RESULTS AND DISCUSSION

Dinitramine was toxic to coldwater and warmwater fishes at concentrations of 0.590–1.52 mg/l. in 96-hr static toxicity tests (Table I). Other terrestrial herbicides with similar uses have toxicities less and greater than dinitramine. Walker (1964) reported a 96-hr toxicity value for atrazine against sunfish species of approximately 5 mg/l. (active ingredient). Mullison (1970), in his review on herbicides, reported that treflan (trifluralin) was toxic to fish at concentrations of 11–210 μg/l.

Dinitramine residues in samples of carp and channel catfish muscle taken immediately after exposure (0 hr) and after 24 hr of withdrawal exceeded the exposure concentration (Table II). The mean concentration of dinitramine residue in carp muscle, ranging from 2.96 to 5.15 μg/g, did not show evidence of elimination during the postexposure period. Residues in channel catfish muscle had declined more than 50% 24 hr after withdrawal, but the concentration was still 20 times the exposure concentration. Persistence beyond 24 hr after withdrawal was not determined. However, dinitramine residues are much more persistent in fish than are residues of TFM (3-trifluoromethyl-4-nitrophenol), quinaldine, and MS-222 (tricaine methanesulfonate) (Hunn and Allen, 1974).

Carp and channel catfish blood plasma contained high concentrations of dinitramine immediately following exposure (Table II). After 24 hr in fresh water, the concentration in plasma decreased from 24.4 to 5.1 mg/l. in carp and from 58.5 to 19.6 mg/l. in channel catfish. The high concentration of the herbicide in plasma as compared with the concentration in water after a relatively short exposure indicates that partitioning of dinitramine across the gill is favored over retention in the water. The partitioning is predictable because of the low solubility of dinitramine in water (1 mg/l.) and its high solubility in organic solvents (57% in acetone) as given in the U.S. Borax Research Technical Data Sheet. Hunn and Allen (1974) stated that solubility in lipids and low solubility in water resulted in rapid uptake of drugs across the gills of fishes.

The concentration of dinitramine in gallbladder bile of the fish after exposure was much higher than the exposure concentration (Table II). However, the magnification was not as great as reported by researchers for other compounds; magnification factors of 124 to 1061 were reported by Lech et al. (1973) for DDT, 2,4-dichlorophenoxyacetic acid, and carbaryl and by Hunn and Allen (1974) for TFM and MS-222. Dinitramine residue concentrations in carp bile are similar in magnitude to those in plasma and may result from equilibrium between plasma and bile.

This preliminary study does not answer all questions regarding what would happen to dinitramine in the aquatic environment. The lack of available information concerning the dynamics of the herbicide in the aquatic ecosystem was a major consideration for doing the study. More work needs to be done using other species and longer withdrawal periods before the dynamics will be understood. However, the data from this study do support two conclusions.

CONCLUSIONS

(1) Residue concentrations of dinitramine in fish muscle, plasma, and bile exceeded the bath exposure concentration and persisted longer than 24-hr postexposure. (2) In several instances, the toxicity of dinitramine to fish was near the limit of solubility of the herbicide in water under our experimental conditions.

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Effect of Sodium Chloride Concentration on the Nitrosation of Proline at Different pH Levels

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Acidity had a determining influence in regard to activation and inhibitory effects of sodium chloride on the rate of nitrosation of proline. In 1 *M* sodium chloride the rate of nitrosation was increased by 325% at pH 0.5 and decreased by 36 and 50% at pH 4.0 and 5.5, respectively. At pH 2.5 a slight inhibiting effect was noticed. Multiple regression analysis showed the best fitted model was of the form, ln (initial rate of nitrosation) = $a + b[\mathrm{NaCl}] + c[\mathrm{NaCl}]^2$.

The carcinogenicity of *N*-nitrosamines has been recognized for some time (Druckrey et al., 1967; Magee and Barnes, 1956). The discovery of volatile nitrosamines in several types of foods treated with nitrite has initiated investigations into the conditions which may promote their formation in food processing and also during digestion (Fan and Tannenbaum, 1973; Bills et al., 1973; Pensabene et al., 1974; Sen et al., 1974; Sander and Burkle, 1969).

