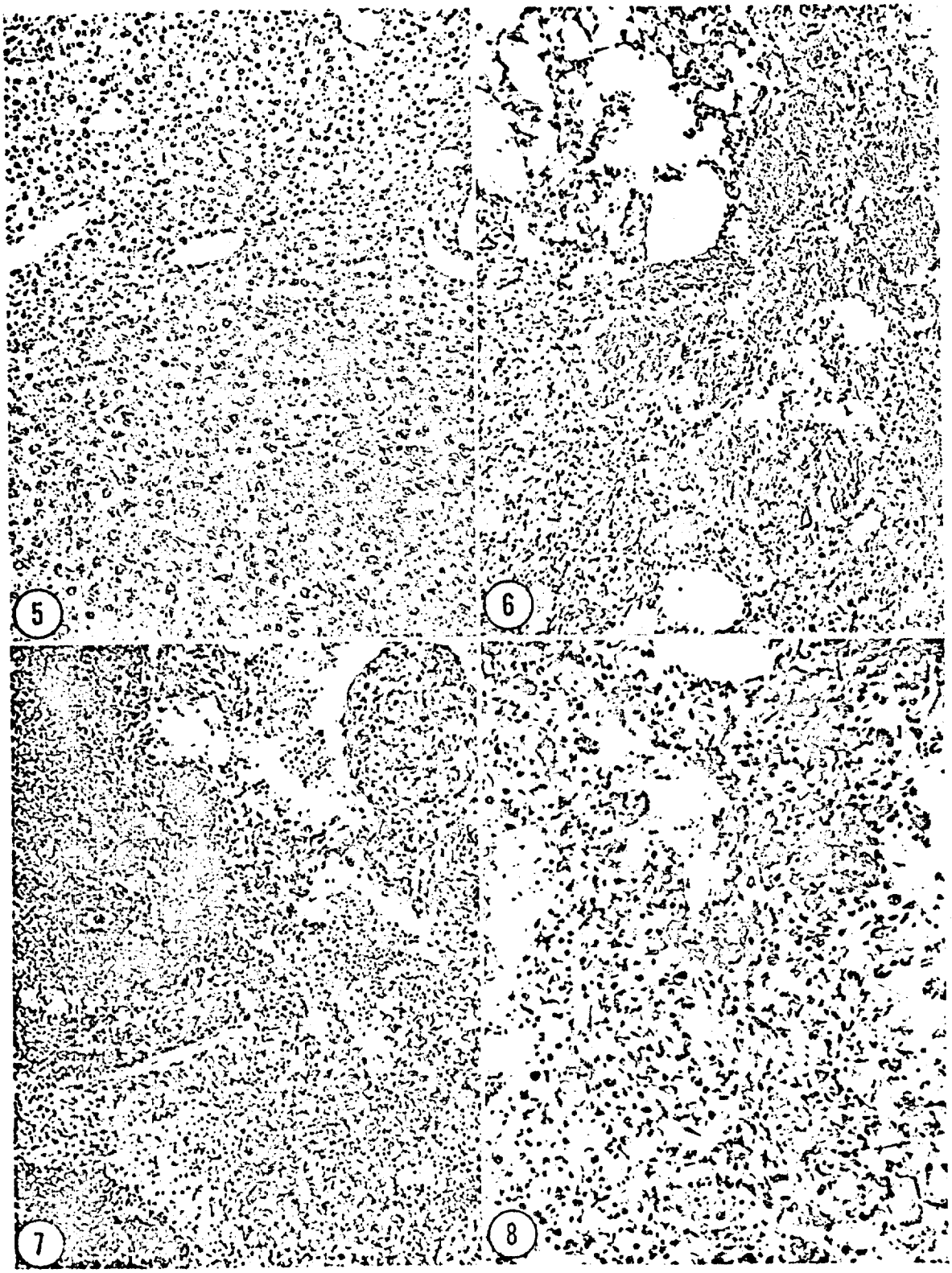
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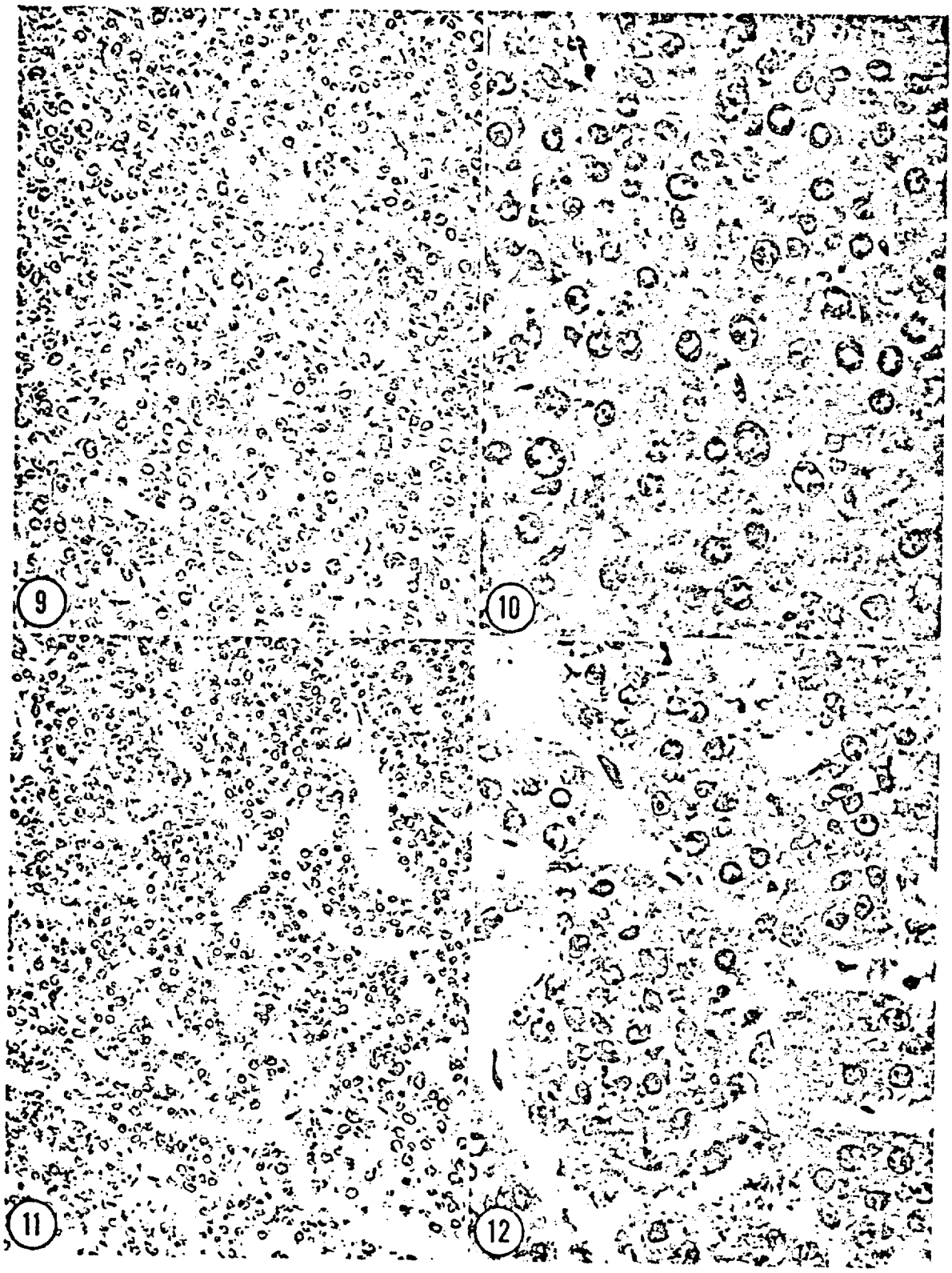
FIG. 9.—Liver. Carcinoma, composed of densely populated atypical cells without any trabecular structure. 58-week-old female mouse treated with 15 μg. of DMN at birth. Hematoxylin and eosin, × 170.

FIG. 10.—Same as Figure 9. × 450.

FIG. 11.—Liver. Carcinoma, composed of neoplastic cells in cordlike arrangement which are separated by dilated vascular spaces. 61-week-old female mouse treated with 30 μg. of DMN at birth. Hematoxylin and eosin, × 130.

FIG. 12.—Same as Figure 11. × 350.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Evidence for an irreversible reaction between nitrite and human methemoglobin

Although nitrite has been used to oxidize hemoglobin to the met form, little is known about nitritomethemoglobin. The magnetic susceptibility of this complex has been determined[1]; and the kinetics of its formation have recently been investigated[2]. There are, to our knowledge, no data on the binding equilibrium. For this reason, we undertook measurements to determine this quantity. We have found that nitrite appears to bind sigmoidally to methemoglobin. This apparent behavior is most probably an artifact arising from a nitrite-induced, irreversible protein change.

Purification of human methemoglobin A, determination of the protein heme iron concentration, and the spectrophotometric binding procedure have been described elsewhere[3]. $NaNO_2$ was obtained from commercial sources and recrystallized from water to remove a yellow contaminant. All experiments were performed in 0.05 M Tris, and the pH was adjusted by varying the cacodylic acid. All absorbances were measured at 22.0° with a Cary 16 spectrophotometer.

The spectrum of nitritomethemoglobin is presented in Fig. 1A. The relatively strong band with a maximum of 538 mμ (uncorrected) and the weak band above 600 mμ are consistent with the known magnetic susceptibility[1], and indicate a large amount of low spin component. The spectral changes accompanying nitrite addition show three isosbestic points above 450 mμ (Fig. 1B) suggesting the presence of only two light absorbing species.

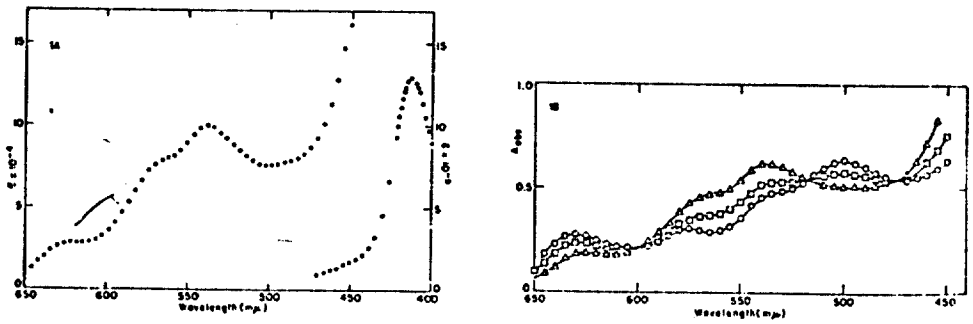
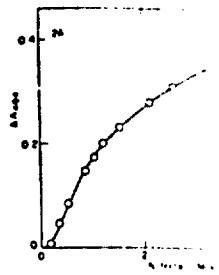


Fig. 1. Spectrum of human nitromethemoglobin A. (A) Spectrum at 22.0° with sample in 0.05 M Tris (cacodylate), pH 7.20. (B) Same conditions as A; heme iron, $7.20 \cdot 10^{-5}$ M. ○—○, no NO_2^-; □—□, $2.05 \cdot 10^{-3}$ M NO_2^-; △—△, $2.05 \cdot 10^{-2}$ M NO_2^-.

The nitrite binding curve is distinctly sigmoidal (Fig. 2A); when the data are replotted using a linearized from of the Langmuir isotherm, the departure from a hyperbolic curve at low fractional saturation is very evident (Fig. 2B). At higher levels of nitrite, complex formation appears to be simple, and apparent equilibrium constants may be calculated.

Reversibility of nitrite binding was performed initially with the following procedure. After addition of nitrite and incubation at 22.2° for 0-3 h to insure equilibration, the solution absorbance was measured. The sample was then diluted 1:1



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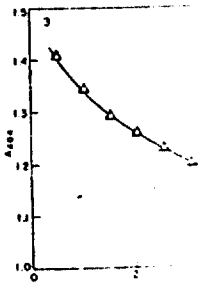

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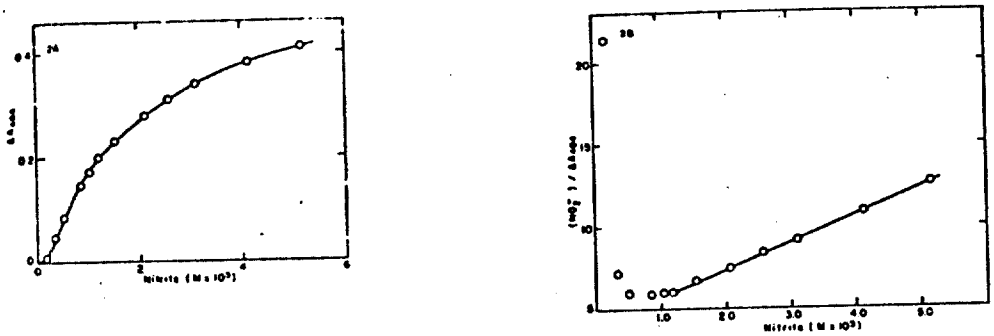


Fig. 2. Nitrite binding to human methemoglobin. A. Binding was performed at 22.0° and in 0.05 M Tris (cacodylate), pH 7.20. Heme iron concentration $5.20 \cdot 10^{-5}$ M. Absorbance measured at 404 mμ. B. The data of A replotted in a linear form of the Langmuir isotherm.

with a stock solution containing no nitrite, but protein at an identical concentration. Thus, the ligand concentration was halved while the protein remained unchanged. After the proper time interval, the sample absorbance was measured again. The results presented in Fig. 3 show that by this procedure the reaction appears completely reversible.

Nitrite binding affinity is pH-dependent, decreasing as the pH is raised from 6.4 to 8.4. The binding constants of other ligands decrease over the same pH range[4]. There is, however, no change in the shape of the binding curve. This is visualized by plotting the fractional absorbance change, $\Delta A/\Delta A_{total}$, *versus* the nitrite concentration normalized with respect to the nitrite concentration at 50% saturation (Fig. 4).

The apparently sigmoidal behavior is of great interest, since no ligand has yet been described which binds to methemoglobin with cooperativity. A sigmoidal curve does not, of itself, indicate site–site interaction[3]. To test for cooperativity, we performed a competitive binding experiment. The rational behind this test has been previously described[3]. It suffices to note that if a ligand, which by itself yields a hyperbolic curve, shows a sigmoidal curve in the presence of nitrite, then the nitrite

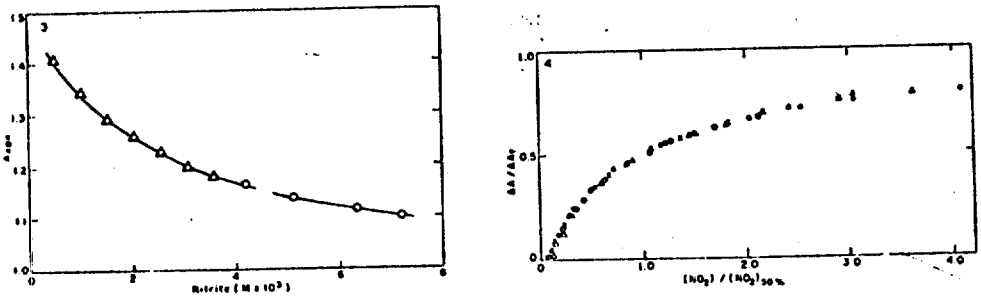
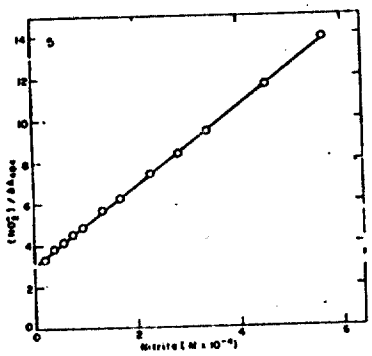


Fig. 3. Reversibility of nitrite binding. The experimental procedure has been described in the text. The conditions are identical to those given in Fig. 2, except the heme iron concentration which is $4.82 \cdot 10^{-5}$ M. ○—○, before dilution; △—△, after dilution.

Fig. 4. Shape of nitrite binding curve as a function of pH. The experimental conditions are those described in Fig. 2 except for pH which was adjusted in each case by addition of cacodylic acid to 0.05 M Tris. The pH and apparent binding constants, respectively, obtained from the 2nd half of the binding data are: ●, 6.05, 870 M^{-1}; ○, 7.12, 410 M^{-1}; ×, 7.61, 250 M^{-1}; △, 7.66, 250 M^{-1}; ▲, 8.22, 150 M^{-1}.

binding occurs with cooperativity. If, however, the competitive curve is hyperbolic, then nitrite does not bind with cooperativity. For the competition experiment we chose cyanate, which was added to a methemoglobin solution at pH 7.20 and 22.0 containing 1.72 mM $NaNO_2$, a concentration sufficient for approximately 50% saturation (Fig. 2). Under these conditions the binding curve is hyperbolic. Therefore, nitrite is not bound to the iron of methemoglobin with site–site interaction.

The results of the competition experiment prompted a reinvestigation of the nitrite binding reversibility. Enough $NaNO_2$ was added to methemoglobin to saturate the heme iron. After storage for one hour at 22° to insure equilibration, the sample was dialyzed extensively at 4° to remove the ligand, and then used as stock in a complete binding experiment. As seen in Fig. 5 the nitrite-treated protein shows a hyperbolic equilibrium with nitrite. This alteration of binding behavior strongly suggests that nitrite treatment irreversibly changes methemoglobin. A nitrite-induced alteration was verified by an observed change in the visible spectrum of the treated protein (Fig. 6).



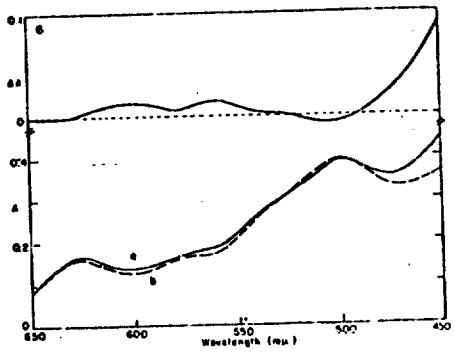


Fig. 5. Nitrite binding to methemoglobin previously treated with nitrite. Sodium nitrite (0.105 g) was added to 21.5 ml of $3.65 \cdot 10^{-4}$ M methemoglobin. After 1 h the sample was dialyzed against seven changes of distilled water (2 l at each change). This stock solution was used in a binding experiment under the conditions of Fig. 2. The data are plotted in a linear form of the Langmuir isotherm.

Fig. 6. Spectrum of nitrite treated protein. Methemoglobin was treated with 0.089 M sodium nitrite as described in Fig. 5. The spectrum of the nitrite treated sample is compared with a control mixed with sodium chloride of the same molarity. Top, difference spectrum; treated *minus* control. Bottom, absolute spectra. ———, nitrite treated; — —, control.

We conclude that nitrite not only binds to the iron of methemoglobin, but also reacts irreversibly either with the apoprotein or the porphyrin group. This secondary, irreversible change is most likely the basis of the apparent sigmoidal "binding" curve, since prior nitrite treatment of the protein abolishes the sigmoid response. The observation of isosbestic points (Fig. 1B), the constant shape of the sigmoidal curve over a wide range of nitrite affinites (Fig. 4) and hence ligand concentration, and the complete reversibility found in the dilution experiment (Fig. 3), suggest that the irreversible alteration occurs concommitantly with binding of nitrite to the heme iron. The nature of the reaction is not known yet, but clearly no major disturbance of the protein structure is involved.

The apparently irreversible change caused by nitrite occurs at very low ligand

concentrations, neutral pH's, and near room temperature. Nitrite has been used to oxidize oxyhemoglobin. The results reported here suggest that nitrite should no longer be used for this purpose.

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Solvent perturbation and accessibility of the tryptophyl and tyrosyl residues in giant keyhole limpets hemocyanin*

Interest in the perturbation behavior of the large subunit proteins and colored proteins[1,2] prompted us to investigate the solution properties of hemocyanin, the large, copper-containing subunit protein responsible for oxygen transport in the hemolymph of mollusks, crustaceans, and several other invertebrates. Electron microscopy of the hemocyanins reveals an ellipsoidal or cylindrical subunit organization of the parent protein, consisting of several hundred subunits[3–5]. A substantial fraction of the subunits constitute the internal core regions of the associated protein. As such, it is reasonable to expect that a large fraction of the hydrophobic tryptophyl and tyrosyl residues should be deeply buried in the internal, solvent-inaccessible regions of the protein. Other inaccessible groups may also be located at areas of subunit contact that may become fully or partly exposed as a result of the dissociation or unfolding of the subunits[6,7]. These expectations are largely borne out by our solvent perturbation studies carried out thus far on giant keyhole limpets *(Megathura crenulata)* hemocyanin. Table I and Fig. 1 present a summary and analysis of our data obtained on native and urea denatured hemocyanin, purchased from Mann Research Laboratories. The data obtained with four perturbants, 90% deuterium oxide, and 20% ethylene glycol, glycerol, and sucrose** indicate that, in neutral

* This work was supported by a research grant (GM 14468) from the National Institute of General Medical Sciences of the National Institute of Health, U.S. Public Health Service.

** These perturbants are known to be the most reliable for such investigations as ours[6,7]. Optical rotatory dispersion measurements on aqueous hemocyanin solutions (at pH 6.8) and in the presence of 90% deuterium oxide, 20% ethylene glycol, and 20% glycerol indicate no significant changes in the mean residue rotation at 233 mμ, suggesting that these perturbants have no adverse effects on the solution conformation of hemocyanin. The mean residue rotations obtained on these solutions in the presence of 0.1 M KCl, 0.01 M $MgCl_2$, and 0.01 M phosphate buffer

concentrations, neutral pH's, and near room temperature. Nitrite has been used to oxidize oxyhemoglobin. The results reported here suggest that nitrite should no longer be used for this purpose.

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Lactic and Succinic Dehydrogenase Activity in Nitrite Toxicosis in the Guinea Pig

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SUMMARY

The influence of sodium nitrite toxicosis on lactic dehydrogenase (LDH) and succinic dehydrogenase (SDH) was determined in the guinea pig. The changes were compared with the effects of hypoxia produced in guinea pigs kept in a sealed chamber containing a mixture of air and nitrogen.

In nitrite toxicosis, activities of LDH and SDH in the myocardium were decreased. In the kidneys, the reduction in activity of both enzymes was associated with focalization of activity. Hyperemia of liver, spleen, kidneys, and lungs and vacuolar degeneration of hepatic cells and renal tubular epithelium occurred in nitrite toxicosis. Prolonged ingestion of sodium nitrite in the drinking water reduced appetite, body weight gain, and hemoglobin concentration.

Most investigators ascribe pathologic changes in nitrite toxicosis to the anoxic state of the tissues. Gross changes result from either the vasodilatory effect of nitrite ions or the oxidation of hemoglobin from the ferrous to the ferric state, with resultant formation of methemoglobin. In either instance, the net effect is tissue anoxia. The effect of this anoxic state has not been studied extensively at the molecular level.

Bodansky[1] observed that reduced phosphopyridine nucleotides, nicotinamide adenine dinucleotide, and nicotinamide adenine dinucleotide phosphate (NADPH) are essential for normal reduction of methemoglobin to hemoglobin in the erythrocyte. In man, an hereditary failure in the production of NADPH has been observed to lead to accumulation of methemoglobin in the erythrocytes.[4,7] Wachstein and Meisel[8] reported cellular enzyme alteration in ischemia. Ziegler[10] concluded that there was marked inhibition of respiratory enzyme capacity to transport electrons in severe hypoxic states.

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Fig. 1.—Myocardium from nontreated guinea pig. Notice strong and discrete reaction of succinic dehydrogenase MTT technique; x 600.

The purposes in the present investigation were to identify any changes in enzymatic activities of LDH and SDH that occurred after nitrite was administered to guinea pigs. The effects of acutely produced hypoxia were also compared to obtain basic information on the pathogenesis of tissue changes in hypoxia of nitrite toxicosis.

Materials and Methods

Fifty-one 1- to 12-month-old guinea pigs were used. Commercial guinea pig pellets[a] were fed ad libitum and fresh cabbage was given as supplement.

Experiment 1.—Twenty guinea pigs were allotted to 3 groups: Group 1 contained 7 guinea pigs, group 2 contained 7, and group 3 contained 6. Group 1 guinea pigs were given 0.5% $NaNO_2$; group 2 guinea pigs were given 1.0% $NaNO_2$; and group 3 guinea pigs were given tap water and were used as controls. Body weight was recorded for each guinea pig at the start of the experiment, during the experiment, and at the end of the experiment. The guinea pigs were treated for a minimum of 2 weeks, with a maximal treatment of 7 weeks for group 2 guinea pigs and of 9 weeks for group 1 guinea pigs.

Hemoglobin concentration was determined by the standard cyanmethmeglobin method, methemoglobin by the method of Evelyn and Malloy as recorded by Hawk *et al.*,[3] and packed cell volume (PCV) by the capillary tube method. Blood was obtained by making a cut into the dorsal metacarpal vein over the edge of the metacarpus. At the end of the experiment, samples were collected by cardiac puncture.

At necropsy, specimens taken from heart, lungs, liver, and kidneys were fixed in 10% buffered formalin for histologic examination. Specimens were frozen in a mixture of solid CO_2 and acetone for histochemical examination. The activities of SDH and LDH were estimated, using monotetrazolium salt (MTT)[b] according to the method of Chason and Pearse.[2] When necessary, differential staining of frozen tissue for fat and glycogen was done, using oil red O and Best's carmine, respectively.

Experiment 2.—Ten guinea pigs were given 1.0% $NaNO_2$ by subcutaneous injection at the rate of 60 mg./kg.; the dose was repeated every hour by injecting 10 to 20 mg. (1 to 2 ml.) of the same solution. Seven other guinea pigs were given one injection at the rate of 60 mg./kg. Nine guinea pigs were used as noninoculated controls. Blood samples were collected

[a] Rockland Laboratory Animal Diets, Tekland, Inc., Monmouth, Ill.

[b] Sigma Chemical Company, St. Louis, Mo.

Fig. 1.—Myocardium from nontreated guinea pig. Notice strong and discrete reaction of succinic dehydrogenase. MTT technique; x 600.

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every hour until death or for a maximum of 5 hours. Laboratory tests and necropsy procedures were done as described for experiment 1.

Experiment 3.—Five guinea pigs, 1 or 2 at a time, were exposed to a mixture of air and nitrogen in a desiccating jar with a 3-L. capacity. Each guinea pig was taken out of the jar *in extremis* for collection of blood. Laboratory investigations and necropsy procedures were done as described for experiment 1.

Results

Experiment 1.—Clinical signs of toxicosis were reduced body weight and appetite. A variable degree of brown discoloration of visible mucous membranes was observed. Hemoglobin concentrations remained within normal limits. Hyperemia of the organs was usually seen on gross and microscopic observations. Vacuolar degeneration was observed in liver and renal tubular epithelium. The level of SDH activity was appreciably diminished in the heart (Fig. 1 and 2) but varied in the other organs examined. The LDH activity in the heart was much less in principals than in nontreated controls (Table 1).

Experiment 2.—Mean concentrations of methemoglobin increased to 7.4 and 6.4 Gm./100 ml. in guinea pigs given one inoculation and in those given multiple injections, respectively, 1 hour after treatment. In guinea pigs given multiple injections, the level was maintained above 4.5 Gm./100 ml. during the 4 hours of observation. Visible mucous membranes were intensely discolored brown. Four hours after the initial injection was given to the multiple-inoculated guinea pigs, the average hemoglobin concentration had decreased from an average of 13.8 to 9.5 Gm./100 ml. The PCV had also decreased from the average of 44.2 to 34.5%. At necropsy, gross pulmonary edema was observed. Histologically, heart, lungs, liver, and kidneys were hyperemic. Histochemically, diminished activities of SDH and LDH were demonstrated, but the decrease in LDH activity in cardiac tissue was most marked and constant (Table 1). Focalization of SDH in the kidney was also prominent (Fig. 3 and 4).

Experiment 3.—Exposure of guinea pigs to air and nitrogen for approximately 2 hours caused a decrease in mean hemoglobin concentration from 13.9 to 11.0 Gm./100 ml. The PCV also

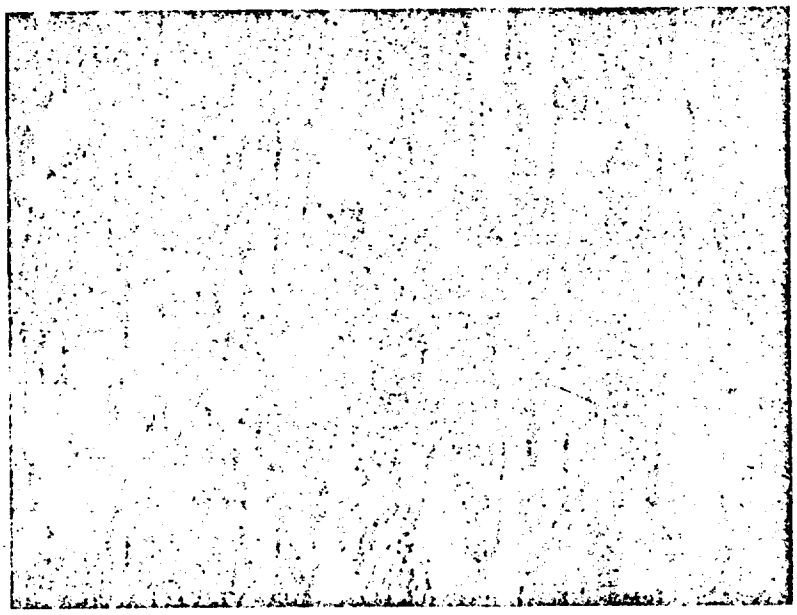

Fig. 2—Myocardium from guinea pig given 10% nitrite solution in drinking water for 4 weeks. Notice comparatively weak reaction of succinic dehydrogenase. MTT technique; x 600.

TABLE 1—Succinic Dehydrogenase and Lactic Dehydrogenase Activities in Heart, Liver, and Kidneys of Guinea Pigs in Nitrite Toxicosis

Tissue	Treatment				
	Control	1.0% $NaNO_2$ in drinking water	0.5% $NaNO_2$ in drinking water	1.0% $NaNO_2$ inoculated subcutaneously	Air and N_2 mixture
SUCCINIC DEHYDROGENASE ACTIVITY					
Heart	4	2.4	3.2	2.3	3.5
Liver	4	2.6	3.2	2.7	3.2
Kidneys	4	2.4	3.0	2.5	2.7
LACTIC DEHYDROGENASE ACTIVITY					
Heart	4	1.4	2.5	1.4	2.0
Liver	4	2.4	3.2	2.8	3.0
Kidneys	4	2.8	2.6	2.5	2.7

Data are average scores for guinea pigs in each treatment group in diminishing order of positivity from 4 to 1.

decreased from 45.5 to 36.6%. Significant gross or histopathologic lesions were not observed. There were decreased LDH activity in cardiac tissue and focalization of LDH in the kidney (Fig. 5). The SDH activity was appreciably diminished only in the kidneys.

Discussion

From the results of tests for SDH and LDH activities, the latter seemed a better index for assessment of nitrite toxicosis. The depletion of cardiac and hepatic LDH agreed with the results of Papadoupoulos *et al.*[5] on the effect of exercise on tissue LDH in the rat. It was not ascertained in our experiments which of the 4 LDH-isoenzymes of the heart was particularly affected. Depletion of cellular enzymes similar to that described by Papadoupoulos *et al.*[5] would be supportive evidence that hypoxia is the mode of action of nitrite on tissues. The histochemically observed decreases in SDH and LDH activities, with focalization, probably represented early signs of cellular involvement. These decreases also were likely related to later degenerative changes observed histologically in the renal epithelium. The similarity in enzymatic reactions between guinea pigs inoculated with nitrite and those made severely hypoxic in the air-

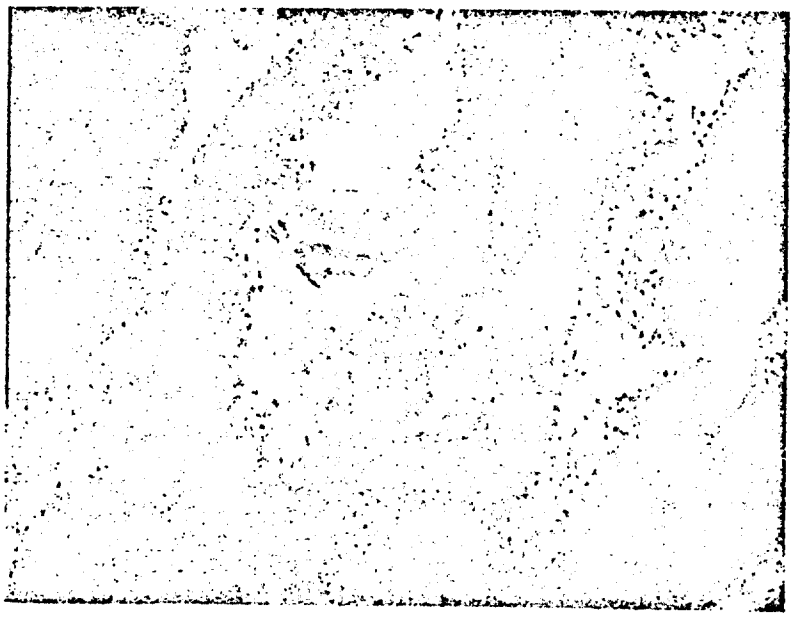

Fig. 3—Kidney from nontreated guinea pig. Notice strong and uniform reaction of succinic dehydrogenase. MTT technique; x 600.

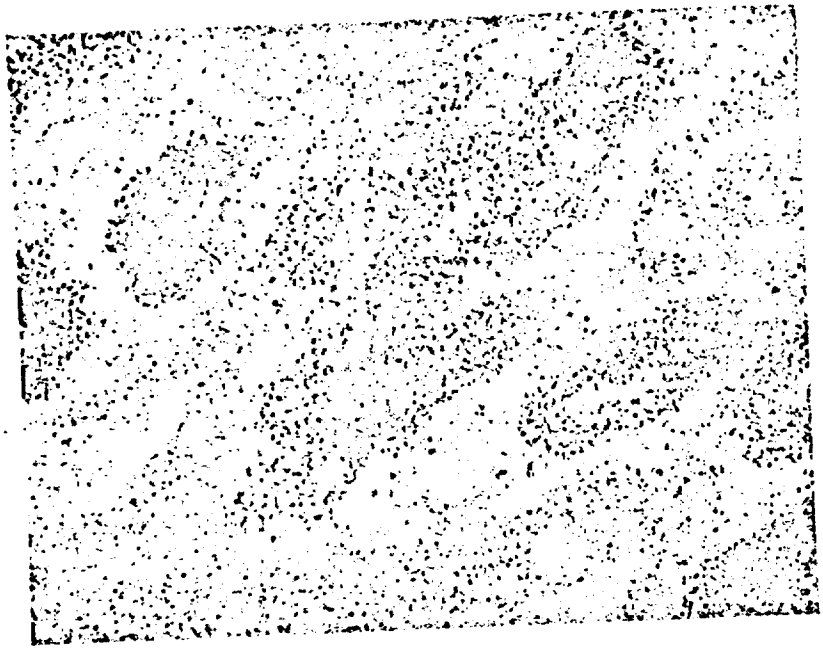

Fig. 4—Kidney from guinea pig inoculated with 1.0% nitrite solution. Notice tendency of positive reaction to localize. MTT technique; x 600.

nitrogen mixture also supported the theory of a similar mode of action.

The inappetence observed in guinea pigs drinking sodium nitrite solutions must have contributed to the body weight loss or impaired weight gain. The observation by Popov[6] that enterokinase and pancreatic lipase in rats are depressed by ingested nitrite or nitrate (or both) may indicate part of the explanation for the observed hypophagia and weight loss. Severe hypoxia has been reported to cause weight loss in rats[10] on ad libitum feeding, and the hypoxia of methemoglobinemia may also have contributed to the weight loss in guinea pigs in the present study. In guinea pigs given 0.5% and those given

Fig. 5—Kidney from guinea pig exposed to mixture of air and nitrogen until moribund. Notice the focalized positive lactic dehydrogenase reaction. MTT technique; x 600.

1.0% $NaNO_2$, hemoglobin concentration and PCV were decreased. Contrary to observations in other species by Winter and Hokanson,[9] relatively high methemoglobinization in animals given 1.0% nitrite did not lead to an increase in hemoglobin concentration. In fact, there was a slight decrease which Atallah[c] had also observed. The dilatory action on the vasculature no doubt contributed to the hyperemia. Determining the effect of nitrite on more of the enzymes involved in electron transport and supply of energy to the cells may elucidate further the mechanism of nitrite toxicosis.

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Long-term Experiment with Canned Meat Treated with Sodium Nitrite and Glucono-δ-lactone in Rats

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(*Received 27 March 1972*)

Abstract—A long-term toxicity experiment was carried out in rats fed a diet containing 40% canned meat treated with either 0·5 or 0·02% sodium nitrite with or without glucono-δ-lactone. This experiment, in which special attention was paid to the detection of a possible carcinogenic effect, was designed to study the possible formation of nitrosamines from nitrite and the secondary amines present in meat, under the conditions of an acid pH and pasteurization. The parameters studied included haematology, clinical biochemistry, histopathology, α-foetoprotein in the serum and the DNA content of the liver cell nuclei. There was no evidence of any preneoplastic change or tumour formation that could be attributed to the feeding of this canned meat.

INTRODUCTION

Most *N*-nitrosamines are known to be powerful carcinogens. Since the discovery by Magee & Barnes (1956) of the carcinogenic activity of dimethylnitrosamine, numerous *N*-nitroso compounds have been tested for their carcinogenic activity. Druckrey, Preussmann, Ivankovic & Schmähl (1967) have presented a detailed review of studies they carried out in rats on the relation between the structure and carcinogenicity of 65 nitrosamines. Striking organotropic effects were observed. The nitrosamines tested, either by the oral or sc route, induced tumours in the liver, kidneys, oesophagus, nasal cavity, stomach, lungs, urinary bladder and central nervous system. The structure of the compound, the dose level and the route of administration often determined the site and type of tumour that developed. The carcinogenic action of *N*-nitroso compounds has also been reviewed by Magee & Schoental (1964) and Magee & Barnes (1967). From the results of Schmähl & Osswald (1967) it is clear that nitrosamines induce tumours in many animal species.

Following the work of Ender, Havre, Helgebostad, Koppang, Madsen & Ceh (1964), the suspicion arose that nitrite could combine with secondary or tertiary amines present in fish and meat to form nitrosamines. In addition, many experiments were carried out on the formation of nitrosamines in the stomach after simultaneous administration of high levels of nitrite and secondary amines (Sander, Schweinsberg & Menz, 1968). Sander (1971) and Sander & Bürkle (1969) reported the induction of malignant tumours by the simultaneous feeding of nitrite and certain basic secondary amines, such as *N*-methylbenzylamine and *N*-methylaniline. In these cases the nitrosamines were assumed to have been formed in the stomach and not in the diet. Sander (1971) also surveyed the possible occurrence of nitrite and secondary amines in the human environment.

In a recent paper by Magee (1971), the possible health hazards of nitrosamines are summarized in relation to the occurrence of these substances in food. In the production of

several meat products, nitrite is added as a preservative and the meat is subsequently pasteurized or sterilized. Sometimes glucono-δ-lactone (GDL) is also added to lower the pH. Heat and a low pH are favourable conditions for the formation of nitrosamines from nitrite and secondary amines. On the other hand the haemoglobin or myoglobin present in the meat serves to neutralize the dangerous properties of the NO^+ ion by reaction with Fe^{2+}. Therefore it was considered to be advisable to carry out a long-term toxicity test, in which special attention would be given to the possible induction of tumours by the ingestion of meat treated in this way.

EXPERIMENTAL

Diets

Preparation of canned meats. The meat for the diets was prepared by the Central Institute for Nutrition and Food Research TNO at Zeist, and consisted of 28% beef, 24% pork meat, 28% bacon, 12% water, 4% starch, 2% NaCl and 0·3% polyphosphate. In addition, the meat for the test diets was treated with 0·5% $NaNO_2$, with 0·5% $NaNO_2$ together with 1% GDL, with 1% GDL alone, or with 0·02% $NaNO_2$ and 1% GDL. The components of the basic meat mixture were mixed and stored in charges of 25 kg in a deep-freeze at −40°C. Before the preparation of the diets, this meat was partially defrosted for one night at 15°C. Thereafter the different components were cut and thoroughly mixed. During this process the nitrite and/or GDL were added. The meat-dough was then canned in tins of 200 g and pasteurized at 80°C for 70 min. During the heating of tins that contained 0·5% $NaNO_2$ and 1% GDL, an effective pressure of 1·25 atm. was used to compensate for the internal pressure caused by a reaction between the nitrite and GDL. The prepared canned meat was stored at −20°C. For the whole experiment five batches each consisting of four charges of 25 kg and one batch consisting of two charges of 25 kg were prepared. The batches were prepared every 4 months.

Analysis of prepared meats. The content of nitrite, fat and water and the pH were determined on each batch after pasteurization (Table 1). The average protein content of the meat used was 12%. It was originally planned to analyse all batches for the presence of nitrosamines, but with the methods available at that time nitrosamine could not be determined in concentrations below 1 ppm. For this reason samples of the different batches were kept. Recently samples of the last batch were sent to Dr. H. Egan of the Laboratory of the

Table 1. *Average pH and nitrite, fat and water content of test and control meats after pasteurization*

Sample	pH	Content of		
		$NaNO_2$ (ppm)	Fat (%)	Water (%)
Untreated meat	6·42 ± 0·14	0·4 ± 0·5	28·6 ± 1·1	54·4 ± 0·5
Meat + 0·5% $NaNO_2$	6·62 ± 0·19	4004 ± 308	28·4 ± 0·7	54·6 ± 0·4
Meat + GDL	5·19 ± 0·10	0·4 ± 0·5	28·2 ± 1·0	53·9 ± 1·0
Meat + 0·5% $NaNO_2$ + GDL	5·71 ± 0·11	1824 ± 296	28·6 ± 1·1	53·4 ± 0·4
Meat + 0·02% $NaNO_2$ + GDL	5·27 ± 0·12	9 ± 3	26·3 ± 0·9	54·1 ± 0·5

GDL = Glucono-δ-lactone
Each value represents the mean of six batches.

Government Chemist, London, for analysis. According to the preliminary results, no nitrosamines were detected in the control meat or in the meat treated with 0·02% $NaNO_2$ and 1% GDL. Meat treated with 0·5% $NaNO_2$ contained 30 ppb* dimethylnitrosamine and <2 ppb diethylnitrosamine, the presence of both nitrosamines being confirmed in this sample by mass spectrometry. Meat treated only with 1% GDL contained <1 ppb dimethyl- and 5 ppb diethylnitrosamine and <1 ppb *N*-nitrosopyrrolidine, while that treated with 0·5% $NaNO_2$ and 1% GDL contained 40 ppb dimethyl- and 14 ppb diethylnitrosamine, 4 ppb *N*-nitrosopiperidine and 4 ppb *N*-nitrosopyrrolidine.

Compounding of diets. At the National Institute of Public Health, the canned meats were defrosted weekly and mixed with a standard diet (Muracon-SSP-Tox) in a weight ratio of 40% meat in SSP-Tox. The various diets were stored under refrigeration until they were given to the animals 3 times/wk. The animal food, Muracon-SSP-Tox, is specially compounded for our laboratory by the firm Trouw and Co., Putten, The Netherlands, and consists of casein, soya protein, maize starch, glucose, cellulose, soya oil, lard, a salt mixture and vitamins.

Animals

Male and female SPF-derived Wistar rats were obtained from the Central Institute for the Breeding of Laboratory Animals TNO, Zeist, The Netherlands. They were housed in wire cages, two animals to a cage. Drinking water was given ad lib. At the beginning of the experiment food was also given *ad lib.*, but when the weight gain became too high the animals were put on rations (about 20 g/day).

Experimental design and conduct

Six groups, each of 30 male and 30 female rats, were fed from just after weaning either on the standard SSP-Tox diet (control group), on a diet containing 40% meat (meat-control group) or on a diet containing 40% meat treated either with 0·5% $NaNO_2$, with 1% GDL, with 0·5% $NaNO_2$ and 1% GDL or with 0·02% $NaNO_2$ and 1% GDL. The animals were weighed weekly during the first 13 wk and every 4 wk thereafter. Food intake was recorded only in wk 1, 2, 5, 9 and 13.

Blood samples for haematological investigations were taken from ten animals in each group after 12, 24, 37, 51, 66, 78 and 91 wk, the haemoglobin level, packed cell volume, numbers of erythrocytes and total and differential leucocytes were recorded and the mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated. The bromsulphthalein (BSP) retention test was used as an index of liver function together with determinations of serum glutamic–pyruvic transaminase (SGPT) activity. These tests were carried out at wk 13 in five males and at wk 26 in five females from each group. SGPT, alkaline phosphatase and urea were determined after 9, 12 and 16 months in blood from male and female rats. Throughout the study, animals were killed if they became moribund. Of the rats dying in this way when about 2 yr old, six from each group were used for determinations of the activities of the hepatic enzymes, aniline hydroxylase, aminopyrine demethylase and glucose 6-phosphatase.

All animals were inspected regularly for the presence of tumours. After 29 months the experiment was terminated, the remaining animals being killed with carbon dioxide.

* b = 10^9 throughout this paper.

All animals that had died or were killed were autopsied and all organs were fixed in buffered 10% formalin or Bouin solution for histopathological examination. The following organs and tissues were studied: liver, kidneys, heart, lungs, brain, spleen, pituitary gland, thyroid, thymus (if any), pancreas, adrenals, testes, ovaries, prostate, uterus, lymph nodes, salivary glands, gastro-intestinal tract (six levels), urinary bladder, spinal cord (three levels), peripheral nerves, skeletal muscles, skin and mammary gland.

At the end of the experiment smears were made from the livers of two, three or four rats in each group, except in that receiving 0·5% nitrite and GDL, from which seven animals were taken. The smears were stained according to the Feulgen technique for DNA. The amount of DNA/cell nucleus was measured microspectrophotometrically with the Zeiss Cytoscan. All animals alive at 29 months were bled and their serum was tested for the presence of α-foetoprotein by double diffusion in 0·8% agarose.

Statistical methods

Statistical analysis was carried out on all parameters except food intake, for which no individual data were available. The statistical calculations were carried out according to the Student's *t* test.

RESULTS

Growth, food intake and mortality

The mean body weights for each group recorded at wk 6, 13, 26, 52, 78 and 104 are given in Table 2. These show that a diet containing 40% meat gave a higher body weight than a standard animal diet. However, the groups receiving meat treated with $NaNO_2$ (0·5%), with or without GDL, generally showed a lower body weight than the meat-control group.

Table 2. *Mean body weight of rats fed diets containing 40% meat treated with nitrite and/or GDL*

Diet	Body weight (g) at wk						
	0	6	13	26	52	78	104
		Males					
Control	48	280**	366***	436***	539***	576***	579
Meat control	51	297	406	487	632	650	622
Meat + 0·5% $NaNO_2$	50	268***	367***	438***	566***	583**	582
Meat + GDL	48	280*	389	462	608	654	651
Meat + 0·5% $NaNO_2$ + GDL	49	251***	344***	421***	535***	578***	589
Meat + 0·02% $NaNO_2$ + GDL	49	280*	383*	457*	594*	638	591
		Females					
Control	46	181	221**	249***	313***	354**	330
Meat control	45	186	240	278	379	409	381
Meat + 0·5% $NaNO_2$	46	179	228	264	355	378	387
Meat + GDL	46	190	241	278	369	392	434
Meat + 0·5% $NaNO_2$ + GDL	46	167***	209***	231***	295***	335***	348
Meat + 0·02% $NaNO_2$ + GDL	47	186	236	272	363	412	395

GDL = Glucono-δ-lactone

Values marked with asterisks differ significantly (Student's *t* test) from those of the meat-control group: $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

The food intake was estimated only during the first 13 wk of the experiment. It was clear hat all animals on the meat diets had a higher food intake than the control animals on a standard laborabory diet, but there were no differences between the meat-control group and the groups receiving meat treated with $NaNO_2$ and/or GDL. The behaviour and appearance of the rats remained good during the first 18 months. Thereafter the mortality increased rapidly in all groups receiving meat (Table 3). Neither $NaNO_2$ nor GDL had any obvious influence on mortality, however.

Table 3. *Cumulative mortality of rats fed diets containing 40% meat treated with nitrite and/or GDL*

Diet	Initial group size	Mortality at month						
		12	15	18	21	24	27	29
Males								
Control	30	1	3	4	5	14	19	24
Meat control	30	0	1	2	9	17	27	30
Meat + 0·5% $NaNO_2$	30	0	1	4	9	14	21	26
Meat + GDL	30	1	1	3	6	18	25	25
Meat + 0·5% $NaNO_2$ + GDL	30	0	0	2	5	15	19	22
Meat + 0·02% $NaNO_2$ + GDL	30	0	1	3	13	18	24	28
Females								
Control	30	0	0	0	4	9	19	24
Meat control	30	1	2	5	10	23	28	30
Meat + 0·5% $NaNO_2$	30	2	2	4	8	18	25	26
Meat + GDL	30	1	1	3	8	18	23	24
Meat + 0·5% $NaNO_2$ + GDL	30	1	3	3	5	14	23	27
Meat + 0·02% $NaNO_2$ + GDL	30	0	1	4	8	17	26	27

GDL = Glucono-δ-lactone

Haematology

In Table 4 only the results for haemoglobin, erythrocytes, total leucocytes and percentages of neutrophils and lymphocytes after 12, 51 and 91 wk are given. No differences, or only incidental ones, were found in the packed cell volume, the numbers of monocytes and eosinophils and the MCV, MCH and MCHC. The number of erythrocytes was lower in the groups receiving nitrite. The haemoglobin content of the blood also showed a tendency towards a lower value in these groups, but the effect was less clear than with the red cell count. Both changes may have been due to the addition of nitrite to the meat. No differences were observed in the numbers of leucocytes. As the animals grew older there was a decrease in the relative number of lymphocytes and an increase in that of neutrophils.

