Large amounts of nitrogen substrates were used in this study in order to enhance the detectability of dimethylnitrosamine, whose formation, as a result, increases more than proportionally to the increase in the concentrations of the precursors (Sander and Schweinsberg, 1972). Although these quantities may not be realistic in terms of the actual levels of the compounds in palmwine, they nevertheless could serve as a model for the study of the mechanism of nitrosamine formation and degradation in fermenting biological systems.

In the studies of Sander (1968) and Alam et al. (1971), bacteria were reported to reduce nitrate to nitrite and subsequently to catalyze the nitrosation of amines. The nitrosation occurred at such pH values which do not permit a spontaneous reaction between the secondary amines and nitrite. The possible involvement of some non-nitrate reducing strains of bacteria in the enzymic nitrosation of secondary amines at neutral pH has also been reported (Hawksworth and Hill, 1971). On the other hand, Collins-Thompson et al. (1972) suggested that the formation, in culture, of dimethylnitrosamine from the corresponding amine and nitrite could be nonenzymic and pH dependent and that catalysis might be due to one or more bacterial products. It would, therefore, seem from our results that the dealkylation of trimethylamine and the reduction of nitrate to nitrite by bacteria and the nitrosation of dimethylamine, either enzymatically by in-dwelling microorganisms or spontaneously in acid pH generated during fermentation, are possible steps in the formation of dimethylnitrosamine in palmwine.

The degradation of dimethylnitrosamine during the fermentation of palmwine, reported here, could also be due to the activities of in-dwelling microorganisms, particularly in the light of the findings of Tate and Alexander (1975) of a possible microbial involvement in the slow decomposition of DMN and other nitrosamines in sewage, soil and lake water and that of Read (1975) of the degradation of DMN by yeast cell suspensions and intracellular extracts. From our results, a safe period for the drinking of palmwine would be after fermentation.

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Received for review December 23, 1977. Accepted August 16, 1978.

Mechanism of *N*-Nitrosopyrrolidine Formation in Bacon

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Evidence is presented to show that *N*-nitrosopyrrolidine in cooked bacon arises by decarboxylation of *N*-nitrosoproline formed very likely by radical nitrosation of free proline in pork belly. A method for measuring *N*-nitrosoproline in raw bacon is described.

In 1962 it was reported (Koppang, 1962) that liver damage was induced in mink by consumption of herring meal treated with nitrite. The causative agent was identified as nitrosodimethylamine (NDMA) (Ender et al., 1964).

The widespread use of nitrite in the preservation of foods, and in particular meats, led to a high level of concern that nitrosamines may be present in foodstuffs as a result of the reaction of the secondary amines with residual nitrite. In the period from 1970 to 1975 there was a rapid development of methodology concerned with the analysis of minute quantities of nitrosamines in foods. At present, there are many methods available which are capable of measuring volatile nitrosamines at the microgram/kilogram level. Excellent reviews have been presented by Foreman and Goodhead (1975) and Scanlan (1975).

Although volatile nitrosamines have been reported in many foods at microgram/kilogram levels, it is only in cooked bacon that the presence of NDMA and *N*-nitrosopyrrolidine (NPYR) has been consistently confirmed (Sen et al., 1973; Crosby et al., 1972). The nitrosamines that are most commonly found in bacon are NPYR and NDMA with the former in preponderance (about 80% of the total volatile nitrosamines). It should be borne in mind, however, that NDMA is the more carcinogenic of the two.

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Table I. Free Amino Acids in Whole Pork Belly and Raw Side Bacon (mg/kg)

	sample									
	pork belly				side bacon					
	1	2	3	4	1	2	2	3	4	5
amino acid	ion exchange	GC	GC	GC	ion exchange	GC	ion exchange	GC	GC	GC
alanine	156	124	106	60	174	143	130	128	122	142
valine	40	20	13	7	68	40	55	24	28	24
glycine	58	62	44	18	87	68	68	64	69	67
isoleucine	21	10	6	2	48	27	22	14	18	16
leucine	41	31	21	12	85	43	50	34	39	39
proline	26	17	22	11	81	41	61	27	22	20
threonine	4	20	17	8		36	79	21	20	18
serine		23	29	7		53	47	35	30	30
methionine	12	6	3		34	13	20	5	10	9
hydroxyproline		2	3		3			4	3	2
phenylalanine	19	13	10		41	19	27	13	16	19
aspartic acid	10	14	12	5	34	25	19	15	13	12
glutamic acid	79	143	144	67	180	224	183	155	141	136
tyrosine	21	8	5		50	16	18	10	10	11
lysine		33	18	16		84	60	33	30	34
arginine	32				90					
tryptophan	5				8					

As part of an overall program to eliminate or suppress nitrosamine formation in cooked bacon, and as a prelude to devising molecules that would act as antinitrosamine agents, the elucidation of a mechanism of formation of NPYR in cooked bacon was undertaken. Our findings constitute the subject matter of the present report.