It has been demonstrated that several anions exert an accelerating effect on the nitrosation of amines in acidic media (Ridd, 1961; Boyland et al., 1971; Boyland, 1971). The order of the accelerating effect was $I^- > SCN^- >$ acetate $> Br^- > Cl^-$ with the chloride ion exhibiting a very

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Table I. Nitrosation of Proline Measured by Two Methods at pH 2.5

[NaCl], M	Nitrosoproline, initial rate, μM/min: Direct spectrophotometric method	Ammonium sulfamate method
0	14.9	11.3
0.1	14.3	11.4
0.25	13.8	9.5
0.50	13.7	10.8
0.75	13.9	11.1
1.00	13.9	9.4

slight catalytic effect at pH 2.0 (Boyland, 1971). The effect of anions appears to result from the formation of covalent nitrosyl compounds, which become the main nitrosating species. In agreement with these findings, Fan and Tannenbaum (1973) demonstrated that chloride and bromide ions (0.1–0.3 M) promoted the formation of nitrosomorpholine at pH 0.5.

In some reports, however, an inhibitory rather than a catalytic effect of chloride was observed. Taylor and Price (1929) observed that 0.05 N potassium chloride has an inhibitory effect on the nitrosation of dimethylamine, but it was not clear at what pH the nitrosation reaction took place. Mirvish et al. (1973) investigated the effect of 0.05, 0.15, and 0.50 M sodium chloride on the nitrosation of sarcosine at pH 1.5, 2.5, and 3.0. The yield of nitrososarcosine was reduced by 34% at pH 2.5 and by 55% at pH 3.5, which was attributed by the authors to activity effects. The presence of 0.05 M sodium chloride in the reacting medium reduced the nitrosation rate of dimethylamine in phthalate buffer at pH 3.4 by 9% (Mirvish, 1970). Fiddler et al. (1973) found no effect of sodium chloride (0.4 M) on the formation of dimethylnitrosamine in a pH 5.6 buffer solution (0.5 M KH_2PO_4 and NaOH).

Since the combined effects of sodium chloride concentration and acidity on nitrosation have not been fully elucidated, we felt it was warranted to initiate a study to clarify this aspect. Accordingly, the purpose of this work was to investigate the effects of sodium chloride concentration on the nitrosation of a secondary amine at different pH levels. As sodium chloride is a common food additive, its effects on nitrosation reactions need to be better understood.

EXPERIMENTAL PROCEDURES

The experiment was carried out with L(−)-proline (Eastman Chemical Co.) as the source of the secondary amine. The rate of nitrosation of proline was investigated at pH 0.5, 2.5, 4.0, and 5.5, in the presence of 0 to 1.0 M sodium chloride. Triplicate runs were done on each sample.

Due to the instability of sodium nitrite under acidic conditions, care was taken to avoid lowering the pH of nitrite solutions until the time of reaction. Stock solutions of proline were adjusted with 0.1 N H_2SO_4 to attain the desired pH after the addition of sodium nitrite. Stock solutions of sodium nitrite were prepared in distilled water (pH approximately 5.4). Standard nitrosoproline was prepared and purified by the method of Lijinsky et al. (1970).

Before the start of the reaction the participants were equilibrated separately at 25°. Five milliliters of the proline solution was mixed with 5 ml of sodium chloride solution in a 50-ml reagent tube with screw-cap (Kimax). Five milliliters of sodium nitrite solution was added, the tube shaken, and the recorder on the spectrophotometer started (Beckman DB-G grating spectrophotometer). Two milliliters of the reacting solution was immediately transferred to a quartz cell, and the absorption recorded for 30 min at 260 nm. The pH 5.5 reaction was very slow and consequently the reaction mixture was held at 25° in a water bath. Aliquots were taken at 24-hr intervals and the absorption was determined. The initial concentrations of sodium nitrite and proline in the reacting medium were 10 and 50 mM, respectively. Sodium nitrite solution was replaced with distilled water in the blank. A new blank was made up for each sample, and was treated as the sample.