Clinical biochemistry

The results of the BSP-retention and SGPT determinations carried out at 3 and 6 months are given in Table 5, while Table 6 presents the results of SGPT, alkaline phosphatase and blood-urea determinations at 9, 12 and 16 months. It is well-known that nitrosamines cause liver damage and if sufficient nitrosamines had been formed in the treated meat, one would have expected abnormalities to have been detected in these liver-function tests (den Tonkelaar & Kroes, 1971). However, no abnormal values were found in the group receiving meat treated with 0·5% $NaNO_2$ and GDL. The results after 9 months showed a tendency

Table 4. *Haematological studies in rats fed diets containing 40% meat treated with nitrite and/or GDL*

Diet	Haematological values in					
	Males at wk			Females at wk		
	12	51	91	12	51	91
	Haemoglobin (g/100 ml)					
Control	15·2	15·4	15·3	15·2*	15·5	14·4
Meat control	15·5	15·4	14·6	14·7	15·4	14·7
Meat + 0·5% $NaNO_2$	14·1*	14·9	13·5	14·6	14·7*	13·8
Meat + GDL	15·4	15·7	14·6	14·9	15·3	15·8
Meat + 0·5% $NaNO_2$ + GDL	14·8*	15·2	14·0	14·6	15·1	14·7
Meat + 0·02% $NaNO_2$ + GDL	14·9	15·4	14·1	15·1*	15·5	15·3
	Erythrocytes (10^6/mm^3)					
Control	7·7	7·7	7·6	7·5	7·3	6·9
Meat control	7·8	7·5	7·4	7·3	7·0	6·7
Meat + 0·5% $NaNO_2$	7·2*	7·0**	7·0	7·1	6·6	6·4
Meat + GDL	7·8	7·4	7·1	7·3	6·9	7·6*
Meat + 0·5% $NaNO_2$ + GDL	7·4*	7·0*	6·9	6·9	6·5	6·9
Meat + 0·02% $NaNO_2$ + GDL	7·6	7·0*	7·0	7·4	6·7	7·1
	Total leucocytes (10^3/mm^3)					
Control	12·4**	8·8	8·7	10·1	6·5	7·1
Meat control	10·9	9·1	8·9	9·4	6·6	6·4
Meat + 0·5% $NaNO_2$	12·4	10·1	9·7	9·7	7·5*	7·1
Meat + GDL	11·2	9·1	10·7	9·2	7·6	7·9*
Meat + 0·5% $NaNO_2$ + GDL	11·3	8·5	8·9	10·7	7·5*	8·0*
Meat + 0·02% $NaNO_2$ + GDL	11·3	10·8*	10·3*	9·0	7·6*	6·6
	Neutrophils (% of leucocytes)					
Control	9·9	15·2	23·6	10·7	11·5	31·6
Meat control	11·7	15·4	25·8	11·0	14·0	27·2
Meat + 0·5% $NaNO_2$	11·1	15·5	23·7	11·3	13·4	28·7
Meat + GDL	10·7	15·4	31·0	10·3	12·0	32·1
Meat + 0·5% $NaNO_2$ + GDL	10·7	17·6	28·2	9·5	14·3	23·9
Meat + 0·02% $NaNO_2$ + GDL	15·5	19·6	30·6	8·9	11·9	30·2
	Lymphocytes (% of leucocytes)					
Control	86·3	78·3	70·5	86·2	83·3	63·1
Meat control	84·1	78·2	67·8	85·9	81·4	67·3
Meat + 0·5% $NaNO_2$	84·8	79·2	71·4	86·3	82·6	67·3
Meat + GDL	83·7	78·7	63·8	85·7	82·7	62·2
Meat + 0·5% $NaNO_2$ + GDL	85·5	77·0	66·2	86·9	81·0	72·7
Meat + 0·02% $NaNO_2$ + GDL	81·3	75·2	63·8	87·2	82·1	64·0

GDL = Glucono-δ-lactone

Values marked with asterisks differ significantly (Student's *t* test) from those of the meat-control group: $*P < 0·05$; $**P < 0·01$.

to a higher alkaline phosphatase value in the groups receiving 0·5% $NaNO_2$. The increases became less marked at later stages and especially after 16 months, although more animals from these groups were used for the later estimations. When the experiment had lasted for about 2 yr, six moribund rats from each group (three males and three females or two males and four females) were killed and used to study the aniline-hydroxylase, aminopyrine-demethylase and glucose-6-phosphatase activity in the liver. Inhibition of these enzymes is

probably a more sensitive indicator of liver damage than the liver function tests performed on serum. There was a wide variation in the values and only a few animals were used from each group but the results did not show any tendency towards inhibition of enzyme activity in the groups receiving 0·5% $NaNO_2$ with or without GDL.

Table 5. *Liver-function tests in rats fed for 3 or 6 months on diets containing 40% meat treated with nitrite and/or GDL*

Diet	Mean values in			
	Males after 3 months		Females after 6 months	
	BSP (m-moles/litre)	SGPT (U/litre)	BSP (m-moles/litre)	SGPT (U/litre)
Control	14·9	16	11·7	11
Meat control	15·1	15	13·9	11
Meat + 0·5% $NaNO_2$	15·5	15	11·0	9
Meat + GDL	15·5	15	14·5	10
Meat + 0·5% $NaNO_2$ + GDL	10·0	15	14·1	8
Meat + 0·02% $NaNO_2$ + GDL	16·6	18	15·8	10

BSP = Bromsulphthalein GDL = Glucono-δ-lactone
SGPT = Serum glutamic-pyruvic transaminase

The serum of each of the five rats/group was used for both determinations. No statistically significant differences were found with Student's *t* test.

During the final months of the experiment a number of animals showed oedema. Therefore in all animals that were killed in a moribund condition or at the end of the experiment, the blood urea was determined. The results are given in Table 7. Although a number of animals showed values that were moderately high (7·1–9·9 m-moles/litre) or high (> 10 m-moles/litre), these were randomly distributed among the groups and no clear effect of the various diets on kidney function could be demonstrated.

Histopathology

All animals that died or were killed were studied histopathologically except for a few in which this was prevented by advanced autolysis. Gross observations revealed no differences between the various groups. The many histological abnormalities observed in the organs and tissues were scattered throughout all the groups and were considered to be abnormalities usually present in the strain of rat used. The most common abnormality was glomerulonephrosis sometimes associated with uraemia and metastatic calcification in several organs, especially the heart. Pronounced hyperplasia of the parathyroids was also found in the same animals. Tables 8 and 9 list only the tumours found in rats that survived for 18 months or more. The incidence of spontaneous tumours was considerable. In the "target organs" for nitrosamine carcinogenesis, notably the lungs, oesophagus, gastro-intestinal tract, liver, kidney, urinary bladder and central nervous system, there were no tumours that could be related to the administration of meat treated with $NaNO_2$ and/or GDL (Table 10).

Table 6. *Clinical chemistry of rats fed diets containing 40% meat with nitrite and/or GDL*

Diet	No. of animals tested	Mean values in Males: SGPT (U/litre)	Males: Alk. Pase (U/litre)	Males: Urea (m-moles/litre)	Females: SGPT (U/litre)	Females: Alk. Pase (U/litre)	Females: Urea (m-moles/litre)
9 months							
Control	5	10	52	7·0	9	53	6·7
Meat control	5	10	63	5·5	13	72	6·0
Meat + 0·5% $NaNO_2$	5	9	77	6·5	9	72	5·8
Meat + GDL	5	15	68	5·8	15	63	5·7
Meat + 0·5% $NaNO_2$ + GDL	5	9	78	6·8	14*	78	5·7
Meat + 0·02% $NaNO_2$ + GDL	5	13	60	6·0	11	53	5·7
12 months							
Control	10	10	38	5·5*	11*	47	4·8
Meat control	5	10	42	4·2	15	48	4·7
Meat + 0·5% $NaNO_2$	10	11	55	4·8	12*	67	4·3
Meat + GDL	5	18	43	5·8*	20	58	5·8
Meat + 0·5% $NaNO_2$ + GDL	10	9	43	4·8	14	65	5·0
Meat + 0·02% $NaNO_2$ + GDL	5	11	40	5·2	15	48	5·3
16 months							
Control	10	14	33	5·8	12	32	5·9
Meat control	5	23	33	5·0	17	37	5·2
Meat + 0·5% $NaNO_2$	10	15	40	4·6	13	33	4·4
Meat + GDL	5	16	30	6·5	15	37	7·8
Meat + 0·5% $NaNO_2$ + GDL	10	13	37	4·8	18	40	5·6
Meat + 0·02% $NaNO_2$ + GDL	5	17	35	7·2	18	32	4·6*

Alk. Pase = Alkaline phosphatase GDL = Glucono-δ-lactone SGPT = Serum glutamic-pyruvic transaminase

Values marked with an asterisk differ significantly (Student's *t* test) from those of the meat-control group: $^*P < 0{\cdot}05$.

Table 7. *Terminal blood-urea values of rats fed diets containing 40% meat treated with nitrite and/or GDL and killed during the last months or at the end of the study (frequency table)*

Diet	No. of rats tested	No. of rats with blood-urea levels (m-moles/litre) of		
		<7	7·1–9·9	>10
Males				
Control	9	3	5	1
Meat control	5	2	1	2
Meat + 0·5% $NaNO_2$	5	3	2	–
Meat + GDL	5	3	1	1
Meat + 0·5% $NaNO_2$ + GDL	9	3	4	2
Meat + 0·02% $NaNO_2$ + GDL	2	–	–	2
Females				
Control	10	3	4	3
Meat control	3	1	2	–
Meat + 0·5% $NaNO_2$	6	5	1	–
Meat + GDL	7	4	3	–
Meat + 0·5% $NaNO_2$ + GDL	6	4	1	1
Meat + 0·02+ $NaNO_2$ + GDL	6	3	1	2

GDL = Glucono-δ-lactone

DNA and α-foetoprotein determinations

Measurement of the DNA content of individual cell nuclei, used as an indicator of preneoplastic changes in the liver, revealed no cells with an abnormal DNA content. The ratio between diploid and tetraploid cells was normal and only a few octaploid cells were found. There were no differences between the groups. Similarly, no positive reactions were obtained when serum samples from all animals killed at the end of the experiment were tested for α-foetoprotein, the presence of which may indicate the existence of liver tumours (Abelev, 1971).

DISCUSSION

As early as 1963, a long-term experiment with nitrite was described by Druckrey, Steinhoff, Beuthner, Schneider & Klärner (1963). Three generations of rats were given 100 mg $NaNO_2$/kg body weight daily in the drinking water. In addition, animals from the F_1 generation were simultaneously given diethylamine in the diet (incorporated in minced meat). A moderately reduced lifespan in all groups (compared with control animals from other experiments) but no influence on haemoglobin content or on the number of erythrocytes was observed. A certain amount (up to 20%) of methaemoglobin was found. Histopathological examination revealed a few tumours, but these were considered to be of spontaneous origin and no indication was obtained that nitrite alone, or in combination with diethylamine, had any carcinogenic or teratogenic effect. In our experiments, $NaNO_2$ at levels up to 0·5% was incorporated in canned meat, with or without GDL, before processing. Growth was inhibited in the groups receiving canned meat with the higher level of added nitrite compared with the group receiving the untreated meat diet. The haematological data suggest that consumption of a meat diet containing added nitrite causes a decrease in the number of erythrocytes. The haemoglobin content of the blood was not affected in a clearly

Table 8. *Tumour incidence in male rats that survived for 18 months or more on diets containing 40% meat treated with nitrite and/or GDL*

Organ	Tumour incidence in rats fed					
	Control diet	Meat-control diet	Meat + 0·5% $NaNO_2$	Meat + GDL	Meat + 0·5% $NaNO_2$ + GDL	Meat + 0·02% $NaNO_2$ + GDL
No. of animals studied ...	25	28	26	27	25	26
Lungs						
Bronchial adenoma	0	0	0	1	0	0
Squamous cell carcinoma	1	0	0	0	0	0
Liver						
Bile-duct carcinoma	0	0	0	1	0	0
Hyperplastic nodules	0	0	0	0	1	0
Kidneys						
Hamartoma	0	0	0	1	0	0
Pituitary gland						
Chromophobic adenoma	6	1	2	1	3	4
Thyroid						
Papillary adenoma	0	0	0	1	0	0
Solid cell carcinoma	0	0	1	0	0	0
Adrenals						
Cortical adenoma	0	0	0	1	1	2
Medullary phaeochromocytoma	2	6	6	2	5	2
Pancreas						
Insuloma	2	0	0	2	0	2
Adenocarcinoma of exocrine system	1	0	0	0	0	0
Adenoma of exocrine system	0	0	0	0	1	1
Testes						
Interstitial cell tumour	1	1	2	0	1	0
Urinary bladder						
Papilloma	0	0	0	0	0	1
Haemo/lymphopoietic system						
Leukaemia	0	0	0	0	0	1
Abdomen						
Sarcoma	0	0	1	0	0	0
Thorax						
Mesothelioma	1	0	0	0	0	0
Ear-duct gland						
Squamous cell carcinoma	0	1	0	0	0	1
Subcutaneous tissue						
Fibroma	2	1	0	0	2	0
Myxoma	0	0	0	0	1	0
Sarcoma	0	1	2	1	0	0

GDL = Glucono-δ-lactone

recognizable manner. The results of Musil (1966), who also studied the effect on the blood of rats of a fairly high nitrite intake over 200 days, point to the same conclusion.

The analytical data show that meat treated with 0·5% (5000 ppm) nitrite still contains about 4000 ppm after processing. In the meat treated with 0·5% nitrite and GDL less than 2000 ppm nitrite is left after processing, while in the group treated with 0·02% nitrite and GDL only about 10 ppm remains. Consequently the experiments were performed with final concentrations of free nitrite of about 1600, 750 and 4 ppm. Haematological data revealed changes in the groups actually receiving 1600 and 750 ppm, so that it is likely that some reaction products of nitrite and meat were involved in this phenomenon and not nitrite as such.

Since nitrosamines are strong hepatotoxic agents, liver damage could be expected if sufficient nitrosamines were formed. However, liver-function tests (the BSP test and the determination of the activity of SGPT) did not indicate any such effect. Microsomal liver enzymes were studied as an indicator of the effect of nitrosamines on the endoplasmic reticulum, but no decrease in the activities of glucose 6-phosphatase, aniline hydroxylase or aminopyrine demethylase was found.

Table 9. *Tumour incidence in female rats that survived for 18 months or more on diets containing 40% meat treated with nitrite and/or GDL*

Organ	Tumour incidence in rats fed					
	Control diet	Meat-control diet	Meat + 0·5% $NaNO_2$	Meat + GDL	Meat + 0·5% $NaNO_2$ + GDL	Meat + 0·02% $NaNO_2$ + GDL
No. of animals studied...	28	25	25	27	27	25
Liver						
Bile-duct adenoma	0	0	0	1	0	0
Hyperplastic nodules	1	0	1	0	2	0
Kidney						
Cortical adenoma	0	1	0	0	1	0
Brain						
Meningioma	1	0	0	0	0	0
Astrocytoma	0	1	0	0	0	0
Pituitary gland						
Chromophobic adenoma	8	7	8	6	7	9
Eosinophilic adenoma	0	1	0	0	0	0
Thyroid						
Papillary adenoma	0	0	1	0	0	0
Adrenal						
Medullary phaeochromocytoma	1	0	2	1	0	0
Pancreas						
Insuloma	0	0	1	0	0	0
Adenoma of exocrine system	0	0	0	0	0	1
Uterus						
Adenocarcinoma	0	0	1	0	0	0
Mammary gland						
Adenoma	5	2	3	1	3	4
Adenocarcinoma	0	0	0	0	0	2
Intestine						
Colonic adenocarcinoma	0	0	0	0	0	1
Haemo/lymphopoietic system						
Leukaemia	0	1	1	1	2	0
Abdomen						
Mesothelioma	1	0	0	0	0	0
Sarcoma	0	0	1	0	0	0
Thorax						
Sarcoma	0	1	0	0	0	0
Ear-duct gland						
Squamous cell carcinoma	0	0	0	1	0	0
Subcutaneous tissue						
Fibroma	0	0	1	1	1	0

GDL = Glucono-δ-lactone

Druckrey *et al.* (1967) studied the carcinogenic effects of 65 different *N*-nitroso compounds in rats, with particular reference to the target organs. In general, symmetrical dialkylnitrosamines produced carcinomas of the liver after oral administration. These authors found also that diethylnitrosamine, at lower dose levels, produced not only liver tumours but also tumours of the oesophagus and nasal cavity. The non-symmetrical dialkylnitrosamines generally produced oesophageal tumours but no liver tumours. On the other hand, cyclic nitrosamines like *N*-nitrosopyrrolidine produced mainly liver tumours. In our own experiments with dimethylnitrosamine (den Tonkelaar & Kroes, 1971) many tumours occurred in the kidneys and lungs in addition to liver tumours, an observation already reported by Magee & Barnes (1962).

Druckrey *et al.* (1967) also studied the dose–response relationship and found that it followed the general formula dt^n = constant, where t is the induction time and d the dosage. This means that a carcinogenic substance present in a diet in a very low concentration shows a very long latent period. Therefore the rats in the experiment described in the present paper were kept as long as possible.

Table 10. *Tumour incidence in "target organs" for nitrosamine carcinogenesis in male and female rats fed diets containing 40% meat treated with nitrite and/or GDL*

Organ	Tumour incidence in rats fed					
	Control diet	Meat-control diet	Meat + 0·5% $NaNO_2$	Meat + GDL	Meat + 0·5% $NaNO_2$ + GDL	Meat + 0·02% $NaNO_2$ + GDL
No. of rats studied...	53	53	51	54	52	51
Lungs	1	0	0	1	0	0
Liver	1	0	1	2	3*	0
Kidneys	0	1	0	1	1	0
Brain	1	1	0	0	0	0
Oesophagus	0	0	0	0	0	0
Intestine	0	0	0	0	0	1
Urinary bladder	0	0	0	0	0	1
Total no. of tumours...	3	2	1	4	4	2

GDL = Glucono-δ-lactone

*Hyperplastic nodules.

No increased tumour incidence was noted in the groups receiving meat with nitrite and/or GDL. The tumours found in the organs that can be considered as target organs for nitrosamine carcinogenesis were equally distributed among all groups. In addition, the measurements of DNA in liver nuclei and the results of the examination of sera for α-foetoprotein did not give any indication of the presence of preneoplastic liver changes.

Preliminary analytical data indicate that the meat treated with 0·5% nitrite and GDL contained about 50 ppb nitrosamines. This level corresponds to 20 ppb in the diet of the rats or a daily intake of 1 μg/kg body weight. In the meat treated with 0·02% nitrite (the normal level of use) in combination with GDL, no nitrosamines at all were detected in the preliminary analyses. Druckrey *et al.* (1967) showed that a daily intake of .75 μg diethylnitrosamine/kg body weight induced a small number of tumours in three target organs, the mean latent period being 840 days. Current experiments in our own laboratory using a subminimal dose level of about 12·5 μg pure diethylnitrosamine/kg body weight/day have so far (after 20 months) yielded no tumours. It is therefore not surprising that no tumours were found after the feeding of meat treated with nitrite and/or GDL, even in the experimental group with the unrealistically high dose level of 0·5% $NaNO_2$ and a lowered pH.

In evaluating the possible hazards of adding nitrite to meat products, it is important to remember that at present it seems impossible to discontinue the use of nitrite because of the danger of botulism that could follow the omission of nitrite from certain meat products. Since it is known (Sander, 1971) that nitrosamines are also formed in the gastro-intestinal tract from nitrite and secondary and tertiary amines, the evaluation of the risk to health from the use of nitrite in meat products should take into account the fact that both groups of substances occur widely in food and in the general environment.

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Essai à long terme sur des rats de viande en boîtes traitée au nitrite de sodium et au glucono-δ-lactone

Résumé—L'article décrit une étude de toxicité à long terme, où des rats ont consommé, à raison de 40% de leur régime, de la viande de conserve traitée soit avec 0,5, soit avec 0,02% de nitrite de sodium avec ou sans glucono-δ-lactone. L'attention s'est portée tout particulièrement sur la détection d'éventuels effets carcinogènes. L'expérience était conçue pour étudier la formation possible de nitrosamines à partir du nitrite et des amines secondaires présentes dans la viande, dans des conditions de pH acide et de pasteurisation. Parmi les paramètres étudiés figurent les données hématologiques, clinico-biochimiques et histopathologiques, la présence d'α-foetoprotéine dans le sérum et la teneur en ADN des noyaux de cellules hépatiques. On n'a relevé aucun indice de modification pré-néoplastique ou de formation de tumeur que l'on aurait pu imputer à la consommation de la viande de conserve en cause.

Langzeitiger Rattenversuch mit Dosenfleisch, behandelt mit Natriumnitrit und Glucono-δ-lactone

Zusammenfassung—Ein langzeitiger Toxizitätsversuch wurde mit Ratten durchgeführt, an die Futter mit einem Gehalt von 40% Konservenfleisch verabreicht wurde, das entweder mit 0,5 oder mit 0,02% Natriumnitrit mit oder ohne Glucono-δ-lactone behandelt worden war. Dieser Versuch, bei dem der Feststellung einer möglichen carcinogenen Wirkung besondere Aufmerksamkeit geschenkt wurde, war dazu bestimmt, die mögliche Entstehung von Nitrosaminen aus Nitrit und den sekundären Aminen im Fleisch unter den Bedingungen eines sauren pH-Werts und der Pasteurisierung zu untersuchen. Zu den berücksichtigten Parametern gehörten Hämatologie, klinische Biochemie, Histopathologie, α-Foetoprotein im Serum und der DNS-Gehalt der Leberzellenkerne. Es gab kein Anzeichen einer präneoplastischen Veränderung oder Tumorentstehung, das der Verfütterung dieses Konservenfleischs zugeschrieben werden konnte.

2308 ✓

NITRATES IN MUNICIPAL WATER SUPPLY CAUSE METHEMOGLOBINEMIA IN INFANT

Joseph Vigil, B.S., Sherman Warburton, B.S., M.P.H., William S. Haynes, M.D., M.P.H., and Leland R. Kaiser, M.A., M.P.H.

A 4-WEEK-OLD INFANT was stricken with methemoglobinemia in the city of B, Colo., on December 3, 1962, and the Tri-County District Health Department was notified. It was provisionally assumed that the infant had developed the disease from ingesting water with a relatively high nitrate content. Serious and occasionally fatal poisonings of infants attributed to ingestion of well water containing nitrates above the Public Health Service recommended standard of 45 mg. of nitrate per liter of water have been reported (*1*). However, few, if any, documented reports of such occurrences incriminate a U.S. municipal water supply, even though many such supplies have exceeded the recommended standard.

Laboratory findings confirmed the provisional diagnosis of methemoglobinemia. The nitrate content of the water ingested by the infant was shown to exceed the Public Health Service recommended standard of 45 mg. per liter, and epidemiologic investigation indicated that the municipal water was the source of the infant's illness.

Water Supply

The city of B is located in the South Platte River Drainage Basin. Traditionally a rural area, with a population of 7,500, it is part of a rapidly expanding metropolitan area. For 75 years the city has depended on the alluvium of the South Platte River aquifer for its water supply. This productive aquifer has wells which yield from 500 to 1,000 gallons of water per minute. The static water table is from 20 to 25 feet below the ground surface. Ten shallow wells from 40 to 60 feet deep were drilled into this aquifer, and the well casings are solid to a depth ranging from 12 to 42 feet. Below the solid casings, perforated casings extend to depths of 60 feet.

Each well is pumped individually, and the water flows directly from the wells into the distribution system. After the demand throughout the distribution system has been satisfied, additional water is stored in concrete storage reservoirs and elevated steel storage tanks located throughout the city. The water is not pretreated, but each well is supplied with chlorination facilities and generally a residual of 0.1 ppm of free chlorine is maintained throughout the system. At times and in certain sections of the city, however, there is insufficient contact time before the water is used by the consumers. All bacteriological samples have been negative for coliform organisms. However, the water is highly mineralized and is contaminated with synthetic detergents. There is also the possibility of organic pollution.

Sources of Pollution

The wells, storage reservoirs, and distribution system are fairly well constructed and protected from surface contamination.

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The city has maintained a good sewage collection system for several years. An excellent complete-treatment sewage plant was completely modernized in 1958. Not all residential units, however, are connected to the municipal sewage system. There are still 121 individual residents using cesspools, septic tanks with leaching wells, and tile leaching fields. These are located in the older section of town near the residence of the infant who contracted methemoglobinemia.

The city of B is situated so that it receives the pollution load from a metropolitan area of approximately 1 million people. This pollution is in the form of sewage and industrial wastes, which receive little pretreatment and are discharged into the Platte River. The water from the river is diverted into numerous canals and irrigation ditches and is used for agricultural irrigation. A number of the ditches flow directly through the city and are so grossly polluted that their stench creates a nuisance.

The aquifer from which the city of B derives its water supply is highly permeable and easily contaminated from surface pollution. It has suffered severe damage from various contaminating substances. These substances generally originate from both sewage and industrial wastes. Sources of wastes include individual sewage-disposal systems; chemicals from chemical industrial plants, oil refineries, and agricultural processes; and stockyards and feedlots. Synthetic detergents, nitrates, chlorides, chlorates, and sometimes chemicals resembling common herbicides have been identified. The nitrates in this aquifer could, therefore, originate from several sources: the river which is highly polluted with biological waste material and is used for extensive agricultural irrigation; nitrogen, urea, and other biological waste products used in commercial fertilizers; and individual cesspools. All of these are potential sources of nitrates and may be contributing factors in the contamination of the aquifer.

Because of the vulnerability of the aquifer and its potential hazard to public health, the Tri-County District Health Department had been concerned for some time. In 1958 the department developed an accelerated sampling and testing program to determine (*a*) the extent of the aquifer damage, (*b*) the main sources of pollution, and (*c*) to what extent this shallow aquifer could be used as a source of domestic water supply. The results indicated that severe damage was occurring in the aquifer and that more intensified efforts were necessary.

Consequently, in 1959 a series of 22 surveillance wells were uniformly established throughout the aquifer. The wells were sampled and a complete analysis was made every 7 months. Two city wells within two blocks of the affected infant's residence were incorporated into this network. In February 1961 one of these wells had a nitrate content of 35 mg. per liter and the other 40 mg. per liter. In September 1961 the same wells showed a nitrate content of 40 mg. and 44 mg. per liter. In April 1962 both wells showed a nitrate content of 52 mg. per liter. At this time a meeting was held with the State health department, and it was agreed to increase the frequency of sampling of the water supply. In October 1962 the medical director of the Tri-County District Health Department notified the city's health officer, city council, and all local physicians and hospital personnel of the danger of using the municipal water for preparation of infants' formula and feeding. By November 1962 the nitrate content of these wells had risen to 62 mg. per liter.

Epidemiologic Investigation

When the Tri-County District Health Department was notified of the case on December 3, a sanitarian was dispatched to the home to determine the circumstances.

The infant's family, of Spanish-American descent, consisted of the parents and two older children. The baby was on a formula of 2 ounces of evaporated milk and 2 ounces of boiled water. The mother (Mrs. L) said the baby had been progressing well while the family lived in another city. When they moved to the city of B, Mrs. L prepared the formula with the same brand of milk and this city's water.

After a few feedings the baby seemed upset and had diarrhea, which the mother attributed to the change of water. Mrs. L felt that this city's water "was hard because when she boiled the water it would leave a film on pans and bottles." To compensate for this she boiled

the water longer, but she thought that this resulted in even more film.

When the infant became quite ill and seemed to have difficulty in breathing, Mrs. L told her husband that they should take the baby to a physician. However, they had no way to get there because their car was not operating. The baby would take only a little water, and Mrs. L gave him some every half hour or so until he quieted down. Later that night when Mrs. L went to check on the baby, he had stopped breathing and was turning blue. Mrs. L picked up the baby and ran to the home of her husband's parents who rushed them to the hospital.

On admission to Colorado General Hospital at 2 a.m. on December 3, the infant was suffering with cyanosis and diarrhea, and the illness was diagnosed as methemoglobinemia. After administration of methylene blue, the baby improved, but he relapsed later and had to be retreated.

Mrs. L was asked to prepare some formula water, and she did this by boiling the water for approximately 20 minutes. The sanitarian took a sample of this water, a sample of previously prepared formula water, and a sample of tapwater.

The tapwater showed a nitrate content of 63 mg. per liter, and the two samples of formula water showed a nitrate content of 73 mg. per liter. The samples were not analyzed for the nitrite ion. It is assumed the rise in nitrates was caused by evaporation, thereby concentrating the nitrates in a smaller volume of water. However, a contributing factor might have been some conversion of nitrites during the boiling process.

When the infant was released from the hospital on December 12, the parents were given a supply of distilled water to be used for his feeding. Since then the child has progressed satisfactorily.

Conclusions

The diagnosis of methemoglobinemia was confirmed by laboratory findings. The infant had not ingested anything other than evaporated canned milk and water, and the water contained a relatively high level of nitrates. Water containing such levels of nitrates has been previously reported to produce serious and sometimes fatal poisonings in infants. We therefore concluded that the infant was stricken with methemoglobinemia by ingestion of water from a municipal water supply which exceeded the Public Health Service recommended standard for nitrates in drinking water.

Followup

Since all wells used by the city of B as a source of municipal water were highly mineralized and were above the Public Health Service recommended standard of 45 mg. of nitrate per liter of water, the Tri-County District Health Department recommended that the city obtain a new source of water which would meet the recommended standards for drinking water. Also, the department, in cooperation with the city council, notified the public and all physicians and hospitals by mail and through the newspapers that the city of B's drinking water exceeds the recommended standards for nitrate content, and it should not be used to prepare formula or for drinking by infants under 6 months of age.

The Tri-County District Health Department has obtained both financial and technical assistance from the State health department and the Public Health Service to perform an extensive survey on underground pollution in the area of the city of B.

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METHEMOGLOBINEMIA*

A CAUSE OF CYANOSIS IN INFANTS AND CHILDREN

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Methemoglobinemia is by no means an uncommon occurrence in infants and children. It should be emphasized that this alarming condition can rapidly lead to death unless the etiology of the cyanosis is suspected. Death due to methemoglobinemia per se is largely inexcusable because of the simplicity and specificity of the treatment.

It was first reported in 1886 when Rayner, quoted by Leader,[1] described 17 cases of methemoglobinemia with the sudden appearance of cyanosis in infants traced to diapers and pads freshly stamped with ink containing nitrobenzene. Accidental poisoning with industrial and medicinal compounds and the continued use of obsolete drugs account for the majority of the cases today. Occasionally, it may be due to sepsis.

Methemoglobin may result from the ingestion, inhalation, or absorption through the skin[2] of the following substances: potassium chlorate, bismuth subnitrate, sulfonamides, particularly sulfanilamide and sulfapyridine, antigas ointment,[3] crayons,[4] well water containing nitrates, aniline, nitrobenzene, acetanilide, aminophenols, antipyrin, ferricyanides, shoe dyes, perfumery, artificial

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2330

flavorings, acetophenacetin, plasmochin, phenylhydrazine, sulfonal, pyrogallic acid and methylene blue. Cases of familial idiopathic methemoglobinemia have been reported.[5] The production of enterogenous methemoglobinemia due to nitrite-forming bacilli has been suggested[6] and disputed.[7]

The striking feature of this condition, particularly in children and infants, is the extreme cyanosis. These small patients cannot describe the sensations of burning and tingling, headache, vertigo and visual disturbances which are concomitant symptoms of methemoglobin intoxication. Therefore, the diagnosis must be made from the objective symptoms.

Methemoglobinemia is most commonly confused with congenital heart conditions, respiratory diseases, cardiac arrhythmias and intracranial injuries. Generally, the diagnosis is not made until the cyanosis fails to disappear under oxygen therapy, or the characteristic chocolate color of the blood is noted in the course of some laboratory procedure.

We wish to discuss five cases of methemoglobinemia due to varied causes, their mechanism of production and treatment.

CASE REPORTS

Case 1. P. M. B., a white girl, aged 6 weeks, entered The Children's Hospital on November 4, 1945. Birth had been normal, and the birth weight was 5 pounds 9¾ ounces. The past history was not unusual, except for a brief period of "diarrhea" immediately after birth which had apparently ceased spontaneously. Two days prior to admission a physician prescribed a pink liquid for a rash on the infant's tongue. On the following day the infant refused her formula and vomited once. She became lethargic but cried out occasionally "as if in pain." On the morning of admission the infant had a peculiar "bluish-yellow" color. The urine was very dark in appearance. Physical examination showed a poorly nourished, deeply cyanotic, infant girl whose skin was bluish-yellow in appearance and whose sclerae were icteric. The lips were cyanotic. The rectal temperature was 102.6° F. The lung fields seemed to be clear. The heart rate was 160 per minute, and there was a questionable murmur. The heart was normal in size, and no arrhythmias were detected. The navel showed evidence of subsiding infection. The liver and spleen were both

palpable 2 cm. below the costal margins. There was a generalized lymphadenopathy. It was thought that the infant had a severe anemia with jaundice due to infection. The infant was given continuous oxygen by tent. Penicillin therapy was immediately instituted.

The cyanosis was not affected by oxygen. Because of the obvious severe anemia, blood withdrawn from the femoral vein for gross examination and cross-matching was chocolate colored. Within thirty minutes all the cells were disintegrated leaving a translucent, brownish fluid. A hemoglobin determination at this time was 6.0 Gm. per cent. There were 1,840,000 red cells per cu. mm. An icterus index could not be done because the blood hemolyzed spontaneously. A blood culture was negative after eight days' incubation. Because of the history of the stomatitis, for which a pink medicine was given, methemoglobinemia due to potassium chlorate poisoning was suspected. The infant was transfused with 35 cc. of whole blood. Eight hours after admission methylene blue (1.5 mg. per kilogram of body weight) was given intravenously and followed by 35 cc. of whole blood. The infant's progress was steadily downward, and the infant expired twelve hours after admission. Autopsy permission was refused. It was later determined that the infant had received 30-35 grains of potassium chlorate over a period of two and one-half days. Death was due to intoxication with this drug resulting in a severe methemoglobinemia and fulminating hemolytic anemia.

Case 2. M. W. J., a white infant girl, aged 13 days, was admitted to The Children's Hospital on December 23, 1946. Seven other siblings were normal. The infant had been a full term baby, normally delivered, and her neonatal history had been uneventful until the day of admission. She lived on a farm where the powdered milk formula was prepared with boiled well water. At 11:00 A.M. on the day of admission the infant suddenly developed cyanosis which became intensified with crying. The infant was placed in continuous oxygen immediately. Physical examination revealed a well developed, well nourished, 10-pound infant girl crying lustily and appearing well except for a profound cyanosis of the entire body which was accentuated in the nail beds and mucous membranes. Rectal temperature was 101° F., pulse rate was 180 per minute, and respirations were 30

per minute. Continuous oxygen therapy seemed to lessen the cyanosis only slightly, and crying, either in or out of oxygen, accentuated the condition. The chest was clear to auscultation. The liver was palpable 3 cm. below the right costal margin. The spleen was not felt. Methemoglobinemia was suspected and corroborated by the chocolate-brown color of the withdrawn blood. The hemoglobin was 13.9 Gm. per cent. Upon shaking, no change in the appearance of the blood was noted, and a comparison with the blood of another infant revealed a dissimilarity in color.

The infant was given 5 mg. of methlene blue intravenously (1 mg. per kilogram of body weight). In thirty minutes some improvement was apparent in the color of the infant, and an additional 2.5 mg. (0.5 mg. per kilogram of body weight) were administered with further improvement in appearance. One-half hour after the second injection blood withdrawn from the femoral vein seemed to have a "normally red" appearance. The hemoglobin determination at this time was 15.1 Gm. per cent. The urinalysis was negative. The cyanosis had disappeared completely by the following morning. The infant was discharged on the third hospital day. No spectroscopic or spectrophotometric methods were available at this time for quantitative analysis for methemoglobin so that the degree of methemoglobinemia was not known. However, a solution of blood gave an absorption band in the region of 600-650 mu when examined in the spectrophotometer which was not present in normal blood. The well water used in the preparation of the formulae given to this infant revealed the presence of nitrates in the amount of 60 parts per million.

Case 3. L. T., a white infant girl, aged 2 months, was referred to The Children's Hospital by the family physician twelve hours after the sudden onset of severe cyanosis. On the day prior to admission the infant became pale and irritable and began to refuse feedings. The birth history was normal. After one month of breast feeding the infant was changed to a powdered milk formula prepared with water obtained from a limestone well on the grandparents' farm. Physical examination revealed a well developed, well nourished infant girl with a rectal temperature of 100.2° F. There was a generalized "heliotrope" cyanosis of the body which was accentuated in the nail beds, finger tips, toes, lips, and several excoriated areas about the buttocks. Respirations were 85 per minute, and a severe tachycardia was present. After the child had been in oxygen a short time, it was noted that the conjunctival mucosae appeared pale. The chest was clear, but evidence of air hunger was characterized by the gasping respirations. No cardiac murmurs could be heard, and the heart was not enlarged to percussion. The liver was soft and palpable 3.5 cm. below the right costal margin. The tip of the spleen was felt. At this time a methemoglobin determination* revealed 5.05 Gm. per cent and a total hemoglobin concentration of 8.5 Gm. per cent.

The infant was given methylene blue intravenously (1.5 mg. per kilogram of body weight) and was returned to the oxygen tent. Thirty minutes later only slight improvement was noted in the color. One and one-half hours after the initial injection, an additional 0.5 mg. per kilogram of body weight of methylene blue was administered intravenously. One-half hour later methemoglobin concentration was 43 per cent (methemoglobin—4.6 Gm. per cent, hemoglobin—10.7 Gm. per cent). Seven and one-half hours after the initial dose of methylene blue, the methemoglobin had fallen to 3.1 Gm. per cent, and the total hemoglobin was 11.9 Gm. per cent. At seventeen and one-half hours the methemoglobin was 0.8 Gm. per cent, and the total hemoglobin was 12.3 Gm. per cent. The infant was normal in color. She had been given 40 mg. of methylene blue and 100 mg. of ascorbic acid orally in this interval. The liver had receded 2.5 cm. Respiratory and cardiac rates were normal. Analysis of the parents' well water containing a brown sediment and thus considered to be "polluted" revealed 0.08 parts per million of nitrates. The water from the grandparents' well was clear and thought to be more potable. It had a nitrate content of 90 parts per million.

Case 4. Girl M., a colored premature infant, weighing 4 pounds 2½ ounces, was born by precipitate delivery. The infant's mother was severely ill at the time from an undiagnosed disease. At operation following delivery, the mother was found to have a ruptured appendix. She subsequently succumbed and at autopsy was found to have peritonitis, pelvic abscesses, and caseous abdominal lymph nodes. The infant was admitted to The Children's

*Methemoglobin determinations were done by a spectrophotometric modification of the method of Evelyn and Malloy using a Coleman spectrophotometer. (See J. Biol. Chem., 126: 665, Dec. 1938).

Hospital on the seventeenth day of life because of failure to do well since birth. Physical examination revealed a normally developed, dehydrated, colored infant appearing acutely and chronically ill. The conjuctivae and buccal membranes were extremely pale, and the infant's hemoglobin was estimated to be about 4.0 Gm. per cent. The chest was apparently clear, and the heart tones were described as being of good quality. There were no cardiac murmurs. The liver and spleen were not palpable. Blood withdrawn from the infant for cross-matching was almost black in color, but upon dilution with saline it was found to be chocolate colored. The diagnosis of methemoglobinemia was made, and a determination spectrophotometrically revealed the presence of practically 100 per cent methemoglobin. Total hemoglobin was 11.6 Gm. per cent. The infant was given 2 mg. of methylene blue per kilogram of body weight intravenously. At the time of the injection, in spite of her naturally dark color, there was cyanosis of the palms, lips and nail beds. The methylene blue was followed by a transfusion of 30 cc. of whole blood. A search for possible agents which might have caused the methemoglobinemia was unsuccessful, and it was thought that sepsis was the most likely possibility. A blood culture revealed the presence of a small gram-negative rod which could not be successfully isolated due to a heavy overgrowth of B. subtilis. A stool culture was negative. The infant was given methylene blue and ascorbic acid orally. Blood withdrawn one hour following the injection of methylene blue was found to contain 4.47 Gm. per cent of methemoglobin and 13.2 Gm. per cent of total hemoglobin. A determination on the morning following admission disclosed 4.45 Gm. per cent methemoglobin and 13.6 Gm. per cent total hemoglobin. Another transfusion of 30 cc. of whole blood was given, and oral methylene blue was continued. The infant was kept in oxygen. She was given 3 cc. of 5 per cent sodium sulfadiazine twice daily by oral route. On the second hospital day the methemoglobin concentration was 2.4 Gm. per cent, and the total hemoglobin was 15.0 Gm. per cent. The Mazzini test was negative. A blood sulfadiazine level on the seventh hospital day was 24.1 mg. per cent. On the eighth hospital day the serum protein was found to be 4.88 per cent, and the infant was given 25 cc. of pooled plasma intravenously without difficulty. One and one-half hours later the

infant was found dead in her crib. A postmortem blood culture failed to grow any organisms after nineteen days' incubation. Postmortem methemoglobin determination was 0.73 Gm. per cent, and the total hemoglobin was 17.9 Gm. per cent. The autopsy findings were compatible with sepsis.

Case 5. E. R., a white boy, aged 2½ years, had been well until the day of admission. Three hours before admission he ate an undetermined number of enteric coated tablets which he found on a neighbor's trash pile. Later he refused his lunch and complained of feeling ill. His mother noticed that he was pale and that his lips were blue. He began to complain of abdominal pain but did not vomit. At this time he was brought to the hospital. Physical examination showed a well-developed and nourished white boy who appeared to be acutely ill with a slate-like coloration of the skin and cyanosis of the lips, mucous membranes and nail beds. He was listless, complained of headache, and disliked sitting up. He was somewhat disoriented. The lungs were clear and respirations did not seem abnormal. The heart rate was 144 per minute and the rhythm regular. There were no murmurs. Blood pressure was 80/54.

The liver was palpable 1½ fingerbreadths below the right costal margin. It was soft and nontender. The spleen was not palpable. Neurological examination was normal. The diagnosis of acute drug intoxication with resultant methemoglobinemia was made, and it was suspected, and later confirmed by analysis, that the tablets were enteric-coated sodium nitrite. A blood sample was obtained, and the child was given 2.2 mg. of methylene blue intravenously per kilogram of body weight. The stomach was washed with normal saline. The material obtained was fluid and had the color of the enteric-coating substance on the tablets. No pieces of tablet were recovered. Four grams of sodium bicarbonate were placed in the stomach. The initial methemoglobin determination revealed 6.5 Gm. per cent and the total hemoglobin level, 12 Gm. per cent. Thirty-one minutes later the methemoglobin concentration had risen to 7.6 Gm. per cent, and the total hemoglobin was 9.45 Gm. per cent. The boy was given an additional one mg. per kilogram of body weight of methylene blue intravenously. Because of the low functional hemoglobin he was placed in an oxygen tent. Two hours following the second dose

of methylene blue examination of the blood revealed 5 Gm. per cent of methemoglobin and 10.5 per cent of total hemoglobin. Forty-five minutes later the cyanosis had largely disappeared. The boy was sitting up in bed and taking fluids by mouth. The pulse rate at this time was 140 per minute and was full and strong. By the following morning the boy was fully recovered.

DISCUSSION

The death of the first patient was unquestionably due to intoxication by potassium chlorate with methemoglobinemia complicated by a fulminating hemolytic anemia. Potassium chlorate in repeated doses has a cumulative effect, since the chlorate ion is not destroyed in the body but is slowly excreted by the kidney. The drug was introduced as an oxidizing germicide, but it has been shown to have little action against bacteria.[8] Unfortunately, its use in the treatment of stomatitis has crept into textbooks.[9] Its use is to be condemned because more effective and less toxic agents are available. Gettler and St. George[10] reported a case of fatal poisoning in a 3-year-old boy for whom potassium chlorate was prescribed as a gargle over the telephone. The medication was mistakenly given orally with death in six hours after the appearance of cyanosis. At autopsy the blood was choclate brown in color and showed methemoglobin spectroscopically. Miller[11] reported an episode of cyanosis in a diabetic woman who had been given 12 grams of bismuth subnitrate over a forty-eight hour period for diarrhea. She was found to have 70 per cent methemoglobin upon admission to the hospital. A 3-week-old infant [12] was given 3 grams in buttermilk for diarrhea with cyanosis and death occurring in twelve hours. In 1907 Boehme[13] reported the case of an 18-month-old child who was given bismuth subnitrate by enema for an x-ray study. A cleansing enema was given following the procedure, but cyanosis due to methemoglobinemia developed and was followed by death. Four other cases[14, 15, 16] due to bismuth subnitrate have been found in the literature, the latest in 1947.[17] Poisoning has occurred from the use of ammonium nitrate as a diuretic.[16] Fortunately, the methemoglobin-forming sulfonamides are used less often today. Sulfanilamide is the worst offender. Hartmann and co-workers[18] followed the production of methemoglobin in infants and children who received sulfanilamide and concluded that while there was an excessive individual variation in the rate and degree of methemoglobin formation, in general, the dosage of sulfanilamide and its blood concentration seemed to have a direct relationship to the methemoglobinemia. Others[19] have failed to find any definite relationship between blood concentrations of sulfanilamide and sulfapyridine and the amount of methemoglobin produced. However, some degree of methemoglobinemia was observed in almost every infant who received either drug.

In February 1945, three premature infants and 32 newborn infants on the pediatric wards of the Louisville General Hospital[20] suddenly developed cyanosis. A careful search for the etiology of the outbreak failed until a nurse recalled that new, freshly stamped diapers had been used in the nurseries before laundering. A second episode occurred in the same hospital eight months later, this time due to unlaundered wash cloths stamped with ink containing aniline and used to wash the excoriated buttocks of three infants with diarrhea.

Stevenson and Forbes[21] reported a 12-month-old infant girl who had become poisoned by oil of mirbane (nitrobenzene) traced to an exterminator spray which had been used on her bed. A similar case report[22] involved 3-week-old twin siblings whose mattress had been sprayed with "Cresco," containing nitrobenzene. Levin[23] reports two cases of his own and 11 of Riviere's following the use of shoe dye on the shoes of infants and children between the ages of 8 months and 11 years.

Recent reports by Ferrant[24], Schwartz and Rector,[25] Comly,[26] and Faucett and Miller[27] concerned infants with methemoglobinemia traced to well water. In the United States, thus far, all reports have come from areas having wells drilled in sandstone or limestone, apparently allowing ready access to the water supply of surface nitrates by seepage through faults in the stone. It is not generally realized that formulae prepared from powdered milk diluted with well water contain three times as much water as do formulae prepared from diluted whole cow's milk. Therefore, the nitrate content of a powdered milk formula may be three times as great.

Recently, reports of idiopathic familial methemoglobinemia have appeared[5, 28]. Idiopathic methemoglobinemia has two characteristics which distinguish it from that of toxic origin. The

methemoglobin is not free in the plasma but is found only in the red cells, and, following exposure to air, it does not readily convert to hemoglobin as does the methemoglobin due to intoxication.[29] As a rule, the conversion of hemoglobin to methemoglobin resulting from toxic drugs occurs within the red cells, and the latter are not injured. High concentrations of certain methemoglobin-forming drugs, however, may result in actual destruction of the corpuscles. This is especially true of acetanilid, acetophenetidin, aniline, nitrobenzene, phenylhydrazine, pyrogallic acid, plasmochin and potassium chlorate. Goodman and Gilman state that "methemoglobin-containing cells apparently are more vulnerable to physiologic stress and to destruction by the reticulo-endothelial system. The result is the disintegration of erythrocytes."[30]

Renal function may be seriously impaired because of the precipitation of hemoglobin and methemoglobin in the renal tubules. Yuile et al.[31] readily produced a pathological lesion resembling "transfusion kidney" by the injection of hemoglobin into rabbits whose kidneys had previously been damaged by a short period of ischemia or by the use of a specific kidney poison, sodium tartrate. This damage was a consistent finding in the presence of acid urine in contrast to the lack of such disturbances when the urine was alkaline. It has been shown that the action of nitrites on blood is inhibited by ammonia.[32]

Darling and Roughton[33] believe that the mechanism of production of methemoglobin is due to the formation of compounds intermediate between reduced (ferrous) hemoglobin and oxidized (wholly ferric) methemoglobin. There are four ferrous atoms in the hemoglobin molecule, and the conversion of one or more atoms to "ferric" methemoglobin leads to an increased affinity of the remaining ferrous atoms for oxygen. Once hemoglobin is transformed to methemoglobin, oxygen is held tenaciously by the red cells and is not available for use by the tissues. It has also been shown that the dissociation curve of the remaining oxyhemoglobin is disturbed, thereby enhancing the anoxemia.[34] These factors thus help to explain the persistence of the cyanosis even after what is sometimes considered to be adequate therapy. It also explains the air hunger which is present in the more severely poisoned patients. Over a variable period of time a spontaneous reversible reaction occurs, and the methemoglobin is reconverted to hemoglobin. Generally, only a fraction (20 per cent to 40 per cent) of the total hemoglobin is available for oxygen transportation to the tissues.

Cyanosis becomes clinically evident when the methemoglobin reaches a level of 3.0 Gm. per cent or 12.3 per cent of the total hemoglobin.[35] Rapid pulse, tachypnea and air hunger occur. Passive congestion of the viscera result from the ineffectual cardiac output associated with the tachycardia. The liver is generally palpable. A severe, underlying anemia is generally found clinically. Rapidly increasing jaundice may be present, especially with those drugs which, in addition, produce liver damage. Cardiac murmurs may be heard but are not common unless there is an associated hemolytic factor to the intoxication. The rapid heart rate and noisy respirations preclude the auscultation of a murmur in many cases.