As a result of this study a number of substances have been found which would block nitrosamine formation in cooked bacon. This work will be reported in full in future publications from these laboratories.

EXPERIMENTAL SECTION

Important Safety Note. *N*-Nitrosamines have been shown to be highly carcinogenic compounds in test animals and all experimental work should therefore be done in a well-ventilated area. Safety gloves should be worn whenever *N*-nitrosamines are being handled.

Analysis of Volatile Nitrosamines. Two methods were used for the analysis of volatile nitrosamines. Both depend on the denitrosation of the nitrosamines by HBr in acetic acid as described by Eisenbrand and Preussman (1970). In the early part of this work the quantitative determination of the liberated nitrite was used as a measure of the nitrosamine concentration. Subsequently, the method of Klimisch and Stadler (1974), involving the measurement of the fluorescence of the reaction products of the amine portion of the nitrosamines with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) after separation by TLC, was employed in our work on the volatiles produced on frying bacon. The combined method has been described in detail in an earlier publication from this laboratory (Cross et al., 1978).

Concentration of Free Proline in Pork Belly and in Side Bacon. The obvious organic precursor of NPYR is the amino acid proline; consequently, we have determined the free amino acid profile of both pork belly and side bacon by the gas chromatographic technique of Gehrke and Roach (1969). A portion of the free amino acids obtained was also analyzed by the standard ion-exchange technique on a Beckman Model 121 amino acid analyzer. The amino acid analyses obtained for four samples of pork belly and five samples of raw side bacon are collated in Table I.

Determination of *N*-Nitrosoproline (NPro) in Raw Bacon. The bacon sample (50 g) was placed in a blender jar with sulfamic acid (about 80 mg). The mixture was extracted at high speed for 4 min using a mixture of water (45 mL) and heptane (75 mL). The homogenized sample was centrifuged at 18000*g* for 20 min, and the liquid layers were removed to a separatory funnel. The residue was reextracted with water (45 mL) and heptane (30 mL) for 2 min. The sample was again centrifuged and the solvent layer added to the funnel. The heptane layer was discarded and the water layer treated with phosphomolybdic acid (2 mL of a solution of 4 g in 5 mL of water). The precipitated protein was removed by centrifugation and the clear aqueous layer filtered through a glass fiber filter under vacuum directly into a 500-mL separatory funnel. The aqueous layer was extracted with dichloromethane (3 × 30 mL). The dichloromethane was discarded and the aqueous layer was adjusted to pH 1 with concentrated hydrochloric acid. The solution was extracted with ethyl acetate (3 × 100 mL) (Hamilton and Ortiz, 1950). The ethyl acetate was dried with anhydrous sodium sulfate (12 g) and transferred to a 500-mL round-bottom flask. The ethyl acetate was removed on a rotary evaporator and the residue dissolved in dichloromethane (5 mL). Ethereal diazomethane was added to form the methyl esters of the nitrosamino acids. The solution was transferred to a 25-mL pear-shaped flask and evaporated through a micro Snyder column to about 0.5 mL. Hexane (5 mL) was added through the column and evaporation continued to remove residual ether and dichloromethane. The hexane solution was applied to a silica gel H column (Cross et al., 1978) and eluted with a mixture of pentane–diethyl ether–dichloromethane (10:3:2, 25 mL). The eluate was discarded. The nitrosamino acids were eluted with the same solvents in the ratio 5:3:2 (25 mL). One-half of the eluate was treated with methyl myristate as an internal standard and analyzed by gas chromatography on either 3% OV-225 or 2% Silar 5 CP on Gas-Chrom Q to determine the quantity of nitrosamino acid present. The remaining half of the eluate was transferred into acetic acid and split with HBr in acetic acid (Eisenbrand and Preussmann, 1970). The amino acids produced were spiked with glycine and leucine as internal standards, converted to *n*-butyl ester heptafluorobutyrates and analyzed by gas chromatography on an SE-30 column.