In infants the cyanosis usually involves the entire body to varying degrees. Older children manifest the cyanosis by blueness of the lips, mucosae and nail beds. Their skin may often have a peculiar slate-gray appearance. If the process is a hemolytic one, oliguria is apt to occur. This may result from the toxic effect of the drug itself or from precipitation of free hemoglobin and methemoglobin in the kidney tubules. The urine should always be alkalinized in these patients as a precautionary measure. Low functional hemoglobin resulting in cerebral anoxia may produce convulsions.

Treatment of the methemoglobinemia should be instituted early in the course of the condition. A 1.0 per cent aqueous solution of methylene blue is given slowly by the intravenous route. The dosage is 1.5 to 3.0 mg. per kilogram of body weight. The drug may be diluted to any desired amount with normal saline. Several of the cases treated in this manner[16, 25] have shown a rather prompt disappearance of the cyanosis within 30 to 60 minutes. This rapid conversion of methemoglobin to hemoglobin has not been true in our experience, several hours being required for the conversion. In addition, with our later cases we have found that the oral administration of methylene blue in doses of 10 mg. every four to six hours is a useful adjunct. Orally, the drug is only one-tenth as effective as it is when used intravenously.

The mechanism of conversion of methemoglobin to hemoglobin

is believed by Hauschild[36] and Wendel[37] to be due to the reversible oxidation reduction or redox system of methylene blue and its colorless form. The latter, according to Wendel, is the effective agent. Colorless (reduced) methylene blue is thought to be produced in the red cells and more actively metabolizing tissues by the action of enzyme systems. The oxidizing action of greater doses of methylene blue is the rationale for the production of methemoglobin in the treatment of cyanide poisoning. Methylene blue is ineffective in the treatment of carbon monoxide poisoning, although it has been recommended and employed for this purpose.[38]

Thionine and ascorbic acid will cause the conversion of methemoglobin to hemoglobin by a redox system similar to methylene blue. Lian[5] and associates first used ascorbic acid for the treatment of familial methemoglobinemia. Hawley et al.[39] found that the addition of sodium bicarbonate enhanced the effect of ascorbic acid by inhibiting its excretion. Reports on the clinical use of a single large dose of ascorbic acid have resulted in the conversion of methemoglobin to hemoglobin in 56 hours.[40] Gibson[41] has shown that glutathione, normally found in red cells, exerts a strong inhibitory effect upon ascorbic acid. He has concluded that the effectiveness of this drug in the treatment of methemoglobinemia would be negligible. The use of ascorbic acid would appear to us to be limited to the treatment of the familial form of methemoglobinemia or to prevent the formation of methemoglobin by those drugs which are known to produce the pigment. In acute cases it is not recommended as an emergency measure.

SUMMARY

1. Five cases of methemoglobinemia in infants and children are reported due to such variable causes as potassium chlorate, nitrates in well water (2), accidental ingestion of sodium nitrite tablets and sepsis.

2. A review of the literature with a report of the common etiological agents which produce this condition is included.

3. The cyanosis must be differentiated from other causes of "blue babies" with which this condition is often confused.

4. Suspected methemoglobinemia can be confirmed by (1) the characteristic chocolate color of the blood; (2) by the spectroscopic examination of the blood revealing the presence of an absorption band in the red region of the spectrum which can be dissipated by the addition of cyanide or alkali; (3) by spectrophotometric determination of the amount of methemoglobin present.

5. The mechanism of production of methemoglobin and the treatment of methemoglobinemia is discussed. Methylene blue should be given intravenously for rapid effect. The recommended dosage is 1.5 to 3.0 mg. per kilogram of body weight. Frequently, the higher dosage will be necessary. The drug can be used orally as an adjunct to parenteral therapy. By this route methylene blue is only one-tenth as effective.

Ascorbic acid might be of value prophylactically to prevent methemoglobin formation. It seems to be useful in the treatment of idiopathic familial methemoglobinemia. In our opinion it is not satisfactory for the treatment of cases due to poisoning.

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POLIOMYELITIS. (Revista Chilena de Pediatria, Santiago, 19: 389, May 1948). Romero and Armijo studied the clinical records of 515 children with poliomyelitis who had been observed in different children's hospitals of Santiago since 1920. The disease was previously sporadic. Since 1935 it has been epidemic. The acuteness of the disease and the number of cases increase every year. The seasonal and age distribution are similar to classical descriptions. Few cases with transitory paralysis or paresis were observed. The bulbar type was rare. In spite of the low mortality, it is believed that in future there may be more serious forms with a tendency for the disease to occur in adults.—*Journal A.M.A.*

MULTIPLE NEURITIS AND POLIOMYELITIS

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(*Continued from the March issue*)

MIASMIC POISONING (*continued*)

Miasmic hematuria was observed during the Civil War and it so closely resembles the "infectious hepatitis" of World War II that it bears consideration in this report. The disease was characterized by jaundice, irritability of the stomach giving rise to uncontrollable vomiting, and a peculiar alteration in the color of the urine, generally attributed by the early writers to the presence of blood. It was regarded by some as a manifestation of malarial poisoning; by others, however, it was thought to be a modification of yellow fever. Faget (1869)[95] pointed out that this disease had frequently been seen in New Orleans but had been generally confounded by medical men with yellow fever.

Berenger-Ferand (1875)[96] showed the existence of a disease in Senegambia which corresponded with the features of the American hematuric fever. He found bile in the urine but no blood corpuscles nor hematin, except in a very small proportion of cases. Several American writers, especially one who signed his name J.H.H. (1875)[97] considered the American hematuric fever to be identical with that described by Berenger-Ferand.

Jaundice is not a common manifestation of multiple neuritis, but it can appear during the course of the disease. The most outstanding example is that resulting from phosphorus, i.e., "acute yellow atrophy". Stechele[98] observed six cases of hepatitis out of 55 cases of poliomyelitis in 1939, and in 3 out of 6 cases in 1940. Reyes[77] (1945) reported the appearance of jaundice in some of his cases of poliomyelitis. The writer[99] has also observed jaundice in a small number of cases of poliomyelitis. Van Rooyan and Kirk[100] observed a simultaneous epidemic of infectious hepatitis and poliomyelitis.

As to the manner in which miasmic poison was disseminated there was some difference of opinion. There was general agreement that it resulted from decaying vegetable matter.

Nordquist[101] states: "The essence of these various and clinically

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Separation and Detection of Volatile and Non-volatile *N*-Nitrosamines

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The detection of small concentrations of *N*-nitrosamines in biological materials requires their prior selective extraction and concentration. A procedure is described for the separation of both volatile and non-volatile *N*-nitrosamines as a class, and their subsequent determination at low levels. *N*-Nitrosamines are adsorbed from aqueous solution on to activated carbon from which many can be desorbed to varying extents by refluxing with methanol. Two sensitive techniques are used for the determination of separated *N*-nitrosamines, namely differential polarography at acid pH and the release of nitrite on ultraviolet irradiation at alkaline pH in the manner of Daiber and Preussmann. The properties of *N*-nitrosamines and related compounds are compared and methods of differentiating between the groups of compounds are discussed.

The polarographic detection of the small concentrations of *N*-nitrosamines likely to be of physiological importance requires their prior selective extraction and concentration. Adsorption on carbon separates effectively both steam-volatile and non steam-volatile *N*-nitrosamines, most of which can be desorbed, at least in part, by refluxing with methanol. This procedure also assists in the separation from other polarographically active nitrogenous compounds, such as *N*-nitramines and *C*-nitro compounds, examples of which remained adsorbed on carbon in refluxing methanol. Differential polarography with and without photolysis under ultraviolet light permits the determination of *N*-nitrosamines in the presence of light-stable contaminants, but with potential interference from unsaturated aldehydes and ketones. Release of nitrite on ultraviolet irradiation at alkaline pH according to Daiber and Preussmann[1] provides a means of differentiation from the latter.

A considerable range of *N*-nitrosamines derived from dialkyl, alkaryl and heterocyclic amines, together with some *N*-nitrosamides studied, are potent carcinogens in a range of species[2,3] so that their detection at low levels in environmental sources is of great importance.

Of the two most sensitive techniques for the detection and determination of *N*-nitrosamines, polarography is more specific than gas - liquid chromatography for such compounds as the half-wave potentials of authentic compounds of this type cover only a limited range of voltages. The specificity of their detection is enhanced in differential polarography in which advantage is taken of the degradation of all *N*-nitrosamines, studied on irradiation with ultraviolet light in solution in 0·2 N hydrochloric acid, to products devoid of a polarographic response at equivalent concentration. Thus a differential polarogram of a *N*-nitrosamine solution with reference to a further aliquot subjected to ultraviolet photolysis comprises a peak that is not influenced by other components stable to such irradiation. Unlike most *N*-nitrosamines, other polarographically active nitrogenous compounds are reduced at the dropping-mercury electrode at both acidic and alkaline pH values. Differentiation between *N*-nitrosamines and unsaturated aldehydes and ketones, between which confusion is possible in differential polarography, can be effected by the photolysis of the former in alkaline solution to nitrite, in the manner of Daiber and Preussmann.[1]

After extraction into diethyl ether, *N*-nitrosodiethylamine has been identified by thin-layer and gas chromatography as a constituent of heated flours and cheese,[4] although Thewlis[5] obtained no evidence of its presence in samples of wheat flour and no accurate determinations of its possible concentration have been published. The identity of the amine moieties contributing to *N*-nitrosamines available in biological systems will not, however, generally be known, and their extraction into lipid solvents from an aqueous environment is by no means always efficient.[6] In detecting and determining small amounts of unknown *N*-nitrosamines in biological systems it is imperative to separate these compounds as a class as

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selectively as possible, as the sensitivity of polarographic detection in homogenates of tissues is poor. Not all *N*-nitrosamines found to be carcinogenic can be separated by distillation in steam but of such compounds examined all were adsorbed from aqueous solution in large measure on activated carbon. After aqueous washing of the separated carbon, most adsorbed *N*-nitrosamines other than those containing phenyl groups and *N*-nitrosodipentylamine can be eluted, at least in part, in boiling methanol. Desorbed *N*-nitrosamines in aqueous methanol can thereafter be detected and determined by differential polarography and by photolysis to nitrite under alkaline conditions.

Experimental

Polarography—

Polarographic observations were made with a Southern Analytical differential cathode-ray polarograph, type A1660. In this instrument, the voltage sweep is applied simultaneously to two identical cells, the output currents of the two being in opposition to each other. The wave heights appertaining to a range of concentrations of various *N*-nitrosamines were observed on the graticule superimposed on the cathode-ray tube used for their display and were used for calibration graphs, in conjunction with the pre-determined amplification factor. The de-oxygenation of solutions was accomplished with a stream of oxygen-free nitrogen.

Ultraviolet photolysis—

Ultraviolet irradiation of solutions of *N*-nitrosamines was undertaken with a Hanovia 1-litre photochemical reactor modified with a quartz vessel constructed to take six samples of 10 ml or less, which was supplied by Quartz Fused Products, Shepperton, Middlesex.

Reagents—

N-Nitrosodimethylamine, diethylamine, dipropylamine and dibutylamine were obtained from Eastman Kodak Ltd., Kirkby, Liverpool; streptozotocin[7] was provided by the Upjohn Company, Kalamazoo, Michigan, U.S.A., and *C*-nitro compounds by the British Petroleum Company, Sunbury-on-Thames. We thank Dr. R. Preussmann of the Forschergruppe Praeventivmedizin, Freiburg, Germany, for samples of the *N*-nitrosamines of methyl-2-hydroxyethylamine, dicyclohexylamine, methyl-4-picolylamine, methylethylamine, methylpentylamine, dipentylamine, dibenzylamine, diethanolamine and *N*-nitrodimethylamine; Professor E. Boyland of the Institute for Cancer Research, London, for *N*-nitrosopyrrolidine, piperidine and *N*-methylaniline; Dr. J. M. Barnes of the M.R.C. Laboratories, Carshalton, Surrey, for *N*-nitrosomethylurea; and Dr. E. K. Weisburger of the National Cancer Institute, Bethesda, Maryland, U.S.A., for 1,4-dinitrosopiperazine. *N*-Nitrosoproline was prepared according to the method of Sander[8] and recrystallised from benzene.

Granular activated carbon 8 to 12 B.S.S. mesh was kindly donated by Sutcliffe, Speakman & Co. Ltd., Leigh, Lancashire.

Nitrite determination—

This was based on the method of Nicholas and Nason.[9]

Results

Properties of *N*-nitrosamines—

Table I records the peak potentials, with reference to the mercury-pool anode, in derivative polarography of a range of *N*-nitrosamines in solution in aqueous 0·2 N hydrochloric acid. The peak potentials were 50 to 80 mV more negative in solution in absolute methanol containing 0·1 volume aqueous 2 N hydrochloric acid. Well formed derivative peaks were observed on polarography of dialkyl, alkaryl and heterocyclic *N*-nitrosamines at a concentration of 0·1 p.p.m., the response disappearing in almost all instances on raising the pH above 2 to 3. Only in the case of 1,4-dinitrosopiperazine was a wave evident at pH 8·4 (potential − 1·34 V) comparable in size with that in 0·2 N hydrochloric acid (potential − 0·76 V). The sensitivities of response of simple dialkyl-*N*-nitrosamines tested were of the same order with millimolar diffusion currents in direct polarography in aqueous 0·2 N hydrochloric acid for *N*-nitrosodimethylamine, diethylamine, dipropylamine and dibutylamine of 26, 26, 39 and 37 μA, respectively. The polarographic waves of all *N*-nitrosamines examined disappeared after irradiation with ultraviolet light in 0·2 N hydrochloric acid within 1½ to 3 hours. Thus a difference polarogram of a solution of a *N*-nitrosamine with reference to an

N-Nitrosamine

N-Nitroso:
Dimethylamine ..
Methylethylamine ..
Methylpentylamine ..
Diethylamine .. ..
Dipropylamine ..
Dibutylamine .. ..
Dipentylamine .. ..
Dibenzylamine ..
Dicyclohexylamine ..
4-Picolylmethylamine
Methyl-2-hydroxyethylami
Diethanolamine ..
Piperidine .. ..
Pyrrolidine .. ..
N-Methylaniline ..
Proline .. .. ..
1,4-Dinitrosopiperazine

* With res
† Based up

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The distillation at a
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In aqueous solution
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method[1] were used in a
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TABLE I

PROPERTIES OF *N*-NITROSAMINES

N-Nitrosamine	Peak potential* in derivative polarography in 0·2 N hydrochloric acid, V	Percentage of nitrosamine recovered on distillation to half volume from 20 per cent. sodium chloride	Percentage of nitrosamine adsorbed by carbon from aqueous solution	Percentage of† nitrosamine eluted from carbon with methanol
N-Nitroso:				
Dimethylamine	−0·94	70	75	75
Methylethylamine	−0·92	70	100	66
Methylpentylamine	−0·83	75	94	28
Diethylamine	−0·88	80	96	94
Dipropylamine	−0·84	85	100	72
Dibutylamine	−0·79	100	100	26
Dipentylamine	−0·78	78	93	0
Dibenzylamine	−0·75	93	94	0
Dicyclohexylamine	−0·86	80	94	16
4-Picolylmethylamine	−0·81	5	100	13
Methyl-2-hydroxyethylamine	−0·93	4	70	70
Diethanolamine	−0·90	0	92	60
Piperidine	−0·79	96	100	84
Pyrrolidine	−0·84	82	95	78
N-Methylaniline	−0·69	96	100	0
Proline	−0·79	0	90	72
1,4-Dinitrosopiperazine	−0·76	0	100	80

* With respect to the mercury pool.

† Based upon the amount of nitrosamine originally treated with carbon.

aliquot subjected to irradiation comprises a peak characteristic of the original before photolysis while polarographically active contaminants not subject to photolysis do not contribute to the resultant wave.

The distillation at atmospheric pressure of an aqueous solution of a simple *N*-nitrosamine to half its original volume leads generally to its concentration in the distillate, particularly if 2 to 3 N sodium hydroxide or 20 per cent. sodium chloride has been added initially. From Table I it is apparent that the introduction of a hydroxyl group into a simple dialkyl-*N*-nitrosamine as in *N*-nitrosomethyl-2-hydroxyethylamine almost completely eliminated the volatility in steam of the former, although the compound remained unchanged in the distillation residue even in the presence of 3 N alkali initially.

The *N*-nitrosamines studied were removed from aqueous 10 p.p.m. solution virtually quantitatively on shaking them for 2 hours with activated carbon (2 g per 100 ml) with the exception of *N*-nitrosodimethylamine and *N*-nitrosomethyl-2-hydroxyethylamine (Table I) for which the efficiency of adsorption was less. The capacity of carbon to adsorb, *e.g.*, *N*-nitrosodiethylamine, persisted at least over the range of pH from that of 0·2 N hydrochloric acid to 0·2 M phosphate buffer at pH 6·0. Adsorption on other reagents such as silica gel and alumina was irregular and rarely complete.

After the sample had been centrifuged and the carbon adsorbent washed with water the elution with boiling methanol (20 ml per gram of dry carbon) proceeded with all *N*-nitrosamines without a phenyl residue except *N*-nitrosodipentylamine, but the proportion recovered varied considerably with the amine moiety of the nitrosamine, as shown in Table I. A second similar desorption with further methanol was sufficient for the quantitative removal from carbon of all *N*-nitrosodipropylamine, but a similar treatment of adsorbed *N*-nitrosodibutylamine raised the proportion adsorbed to only 50 per cent.

In aqueous solution or in methanol with or without water, *N*-nitrosamines are photolysed to yield inorganic nitrite at an alkaline pH. When the conditions of Daiber and Preussmann's method[1] were used in aqueous sodium carbonate, 80 to 100 per cent. of one nitrogen atom involved in the nitrosamino group was liberated within 3 hours. In the presence of methanol, nitrite released was itself subject to photolytic degradation and its release from an aqueous methanolic solution of sodium carbonate increased initially up to an optimum period of irradiation of about 15 minutes, after which the inorganic nitrite detected declined to zero. A maximum release of up to 40 per cent. of one nitrogen atom as nitrite provided confirmation of the results of Daiber and Preussmann[1] for a more restricted range of *N*-nitrosamines.

PROPERTIES OF RELATED COMPOUNDS—

Both of the *N*-nitrosamides studied (Table II) gave initially strong polarographic waves in 0·2 N hydrochloric acid, but that of *N*-nitroso-*N*-methylurea disappeared within a few hours because of its instability. A polarographic response in this electrolyte was common to a range of other compounds representing various nitrogenous types and also to unsaturated aldehydes and ketones, but acetaldehyde gave no response. Unlike the majority of *N*-nitrosamines, most other nitrogenous compounds gave polarographic waves also under alkaline conditions, the sensitivity of response often being greater than at acidic pH and at a more negative potential after the addition of borax to pH 8·4. *N*-Nitrodimethylamine gave a polarographic wave only at alkaline pH. Unsaturated aldehydes and ketones resembled the simpler *N*-nitrosamines in that their polarographic response disappeared on raising the pH in this manner.

None of the compounds tested could be differentiated from *N*-nitrosamines by ultraviolet photolysis in 0·2 N hydrochloric acid, as all were susceptible under the conditions used and all were strongly adsorbed by activated carbon. However, none of the other nitrogenous compounds could be detected polarographically in methanol after attempted desorption from carbon in the refluxing solvent. The unsaturated aldehydes investigated were partially released into this solvent but the unsaturated ketones resisted desorption by methanol completely.

In solution in aqueous sodium carbonate, all nitrogenous compounds tested yielded nitrite on irradiation with ultraviolet light; pentyl nitrite reacted as inorganic nitrite also without photolysis. However, this reaction provided a means of differentiating between *N*-nitrosamines and unsaturated aldehydes and ketones, between which confusion could otherwise arise polarographically.

TABLE II

PROPERTIES OF COMPOUNDS RELATED TO *N*-NITROSAMINES

Substance	Peak potential* (V) in derivative polarography in aqueous: 0·2 N hydrochloric acid	Peak potential* (V) in derivative polarography in aqueous: pH 8·4 buffer	Elution from carbon with methanol	Photolysis in alkali to nitrite
N-Nitrosamides—				
N-Nitroso-*N*-methylurea ..	−0·68	−1·06	Unstable	+
Streptozotocin	−0·40 −0·62	−1·06	−	+
N-Nitramines—				
N-Nitrodimethylamine ..	—	−1·34	+	+
ω-Nitroarginine	−0·54	−1·15	−	+
C-Nitro compounds—				
Nitromethane	−0·82	−1·00	−	+
1-Nitropropane	−0·73	−0·95	−	+
Alkyl nitrite—				
Pentyl nitrite	−0·99	−1·10	−	Reacts as nitrite
Alkyl nitrate—				
Ethyl nitrate	−0·77 −1·02	No wave	+	+
Unsaturated aldehydes—				
Crotonaldehyde	−1·03	No wave	+	−
Cinnamaldehyde	−0·66	−1·12	−	−
Furfuraldehyde	−0·99	−1·45	+	−
Unsaturated ketones—				
α-Ionone	−1·01	No wave	−	−
β-Ionone	−0·86	No wave	−	−

* With respect to the mercury-pool anode.

RECOMMENDED PROCEDURES FOR THE SEPARATION AND DETECTION OF *N*-NITROSAMINES IN BIOLOGICAL MATERIALS—

In the absence of excessive lipid, aqueous homogenates of biological materials should contain both steam-volatile and non steam-volatile *N*-nitrosamines which can be separated from the residual matrix by high speed centrifugation.

Simple volatile *N*-nitrosamines such as *N*-nitrosodimethylamine, -dipentylamine, -dibenzylamine and -piperidine are readily separated by fractional distillation of aqueous slurries containing salt and methanol. For the removal of both volatile and non-volatile

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N-nitrosamines, clarified aqueous homogenates are stirred for 2 hours with granular activated carbon (2 g per 100 ml); the efficiency of the removal of *N*-nitrosamines from solution is impaired by the presence of lipids. Aqueous washing of the separated carbon does not result in appreciable loss of adsorbed nitrosamines but allows the removal of traces of inorganic nitrite possibly remaining from the original material examined; the polarography of *N*-nitrosamines must proceed in an acidic environment, which could promote the formation of artifacts if secondary amines are concurrently extracted.

After two or three washings of the carbon adsorbent with distilled water and decantation of residual liquid, *N*-nitrosamines are desorbed in refluxing methanol for 2 hours. Examination by derivative polarography of the methanolic eluate containing 0·2 N hydrochloric acid reveals waves attributable to *N*-nitrosamines at potentials within the relevant range, the concentration being determined by the response on addition of an authentic *N*-nitrosamine, preferably with a peak potential close to that observed. The disappearance of the wave without the appearance of a further wave at a more negative potential on addition of borax to pH 8·4 provides additional evidence of the presence of an *N*-nitrosamine. Similarly, the wave of an *N*-nitrosamine at acid pH should disappear on irradiation with ultraviolet light for 2 hours.

The determinations of inorganic nitrite on aliquots of methanol eluates containing 0·1 per cent. of sodium carbonate before and after ultraviolet irradiation for 15 minutes will indicate an increase caused by exposure to ultraviolet light if one or more *N*-nitrosamine is present. The reaction is complex and the yield of nitrite is dependent upon the presence of other compounds of biological origin. For the determination of *N*-nitrosamines present, it is therefore necessary to determine the yield of nitrite from an authentic *N*-nitrosamine in the same environment on making incremental additions to further aliquots of the methanol eluates. According to the results presented by Daiber and Preussmann,[1] the yield of nitrite on alkaline irradiation of various *N*-nitrosamines is a linear function of their molar concentrations up to at least 0·2 mM.

Discussion

The problem of the determination of small levels of *N*-nitrosamines in biological sources is virtually unique in that it requires the detection of many such compounds all of potential physiological importance but with different physical properties and with only a nitrosamino group or groupings in common. It will be apparent from the foregoing that the methods of detection advocated are not unequivocal in characterisation and provide an incomplete extraction at best. Thus the results obtained are most likely to represent underestimates of levels at which *N*-nitrosamines are present, although the absolute concentrations in environmental sources are seldom of great concern.

Other nitrogenous compounds simulating *N*-nitrosamines are unlikely to be formed without relatively drastic conditions of reaction and their confusion for *N*-nitrosamines would introduce an error on the side of safety. Some of the *N*-nitrosamines that would be lost when using the separation techniques involving adsorption on carbon, such as *N*-nitrosodiphenylamine, are not considered to be carcinogenic.[2] However, despite its obvious limitations, the method provides a means of analysis of many *N*-nitrosamines which would not be detected by the customary distillation technique, but which are nevertheless of potential physiological significance.

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2339

Survey of Literature Relating to Infant Methemoglobinemia Due to Nitrate-Contaminated Water

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METHEMOGLOBINEMIA is due to the presence of methemoglobin in the blood. It may result from the reaction of any one of several oxidizing agents with the hemoglobin normally present in the blood. Unlike the oxygen in hemoglobin, that in methemoglobin is so firmly bound that the methemoglobin cannot function as an oxygen carrier by alternate oxygenation and deoxygenation. Thus, the result may be anoxemia with serious consequences or even death.

Although methemoglobinemia may result from congenital heart diseases, or from the ingestion, inhalation, or absorption, or the medicinal administration, of any one of several drugs or chemicals, an important cause of cases in infants is the ingestion of water high in nitrate. This review of the literature is concerned with the latter cause.

HISTORICAL

According to Eusterman and Keith,[21] Gamgee[28] demonstrated as early as 1868 that the addition of amyl nitrate to blood could result in the formation of methemoglobin. These same authors noted that Binz and Geringer[4] reported in 1901 that the administration of large doses of nitrates to animals may cause the formation of methemoglobin. Medovy, Guest, and Victor[51] credit Stokvis[68] and Talma[69] with describing cases of methemoglobinemia in 1902.

In reviewing the literature, Comly[13] mentioned that van den Bergh and Gutterink[71] reported in 1906 that nitrites could be formed in the bowels from nitrates, and that the nitrite was more likely to be absorbed if the intestine was damaged. These authors noted a few instances in which a nitrite-producing bacillus was recovered from a stool or sputum of individuals having sulfhemoglobinemia. However, it remained for Zobell[81] to demonstrate *in vitro* that many organisms commonly found in the gastrointestinal tract of man are capable of reducing nitrates to nitrites.

In 1928 Barker and O'Hare[3] described a case of cyanosis in a patient who had been treated with ammonium nitrate. The following year, Eusterman and Keith[21] reported two cases of methemoglobinemia which occurred among a large number of adults administered ammonium nitrate for diuretic purposes. However, after two dogs, to which they administered intravenous injections of the drug, failed to show symptoms of methemoglobinemia, they concluded these cases were due to abnormal metabolic processes in the two patients. In 1933, Roe[60] reported the death of a month-old infant who had been treated with bismuth subnitrate. He was the first to associate nitrates with serious or even fatal cases of infant methemoglobinemia. One year later

Chen, Rose, and Clowes [12] demonstrated that methemoglobinemia could be produced by intravenous injections of sodium nitrate.

Thus, the way was paved for Comly,[13] who in reporting on two cases of infant methemoglobinemia in 1945 concluded that methemoglobinemia may occur in an infant following ingestion of water high in nitrates, especially if the infant is suffering from gastrointestinal disturbances. Maxcy,[47] writing in 1950, noted that subsequent observations have established the validity of Comly's hypothesis.

MEDICAL ASPECTS

Cause—Although methemoglobinemia in infants may be caused by congenital heart diseases,[13, 20, 21] by absorption of dyes used for laundry marking,[31, 61, 78] and by drugs administered in medical treatment,[60, 76] many of the cases reported since 1945, when Comly [13] published his conclusions that infant methemoglobinemia may be due to ingestion of high nitrate water, have been diagnosed as having been due to nitrates in the water used in the feeding formula.[8, 11, 23, 24, 48, 50, 59, 66, 84, 85] Donahue [18] alone has expressed the belief that infant methemoglobinemia may result from nitrates ingested in the mother's or cow's milk. However, no positive test for nitrates could be demonstrated in the milk from two cows fed for a period of 10 days on water containing 114 p.p.m. nitrate nitrogen.[63]

Susceptibility—Comly's hypothesis on infant methemoglobinemia induced by ingestion of high nitrate water caused considerable speculation concerning the effect of age and other factors on susceptibility. This led to a series of experiments conducted by Cornblath and Hartmann.[15] They fed several infants on a formula containing an artificial well water of high nitrate-nitrogen concentration, but failed to produce apparent methemoglobinemia. Further investigation revealed that nitrate-reducing organisms, which they cultured from saliva and gastric juices of infant cases, were active only at a pH level above 4.9. On checking the gastric juices of infants, who previously had developed appreciable levels of methemoglobin, they found no free acid and pH values above 4. Based on this evidence Cornblath and Hartmann concluded that the lack of acidity of the gastric juices of a newly born infant may permit nitrate-reducing organisms to grow in the upper gastrointestinal tract and, thus, to reduce nitrates to nitrites before the former can be completely absorbed. In an older infant the acidity of the gastric juices inhibits the growth of the nitrate-reducing bacteria.

The work of Marriott, Hartmann, and Senn,[45] who investigated the acidity and bacterial flora of 200 infants suffering from diarrhea, supports this hypothesis. The pH of the stomach contents of well infants varied from 2.0 to 5.0, and averaged 3.7; that for infants suffering from bacillary dysentery ranged from 2.0 to 5.0, and averaged 3.0; but that for infants with nonspecified diarrhea averaged 5.6, and varied from 4.6 to 6.5.

Robertson and Riddell [59] considered the total amount of hemoglobin present in the blood and the energy of the enzyme system which operates to reduce methemoglobin to hemoglobin as important. Several observers [13, 61, 71] have suggested that the nitrite formed in the upper gastrointestinal tract is more readily absorbed into the blood system when the mucosa is damaged.

In an analysis of 94 cases, Bosch, *et al.*,[8] noted that the period of exposure to a feeding formula containing high nitrate water varied from 1 to 30 or more days.

Apparently, some of the factors which determine whether or not an infant may have nitrate-induced methemoglobinemia are: (a) nitrate-nitrogen content and

amount of water ingested daily; (b) duration of exposure to a feeding formula containing high nitrate water; (c) presence and activity of nitrate-reducing bacteria in the upper gastrointestinal tract—which is affected by the pH of the gastric juices; and (d) the condition of the mucosa.

Physiological Effects—The characteristic symptoms ordinarily associated with infant methemoglobinemia are the greyish- or brownish-blue coloration, which usually is first noticeable around the lips, spreads to the fingers and toes and over the face, and may eventually cover the entire body.[8] Other symptoms which may be noticed are drowsiness and an increased rate of respiration.[48] Although not always present, methemoglobinemia is commonly accompanied by diarrhea or other gastrointestinal disturbances.[10]

The cyanosis—the greyish- or brownish-blue coloration—is attributed to the anoxemia resulting from the decrease in the oxygen-transporting capacity of the blood.[26] The nitrites, absorbed by the blood, react with the hemoglobin to form methemoglobin. Conant[14] notes that this is an oxidation reaction in which the ferrous iron in hemoglobin is oxidized to ferric iron in methemoglobin. In the latter state the oxygen is so firmly bound that methemoglobin cannot function in the manner of hemoglobin in the transfer of oxygen. Ordinarily this reaction is reversible and occurs without permanent injury to the red blood cells.

According to Robertson and Riddell,[59] methemoglobinemia is usually first noticeable in infants when 10 per cent of the hemoglobin has been transformed to methemoglobin, and severe cases may result when it increases to 20 per cent. However, recovery has occurred in cases where the methemoglobin level has been observed to be as high as 57 per cent.[64]

Diagnosis—Diagnosis of cases of infant methemoglobinemia as being due to the ingestion of nitrate-contaminated water may be either presumptive or absolute. According to Bosch, *et al.*,[8] a presumptive diagnosis is justified when the physical findings are typical and either the freshly removed blood has the chocolate brown color associated with methemoglobin, or spontaneous disappearance of the cyanotic condition occurs within 48 hours after changing the water used in the feeding formula, provided an analysis of the water originally used in the feeding formula shows it to have a nitrate-nitrogen content in excess of 10 or 20 p.p.m.

An absolute diagnosis requires the demonstration of appreciable amounts of methemoglobin in the blood by either a spectroscopic examination or the photoelectric colorimetric method of Evelyn and Malloy,[22] together with analytical results showing at least 10 or 20 p.p.m. of nitrate nitrogen in the water used in the feeding formula. Finch[26] cautions that the blood should be examined within 1 or 2 hours after withdrawal since on standing methemoglobin may be spontaneously reduced to hemoglobin.

Treatment—In general, treatment of infants having nitrate water-induced methemoglobin has consisted of the discontinuance of the use of nitrate-contaminated water and, in the more severe cases, the intravenous injection or oral administration of methylene blue. Oxygen therapy, also the administration of such drugs as ascorbic acid, thiamine, and niacin, has been suggested, but for one reason or another none of these treatments has found general use.

In cases where a water of permissible nitrate content could not be obtained, Comly[13] recommended feeding whole milk, preferably acidified and boiled. Stafford[66] suggested that the new-born infant be retained in a hospital until the safety of the water to be used in the feeding formula has been determined.

Various observers[29, 51, 66] have reported the recovery of infant methemo-

globinemia cases following the change from use of nitrate-contaminated water to one relatively free from nitrates. In several instances, an infant has again become cyanotic when returned to his original feeding formula containing high nitrate water.[13, 61, 66] Bosch, *et al.*,[8] implied that spontaneous recovery should occur in 48 hours after discontinuance of use of a feeding formula containing high nitrate water, while Halpern and Dubost[33] stated that such recovery will occur in 4 days.

In 1939, prior to Comly's hypothesis of high nitrate water-induced methemoglobinemia, Wendel[79] reported on the effectiveness of methylene blue in treating sodium-nitrite-induced methemoglobinemia in dogs. He found that an intravenous injection of 0.1 to 0.2 ml. of a 1 per cent solution (1 to 2 mg.) of methylene blue per kilogram of body weight resulted in a rapid decrease in the methemoglobin level. In studying the molecular ratio of the hemoglobin produced per unit of methylene blue added, he could not explain the reaction on the basis of the reducing action of methylene blue. Thus, he concluded that it was a catalyst which accelerated the rate with which the enzyme systems of the red cells normally reduced methemoglobin to hemoglobin.

Warburg, *et al.*,[74] according to Stafford,[66] found that an overdose of methylene blue may produce methemoglobinemia. However, Finch[26] does not consider this important, since dosages up to 10 mg. per kilogram of body weight do not produce significant amounts of methemoglobin. It is at least significant that intravenous injections of 1 to 2 mg. per kilogram of body weight have resulted in the disappearance of cyanotic conditions in numerous cases in a matter of 30 minutes or less.[11, 13, 23, 24]

In his studies, Wendel[79] also noted that oral administration was effective. Stafford[66] stated the dosage used in oral administration should be 10 to 20 mg. of methylene blue per kilogram of body weight. This is about 10 times the amount used in intravenous injections. He reported favorable results following treatment by oral administration.

Prevention—In general, the prevention of infant methemoglobinemia due to nitrate-contaminated water is largely one of education. If the infant's parents and the doctor in the case are informed of the problem, steps are usually taken to prevent nitrate-induced methemoglobinemia.

Water for use in preparing the infant's formula should contain not more than 10 or possibly 20 p.p.m. nitrate nitrogen.[13, 47] In areas where there is the least question concerning the nitrate content of the water to be used, a sample of that water should be taken and analyzed. If it is high in nitrates, the infant should be retained at the hospital,[66] the feeding should be whole milk without addition of any water,[13] or water having a safe nitrate concentration should be obtained and used in preparing the formula.

An intensive program has resulted in a marked decrease in the reported cases. For example, in Minnesota the reported cases were 55 in 1947, 52 in 1948, but only 12 for the first 6 months of 1949.[8]

In some cases it may be possible to secure a low nitrate water by constructing new wells, properly located and protected from surface contamination. However, this problem appears to require further investigation.

Finally, reporting of methemoglobinemia in infants should be required. Information on the extent of the cases and the regions in which they occur would prove helpful in its prevention.

MORBIDITY AND MORTALITY

Metzler[52] noted that methemoglobinemia is not one of the reportable diseases; and that the cases voluntarily reported are a poor indication of the true incidence. The most complete informa-

tion on the morbidity and mortality among infants due to methemoglobinemia resulting from ingestion of nitrate-contaminated water is the summary of reported cases and deaths included in the report of the Committee on Water Supply, Engineering Section, American Public Health Association.[89] Table 1 reproduces in part these data. It is based on a questionnaire sent to the 48 states and Alaska and Hawaii, to which all but one state replied. Special emphasis was placed on restricting the data to those cases definitely associated with nitrate-contaminated water. Apparently, the reported cases cover a period from 1945, or even earlier, to the dates of the replies to the questionnaires.

A total of over 278 cases and of 39 deaths are noted. Geographically the states reporting cases center around Iowa, where the first case associated with nitrate-contaminated water was recognized. Except for the north central group, only 4 states—California, Georgia, New York, and Virginia—reported cases. Infant methemoglobinemia due to high nitrate waters has also been reported for Canada[50, 59] and Belgium.[24]

Probably the most intensive study of nitrate-induced infant methemoglobinemia is that for Minnesota, which was reported by Bosch, *et al.*[8] It is interesting to note that familiarity with the problem by both physicians and parents apparently has led to a marked decrease in the reported cases.

WATER SUPPLY ASPECTS

Permissible Nitrate-Nitrogen Concentration—The permissible nitrate-nitrogen concentration in water which may cause infant methemoglobinemia when used in a feeding formula is dependent on the individual's susceptibility, the increase in nitrate-nitrogen concentration due to boiling the water, the quantity of boiled water consumed per day per unit weight of the infant, the duration of exposure to the high nitrate water, and possibly other factors.[8, 15, 47]

Comly[13] considered it inadvisable to use well water containing more than 10 or 20 p.p.m. nitrate nitrogen in pre-

TABLE 1

Reported Cases of Nitrate Water-Induced Infant Methemoglobinemia Classified According to Nitrate-Nitrogen Concentration of Water Used in Feeding Formula

State	Methemoglobinemia Reported Cases	Reported Deaths	Number of Cases Associated with Indicated Ranges of NO_3-N Conc. (P.P.M.) 0–10	11–20	21–30	31–50	51–100	100+	No. of Cases for Which Data Are Available	Supplementary Reference
California	1	0	0	0	0	0	1	0	1	
Georgia	6	3	—	—	—	—	—	—	0	Frith[27]
Illinois	75	6	0	1	2	2	12	11	28	
Indiana	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	Metzler[33]
Michigan	7	0	0	0	0	0	0	7	7	
Minnesota	139	14	0	2		25	53	49	129	Bosch et al.,[8]
Missouri	2	0	0	0	0	0	0	2	2	
Nebraska	22	1	0	1	0	4	9	8	22	
New York	2	0	0	0	0	0	1	0	1	
North Dakota	9	1	0	1	1	0	0	6	8	
Ohio	0	0	0	0	0	0	0	0	0	
Oklahoma	0	0	0	0	0	0	0	0	0	
South Dakota	Several	0	—	—	—	—	—	—	—	
Texas	0	0	0	0	0	0	0	0	0	
Virginia	1	0	0	0	0	0	1	0	1	
	278+	39	0	5		36	81	92	214	
Per cent of Total			0.0	2.3		16.8	37.8	43.1	100.0	

Principal Source: Progress Report of Committee on Water Supply, A.P.H.A., Water Supply: Nitrate in Potable Waters and Methemoglobinemia. *A.P.H.A. Yearbook* 40, 5:110 (May), 1949–1950.

TABLE 2

High Nitrate Waters, Their Geographical Distribution and Total Reported Cases of Infant Methemoglobinemia

State	Total No. Methemo. Cases Reported[1]	Year	Type of Supply	No. of Samples Examined	Per cent of Samples Examined within Indicated NO_3–N (P.P.M.) Range 0–10	11–20	21–50	50+	Reference
California	1	—	—	—	—	—	—	—	
Georgia	6	—	—	—	—	—	—	—	
Illinois	75	1946		7,000	80±	←	20±	→	(76)
Indiana	1	—	—	146	←	98.7 →	← 1.3	→	(52)
Iowa	69	1939	Dug Wells	454	66.5	10.6	14.3	8.6	(38)
			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±	→	(7)
			> 100′ deep	370	←	96.5 →	← 3.5	→	
Kansas	13	—	Rural Wells	—	67±	← 33±		→	(52)
Massachusetts	0	1948	Private	—	—	← 47 samples		→	(89)
Michigan	7	—	—	2,847	93.0	←	7.0	→	(48)
Minnesota	139	1947–9	Municipal	514	96.9	←	3.1	→	(8)
				2,912	84.9	←	15.1	→	
Missouri	2	—	—	—	—	—	—	—	
Nebraska	22	1948–9	Municipal	416	95.0	←	5.0	→	(83)
			Non-municipal	2,687	79.8	←	20.2	→	
			School	258	95.0	←	5.0	→	
			Farm	275	78.0	←	22.0	→	
New York	2	1943	—	2,864	96.1	3.3	0.6	0.0	(89)
		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	
North Dakota	9	—	—	509	84.9	←	15.1	→	(1)
		—	—	287	84.0	←	16.0	→	(72)
Ohio	0		Rural Wells	10,500	90.5	5.0	3.5	1.0	(75)
			State Park Wells	400	100.0	0.0	0.0	0.0	
Oklahoma	0	—	—	415	Av. NO_3–N Conc. = 28 p.p.m.				(89)
South Dakota	Several	—	—	—	—	—	—	—	
Texas	0	—	Rural Wells				Numerous		(89)
Virginia	1	—	Rural Wells	—	—	—	—	—	

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

paring an infant's feeding formula. Data presented in Table 2 indicate that no cases were reported which were associated with water containing 10 p.p.m. nitrate nitrogen or less, and the nitrate-nitrogen content was less than 20 p.p.m. for only 2.3 per cent of the cases for which data were available. It should be noted, however, that in many of these cases clinical data were insufficient for definite diagnosis, and samples of water for the analysis were sometimes collected several months following the occurrence of the case.

Although 10 p.p.m. nitrate nitrogen has been suggested as the permissible level,[13, 47] the A.P.H.A. Committee[89] points out that most of the cases studied were associated with nitrate-nitrogen concentration in excess of 40 p.p.m. and comments that it is "impossible at this time to select any precise concentration of nitrates in potable waters fed infants which definitely will distinguish between waters which are safe or unsafe. . . ."

Geographical Distribution—The geographical distribution of high nitrate waters is widespread. Twenty of 37 states, which replied to this item on the A.P.H.A. Committee's questionnaire, reported wells yielding water containing in excess of 50 p.p.m. nitrate nitrogen.[89] Results of fairly extensive surveys, indicating the occurrence and concentrations of high nitrate waters, are available in the literature for Illinois,[76] Iowa,[38] Michigan,[48] Minnesota,[8] Nebraska,[83] New York,[89] and Ohio.[75] The available data are summarized in Table 2.

Although the information presented in the literature is insufficient to permit a sound analysis of the problem, it does

indicate that waters of high nitrate-nitrogen concentration occur in a sufficient number of instances to anticipate cases of nitrate water-induced methemoglobinemia in Illinois, Iowa, Michigan, Minnesota, Nebraska, Ohio, Oklahoma, and possibly New York and Texas. The absence of any reported cases of infant methemoglobinemia in Ohio, Oklahoma, and possibly Texas, does not appear to be due to the lack of occurrence of high nitrate waters.

Types of Supply—High nitrate waters have been found most frequently in private wells serving rural homes. However, water containing in excess of 10 p.p.m. nitrate nitrogen has been reported for wells supplying municipal water works in Illinois,[49, 76] Kansas,[52] Minnesota,[8] Nebraska,[83] and New York.[89] Available evidence indicates that surface supplies do not offer a problem except in very unusual cases.[49, 75, 89]

Construction of Wells—Most cases of infant methemoglobinemia have been associated with water from dug wells. Bosch, *et al.*,[8] noted that in 125 of 129 cases investigated the water came from dug wells. Data reported for Iowa[38] show nitrate-nitrogen concentrations over 10 p.p.m. in water samples from 39.7 per cent of dug wells, 21.5 per cent of the bored wells, and only 4.5 per cent of the drilled wells.

The location and physical features, also the nature of the water-bearing strata, may be important. Nearby sources of pollution were believed to be significant by 12 of 22 states reporting on this item in the A.P.H.A. Committee's questionnaire.[89] Bosch, *et al.*,[8] noted that 83 of 129 wells involved in cases of methemoglobinemia were within less than 50 feet from a barnyard, pigpen, privy, cesspool or other source of animal or human contamination. It may be significant to note the marked difference, shown in Table 2, in the percentage of high nitrate waters from school and farm wells in Nebraska, also that between state park wells and rural wells in Ohio.

In many cases farm wells, and particularly dug wells, are inadequately protected against contamination by surface water or water percolating but a short distance through the soil. Thus, as might be expected, several states have reported a high percentage of the high nitrate waters to be positive for coliform organisms.[38, 50, 75, 76] However, there appears to be no relationship between nitrate concentration and bacterial numbers.

Sources of Nitrate Contamination—According to Sarles, *et al.*,[63] the principal sources of nitrogenous matter in the soil are the decomposition products of plants, animals and microörganisms; the liquid and solid wastes of animal metabolism; and fertilizers added to enrich the soil. The possibility of geological formations containing appreciable amounts of nitrates must also be considered.

It may be significant that 12 of 22 states replying to this item on the A.P.H.A. Committee's questionnaire expressed the opinion that nearby sources —barnyards, pigpens, privies, cesspools, etc.—were important in causing high nitrate waters.[89] Such a belief is substantiated by the variation in nitrate-nitrogen content of waters from wells, such as noted by Borts,[7] Maxcy,[47] and in the New York studies.[89] However, Metzler[52] observed only seasonal change in the nitrate content of 4 municipal wells, and Bosch, *et al.*,[8] noted no appreciable variation in the nitrate content of samples taken bi-weekly from each of 5 wells, or in samples from 2 wells taken after more than an hour of continuous pumping. Two wells, a 30-foot dug well and a 71-foot drilled well, were reconstructed in an attempt to exclude nitrate contamination, but no significant reductions in the nitrate content of their waters were noted.

The A.P.H.A. Committee's report[89] notes that high concentration of nitrates

in waters in rural wells, which usually were poorly constructed, shallow, dug wells, did not seem to be associated with nearby sources of pollution. Weart[76] notes that the upper 40 inches of soil may contain 16,000 lbs. of organic nitrogen per acre. Sarles, Frazier, and McCarter[63] discuss the decomposition of nitrogenous matter. Proteins and nitrogenous matter are first hydrolyzed into amino acids or other water-soluble products consumed by bacteria which give off ammonia. Nitrification may occur through the action of *Nitrosomonas* or *Nitrosococcus* bacteria, which in the presence of air oxidize ammonia to nitrous acid; and by *Nitrobacter*, which oxidizes the nitrous acid to nitric acid.

Since bacteria are essential in the production of nitrates from organic nitrogen, factors influencing their activity affect the nitrate content of the soil. Sarles, *et al.*,[63] note that nitrification takes place only when the soil contains buffering substances which neutralize the nitric acid and maintain a pH around 6.5 to 8.0; when aerobic conditions, such as result from plowing, cultivating, etc., are maintained; when soil contains only about 50 per cent of its water-holding capacity; when ammonia salts and other nutrient elements are present in the soil; and at temperatures above 50° and below 100° F.

ANALYTICAL PROCEDURES

There are several procedures by which the nitrate content of water may be determined. Most laboratories use a screening test to differentiate between low and high nitrate concentrations, and then determine the nitrate nitrogen in high nitrate waters by a more accurate procedure.

One nitrate screening test is that developed by Riehl,[75] which he adopted from "Qualitative Analysis by Spot Tests" by Fritz Feigl. Another screening procedure is that developed by Bray[9] and used by Illinois. Both of these procedures are spot plate tests which may be used in the field to prevent overburdening the laboratory with large numbers of samples of low-nitrate waters.

Metzler[52] reports that the brucine procedure is used in Kansas. Local health departments have been equipped with field test kits with which on-the-spot tests are made to determine whether the nitrate-nitrogen concentration is within the 10 p.p.m. limit usually considered the maximum permissible level.

More precise methods for use in the laboratory are the phenol-disulfonic acid and reduction methods as given in *Standard Methods*.[88] Still another method which can be used is the brucine method.[36, 55, 80] For precise work it would seem desirable to use a photoelectric colorimeter for comparing the color developed in samples with that of standard concentrations.

FURTHER INVESTIGATIONS NEEDED

Maxcy,[47] in a report adopted by the Committee on Sanitary Engineering and Environment, Division of Medical Sciences, National Research Council, notes that there is urgent need for further study before comprehensive measures of prevention can be advocated. Such a study should consist of observations in various parts of the United States by the same unit.

Some of the factors which should be investigated are the permissible level of nitrates in waters fed to infants; the factors which predispose some infants to methemoglobinemia, whereas other infants consuming equally high nitrate waters are unaffected; the health of infants routinely consuming water containing high nitrate in regions where methemoglobinemia has not been reported; the fluctuations, if any, in the nitrate content of waters from selected wells, including factors which influence such variations.[47, 89]

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(Numbers refer to numbered references in main reference list)

HISTORICAL

3, 4, 12, 13, 21, 28, 41, 47, 51, 60, 68, 69, 71, 81.

MEDICAL

Cause

8, 11, 13, 18, 20, 23, 24, 31, 48, 50, 53, 58, 59, 60, 61, 66, 73, 78, 84, 85.

Susceptibility

7, 8, 13, 15, 45, 46, 56, 61.

Diagnosis

8, 21, 26

Treatment

5, 8, 11, 13, 23, 24, 25, 26, 33, 34, 35, 37, 41, 51, 54, 61, 66, 74.

Prevention

8, 13, 47, 52, 66.

MORBIDITY AND MORTALITY
8, 27, 47, 52, 83, 89.