Recoveries of NPro added to non-nitrite bacon at the 1 ppm level varied in seven samples from 22 to 35% by the methyl ester method with an average of 29%. By the amino acid determination recoveries varied in 14 samples

Table II. Concentration of Volatile Nitrosamines in the Rasher and the Cook-Out Fat from Fried Bacon

sample	total volatile nitrosamines, μmol × 10^{-2}/kg	
	cook-out fat	rasher
1	70	23
2	4	2
3	31	20
4	58	23
5	58	30
av	44	20

Table III. Effect of Frying Time on Nitrosamine Concentration in Bacon Fat

frying time, min	nitrosamine, μmol × 10^{-2}/kg
8	27
12	35
16	25
20	20

from 29 to 39% with an average of 35%.

Frying Conditions. The temperature of the cook-out fat produced on frying side bacon was measured by placing a Pacific Transducer Corporation contact thermometer at the center of the electric frying pan and surrounding it with bacon. The bacon was placed in the pan with slices overlapping so that the lean edge of one slice overlapped the fat edge of the adjacent slice. Ten slices were fried at one time. Starting with a cold frying pan, the control was set at the 360 °F setting, and time and temperature were measured. After 4 min, the bacon was turned for the first time. Thereafter it was turned frequently until frying was complete. The maximum temperature, fluctuating between 167 and 172 °C, was reached after 5 min of frying.

Concentration of Nitrosamines in the Rasher and the Cook-Out Fat from Fried Bacon. Several samples of side bacon were fried as described above and a comparison of the concentration of nitrosamines in the rasher and cook-out fat was made. These data are presented in Table II showing that the concentration in the cook-out fat is approximately twice that in the rasher. In most of our subsequent work we analyzed only the cook-out fat. The rashers were analyzed by the procedure described in an earlier publication (Cross et al., 1978) with the modification that the first distillation was omitted. The rasher was ground in a blender for 3 min with cold 3 N NaOH before the vacuum distillation. Analysis of 14 samples containing 10, 20, and 50 μg/kg of NPYR gave an average recovery of 48% by the colorimetric procedure.

Dependence of the Concentration of Nitrosamines in Cook-Out Fat on Frying Time. In a study of the effect of frying time on nitrosamine level, packaged bacon sufficient for four samples was sampled sequentially to give four lots of bacon which were more or less equivalent. The slices of each of the packages of bacon were distributed consecutively between four new packages to achieve this result. The samples were fried for periods of 8, 12, 16, and 20 min. After 4 min of frying, the slices were turned frequently until the frying was finished. The cook-out fat was collected and analyzed for nitrosamines. The results are presented in Table III. Similar results comparing the effect of grilling and frying of bacon are presented in Figure 1 (Coleman, 1976b).

Decarboxylation of NPro to NPYR. A solution of *N*-nitrosoproline (60 mg), prepared by the method of Sander (1967), and sodium chloride (12 g) in water (62 mL) were added to freshly ground pork belly (620 g). The solution was well dispersed through the meat by mixing.

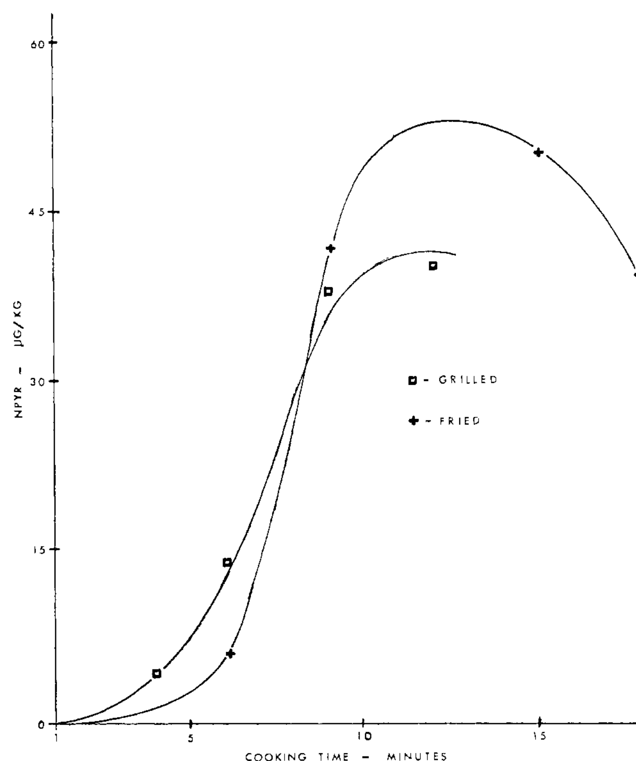


Figure 1. The effect of grilling and frying time on NPYR production in bacon.