WATER SUPPLY ASPECTS
Critical Nitrate Nitrogen Level
13, 15, 47.
Geographical Distribution
1, 8, 17, 30, 52, 83, 89.
Types of Supplies
8, 47, 75, 83, 89.
Well Construction
7, 8, 47, 52, 75, 76, 89.
Sources of Nitrates
7, 8, 44, 52, 63, 75, 76.

ANALYTICAL PROCEDURES
9, 39, 40, 57, 65, 75, 88.

FURTHER INVESTIGATIONS NEEDED
47, 89.

International VD Union Meets

The 28th General Assembly of the International Union against the Venereal Diseases was held in Paris, May 21–25, 1951. It commemorated the 50th anniversary of the founding of the French Society for Moral and Sanitary Prophylaxis by Alfred Fournier. Dr. E. H. Hermans, Netherlands, president of the Union, presided, and Dr. Andre Cavaillon, secretary-general of the Union and the French Society acted as hosts. The Assembly was attended by 52 delegates from 28 countries, as well as by observers from the World Health Organization, the League of Red Cross Societies, the International Abolitionist Federation, the International Alliance of Women, and others.

Much of the Assembly's program was devoted to the discussion of sex education. Among the numerous papers presented was a report by the American Social Hygiene Association on "Progress and Trends in Sex Education in the United States." The agenda also included a joint session of Assembly delegates, doctors, educators, psychologists, and social workers on this subject.

Resolutions unanimously adopted by the Assembly propose the establishment of a Union Liasion Office in the future, possibly in Geneva; and the preparation of a new statement of Union objectives, with program and budget. These resolutions enable the Union to meet today's challenges better equipped to fulfill its responsibilities as the international voluntary agency in its field.

The United States delegation, chaired by Bailey B. Burritt of the American Social Hygiene Association's Executive Committee included: Mrs. Burritt, Dr. Robert H. Bishop, Jr. (A.S.H.A. Executive Committee and Board of Directors) and Mrs. Bishop, Dr. William A. Brumfield, Jr. (deputy health commissioner of New York State and chairman of A.S.H.A. Committee on International Activities), Lt. Col. Louis Kossuth (attached to General Eisenhower's staff in Paris), Irving J. Fasteau (Permanent Attache for Social Affairs, American Embassy, Paris), and Dr. Robert D. Wright (professor of social and environmental medicine, University of Virginia).

Biochemical Journal Vol 81 (1961). 2341

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Metabolism of Nitrate by Cattle

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Nitrate toxicity has been observed frequently in ruminants fed on a variety of plant materials high in nitrate (Bradley, Beath & Eppson, 1939; [illegible]rsby & Farwell, 1950; Newson, Stout, Thorp, Barber & Groth, 1937; Savage, 1949; Whitehead, [illegible] & Moxon, 1948; Whitehead & Moxon, 1952). Davidson, Doughty & Bolton (1941) described the toxicity symptoms in nitrate poisoning in detail. Bradley *et al.* (1939) indicated that the symptoms of nitrate toxicity are associated with the high levels of methaemoglobin and hence the low oxygen-carrying capacity of the blood of affected animals. Lewis (1951) demonstrated that micro-organisms in the rumen of sheep reduce nitrate to ammonium via with nitrite as an intermediate; enough nitrite was transferred to the blood to convert a considerable fraction of the haemoglobin into methaemoglobin. Sund, Wright & Simon (1957) reported that a non-contagious abortion observed among cattle pastured on marsh land in central Wisconsin apparently was a manifestation of nitrate toxicity.

The work presented here was designed to study the time-course of nitrate reduction in the rumen, the changes induced in the blood by metabolites of nitrate, and the disposition of [^{15}N]nitrate by the animal.

MATERIALS AND METHODS

The experimental animals were 700–800 lb. Hereford cows with permanent rumen fistulas which were fitted with [illegible]. A known amount of KNO_3 in 500 ml. of water was added through the rumen fistula each morning before the cow was offered hay, concentrates and water *ad lib.* The conversion of haemoglobin into methaemoglobin in the blood was determined daily about 3 hr. after the administration of KNO_3. In the experiments with ^{15}N, a solution of $K^{15}NO_3$ was added to the rumen through the fistula in a cow which showed about 15% methaemoglobin in its daily samples of blood. Throughout the day that $K^{15}NO_3$ was added, periodic samples were taken of rumen liquor, blood and urine. Blood samples were removed from the jugular vein. Analysis for methaemoglobin was performed immediately on freshly drawn blood, and the remaining blood was treated with sodium oxalate to prevent clotting. Urine samples obtained with a catheter were preserved with toluene in a refrigerator until analysed. The rumen liquor withdrawn through the fistula was strained through 2 layers of cheese cloth; it then was clarified by the method of Lewis (1951), and the clear supernatant solution was used for analysis.

Analyses for nitrate, nitrite, urea and ammonia in the rumen liquor, blood and urine were made by the methods described in the A.O.A.C. handbook (Lepper, 1945). ^{15}N was determined with a Consolidated–Nier isotope ratio mass spectrometer. Methaemoglobin and total haemoglobin were determined by the method of Evelyn & Malloy (1938). The blood sample treated with oxalate was centrifuged, and the cells were washed twice by centrifuging from 0·85% NaCl and then were refrigerated until used. Samples (10 ml.) of the supernatant fluid were stirred while to each were added 3 ml. of 0·5M-$Ba(OH)_2$ and 3 ml. of 0·5M-$ZnSO_4$ to precipitate the proteins. The precipitate was removed by centrifuging, and the clear supernatant fluid was used for analysis of ammonium, nitrite and nitrate ions.

Haemin and globin were separated and purified essentially as described by London, Morell & Kassenaar (1958).

The washed red cells were lysed in cold deionized water, and the lysed suspension was filtered with stirring into 12 vol. of chilled acetone containing 1·2 ml. of 12 N-HCl/100 ml. The precipitated globin was filtered and then washed with chilled acetone. The washings were added to the filtrate carrying the haemin in solution. The globin was purified by dissolving the crude precipitate in water, adding 14% trichloroacetic acid to complete precipitation, and washing the new precipitate twice with 7% trichloroacetic acid and once with distilled water. The globin was dissolved in N-NaOH, and the precipitation and washings were repeated. The precipitate finally was washed once with a mixture of equal volumes of acetone and ether, twice with acetone and once with ethanol before drying. The dried white powder was hygroscopic.

The haemin was recovered from the acetone solution by addition of one-third volume of deionized water followed by vacuum evaporation, filtration and washing with two portions of cold water. It was purified further by dissolving it in acetone and repeating the precipitation and washings before drying it over $CaCl_2$ in a vacuum.

RESULTS

Metabolism of nitrate added directly to the rumen

The concentrations of ammonium, nitrite and nitrate ions in the rumen liquor were followed periodically in a cow given 70 g. of KNO_3 through a rumen fistula. The nitrate concentration decreased rapidly whereas ammonium and nitrite increased (Fig. 1). Ammonium and nitrite ions were at their maximum concentrations about 3 hr. after nitrate was administered.

Although rather high concentrations of nitrite were produced in the rumen, very little methaemoglobin was detected in the blood, and appreciable concentrations appeared only after several days of treatment (Table 1). After 16 days, the daily dose of KNO_3 was increased to 100 g. As indicated in Table 1, the percentage conversion into methaemoglobin approached 15% after a few days on 100 g. of KNO_3 a day. In later experiments, the animals were 'conditioned' by giving them 100 g. of KNO_3 daily until their blood carried about 15% methaemoglobin 3 hr. after treatment.

Experiments were performed with $K^{15}NO_3$, so that the nitrate nitrogen and products derived from it could be traced. A solution of 120 g. of $K^{15}NO_3$, 1·7 atom % ^{15}N excess, was introduced into the rumen through the fistula of a 'conditioned' cow in the morning before feeding. Analyses from the first experiment are recorded in Figs. 2 and 3. Fig. 2 indicates the accumulation of methaemoglobin in the blood of the treated cow and the [illegible] concentrations of the nitrate plus nitrite fractions and the ammonia fraction in the rumen liquor. A peak value of 16% for blood methaemoglobin was recorded 3 hr. after labelled nitrate was supplied; this peak appeared about an hour after the peak concentration of ^{15}N in the ammonium

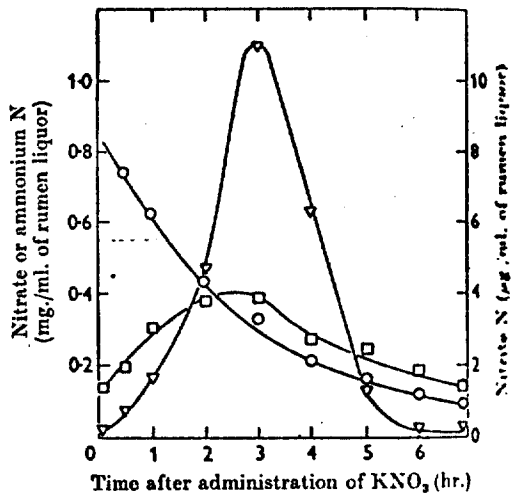

Fig. 1. Reduction of nitrates in the rumen of a cow receiving 70 g. of KNO_3 via rumen fistula. ○, Nitrate nitrogen; ▽, nitrite nitrogen; □, ammonium nitrogen.

Table 1. *Induction of methaemoglobin formation in the blood of cattle by repeated dosing with potassium nitrate*

Days of treatment*	Time after dosing (hr.)	Percentage conversion into methaemoglobin	Days of treatment†	Time after dosing (hr.)	Percentage conversion into methaemoglobin
0	0	0·26	16	6	0·62
1	0	0·25	19	6	2·44
1	4	0·20	20	4	11·00
3	4	1·20	20	6	1·70
4	6	0·00	23	3·5	10·50
5	2	0·00	23	5	7·80
5	4	1·20	23	6	6·40
5	6	0·30	25	3	14·30
9	4	4·65	27	3	14·00
12	5	1·87	28	4	14·20
12	6	1·68	—	—	—

* Animal received 70 g. of KNO_3 daily.
† Animal was shifted to 100 g. of KNO_3 on day 16 and received this amount daily thereafter.

...of the rumen was reached. The decrease in ^{15}N concentration in the nitrate plus nitrite fraction followed a more precipitous curve than that observed earlier for the disappearance of unlabelled nitrate from the rumen (Fig. 1).

Fig. 3 indicates the appearance of ^{15}N in the fractions recovered from urine; the curve for methaemoglobin formation in the blood is included for reference. The total nitrogen carried a higher concentration of ^{15}N and reached a peak concentration of ^{15}N sooner than urea or ammonium nitrogen. Urea and ammonium ions reached their highest concentrations of ^{15}N at almost the same time; the urea had the higher concentration of ^{15}N.

The blood samples in the first experiment were too small to provide sufficient nitrogen for proper mass analysis of fractions; the protein nitrogen diluted the ^{15}N excessively in the total blood nitrogen. Adequate samples of blood (125 ml.) for mass analysis on fractions were taken in the second experiment. Fig. 4 shows the ^{15}N concentrations in various nitrogenous fractions from the blood of a cow that received $K^{15}NO_3$ in Expt. 2. ^{15}N accumulated quickly in the nitrate plus nitrite fraction of the blood and remained high during the 8 hr. period of sampling (note that these values in Fig. 4 are divided by 10). The methaemoglobin level approached a maximum of 22 % at 3 hr. The highest concentration of ^{15}N in the ammonium ions in the blood also was observed near 3 hr., when its atom % ^{15}N excess was about a twelfth of that in the nitrate plus nitrite fraction. The ^{15}N in haemin reached a peak concentration more rapidly than did the ^{15}N of the ammonium ion. The globin fraction showed no detectable increase in ^{15}N above its normal abundance, although the haemin

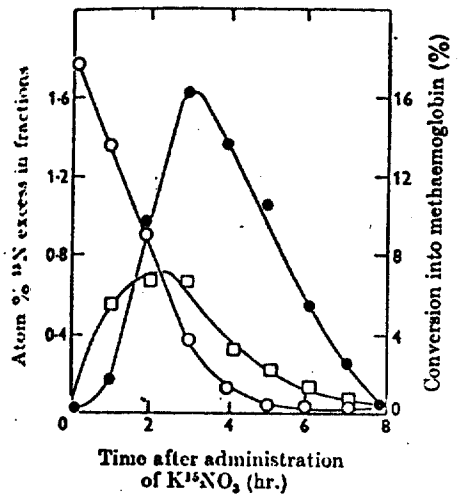

Fig. 2. Reduction of nitrate in a cow's rumen and formation of methaemoglobin in the blood. 120 g. of $K^{15}NO_3$, 1·7 atom % ^{15}N excess, was added to the rumen. Expt. 1: ○, nitrate nitrogen plus nitrite nitrogen; □, ammonium nitrogen; ●, percentage conversion into methaemoglobin.

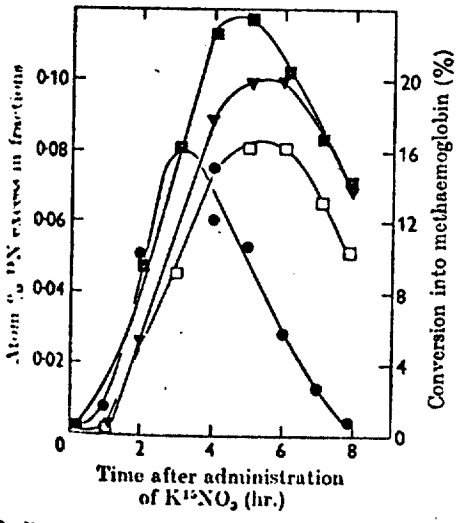

Fig. 3. Formation of methaemoglobin in the blood and accumulation of ^{15}N in the nitrogenous compounds of the urine of a cow receiving 120 g. of $K^{15}NO_3$, 1·7 atom % ^{15}N excess, through a rumen fistula. Expt. 1: □, ammonium nitrogen; ▼, urea nitrogen; ■, total nitrogen; ●, percentage conversion into methaemoglobin.

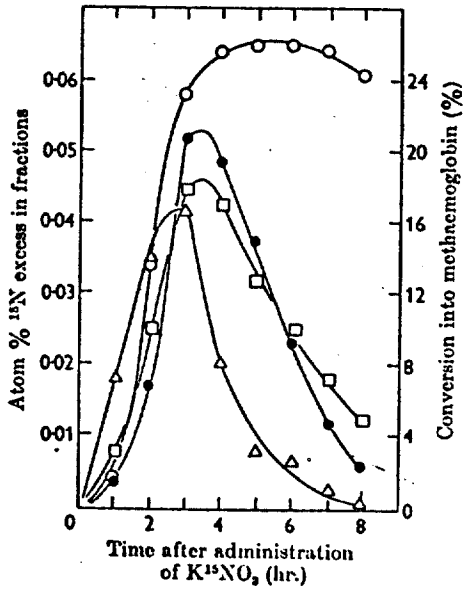

Fig. 4. Formation of methaemoglobin in the blood, and accumulation of ^{15}N in the nitrogenous fractions of the blood of a cow receiving 120 g. of $K^{15}NO_3$, 1·7 atom % ^{15}N excess, through a rumen fistula. Expt. 2: ○, atom % N^{15} excess × 10^{-1} of nitrate plus nitrite nitrogen in plasma; □, ammonium nitrogen in plasma; △, haemin; ●, percentage conversion into methaemoglobin.

retained its label despite the vigorous treatment involved in its separation and purification.

The results of urine analysis are presented in Fig. 5. Again the ^{15}N in the total nitrogen (which includes nitrate and nitrite) rose more rapidly and to a higher level than in urea or ammonium nitrogen. The concentrations of ^{15}N in the total, urea and ammonium ion fractions were highest near 4 hr. and declined to only about half their peak concentrations by 8 hr. The maximum conversion of haemoglobin into methaemoglobin in the blood occurred shortly before the maximum concentration of ^{15}N was reached in the urine nitrate.

Reactions of nitrate and nitrite with blood

As the haemin from blood of cows given $K^{15}NO_3$ accumulated ^{15}N, it appeared worth while to study the binding of nitrite *in vitro*. To 5 ml. of fresh blood plus oxalate was added 750 μg. of $^{15}NO_2^-$, 3·5 atom % ^{15}N excess, and the mixture was refrigerated overnight. The methaemoglobin concentration was determined, and the cells were separated and washed by centrifuging until the washings gave a negative test for nitrite. The globin and haemin were separated as described, samples of each were digested and their ^{15}N concentrations were determined. The blood containing 51 % methaemoglobin showed no detectable ^{15}N in its globin, but the haemin had 0·023 atom % ^{15}N excess. This demonstrated that the nitrogen from nitrite was bound firmly enough to the haemin of methaemoglobin to withstand displacement by acetone containing 1·2 ml. of 12 N-HCl/100 ml. The nature of this binding is not known, and no attempt was made to determine whether it was accompanied by an alteration in the composition of the haemin molecule.

Whole blood, cells suspended in 0·85 % NaCl and lysed cells, each containing about 50 % methaemoglobin, were shaken overnight in pure O_2 in an effort to displace nitrite. Although the percentage of methaemoglobin decreased in each case no detectable nitrite or NH_3 was released into the medium. Treatment of other samples of the same materials with 2·5 % neutralized NaCN was followed immediately by disappearance of methaemoglobin, but no significant quantities of nitrite or NH_3 appeared in the medium.

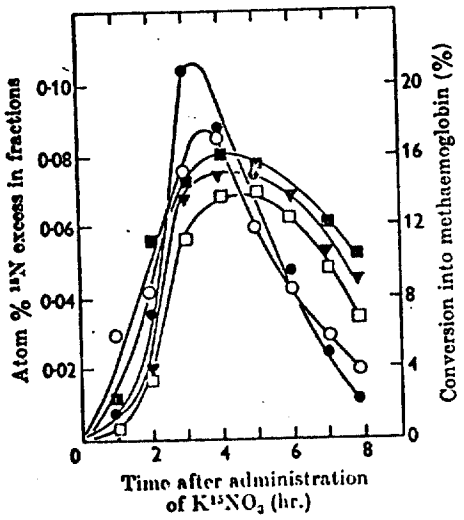

Fig. 5. Formation of methaemoglobin in the blood, and accumulation of ^{15}N in the nitrogenous fractions of urine, from a cow receiving 120 g. of $K^{15}NO_3$, 1·7 atom % ^{15}N excess, through a rumen fistula. Expt. 2: ○, nitrate nitrogen; □, ammonium nitrogen; ▼, urea nitrogen; ■, total nitrogen; ●, percentage conversion into methaemoglobin.

DISCUSSION

It is generally recognized that the toxicity of nitrate for ruminants depends upon its reduction to nitrite in the rumen and the combination of the nitrite with haemoglobin to form methaemoglobin in the blood. In addition to the acute symptoms that accompany nitrite poisoning, other conditions such as a non-contagious abortion in cattle (Sa[illegible] *et al.* 1957), have been attributed to ingestion of materials high in nitrates. The current studies re-emphasize the very rapid conversion of nitrate into nitrite and ammonium ions in the rumen. The dense microbial flora of the rumen maintains reducing conditions and can reduce added nitrate without delay. Fig. 1 shows the rapid disappearance of nitrate added directly into the rumen of a cow through a fistula, a disappearance which was accompanied by appearance of a maximum concentration of ammonia and nitrite within 3 hr. Our approximations indicated that at this time about 14 % of the added nitrate had been converted into nitrite, but such calculations are subject to substantial corrections for normal pool and absorption of nitrate, nitrite and ammonia.

Despite the fact that nitrite was formed quickly in the rumen with no evident need for adaptation of the microflora, toxicity to the animal was delayed and very little methaemoglobin appeared in the blood for several days after daily additions of nitrate to the rumen were initiated. The nature of this adaptation is not clear. Considerable quantities of nitrate appeared in the blood (Fig. [illegible]) but it seems questionable that substantial reduction of nitrate is accomplished in the blood. One cannot ignore the fact that the time of heavy conversion of nitrate into nitrite in the rumen (Fig. 1) very nearly coincided with the time of maximum methaemoglobin formation in the blood.

Fig. 2). Further, the concentration of nitrate increased in the blood (Fig. 4) beyond the time of a precipitous decrease in percentage of methaemoglobin and the similar decrease in nitrite concentration in the rumen. Data still are insufficient to account accurately for the amount of nitrite formed in the rumen and in the blood, but Pfander, Hamer, Ellis & Muhrer (1957) found very little toxicity from nitrate added intravenously, an observation that supports the primary formation of nitrite in the rumen rather than in the blood.

[^{15}N]Nitrate was added directly into the rumen to yield more detailed and specific information on nitrate reduction in the rumen, the transfer of nitrate and its products to the blood stream, and their elimination in the urine. The ^{15}N concentration of the nitrate plus nitrite fraction decreased rapidly (Fig. 2). The ^{15}N of the ammonium fraction increased as that of the nitrate plus nitrite decreased. After about 2·5 hr., the ^{15}N concentration of the ammonium ions remained higher than that of the nitrate plus nitrite ions. This response likely came from a differential dilution of the ions; the ingested feed carried much more nitrate than it did nitrate or ammonia, so that dilution of the ^{15}N in ammonia occurred more slowly than the dilution in the nitrate plus nitrite.

The maximum concentration of nitrite in the rumen appeared about 3 hr. after nitrate was added to the rumen (Fig. 1), and the maximum conversion of haemoglobin into methaemoglobin occurred at almost the same time (Fig. 2). One would expect very little delay in methaemoglobin formation once the nitrite reached the blood stream. The data support the idea that the bulk of the nitrite (and by implication ammonia and nitrate) is absorbed directly into the blood from the rumen rather than from lower sections of the digestive tract. The virtual coincidence of attaining a maximum nitrite concentration in the rumen and a maximum methaemoglobin level in the blood indicates that the absorption is very rapid.

In relation to the rapid drop in total nitrate nitrogen in the rumen (Fig. 1) and ^{15}N in the nitrate plus nitrite of the rumen (Fig. 2) it is interesting to observe (Fig. 4) the persistence of a high concentration of ^{15}N in the nitrate plus nitrite fraction in the blood plasma 4–8 hr. after the administration of potassium [^{15}N]nitrate. The rapid decrease in the percentage methaemoglobin after 3–3·5 hr. (Fig. 2–4) suggests that the nitrate plus nitrite fraction of the plasma consisted mostly of nitrate, and that the conversion of nitrate into nitrite in the blood probably was negligible.

The ^{15}N concentration of the ammonium ions always was far less than that of the nitrate plus nitrite of the plasma (Fig. 4), although they were approximately equivalent in the rumen after 2·5 hr. (Fig. 2). More favourable absorption of nitrate plus nitrite than of ammonium ion from the rumen into the blood, or more extensive isotopic dilution of ammonium than of nitrate plus nitrite in the blood, could explain the observations.

The peak concentration of ^{15}N in the haemin appeared before the maximum total conversion into methaemoglobin (Fig. 4). This suggests that the ^{15}N concentration of the nitrite in the rumen was at a maximum before the absolute concentration of nitrite in the rumen reached a maximum under our experimental conditions. It again emphasizes the rapidity with which nitrite is absorbed from the rumen into the blood. Land & Virtanen (1959) reported incorporation of ^{15}N from ammonium nitrate into milk protein an hour after feeding with the labelled compound.

The appearance of maximum concentrations of ^{15}N in the total nitrogen fraction of the urine was earlier than for the urea and ammonium nitrogen (Fig. 3) probably because the total nitrogen included nitrate. The concentrations of ^{15}N in urea and ammonium ions rose and fell together and reached peak values at approximately the same time (5–6 hr. after administering potassium [^{15}N]-nitrate). Fig. 5 emphasizes that the highest concentration of ^{15}N in nitrate appeared near the time of greatest conversion of haemoglobin into methaemoglobin. Again these appeared before the highest concentrations of ^{15}N appeared in the total nitrogen, urea nitrogen and ammonium nitrogen. Comparison of Figs. 3–5 suggests that there was a lag of as much as an hour between the times the highest concentrations of ^{15}N were observed in the urine compared with the blood. In part, this may reflect inherent delay in sampling the urine rather than a true lag in equilibration between the two fluids.

SUMMARY

1. A cow receiving a direct addition of 70–100 g. of potassium nitrate a day into the rumen through a fistula showed no methaemoglobin in the blood for several days. This delay in methaemoglobin formation could not be attributed to failure of the micro-organisms of the rumen to form nitrite, for they immediately showed vigorous nitrate reduction.

2. Potassium [^{15}N]nitrate was added to the rumen to trace the formation, absorption and elimination of nitrate and the compounds formed from it. Nitrate, nitrite and ammonium ions were absorbed in considerable quantities directly from the rumen into the blood. The highest concentrations of ^{15}N in the ammonium ion in the blood appeared 3–4 hr. after potassium [^{15}N]nitrate was added to the rumen, whereas the highest ^{15}N concentration in the nitrate plus nitrite fraction

appeared in the blood after 5–6 hr., and the high concentration persisted.

3. The curves for excretion of the ^{15}N-labelled total, nitrate, urea and ammonium nitrogen fractions in the urine lagged behind their corresponding curves for appearance and disappearance of these components in the blood by about an hour.

4. The formation of methaemoglobin in the blood followed the time course of nitrite formation in the rumen rather closely, suggesting that nitrite was passed rapidly and directly from the rumen to the blood. There was no direct evidence for nitrite formation from nitrate in the blood.

5. Nitrite was bound tightly to the haem of methaemoglobin, for it could be recovered in haem that had been subjected to treatment with acetone containing 1·2 ml. of 12N-hydrochloric acid/100 ml.

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Plant Polyphenols

4. HYDROXYCINNAMIC ACID–SUGAR DERIVATIVES*

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Hydroxycinnamic acids, which occur in most higher plants, are almost always present in bound form and are liberated from plant extracts by hydrolysis with acid or alkali. Of the organic substances known to occur in combination with hydroxycinnamic acids, quinic acid is the one most frequently mentioned in the literature: thus chlorogenic acid (a quinic ester of caffeic acid) appears to be almost ubiquitous and at least four other caffeoyl quinic acids are known. *p*-Coumaroylquinic acid is also reported to occur with some frequency (Cartwright, Roberts, Flood & Williams, 1955; Griffiths, 1959). In contrast, few simple sugar derivatives have been described (Karrer, 1958) and, with the exception of *o*-coumaroylglucoside (melilotoside) (Charaux, 1925), none has been fully characterized.

In view of current interest in the biosynthesis (McCalla & Neish, 1959), natural distribution (Bate-Smith, 1954; Herrmann, 1958) and biological function (Lee & Le Tourneau, 1958; Rabin & Kl[illegible] 1957) of the hydroxycinnamic acids, a study was made of their derivatives occurring in the potato and a number of other higher plants. Besides the *p*-coumaroylglucose and caffeic acid 3-β-glucose found in potato berries (Corner & Harborne, 19[illegible]) seven other naturally occurring cinnamic acid glucose derivatives have been found. Further simple glucose esters have been identified in nearly a hundred plant species.

The finding that glucose esters are of [illegible] occurrence suggests that they may be formed during the metabolism of free hydroxycinnamic acids in plant tissues. Previous work has indicated that phenolic glucosides (Kosuge & [illegible] 1959) and quinic esters (Levy & Zucker, 196[illegible]

* Part 3: Harborne & Sherratt (1961).

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A Research Note

NITROSAMINES AND THE INHIBITION OF Clostridia IN MEDIUM HEATED WITH SODIUM NITRITE

INTRODUCTION

PERIGO et al. (1967) and Perigo and Roberts (1968) described the formation of an inhibitor for the growth of several species of *Clostridium* during the sterilization of a culture medium in the presence of $NaNO_2$. They concluded that nitrite reacted with a component of the medium and the inhibitory activity differed from that of nitrite alone. The nature of the inhibitor is not known. It was further shown by Johnston et al. (1969) that in meat suspensions heated with $NaNO_2$ the inhibitory action was due to NO_2^-, although meat heated for 4 days at 80°C required less NO_2^- to inhibit *Cl. botulinum* than suspensions of meat not receiving the additional heat treatment. The extended heat treatment of the meat may have caused decomposition of the proteins with liberation of amino acids, peptides, and possibly, amines. Furthermore, the medium used by Perigo et al. contains yeast extract, beef extract and enzymic digests of meat thus making it rich in amines and amino acids. Nitrite reacts with amines and amino acids forming N-nitroso compounds, either N-nitrosamines or N-nitrosamides which are toxic and carcinogenic to animals and mutagenic to various species of microorganisms (Adelberg et al., 1965; Marquardt et al., 1963). We investigated the possibility that the "Perigo inhibitor" might be certain volatile N-nitroso compounds.

MATERIALS & METHODS

THE MEDIUM of Perigo et al. (1967) was used at pH 7.0 to reduce possibility of interference by HNO_2 at lower pH levels. Tryptone, peptone, yeast extract, and beef extract were obtained from Difco Labs. Sodium nitrite was added to tubes of medium so that, by doubled dilution, levels ranging from 640 to 1.25 ppm were obtained. In one set of conditions the NO_2^- was autoclaved in the medium at 15 psig for 15 min; in a second set of conditions the medium and NO_2^- solutions were autoclaved separately, then mixed aseptically. *Cl. botulinum B1218* (USDA culture) was grown at 35°C for 24 hr in thioglycollate medium (Difco), diluted 10-fold with water, and one drop added to each tube containing 4 ml of the Perigo medium. The medium was overlayered with for 18 hr and the minimum inhibitory concentration (MIC) taken as the tube in which there was no visible growth or gas production.

To analyze for the presence of nitroso compounds a batch of medium was autoclaved at 15 psig for 20 min, NO_2^- added at 640 ppm and the whole re-autoclaved at 6 psig for 20 min. 200 ml of heated culture medium was extracted 2× with 200 ml each time of CH_2Cl_2 after saturation with NaCl. The combined extracts were treated with anhydrous Na_2SO_4 and concentrated to 5 ml in a Kuderna-Danish concentrator using a steam bath. The solution was washed 2× with 5 ml each 0.1M HCl to remove some interfering compounds, dried with anhydrous Na_2SO_4 and concentrated to 0.5 ml. The concentrate was analyzed for certain volatile nitrosamines by gas chromatography, using a Varian Model 1740 instrument equipped with two 9 ft × 1/8 in. OD stainless steel columns packed with 15% Carbowax 20M-TPA on 60–80 Gas-Chrom P. The standard flame ionization detector was modified for use as an alkali flame ionization detector as described by Howard et al. (1970). Flow conditions were: helium, 58; hydrogen, 45; and air, 188 ml/min. Injector port and detector temperatures were 190° and 250°C, respectively. Column temperature was programmed from 115–185°C at 4°/min.

Confirmation of nitrosamines was carried out with the same gas chromatograph connected to a DuPont Model 21-492 mass spectrometer. The GC column was maintained at 115° and injection port and detector temperatures at 200° and 230° respectively. The column effluent was split 1:1 and passed into the mass spectrometer via an inlet line heated to 200°C. The mass spectra were obtained at an ionizing voltage of 70 ev and an ion source temperature of 200°C.

RESULTS & DISCUSSION

THE RESULTS OBSERVED on growing the *Cl. botulinum* in the nitrite-treated media confirmed the observations of Perigo et al. (1967) and Perigo and Roberts (1968). The MIC of NO_2^- in the medium autoclaved with NO_2^- was 40 ppm; in the medium to which NO_2^- had been added aseptically, the MIC was 640 ppm. An inhibitor of the growth of clostridia was formed under the conditions described.

An extract of a batch of medium prepared with 640 ppm NO_2^- (subsequent testing with *Cl. botulinum* showed the MIC was 10 ppm) was examined for the retention times similar to those for N-nitrosodimethylamine, N-nitrosomethylethylamine, and N-nitrosomethylpropylamine. However, structure confirmation with the mass spectrometer showed that none of these components contained a nitrosamine.

The inhibitory activity of a number of nitrosamines against *Cl. botulinum* was examined. The following nitroso compounds were placed into solution (200 mg in 10 ml water) and sterilized by Seitz filtration: N-nitrosodimethylamine (DMNA), N-nitrosodiethylamine, N-nitrosoproline, N-nitrosomorpholine, N-methyl-N-nitrosoethylamine, N-nitrosodiethanolamine, 1-nitrosopiperidine, N-nitrosodiphenylamine, N-methyl-N-nitrosoaniline and N-nitrosomethylurea. These nitroso compounds were prepared and purified in the laboratory. All preparations except the last three were added to the culture medium, at pH 7.0, to give solutions containing 10, 100 and 1000 ppm nitroso compound. The last three compounds were too insoluble so saturated solutions were prepared and two $\log_{10}$ dilutions made. Inoculation

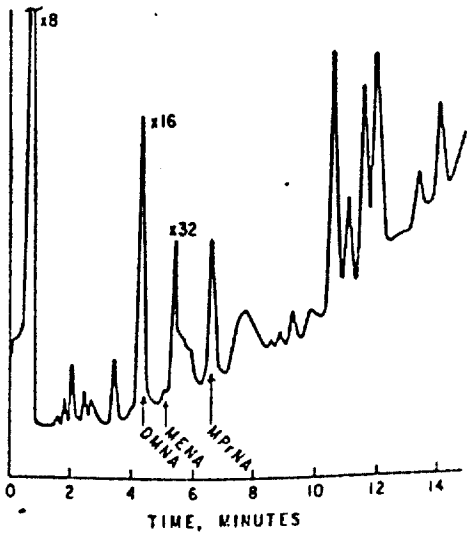

Fig. 1—Gas chromatogram of the extract of culture medium. Arrows indicate retention times for N-nitrosodimethylamine (DMNA)

and incubation were as described. None of the nitroso compounds showed inhibitory action against *Cl. botulinum* even at the highest concentration tested.

The "Perigo inhibitor" does not appear to be a nitrosamine detectable under the experimental conditions used, nor do the several nitrosamines tested appear to be inhibitory to the growth of clostridia.

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JFS APPLIED SCIENCE and ENGINEERING

2360

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THE EFFECT OF SODIUM NITRITE ON THE FLAVOR OF FRANKFURTERS

INTRODUCTION

THE CURING OF MEAT and comminuted meat products with salt and an alkali nitrate salt is an ancient process originally intended as a method of preservation. Changes in the color and appearance of the product occur in the process. In 1891 Polenski demonstrated that the nitrate in the cure was reduced to nitrite as a result of bacterial action. The red color of the product, which was considered a characteristic of cured meat, was found by Tisskalt (1899) to be formed in the presence of nitrite. Kerr and co-workers (1926) established the limits of sodium nitrite concentration in the cure that would yield a satisfactory product, and these limits are part of the legal requirements for cured meats in use today. The work of Kerr et al. (1926), however, was primarily directed toward the development of the cured color in the product. They noted that the flavor and quality of the products were equivalent to those prepared in the customary fashion, i.e., with nitrate only, but there was no investigation of the effect of the nitrite ion on the flavor of the product.

The relationship of nitrite to flavor was first described by Brooks et al. (1940) in a study of the use of nitrite in the cure of bacon and ham. Although they presented no taste panel data, these authors stated that the panel showed a preference for meat cured with nitrite. (It must be noted, however, that in a number of experiments very little differences in flavor were found among the variously treated bacons.) Barnett et al. (1965) reported on an extensive study of the factors affecting cured ham flavor. In a study on the concentration of nitrite in the pumping pickle, they found the panel had an equal preference for hams pumped with pickle containing the usual nitrite concentration (1.5g sodium nitrite per liter) and those in which pickle with 0.1g sodium nitrite per liter had been used. Recently Cho and Bratzler (1970) studied the effect of nitrite and smoke on the flavor of cured pork roasts, reaching the conclusion that more cured flavor was present in the roasts cured with nitrite.

The effect of nitrite on the flavor of frankfurters, a comminuted product of beef, pork and various spices, is reported in this paper.

MATERIALS & METHODS

FRANKFURTERS were prepared by a standard procedure according to the following formulation: lean beef–45%; pork (50% lean)–55%; and ice–25% of total beef and pork weight. The cure salts were added in the following quantities per pound of total meat: salt–11.4g; sugar–9g; commercial spice preparation–2.4g; sodium ascorbate–0.24g; sodium nitrate–0.574g; and sodium nitrite (when added)–2.070g. The frankfurters were cooked in a Dry-Sys smokehouse, using a 90-min program of increasing heat and controlled humidity. When smoking was desired, smoke, generated in a Mepaco apparatus from commercial hickory sawdust, was led into the smokehouse for the entire 90-min cooking period.

The frankfurters were stored at 5°C overnight and submitted to a taste panel for evaluation. The panel consisted of 23 judges who had been testing frankfurter flavor for several years. Frankfurters were heated for 5 min in water that had been brought to a boil, cut into ½-in. pieces and kept warm in a double boiler over hot water. Judging was carried out in individual booths under low intensity green light. While red light did cover up differences in color among the frankfurters, it was still possible to distinguish among them by the intensity of the dark color visible. This was alleviated to some extent by the use of the green light.

Types of tests used

Triangle test. The two samples to be compared were given as the odd sample an approximately equal number of times in a random fashion and were positioned randomly in the triangle to prevent sample or positional bias. Statistical significance of the results of these tests was determined from the table in the book by Amerine et al. (1965).

Scoring test. The panelists were presented with a standard of untreated frankfurter having a given value of "0" for poor flavor and were asked to score test samples for "frankfurter" flavor, on a scale of 0 to 10, compared to the standard. A duplicate of the standard was included as a hidden control. Analysis of variance was carried out as described in Steel and Torrie (1960).

Table 1—Triangle test evaluation of the flavor of frankfurters prepared with cure in which sodium nitrite was either present or absent

Experiment	Conditions	No. correct/ No. judges
1	Cooked, no smoke; + NO_2 vs. no NO_2	15/22***
2	Cooked, no smoke; + NO_2 vs. no NO_2	28/36***
3	Cooked, no smoke; + NO_2 vs. no NO_2	18/24***
4	Cooked, smoke; + NO_2 vs. no NO_2	11/17**
5	Cooked, smoke; + NO_2 vs. no NO_2	13/24*
6	Cooked, smoke; 50% NO_2 vs. no NO_2	12/17**
7	Cooked, smoke; 50% NO_2 100% NO_2	9/17NS

*p = .05; **p = .01; ***p = .001; NS = not significant

RESULTS & DISCUSSION

FRANKFURTERS prepared without sodium nitrite in the cure, and cooked but not smoked had an unpleasant grey color. Comments were made by the judges that

Table 2–Scaling test evaluation of the flavor of cooked or smoked frankfurters prepared with cure in which sodium nitrite was either absent or present

		Nitrite	
		No	Yes
Smoke	No	0.65[a]	3.85
	Yes	5.05	4.65

Analysis of Variance

Sources	df	SS	MS	F
Total	79	679.8		
Judges	19	155.3	8.17	1.63NS
Treatment	3	239.2	79.73	15.93**
Smoke vs. no smoke	1		135.20	27.01**
Nitrite vs. no nitrite	1		39.20	7.83**
Smoke × nitrite	1		64.80	12.95**
Error	57	285.3	5.00	

[a]Averages of scores of judges on a scale of 0 = no hot dog flavor, to 10 = excellent hot dog flavor
**Significant at p = .01; NS = not significant

Table 3–Scaling test evaluation of the flavor of cooked or smoked frankfurters prepared with cure containing various concentrations of sodium nitrite

		Nitrite		
		0	50%	100%
Smoke	No	1.18[a]	4.33	–
	Yes	5.25	5.59	5.02

Analysis of Variance

Sources	df	SS	MS	F
Total	84	472.92		
Judges	16	29.32	1.83	NS
Treatments	4	217.97	54.49	15.40**
Cooked vs. smoked	1		130.73	37.14**
0 vs. 50% NO_2 (cooked)	1		84.47	24.00**
Nitrite in smoked franks				
Linear	1		0.43	NS
Quadratic	1		2.36	NS
Error	64	225.61	3.52	

[a]Averages of scores of judges on a scale of 0 = no hot dog flavor, to 10 = excellent hot dog flavor
**Significant at p = .01; NS = not significant

this type of preparation had an unappetizing cooked pork flavor. When the frankfurters without sodium nitrite were smoked as well as cooked, the surface was brown (the intensity varying with degree of smoke applied) as a result of the deposition of smoke components. The interior of such franks, however, was still grey in color. The hardened, denatured protein skin of the franks could be removed easily. A red pigment was noted on the surface of the grey, underlying meat, which on analysis with the Cary Spectrophotometer was identified as nitrosomyoglobin. Smoke appears to contain sufficient oxides of nitrogen to penetrate the sausage casing into which the meat is stuffed and to react with myoglobin to form the nitrosated pigment.

A change in frankfurter flavor noticeable to the judges was brought about by eliminating sodium nitrite from the cure. Experiments 1 to 5 in Table 1 show that a statistically significant number of judges could distinguish between the flavors of franks prepared with or without sodium nitrite. While this was particularly true with the franks that were cooked only (Experiments 1–3) smoke did not prevent the selection of the correct odd sample, although the number of correct responses was somewhat lower (Experiments 4–6).

To quantitate the differences in frankfurter flavor indicated by the triangle test, a scoring procedure was applied. Frankfurters were prepared with and without sodium nitrite. One half of each batch was cooked only and the other half smoked as well. The results of triangle tests to detect differences in flavor among the samples are shown in Experiments 3 and 5 in Table 1. Table 2 shows the scores and analysis of variance for the flavors of the four preparations compared to a control sample of no nitrite–no smoke–treated frankfurter with a value of "0" for frankfurter flavor. In the absence of smoke there was a highly significant difference in flavor produced on the addition of nitrite. When the frankfurters were smoked, however, there was essentially no difference in the scores of the untreated and nitrite-treated franks. The analysis of variance demonstrated the statistically significant interaction between smoke and nitrite treatment.

The effect of nitrite concentration was explored by preparing frankfurters with no nitrite, with the full amount of sodium nitrite normally used (100% nitrite), and with half this concentration of sodium nitrite (50% nitrite). Half of each batch was cooked, the other half was cooked and smoked. Triangle tests showed that the judges could distinguish between the flavors of the franks with no nitrite and 50% nitrite, but although there was a trend to distinguish between the flavors of the franks treated with 50% and 100% nitrite concentration, the values were not significant at the 5% level (Table 1, Experiments 6 and 7). The frankfurters used in these tests were smoked; triangle tests with the franks that had been cooked only were not carried out since, on the basis of the previous tests, there was very little difficulty in distinguishing between untreated and nitrite-treated franks.

The scores of scaling tests of the flavors of these preparations, and the analysis of variance in the data, are shown in Table 3. In the absence of smoke there was a significant difference in the values assigned to the untreated and 50% nitrite-treated franks. (Although the 100% nitrite-treated, cooked-only franks were not tested it is anticipated that there would not be a significant difference between this preparation and the 50% nitrite-treated franks.) The presence of smoke resulted in similar scores (no significant difference) for the franks receiving no nitrite and those prepared with the two levels of sodium nitrite. The highly significant statistical difference among treatments was analyzed into single degree-of-freedom contrasts. The difference in scores between the cooked-only and the cooked and smoked samples is statistically significant. The scores of the flavors of the smoked franks treated with the various concentrations of nitrite were not significantly different but there appears to be a trend that suggests their relationship can be described by a quadratic expression, indicating the presence of a point of maximum flavor.

A consumer-type test was carried out on a group of visitors to the laboratory consisting of children and adults, male and female. They were requested to indicate their preference between a pair of smoked frankfurters prepared with and without nitrite in the cure; 44 out of 55 of those participating preferred the flavor of the frankfurter with nitrite.

It is interesting to note that in triangle Experiments 4–6 (Table 1) in which the frankfurters were smoked, and in the experiments with cured pork described by Cho and Bratzler (1970), the judges were able to detect the effect of nitrite on the flavor of the product; the application of smoke apparently did not affect the flavor. However, when the frankfurters were subjected to a scaling test (Experiment 3, Tables 1 and 2; Experiment 6, Tables 1 and 3), the flavors of the smoked products were judged approx-

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Gypsy Moth Control with the Sex Attractant Pheromone

Mass trapping or permeation of the air with pheromone can prevent male gypsy moths from finding mates.

Morton Beroza and E. F. Knipling

The gypsy moth [*Porthetria dispar* (L.)], a serious defoliator of forest, shade, and orchard trees in northeastern United States, is spreading rapidly to the South and gradually to the West nd threatens to become a national problem.

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There is deep division among scientists, administrators, environmentalists, and public officials about whether its spread can be stopped or should be stopped. On some occasions we read of citizens and township officials begging for relief from the moth's depredations; at other times it is claimed that after the initial flareup damage can be small, and that we should learn to live with the insect and find ways to minimize the damage rather than attempt to halt expansion of the present infestation. The way in which the gypsy moth problem has been handled has been criticized (*1*), but the critics have not come up with practical and ecologically acceptable solutions.

In this article we describe the problem and discuss the possibilities of using the recently identified sex pheromone of the gypsy moth (*2*) to combat this insect.

History

The gypsy moth, a native of Europe, Asia, and North Africa, was brought to Medford, Massachusetts, in 1869 for the purpose of producing silk for local industry; unfortunately, some insects accidentally escaped. The moth became established, but was largely unnoticed until 20 years later when there was a devastating population explosion. The following comment of a local resident is typical (*3*): "In 1889 the walks, trees and fences in my yard and the sides of the house were covered with caterpillars. I used to sweep them off with a broom and burn them with kerosene, and in half an hour they were

imately the same whether or not sodium nitrite had been used in the cure. The triangle test is purely a difference test, indicating in this case that an effect–some effect–differentiates the two samples. The scaling procedure requires a value judgement of the flavor, taking into account all factors, psychic and physical, that enter into such a judgement. Thus, while it is of interest to know that a difference can be detected, it would appear to be of greater importance that the judges found no significant difference in the flavor of smoked frankfurters in the presence or absence of sodium nitrite.

It is also of interest to note that the presence of a commercial frankfurter spice formulation was not sufficient to impart a good frankfurter flavor in the absence of sodium nitrite in the cure.

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THE EFFECT OF ASCORBIC ACID ON THE OXIDATION OF HEMOGLOBIN AND THE FORMATION OF NITRIC OXIDE HEMOGLOBIN[a]

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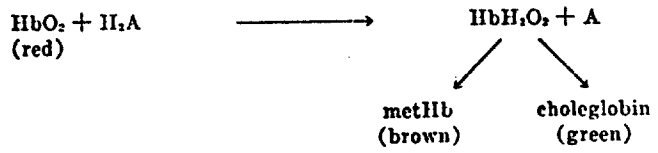
(Manuscript received June 9, 1951)

In previous work from this laboratory *(1)* it was found that the addition of ascorbic acid to hemoglobin solutions in the absence of nitrite resulted in rapid discoloration and greening, whereas in the presence of nitrite the ascorbic acid brought about fixation of nitrite, producing a comparatively permanent "cured meat" color. Because of the possible importance of these reactions to problems of color retention in meat, the literature on this subject has been reviewed and further experiments are here reported.

The known reactions of the hemoglobin pigments with ascorbic acid are as follows:

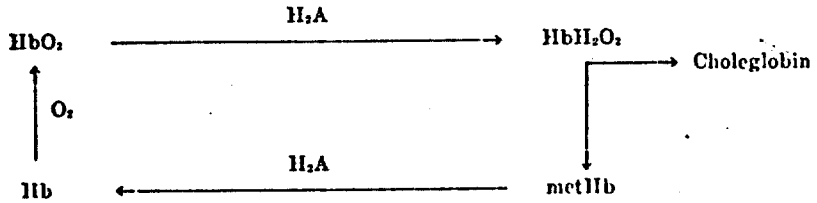
1. Reduced hemoglobin (Hb), purplish red in color, does not react with ascorbic acid (H_2A) in the absence of oxygen.

2. Oxyhemoglobin (HbO_2), bright red in color, undergoes a series of reactions with ascorbic acid *(1)*. The latter donates hydrogen, resulting in an unstable hydrogen peroxide derivative of hemoglobin (HbH_2O_2). The subsequent oxidation of the hemoglobin molecule proceeds in 2 directions. A relatively small portion is converted to a green decomposition product, choleglobin, which eventually precipitates as a green hemochromogen. The remaining HbH_2O_2 oxidizes to methemoglobin (metHb). The reaction may be expressed as follows:



3. Methemoglobin, the ferric hemoglobin, brownish in color, is reduced by ascorbic acid to Hb, the original ferrous hemoglobin *(3)*, which in the presence of oxygen becomes bright red HbO_2.

Reactions 2 and 3 can go on simultaneously. In fact, Lemberg and Legge *(4)* postulate a continuation of the cycle:



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The choleglobin concentration builds up as the cycle repeats itself. This work by Lemberg and Legge as well as the observations on reduction of metHb *(3)* were carried out under physiological conditions of temperature, pH, etc. The reactions have not been studied under meat storage conditions.

4. Nitric oxide hemoglobin (NOHb), the bright pink, heat stable pigment of cured meats, is readily formed in the presence of ascorbic acid and nitrite *(1)*. This reaction has not been thoroughly studied. Presumably NOHb, like COHb *(6)* is incapable of forming a hydrogen peroxide derivative with hydrogen donors such as ascorbic acid, and is therefore not oxidized to metHb and choleglobin by ascorbic acid (although both NOHb and COHb are rapidly oxidized by the direct addition of hydrogen peroxide). Under these conditions the reducing action of ascorbic acid on metHb would be expected to predominate. This is important both in color fixation (since nitric oxide reacts with Hb but not with metHb) and also in protection of cured meat color, since NOHb is readily oxidized to metHb *(7)*.

The experiments reported in the present paper extend the observations on ascorbic acid and the hemoglobin pigments over a range of temperatures, concentrations, and pH values of interest in meat preservation.

METHODS

Preparation of hemoglobin. The hemoglobin solutions used in these experiments were prepared from washed red cells from defibrinated hog or beef blood. The cells were laked with 4 parts of distilled water and stored in a freezer at −20°C. At the time of use the pH was adjusted to 5.6 with HCl and the stroma material filtered off before final dilution to the desired concentration.