Table IV. Effect of Added Proline on the NPYR Content of Fried Bacon

sample	proline added, mg/kg	NPYR, μg/kg
1	0	32
2	50	75
3	100	100

The product contained 87 mg/kg of NPro. After the product was allowed to stand for 3 h in the refrigerator, the meat was made into patties and fried. The cook-out fat was analyzed for nitrosamines by the colorimetric method (Cross et al., 1978).

Effect of Added Proline on the NPYR Level in Fried Bacon. Three 1-lb packs of bacon were sequentially divided into three samples. One sample was streaked with water (4 mL) and the other two with L-proline in water. The bacon was fried and the cook-out fat analyzed for NPYR by the densitometric technique. The data are presented in Table IV.

Decomposition of L-Proline and *N*-Nitroso-L-proline in Lard. A mixture of powdered L-proline (6.4 mg) or *N*-nitroso-L-proline (7.0 mg) in lard (12.7 mg) was scanned in a Perkin-Elmer DSC-1 calorimeter from 80–175 °C at a rate of 10 °C/min.

Collection and Analysis of Volatiles Produced on Frying Side Bacon. Two methods were used to collect the volatiles from frying bacon. In the first method we collected the volatiles to determine the distribution of nitrosamines between the vapor and the cook-out fat in a manner similar to that reported by Gough et al. (1976). The exact details of the method used and the results of our work are available in the supplemental section (see Supplementary Material Available paragraph).

A second method of collection was used to determine the concentration of volatile nitrosamines in the vapor over bacon as the frying progressed. The electric frying pan lid was drilled to accept a polyethylene nipple which was

Table V. Nitrosamines in the Vapor over Frying Bacon

sample		nitrosamines by TLD, μg	
		NDMA	NPYR
1	A	0.15	0.23
	B	0.14	0.06
	C	0.18	0.38
	D	0.63	1.23
	E	0.94	2.06
cook-out fat	F	2.35	10.05
		2.31[a]	7.89[a]
2	A	0.22	0.20
	B	0.21	0.07
	C	0.79	0.74
	D	0.46	0.67
	E	2.05	2.29
cook-out fat	F	4.26	7.05

[a] Analysis done in duplicate.

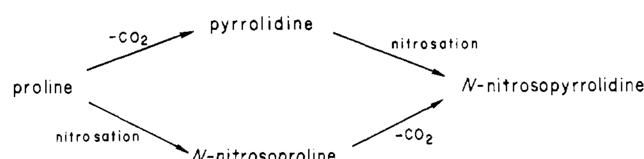
connected through a 4-cm piece of black rubber tubing to an adaptor to a straight condenser containing a spiral cooling surface. The outlet was connected to a Perkin triangle which was calibrated at 5- and 10-mL volumes so that fractions could be taken during frying. A water aspirator was connected to the triangle through a variable leak.

Four pounds of bacon was sequentially sampled so that ten more or less equivalent slices could be used at one time. In order to cook the 4 lb of bacon, the frying was repeated six times. The bacon was placed in the cool frying pan and the apparatus connected so that fractions of the distillate could be taken throughout the frying. The bacon was cooked for 12 min without turning. When the vapor from the cooking bacon began to escape from around the lid of the frying pan the vacuum was adjusted until it just stopped. Thirteen 5-mL fractions were taken; the 14th fraction contained the amount collected until the end of the frying period. The condensing system was rinsed well with water after each batch of bacon was cooked. Combining the corresponding fractions of the six batches, 13 fractions were obtained, of 30 mL, plus a variable 14th (30 mL). By further combining adjacent fractions in groups of three we obtained four fractions of 90 mL each plus the variable fifth fraction (90 mL) (referred to as samples A to E in Table V).

For every 100 mL of distillate we added sodium chloride (20 g) and 10 N sulfuric acid (1 mL). The solution was extracted with dichloromethane (4 × 25 mL). The combined dichloromethane layer was backwashed with 1.5 N sodium hydroxide (2 × 8 mL), dried with anhydrous sodium sulfate, and further cleaned up by our usual Silica Gel-H procedure (Cross et al., 1978). The nitrosamines were treated with hydrogen bromide in acetic acid to remove the nitroso group. The resulting hydrobromides, after removal of the acetic acid on a rotary evaporator, were converted to the NBD derivatives, which were separated by thin-layer chromatography, and quantitated by fluorescent densitometry. No corrections were made for nonquantitative recovery of nitrosamines in the described procedure. The results are presented in Table V.

Effect of Frying Bacon in an Uncovered Pan and in a Vented, Covered Pan on the Nitrosamine Concentration in the Cook-Out Fat. Four pounds of sliced bacon were sequentially sampled into two lots of 2 lb each. One lot was fried for 12 min, the bacon being turned only once at 8 min. The vapors were allowed to escape into a fume hood. The other lot was fried under the same regime but in a covered, vented pan operated as in our vapor collection method described above. The cook-out fat from each of the two samples was analyzed for volatile nitrosamines.

Scheme I



RESULTS AND DISCUSSION

A number of possible precursors of NPYR suggest themselves. These include proline, pyrrolidine, spermidine, putrescine, ornithine, and collagen (Bills et al., 1973; Huxel et al., 1974; Gray and Dugan, 1975; Coleman, 1978). Most of the work, however, is based on studies in simulated model systems which, while useful, may not necessarily reflect the true situation in bacon. Although there are few data on the occurrence of amines in bacon, it appears from the work of Patterson and Mottram (1974) that naturally occurring pyrrolidine is not the precursor of NPYR. Singer and Lijinsky (1976) did not detect significant quantities of pyrrolidine in meat samples.

Since proline occurs naturally in many foods and is present at substantial levels in connective tissues, it appeared to us to be the most likely organic precursor of NPYR. We have consequently examined the free amino acid profile of both pork belly and side bacon by the standard ion-exchange technique and by the gas chromatographic method of Gehrke and Roach (1969). The results for four samples of pork belly and five samples of raw side bacon are collated in Table I and show a free proline content in pork belly of 11–26 mg/kg and in raw side bacon of 20–81 mg/kg. These values are in very good agreement with the values reported in the literature. Thus, Nakamura et al. (1976) found the proline content of bacon to be 25–72 mg/kg, while Gray et al. (1977) reported an average value of 24 mg/kg. In the case of pork belly, Lakritz et al. (1976), as well as Gray and Collins (1977), published data in agreement with our findings.

Since the amount of free proline in bacon is several orders of magnitude more than required to account for the NPYR formation in the microgram/kilogram range, it is very likely the single largest source of NPYR production in bacon, with minor, if any, contribution from the connective tissues which are rich in proline and may break down on heating to liberate the amino acid. Compelling, if not conclusive, evidence rests on the observation that when free proline is added to bacon there is a proportionate increase in NPYR formation (Table IV). One can calculate from the apparently linear relationship that, if the source of NPYR in fried bacon is free proline, then the bacon used in this experiment must have contained about 48 mg/kg of free proline, in good agreement with the experimental data presented above. Coleman (1978) has also established a linear relationship between the level of free proline present at cooking with the level of NPYR found in cook-out fat. By working with C_{14} labeled proline, Hwang and Rosen (1976) have similarly come to the conclusion that proline is the major precursor of NPYR. The conversion of proline to NPYR could conceivably occur by either of the two pathways shown in Scheme I.

On the basis of the evidence presented below, the lower pathway involving the intermediacy of *N*-nitrosoproline appears to be the more likely one, both the nitrosation and decarboxylation occurring during the cooking process.

The only difference between the two routes is in the order in which nitrosation and decarboxylation occur. In

the top pathway, proline first undergoes decarboxylation to pyrrolidine, which then undergoes nitrosation to NPYR, whilst in the lower route, nitrosation takes precedence over decarboxylation. An indication of the relative ease of decarboxylation of proline and NPro was obtained from differential scanning calorimetric studies. Whilst no thermal change was observed with L-proline in the 80–175 °C range, the NPro underwent an endothermic change at 104 °C indicative of its melting, followed by rapid decomposition with evolution of gas at 113 °C. The conversion of NPro to NPYR is thus, relatively speaking, more facile than the transformation of proline to pyrrolidine. The lower pathway, involving intermediacy of NPro is thus the more likely route.

Although NPro decarboxylates more readily than proline, the decarboxylation process under bacon frying conditions is by no means a facile one. Thus, when ground-up uncured pork belly spiked with NPro at a level of 87 ppm was made into patties and fried, the yield of resultant NPYR found in the cook-out fat was only 0.16%. This explains why NPYR is formed in cooked bacon in the microgram/kilogram range although free proline is present in milligram/kilogram quantities. The decarboxylation of NPro is thus the yield-limiting step in the conversion of proline to NPYR.