Total hemoglobin in the diluted solutions was measured by the method of Evelyn and Malloy *(2)*. The method consists of an initial oxidation of all the hemoglobin pigments present to the ferric state with ferricyanide, followed by formation of methemoglobin cyanide, the absorption of which is read in the Evelyn photoelectric colorimeter using a 540-mμ filter. Values for total hemoglobin by this method did not change much even when choleglobin formation was far advanced and the solutions were green in color, provided that precipitation had not occurred.

Oxidative discoloration of hemoglobin solutions. The oxidation of hemoglobin under the conditions of these experiments resulted in a mixture of metHB, ferrous and ferric choleglobins, and possibly other oxidation products. The method proposed for the quantitative analysis of such complex mixtures *(5)* was considered too involved for the rapid analysis of a large number of samples. A simpler test was therefore devised which would indicate the approximate extent of discoloration or loss of red color.

Both HbO_2 and NOHb absorb strongly in the 540-mμ region; neither shows peaks or high general absorption between 600 and 700 mμ. The oxidation products of hemoglobin, on the other hand, absorb much more strongly in the latter region. MetHb has a pronounced peak at 630-635 mμ, ferrous choleglobin at 628 mμ, and ferric choleglobin at 670 mμ *(4)*.

It was found that percent transmission readings on an Evelyn photoelectric colorimeter with a 635-mμ filter set at 100% transmission with distilled water were a useful index to the visual appearance of hemoglobin solutions. The values, of course, are not specifically related to any particular compound; all of the oxidation products mentioned contribute to absorption in this region. Choleglobin absorbs to a greater extent than metHb. Under the rather extreme set of conditions where 2 samples in the same experiment oxidize along different pathways, the one in which the oxidized product is largely choleglobin will appear to retain more red color than the sample oxidized mainly to metHb when both have the same percent transmission at 635 mμ. In general, however, the transmission readings of such oxidizing solutions parallel rather closely the retention of red color as observed visually. The transmission readings are used throughout this

study to indicate both loss of red color through hemoglobin oxidation, with a consequent drop in transmission, and color fixation in nitrited hemoglobin solutions, which is accompanied by a reduction of metHb and increase in transmissions.

Taken in conjunction with metHb values, the transmission readings at 635 mμ give an indication of the extent of choleglobin formation. That is, a fall in transmission, accompanied by constant or diminishing metHb, is assumed to be due largely to formation of choleglobin.

MetHb was measured by the method of Evelyn and Malloy *(2)*. Since an initial reading at 635 mμ is used in this method, the same solutions used for the transmission values could then be converted to the cyanide derivative for metHb determinations, by the addition of a drop each of 12% acetic acid and 5% KCN to 10 ml. of the hemoglobin preparations in colorimeter tubes. The formula for calculation of metHb concentration, after revision of the Evelyn and Malloy constant for differences in dilution, becomes:

MetHb, % equals $(L_1 - L_2)/2.77$, where L_1 and L_2 are optical densities at 635 mμ before and after converting to methemoglobin cyanide.

All hemoglobin experiments were carried out in 0.02*M*. phosphate buffer, pH 5.6. Ascorbic acid was adjusted to this pH before adding to the buffer, or, more conveniently, a mixture of sodium ascorbate and ascorbic acid in the right proportions to give this pH (97 parts salt to 3 parts acid) could be used without preliminary pH adjustment, making due allowance for the lower ascorbic content of the sodium compound.

TABLE 1

Effect of temperature and concentration of ascorbic acid on oxidation of hemoglobin solutions *

Time	Temperature	H_2A concentration	Methemoglobin as % of total Hb (Initial is 13)	% tranmission at 635 mμ (Initial is 65)	Visual appearance
hours	°*C.*	%			
1	40	0	32	47	dull red
		0.02	15	47	dull red
		0.10	1.2	20	greenish purple
		0.50	0.4	16	greenish purple
24	24	0	39	42	brownish red
		0.02	9.7	64	bright red
		0.10	9.3	12	green
		0.50	5.7	10	green
24	4	0	21	61	red
		0.02	11	71	bright red
		0.10	10	72	bright red
		0.50	4.6	26	brown purple
24	−20	0	23	58	red
		0.02	30	48	dull red
		0.10	27	45	dull red
		0.50	7.5	21	brown purple

* Hb concentration is 0.28%.

RESULTS AND DISCUSSION

Effect of ascorbic acid in the absence of nitrite. Of the variables studied, temperature and concentration of H_2A were of greatest importance in determining both the kind and rate of change. Table 1 summarizes results of an experiment illustrating the effect of both variables.

At 40°C. all concentrations of H_2A used brought about rapid discoloration, the solutions passing through a brownish purple stage, then turning green, followed within a few hours by precipitation of the green cholehemochromogen described by Lemberg and Legge *(4)*. The control

samples discolor at a much slower rate and the discoloration is the typical brownish red of metHb. MetHb is reduced very rapidly by higher concentrations of H_2A at this temperature and it is evident that the discolorations obtained with H_2A are largely due to choleglobin formation.

At room temperature, the lowest concentration of H_2A (0.02%) protected the color of the solution. At this concentration, the experimental sample remained better than the control indefinitely. The next higher concentration was protected during the first hour, but later turned green. The highest concentration greened very rapidly. No precipitates formed at any concentration at this or lower temperatures with fresh hemoglobin preparations. However, after prolonged freezer preservation of the stock solutions partial denaturation of the proteins seemed to occur and precipitates did form at room temperature.

In the refrigerator, both 0.02 and 0.10% H_2A protected indefinitely as compared to the control. Transmission readings at the end of 24 hours indicate that these samples were not only better than the control samples at this time, but also better than the original hemoglobin solution at the start of the experiment, due to the reduction of some metHb originally present. Choleglobin formation was apparent only at the highest concentration of H_2A.

TABLE 2

Effect of salt on color retention in hemoglobin solutions[a]

Added constituents	Transmission at 635 mμ[b] after 24 hours at		
	24°C.	4°C.	−20°C.
Control	35	57	48
NaCl, 2%	25	50	41
H_2A, 0.1%	5	66	32
NaCl plus H_2A	2	49	28

[a] The Hb concentration was 0.36%.
[b] Initial transmission was 58.

H_2A at all concentrations brought about a discoloration of frozen samples. However, except at the highest concentration, the discoloration was due more to metHb than to choleglobin formation. When the frozen samples were thawed and allowed to stand at room temperature for several hours, transmission of the control continued to decrease, whereas transmission increased in samples containing 0.02% and 0.10% H_2A, so that the latter became distinctly redder than the control. Discoloration with 0.5% H_2A was due to choleglobin and was not reversed on thawing. In this and other experiments to be reported on frozen solutions, it must be remembered that the freezing process not only lowers the temperature but also concentrates all soluble constituents by removal of water as ice.

Evidently the higher the temperature and the higher the concentration of H_2A the greater is the likelihood of discoloration. At lower concentrations and lower temperatures (down to the freezing point) H_2A protects the color.

The addition of 2% salt (C. P. sodium chloride) to hemoglobin solutions brought about more rapid oxidation of the hemoglobin at all temperatures and in the presence and absence of H_2A (Table 2).

Variations of pH within the extreme limits 6.4 to 5.2 of normal meat also affected the rate of oxidation of hemoglobin solutions. The lower the pH the more rapid the fall in transmission at 635 mμ, both in plain hemoglobin solutions and in the presence of H_2A.

Experiments with nitrite. In preliminary work on the development of "cured meat color" with H_2A, it was found that the use of nitrates had no effect on the color under the conditions of these experiments. Nitrates are slowly reduced in meats by bacterial action during prolonged curing processes. This reduction is not accomplished in hemoglobin solutions in the short times necessary to develop the cured meat color with H_2A. For this reason only nitrite was added (as $NaNO_2$) and the concentration selected unless otherwise stated was 0.02%, the upper limit of nitrite allowed in meat. Sugar (dextrose) like nitrate, had little or no effect on formation of NOHb.

The addition of nitrite alone to hemoglobin solutions caused an immediate large drop in transmission and a concurrent increase in metHb. Nitrite is known to bring about metHb formation with both HbO_2 and Hb *(4)*. After the first large drop, transmission continued to drop at a much slower rate over a period of some hours, but the metHb usually decreased. Evidently the oxidation of hemoglobin brought about by nitrite does not stop with metHb.

The initial drop in transmission and increase in metHb were not prevented by adding H_2A prior to or together with the nitrite unless a large concentration (0.5%) of H_2A was used or the pH was lowered. The reduction of metHb by H_2A is a much slower process than the oxidation of hemoglobin by nitrite under most conditions. Full color development could not be attained if the addition of H_2A was delayed until the oxidation of a considerable fraction of the hemoglobin proceeded beyond the stage of metHb.

Figure 1 shows results of a typical experiment in which 0.02% nitrite was added to a hemoglobin solution. Only transmissions are plotted. MetHb concentration increased immediately from 16 to 32%. Addition of 0.5% H_2A at this point resulted in gradual reduction of the metHb over the two-hour period of increasing transmission and formation of NOHb. At the peak of this curve, no metHb remained. The addition of the same concentration of H_2A to another aliquot of the hemoglobin-nitrite solution after it had stood for 2 hours and lost approximately half of its metHb, resulted in the same type of curve, but with much lower peak transmission. Again metHb had completely disappeared when the peak was reached. In subsequent experiments the nitrite and H_2A were added together.

All factors previously found to accelerate oxidation of hemoglobin in the absence of nitrite, accelerated color fixation in the presence of nitrite.

Temperature had a marked effect on the rate of NOHb formation. In the refrigerator color fixation was slow and often incomplete, whereas under the same conditions a more brilliant color developed rapidly at the oven temperature of 45°C. If allowed to remain at this high temperature subsequent fading of the color was also rapid.

Freezing of solutions of hemoglobin and nitrite in the absence of H_2A caused intense oxidation and greening of the solution. However, when

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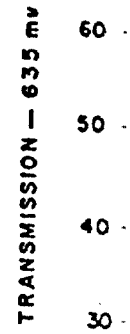

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Salt accelerated NOHb formation and raised the peak of transmission under all conditions. Some effects of temperature, freezing, and the addition of salt on transmission are shown in Table 3.

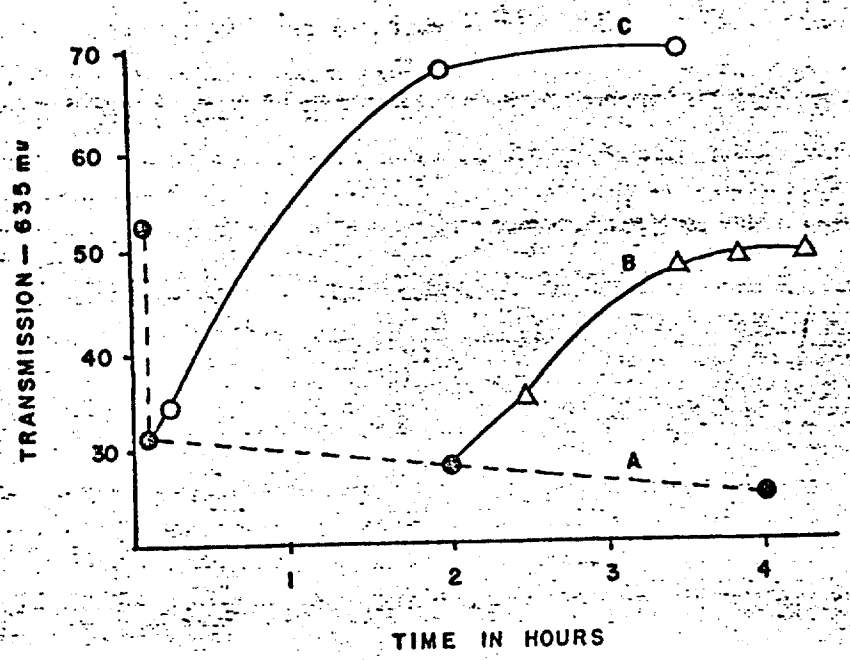

Figure 1. Formation of NOHb on addition of 0.5% H_2A to nitrited hemoglobin solutions at 24°C. Solution contained 0.38% total Hb (of which 16% was metHb) and 0.02% nitrite. A, nitrite alone; B, H_2A added after 2 hours; and C, H_2A added after 5 minutes.

Increasing concentrations of H_2A, at least up to 0.5%, accelerated NOHb formation and gave higher peak values (Figure 2). The lowest concentration of 0.02% was not sufficient to reduce all of the metHb formed by the nitrite. Color fixation was long delayed and very incomplete. With both 0.1% and 0.5% H_2A, metHb was completely reduced, but the reduction took somewhat longer with 0.1% and since some further oxidation products of hemoglobin had formed during this time, the peak was slightly lower. The difference between peak color attained with these 2 concentrations of H_2A was too small to be detected visually.

Proper color fixation also seems to be a function of the concentration of hemoglobin and of nitrite; that is, at low concentrations of either, color fixation requires higher concentrations of the other. At hemoglobin concentrations of approximately 0.2% color fixation occurred at nitrite concentrations as low as 0.001% in the presence of 0.1% H_2A. When hemoglobin was further reduced to 0.05%, a higher nitrite level of 0.02% was necessary to secure proper fixation.

Higher hydrogen ion concentrations increased rate and peak of color fixation. At room temperature, with 0.1% H_2A and 0.02% nitrite, the

times required to reach peak color and the highest transmissions attained were as follows: pH 5.2, transmission 76 after 40 minutes; pH 5.8, 68 in 3 hours; pH 6.4, 43 in 7 hours.

TABLE 3

Effect of salt and temperature on development of cured meat color by ascorbic acid [f]

Temperature	Added salt and H_2A	Transmission at 635 mμ [g]	
		2 hours	24 hours
°C.			
45	Control (nitrite only)	24	9
	Salt, 2%	20	precipitate
	H_2A, 0.1%	74	43
	Salt + H_2A	73	45
24	Control	34	17
	Salt, 2%	28	11
	H_2A, 0.1%	51	37
	Salt + H_2A	71	68
4	Control	42	36
	Salt, 2%	37	22
	H_2A, 0.1%	57	53
	Salt + H_2A	67	61
—20	Control	....	8
	Salt, 2%	....	7
	H_2A, 0.1%	....	58
	Salt + H_2A	....	68

[f] All solutions contained initially 0.36% total Hb (of which 15% was metHb) and .02% nitrite.
[g] The initial transmission of the Hb solution, before addition of nitrite, was 58.

Mechanism of NOHb formation with H_2A. The following steps are suggested as the probable mechanism of NOHb formation with nitrite and H_2A, in line with the experimental facts which have been presented. These apply only to color fixation with H_2A, not to the normal meat curing process.

1. HbO_2 is converted to metHb by nitrite. This is probably an essential step in the process, since HbO_2 cannot combine with NO.
2. MetHb is reduced to Hb by H_2A. This is relatively slow as compared to Step 1. The reduction closely parallels color fixation and probably determines the rate of NOHb formation.
3. Hb unites with NO (formed from NO_2 under reducing conditions in an acid medium). This, like Step 1 is a rapid reaction *(7)* and is probably not a limiting factor.

Factors which aid in color development, that is, salt, hydrogen ions, heat, freezing, etc., may do so by catalyzing the reduction of metHb by H_2A but the data presented in this paper are not sufficient to establish this point.

SUMMARY AND CONCLUSIONS

The known reactions of ascorbic acid with heme pigments are reviewed. A simple method is described for following: 1, discoloration due to oxidation in hemoglobin solutions; and 2, development of nitric oxide hemoglobin in nitrited solutions.

In the absence of nitrite, ascorbic acid protects hemoglobin solutions when it is added in low concentrations and at low temperatures. At high concentrations and high temperatures it brings about oxidation and discoloration. Freezing, salt, and low pH values accelerate the oxidation.

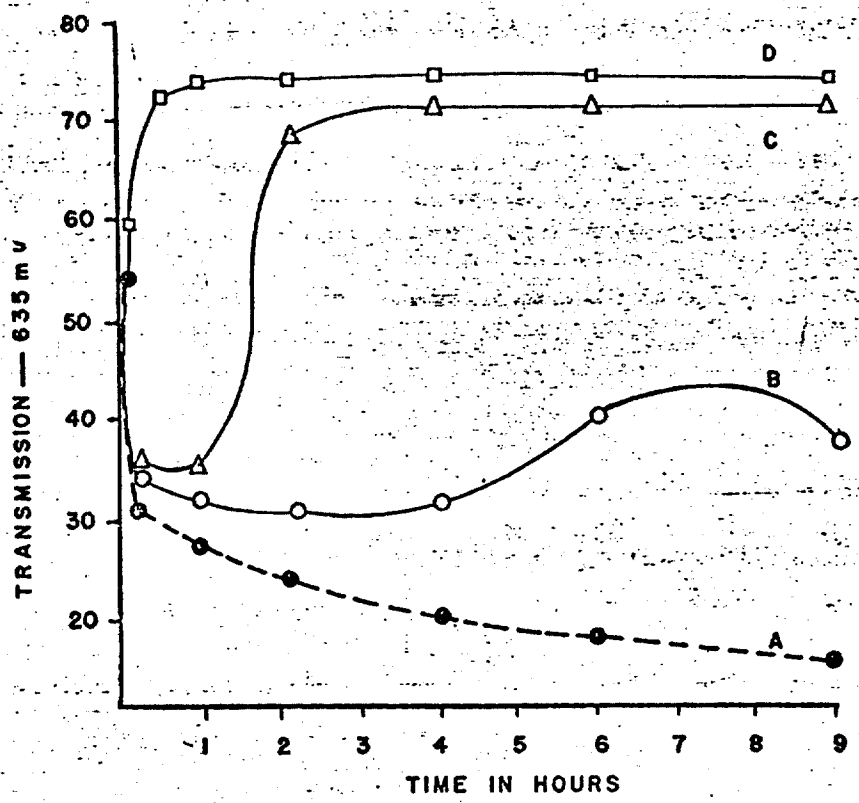

Figure 2. Color development with various concentrations of H_2A at 45°C. Solution contained 0.41% total hemoglobin (of which 11% was metHb) and 0.02% nitrite. A, no H_2A; B, 0.02% H_2A; C, 0.10% H_2A; and D, 0.50% H_2A.

Nitrite brings about a rapid oxidation of oxyhemoglobin to methemoglobin and later to other oxidation products. The addition of ascorbic acid with nitrite causes rapid development of nitric oxide hemoglobin, the cured meat pigment. Nitrates and sugar have no effect on the reaction. Salt, low pH values, high temperatures, and freezing accelerate the color-fixation. The possible mechanism is discussed.

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2366

Part V. Pharmacodynamics of carcinogenic azo dyes, aromatic amines, and nitrosamines

In this review we touch upon factors affecting the carcinogenicity of three broad classes of chemicals: the carcinogenic azo dyes, the aromatic amines, and the alkyl nitrosamines. Present knowledge of their mode of action indicates that they share some points in common but differ in other respects.

1. All three types of compounds presumably require metabolic activation by the host. In part, species differences in relative carcinogenicity are accounted for by the varying efficiency with which this is accomplished. With the azo dyes the active intermediate is as yet unknown; with the aromatic amines it seems to be an N-hydroxy derivative; with the alkyl nitrosamines, the evidence points to active diazoalkanes.

2. All carcinogens combine firmly with a tissue receptor, and search for its identity is actively under way. So far, some combinations of azo dyes with proteins have been demonstrated. Compounds of unknown structure between metabolites of certain aromatic amines and alkyl nitrosamines with proteins and also with ribonucleic acid have been observed. The specific mechanisms are not yet known. Conceivably different types of carcinogens may affect different receptors in the host cells. The common points these reactions have is that (a) the block due to the carcinogen-receptor adduct is not lethal, (b) the block is rather durable or is renewed at regular intervals to maintain this condition for an extended period of time, (c) the block elicits reaction on the part of the host system trying to overcome this restraint at the molecular level.

3. Carcinogens do not act in a vacuum but in a host with numerous feedback control systems to maintain homeostatic balance. Disturbance of these systems by the carcinogen elicits, mobilizes, or strengthens endogenous growth-controlling factors which play an important role in the development of the tumor. An understanding of these complex phenomena is essential for the complete comprehension of the initiation and development of the neoplastic process.

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This paper will consider certain aspects of the carcinogenicity of three classes of chemical carcinogens: (1) the azo dyes, (2) the aromatic amines, and (3) the nitrosamines, which are models of compounds presumably requiring metabolic activation. In contrast, the carcinogenic hydrocarbons* are probably the actual car-

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*See article by Falk and Kotin, page 88.

cinogen or close (epoxide ?) to it. By definition, a compound requiring metabolic activation is one which when administered to animals is very likely not carcinogenic by itself ("procarcinogen") but requires transformation in the host to become a "proximate" carcinogen—a sort of lethal synthesis. In this concept, the proximate carcinogen is the material which actually interacts with appropriate tissue receptors and thus sets in motion the complex series of phenomena eventuating in neoplasia.

The discussion of these three classes of carcinogens hopefully will bring out principles of their mode of action which may be extended to the operation of other types of carcinogens.[94] As will be apparent, there are some fundamental differences with respect to the proximate agent derived from the compounds administered and also in regard to the cellular entity attacked by this proximate carcinogen.

It will be impossible to do full justice to the authors of the extensive and intensive studies performed in this area. Our aim is to provide a glimpse of the more striking developments and arguments from our personal viewpoint.

General observations

Experimental investigations in chemical carcinogenesis, whether they be extensions of chronic toxicity tests which result in the development of neoplastic lesions or studies specifically designed to reveal carcinogenicity with certain materials, have permitted the formulation of certain general conclusions. Tumor formation depends on species, strain, age, diet, sex, and hormonal status of the test animal, on the dosage, frequency, and mode of administration, and, of course, on the structure of the compound. Sometimes even the criteria for specifying the end point play a role. Some workers recognize carcinogenic activity only if the lesion can be classified as malignant, whereas others will ascribe activity to a material inducing abnormal growth under certain limited conditions. The animals used most frequently in cancer induction experiments are rats or mice. Broadly speaking, mice are often selected for tests involving application of materials to the skin with tumor development at that point; rats find more application for oral intake of materials with visceral tumor development, or for subcutaneous injection resulting in sarcoma induction at the site. In recent years, hamsters have taken a prominent place in the armamentarium of the oncologist; rabbits excel in their sensitivity to certain mixtures derived from petroleum oils applied to the skin; dogs have been used because they respond like humans to treatment by certain azo dyes and aromatic amines with production of bladder tumors. On the other hand, the guinea pig is well known for its relative resistance to a carcinogenic challenge and is, therefore, of little importance.* However, this slight sensitivity of the guinea pig has made it an interesting tool in comparative studies on mechanism, since an understanding of the fundamental reasons for the low susceptibility of this species provides some clues on the manyfold factors involved in the neoplastic process.

Development of inbred strains of rats and mice in the last few decades has provided tools which show specific and exquisite sensitivity in respect to one or more characteristics. They offer advantages because of the reproducibility of the experiments and reliable information on spontaneous neoplasms. However, consideration must be given to the fact that some inbred lines have been obtained and selected for reasons which are not related to carcinogenic bioassays.

In comparative experiments oncologists make use of the following criteria: per cent of animals with tumors; location, morphology, and identity of the tumors; number

*Recently, however, Argus and Hoch-Ligeti observed that malignant, metastasizing carcinomas of the liver and also tumors in the lung and gallbladder were induced rapidly in guinea pigs by diethylnitrosamine. "Induction of malignant tumors in the guinea pig by oral administration of diethylnitrosamine," J. Nat. Cancer Inst., 1963. See also Druckrey, H., and Steinhoff, D.: Erzeugung von Leberkrebs an Meerschweinchen, Naturwiss. 49:497-498, 1962.

of multiple tumors; latent period to first tumor; and average latent period.[9] Other things being equal, the latent period is a function of the life-span of the animals used. Thus, with long-lived animals such as dogs or monkeys, tumor formation occurs only after many years. For this reason, such species find application only in special cases. On the other hand, intensive search among the short-lived species for susceptibility to carcinogens may turn up useful new ones for the bioassay of chemicals.

Another principle generally applicable is that the younger the animals are when first exposed to the drug, the more sensitive they are to most carcinogens. In part, this greater susceptibility is due to the obvious fact that younger animals can be exposed to the chemical for a longer period of time than older ones within the limits of their life-span. Additionally, it was shown that, by and large, newborn animals are more sensitive to both viral and chemical carcinogenesis.[40] The basic reasons are not well understood. They may involve biochemical induction of certain enzymes or synthesis or deletion of other factors, early immunological adaption to latent tumor cells, or other reasons. In any case, it seems significant that even with the higher sensitivity of newborn or weanling animals, the tumors induced arise no sooner than the time of sexual maturation. Possibly the profound alterations in the physiology, biochemistry, and morphology of the host attending to this process may play a role in the early occurrence of tumors in animals treated with certain chemicals.

The compounds discussed here are generally most active after prolonged oral intake. Single dosing is rarely effective, owing chiefly to the rapid excretion of the compounds or their metabolic products. Under special conditions, induction of tumors after a single large dose was observed.[36, 49] Many carcinogens have relatively low acute toxicities but are definitely toxic in chronic treatment. As may be expected, tumor induction is higher and the latent period shorter, the larger the dosages of the material administered. However, the highest tolerated level will sometimes give a less than maximal response owing to inhibition of growth of the host and possibly also of the induced tumor.[33]

In experiments leading toward an understanding of the mode of action of chemical carcinogens, the obvious approach is the determination of the constituent of the host which is first affected by the treatment. Biochemists and pharmacologists interested in oncology have performed innumerable studies directed to this end. However, it must unfortunately be concluded, at this time, that no case is known in which the point of attack of the carcinogenic agent on a definite tissue constituent is understood. Examination of the patterns of activities or concentrations of enzymes, nucleic acids, and other body constituents during the precancerous period has not pointed a finger at any one of these as uniquely related to the carcinogenic process.* The frustrating aspect of these studies is that it is well known that the induced tumors do exhibit a distinct constitution and metabolic pathways often quite different from those of the normal tissues of origin.[13, 27] On the basis of such findings Greenstein[25] has coined the concept of convergence of enzyme patterns typical of the neoplastic state. However, the recent utilization of minimal deviation tumors,[24] that is, tumors which differ from their normal tissue of origin in only a few characteristics, has rekindled the hope that studies of this type may yet lead to an understanding of the primary lesion during carcinogenesis.

Carcinogenic azo dyes

Carcinogenicity and chemical structure. Although the reversible proliferative effect of certain complex azo dyes, such as scarlet red, was discovered early in the century, the carcinogenicity of azo dyes (Fig.

*Note the recent review by Reid, E.: Significant biochemical effects of hepatocarcinogens in the rat, Cancer Res. 22:398-430, 1962.

1) was first established conclusively in 1931 by Yoshida. These compounds generally caused liver tumors in rats and mice.[51] As was the case with the carcinogenic hydrocarbons, the initial discovery of the carcinogenicity of one typical azo dye led to a flurry of efforts to test other compounds of this class. Thus, a sizable body of information on chemical structure versus carcinogenicity in this series became available.[2, 61] First of all, it may be noted that compounds of the type of *o*-aminoazotoluene* are more carcinogenic in mice of certain strains and less carcinogenic in rats, whereas the reverse holds for compounds of the type of DAB (4-dimethylaminoazobenzene).† This difference persists among the analogs of the respective dyes and appears to be of quantitative rather than qualitative nature. Thus, among the aminoazotoluenes, the 2′,3-dimethyl derivative is a strong carcinogen in mice but weak in rats, whereas the 2,3′-dimethyl compounds, which are fairly weak in mice, show no activity in rats. Furthermore, compounds related to 4-dimethylaminoazobenzene require at least one methyl group on the amino nitrogen to exhibit carcinogenic activity for the liver. On the other hand, the aminoazotoluenes do not, and, in this case, the acetyl or diacetyl derivatives are also carcinogenic. There appear to be no explanations for these discrete but definite differences.

The largest series of comparative experiments on the carcinogenicity of derivatives of 4-dimethylaminoazobenzene in rats were performed in the laboratories of E. C. and J. A. Miller. A detailed exposition of all the ramifications of these studies cannot be accomplished in this article and the reader is referred to appropriate reviews.[55, 61] However, the chief conclusions derived from this work are as follows: Substitution at all positions in the ring bearing the dimethylamino group reduces or

*Chemical Abstracts nomenclature: 4-*o*-Tolylazo-*o*-toluidine.

†Chemical Abstracts nomenclature: N,N-dimethyl-*p*-phenylazoaniline.

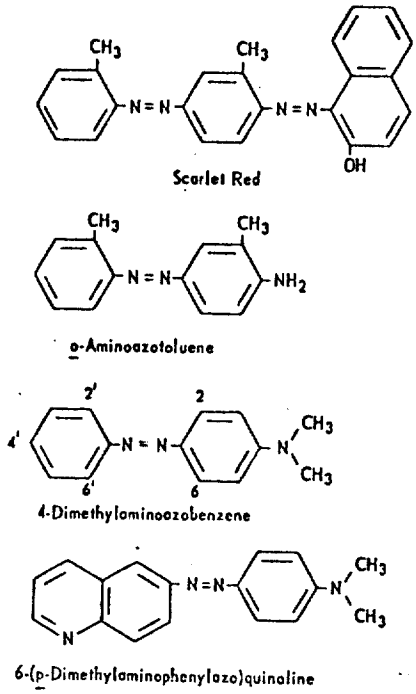

Fig. 1. Formulas of azo dyes.

abolishes carcinogenicity with the exception of a fluorine at the 2-position. In the other ring, substitution of the 3′-position with NO_2, Cl, CH_3, F, and even CN or COOH gives active compounds. In fact, the 3′-methyl or 3′-fluoro derivatives are among the more active of this class of compounds. Replacement of suitable groups at the 2′-position is somewhat less effective and substitution at the 4′position gives rise to compounds of even lower activity. In this connection, substitution on the 4′carbon atom by chloro groups lowers and by bromo groups abolishes the carcinogenicity. This may be due to steric effects owing to the increasing thickness of the molecule which for this reason may not fit into the appropriate tissue receptor. Alternatively, the decreased effect may also be related to the lower absorption of the less soluble dye fed in the diet.

A special case and exception to these rules are observed when the substituents are fluorine atoms. Thus progressive replacement of the ring hydrogens by fluorine atoms at appropriate positions gave

compounds that were either as active or more active than the parent DAB. However, the derivatives were inactive when the fluorine was on the 2 and 6 positions. From this ingenious approach it was concluded that blocking of the 2- (or 6-) position by fluorine covered a point on the ring of the azo dye which was somehow involved in the initiation of the carcinogenic effect.

Recently Brown and associates[9] interchanged one of the phenyl rings in dimethylaminoazobenzene by a quinoline or a quinoline-*N*-oxide residue. Several of the compounds so obtained, particularly those where the dimethylaminophenylazo group was attached to the 5- or 6-position of the quinoline ring, were extremely potent hepatocarcinogens. They were about 20 times more active than any azo dye previously tested. Future application of these compounds to studies on the possible existence of a threshold* in chemical carcinogenesis might prove worthwhile.

Whereas most azo dyes so far mentioned affect chiefly the liver of mice and rats, Miller and Miller[63] observed that the simple addition of a 3-methoxy group in 4-dimethylaminoazobenzene led to a compound which produced ear duct tumors but was not strikingly active toward the liver. Also, the demethylated compound was as effective as the N-methyl derivatives. This compound thus constitutes a bridge between the azo dyes and aromatic amines. The corresponding 2-methoxy compound was not carcinogenic, again pointing to the need for a free 2-position.

Certain azo dyes are carcinogenic even if they do not contain an amino or dimethylamino grouping. Thus, 2,2′-azonaphthalene is highly active, 1,1′-azonaphthalene somewhat less, and 1,2′-azonaphthalene appears to be noncarcinogenic. This class of compounds also includes some used commercially prior to the discovery of their carcinogenicity, for example, Poncean 3R.[26]

Modifying factors. It was discovered earlier that the outcome of experiments leading to tumor formation by 4-dimethylaminoazobenzene can be markedly affected by dietary conditions.* Thus, the carcinogenicity can be greatly reduced by a high riboflavin and also by a high protein diet. Apparently, the riboflavin levels mediate the destruction of the dye and concomitant reduction of the effective level of the carcinogen[56, 61] by means of a flavin-adenine nucleotide requiring enzyme which reduces the azo linkage of the dye. Along similar lines, the amount of azo dye available appeared to be reduced by simultaneous administration of metal ions such as cobalt, nickel, or copper, but manganese, zinc, molybdenum, and cadmium had no effect.[11, 34] In fact, Howell[34] suggested that metal ions play a more specific role in this connection. We have noted that certain derivatives of the azo dye can chelate metal ions.†

The joint administration of 3′-methyl-4-dimethylaminoazobenzene‡ and 2-acetylaminofluorene resulted in a synergistic effect. Another liver carcinogen, ethionine, given simultaneously with azo dye had only a slight effect, but feeding these two compounds alternately led to a reinforcement of the individual action.[56] On the other hand, the combination of a polynuclear carcinogenic hydrocarbon such as 3-methylcholanthrene or dibenz[*a*,*h*]anthracene and also of some of the noncarcinogenic analogs had a pronounced inhibiting effect on the carcinogenicity of dimethylaminoazobenzene.[58] This unusual and at that time perhaps unexpected result has engendered considerable efforts at a rational explanation which have recently borne fruit. It was, indeed, discovered that certain polynuclear hydrocarbons and also some chemically unrelated materials such as phenobarbital possess the faculty for inducing enzymes concerned with the metab-

*See article by Mantel, page 104.

*See article by Kraybill, page 73.

†Weisburger, J. H., Grantham, P. H., and Weisburger, E. K.: Biochem. Pharmacol. (in press). (Fed. Proc. 19: 398, 1960.)

‡Chemical Abstracts nomenclature: *N,N*-dimethyl-*p*-(m-tolyl)azoaniline.

olism and detoxification of the azo dyes, thus resulting in a lower effective level of the dye and consequently decreased tumorigenic effect. These and other aspects of this intriguing problem have been summarized by Conney and Burns.[12]

The carcinogenicity of the azo dyes seemed to be affected by the sex of the animal less than is the case with some of the other carcinogens. However, with *o*-aminoazotoluene, female mice showed a higher incidence of liver tumors than males. On the other hand, with 4-dimethylaminoazobenzene a few investigators noted more hepatomas in male rats when compared with females, although in many cases both sexes exhibited similar incidences and latent periods. Since the maximally tolerated levels of the carcinogens were used, it is probable that whatever differences might have been inherent in the system were swamped.[21] Adrenalectomy had a protective effect, especially if adrenal regeneration were inhibited by administration of deoxycorticosterone. Hypophysectomy dramatically reduced the incidence of hepatoma, but tumor formation could be restored by the administration of ACTH or growth hormone.[25] Along these lines, it was observed that chlorpromazine, a tranquilizer, also inhibited carcinogenesis, an action which may be based on its property to induce pituitary atrophy.[22, 43, 52] Reserpine, however, accelerated carcinogenesis by azo dye.[43]

Metabolism studies. Shortly after the definite discovery of the carcinogenic effect of various chemicals, studies on their metabolism were undertaken with the ultimate aim of understanding their mode of action. The more immediate purposes of such researches were twofold: (1) determination of the various metabolites with respect to less active detoxification products and also possible materials with higher activity which might actually be the proximate carcinogens; (2) determination of the nature of the tissue receptor actually involved in the initiation of the carcinogenic process. Most fundamental studies of the nature of drug action have, of course, this dual aspect.

Early studies on the metabolism of a typical azo dye have indicated that the molecule is subject to at least three different modes of metabolism (Fig. 2): oxidative demethylation of the N-methyl groups, reductive splitting of the azo linkage, and hydroxylation at several selected positions of the aromatic rings. Some of these metabolites were identified in the urine, others in the tissues. However, none of those presently known are more carcinogenic or even as carcinogenic as the compound administered. Thus, those materials are most likely simple detoxification products which are not directly involved in the carcinogenic process.

These studies have led, however, to a highly significant observation which has resulted in important developments in this field. Indeed, in 1947 Miller and Miller reported that the livers of rats fed 4-dimethylaminoazobenzene had a yellow color which turned pink in acid, a behavior similar to that of the free azo dye. This "bound" dye was, nevertheless, not extractable from the liver under any of the many conditions tried but was released only upon hydrolysis of the liver proteins. The level of the protein-bound dye in the livers of animals fed azo dye continuously reached a maximum at about 3 to 5 weeks and then declined progressively. However, the hepatomas induced by the dye were not stained. The conclusion was drawn that the hepatoma cells derived from the parenchymal cells did not contain the protein component capable of fixing the azo dye.

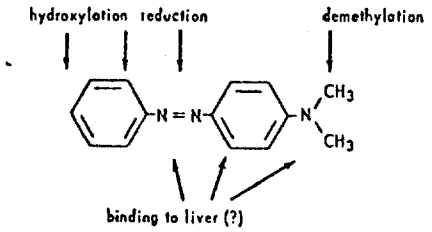

Fig. 2. Main sites of metabolic transformation in 4-dimethylaminoazobenzene.

From further studies along these lines, the protein deletion hypothesis was evolved by the Millers and also by Potter,[73a] and by Rusch,[74] each approaching this problem from a different viewpoint. The hypothesis postulates that during the transformation from normal to cancerous tissue, certain proteins with growth-control functions may be lost during the progress of the neoplastic transformation. Further experimental observations on protein binding have produced suggestive correlations between this phenomenon and carcinogenesis. Thus, with the azo dyes, binding was found only in the liver, the primary site affected by the carcinogen, although at later periods small amounts of binding were also noted in the adrenals. Species and strains of animals that were not responsive to the carcinogenic effect likewise only showed low levels of bound dye. Furthermore, these studies were extended to other types of carcinogens, and it was found that the material administered or a metabolite thereof (as measured by isotope labeling, for example) were bound to tissues of the host animals. In these other cases, correlations could be made between carcinogenicity and the extent of binding. There were, however, some exceptions. Materials which were not carcinogenic gave rise to protein binding; or tissues in which tumors are not normally induced contained bound carcinogen. On the other hand, there is no record of a carcinogen which does not give rise to tissue-bound materials. Miller and Miller[62] concluded that formation of protein-bound derivatives may be a necessary, but not sufficient, condition for the production of the neoplastic transformation. In other words, the bound carcinogen may simply be an initiating step which must be followed by subsequent reactions controlled by and subjected to other conditions before the malignancy may become apparent. A number of laboratories have exerted considerable effort to define chemically the components of protein-bound carcinogen. From the viewpoint of the dye-derivative bound and the nature of the tissue receptor, evidence accumulated that there are actually several such materials. Techniques have not yet been developed which are powerful enough to probe into the nature of the bond between dye and particulate parts of cells, although this may be the area of fundamental importance in which the primary interaction between the carcinogen and the tissue occurs. However, the rapid progress being made in bio- and histochemical methodology may permit such studies in the near future. Examination of the binding of carcinogen to the soluble component of cells, which may reflect in part similar interactions in the particulate phases, has been achieved. Thus, ion exchange chromatography, starch gel and column electrophoresis have provided a few leads.[79, 91, 93] By the latter technique, Sorof and co-workers[79] have demonstrated bound azo dye in a discrete number of fractions which they labeled "slow h_2 proteins." These proteins exhibited initial increases in amount followed by a progressive decrease during the precancerous period. The induced tumors were devoid of this class of proteins. Hence, they seem to show the behavior expected during carcinogenesis in the light of the protein deletion hypothesis. Further refinements of these techniques are in order so that the exact nature of the materials involved can be determined. Once individual proteins containing bound dye are isolated, determination of their biologic function and chemical structure[29, 31] may eventually lead to a fuller understanding of the phenomenon of protein binding. In the meantime, examination of the cruder protein fractions after hydrolysis suggested, as may be expected, the presence of many amino acids. Terayama and co-workers[42] concluded that the azo dye may be bound through tyrosine. However, Hughes[37] decided from his experiments involving administration of carbon-14-labeled amino acids followed by isolation and hydrolysis of the protein-bound dye that probably tryptophan, valine, methionine, or cysteine but not tyrosine were the amino acids in-

volved in the protein-bound dye. In regard to the dye component bound, several investigators isolated methylaminoazobenzene and a "polar dye." Other experimental evidence indicates that the binding may occur either through the 2-position or the N-methyl group or both. Recently Miller and Miller[62] reported that the bound dye may contain sulfur, possibly from a sulfur-containing amino acid. In any case, neither the protein part nor the azo dye part nor even a proximate carcinogen derived from the azo dye have been unambiguously identified. Active work in a number of laboratories may shed more light on this problem in the future.

Aromatic amines and derivatives

Although there was slight evidence, based on four cases, for the carcinogenicity of some aromatic amines toward the human bladder as early as 1895 ("aniline cancer"), it was not until 1938 that Hueper[35] and associates definitely proved 2-naphthylamine to be a bladder carcinogen in the dog. From that date onward, the carcinogenicity in certain species of other aromatic amines such as benzidine, xenylamine (4-biphenylamine), 2-fluorenamine, 4-stilbenamine, some derivatives of these compounds, and closely related chemicals (Fig. 3) was discovered.[56a] In certain cases, the recognition of these effects came only after a study of epidemiological and occupational[35] data* which were then confirmed in the laboratory. Fortunately, the powerful carcinogenicity of 2-fluorenamine and its acetyl derivative was revealed during chronic toxicity tests prior to their use as insecticides, and, therefore, these compounds were never employed commercially. Other aromatic amines, however, were materials of some importance as intermediates in the dyestuff, rubber, and related industries. Some of the exposed workers developed bladder cancers after a long latent period.

Experimental application of most car-

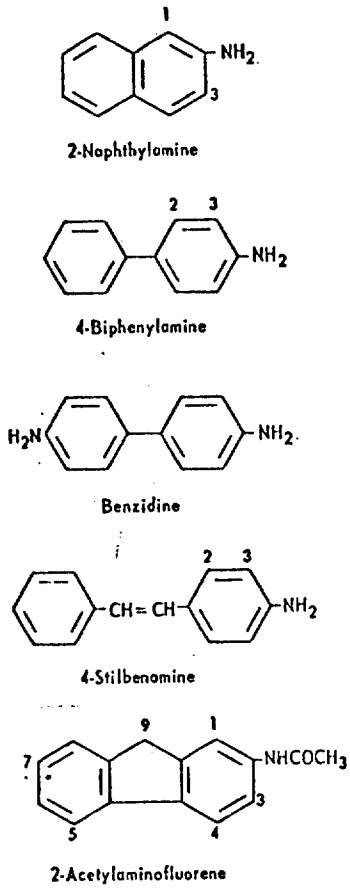

Fig. 3. Formulas of some carcinogenic aromatic amines or derivatives.

cinogenic aromatic amines (as such, or as acetyl derivatives) by skin painting or intraperitoneal or subcutaneous injection in suitable animals rarely results in tumor formation at the point of application (except for 4-stilbenamino derivatives), but instead gives rise to visceral tumors at characteristic sites. From the fact that tumors are generally *not* produced at the point of entry, the tentative conclusion is drawn that the materials themselves are not really the proximate carcinogen, but require metabolic activation.

With most of the aromatic amines, continuing oral administration is considered to be the most effective way of eliciting the carcinogenic action. Industrial exposure and absorption of these materials presumably arises by the percutaneous route,

*See article by Hueper, Clin. Pharmacol. & Therap. 1:776-814, 1962.

but under certain hygienic conditions entry via the respiratory and oral passages may also occur. In rats and mice the carcinogenic amines most often affect the liver. Some compounds also cause tumors in the intestines, bladder, kidney, ear duct, or mammary gland, and, indeed, a few compounds are effective in the extrahepatic tissues only. Under suitable conditions, the more active compounds will lead to tumor formation in as little as 3 to 4 months. The less potent materials may require as long as 18 to 24 months, which sometimes complicates the interpretation of the results because of the occurrence of spontaneous tumors at similar sites. As in the case of the azo dyes, the carcinogenicity of individual aromatic amines varies a great deal from species to species. Some of the divergence is ascribable to the specific metabolic pathways undergone by the compound in a given animal species. In other cases, the differences are more deep-seated and rest on the genetic environment of the host. Specific nutritional factors appear to play less of a role than in the case of the azo dyes. However, the operation of certain dietary influences can alter somewhat the outcome of the carcinogenic process.

In view of the production of bladder tumors by several of the aromatic amines, Jull, Bonser, and others have made use of the original technique of implanting a compound in a pellet of cholesterol or of paraffin wax in the bladders of mice or rats.[7a, 8] These methods have been applied by Boyland[8] and co-workers in London, and Price and associates[10] at the University of Wisconsin. Thus, compounds derived from aromatic amines could be tested directly on the bladder epithelium. Such experiments theoretically permitted discrimination between materials that are direct acting or which might normally reach the bladder after prior metabolism in the liver and other organs. A considerable body of information was accumulated regarding the carcinogenicity of these compounds under these precise conditions. However, some drawbacks are inherent in this procedure: (1) Tumor formation cannot be readily detected and animals must be killed for autopsy at arbitrary times before maximal effects have taken place. (2) Control animals bearing the vehicle alone also show papilloma and carcinoma formation and bioassay of rather weak materials is difficult. (3) Elution of the test material from the implanted pellet occurred rather more rapidly than may have been expected,[19] and hence exposure of the bladder may be only temporary with some of the compounds. With powerful carcinogens and materials of low diffusibility, this technique has, however, given some useful leads.

Carcinogenicity and chemical structure. Whereas 2-naphthylamine is a powerful carcinogen, the 1-isomer is much less active. Similarly, in the biphenyl series, the 4-amino derivative exhibits appreciable carcinogenicity whereas the 3- and 2-isomers are much less or even not carcinogenic.[60] With the interesting carcinogen, 2-acetylaminofluorene,* the isomeric 1- and 3- compounds are not as active,[67] while 4-acetylaminofluorene is not carcinogenic at all.[67, 75] With other ring systems, 2-anthramine, 2-phenanthrylamine,[64] 4-stilbenamine are all carcinogenic.

All these aromatic amines have in common the fact that the amino group is located in the *para* position to the biphenyl link or the ring annelation. A rational explanation for these structure-activity relationships is presently not available. It must be concluded, however, that an amino group at such positions in a polynuclear ring system may very likely confer carcinogenic properties upon a compound. Thus, due care in handling these materials is indicated even if their actual potency has not yet been evaluated. Furthermore, use as organic intermediates, especially in commercial applications of compounds with structures other than those with the amine group in this preferred position, is recom-

*Chemical Abstracts nomenclature: N-2-fluorenylacetamide.

mended. Very likely, the ingenuity of the organic chemist will devise ways of obtaining final products with desired properties and yet take into account presently available knowledge on structure versus carcinogenicity. Such caution seems especially indicated if aromatic amines are used to synthesize azo dyes which may gain entrance to the human organism, since these dyes can be reduced back to the original amines in vivo.

The bioassay of numerous derivatives and analogs of the carcinogenic aromatic amines has provided some leads and criteria for activity in this series of compounds. Thus, substitution of a methyl, a methoxy group, or of a fluorine atom often results in the maintenance or even in an increase of the carcinogenic activity of the basic compound. This is especially so when the substituent blocks an open *para* or an extended *para* position. However, methyl derivatives sometimes are less active, perhaps owing to metabolic detoxification to an inactivating carboxyl residue. Ring hydroxy derivatives are usually much less active because they undergo ready elimination after conjugation with sulfuric or glucuronic acid. An apparent exception is the activity of such materials by the bladder implantation test, discussed previously, when the hydroxy group is *ortho* to the amino group. Under those conditions a number of compounds such as 1-amino-2-naphthol, 2-amino-1-naphthol, and even naturally occurring metabolites of tryptophan (3-hydroxykynurenine, 3-hydroxyanthranilic acid) have induced bladder tumors.[5] Insertion of the heavier halogen atoms, chlorine, bromine, and iodine, often results in a decrease or loss of activity. In some instances this may be due to the less soluble nature of the resulting compounds, which cannot be absorbed from the gastrointestinal tract. Additionally, the larger size of the halogen, especially bromine and iodine, may increase the thickness of the molecule to such an extent that it cannot fit into the specific tissue receptor site and thus initiate the carcinogenic reaction. A possible exception is 3-iodo-2-acetylaminofluorene,* which induced ear duct tumors in rats.[67] Addition of a second amino group, as in benzidine[68] or 2,7-diacetylaminofluorene,†[68] gave a more active material.

In most species the mono- or diacetyl derivatives or the N-methyl or N,N-dimethyl derivatives exhibit activity approximating that of the free amines. These substituents on the nitrogen can be readily removed by the host animal by hydrolytic or oxidative mechanisms. However, substituents which are not subject to biochemical elimination such as the benzoyl or tosyl derivatives usually are only slightly active or inactive. It can be concluded that to be carcinogenic, aromatic amines must have substituents on the nitrogen which are biochemically labile.

A recent study describes the carcinogenicity of 2-acetylaminofluorene bearing a fluorine substituent at each of the seven free ring positions.[54] All of these compounds were active to some extent in the rat to at least one organ. If later tests also show carcinogenicity in a 1,3-difluoro compound, there is perhaps no requirement for a free ring position in 2-fluorenamine, in contrast to azo dye, where the 2-position was involved. This has a bearing on concepts of the structure of the proximate carcinogen bound to the sensitive tissue receptor.

Factors affecting the carcinogenicity. Although the carcinogenicity of aromatic amines such as 2-naphthylamine, benzidine, or 4-biphenylamine in animals has provided the experimental evidence needed to implicate these materials in the occurrence of occupation-connected cancers in humans, these compounds have found relatively less use in fundamental studies. On the other hand, 2-acetylaminofluorene which has had no commercial application has undergone intensive study in many

*Chemical Abstracts nomenclature: *N*-(3-iodo-2-fluorenyl)acetamide.

†Chemical Abstracts nomenclature: *N,N'*-2,7-fluorenylenebisacetamide.

laboratories. Thus, the literature contains a large body of data on the effect of many substances on the carcinogenicity of this compound. The interested reader is referred to recent reviews on this subject.[4, 62, 90] Only a few pertinent phenomena will be discussed here.