In view of the implied intermediacy of NPro in the preferred scheme of conversion of proline to NPYR, we have looked for the nitrosamino acid in raw bacon. Towards this end, a procedure was developed for measurement of nonvolatile nitrosamines in raw bacon, in which, after a suitable clean-up procedure, NPro was quantitated by GC as the methyl ester. In an alternative method, the same nitrosamino acid was cleaved with HBr and acetic acid according to the method of Eisenbrand and Preussmann (1970) and the resulting proline quantitated once again by GC as the *n*-butyl ester heptafluorobutyrate. Agreement between the two methods was good, although recoveries of NPro at the 1 ppm level by both methods were low (29–39%). The amount of NPro detected by us in the raw bacon was in the 40 μg/kg range by both the methods. This compares favorably with the results of Ivey (1974), who found 13–46 μg/kg of NPro in five out of six samples of bacon. These data, however, are not in agreement with those of Kushnir et al. (1975), who reported NPro in the milligram/kilogram range in raw bacon. Even the high levels of NPro claimed by Kushnir would only account for about 2 μg/kg of NPYR in the fried bacon fat considering that the yield of decarboxylation is only ca. 0.16%. Clearly, preformed NPro in raw bacon is not the main precursor of NPYR found in cooked bacon. Sen et al. (1976) and Tannenbaum et al. (1977) have come to similar conclusions. However, it should be borne in mind that this by no means rules out the intermediacy of NPro, which could be formed at higher temperatures during the cooking process.

Having established the likely organic precursor of NPYR as free proline and the overall pathway for its conversion to NPYR, attention was next focused on the nitrosation step. The formation of NPYR in cooked bacon is a thermally induced process. Thus, we as well as others (Fazio et al., 1973; Crosby et al., 1972; Sen et al., 1973; Fiddler et al., 1974; and Telling et al., 1974) have looked for and found little or no NPYR in raw side bacon. The cooking conditions, however, have an important bearing on the yield. This is clearly shown in Figure 1.

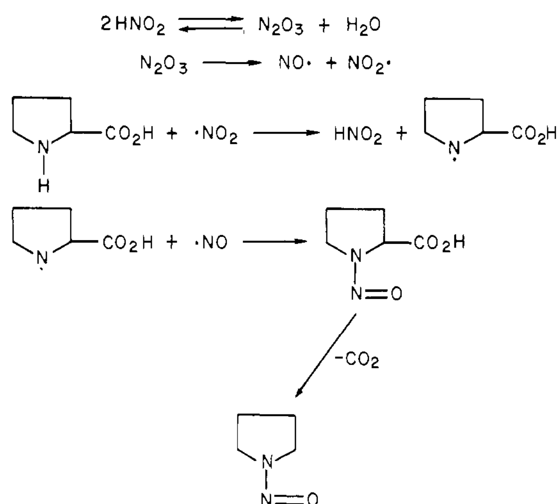
From Table III and Figure 1 it can be seen that the maximum amount of nitrosamine is produced when the bacon is fried or grilled for about 12 min. During the initial 4 min of heating, very little nitrosamine is found in the rendered fat, after which it increased sharply reaching a maximum at around 12 min and then beginning to decline. It is interesting to note that at 8 and 16 min, roughly the same amount of NPYR is detected in the cook-out fat, but for different reasons. At 8 min the formation of NPYR is not complete, while at 16 min some of the NPYR formed is either destroyed or lost in the vapor. It has been found that the temperature during the first few minutes of frying barely exceeds the boiling point of water. During that time, therefore, the bulk of the water is being boiled off and it is only after the major portion of water in the system is expelled that the nitrosamine formation speeds up. Two explanations can be offered for the initial low formation of nitrosamines: (1) that the nitrosamines are actually formed at about 100 °C but, being steam-volatile, are removed with the expelled water, or (2) that the nitrosation occurs at temperatures greater than 100 °C after the major portion of the water is removed and therefore occurs in the fat phase.

When ground pork containing 70 μg/kg of NPYR was fried no nitrosamines could be detected in the cook-out fat, presumably because it was expelled in the vapor due to its well-known steam volatility. In marked contrast, the frying or grilling of bacon does produce measurable quantities of NPYR. It would therefore appear that very little nitrosamine is present while the water is being expelled during cooking. This was also clearly shown in subsequent experiments in which the volatiles produced on frying side bacon were collected.

In the first method of collection, total volatiles were collected in one lot and analyzed. Our colorimetric procedure (Cross et al., 1978) was not applicable to the analysis of nitrosamines in the volatiles due to the contamination with smoke constituents which inhibited color formation. The TLD method was therefore used. The cook-out fat was also analyzed. The results showed that up to 62% of the NDMA and 32% of the total NPYR produced on frying bacon appeared in the vapor. The possibility that the frying of bacon in a vented covered pan results in refluxing and distillation of the nitrosamines from the fat was ruled out since bacon fried normally in an open frying pan and in a vented covered pan showed no differences in the nitrosamine levels in the cook-out fat (45 vs. 42 μmol × 10^{-2}/kg, respectively). Recently, results of two studies carried out along similar lines have appeared. Sen et al. (1976a) found, on average, about 70% of the total NDMA and 50% of the total NPYR in the vapor produced during cooking, in reasonable agreement with our results. In contrast, Gough et al. (1976) reported that the majority of their samples showed >85% of the NDMA and >75% of the NPYR in the vapors. It is possible that the disagreement with the data presented by the English workers is due to the different types of bacon used. In our experiments we used side bacon containing about 30% moisture and 60% fat. This is the type of bacon commonly consumed on the North American Continent, whilst Wiltshire bacon, which contains the eye muscle, was used by the English workers.

The second method of collection was used to determine the concentration of volatile nitrosamines in the vapors over bacon, as the frying progressed. The distillate was collected in small fractions and analyzed by our TLD method (Cross et al., 1978). The results in Table V show that both NDMA and NPYR increase substantially as the cooking proceeded, the quantities present in the last two fractions being up to ten times more than those in the earlier fractions. This strongly suggests that *nitrosamine*

Scheme II



formation during frying of bacon occurs essentially, if not entirely, in the fat phase, after the bulk of the water is removed and therefore by a radical rather than an ionic mechanism. The latter is an important point to stress since it has a strong bearing on the search for suitable substances that would block nitrosamine formation in bacon. In light of this finding, the reduced yields of nitrosamines observed in grilling vs. frying (vide supra) become understandable. During grilling the cook-out fat runs out of the heated area and therefore never reaches the same high temperatures as during pan frying of bacon.

It is tempting to speculate that the following situation obtains in bacon during cooking, namely that nitrous acid is converted essentially into N_2O_3 by continuous removal of water and the latter species then undergoes dissociation at higher temperatures (>100 °C) to nitric oxide and NO_2. Current work by Challis et al. (1976) has shown that the oxides of nitrogen act as nitrosating agents several orders of magnitude faster than nitrous acid itself. Since nitric oxide is relatively stable, it is very likely that the NO_2· radical acts as the chain initiator and abstracts the amino proton from proline to give a radical which then combines with the NO· radical to give *N*-nitrosoproline as shown in Scheme II.

Based on the mechanism of formation of nitrosopyrrolidine in bacon shown in Scheme II, one would expect that a good nitrosamine blocking agent in bacon should satisfy inter alia the following requirements: (1) serve as a good NO· radical trap, (2) be fat soluble (lipophilicity), (3) be non-steam volatile, and (4) be stable up to the maximum frying temperature of about 174 °C.

Further work to be reported from these laboratories and recent patents on the use of the antioxidant ethoxyquin (Coleman, 1976a) and its analogues (Bharucha and Coleman, 1978) in blocking nitrosamine formation in bacon fully substantiate this view.

CONCLUSIONS

(1) Free proline, which is present in milligram/kilogram quantities in pork belly, is the most likely organic precursor of NPYR in cooked bacon. Addition of proline as such to bacon results in a proportionate increase in NPYR, suggesting that free proline rather than nitrite is the limiting factor in NPYR production.

(2) Raw bacon is essentially free of volatile nitrosamines.

(3) Time and temperature have a significant effect on the yield of nitrosamines. Grilling, by its nature, produces less nitrosamines than frying.

(4) The amount of nitrosamines in the cook-out fat is roughly twice that in the rasher.

(5) Of the two possible pathways for the conversion of proline to NPYR, the one involving the intermediacy of NPro is favored.

(6) There is not sufficient preformed NPro in raw bacon to account for the NPYR formed on cooking.

(7) The decarboxylation of NPro to NPYR under bacon-frying conditions proceeds very poorly (ca. 0.16% yield) and most probably therefore is the yield-limiting step.

(8) During the frying of bacon up to 62% of the NDMA and 32% of the total NPYR produced appear in the vapors.

(9) Nitrosation in bacon occurs on frying largely after most of the water is expelled from the system. It therefore occurs in the fat phase and presumably by a radical rather than an ionic mechanism.