2-Acetylaminofluorene is an unusual carcinogen in that it causes tumors at a great diversity of sites in a sensitive species. Slight variation in the structure of the compound sometimes reinforces the carcinogenicity at certain sites and abolishes it at others. Thus, in rats the closely related 4-acetylaminobiphenyl cannot produce liver tumors but does give rise to mammary tumors and, interestingly, uterine tumors in female rats.[69] The joint administration of 2-acetylaminofluorene and 4-acetylaminobiphenyl* has a simple additive effect. On the other hand, the combined intake of tannic acid and 2-acetylaminofluorene has a synergistic effect on the liver.[69]

Additional tryptophan in the diet of certain strains of rats receiving 2-acetylaminofluorene appears to decrease the incidence of liver tumors and lengthen the latent period of their occurrence while simultaneously leading to cancers of the bladder. This interesting redirection of the carcinogenicity has received considerable attention without receiving a clear-cut explanation.[90] It is possible, however, that the formation of bladder tumors in the presence of tryptophan plus carcinogen may reflect the delayed formation of liver tumors (average survival 400 days) which permits carcinogenesis to occur in extrahepatic organs, such as the bladder, in a sensitive strain. Without the additional tryptophan, most of the rats die of hepatoma after a relatively short latent period (283 days). Other dietary factors exert relatively little influence on the carcinogenicity of 2-acetylaminofluorene except the well-known and generally applicable inhibition owing to direct or indirect dietary restriction, as developed by Tannenbaum and discussed by Kraybill.*

Chronic administration of methylcholanthrene and certain other carcinogenic or noncarcinogenic polynuclear hydrocarbons together, with 2-acetylaminofluorene has a dramatic and unexpected inhibiting effect on the carcinogenicity at all sites owing to alteration of the metabolism of the compound (see below).[55]

Exogenous and endogenous hormonal factors exhibit appreciable and specific effects on the carcinogenicity of 2-acetylaminofluorene and probably also of other aromatic amines (although experimental evidence for the latter are rather scanty). Thus compounds such as 2-acetylaminofluorene or 4′-fluoro-4-acetylaminobiphenyl† lead chiefly to liver cancers in male rats and mammary cancers in female rats. Some of the neoplasms in other tissues such as ear duct or Harderian gland have not been definitely sex-linked. Carcinogenesis in the liver of male and female animals is accelerated by the addition of androgen, estrogen, and of anabolic hormones.[4, 21] Adrenalectomy appears to abolish the carcinogenicity of 2-acetylaminofluorene for liver, especially if regrowth of adrenal accessories can be prevented.[72] However, addition of testosterone to adrenalectomized animals fully compensates and in fact reinforces carcinogenicity to the liver. Likewise, administration of cortisone results in potentiation either in adrenalectomized[21] or in normal animals.[32] Other alterations of the hormonal status of the host have pronounced effects in the outcome of the experiments. Thus, thyroidectomy completely abolishes the sensitivity of the liver to 2-acetylaminofluorene.[4] Along similar lines, administration of thiouracil also protects the liver but in turn directs the carcinogenic effect to the thyroid gland.[71] Removal of the pituitary gland similarly inhibits the sequence of

*Chemical Abstracts nomenclature: 4-phenylacetanilide.

*See article by Kraybill, page 73.

†Chemical Abstracts nomenclature: 4-(p-fluorophenyl) acetanilide.

steps leading to carcinogenesis in liver and mammary gland,[50] but with 2-acetylaminofluorene neoplasms still appear in the ear duct. Thus, disturbance of the hormonal pattern had a selective effect. Bielschowsky[1] believes that thyroidectomy acts indirectly via atrophy of the acidophil elements in the pituitary, which apparently play a role in the carcinogenic process. Partial restoration of the carcinogenic effect was secured by administration of growth hormone to hypophysectomized animals. In a naturally occurring genetic variant of the hypophysectomized animal, the pituitary dwarf mouse, which suffers from multiple endocrine deficiencies, the carcinogenic process was greatly slowed down as compared to normal equivalents.[6] Alloxan diabetic animals also are less responsive to the carcinogenic effect.[5] It is evident from the few examples cited that hormonal factors play an important role in the carcinogenic process initiated by 2-acetylaminofluorene and probably also in the case of other aromatic amines:

It is well known that the entire organism is in a state of delicate homeostatic balance with respect to endogenous hormonal factors. This equilibrium is a function of age, sex, genetic constitution, diet, and other environmental factors. Alteration in any one of the hormonal levels by whatever means is followed by attempts on the part of the organism to restore the balance to its original physiologic level. The success achieved depends on the severity of the factor causing the original imbalance. Failure to regain normal conditions and prolongation over an extended period of time of hormonal imbalance by itself has given rise to tumor formation in certain hormonal target organs. Furthermore, in the elegant metabiotic experiments of Bielschowsky,[1] artificially induced abnormal levels of endogenous factors have redirected the carcinogenicity of 2-acetylaminofluorene to target organs of the hormones, as the ovary in females or as the seminal vesicles in males. In this case, the presence of the chemical carcinogen no doubt accelerated the process, for the induction of this type of tumor can be achieved by hormones alone.[23, 38, 45] However, presently known hormones are not necessarily always involved in the development of the neoplastic process. Thus, Lorenz and Shimkin[44] observed similar patterns of carcinogen concentration in the lungs of strain A and strain C57bl mice even though the former strain was much more susceptible to the induction of pulmonary tumors.

The few examples illustrate the close cooperation between endogenous or exogenous hormonal or other growth factors and chemical carcinogens in the production of neoplasia. Present concepts suggest that the chemical carcinogen plays an important role in the formation of "latent tumor cells." Further growth and progression of these is controlled by various hormonal or other endogenous factors. Optimal induction of neoplasms at selected sites appears to require the concerted action of both chemical carcinogen and endogenous growth factors. Carcinogenesis of the mammary gland has been considered in such terms by Bonser and associates.[7b] In special experimental designs, tumors were developed by croton oil,[73c] Tween 60, carboxymethylcellulose, or carrageen,[86b] in mice or rats fed 2-acetylaminofluorene, at the sites treated by these varied "promoting agents."

Prevention of the neoplastic reaction can conceivably be achieved by the elimination of chemical carcinogens from the environment, by redirection of their metabolic pathways, or by control or modification of growth-promoting factors in the host. Considerable efforts are being exerted at the present time to study these phenomena in greater detail and to obtain insight into their operation at the molecular level.

Metabolism studies. Basically, the carcinogenic aromatic amines undergo similar metabolic reactions in vivo and in vitro to those of any other aromatic compound.[82] These biochemical reactions include ring hydroxylation at selected positions and conjugation of the phenolic products with

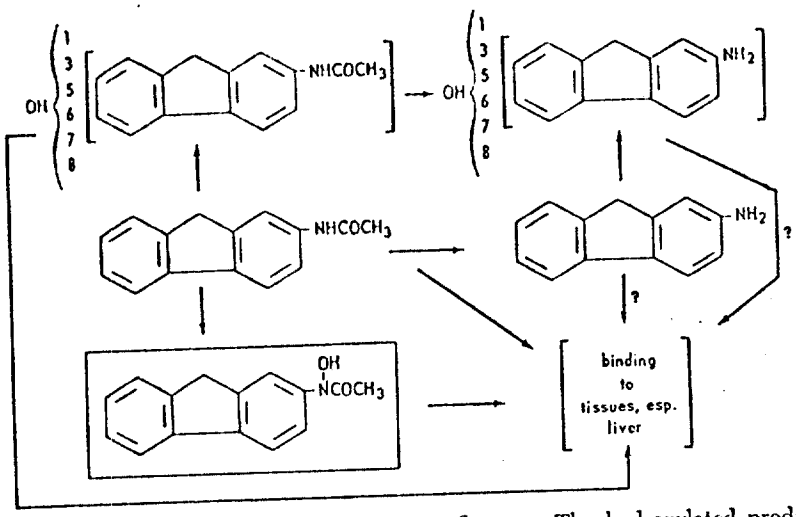

Fig. 4. Main metabolites of 2-acetylaminofluorene. The hydroxylated products occur in urine chiefly as glucosiduronic acids, the 7-hydroxy compound also as a sulfuric acid conjugate (see text).

sulfuric and glucuronic acid; reversible acetylation of the amino group (except in dogs which only deacetylate); oxidative removal of any alkyl amino group; formation of mercapturic acids or analogous sulfur derivatives; potential formation of epoxy type compounds; oxidation or hydroxylation on the nitrogen, and combination of some of these intermediates with tissue components such as proteins, nucleoproteins, or nucleic acids.

Some 19 metabolites of 2-naphthylamine have been identified in various quantitative relationships depending on the animal species used.[8] The more prevalent reaction consists in hydroxylation at the 6- or at the 1-position. The former occurs chiefly in species immune to the carcinogenic effect, whereas the latter is found more in sensitive species. Bioassay of the fundamental structures involved, namely, 2-amino-6-naphthol and 2-amino-1-naphthol by the bladder implantation test, has demonstrated the absence of carcinogenicity with the 6-hydroxy derivative while some activity was attached to the 1-hydroxy compound. This might be a proximate agent derived from 2-naphthylamine, itself inactive in this test. More recently, hydroxylation on the nitrogen has been observed[58] and the corresponding 2-naphthylhydroxylamine has been found carcinogenic.* The oxidative combination of 2-amino-1-naphthol with protein and bladder constituents has been demonstrated.[3]

As is true with biologic studies, the biochemical transformations of 2-acetylaminofluorene have undergone more penetrating scrutiny than those of most other carcinogens. Briefly, the chief reactions include hydroxylation at the various ring positions (Fig. 4) followed by conjugation with sulfuric or glucuronic acid. Furthermore, an important new reaction discovered in the laboratory of the Millers,[59] consists in hydroxylation of the amino nitrogen. Interestingly, *N*-hydroxy-2-acetylaminofluorene† occurs in the urine in very small amounts after a single dose of the parent compound. However, upon continued intake of acetylaminofluorene, the amount of *N*-hydroxy derivative increases until it amounts to about 20 per cent of the dose in rats. In rabbits, even a single dose gives rise to some *N*-hydroxy derivative but re-

*Private communication from Dr. E. Boyland, London, and Dr. D. Clayson, Leeds (cf. Brit. Emp. Cancer Campaign 39:81, 1961).

†Chemical Abstracts nomenclature: *N*-Hydroxy-2-fluorenylacetamide.

peated dosing gives rise to increasing quantities finally contributing 30 per cent of the dose.[39] Recent studies with 4-biphenylamine,[65] 2-naphthylamine, and other aromatic amines have shown that *N*-hydroxylation is not restricted to 2-acetylaminofluorene but seems to be a general reaction occurring with substantially all aromatic amines[41] under the proper conditions.

The proportion of hydroxylated metabolites produced depends on the species. Thus, guinea pig urine contains mainly the 7-hydroxy derivative of 2-acetylaminofluorene,[59] rabbit urine, the 7-hydroxy and *N*-hydroxy derivatives; whereas rat urine contains all of the hydroxylated metabolites in varying proportions. The data suggest that there are at least three different enzyme systems concerned with these hydroxylations. One would affect chiefly the 7-position (an extended *para* position). This is the only system present in the guinea pig. Another, apparently inducible and present in rats, rabbits, and other susceptible species, would affect hydroxylation on the nitrogen. A third enzyme system would be concerned with hydroxylation at the *ortho* positions, that is, on carbons 1, 3, 4, 5, and 8, available mainly in the rat. Only the 7-hydroxy derivative, of all these hydroxylated metabolites, is excreted as a sulfuric acid ester; more of this conjugate is formed in the rat than in the rabbit. All the other hydroxylated derivatives are eliminated as glucuronides. Urine contains rather small amounts of the free compounds.[58, 59]

Bioassay of certain of these hydroxylated metabolites in rats, the species most sensitive to the carcinogenic effect of 2-acetylaminofluorene, has shown that the 1-, 3-, 5-, and 7-hydroxylated derivatives are inactive or certainly much less active than the parent compound.[57] However, the *N*-hydroxy-2-acetylaminofluorene not only is more carcinogenic than the precursor but also causes sarcomas at the point of injection.[57] It would appear, therefore, that this material (see box in Fig. 4) may be considered to be a proximate carcinogen derived from 2-acetylaminofluorene. Additional evidence for this concept can be found in the fact that rats treated jointly with 3-methylcholanthrene and 2-acetylaminofluorene, by which carcinogenicity is inhibited, do not form appreciable amounts of the *N*-hydroxy derivative. However, animals treated with methylcholanthrene and the *N*-hydroxy derivative do undergo carcinogenesis.[59] Administration of the *N*-hydroxy compound to guinea pigs, which cannot synthesize it, leads to tumor formation in this resistant species, albeit after a long latent period. Furthermore, subcutaneous injection of the insoluble copper chelate of the *N*-hydroxy compound, a procedure giving slow release of the active material, gave rise to sarcomas at the site.[57]

By means of isotope techniques the binding of radioactivity from labeled 2-acetylaminofluorene to a variety of tissue proteins could be demonstrated. The amount of activity bound was high in the rat, low in the guinea pig, intermediate in some other species, thus paralleling the relative carcinogenicity of the compound in these species,[55] and similar to the findings made with the carcinogenic azo dyes. However, radioactivity was bound to some tissues (e.g., heart) of the rat which rarely if ever undergo carcinogenesis.[60] Furthermore, the liver proteins of young female rats contained similar levels of bound isotope to those of male rats although after sexual maturation the males exhibited slightly higher levels than the females.* Hypophysectomized* or thyroidectomized[60] rats had liver proteins with similar although not identical amounts of carbon[14] as compared to their controls, indicating perhaps that in this system the active carcinogen combined with the proper tissue receptor to form the latent tumor cell. However, absence of growth promot-

*Weisburger, J. H., and Weisburger, E. K.: Endogenous and exogenous factors in chemical carcinogenesis by *N*-2-fluorenylacetamide, Acta Unio internat. contra cancrum (in press). Manuscript submitted for publication.

ing factors may account for the failure of neoplasia to develop. Indirect but persuasive evidence on this point is supplied also by the stimulation of amino acid incorporation into liver microsomes, elicited by 2-fluorenamine, which was not present in adrenalectomized rats.[4b] Thus, the increased protein synthesis called for by the carcinogen was controlled by some hormonal factors.

Current studies along those lines aim to refine the processes and attempt to gain an understanding of the exact mode of combination between carcinogen and tissue. This involves determination of the chemical structure of the compound bound. Present evidence indicates that several materials derived from 2-acetylaminofluorene are bound. It also involves the tissue receptor affected. Studies thus far indicate that a number of different proteins,[78, 79, 86, 87] RNA (ribonucleic acid),[53] and perhaps even deoxyribonucleic acids are involved. Not all of these combinations may be specifically related to the carcinogenic process.

It will be important to discover the interaction characteristic of the neoplastic reaction.

Nitrosamines

Carcinogenicity and chemical structure. During a study of factors possibly responsible for liver damage in industrial workers, Magee and Barnes[48] quite recently discovered that feeding dimethylnitrosamine, a useful organic solvent, to rats led to development of hepatic tumors. Further experiments showed that this compound could also induce lung and kidney tumors.[1a, 49, 76, 95] Upon investigation of the activity of analogs, diethylnitrosamine,[1a, 14, 81] dipropylnitrosamine, and diamylnitrosamine[16, 18] were also found to be carcinogenic, usually not at the point of application. Diethylnitrosamine interestingly caused lung tumors in hamsters but only inflammatory changes in the lungs of rats.[15]

Guinea pigs were recently found highly susceptible to the carcinogenic effect of this compound.*

In addition, *N*-nitroso derivatives of piperidine, piperazine, morpholine, and *N*-methyl-*N*-nitrosoaniline also possessed this carcinogenic potency.[17, 18] However, *N*-phenyl-*N*-nitrosoaniline (diphenylnitrosamine),[1a] diisopropylnitrosamine, and dimethylhydrazine,[1a] a possible reduction product of dimethylnitrosamine, had no activity. Certain related compounds, *N*-nitroso-*N*-methylurea or *N*-nitroso-*N*-methylurethane[19, 57] led to production of carcinoma of the forestomach in rats, a relatively rare lesion. Simultaneous administration of both dimethylnitrosamine and 4-dimethylaminoazobenzene had an antagonistic effect and inhibited liver cancer formation.[42] Possibly intermediates derived from one compound adversely affected the "activation reaction" of the other.

A naturally occurring product, consumed by the inhabitants of Guam and other Far Eastern regions, is the cycad nut *(Cycas circinalis).* These nuts contain glucosyloxyazoxymethane, a structure closely related to the dialkylnitrosamines.[73b] Administration of diets containing 1 per cent of the nuts to rats gave rise to a high yield of hepatomas and renal tumors.† It would seem important to confirm the locally prevailing notion that an above-average incidence of hepatomas occurs in natives of the Guam area.

Metabolic Studies. Even though the carcinogenicity of dimethylnitrosamine and related materials was discovered a short time ago, considerable progress was made in elucidating their fate in animals and possible mode of action. The metabolism in rats and mice was followed by means of a polarographic method,[16] and by means of C^{14}- and N^{15}-labeled material.[50] Much of the isotope from C^{14}-dimethylnitrosamine

*See reference to Argus and Hoch-Ligeti, footnote page 111.

†Personal communication from Dr. G. Laqueur NIAMD, Bethesda, Md. (Cf. 4th Congress, Intern. Acad. of Pathology, Zurich, July, 1962; Pub. Health Rep. 77: 615-616, 1962.)

was respired as carbon dioxide and the remainder was distributed throughout the tissues. With N^{15} as the label the various liver cell fractions (nuclei, mitochondria, microsomes, liver protein, and nucleic acids) were evenly but only slightly labeled. There was a large concentration of N^{15} in urinary urea and free bases. These data indicated the major part of a dose was degraded and only small amounts were incorporated into tissues. Further studies on the effect in vivo showed that it lowered the incorporation of C^{14}-labeled amino acids into liver proteins by about 50 per cent but not into kidney and spleen proteins.[47] Recently Emmelot and co-workers,[20, 66] demonstrated that cysteamine or cysteine prevented this effect on amino acid incorporation.

The incorporation of P^{32} into various phosphorus-containing fractions of rat liver, except for ribonucleic acid, was not significantly altered.[47] Thus, it appeared that dimethylnitrosamine interferes in protein synthesis in the liver cells, probably as a result of the production of fraudulent ribonucleic acid (see below).

In vitro studies demonstrated further that metabolism of dimethylnitrosamine occurred only in liver slices or homogenates in the presence of oxygen, and not in other tissues.[52] The microsomal and soluble fractions of liver were both necessary for the metabolic breakdown. Dialysis of homogenates removed the activity but addition of tri- or diphosphopyridinenucleotide, especially the former, partially restored the ability to metabolize the compound. This behavior is in accord with that expected for an oxidative demethylation.

The results of these investigations led to the premise that dimethylnitrosamine was demethylated in vivo, with the formation of an active methyl compound, perhaps diazomethane. Magee and associates[51] made the important observation that methylated histidines were formed in the protein of rat liver slices incubated with dimethylnitrosamine. Furthermore, an unnatural purine, namely 7-methylguanine, appeared

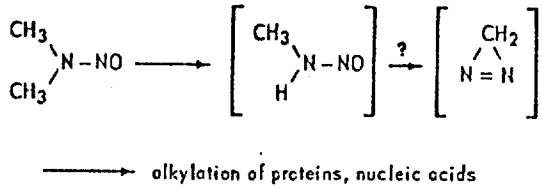

Fig. 5. Postulated intermediates in the metabolism and activation of dimethylnitrosamine, giving materials capable of alkylating proteins and nucleic acids.

in the liver ribonucleic acid of rats injected with C^{14}-labeled material.[50] Considering that diazomethane was carcinogenic,[77] one explanation (Fig. 5) for the carcinogenicity of dimethylnitrosamine could be that it is converted to an active methyl compound (although not necessarily a diazoalkane) which leads to synthesis of an abnormal ribonucleic acid. Thus the genetic mechanisms and cellular information transfer processes are altered. Host response to these abnormal conditions may eventually result in tumor development. Other theses may be evolved with time, but this concept seems in accord with the observed facts.

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Observations on the Detection of Dimethyl- and Diethyl-nitrosamines in Foods with Particular Reference to Alcoholic Beverages

239

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Simple polarographic and gas chromatographic methods commonly used for the detection of nitrosamines in model systems have been found to be inadequate when applied to a potable spirit. Although these methods indicated the possible presence of nitrosamines in the spirit, gas chromatography combined with mass spectrometry showed that compounds other than nitrosamines were responsible for the putative chromatographic peaks. After isolation by preparative gas chromatography, the compound responsible for the polarographic activity, interpreted as nitrosamine in the unconcentrated spirit, was shown to be furfural.

Introduction

NITROSAMINES are now well established as carcinogenic compounds[1] and, because of their possible formation from secondary amines, much experimental work has been devoted to their detection in foods. Their presence has been suggested in fish,[2] fish meal,[3,4] wheat kernels,[5,6,7,8] dairy products[4,8,9,10] and meat,[2,3] as well as in products of the Maillard reaction,[11] with consequent implications for cooked or processed foods.

Much of this work, with some exceptions,[12,13] is based on polarographic, colorimetric and chromatographic methods. The first of these methods leaves much to be desired from the point of view of specificity, and even gas chromatographic evidence based solely on reference compounds is doubtful. When approaching levels of 1 ppm or less, at which nitrosamines are claimed to be a significant health hazard, the number of volatile constituents of foods is very large,[14,15] and it is highly probable that more than one substance can be found with a retention time similar to any nitrosamines so far postulated, particularly if no preliminary selective separation has been carried out. Positive identification of the substances responsible for such peaks can be obtained by combining gas chromatography with mass spectrometry, and this technique has been applied to the analysis of a British potable spirit for dimethyl- and diethyl-nitrosamines in view of the report suggesting the presence of dimethylnitrosamine in similar African preparations.[16]

Experimental

Techniques

Polarography

Half-wave potentials of 5-ml samples in the presence of 0·2 N-HCl were measured against a saturated calomel electrode using an Evershed pen-recording polarograph.

Gas chromatography and preparative gas chromatography

Gas chromatography (g.c.) was carried out using an F & M 810 gas chromatograph fitted with flame ionisation detectors and a 12 ft × ¼ in copper column packed with 10% Carbowax 20M on 60 : 80 mesh Chromosorb W, and programmed at 8°c/min from 65 to 210°c with a nitrogen flow of 40 ml/min. The instrument for preparative g.c., which was constructed in the laboratory,[17] was fitted with a 20 ft × ¾ in 5% Carbowax 20M column and programmed from 100 to 210°c at 4°c/min., with a nitrogen flow of 500 ml/min. Trapping was accomplished by using specially designed traps.[18]

Gas chromatography–mass spectrometry

An LKB 9000 instrument for g.c.–m.s., equipped with a g.c. column identical to that used for simple g.c. was used for recording mass spectral data. Various chromatographic operating conditions were used, e.g., a temperature programme rate of 6°c/min between 65° and 210°c, and a helium flow of 30 ml/min.

Reagents and samples

Synthetic solutions

Two standards were prepared in ethanol/water (35/65), the first containing 1 ppm each of dimethylnitrosamine and diethylnitrosamine, and the second 2 ppm of each nitrosamine, and in addition palmitic acid (420 ppm) and acetic acid (50 ppm) to provide total acidity and pH comparable to that of commercial spirits. The solutions were concentrated by distillation almost to exhaustion under vacuum (40 torr) and each distillate was collected in an ice-cooled receiver and two solid carbon dioxide–acetone traps in series. Possible photolytic effects were reduced to a minimum by wrapping the apparatus in aluminium foil. The contents of the traps were combined, neutralised with saturated potassium carbonate solution, salted (300 g/l sodium chloride) and extracted once with 300 ml of dichloromethane and then twice with 150 ml. Because of the high ethanol content, distillates had to be diluted with an equal volume of water to prevent complete miscibility with the dichloromethane. The extracts were dried with sodium sulphate (200 g/l) followed by calcium sulphate (200 g/l).[19] The bulk of the solvent was removed through a 60 cm × 2·5 cm fractionating column, packed with stainless steel mesh (Bagg, Cosland Ltd.,) operating under a reflux ratio of 2 : 1; the residue was then transferred to a similarly packed and operated 30 cm × 2 cm column. Both extracts contained large amounts of ethanol which were removed by raising the temperature of fractionation. Light was excluded as much as possible during all stages of concentration and the final concentrates (500 μl) were stored under nitrogen in sealed ampoules wrapped in aluminium foil. The solvents used were all B.D.H. laboratory grade reagents purified by fractionation through the 60 cm × 2·5 cm stainless steel column.

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Commercial spirit

The material used was an authentic potable spirit of 70° proof, purchased through a retailer. It was selected from 12 branded products as possessing the potentially highest nitrosamine content, as indicated by polarography. The spirit was concentrated as above but the distillate was collected in two fractions, the first up to 31°c and the second at 31–32°c (Fig. 1). Each fraction was examined separately. To reduce the number of components recovered by dichloromethane, a preliminary extraction was made with pentane; tests with standard solutions showed that dimethyl- and diethyl-nitrosamine were virtually insoluble in pentane and were extracted almost entirely into the dichloromethane.

Examination of samples

The extracts and samples taken during distillation and extraction were examined by g.c. and polarography. Polarographic examination of the distillates was carried out directly on 5-ml samples; for the extracts the requisite amount of each, determined by its degree of concentration, was first diluted to 5 ml with the appropriate extracted distillate.

Samples of dimethyl- and diethyl-nitrosamine, the extracts of the standard solutions and the dichloromethane and pentane extracts of the commercial spirit were analysed by g.c.–m.s. and mass spectra were recorded. In addition to scanning the visible g.c. peaks of the dichloromethane extracts as they emerged, mass spectra were also recorded continuously over the period when dimethyl- and diethyl-nitrosamine would have appeared, irrespective of whether g.c. peaks were observed or not. The dichloromethane extract which showed most polarographic activity at −0·91V was further examined by g.c.–m.s. with the mass spectrometer set to monitor specifically for the molecular ions of the two nitrosamines. Subsequently, the same extract was subdivided into eluent fractions by preparative g.c., each fraction was regenerated into 5 ml 20% ethanol solution and examined for polarographic activity.

Results and Discussion

Prior to concentration, polarographic examination of the standard solutions of the nitrosamines showed that they could be detected by their half-wave potentials at about 0·91V down to an estimated 1 ppm. Gas chromatographic examination of the same solutions on the Carbowax column showed that the two nitrosamines could be separated satisfactorily with a detection limit of 5×10^{-2} ppm, and without apparent decomposition. By comparing peak heights before and after the distillation, extraction and concentration procedures recovery of the two nitrosamines was shown to be of the order of 20% and 30%, respectively. The presence of the added organic acids had no apparent effect on recovery. Examination of the extracts by g.c.–m.s. showed that both nitrosamines

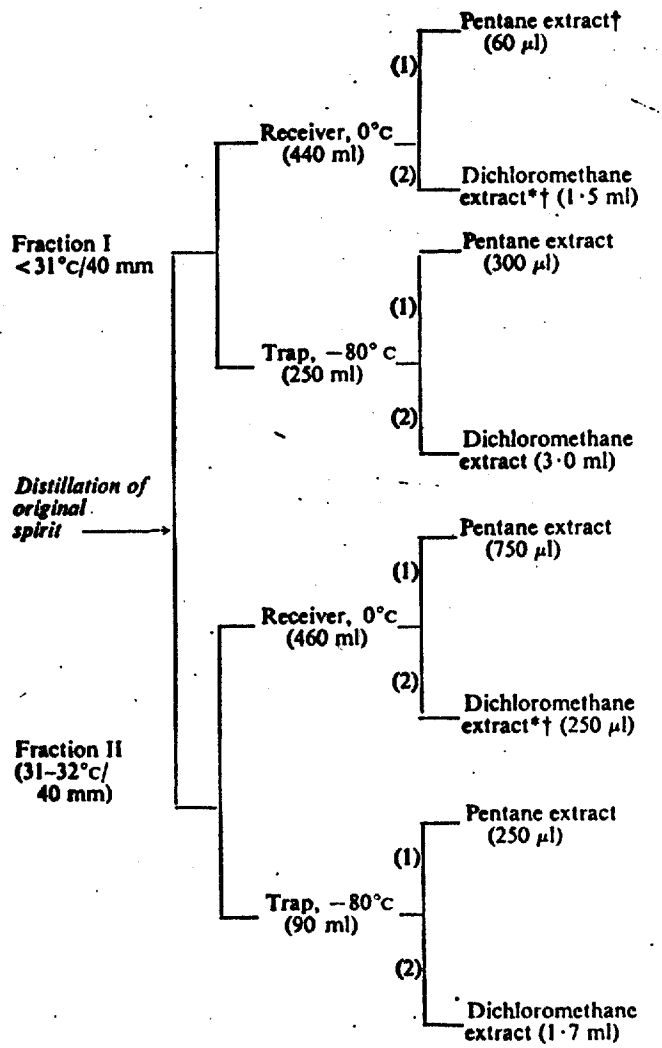

FIG. 1. *Distillation and extraction of original spirit*

(1) and (2) refer to the sequence of extraction
* extracts showing greatest polarographic activity
† extracts examined by g.c.–m.s.

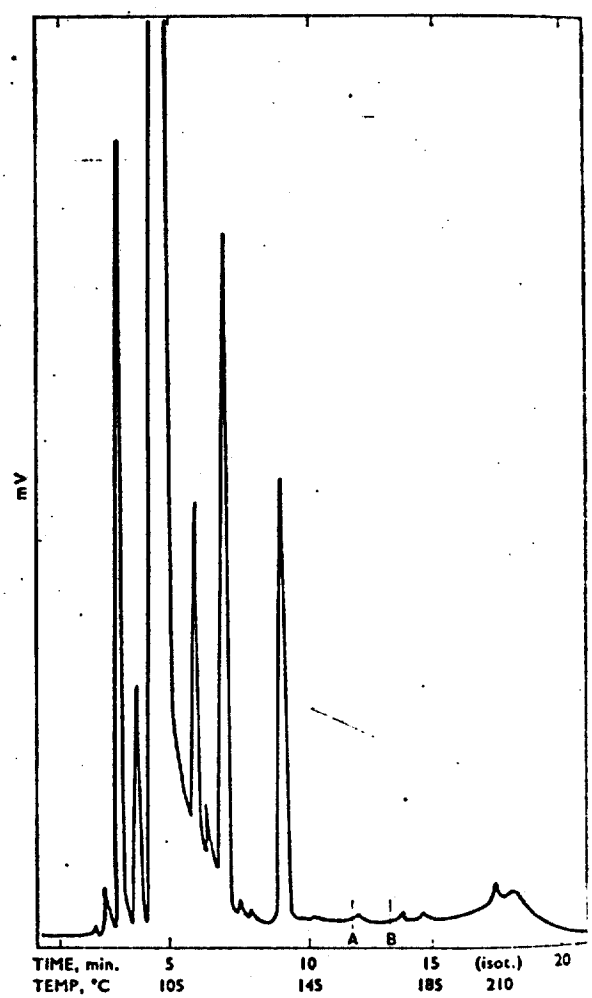

FIG. 2. *Chromatogram of spirit (direct injection)*

A and B indicate the positions at which dimethyl- and diethyl-nitrosamine would appear, respectively. Conditions: 12 ft × ¼ in Carbowax 20M on 60 : 80 mesh Chrom W, temperature programmed from 65–210 c at 8°c/min and with a flow of 40 ml/min. 1 μl sample.

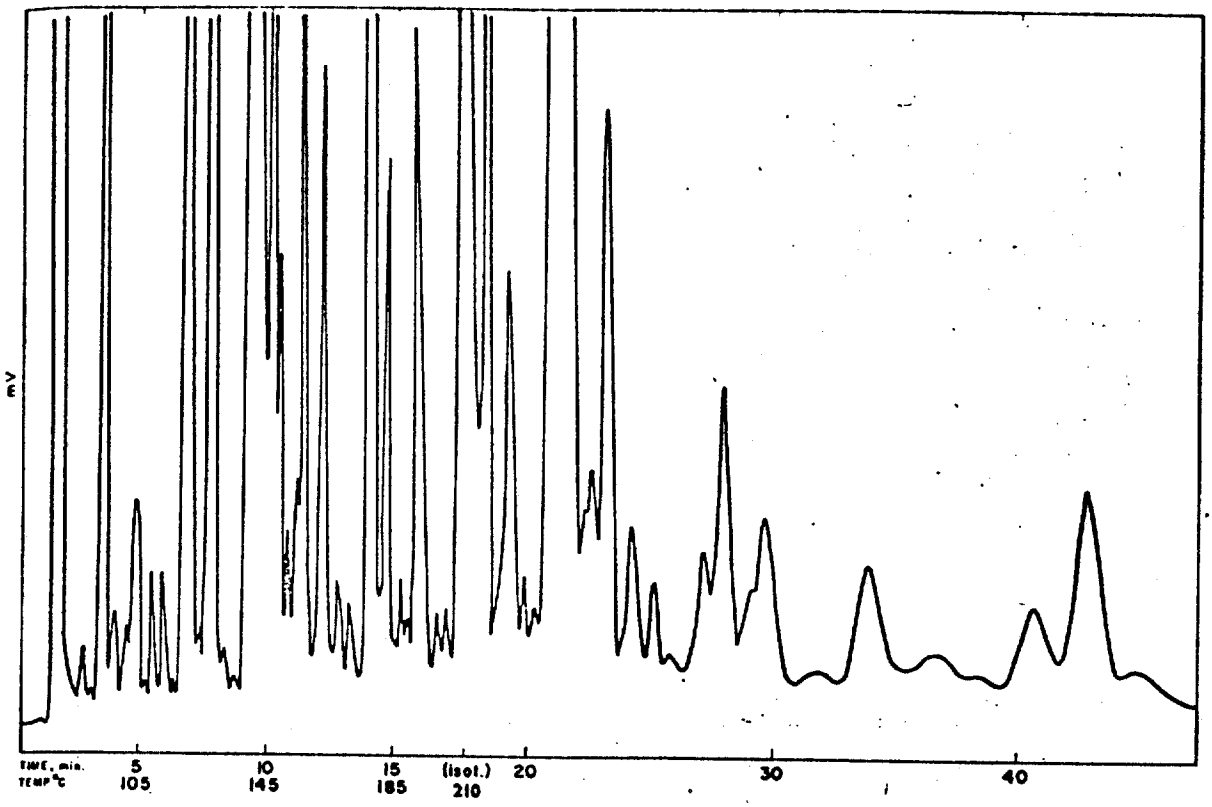

FIG. 3. *Chromatogram of pentane extract from the ice trap of fraction I*

Conditions: As for Fig. 2

ated at their expected retention times and gave good mass spectra, similar to those published.[20] The molecular ions were the most abundant species recorded, with mass-to-charge ratios (m/e) of 74 and 102, respectively. The spectra were recorded with the electron multiplier attenuated by a factor which, considered with the 100-fold sensitivity range of the 3-channel galvanometer recorder afforded theoretical detection limits for the pure compounds of the order of $1 \cdot 10^{-4}$ ppm.

Polarographic examination of the commercial spirit before concentration showed a small current at the same half-wave potential as that of nitrosamines. Interpreting this as nitrosamine, it corresponded to $2 \cdot 7 \pm 0 \cdot 5$ ppm. Examination by g.c. (Fig. 2) showed a trace component with retention characteristics only slightly different from those of dimethylnitrosamine, and calculated to be present at a maximum concentration of 5 ppm in the original spirit. The peak, however, also correlated satisfactorily with hexanol, a known

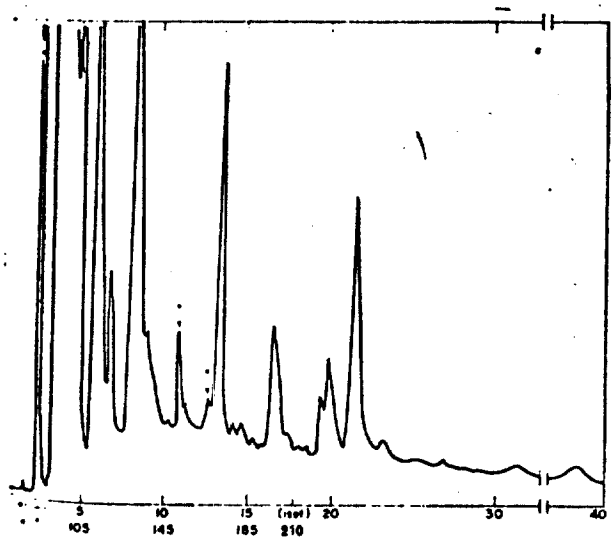

FIG. 4. *Chromatogram of dichloromethane extract from the ice trap of fraction I*

Possible nitrosamine peaks are indicated by *. Conditions: As for Fig. 2

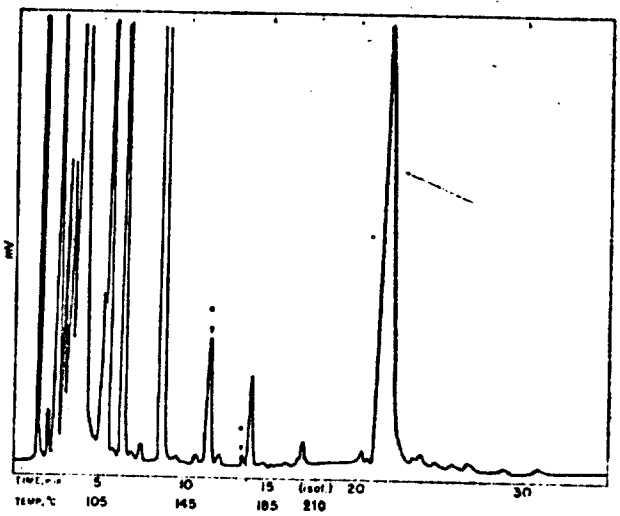

FIG. 5. *Chromatogram of dichloromethane extract from the ice trap of fraction II*

Possible nitrosamine peaks are indicated by *. Conditions: As for Fig. 2

constituent of fermented products, and comparison of the retention behaviour of hexanol with that of dimethylnitrosamine showed that under these particular g.c. conditions, the latter would elute as a shoulder on the leading edge of the hexanol peak. Similarly, another peak, also at approximately 5 ppm, was present in the chromatogram of the spirits which could have been interpreted on the basis of retention data as diethylnitrosamine.

Gas chromatographic examination of the extracts from the spirits (Figs. 3, 4 and 5) showed that the largest number of components occurred in the pentane extract. This preliminary extraction, therefore, considerably reduced the number of volatiles which would otherwise have been extracted by the dichloromethane and hence confused the detection of possible nitrosamines.

No indication of either nitrosamine could be found by g.c.–m.s. in either of the two dichloromethane extracts. The spectra of all other peaks in the extracts were examined for the possible presence of higher aliphatic nitrosamines and nitrosoproline, but again no evidence of these compounds could be found. Hexanol was confirmed as the component detected in the unconcentrated spirit with a retention time similar to that of dimethylnitrosamine, although traces of ethyl lactate were also found in one extract. Ethyl octanoate was identified as the compound with a similar retention time to that of diethylnitrosamine. No mass spectrometric examination was made of the solid carbon dioxide–acetone trap extracts as these gave gas chromatograms which were weaker but similar to those of the other extracts.

During the continuous monitoring of the g.c. effluent specifically for ions of m/e 74 and 102, several responses (peaks) were recorded, two of which occurred at retention times corresponding to dimethylnitrosamine and diethylnitrosamine. Both peaks were extremely small, even with the mass spectrometer operating at maximum sensitivity. Complete mass spectra were recorded immediately and the compounds being eluted were identified as hexanol and ethyl octanoate, respectively. The ion m/e 102 occurred in greater abundance than m/e 74 and was attributed to the ^{13}C isotope fraction of the major ion m/e 101 present in the spectrum of ethyl octanoate. The ion m/e 74 cannot be accounted for specifically in the same way, but it does occur as a very minor fragment ion in the spectrum of hexanol. Even if both ions had been due entirely to the respective nitrosamines, comparison of their intensities with those recorded for the authentic compounds in the control experiment indicated that they could only have represented quantities of the order of 5×10^{-3} ppm dimethylnitrosamine and 5×10^{-2} ppm diethylnitrosamine in the original spirits. These amounts are entirely inadequate to account for the observed polarographic activity of the unconcentrated spirits. After distillation, this activity was greatest in fraction I, although it was also present in fraction II. It was completely removed by the extracting solvents, greatest activity occurring in the dichloromethane extracts.

Polarographic examination of the eleven fractions isolated by preparative g.c. from the extract possessing most activity showed that activity was confined to the sixth g.c. fraction only. Examination of the data from the appropriate region of the g.c.–m.s. analysis of the extract showed that furfural was the major constituent of this fraction. Ultra-violet spectroscopic examination of the fraction confirmed the presence of furfural and the absence of nitrosamines. An authentic sample of furfural was shown to have a half-wave potential of −0·91V, similar to nitrosamines; this similarity has recently been confirmed by other workers.[21]

Conclusion

These results emphasis the limitations and dangers inherent in the use of simple polarographic or gas chromatographic procedures for the analysis of nitrosamines and show that the use of highly discriminating techniques such as gas chromatography–mass spectrometry are essential for the accurate identification of nitrosamines in a multi-component system.

No evidence for the presence of nitrosamine compounds has been found in a sample of commercially produced potable spirits.

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2400

Nitrate in Foods and Its Relation to Health[1]

J. K. WILSON[2]

IN 1943, the author (5)[3] pointed out that nitrates in the food of animals may be reduced by bacteria to nitrites and that these are likely to cause poisoning through combination with the hemoglobin of the blood. The object of this paper is to present data demonstrating that nitrates often are present in many vegetable foods and in concentrations sufficient to cause poisoning, particularly with infants. In this respect they seem to parallel carbon monoxide and hydrocyanic acid.

A few papers dealing with the presence of nitrate in various crops, including vegetables, are available. Some of the pertinent ones were reviewed by Wilson (5) in his paper dealing with nitrate in plants and its indirect toxicity to animals. The nitrate content of such foods as beets, broccoli, cabbage, carrots, celery, and the fruits of cucumber and watermelon was included in his study.

Following his work, Gilbert, *et al.* (2) made analyses of many plants for their content of nitrate and reemphasized the effect of this material on human health, pointing out, as did Wilson (5), that the eating of sufficient food of this type in lethal amounts is not probable with adults. They state that the ingestion of a vegetable dinner using foods containing as much nitrate as was found by them could be considered somewhat hazardous. Recently, Weart (4), working as a sanitary engineer in Illinois, drew attention to a new disease called infant methemoglobinemia which was said to be due to too much nitrate in the drinking water. In Illinois alone 33 cases of the disease with 5 deaths were reported in 1947. A maximum safety limit of 10 parts of nitrate per million of water has been set by certain authorities.

Procedure

The procedure for ascertaining the nitrate content of the various samples of vegetable products was primarily the same as that followed previously (5). A modification consisted in the addition of activated charcoal to the solution after neutralization with ammonium hydroxide, shaking, filtering, and then comparing the color of the filtrate with the standard. The samples of baby foods and some of the green vegetables were purchased from the local markets in May and June 1948. The data in Table 1 were taken from a previous paper (5). In all cases the content of nitrate is given as parts in a million parts (ppm) of the sap or expressed juice of the plants.

Presentation of Data

The nitrate content of any food is a direct measure of the potential amount of nitrite that may appear, and the toxicity that follows will depend on the rapidity of the reduction. The data in the tables may be us as an indication of what may happen. In Table 1, given the nitrate content of green vegetables grow at Ithaca, N. Y. It can be seen that only tomato contained no nitrate and that 7 of the 14 vegetab contained at least 1,000 ppm in the juice. Broco cabbage, and cauliflower were found to contain 1,0 ppm or more in their juice, and rhubarb contained much as 5,454 ppm.

TABLE 1.—*Nitrate in green vegetables grown at Ithaca, N. Y.*

Vegetable	Nitrate, ppm
Beets	1,333
Broccoli	785
Broccoli	3,846
Cabbage	2,222
Carrots	442
Cauliflower	2,000
Celery	1,212
Cucumber	121
Lettuce (head)	1,250
Lettuce (curly)	870
Muskmelon	433
Rhubarb	5,454 and 1,0
Tomatoes	0
Watermelon	100 and 1,0

The nitrate content of green vegetables purchas from a local market, as well as that of certain "qu frozen foods", is given in Table 2. It is apparent th the nitrate content of different samples of the sa vegetable varies considerably, going as high as ppm for quick frozen spinach. This sample of spin contained only 15.4% dry material so that if the nitr present was expressed as potassium nitrate and th calculated as percentage of the total solids it would equal to 3.84%, or to 3.24% as potassium nitri

The variations in nitrate content of certain vegetab can be seen also from the data listed in Table 3. O

TABLE 2.—*Nitrate in the juice of green vegetables and "quick fro foods" for sale at Ithaca, N. Y., in May 1948.*

Vegetable	Nitrate, ppm
Asparagus	50
Cabbage	1,200
Cabbage	186
Carrots	325
Carrots (diced, quick frozen)	181
Celery (fa)	1,240
Celery (pascal)	3,232
Lettuce (curly)	1,818
Lettuce (head)	400
Potatoes (new)	63
Rhubarb	1,818 and 4,54
Radish	303
Radish	1,176
Spinach	300
Spinach (plant 1)	2,353
Spinach (plant 2)	1,6
Spinach (plant 3)	2,006
Spinach (quick frozen)	3,636

[1]Contribution from the Department of Agronomy, New York State College of Agriculture, Ithaca, N. Y. Received for publication August 14, 1948.

[2]Professor of Agronomy. This paper was prepared shortly before Professor Wilson's death.

[3]Figures in parenthesis refer to "Literature Cited", p. 22.

TABLE 3.—*Nitrate content of strained baby foods obtained on the market.*

Food	Nitrate, ppm
Green beans	37
Beets	333, 750
Carrots	3, 0
Peas	0
Spinach	616
Spinach	833
Squash	0, 0
Tomatoes	0
Vegetable soup	37

sample of strained beets labeled baby food contained 333 ppm, whereas another contained 750 ppm. Likewise, a sample of spinach contained 616 ppm, whereas another contained 833 ppm. This is taken to mean that the nitrate content of any vegetable will vary, perhaps, depending on the amount of nitrate that is accessible to the growing plant.

It was observed that quick frozen spinach had some ice crystals and considerable frost in the package with the green spinach. In one case, therefore, these were allowed to melt and drain away before the tissue was pressed for its juice. The sample of juice finally taken contained 6,666 ppm of nitrate which is equivalent to [illegible] of potassium nitrate on a dry basis.

Discussion

The data presented in this paper record the content of nitrite in many vegetables and vegetable foods that are consumed by humans and carries the idea that each ion of nitrate that was found is a possible source of nitrite that is about ten times more toxic than the nitrate from which it came. If the nitrate ions were not reduced to nitrite, little or no concern should be felt about its concentration in foods or drinking water. Because an unknown quantity of the nitrate may be reduced to nitrite, these data should be of timely importance.

It is apparent from the data that probably most succulent leafy vegetables contain some nitrate in their juice and this is present very likely in proportion to the quantity that was available to the growing plant. The edible parts of spinach, rhubarb, cauliflower, turnips, and cabbage may contain in their juice at least the equivalent of 15,352 ppm of potassium nitrate, which is equivalent to approximately 9.4% of the dry weight. As much as 10.2% (1) and 11.1% (2) of the dry weight of spinach and turnips respectively have been reported. This nitrate may be carried with the leafy material and appear in fresh vegetables from the garden, in those offered for sale on the market, or in strained baby foods. Such quantities may be larger if the vegetables are allowed to wilt some before they are analysed or if their moisture is spontaneously released when the plants are in the field. Many other factors can operate to modify this content of nitrate (1,2), and data indicate that the fruit contains much less nitrate than supporting tissues carry (5). This may be true particularly of tomatoes, squash, ears of corn, green peas, and similar foods. These quantities of nitrate are paralleled by similar quantities in numerous other plants. Mayo (3) reported that potassium nitrate represented 25% of the dry weight of certain samples of corn stalks.

Weart (4) reported cases of hemoglobinemia in infants caused by nitrate in the drinking water and suggested the maximum safety limit of 10 parts of nitrate nitrogen per million parts of water. He pointed out that doctors in Illinois have been advised not to prescribe feeding formulas involving water unless the nitrate content of the water is known to be within this limit. If the formulas contain vegetables such as those analysed and reported on in this paper, it is clear that the nitrates of such vegetables as spinach, lettuce, celery, turnips, and certain others may be far more potent in producing hemoglobinemia than that of the drinking water. Certainly the nitrate in the vegetables would augment that which may be present in the water. It may be suggested that in some cases the nitrate in the vegetable foods may be solely responsible for this ailment of babies and may cause other disturbances not now properly diagnosed, for the nitrate may be 200 times stronger in such foods than that which was set as the safe standard for the drinking water.

More date are needed to appraise properly the effect of nitrate in foods on adults. It is well known that nitrate in feeds is associated with cornstalk disease, oat hay poisoning, borna disease of horses, grass tetany in cattle, and bloat in certain types of animals. Animals that ingest feed containing toxic but not lethal amounts of nitrate may abort the forthcoming offspring. Feeds that contain 1.5% of potassium nitrate were known to effect such a result although few grass symptoms in the mother were observed (2). Calculated on the dry basis, several foods listed in this paper contained as much or more nitrate than that present in feed which was injurious or lethal to animals.

If it is assumed that 1 gram of potassium nitrate ingested at a single meal is toxic, a basis is available for evaluating the effect of nitrate on human adults. Using the findings recorded in this paper it would be necessary to ingest only 3.9 ounces of rhubarb to get a toxic quantity of nitrate. If one uses the findings of other workers and makes similar calculations, about 2.3 ounces of spinach, turnips, or certain other vegetables would supply the toxic quantity. The present practice of applying large applications of nitrate of soda to crops in order to produce succulent material with a bright green color and to obtain heavier yields may be responsible in most cases for the high content of nitrate in these foods.

Summary and Conclusions

Leafy vegetables, frozen foods, and prepared baby foods were analysed for their content of nitrate. From the findings it is suggested that the nitrate in such foods may contribute to hemoglobinemia found in infants and may produce certain toxic, if not lethal, conditions in adults. The high content of nitrate in the foods may be attributed in many instances to the application of nitrogenous fertilizers, especially nitrate of soda, to the growing crops.

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2407

NITRITE POISONING OF PIGS.

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SUMMARY.

Heavy mortality in pigs fed on soup prepared by cooking beef and offal in well water was investigated on two farms. The well water on each farm had a high nitrate content, equivalent to 2970 and 1740 p.p.m. expressed as sodium nitrate. Batches of these soups which were analysed contained nitrites equivalent to 1127 and 393·6 p.p.m. sodium nitrite.

It is considered that the mortality which occurred in pigs on these farms was due to nitrite poisoning and that the nitrite was derived from the nitrate in the well waters.

The toxicity of sodium nitrite for pigs was observed by dosing after 18 hours fasting with a 1 per cent. aqueous solution. Doses of 0·05, 0·06, 0·07, and 0·08 gm. sodium nitrate per kgm. body weight produced moderate to severe non-fatal methaemoglobinaemia. Two pigs weighing 61 and 80 kgm. died two hours after they had been dosed at the rate of 0·09 gm. per kgm.

Soups were prepared in the laboratory by boiling beef, bones and meat scraps in well water from one of the affected farms. The water contained nitrates equivalent to over 2300 p.p.m. sodium nitrate and nitrites equivalent to less than 10 p.p.m. sodium nitrite. In two trials soups containing 1430 and 1245 p.p.m. sodium nitrite were obtained.

See Errata - p. 84

A122819

INTRODUCTION.

The ability of ingested nitrite to reduce haemoglobin to methaemoglobin and thus to impair the oxygen capacity of the blood has long been known. Toxic symptoms appear when about 50 per cent. of the haemoglobin has been reduced to methaemoglobin (Nicholson, 1945).

Poisoning of cattle by oaten hay containing nitrate was described by Bradley, Eppson and Beath (1940) and Davidson, Doughty and Bolton (1941). Williams and Hines (1940) showed that the toxicity of the plant *Salvia reflexa* (mint weed) is due to nitrate. The nitrate is reduced by enzymes in the plant or in the rumen or liver of the animal to the more toxic nitrite. Gwatkin and Plummer (1940) showed that pigs dosed, after 24 hours' starvation, with potassium nitrate solution at the rate of 2·5 gm. of the salt per kgm. body weight suffered no ill effects, while 3·7 gm. per kgm. was fatal. Potassium nitrite given under similar conditions was much more toxic, as little as 0·25 gm. per kgm. being fatal.

McIntosh, Nielson and Robinson (1943) showed that the toxicity of cooked mangels fed to pigs was due to the presence of nitrite. They found that, to prevent the formation of toxic amounts of nitrite, it was necessary to cook the mangels at or near 100 deg. C. for at least two hours. McIntosh (1950) states that in this work mangels containing 6050-7360 p.p.m. sodium nitrate were cooked in a town water supply which, though not tested, probably contained minute traces only of either nitrate or nitrite.

In Queensland, at various times, heavy mortality has occurred in pigs fed cooked vegetables (most often pumpkin or mangels) or cooked meat or offal. None of the cases in which cooked vegetables were implicated has been investigated with nitrite poisoning in mind, but the New Zealand work of McIntosh *et al.* suggests that these Queensland outbreaks might have been due to nitrite poisoning.

The observations reported here relate to poisoning caused by soup made from boiled meat and offal. On two farms where detailed observations were made it was found that the water in which the offal was boiled had a high nitrate content. When offal was boiled in these waters nitrite was produced in amounts sufficient to render the food toxic to pigs.

Observations are recorded here on:—

(1) Natural outbreaks of nitrite poisoning in pigs fed on cooked offal.

(2) The toxicity of sodium nitrite for pigs.

(3) The toxicity of cooked offal prepared experimentally with well water which had a high nitrate content.

METHODS OF ANALYSIS.

Nitrates were determined by a modification of Ulsch's method in which nitrate was converted to ammonia with sulphuric acid and reduced iron, the ammonia being distilled by the usual Kjeldahl method, using magnesium oxide instead of sodium hydroxide to liberate ammonia. Devarda's alloy was not available for the reduction.

Nitrites were estimated by 1940 AOAC methods of analysis (Association of Official Agricultural Chemists, 1940).

Nitrate and nitrite levels are expressed throughout as the equivalents in parts per million of sodium nitrate and sodium nitrite respectively.

Haemoglobin determinations were made by J. M. Harvey in the Department's Chemical Laboratory by measuring the oxygen capacity of the blood in a Van Slyke manometric gas analysis apparatus.

CASE REPORTS.

Farm A (Beaudesert District).

This herd was fed on boiled slaughteryard offal, bones and meat scraps (mostly beef). Water was obtained from two sources—an earthen tank (or dam) and a well 135 feet deep. There was a local belief that pigs could not be fattened at this piggery owing to the toxicity of the well water. Consequently the owner, a butcher, used the piggery only when he had an excess of offal. Further, he did not use the well water unless the dam was dry. The offal was cooked principally to secure the tallow.

Information from the owner showed that in 1945 the dam dried up and offal was boiled in well water and fed to the pigs. Six pigs died during the following week and pig fattening was then abandoned for some months.

About April, 1946, he again penned pigs for fattening and used water from the dam to boil the offal. No sickness or deaths occurred. By July 1 the dam was empty and well water was brought into use. The herd appeared healthy at feeding time on the evening of July 6, but on the following morning 16 of a herd of about 30 were dead. Analyses of samples submitted by the local Inspector of Stock showed that no arsenic or heavy metals were present in the stomach contents and that the waters were low in total chlorides.

As butcher's offal was used it was suspected that beef pickled with nitrate may have been included in the offal. Analysis confirmed nitrite in all specimens, as follows:—

Well water—8·9 p.p.m. sodium nitrite.

Well water stored in iron tank—69·0 p.p.m. sodium nitrite.

Stomach contents of dead pig—3·0 p.p.m. sodium nitrite.

The water samples were received in 1 lb. glass jars with metal screw caps. The nitrites in the sample from the iron tank rose in a few days from 69 p.p.m. to 189 p.p.m., and later to 234 p.p.m. Nitrates were present in large quantities, viz., 2888 and 2324 p.p.m. sodium nitrate in the well and tank waters respectively.

The piggery was visited on July 19, 1946, to observe the boiling processes and to examine the possibility of the nitrate in the well water being derived from contamination of the well by refuse. It was found that the position of the well precluded external contamination. Field tests showed that the nitrites were higher in well water stored in a 400-gallon iron tank than in the water as it was pumped from the well. The dam water was free from nitrite. Laboratory analyses (Table 1) showed that the total chlorides calculated as sodium chloride were the same in both waters (viz., 761 p.p.m.), indicating that the increase in nitrite was not due to evaporation. Two batches of soup composed of offal, bones

Table 1.

CHEMICAL ANALYSES OF SAMPLES COLLECTED AT FARM A.

Date.	Sample.	$NaNO_2$ p.p.m.	$NaNO_3$ p.p.m.	NaCl p.p.m.
July, 1946 ..	Well water direct from delivery pipe of pump*	6·1	2970	761
	Well water stored in iron tank	41·6	2890	761
	Soup during boiling	1127	..	..
	Soup cooled and tallow removed ready for feeding	1225	..	..
July, 1947 ..	Well water direct from delivery pipe of pump	5·0	1970	569
	Well water stored in iron tank	100	2110	627
	Soup ready for feeding	2·5	trace	..
	Dam water being used for preparation of soup	nil	nil	..

* All samples were taken in glass bottles with cork stoppers and were filled to leave no air-space.

and beef scraps were also high in nitrite. A batch which had been boiling for several hours and was still boiling when sampled contained 1127 p.p.m. sodium nitrite. A soup which had cooled and from which the tallow had been skimmed contained 1225 p.p.m. sodium nitrite.

The herd was being fed entirely on cooked offal diluted with an equal part of dam water, so that the final nitrite content would be 500-600 p.p.m. sodium nitrite (500 p.p.m. = 35 grains per gallon).

There had been no deaths from July 7 until the afternoon before the visit, when one pig died. A post-mortem examination was done on this animal about 18 hours after death, but the carcase was so decomposed as to be of little value. The liver contained 2·9 p.p.m. and the stomach content 7·8 p.p.m. sodium nitrite.

Experiments reported below showed that 0·09 gm. sodium nitrite per kgm. body weight may kill pigs. The pigs on farm A weighed about 45 kgm., hence 8·1 litres of a soup containing 500 p.p.m. sodium nitrite could be fatal to such animals. As no other feed was given it is likely that this amount of soup would be consumed.

The nitrite in the cooked offal was evidently derived from the nitrate in the well water. It will be noticed that some nitrite was produced during the storage in the iron tank and that a great increase in nitrite occurred when the water was boiled with meat in large iron vessels over wood fires.

Heavy rain (18·17 inches) fell between January 18 and February 1, 1947. On February 10 the well water contained 9·5 p.p.m. sodium nitrite and 2433 p.p.m. sodium nitrate, indicating very little dilution by the heavy rain. On February 26 the well water contained 8·4 p.p.m. sodium nitrite and 2331 p.p.m. sodium nitrate. The well was still high in nitrate (1970 p.p.m.) in July, 1947, and soup being prepared at that time with dam water contained only negligible nitrite, viz., 2·5 p.p.m. (Table 1).

Farm B (Pittsworth District).

This herd consisted of 80 mixed pigs fed on whey, crushed sorghum, wheat, barley and wheaten or lucerne hay. On August 9, 1946, beef scraps were boiled in water pumped from a deep bore and fed to pigs. Each day thereafter this beef-soup was reboiled in well water and some was fed. On August 12, 14 store pigs 3-5 months old died within 2½ hours of feeding. Analyses of samples collected on the day of this mortality by the local Inspector of Stock resulted as follows:—

Bore water—1740 p.p.m. sodium nitrate;
0·37 p.p.m. sodium nitrite.

Beef soup—1690 p.p.m. sodium nitrate;
393·6 p.p.m. sodium nitrite.

Stomach contents of dead pig—4·9 p.p.m. sodium nitrite.

Inflammation of the gastric mucosa, with many haemorrhages from pin-point up to 2 cm. in diameter, was found in a dead pig examined by the Inspector of Stock. No other lesions were noticed. The characteristic feature of nitrite poisoning is the brown colour imparted to the blood by methaemoglobin, but this may disappear a few hours after death.

It will be noted that the beef soup was boiled three times before deaths occurred. The final product contained 393·6 p.p.m. (28 grains per gallon) sodium nitrite. Assuming that these pigs weighed 25 kgm. and that 0·09 gm. of sodium nitrite per kgm. body weight constitutes a fatal dose, then about 5½ litres of this beef soup would be fatal. McIntosh *et al.* (1943) stated that boiled mangels containing 20 grains sodium nitrite per gallon (i.e. 286 p.p.m.) are dangerous to small pigs.

THE TOXICITY OF SODIUM NITRITE.

Pigs varying in weight from 48 to 80 kgm. were dosed experimentally with sodium nitrite. The experimental animals were in good condition and had been fed in pens on crushed grain containing about 9 per cent. meatmeal and 10 per cent. lucerne chaff for several weeks before they were used. The morning feed was omitted on the day the pigs were dosed. A small feed of crushed grain was given about two hours after treatment. The results are summarized in Table 2.

Table 2.

EFFECT ON PIGS OF 1% SOLUTION OF SODIUM NITRITE GIVEN BY STOMACH TUBE.

Dose Rate gm./kgm.	Animal No.	Weight kgm.	Haemoglobin (gm. per 100 ml.)			Results.
			Pre-treatment.	3 hr.	5 hr.	
0·04	487	73	..	..	..	Non-fatal methaemoglobinaemia 2–4 hours after treatment. Recovered in 6–7 hours
0·045	581 (capsule)	73	..	..	..	
0·045	485	66	..	..	..	
	486 (capsule)	73	14·6	9·4	13·0	
0·05	484	53	14·2	8·7	11·3	
0·06	488	48	..	..	..	
0·07	488	51	..	..	..	Severe non-fatal methaemoglobinaemia 2–4 hours after treatment. Recovery in 7 hours
0·08	487	75	..	..	..	
0·09	484	61	..	..	..	Dead in 2 hours
	486	80	..	..	..	

The effect of sodium nitrite given in the solid form in gelatin capsules was compared with the effect of a 1 per cent. aqueous solution given by stomach tube. A pig dosed by capsule developed similar, but slightly less intense, symptoms to those shown by a pig dosed with a 1 per cent. solution at the same dose rate—viz., 0·04 gm. sodium nitrite per kgm. body weight. At a dose rate of 0·045 gm. per kgm., administration by capsule and stomach tube produced symptoms which were similar in intensity and in the time of their development and eventual disappearance. Subsequent observations were made by dosing by stomach tube with the 1 per cent. solution.

The lowest dose rate used—viz., 0·04 gm. per kgm.—produced definite symptoms of methaemoglobinaemia. About two hours after treatment the skin over the whole body was blanched. The pigs became docile and lethargic but they ran normally when chased. The conjunctival vessels were a definite nut-brown colour. About five hours after treatment these symptoms had disappeared and the animals appeared normal.

Single pigs were then dosed with 0·05, 0·06, 0·07, and 0·08 gm. per kgm. Symptoms of methaemoglobinaemia developed in all cases and became more severe as the dose rate was increased, but none of these doses was fatal. Symptoms were at their height three to four hours after treatment. Blanching of the skin was the first visible symptom, and it was easily seen when there were treated and untreated pigs in the same pen. (All the experimental pigs were Large Whites.) The higher dose rates produced methaemoglobinaemia, which was so marked that distinctly coffee-coloured blood was visible in the conjunctival vessels. Respiration became rapid and shallow and the pigs were very disinclined to exert themselves.

Two pigs weighing 61 and 80 kgm. were then dosed at the rate of 0·09 gm. per kgm. Both pigs developed severe methamoglobinaemia and died two hours after treatment. There was no struggling until about five minutes before death.

Autopsy immediately after death of the two fatal cases revealed similar lesions in each animal. The subcutaneous blood vessels were engorged with chocolate-coloured blood. The heart was in diastole. The lungs were pale and contracted and approximately 20 sub-pleural petechiae were found about the hilus of each organ. Congestion and petechiation was noted on the glandular mucosa of each stomach. The duodenum was catarrhal but the remainder of the small and large intestines of each pig appeared normal. The livers and kidneys were greatly engorged with chocolate-coloured blood and the spleens were contracted. Liver, kidneys, urine and contents of the alimentary tract of these two pigs were analysed for nitrite. The results of these analyses are shown in Table 3.

Table 3.

PIGS DOSED WITH 1% SOLUTION SODIUM NITRITE: SODIUM NITRITE CONTENT OF ORGANS FROM FATAL CASES.

Pig No.	Specimen.	Total Weight gm.	Weight of Sample gm.	$NaNO_2$ p.p.m.
484	Stomach content	566	566	887
	Small intestinal content	336	336	2·96
	Large intestinal content	not weighed	762	3·45
	Liver..	1816	681	less than 1
	Two kidneys	227	227	less than 1
	Urine	about 100	..	less than 1
486	Stomach content	1589	1589	250
	Small intestinal content	364	364	4·0
	Large intestinal content	3458	790	3·2
	Liver..	2000	454	less than 1
	Two kidneys	252	252	less than 1
	Urine	nil	nil	..

EXPERIMENTS WITH COOKED OFFAL.

Observations were made on the nitrite and nitrate content of beef soups prepared experimentally with well water from farm A. The toxicity of the soup was tested by feeding it to pigs.

The soup was made by boiling beef scraps (30 lb.), usually rib and shin bones and a head, for four to five hours in well water. The vessels used were an iron petrol drum (44 gallons) and a kerosene tin. The latter was a 4-gallon iron vessel coated with tin by dip galvanizing.

Table 4.

NITRITE CONTENT OF SOUPS PREPARED BY BOILING BONES AND OFFAL IN WELL WATER FROM FARM A.

Trial.	Treatment.	Vessel.	$NaNO_2$ p.p.m.
1	First boiling	Iron drum	..
	Second boiling	Iron drum	..
	Third boiling	Iron drum	..
	Fourth boiling	Kerosene tin	720
2	First boiling	Iron drum	258·8
	Second boiling	Iron drum	241·6
	Third boiling	Iron drum	156·0
	Fourth boiling	Kerosene tin	1060·0
	Fifth boiling	Kerosene tin	..
	After cooling overnight	Kerosene tin	1430·0
3	First boiling	Iron drum	162·6
	Second boiling	Glass flask	101·0
	Growth of air-borne micro-organisms, 7 days at room temperature	..	9·9
4	First boiling	Iron drum	15·5
	Second boiling	Iron drum	23
	Third boiling	Kerosene tin	1070
	After cooling overnight	Kerosene tin	1245

The nitrite content of the soup was estimated after each boiling (Table 4). When the content had reached a level considered likely to be toxic, pigs which had been fasted overnight were dosed by stomach tube. The amount of soup administered was determined by its nitrite content and the body weight of the pig. It was intended to dose at the rate of 0·09 gm. per kgm., but in both trials where death resulted the nitrite content of the soups increased during the interval between the last estimation and drenching so that the doses given exceeded 0·09 gm. per kgm. (Table 5). The usual morning feed was omitted prior to drenching.

Blood haemoglobin estimations were made before and at intervals after dosing. Pigs which died were autopsied immediately after death.

Trial 1.

The water used in this trial had been collected at farm A six months previously. It contained at the commencement of the trial 2·06 p.p.m. sodium nitrite and 2783 p.p.m. sodium nitrate.

The soup was boiled in an iron drum, but the nitrite content did not rise to a level considered toxic for pigs. After three boilings the volume was considerably reduced. The soup was then transferred to a kerosene tin and again boiled. This fourth boiling increased the nitrite content to 720 p.p.m. Three litres of this soup were given to a pig (no. 492) weighing 37 kgm. The dose rate was 0·058 gm. sodium nitrite per kgm., but no adverse effects were noted.

Table 5.

EFFECT OF EXPERIMENTALLY PREPARED SOUPS (TABLE 4) ON PIGS.

Trial.	Pig No.	Weight of Pig kgm.	Soup.		Dose Rate of $NaNO_2$ gm./kgm.	Haemoglobin (gm./100 ml.)		Result.
			Nitrite Content p.p.m.	Vol. Dosed ml.		Pre-treatment.	Just before Death.	
1	492	37	720	3000	0·058	..	..	No adverse effects
2	493	46	1430	4000	0·124	12·1	1·5*	Death in 51 minutes
2	496	38	1430	3200	0·120	10·7†	2·6	Death in 40 minutes
4	495	82·1	1245	6900	0·105	17·8	5·3	Death in 63 minutes
4	492	76·2	1245	3500	0·059	13·5	10·3‡	Non-fatal methaemoglobinaemia

* Sample taken just prior to death was showing a tendency to clot, so result is not accurate.

† The pre-treatment sample of approximately 5 ml. of blood was added to 0·5 ml. of aqueous sodium citrate, so the figure given is about 10% low. Dried sodium citrate was used for all other blood samples.

‡ Sample taken 2 hours after dosing.

Trial 2.

The water used in this trial (and in trials 3 and 4) was also from farm A and contained 8·4 p.p.m. sodium nitrite and 2331 p.p.m. sodium nitrate. Soup was prepared as in trial 1 and boiled for four hours in the iron drum. The first boiling increased the nitrite content to 258·8 p.p.m., but further boiling decreased the concentration, so that after the third boiling it was down to 156 p.p.m. The soup remaining in the drum was stored in glass jars for seven days, during which time there was a profuse bacterial growth. The soup was then transferred to a kerosene tin and boiled for the fourth time for four hours. The nitrite content rose to 1060 p.p.m. The soup was boiled a fifth time and then two pigs were each dosed with a quantity estimated to contain 0·09 gm. sodium nitrite per kgm. Subsequent analyses showed that the last boiling and cooling had increased the concentration to 1430 p.p.m. sodium nitrite. The pigs therefore

received a dose of 0·12 gm. per kgm. Both pigs developed severe methaemoglobinaemia. Death occurred 40 minutes (pig. no. 496) and 51 minutes (pig no. 493) after dosing.

Post-mortem examinations done immediately after death showed similar lesions in both pigs. The subcutaneous vessels were engorged with chocolate-coloured blood. The heart was in diastole but showed no macroscopic lesions. The lungs were pale and contracted, with a few subpleural petechiae. The stomach of one pig showed a marked catarrhal inflammation. The glandular mucosa was congested and seemed to strip rather readily. Inflammation of the duodenum was most marked and extended for several feet. The liver and kidneys were engorged with coffee-coloured blood. Samples of liver, spleen, kidney, stomach and intestinal contents were collected for chemical analysis (Table 6).

Table 6.

PIGS DOSED WITH EXPERIMENTAL COOKED OFFALS: SODIUM NITRITE CONTENT OF ORGANS FROM FATAL CASES.

—	Pig No.	Volume of Soup and Nitrite Content.	Weight of Pig kgm.	Dose Rate gm./kgm.	Specimen.	$NaNO_2$ p.p.m.
Trial 2	493	4000 ml. containing 1430 p.p.m. $NaNO_2$	46	0·124	Stomach content ..	7·6
..	..	..	..	..	Intestinal content ..	6·6
..	..	..	..	..	Liver..	less than 1
..	..	..	..	..	Kidney	1·75
..	..	..	..	..	Spleen	1·6
Trial 2	496	3200 ml. containing 1430 p.p.m. $NaNO_2$	38	0·120	Stomach content ..	101
..	..	..	..	..	Intestinal content ..	5·7
..	..	..	..	..	Liver..	less than 1
..	..	..	..	..	Kidney	2·75
..	..	..	..	..	Spleen	3·5
Trial 4	495	6900 ml. containing 1245 p.p.m. $NaNO_2$	82·1	0·105	Stomach content ..	478
..	..	..	..	..	Small intestinal content	514·5
..	..	..	..	..	Large intestinal content	less than 1
..	..	..	..	..	Liver..	less than 1
..	..	..	..	..	Kidney	less than 1
..	..	..	..	..	Spleen	less than 1

Trial 3.

As trials 1 and 2 indicated that repeated boilings in the iron drum did not raise the nitrite content to a toxic level, it was thought that perhaps bacteria may have been responsible for the reduction of nitrate to nitrite, so the following experiment was undertaken.

A sample (about 2 litres) was taken from a batch of soup which had been boiled once for four hours in the iron drum. The nitrite content was 162·6 p.p.m. The sample was boiled for one hour in a flask and then exposed to contamination by air-borne micro-organisms for seven days. At this time the nitrite content was 9·9 p.p.m., compared with 101·0 p.p.m. immediately after boiling.

This experiment indicates that the increase in nitrite was not due to micro-organisms.

Trial 4.

Two boilings in the iron drum reduced the volume of soup from 11 gallons to 2¾ gallons. The nitrite content increased from 9 p.p.m. in the original water to 20 p.p.m.

The soup was then transferred to a kerosene tin and boiled for the third time. The nitrite content rose to 1070 p.p.m. After the soup had stood overnight in the tin, the nitrite content increased further to 1245 p.p.m.

This soup administered to pig no. 495 at the rate of 0·105 gm. per kgm. produced methaemoglobinaemia and death in 63 minutes. Observations on this animal were:—

Pig. No. 495. Weight 181 lb. (82·1 kgm.).

10.0 ..	Dosed by stomach tube with 6900 ml. of soup containing 1245 p.p.m. sodium nitrite.
10.5 ..	Urinated.
10.28 ..	Urinated.
10.29 ..	Paddling gait, respiratory rate 68 per minute.
10.35 ..	Respiratory rate 80 per minute.
10.37 ..	Animal lay down.
10.39 ..	Animal rose and defaecated.
10.40 ..	Restless; skin blanched.
10.47 ..	Vomited twice; quantity (estimated) 300-400 ml.
10.49 ..	Vomited about 200 ml.
10.50 ..	Marked ataxia.
10.51 ..	Went down and struggled.
10.53 ..	Buccal mucosa very cyanotic; marked respiratory distress.
10.55 ..	Animal lying on its side paddling; dyspnoeic.
11.03 ..	Death.

A post-mortem examination done within 30 minutes of death presented lesions identical with those described in trial 2. Liver, kidneys, spleen and contents of the alimentary tract were analysed for nitrites; the results are shown in Table 6. The remainder of this batch of soup (3,500 ml.) was administered to pig no. 492. The animal weighed 76·2 kgm. so the dose rate was 0·059 gm. sodium nitrite per kgm. Non-fatal symptoms of methaemoglobinaemia were exhibited.

The Reduction of Nitrate to Nitrite in Well Water.

The original well water from farm A contained approximately 10 p.p.m. sodium nitrite and 2800 p.p.m. sodium nitrate. It was thought at first that boiling with offal was sufficient to reduce the nitrate, but this was not substantiated by the experiments described above. Certain bacteria are capable of reducing nitrate to nitrite. It was considered that the increase in nitrite concentration in trial 2 may have been brought about in this manner, but this was not confirmed in trial 3.

Boiling in the iron drum resulted in an initial increase in nitrite followed by a decrease (trial 2). Concentration by boiling in this vessel did not increase the nitrite content. However, when the mixture was transferred to a kerosene tin and boiled there was, on each occasion (i.e., in trials 2 and 4), a marked increase in nitrite. It was felt that the tin coating of the kerosene tin may have been responsible for the conversion of nitrate to nitrite either by acting as a catalyst or by preventing the complete reduction of nitrate to ammonia.

DISCUSSION.

The evidence presented indicates that the field outbreaks were due to nitrite poisoning resulting from feeding offal cooked in water with a high nitrate content.

The mechanism of the reduction of nitrate to nitrite is still obscure. Suckling (1944) stated:—"Many waters containing nitrates, if allowed to stand in contact with iron, zinc or lead in pipes or cisterns, act upon the metal, the nitrates being in part reduced to nitrites and ammonia." This might explain the increase in nitrite in the water samples kept in the glass jars with metal screw caps and also in the water stored in the iron tank at farm A.

The experiments reported here in which offal was boiled in an iron drum and in a kerosene tin do not duplicate the conditions at farm A, where the boiling was done in a cast iron vat. Also, in the field excess soup was retained and boiled with the next batch of offal. Further chemical experiments are being done on the factors concerned in the production of nitrite from these waters.

The excess nitrate in the soup did not appear sufficient to be toxic, because in trial 1 pig no. 492 received 0·058 gm. per kgm. sodium nitrite plus the unreduced nitrate and suffered no ill effects.

From their work on nitrite poisoning in pigs fed on cooked mangels, McIntosh *et al.* (1943) concluded:—"Mangels cooked at temperatures below boiling point, or for too short a time at boiling point, were shown to be dangerous to pigs, owing to the amount of nitrite formed during cooling. . . . Production of nitrite on cooling may be avoided by boiling mangels for at least two hours, at or near boiling point." McIntosh (1950) points out that in their experiments

no nitrite was present at the completion of the cooking. All the nitrite was produced during cooling and its presence or absence depended solely on the temperature during cooking. In cooked offal, on the other hand, toxic levels of nitrite are not avoided by cooking at 100 deg. C., because a vat at farm A which had been boiling for several hours and was still boiling when sampled, contained 1127 p.p.m. sodium nitrite (Table 1).

In Queensland, four natural well waters, besides those from farms A and B described above, have been encountered with nitrate contents ranging from 270 to 4000 p.p.m. There is a record also of a sub-artesian water analysed in the Department's Chemical Laboratory in 1930 containing the equivalent of 2380 p.p.m. sodium nitrate. This water was submitted as the suspected cause of deaths in sheep, but the particulars of the outbreak could not be found. The observations reported above indicate the danger of using such waters to prepare cooked offal for feeding to pigs. Such waters may be dangerous also to human beings, particularly infants. Faucett and Miller (1946) record three cases of methaemoglobinaemia in infants fed milk mixtures diluted with well waters high in nitrate. The water used by two patients contained 70 mgm. nitrate nitrogen per litre (i.e., 425 p.p.m. sodium nitrate) and a trace of nitrite nitrogen per litre. In the other case the well water contained 300 mgm. nitrate nitrogen per litre (i.e., 1821 p.p.m. sodium nitrate) and a trace of nitrite nitrogen per litre. Ferrant (1946) reported two cases of methaemoglobinaemia in newborn infants fed a powdered milk formula made up with boiled well water. In the first case the water contained 497 p.p.m. nitrate (i.e., 681 p.p.m. sodium nitrate) and 0·114 p.p.m. nitrite (i.e., 0·171 p.p.m. sodium nitrite), and in the second case 180 p.p.m. nitrate (i.e., 247 p.p.m. sodium nitrate) and a trace of nitrite.

Gwatkin and Plummer (1946) showed that pigs dosed with 10 per cent. solution of potassium nitrite after 24 hours fasting died of methaemoglobinaemia, whereas pigs given a similar dose rate without previous fasting suffered only slight ill effects. The presence of food in the stomach probably prevents fatal anoxaemia from sudden conversion of haemoglobin to methaemoglobin by delaying absorption of nitrite. It seems, therefore, that in the field mortality from nitrite poisoning may be greatly influenced by the amount of ingesta in the stomach when the nitrite-containing feed is given.

Nitrite poisoning can be diagnosed by the distinct brown colour imparted to the blood by methaemoglobin. The brown colour is readily detected in sick or freshly dead animals but it may not be detected if the autopsy is done several hours after death, because the methaemoglobin is converted by bacteria back to haemoglobin (Williams and Hines, 1940).

The nitrite content of specimens from pigs which died of experimental nitrite poisoning (Tables 3 and 6) indicates that the stomach content is the most suitable specimen for diagnosis, though there is a wide variation in the amount of nitrite recovered. The stomach contents of three pigs which died after dosing with experimental cooked offals contained 7·6, 101 and 478 p.p.m. sodium nitrite (Table 6). The variation is presumably due to the action on the

nitrite of enzymes and bacteria. Thus the presence of only a small amount of nitrite in the stomach or other organs does not necessarily exclude a diagnosis of nitrite poisoning.

Symptoms and lesions of fatal nitrite poisoning were produced in three pigs dosed with these soups in amounts corresponding to 0·105, 0·120 and 0·124 gm. sodium nitrite per kgm. body weight.

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Effects of Long-Term Feeding of Nitrate, Nitrite, or Hydroxylamine on Pregnant Dairy Heifers

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SUMMARY AND CONCLUSIONS

Fifteen heifers were fed sodium nitrate daily, in addition to a balanced ration, from the time they were 2 months pregnant until they had calved or aborted. Four heifers were fed sodium nitrite and 4 hydroxylamine hydrochloride under the same schedule. The dosage of nitrate fed one group was adjusted to maintain daily peak methemoglogin levels at 20 to 30% of total hemoglobin; methemoglobin levels of the other group on nitrate and those fed nitrite were generally maintained at 40 to 50% or higher. Levels of hydroxylamine were adjusted to maintain the packed cell volume (PCV) between 25 and 30.

Three abortions, one of which was caused by vibriosis, occurred in the groups fed nitrate or nitrite. Total hemoglobin and red blood cell concentrations increased in heifers fed nitrate or nitrite in proportion to blood methemoglobin levels. Fluctuations in serum nitrate and nitrite correlated with those in methemoglobin. Elevated red blood cell and hemoglobin concentrations occurred in most of the calves of heifers maintained at high methemoglobin levels. The heifers given hydroxylamine developed moderate to marked anemia but calved normally.

The feeding of nitrate, nitrite, or hydroxylamine had no apparent effect on serum levels of calcium, magnesium, phosphorus, or carotene. Serum vitamin A levels were generally low in heifers fed nitrate or nitrite, but a causal relationship could not clearly be established.

Significant lesions were not observed on gross pathologic examination of the heifers maintained at high methemoglobin levels. Degenerative changes in the coronary arterioles and necrosis of the renal glomeruli were observed to a variable extent in histologic examination of tissues from these heifers.

These results indicate that heifers given an adequate ration can tolerate the ingestion for prolonged periods of quantities of nitrate or nitrite sufficient to convert 40 to 50% or more of total hemoglobin to methemoglobin, with no detrimental effects on the maintenance of pregnancy. Hydroxylamine in dosages sufficient to produce anemia also failed to affect pregnancy.

The toxic effects of nitrate and its metabolites upon ruminants have been studied increasingly during the past several years. Evidence has accumulated during this time for [3,21,25] and against [5,6,17] the hypothesis that abortions in cattle may result from chronic nitrate intoxication. These observations were made in widely separated geographical areas, on cattle varying greatly in nutritional status and under widely divergent methods of management. Such factors may account in part for this lack of agreement.

The present studies were conducted primarily to determine whether abortions could be produced by nitrate or its metabolites when fed over a prolonged period to heifers on a good plane of nutrition. In preivous studies [31] relatively large amounts of nitrate and small quantities of nitrite and hydroxylamine had been detected in

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the serum of heifers given sodium nitrate solutions orally. The feeding of nitrite and hydroxylamine was therefore included in these experiments in an attempt to differentiate effects due to these various substances.

It was intended in this study to regulate the dosage of each chemical so as to produce a comparable level of toxicity in the cattle within each treatment group, and to establish these levels below those required to produce signs of acute poisoning. Previous work[5,31] had demonstrated considerable variation in the toxic effects produced in heifers fed nitrate at the same dosage rate per unit of body weight. In view of this, a measure of one of the systemic effects produced by feeding these chemicals was considered a better criterion for the type of comparison desired than was the dosage on a body weight basis. Dosages of nitrate and nitrite were therefore regulated to produce predetermined levels of blood methemoglobin; those of hydroxylamine were adjusted to maintain a certain degree of depression of the PCV.

Materials and Methods

The Holstein-Friesian and Guernsey heifers, approximately 16 to 18 months old, used in these experiments were fed a daily ration of corn silage (15 to 20 lb.), good quality grass and alfalfa hay (about 15 lb.), and a combined grain mixture and mineral supplement * containing 250 I.U. of vitamin A palmitate per pound (6 to 8 lb.). Of the 23 heifers used, 6 had been bred naturally before being purchased; the rest were bred artificially with semen from an Aberdeen Angus bull. They were placed on experiment when 2 months pregnant except for heifers 16 and 20, pregnant 4 and 3 months, respectively. The heifers were kept in a barn and stanchioned throughout the course of the trial.

Fifteen heifers were fed sodium nitrate, 4 sodium nitrite, and 4 hydroxylamine hydrochloride. The daily dosage for each heifer was given in 4 equal parts, mixed with silage (nitrate and nitrite) or grain (hydroxylamine), at 7:30 and 10:30 a.m. and at 1:30 and 4:30 p.m. Daily feeding was continued until the heifers aborted or calved, or until the day of necropsy, except for temporary withdrawal of the chemicals in a few instances of anorexia or when signs of excessive toxicosis were observed.

Dosages of nitrate and nitrite were regulated weekly on the basis of the daily maximum methemoglobin percentage of total hemoglobin. Nine heifers given nitrate (group II, heifers 2, 4, 8, 14, 19, 20, 25, and 26) and those on nitrite (6, 10, 24, and 31) were to be maintained at a 40 to 50% methemoglobin level; the other 6 on nitrate (group I, heifers 1, 5, 11, 17, 23, and 28) at a 20 to 30% level. The dosage of hydroxylamine was regulated weekly by hematocrit readings, an attempt being made to depress the PCV and maintain it between 25 and 30.

Oxalated blood samples were obtained from each heifer once a week, betwen 2:00 and 3:00 p.m. Methemoglobin and total hemoglobin concentrations were determined by the method of Evelyn and Malloy.[8] The PCV was measured weekly and total red blood cell counts made biweekly. Serum nitrate and nitrite concentrations were generally determined on all samples, but hydroxylamine was measured only in samples from heifers given that chemical. Nitrate was determined by the method of Westland and Langford,[30] using fresh strychnidine reagent each day. Nitrite and hydroxylamine were determined as previously described.[31]

Concentrations of calcium, magnesium, phosphorus, vitamin A, and carotene were determined every 3rd week in serum separated from clotted blood samples. Calcium and magnesium were measured by the method of Todd,[26] phosphorus by the method of Fiske and SubbaRow,[9] and vitamin A and carotene by that of Boyer *et al.*[2]

Postmortem examinations were performed on heifers 8, 10, 14, 20, 24, 25, 26, and 31 within 10 days after parturition or abortion, and on heifers 2 and 4 within 2 hours after death from acute nitrate poisoning. Calves were necropsied within 24 hours after birth. Histologic sections were prepared from lung, liver, kidney, spleen, lymph nodes, brain, heart and skeletal muscle, intestine, pancreas, mammary gland, thyroid, uterus, and placenta tissues. Tissue specimens were fixed in a 10% formalin solution, sectioned at 6 μ, and stained with hematoxylin and eosin.

Statistical analyses were performed according to standard methods outlined by Snedecor.[23]

Results

HEIFERS FED SODIUM NITRATE

Blood Studies.—For purposes of statistical analysis, data from heifer 19 were included with those of group I heifers, and those from heifer 1 were excluded because she had aborted in the 5th month of pregnancy. Over-all means and ranges of values for total hemoglobin, percentage of methemoglobin, PCV, red blood cell counts, and serum nitrate and nitrite are listed for each heifer (Table 1).

Total hemoglobin concentrations increased in all heifers, generally plateaning after the 5th or 6th week of treatment and undergoing more random variations there-

* An analysis of this mixture, on a dry matter basis, is as follows: protein, 18.62%; nitrogen-free extract, 62.01%; ether extract, 3.82%; crude fiber, 9.50%; total ash, 6.02%; Ca, 0.70%; P, 0.77%; and Mg, 0.23%.

TABLE 1—Mean and Range of Values for Total Hemoglobin, Percentage of Methemoglobin, Packed Cell Volume, Red Blood Cell Counts, and Serum Nitrate and Nitrite in Heifers Fed Sodium Nitrate

Heifer No.	Total hemoglobin (Gm.)		Methemoglobin (% total hemoglobin)		Packed cell volume		Red blood cells (millions/cmm.)		Serum nitrate ($\mu g. NO_3$-N/ml.)		Serum nitrite ($\mu g. NO_2$-N/ml.)	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
Group I *												
5	11.9–19.1	14.8	16.5–42.5	28.3	44–70	53	4.78–10.05	7.92	4–37	21	0.4–1.2	0.5
11	10.8–15.7	13.4	17.2–77.0	29.8	40–57	47	4.97– 8.98	7.44	10–45	24	0.4–1.8	0.7
17	11.3–16.4	13.9	10.6–40.7	27.8	43–61	51	6.11– 8.57	7.32	10–60	22	0.4–1.6	0.8
19	11.4–15.3	13.6	6.3–55.8	22.0	42–53	46	5.11– 7.88	6.62	1–34	18	0.3–1.4	0.5
23	10.6–16.6	13.7	6.5–48.5	26.6	42–63	49	5.10– 9.84	7.66	14–56	32	0.4–2.0	0.8
28	11.3–15.4	13.0	9.0–54.6	30.1	38–49	44	4.50– 9.84	6.56	12–41	24	0.3–2.1	0.6
Group II **												
2	9.0–19.2	17.0	10.8–77.0	51.8	42–66	60	5.99–10.13	8.44	4–66	32	0.4–2.1	0.9
4	10.8–18.6	16.5	32.0–70.0	49.6	37–61	55	6.02–10.15	8.21	16–52	33	0.4–1.9	0.8
8	9.1–19.2	16.7	26.9–61.7	46.1	42–69	61	5.02–11.80	8.48	10–36	22	0.3–2.9	0.7
14	14.0–20.2	18.2	11.4–67.0	49.4	50–70	62	4.55–11.55	8.59	16–52	33	0.4–1.2	0.6
16	10.8–20.0	15.8	30.5–64.5	47.7	30–65	53	5.33– 9.40	7.67	9–44	25	0.3–1.6	0.8
20	11.8–16.5	15.1	5.8–62.0	38.6	42–59	53	5.11– 8.75	7.27	16–52	31	0.4–1.7	0.7
25	13.7–17.9	15.9	23.4–67.2	45.4	44–65	54	5.17– 9.61	7.74	9–43	33	0.3–1.0	0.6
26	12.0–19.2	16.2	25.1–63.2	44.4	40–59	52	5.09–11.34	8.36	12–72	29	0.4–0.6	0.5

* Low-level treatment. ** High-level treatment.

after. Weekly dosage increases were necessary until an equilibrium had been achieved within the desired ratio of methemoglobin to total hemoglobin. Although wide variations occurred in each heifer in the range of methemoglobin percentages recorded, these were usually maintained within the desired range after the first several weeks of treatment in all heifers except 19 (Table 1). In group II heifers the great increase in hemoglobin concentration made it possible to raise the proportion of methemoglobin to even higher levels than had originally been planned. Some heifers in this group were maintained for prolonged periods at levels of methemoglobin exceeding 55% of the total hemoglobin (Table 2).

Great variation existed among the heifers in the dosage of nitrate required to produce the desired amount of methemoglobin, a variation related inversely with that in the rate of eating of the silage mixture. In the 18th week, for instance, 17.3 Gm. of nitrate per hundredweight was sufficient to produce 62.0% methemoglobin in heifer 2; whereas heifer 20, with 36.5% methemoglobin, consumed 31.4 Gm. per hundredweight.

Variations in methemoglobin levels during the day were measured in 11 heifers by determinations made on samples taken just prior to the 7:30 feeding and at 2:30 and 9:30 p.m. The morning values were on the average 42% as high as those in the afternoon, and ranged from 28 to 63%. Those from the evening averaged 74% of those from the afternoon, with a range of 44 to 96%. On the basis of these results, methemoglobin levels measured between 2:00 and 3:00 p.m. were considered to represent the approximate daily maximum.

Mean values for total hemoglobin, PCV, and red blood cell counts were significantly greater in heifers of group II than in group I ($p<0.01$). Mean serum nitrate values were also higher ($p<0.02$), but nitrite values were not significantly different. When pretreatment values were compared with the means of those obtained between the 16th and 20th weeks of treatment, hemoglobin, PCV, and red blood cell counts had risen significantly in heifers of group II ($p<0.01$). In group I heifers, hemoglobin and PCV values had also risen significantly ($p<0.05$), but red blood cell counts had not.

The relationships were calculated for each heifer between the successive weekly variations in methemoglobin concentration (Gm./100 ml.) and the corresponding variations of serum nitrate and nitrite and of total hemoglobin. These regressions were pooled after it had been established that they were not heterogeneous between the 2 treatment groups or among the heifers within each group. Significant correlations were then found between the variations in methemoglobin and those in

TABLE 2—Total Hemoglobin and Percentage of Methemoglobin in Heifers 2, 4, and 14 During Successive Weeks of the Feeding Trial

	Heifer 2		Heifer 4		Heifer 14	
Week	Total hemoglobin (Gm.)	Methemoglobin (% total hemoglobin)	Total hemoglobin (Gm.)	Methemoglobin (% total hemoglobin)	Total hemoglobin (Gm.)	Methemoglobin (% total hemoglobin)
1	9.0	28.0	10.8	39.0	15.1	39.2
2	12.3	10.8	13.4	35.3	15.3	17.6
3	12.6	35.3	14.4	35.5	16.4	47.5
4	13.6	40.0	15.2	36.3	16.6	11.4
5	14.0	55.0	14.9	39.8	16.8	53.0
6	16.8	39.7	16.2	32.0	18.2	30.6
7	17.9	49.6	18.2	35.5	16.8	53.2
8	18.0	55.6	17.9	47.8	.....	.....
9	16.8	.....	16.0	41.5	18.0	56.5
10	17.5	55.0	15.6	56.0	17.7	32.8
11	17.5	67.5	16.8	64.4	19.2	59.5
12	17.8	59.5	17.9	58.0	18.9	52.8
13	19.0	77.0	.....	.....	17.5	49.2
14	.....	.....	15.3	62.0	17.5	53.2
15	15.4	62.4	16.4	46.2	19.6	65.5
16	17.9	46.4	17.1	46.5	18.6	52.7
17	18.2	58.0	14.9	48.4	20.2	58.5
18	18.7	59.8	15.4	52.9	.....	.....
19	17.7	61.0	15.8	51.8	19.4	51.8
20	17.9	49.1	16.8	48.8	19.0	52.4
21	17.1	69.5	16.0	49.5	19.2	54.7
22	18.2	55.4	16.4	60.4	18.6	60.1
23	17.1	59.2	.....	.....	17.9	51.1
24	.....	.....	18.0	41.2	18.8	58.8
25	18.2	52.1	17.8	52.2	18.2	58.0
26	16.9	55.4	17.1	68.8	20.2	67.0
27	17.9	51.8	17.3	59.5	19.6	42.2
28	19.2	43.8	17.9	60.9	19.6	53.0
29	17.5	50.2	17.7	46.0	19.0	47.0
30	17.5	51.0	18.6	70.0	.....	.....

nitrate ($r = 0.41$; $p < 0.01$), nitrite ($r = 0.36$; $p < 0.01$), and hemoglobin ($r = 0.20$; $p < 0.01$).

The mean and range of values obtained during the feeding trial for serum calcium, magnesium, phosphorus, carotene, and vitamin A are listed (Table 3).

Total hemoglobin, percentage of methemoglobin, PCV, red blood cell counts, and serum nitrate levels are presented for 5 calves of group II heifers and 2 from those given nitrite (Table 4). Hemoglobin and PCV values of this group were significantly higher ($p < 0.01$) than those determined for 13 control calves within the 1st day of birth. Red blood cell counts did not

TABLE 3—Mean and Range of Values for Serum Calcium, Magnesium, Phosphorus, Carotene, and Vitamin A in Heifers Fed Nitrate

	Calcium (mg./100 ml.)		Magnesium (mg./100 ml.)		Phosphorus (mg./100 ml.)		Carotene (μg./ml.)		Vitamin A (μg./100 ml.)	
Heifer	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
GROUP I *										
5	8.6–10.1	9.5	2.2–3.0	2.5	5.9–8.1	6.8	3.9–7.7	6.7	3.5–14.3	9.2
11	8.7– 9.6	9.2	2.1–2.8	2.4	4.8–6.8	5.6	2.8–3.8	3.4	13.3–23.6	17.3
17	8.6– 9.6	9.2	2.1–2.7	2.5	4.3–7.1	5.8	4.6–7.9	6.5	10.2–14.5	12.6
19	8.2– 9.9	9.2	2.4–3.6	2.9	4.8–7.7	6.0	5.3–7.5	6.3	10.9–21.0	14.0
23	7.6– 9.9	9.1	1.9–3.4	2.6	5.4–8.1	6.8	6.3–8.5	7.1	7.0–17.2	13.7
28	8.6– 9.8	9.4	2.1–2.6	2.2	5.0–7.7	6.5	1.6–3.9	2.9	8.4–27.2	17.7
GROUP II **										
2	9.0–10.6	9.8	1.9–2.9	2.3	4.9–7.4	6.2	2.7–3.9	2.9	9.7–19.6	14.6
4	9.2–10.1	9.6	2.3–2.9	2.4	5.8–7.4	6.4	3.2–4.1	3.7	3.5–19.4	13.6
8	8.2–10.9	9.3	2.1–2.9	2.4	5.4–7.8	6.3	2.6–4.5	3.2	8.4–18.3	13.2
14	8.6–10.3	9.6	2.1–3.8	2.8	4.4–7.5	6.5	3.3–7.5	4.0	6.0–18.2	12.5
16	8.5– 9.6	9.2	2.2–2.6	2.4	5.4–8.0	7.0	4.6–6.4	5.4	10.8–14.6	13.2
20	8.5–10.3	9.2	2.0–2.9	2.4	5.2–7.0	6.4	5.3–7.8	6.3	7.0–11.5	11.1
25	8.4– 9.8	9.3	2.2–3.1	2.6	5.1–7.5	6.4	1.6–4.6	2.1	10.9–21.0	14.9
26	8.9–10.2	9.3	2.2–3.6	2.7	4.4–6.6	5.6	3.0–7.3	4.0	9.4–19.1	13.6

* Low-level treatment. ** High-level treatment.

TABLE 4—Total Hemoglobin, Percentage of Methemoglobin, Packed Cell Volume, Red Blood Cell Counts, and Serum Nitrate in the Calves of Some Heifers Fed Nitrate and Nitrite

Calf	Total hemoglobin (Gm.)	Methemoglobin (% total hemoglobin)	Packed cell volume	Red blood cells (millions/cmm.)	Serum nitrate * (μg.NO_3–N/ml.)
2	11.0	6.8	43	7.72	....
4	14.5	0.6	60	10.09	....
10	12.0	2.6	41	7.08	....
14	16.3	1.1	55	10.90	23
24	16.4	0.8	55	10.36	....
25	16.9	1.8	57	12.04	26
26	14.5	1.5	48	9.20	15

* Determined only in calves 14, 25, and 26.

differ significantly between the groups. Nitrite could not be demonstrated in the serums of calves 14, 25, and 26.

Clinical Observations.—Two heifers in this group aborted. Heifer 1 aborted in the 5th month of pregnancy, and *Vibrio fetus* was isolated from the fetal stomach contents. Heifer 8 aborted during the 8th month of pregnancy; bacteriologic examination was negative. The fetal blood contained less than 2% methemoglobin.

Two heifers were inadvertently killed by acute nitrate poisoning. Heifer 4 died several days after she had calved, and heifer 2 died on the day before she was due to calve. Heifers 14 and 25 had mild to moderate signs of nitrate intoxication in the middle or late afternoon on several occasions. Treatment was withheld at these times and nitrate feeding discontinued. These incidents occurred after a temporary change to feeding grass silage had been made.

Heifer 11 once had signs of acute nitrate poisoning and was treated intravenously with a methylene blue solution. Heifer 5 collapsed suddenly one morning while eating. The cause of this was not established, although acute nitrate poisoning was excluded, and the heifer was normal thereafter.