(10) The essential but probably not the only requirements for a potential anti-nitrosamine agent in bacon are: (a) ability to trap NO· radicals; (b) lipophilicity; (c) non-steam volatility; and (d) heat stability up to 174 °C (maximum frying temperature).

Supplementary Material Available: The collection of the vapor in one fraction over frying bacon is presented for six samples. Analytical data for these six samples giving distribution of cook-out fat, cooked rasher, and distillate are included. The nitrosamine amounts in three portions are presented in tabular form. The method used to obtain the three fractions is described (4 pages). Ordering information is given on any current masthead page.

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Received for review January 3, 1978. Accepted August 14, 1978.

Isolation and Partial Purification of a Red Tide (*Gymnodinium breve*) Cytolytic Factor(s) from Cultures of *Gomphosphaeria aponina*

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Material(s) that is cytolytically active toward the red tide organism *Gymnodinium breve* has been isolated from the blue-green alga *Gomphosphaeria aponina*. A cytolytic assay was developed and is described. Optimum yields of cytolytic material(s) were obtained by extraction of cultures of *G. aponina* with chloroform at neutral pH. Purification of the cytolytic material(s) was attempted using chloroform–methanol mixtures with best results obtained with elution on activated alumina (WB-2) columns, silica thin-layer chromatography, and silica high-performance liquid chromatography. Considerable losses of activity and specific activity were noted during purification, but the activity and specific activity could be restored by pooling fractions obtained in the separations. Mass spectral analysis of a purified fraction with maximum activity indicated a significant fragment with probable formula of $C_{29}H_{49}O$.

Enthusiasm for marine aquaculture in the near-shore waters of the Gulf of Mexico is undoubtedly mitigated by a number of factors, including the absence of agents suitable for managing periodic red tides. In Florida's coastal waters, for example, red tides are associated with periodic, sporadic blooms of the unarmored dinoflagellate, *Gymnodinium breve* (cf. Martin and Martin, 1976a), which result in massive mortalities of marine animals. The exact cost of these outbreaks is uncertain, but the estimated cost of the 1971 outbreak that lasted for 3 summer months and covered a coastal area of seven counties was $17 000 000 (Habas and Gilbert, 1974).

Some screening studies have been concerned with chemicals that would be effective in managing *G. breve*. Marvin and Proctor (1964) examined 4800 chemicals for this purpose, though expense and other considerations apparently limited follow-up studies.

We believe that isolation of cytolytic material(s) (provisionally called "aponin") from cultures of the blue-green alga, *Gomphosphaeria aponina*, represents a potentially promising approach to management of red tides (Kutt and Martin, 1975; Martin and Martin, 1976a). First, aponin (and the associated organism) was isolated from a 1973 red-tide outbreak (Kutt and Martin, 1974, 1975). In addition, it has been demonstrated (McCoy and Martin, 1977) that crude aponin was not ichthyotoxic to test fish, *Poecilia sphenops*, and, in fact, the ichthyotoxicity of *G. breve* cultures was mitigated at certain concentrations of crude aponin. More recently, it was demonstrated that concentrations of aponin that were effectively cytolytic toward *G. breve* cultures did not kill brine shrimp, *Artemia salina* (Eng-Wilmot and Martin, 1978b).

The present report describes the optimization of aponin isolation, development of an effective cytolytic bioassay, and fractionation of the crude isolates into two chromatographically pure biologically active fractions.

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EXPERIMENTAL SECTION

Organism Source and Culture. Unialgal cultures of *Gymnodinium breve* were obtained through the courtesy of W. B. Wilson and S. M. Ray (Texas A&M Marine Institute, Galveston) and were maintained as axenic stock cultures in either artificial sea water or natural aged sea water, salinity, *S*, 33 ppt enriched with modified B-5 supplements as described by Brydon and co-workers (1971). *Gomphosphaeria aponia* was isolated from environmental samples (Kutt and Martin, 1975) and identified by Dr. C. J. Dawes (cf. Dawes, 1974). Unialgal bacterial-free stock cultures were maintained in suitably enriched artificial sea water, *S*, 28 ppt (Eng-Wilmot and Martin, 1978a). Large-scale semicontinuous cultures of *G. aponina*, grown under optimized culture conditions (Eng-Wilmot and Martin, 1977b), served as the organism source in these studies.

The microorganisms were enumerated with a Coulter Counter (Model B) equipped with a C-1000 Channelyzer and 100-μm aperture, with electronic and threshold set-

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