Vulvar edema was evident in all heifers except 28, being more marked and beginning earlier (4th to 5th month of gestation) in some of the Guernsey heifers (5, 16, 17, 20, and 23). In the others, mild to moderate edema became evident several weeks before calving.

The nonpigmented mucous membranes of all the heifers became brownish gray and remained so throughout the course of the feeding period. This condition was much more marked in the heifers of group 11.

Pathologic Findings.—The only lesions observed on examination of the heifers in group II were a light brown discoloration of the lungs, a general darkening of the muscle tissue, and the discolored mucous membranes. These changes were more evident in heifers 2 and 4 and were considered to result from hypoxia and the presence of methemoglobin in the blood. Heifer 8 had a placentitis due to retained fetal membranes.

Histologic changes were observed in the hearts and kidneys. Vacuolation in the medial layer of the coronary arterioles was observed in all heifers but was more marked in 8 and 26. Focal accumulations of lymphocytes and plasma cells occurred in the myocardium of heifers 2, 4, 14, and 26. Glomerular lesions, observed in all kidneys, were more intense and more widely distributed in heifers 4, 8, 14, and 25. Changes appeared to progress from capillary distention and transudation of proteinaceous material to necrosis of portions of the glomerular tuft (Fig. 1). Minor tubular degeneration and foci of hemorrhage were observed occasionally.

Significant pathologic changes were not found in the tissues of the calves of these heifers and their fetal membranes or in the aborted fetus from heifer 8.

HEIFERS FED SODIUM NITRITE

One heifer (6) of this group had to be taken off experiment after 10 weeks, because she could not be made to eat enough nitrite to raise her blood methemoglobin level significantly. Methemoglobin percentages in heifers 10, 24, and 31 averaged 37.4, 43.2, and 31.8, respectively. In the later period of the experiment, these heifers consumed 14.5 to 17.0 Gm. of nitrite per hundredweight daily. Hemoglobin, PCV, and red blood cell changes generally

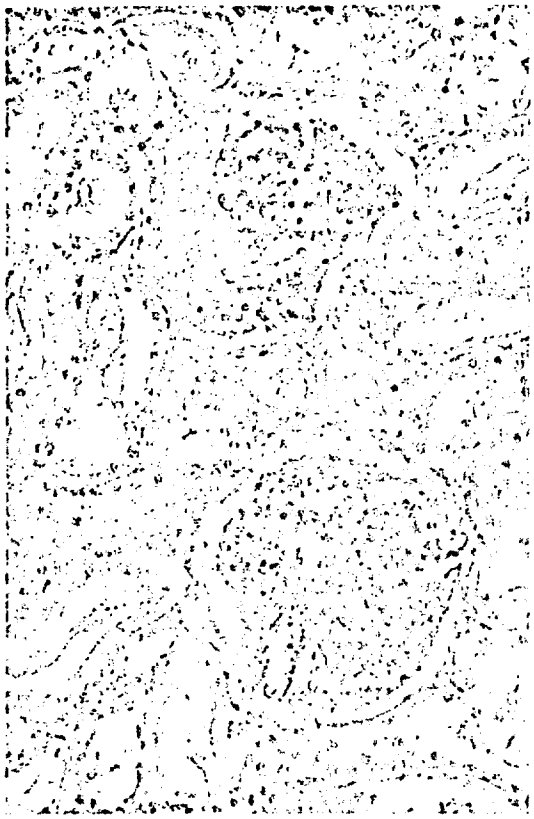

Fig. 1—Glomerular necrosis in heifer 14, fed sodium nitrate. H & E stain; x 125.

paralleled those of the heifers fed nitrate. Serum levels of nitrate, nitrite, calcium, magnesium, phosphorus, carotene, and vitamin A were also similar between the groups.

Heifer 31 aborted during the 8th month of gestation. Bacteriologic and pathologic examinations of the fetus and fetal membranes yielded no significant findings.

Tissue changes in heifers 10 and 24 resembled those previously described. Lesions were not observed in their calves or in the fetal membranes.

HEIFERS FED HYDROXYLAMINE

Total hemoglobin, PCV, and red blood cell counts were depressed in the heifers fed hydroxylamine (Table 5). Percentages of methemoglobin were generally under 10% and rarely exceeded 15%. Daily dosages of hydroxylamine varied from 40 to almost 60 mg. per kilogram of body weight. The compound was withheld from heifer 9 for 2 periods of 3 weeks each, when her PCV had decreased to 25 in one instance and to 22 in the other. These heifers had no clinical signs of illness attributable to the chemical, and all delivered normal calves.

Nitrate was sometimes found in the serums of these heifers, almost always in concentrations less than 2.0 μg. NO_3-N per milliliter. Trace amounts of hydroxylamine were demonstrated in only a few instances. Serum levels of calcium, magnesium, phosphorus, and carotene were similar to those of heifers fed nitrate. Mean serum levels of vitamin A during the course of the treatment period ranged from 17.2 to 19.2 μg. per 100 ml. in these 4 heifers. These mean values were as a whole significantly greater than those of the heifers fed nitrate ($p < 0.01$).

Discussion

It was considered that abortions produced in cattle as a result of nitrate ingestion could occur either through a direct toxic activity, such as the hypoxia produced by the effects of nitrite, or more indirectly, through an interference by nitrate or its metabolites with other physiologic functions, such as vitamin A or iodine metabolism. Since other prerequisites besides nitrate ingestion might be required for these effects, especially of the latter type, to occur, the negative results of this trial must be interpreted on a restricted basis. It has been shown, nevertheless, that heifers given a balanced ration can maintain normal pregnancies despite ingestion for prolonged periods of quantities of nitrate or nitrite sufficient to convert 40 to 50% or more of the hemoglobin to methemoglobin. Since specific causes

TABLE 5—Total Hemoglobin, Packed Cell Volume, and Red Blood Cell Counts in Heifers Fed Hydroxylamine

Heifer	Time	Hemoglobin (Gm.)*	Packed cell volume	Red blood cells (millions/cmm.)
3	Pretreatment	12.5	44	7.01
	Treatment (range)	9.4–14.0	26–41	3.32–5.98
	Treatment (mean)	10.8	31	4.43
7	Pretreatment	13.3	43	6.00
	Treatment (range)	8.5–14.2	26–45	3.06–7.41
	Treatment (mean)	11.0	33	4.47
9	Pretreatment	12.7	39	5.09
	Treatment (range)	6.7–12.9	22–41	2.86–6.58
	Treatment (mean)	9.3	30	4.07
13	Pretreatment	14.5	52	7.48
	Treatment (range)	8.8–13.7	24–45	3.36–6.29
	Treatment (mean)	10.6	33	4.59

were not found for the abortions of heifers 8 and 31, the possibility that nitrate toxicosis was involved cannot be excluded; however, the failure of any other heifers in these treatment groups to abort makes it more likely that these abortions were from unrelated causes.

Available data do not permit an accurate estimation of what would have constituted a lethal dosage of nitrate for the individual heifers under the conditions of this experiment. It was significant, however, that under stress of a sudden feed change, and with no increase in nitrate dosage, 2 heifers of group II died of acute nitrate poisoning and 2 others probably would have, had nitrate feeding been continued. It is probable that in this group of heifers the adaptive mechanisms for reducing nitrite in the rumen and compensating for its hypoxic effect on the system were approaching their limit.

Because one effect of hypoxia is to stimulate erythropoiesis,[20] the raised hemoglobin and red blood cell concentrations in heifers fed nitrate or nitrite were presumed to have resulted largely, if not entirely, from the effects of the nitrite-induced hypoxia. The significantly greater response in the heifers of group II and the positive correlation between methemoglobin and total hemoglobin formation support this hypothesis. It was not proved in this study whether the hemoconcentrations observed could have been due to intravascular fluid loss; however, there was no evidence of dehydration in these heifers, and blood volumes of other heifers receiving similar treatment have been shown to increase.*

The erythropoietic stimulation which apparently occurred in some of the fetuses of heifers fed nitrate or nitrite (Table 4) may have resulted from a diminished concentration of oxygen available at the placental site of interchange, or from a deficiency in functional fetal hemoglobin, resulting from methemoglobin formation. In view of the lack of abnormal amounts of methemoglobin in the blood of these calves shortly after birth, it appears possible that insufficient nitrite entered the fetal circulation to produce the latter type of response.

Wang *et al.* found that after the administration of nitrate to cattle, blood concentrations of methemoglobin and of nitrate plus nitrite nitrogen increased proportionally until peak methemoglobin levels had been attained.[27] Since the nitrogen measured was probably almost entirely from nitrate, the present correlation between serum nitrate and blood methemoglobin may be explained in this manner. It is not clear, however, in what proportion the nitrate in the blood represents that absorbed from the rumen and that formed from nitrite during the conversion of oxyhemoglobin to methemoglobin.[15] Such a mechanism probably accounts for the nitrate detected in the serums of heifers fed sodium nitrite.

Decreased liver storage of vitamin A[7,18,33] as well as signs of vitamin A deficiency[18,33] have been produced in rats by feeding nitrate or nitrite. Carotene conversion to vitamin A is also depressed in hypothyroidism,[13,19] and nitrate has been shown to interfere to some extent with thyroid function.[1,29,32] In cattle and sheep, feeding nitrate has caused lowered plasma[16] or liver[10] vitamin A levels or had no significant effect,[4,6,11,22,28] but signs of vitamin A deficiency have not been observed. Despite attempts to provide diets adequate in vitamin A and carotene content, serum vitamin A levels of the heifers fed nitrate or nitrite in the present study were often very low, in many instances below 10 μg. per 100 ml. The transient collapse of heifer 5 may have resulted from an increased pressure of the cerebrospinal fluid due to vitamin A deficiency.[14] The significant difference in average serum vitamin A levels between heifers fed nitrate and those fed hydroxylamine may have indicated a depression in circulating vitamin A which was due to nitrate feeding; however, the effect of nitrate or nitrite on the vitamin A status of these heifers cannot be critically evaluated because of the lack of control heifers.

The degree to which the toxic effects of nitrate or its metabolites were responsible for the histologic changes observed in these heifers is uncertain. The long-term injection of nitrite into rats produces sclerotic changes in the arteries and arterioles of various organs, including the heart, brain, lungs, and kidneys.[12] Whether the relatively mild degenerative changes in the coronary arterioles of these heifers were of related origin is uncertain.

* Davison, K. L.; Department of Animal Husbandry, Cornell University, Ithaca, N.Y.: Personal communication, April, 1963.

The cause of the glomerular lesions is unclear. There appeared to have been a relatively recent and acute toxic effect on the glomerular capillaries. The focal nature of the lesions, often observed, suggests that the necrotic zones may have represented infarcts, although evidence of thrombosis or embolism was not found in the glomeruli. Of the 4 heifers in which these lesions were most marked, 3 had retained their fetal membranes, and it is conceivable that the glomerular damage was associated with an effect of this toxic state. Available evidence indicates that the electrocution process, by which most of these heifers were killed, did not produce these changes.

Serum calcium levels in all of these heifers fell in the lower range of normal values or were occasionally somewhat below this range.[24] In most of the heifers, especially the Guernseys, serum phosphorus levels during the first 2 to 3 months of the feeding trial were in the high range or higher than normal and then gradually decreased. These trends were observed before treatments began and appeared to be unaffected by the type or intensity of treatment or by the Ca:P ratio in the feed (maintained at approximately 2:1). Serum magnesium levels increased gradually in most of the heifers but with few exceptions did not exceed normal[24] serum concentrations.

The over-all response of heifers fed nitrite did not differ essentially from that of those fed nitrate, and surprisingly large doses of nitrite were tolerated; however, the presence of unexpectedly large quantities of nitrate in the blood of heifers fed nitrite precluded the attempt to differentiate any changes produced by nitrate per se. The feeding of hydroxylamine produced no evidence of its potential hazard to the maintenance of pregnancy.

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2418

Nitrates, Nitrites, and Nitrosamines

Extensive research is needed to establish how great a food hazard these nitrogenous substances present.

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The potential hazards of nitrosamines [illegible]xicants formed in, or as a result [illegible]ating, certain foodstuffs have been described in newspapers (*1*), in testimony before congressional committees (*2*), in technical articles (*3*), and in consumer publications (*4*). We believe it appropriate therefore to review the status of knowledge of these substances to gain perspective and to distinguish between actual and potential occurrence of these compounds or their formation in vivo (or both). Some authors have carried out test tube reactions between nitrite and secondary amines and have indicated that these precursor substances are components of a number of foods we eat or drugs we take (*5*). However, it does not necessarily follow that by either in vitro or in vivo mechanisms we are being exposed to nitrosamines from a variety of sources. Evidence for the presence or formation of nitrosamines in foods is limited and some of the earlier reports of nitrosamines in human nutrients may have been based on inadequate analytical procedures (*6*). Furthermore, because [illegible]macological data on the action of various nitrosamines are incomplete at this time, we believe that it is not yet possible scientifically to ascertain the true danger from nitrosamines in our environment. The demonstrated carcinogenicity of many of these compounds, however, indicates that well-planned, long-range research is mandatory to obtain the needed answers.

Not only may nitrites conceivably serve as reactants with amines or amides to form toxic nitroso compounds, but the nitrites—and their precursor nitrates—have themselves been implicated in causing toxicity in animals and humans, and particularly in children. Ecologists have expressed concern regarding nitrate concentrations in the environment and the effect on the food and water supply.

Nitrates

Our major intake of nitrates in foodstuffs comes primarily from vegetables or water supplies that are high in nitrate content, or from nitrates used as additives in the meat-curing process.

Nitrates are natural constituents of plants. They are present in large quantities in many vegetables, but they occur in only minor amounts in fruit (*7*). Spinach, beets, radishes, eggplant, celery, lettuce, collards, and turnip greens are among the vegetables that generally contain very high concentrations of nitrates (*7*). The nitrate content of some samples may be more than 3000 parts per million. However, the absolute values reported vary extremely because of genetic, environmental, sampling, and maturity factors.

The most important factors that favor large accumulation of nitrate in vegetables include (i) a nitrate-rich environment such as may be caused by high levels of fertilization, especially during the ripening period (*8*); (ii) species that are prone to accumulate nitrate (*9*); (iii) plant nutrient deficiencies (such as that caused by a lack of molybdenum) (*10*); (iv) conditions of reduced light intensity during maturation (*11*); (v) lack of water (*12*); and (vi) plant damage from chemical treatments (*13*). Interactions among these factors complicate the picture and account for the wide ranges of values reported for nitrate of the same vegetables.

A person is likely to consume as much or more nitrates from his vegetable intake as from the cured meat products he eats (*14*). Sodium or potassium nitrate is permitted as an additive to meat products in the United States at levels of 2¾ ounces to 3½ ounces per 100 pounds of meat (*15*). In some countries, not including the United States, addition of small amounts of nitrate is permitted in the manufacture of some varieties of cheese (*7*).

Concern has frequently been expressed about the high nitrate content of some water supplies, particularly those from wells. This nitrate comes from many sources, including precipitation, soil and rock, agricultural use of fertilizers, nitrogen fixation by microorganisms and plants, and especially decomposition of plant and sewage wastes followed by the leaching of nitrates into groundwater. Health agencies have issued reports that, for water to be safe for domestic use, the concentration of nitrate should not exceed 10 parts per million expressed as nitrate-nitrogen (*16*). Numerous examples may be found in which the nitrate content of well water is in excess of this amount (*17*). This situation presents some hazard of potential toxicity. However,

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the usual nitrate intake of an adult may be less from the water supply than from either the vegetables or meat products ordinarily consumed (*7*).

In the quantities normally occurring in food or feed, nitrates become toxic only under conditions in which they are, or may be, reduced to nitrites. Otherwise, at reasonable concentrations, nitrate ions are rapidly excreted in the urine. The nitrites, then, constitute the principal toxic agent, and high intake of nitrates constitutes a hazard primarily under conditions that are favorable for their reduction to nitrite. Four such situations in which this may occur are well documented.

1) The microbial environment in the rumen of cattle causes reduction of nitrate to nitrite. Subsequent absorption of the nitrite ion may result in toxicity to cattle. Hence a danger is present when feeds or water of high nitrate content are consumed (*18*). In the horse, the enlarged cecum and colon also provide a location for microbial reduction of nitrate, and subsequent toxic effects (*19*).

2) The lesser stomach acidity of infants under about 4 months of age may permit the growth of microorganisms that can reduce nitrate to nitrite. As a result, providing water of high nitrate content to infants is a real hazard. Numerous deaths from this cause have been recorded (*20*).

3) When spinach, whether processed or unprocessed, is stored under conditions that permit the growth of microorganisms, nitrate may be reduced to nitrite. A number of cases of toxicity in infants have been reported from spinach left at room temperature for some time after cooking or after a jar of baby food was opened. Conceivably, such nitrite toxicity may also develop in other vegetables or in prepared foods of high nitrate content, but most of the cases reported deal particularly with spinach (*21*).

4) Reduction of nitrate to nitrite has occurred in damp forage materials that were high in nitrate content. Ingestion by livestock proved toxic (*22*). Also, release of oxides of nitrogen from ensiled forages may be hazardous to man and animals (*23*).

In the curing of meat, some of the added nitrate is usually reduced to nitrite but authentic cases of toxic effects from added nitrate only were not found.

Thus, nitrates are not toxic per se, but may under some circumstances be the starting point for a chain of reactions that result in the conversion to toxic substances. Hence, prudence dictates that we monitor the nitrate content of our foods, feeds, and water supplies, divert any samples of particularly high content into channels where they will do no harm, and be aware of actions that can lead to decreasing the use and amount of nitrate in foods when such decrease becomes warranted.

Nitrites

The proved toxicity of nitrites is due primarily to their interaction with blood pigment to produce methemoglobinemia, and their presumptive toxicity relates to their possible reaction, under normally encountered situations, with amines or amides to form toxic nitroso compounds.

Hemoglobin (Hb), the respiratory pigment containing Fe(II), normally transports oxygen to the tissues as a loose complex—oxyhemoglobin ($Hb \cdot O_2$). After the iron is oxidized to the ferric state, the pigment loses its ability to transport oxygen and forms a brown compound, methemoglobin (MetHb). The presence of nitrite in the blood results in MetHb formation. When the methemoglobin concentration exceeds 70 percent, asphyxia occurs, although at lower levels the reaction is reversible (*24*). The nitrite is very strongly bound to the heme of methemoglobin (*25*).

Although there are a number of instances in cattle of nitrite poisoning from water or forage, or in infants from nitrite-containing spinach, the incidence of toxicity is really quite low and the intakes required for serious toxic effects are usually large. As was mentioned in the previous section, the nitrites are in these instances derived from reduction of nitrates. Poisoning of adult humans by nitrite apparently has not been a problem. However, accidental addition of excessive amounts of nitrite to foods has led to instances of poisoning of both adults (*26*) and children (*27*).

Nitrites are more toxic than nitrates, and restriction of the daily intake for man to 0.4 milligram per kilogram of body weight is recommended (*28*). However, nitrites have been used therapeutically as medication for vasodilation and as an antidote for cyanide poisoning in doses of 30 to 300 milligrams without severe toxic effects (*29*). Consumption levels that may cause long-term hazards in man have not been established.

Nitrates and Nitrites in Cured Meat and Fish

The principal source of nitrite in our diets is processed (cured) meat or fish, and nitrites are considered a potential reactant precursor for nitrosamines.

Originally meat was cured in brine containing potassium nitrate as one of the ingredients. However, the actual curing agent was found to be the nitrite produced by the bacterial reduction of the nitrate salt. When it was shown that nitrite could be substituted for nitrate in the cure solution with the production of a more uniform and completely satisfactory product in a shorter period of time, a legal limit of no more than 200 milligrams of residual nitrite (calculated as $NaNO_2$) per kilogram of meat was established (*30*). The action of nitrite in the cure process is threefold: (i) formation of characteristic color, (ii) production of cured flavor, and (iii) antibacterial activity.

The role of nitrite in color formation is to furnish nitric oxide which reacts with myoglobin (Mb) to give nitrosylmyoglobin (MbNO) the red-pink pigment of cured meat. Although the fate of nitrite after addition to meat is not fully known, the following reactions have been postulated to occur in the presence of added reductants (AH_2) (*31*).

$$NaNO_2 + H^+ \longrightarrow HNO_2 + Na^+$$
$$2HNO_2 \longrightarrow N_2O_3 + H_2O$$
$$N_2O_3 + AH_2 \longrightarrow AHNO + HNO_2$$
$$AHNO \longrightarrow AH \cdot + NO$$
$$NO + MetMb \longrightarrow MetMbNO$$
$$MetMbNO + AH_2 \longrightarrow MbNO$$

In the presence of ascorbic acid, cysteine, quinones, or other reductants, nitrous acid, formed from the nitrite ion, is reduced to nitric oxide, which forms a complex with metmyoglobin (MetMb). The nitrosylmetmyoglobin (MetMbNO) is reduced by ascorbic acid to nitrosylmyoglobin. Nitrosylhemochrome, the cure pigment, results when the product is heated.

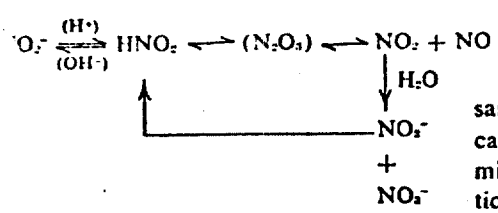
Another mechanism for the production of nitric oxide (NO) proposes (*32*) the reduction of nitrite mediated by reduced cytochrome c [Fe(II)Cyto c] and reduced nicotinamide adenine dinucleotide (NADH):

$$NO_2^- + Fe(II)Cyto\ c \longrightarrow NOFe(III)Cyto\ c$$
$$NOFe(III)Cyto\ c + NADH \longrightarrow NO + Fe(II)Cyto\ c$$

Cured meat flavor is a function of the activity of nitrite on meat components. While emphasis has been placed on the reaction of nitrite with meat pigments, the so-called "cosmetic effect," and on the antibacterial action the compound, little attention has been paid to the development of the characteristic cured flavor. However, there have been several reports on the effect of nitrite on the flavor of bacon, ham, and frankfurters (*33*). Initial studies indicate that flavor can be obtained at concentrations of nitrite lower than the legal limits. There is no information available at this time, however, about the reactions or the meat components involved.

The antibacterial effects of nitrite appear to be necessary to maintain the stability of cured canned meat products exposed to less-than-sterilizing heat treatment. The growth of *Clostridium botulinum* and toxin production are inhibited. While the mode of action of nitrite is still unknown, it has been reported that division of vegetative cells does not occur in the presence of this ion (*34*). Growth of surviving organisms is also inhibited by the action of residual nitrite.

Inhibition of the growth of *C. botulinum* in these meat products is a complex phenomenon involving interaction of the number of spores present, the amount of heat applied, and the concentrations of sodium chloride and sodium nitrite used (*35*). The interdependence of these factors is so great that minor changes in the conditions of one may require balancing modifications in all the others. The *p*H of the product may also play an important role in the bactericidal effect of nitrite, although it is difficult to adjust the *p*H of meat. It has been shown in model systems that there is approximately a tenfold increase in antibacterial effect with a decline of one *p*H unit in the range of *p*H 7.5 to 6.0 (*36*). Under the more acid conditions, larger concentrations of undissociated nitrous acid are available and, according to Shank *et al.* (*37*), this is the molecular species responsible for inhibition of the growth of clostridia. The dynamics of nitrous acid formation may be shown as a cyclic reaction:



Concomitant oxidation-reduction reactions lead to the formation of nitric oxide and nitrogen dioxide. The latter reacts with water forming nitrate ion and nitrite ions, which reenters the cycle. At the *p*H of greatest antibacterial activity (*p*H 4.5 to 5.5) the amount of nitrous acid present is at a maximum.

A number of episodes of botulism in recent years were caused by consumption of improperly handled or improperly processed fish or fish products (*38*). Control of *C. botulinum* in these products is attained by the use of sodium nitrite cure preparations. The preservation of fresh fish fillets or lightly smoked saltwater fish—such as salmon, tuna, halibut, and cod (*39*)—as well as freshwater fish—chub, for example (*38*)—has been described. Federal regulations limiting the residual nitrite in smoked, cured tuna to 10 mg/kg and in sable, salmon, and shad to no more than 200 mg/kg have been established. The nitrite content of smoked chub must be not less than 100 mg/kg (*30*).

The use of nitrite in the preservation of fish may result in the formation of nitrosamines. Certain species of fish, particularly of saltwater fish (*40*), contain large quantities of trimethylamine, trimethylamine oxide, and dimethylamine which may react with nitrite to form *N*-nitrosodimethylamine.

N-Nitroso Compounds

The reaction of nitrite with some classes of amines is a matter of public health interest at this time. Nitrosamines have been used as intermediates in a number of industrial processes (*41*); hence exposure to these compounds may be more widespread than assumed. Animal studies have shown that *N*-nitrosamines and the related *N*-nitrosamides are carcinogenic (*42*), and they may be mutagenic and teratogenic (*43*) as well. Nitrosamines having a wide variety of molecular structures are carcinogenic; of the approximately 100 compounds tested, about 75 percent produced lesions in test animals. Various tissues respond to the action of these compounds, some of them specifically to a certain nitrosamine or to groups of nitrosamines. The effects of nitrosamines can be elicited by several routes of administration—oral, intravenous, inhalation, subcutaneous, intraperitoneal, and topical. Local sarcomas have been observed rarely at the site of injection; the carcinogenic activity usually occurs elsewhere. It would seem, therefore, that the nitrosamines themselves are not carcinogens but may behave as carcinogen precursors (*44*). Studies with radioactive tracers suggest metabolic degradation of the nitroso compound to form an alkylating radical or ion that attacks the 7-position of guanine in nucleic acids. Although no cancer in man has yet been traced to nitrosamines as causative agents, the experimental results in animals suggest that these compounds also would be carcinogenic to man.

The acute toxicity or eventual carcinogenicity of the nitrosamines may, depending on the compound and the circumstances, show itself at very low dosages (*43*). Results of feeding studies are not yet decisive as to precise dose-response relationships. In a study with rats given a single dose, concentrations of *N*-nitrosodimethylamine greater than 5 ppm induced tumors in more than 70 percent of the animals, whereas continuous feeding of 1 ppm in the diet has been suggested as a threshold dose (*45*).

N-Nitrosamines, principally *N*-nitrosodimethylamine, have been reported in a number of foods. However, improved analytical procedures and recognition of artifacts in the preparations have now led to questions concerning the validity of these reports.

Nitrosation has in the past been considered to occur with secondary amines only. Recent studies have indicated that nitrosamines are also formed from tertiary amines and quaternary ammonium compounds that occur naturally in foods (*46*) and drugs (*47*).

The nitrosation reaction may proceed chemically or it may occur as a result of the metabolic activity of microorganisms. Ease of nitrosation, chemically, may be influenced by many factors, including the basicity of the amine (*48*), *p*H (*49*), substrate concentration (*49*), and the presence of some inorganic ions (*50*). The thiocyanate ion is of particular interest. This ion is normally present in human saliva in amounts ranging from 12 to 33 mg/100 ml (higher amounts are found in the saliva of smokers) and is capable of increasing the rate of nitrosation of morpholine (*51*).

In addition to the potential of formation of nitrosamine in processed foods, there may be nitrosamine forma-

tion in the gastrointestinal tract from ingested nitrite and secondary amines or their precursors (*52*). In several in vitro studies nitrosamine was formed from amines and nitrite in media in which intestinal microorganisms were growing (*53, 54*). In other tests, with human patients exhibiting conditions in which there was no, or only low, stomach acidity, nitrosation of diphenylamine, a nontoxic easily nitrosated amine, occurred (*55*). The appearance of tumors in various organs or the methylation of the 7-position in the nucleic acid guanine in rats that had ingested amines or amides and nitrite was considered presumptive evidence for the formation of nitroso compounds (*56*). The role of the intestinal bacteria in nitrosation under these conditions, however, is still not clear. Under healthy conditions the human stomach and upper gastrointestinal tract contain very few organisms (*57*), but when the acidity decreases, conditions may be favorable for the presence of nitrate-reducing, nitrosating bacteria. Nitrates and nitrites ingested with food or water are normally absorbed very rapidly from the stomach and upper gastrointestinal tract, appearing eventually in the urine. Thus, though the residence time of the nitrates and nitrites may be too short for gastric involvement, the potential exists for bladder involvement. It has been shown that rats with experimentally induced bladder infections of *Escherichia coli* excreted nitrosated piperidine after they had ingested the amine and a water solution of nitrate (*53*).

Correlation between many preformed nitrosamines and tumor induction in animals has been demonstrated. However, the relation between nitrite (nitrate) and amines (or their precursors), the end formation of nitrosamines in foods or in vivo, and their carcinogenic effect is still tenuous. While nitrosamine formation occurs more or less readily in vitro, in the normal, healthy human gastric conditions do not seem to be favorable for nitrosamine formation. At this time there is not enough information concerning the naturally occurring amines and their precursors, the *p*H changes in the stomach during digestion, the rate of absorption of the reactants, or the role of the intestinal flora.

The number of reports of nitrosamines in foods confirmed by mass spectrometry, the only procedure currently recognized as definitive, is very limited. Important gaps in our information exist. In addition to lack of information about all the compositional factors of the foods, we are also ignorant of the processing factors that could lead to nitrosamine formation.

Other environmental factors have been mentioned as potential sources of nitrosamines. Tobacco smoke has been implicated in the development of lung cancer. The tobacco plant is rich in amines, and an even larger number of these compounds has been identified in tobacco smoke as a result of the pyrolysis of the nitrogenous constituents of the plant (*58*). Nitrate is also present either as a plant constituent or it may have been added during processing. Under these conditions the presence of nitrosamines has been reported in tobacco (*59*) and in tobacco smoke (*58, 60*). However, reports of nitrosamines in tobacco smoke should be evaluated for reliability of analytical methods used, and the possibility that they may be formed (artifacts) in the collecting traps should be considered (*58*).

A number of commonly used drugs that are taken either in large doses or for long periods of time contain secondary amine groups or structures that may be amine precursors. There is some interest in the potential internal nitrosation of such drugs if ingested with nitrite or water containing high levels of nitrate. Model studies (*47*) reacting nitrite with oxytetracycline and antipyrine yielded *N*-nitrosodimethylamine, and *N*-nitrosodiethylamine was obtained when the drug disulfiram was used. Although no nitrosamine could be isolated from the system containing nitrite and tolbutamide, nitrosohexamethyleneimine was formed from tolazamide. Extended studies with more drugs and investigation of the applicability of model systems to the human experience are needed.

Summary

We are faced with evaluating the potential hazard of nitrate, nitrite, and nitrosamines in our environment. The extent of real danger is not yet known, but deliberate consideration of the available information would suggest that the hazard is not sufficiently great to cause alarm. There may be some who advocate immediate elimination of or drastic reduction in amounts of nitrite or nitrate in cured meat and fish. Before actions such as these would be taken, we should be very sure that we are not foregoing the needed preservative effects of nitrite, which protects us against serious outbreaks of food poisoning. We could be replacing one hazard by another, more serious one.

There is under way in the scientific community a commendable amount of research on many aspects of this important problem. This effort is necessary because so little is known of the possible in vivo synthesis of nitrosamines.

Thus we should continue to regard nitrites and nitrosamines as possible important toxicants but should be cautious about taking any action relative to modification of our food supply until we are sure the action is needed, justified, and proper.

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2465

Renal and Pulmonary Tumors in Rats Fed Dimethylnitrosamine*

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SUMMARY

Renal and pulmonary neoplasms developed in male and female Sprague-Dawley rats fed a diet containing 125 ppm dimethylnitrosamine (DMN) for longer than 80 days. The tumors found in the kidneys can be classified into two types: (*a*) benign solid and cystic adenomas occurring in rats fed DMN for less than 160 days; (*b*) anaplastic (possibly malignant) epithelial tumors in animals treated longer than 160 days. Characteristic nuclear alterations were observed in the cells of the proximal convoluted tubules and are interpreted as the earliest morphological manifestation in the kidney of DMN intoxication.

Stimulated by the observation of the occurrence of two cases of cirrhosis of the liver among workers handling dimethylnitrosamine (DMN), Barnes and Magee (1) studied this compound in experimental animals, particularly in rats. These authors found that, when DMN was fed in a concentration of 50 ppm in a normal diet, primary liver neoplasms, seven of which showed metastases, developed between 26 and 42 weeks in nineteen of twenty animals (11). Recently, the same authors reported the occurrence of renal tumors in a relatively high incidence after feeding DMN to rats (12).

During a study of precancerous changes in the liver, with the use of this compound, among others, multicentric tumors arising in kidneys and in lungs were observed before the findings of Magee and Barnes (12) were known. These observations are reported in confirmation of those of the latter authors.

MATERIALS AND METHODS

Male and female Sprague-Dawley rats, 78 in number, with an average body weight of 100 gm., were placed on the standard low choline diet (0.03 per cent) used in this laboratory (6), supplemented by 125 ppm of DMN,[1] while 33 animals were kept as controls on the basal diet alone. Subtotal hepatectomy was performed in twenty animals (thirteen experimentals and seven controls), and sham laparotomy in 23 (seventeen experimentals and six controls) before feeding of the toxic diet.[2] Of the 111 rats employed, twenty died spontaneously and were excluded from the study.

The rats were sacrificed by cardiac exsanguination under ether anesthesia. Body weights at sacrifice are given in Table 1. After gross inspection tissues were fixed in formalin or Carnoy's mixture, dehydrated, and embedded in paraffin. The following staining methods were used: hematoxylin-eosin, chromotrope-2 R aniline blue, Gomori silver impregnation, methyl green-pyronine, periodic acid-Schiff (PAS) reaction after diastase digestion, and toluidine blue. In some cases reactions for different enzyme activities (alkaline phosphatase at pH 9.2, adenosine triphosphatase) were performed.

RESULTS

Animals fed DMN for less than 80 days did not show any neoplastic process in the kidneys, although some atypism was seen in the cells of the cortical tubules.

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[1] The DMN was obtained from Delta Chemical Works, New York, the fraction distilling at 150° C. (uncorr.) being used.

[2] We are indebted to Dr. Kenneth Weinbren for his help with this phase of the study.

Small nodules, visible only microscopically, were found in the kidneys of six of eighteen rats treated for a period of 80–120 days, and in six of ten animals fed DMN for 120–160 days (Tables 2 and 3). Seven of eight animals, which were on the diet for more than 160 days, developed nodules varying from barely visible up to 9 mm. in diameter. The smaller nodules appeared as solid, well outlined structures of whitish-grey color, localized exclusively in the renal cortex. The larger neoplasms occupied extensive parts of the organs (Fig. 1) leaving only small areas of compressed renal tissue between them. These tumors displayed areas of hemorrhage and necrosis. They extended into the medulla and caused a bosselated appearance of the kidney without invading the capsule.

Microscopically the tumors present two different types. All the neoplasms arising in rats fed DMN for less than 160 days, i.e., all the nodules visible only microscopically, looked very similar. They consisted of nests and cords of large cells without lumen formation. The cytoplasm showed basophilic staining, and the nuclei were large, vesicular, and somewhat irregular in shape. Mitoses were common and sometimes atypical. The nuclei contained large nucleoli, often increased in number. Within these nodules smaller groups of cells were separated by a delicate collagenous stroma. The nuclei of the stromal cells could be easily differentiated from the tumor cells and correspond to typical fibroblasts and fibrocytes. The outline against the surrounding tissue was sharp, the tubules in the vicinity being slightly compressed. Nodules of larger size showed central necrosis. These solid nodules were combined in some cases with cysts of different size which resembled dilated tubules except for the quality of their epithelium (Fig. 2). The lining cells were similar to those forming the solid nodules. They were often arranged in two or more layers without regularity, sometimes forming papillary projections into the lumina. These cysts were also found without connection to solid structures. The arrangement of the stroma was the same as described above. Toluidine blue staining produced a

TABLE 1

AVERAGE BODY WEIGHTS OF EXPERIMENTAL AND CONTROL RATS AT SACRIFICE

Animals	Period (days)			
	0–79 (gm.)	80–120 (gm.)	121–160 (gm.)	161–224 (gm.)
Controls	214±27 (14)*	288±61 (7)	370±74 (2)	358±84 (3)
Experimentals	173±24 (19)	236±35 (13)	244±40 (9)	217±23 (7)
Partial hepatect., controls	243± 3 (2)	344±39 (2)	382 (1)	
Partial hepatect., experimentals	147±29 (5)	220±19 (5)	226 (1)	

* Means ± average deviation. Parentheses indicate number of animals sacrificed in each period.

TABLE 2

SURVIVAL TIME AND TUMOR INCIDENCE OF RATS FED DMN FOR VARYING PERIODS OF TIME

Period (days):	0–79	80–120	121–160	161–224
	Experimentals			
No. surviving	57*	37	23	8
No. dying	8	1	1	0
No. with kidney tumors	0/19	4/13	5/9	7/8
No. with lung tumors	0/19	0/13	2/9	5/8
No. with liver damage	5/19	11/13	6/9	8/8
	Controls			
No. surviving	26	12	5	3
No. dying	0	0	0	0
No. with kidney tumors	0/14	0/7	0/2	0/3
No. with lung tumors	0/14	0/7	0/2	0/3
No. with liver damage	0/14	0/7	0/2	0/3

* Denotes no. of animals surviving until time of sacrifice.

slight metachromasia of the cells of both the solid and the cystic tumors. In addition to the unquestionable neoplastic changes, solid nests of cells were found, not exceeding the size of a single tubule and not traversed by septa of connective tissue (Fig. 3). They consisted of the same large basophilic cells with large, pale, irregularly shaped nuclei. They could be found in animals treated for 80–120 days with DMN, even in the absence of tumors.

The second type of renal neoplasm developed in rats fed DMN for more than 160 days and consisted mostly of nests and groups of cells with a great variation in size and shape, separated quite irregularly by broad bands of connective tissue. The nuclei showed considerable polymorphia and polychromasia. Mitoses were numerous and often atypical. Large areas of these more anaplastic tumors were necrotic or were destroyed by extensive hemorrhages. In some cases, however, PAS staining after diastase treatment revealed an architecture similar to that of the renal cortex, namely, groups of tumor cells surrounded by thin PAS-positive membranes, very similar to those surrounding the renal tubules as basement membranes. In other cases the neoplasm consisted of an adenomatous papillary tissue with more advanced anaplasia, but somewhat reminiscent of the previously described cystic structures. These anaplastic types of tumors did not show any metachromasia.

TABLE 3

SURVIVAL TIME AND TUMOR INCIDENCE OF PARTIALLY HEPATECTOMIZED RATS FED DMN FOR VARYING PERIODS OF TIME

Period (days):	0–79	80–120	121–160
		Experimentals	
No. surviving	11	6	1
No. dying	2	0	0
No. with kidney tumors	0/5	2/5	1/1
No. with lung tumors	0/5	1/5	0/1
No. with liver damage	2/5	3/5	1/1
		Controls	
No. surviving	5	3	1
No. dying	2	0	0
No. with kidney tumors	0/2	0/2	0/1
No. with lung tumors	0/2	0/2	0/1
No. with liver damage	1/2	2/2	1/1

Most of the rats with tumors showed disseminated focal alterations of the epithelium of the cortical tubules. Large, sometimes monstrous nuclei exhibited an unusual variation in size, shape, and chromatin content (Fig. 4). These changes were most prominent in the proximal convoluted tubules. They could also be found in tumor-free animals treated with DMN for less than 80 days, but were absent from the controls.

Of the animals studied both with and without renal tumors, eight treated with DMN for more than 111 days developed multiple nodules in the lungs, ranging in size up to 1 mm. Histologically they consisted of epithelial tissue arranged in somewhat alveolar structure (Fig. 5). The cells were medium-sized, the nuclei polymorphic and varying in chromatin content. Bronchi were involved and were partly destroyed by the neoplastic tissue (Fig. 6). In one animal fed DMN for 209 days, a pulmonary neoplasm with a diameter of 1 cm. was observed. Microscopically, this was an anaplastic immature epithelial tumor arising from the bronchial lining, showing gradual transition to the normal epithelium. Histochemical demonstration of alkaline phosphatase activity showed a weak positive reaction in the cells of this tumor. No positive reaction was found in any of the renal tumors.

Other investigated organs, such as lymph nodes, lower uninary tract, and pancreas, did not show neoplastic changes. In the livers of animals treated for more than 15 weeks, however, hyperplastic and cystic nodules could be observed in a relatively high incidence.

Four rats were returned to stock diet (Rockland rat pellets) after a treatment of 202 days. One of them was found dead 9 weeks after the change of the diet and could not be used for further study. The three remaining animals were sacrificed after 22, 63, and 93 days, respectively. All of them had grossly visible kidney and lung tumors.

The effect of partial hepatectomy on the incidence of tumors in kidney and lung was negligible.

DISCUSSION

Spontaneous renal tumors in rats are rare. A few malignant embryonic nephromas, carcinomas,

FIG. 1.—Kidney of rat fed DMN for 217 days. Large cortical tumors extending into the medulla. Toluidine blue. ×5½.

FIG. 2.—Kidney of rat fed DMN for 139 days. Solid tumor between arrows. Cystic lesion nearby arising from dilated tubules. H. & E. ×150.

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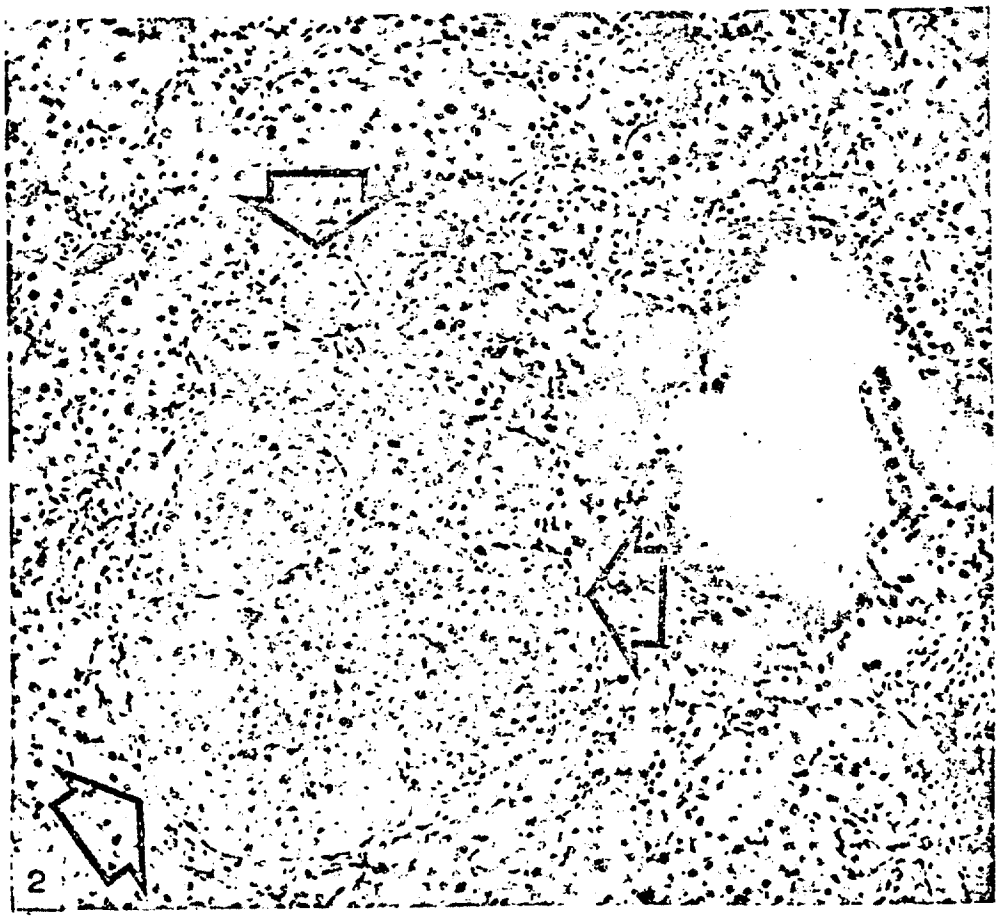

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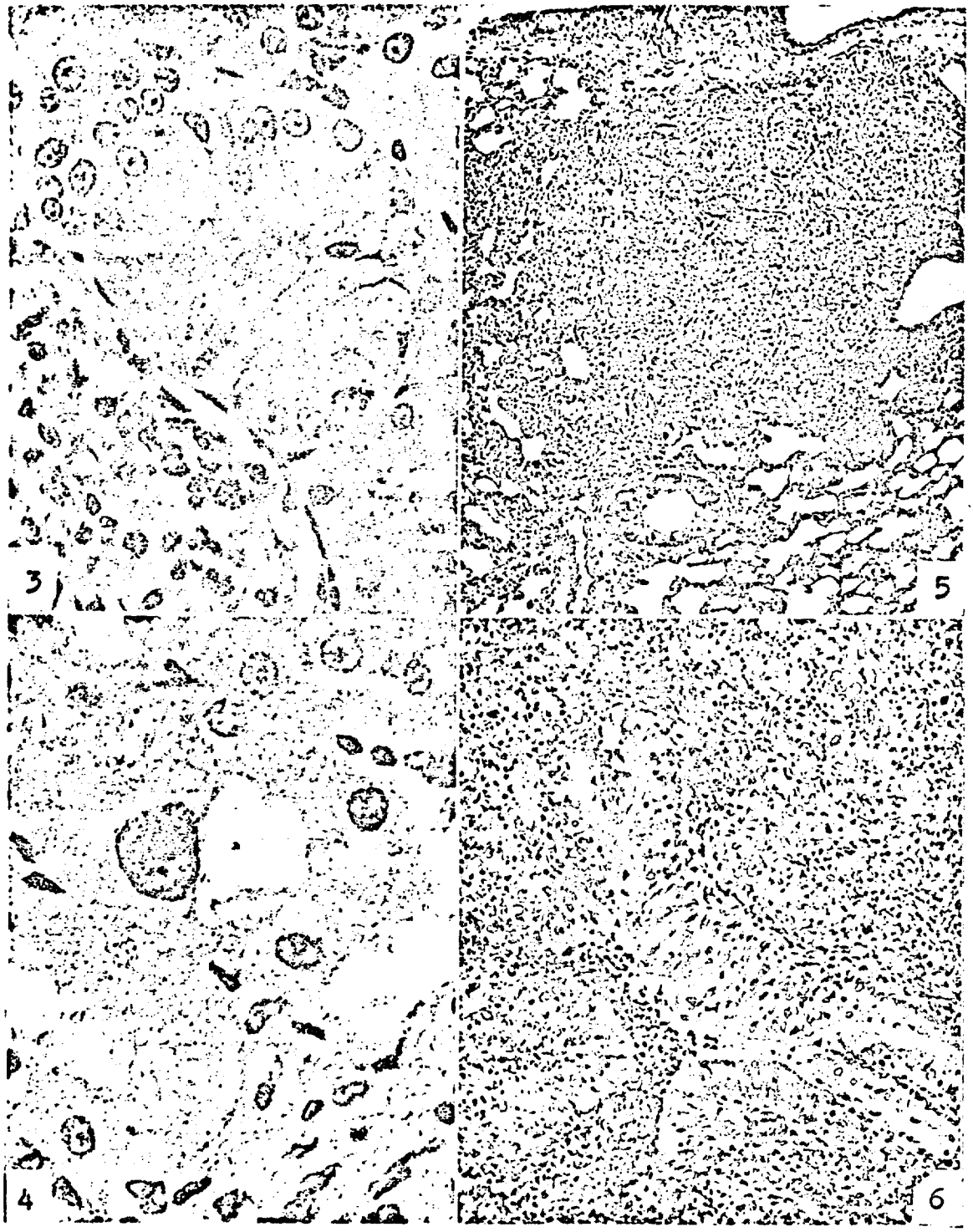
FIG. 3.—Kidney of rat fed DMN for 86 days. Small solid cell nest not exceeding the size of a single tubule. H. & E. ×600.

FIG. 4.—Kidney of rat fed DMN for 106 days. Proximal convoluted tubule. Nuclear atypism. H. & E. ×950.

FIG. 5.—Lung of rat fed DMN for 167 days. Epithelial tumor with alveolar structure. H. & E. ×40.

FIG. 6.—Lung of rat fed DMN for 224 days. Tumor tissue invading a bronchus. H. & E. ×150.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and sarcomas have been reported (3, 4, 16–18). McCoy (15) found eleven kidney tumors, four of which were adenomas, in a series of 100,000 rats. More recently, the development of renal neoplasms following lead intoxication (19) and treatment with 4′-fluoro-4-aminodiphenyl (14) has been described. As mentioned above, Magee and Barnes (12) found a high incidence of tumors in the kidneys of rats after DMN feeding. These authors classified them into two types: benign adenomas, developing in older animals, and malignant anaplastic adenocarcinomas occurring in younger rats within a shorter period after the treatment. These results differ from our findings, as we observed the anaplastic types only in the older animals treated for a longer period. The difference in the dosage and the strain of rats used may have been responsible for the different results.

From a comparison of our results with the histologic description and the photographs published by others, it appears that most of the experimental kidney tumors present a similar appearance independent of the causative agent. Even spontaneous renal neoplasms observed by Eker (5) in an inbred strain of Wistar rats do not differ significantly.

The tumors found in the lungs were considered to be primary lesions, since the architecture was somewhat similar to the pulmonary parenchyma, and dissimilar to the renal tumors. Moreover, transition of lung tissue into tumor, infiltration, and loss of a sharp border were observed; lung tumors were found in rats in the absence of renal neoplasms; and histochemically a different enzyme pattern from that of the renal tumors was noted.

Biochemical studies have revealed (*a*) that DMN is rapidly metabolized by liver slices or homogenates and little or not at all by preparations of kidney or other organs (13), (*b*) that protein synthesis in the liver, but not in the kidney or spleen, is markedly decreased by DMN (10), and (*c*) that the microsomal fraction of the liver is primarily involved both in the degradation of DMN (13) and the metabolism of azo dye carcinogens (7, 9). Little is known about the metabolic products of DMN (8), however, and its mechanism of action has not been elucidated.

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