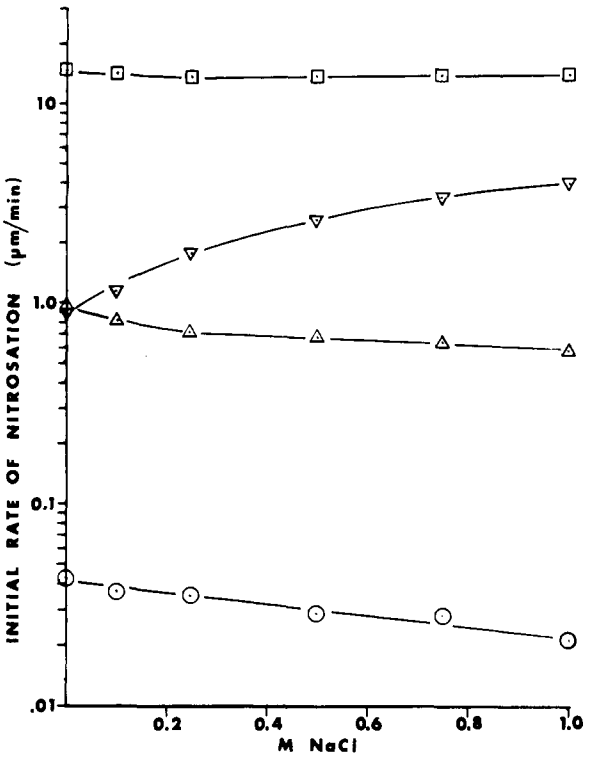

Figure 1. Effect of sodium chloride concentration on the rate of nitrosation (logarithmic scale) of proline at different pH levels: (▽) pH 0.5; (□) pH 2.5; (△) pH 4.0; (○) pH 5.5.

The direct spectrophotometric procedure as described above was tested against a similar assay by Mirvish et al. (1973), for nitrosoproline, in which the reaction was stopped before measuring the absorption. The latter method was used at pH 2.5 at all salt concentrations. The reaction was quenched at 5-min intervals by adding 5 ml of 200 mM ammonium sulfamate in 2 N H_2SO_4. After a 5-min equilibration period the absorption of the sample was measured at 260 nm. For the zero time samples, ammonium sulfamate was added before sodium nitrite. All samples were run in duplicate.

The rate of nitrosation of proline was determined from initial rates, since nitrite in open vessels is unstable upon prolonged incubation (Fan and Tannenbaum, 1973). The rate was estimated from the slope of the initial part of the recorded curve. The molar absorptivity at 260 nm for nitrosoproline was found to be 1985 at pH 0.5, 2150 at pH 2.5, 2865 at pH 4.0, and 2820 at pH 5.5. Absorption at 260 nm instead of 238 nm was used to reduce the interference by nitrite (Mirvish et al., 1973). The stability of nitrosoproline to the ultraviolet light in the spectrophotometer was checked at pH 0.5, 2.5, and 4.0 by leaving a sample of standard nitrosoproline in a quartz cell in the sample compartment for 1 hr. No change in absorbance was observed.

RESULTS

The assay used for measuring the rate of formation of nitrosoproline, the direct spectrophotometric method, was compared at pH 2.5 to the procedure by Mirvish et al.

Table II. Regression Models Describing the Relationship between Sodium Chloride Concentration and Rate of Nitrosation of Proline at Different pH Levels

pH	Model
0.5	ln (rate)[a] = −0.01 + 2.77[NaCl] − 1.32[NaCl]^2
2.5	ln (rate) = +2.69 − 0.25[NaCl] + 0.19[NaCl]^2
4.0	ln (rate) = −0.08 − 0.86[NaCl] + 0.42[NaCl]^2
5.5	ln (rate) = −3.19 − 0.59[NaCl][b]

[a] Natural logarithm. [b] Quadratic term not significant.

Table III. Statistical Description of the Regression Models

	pH 0.5	pH 2.5	pH 4.0	pH 5.5
Mean-squares total	0.3117	0.00146	0.0313	0.0574
Mean-squares regression	2.6042	0.00674	0.2420	0.4551
Mean-squares residual	0.00607	0.000751	0.00318	0.00434
t values of the quadratic term [NaCl]^2	−6.93	2.90	3.07	−0.24

(1973), which will be referred to as the ammonium sulfamate method (Table I). The ammonium sulfamate method gave slightly lower values for the rates of formation of nitrosoproline, and the intrinsic variability in the ammonium sulfamate assay was larger than for the direct spectrophotometric method. As a consequence, a small but significant correlation between sodium chloride concentration and rate was detected at pH 2.5 using the direct spectrophotometric method, whereas no significant correlation was observed when the ammonium sulfamate assay was used.

The effect of sodium chloride on the rate of nitrosation of proline at different pH levels is shown in Figure 1. Strong activation was observed at pH 0.5, very slight inhibition at pH 2.5, and moderate inhibition at pH 4.0 and 5.5. Maximum effects occurred in 1 M sodium chloride in which the rate of nitrosation was increased 325% at pH 0.5 and decreased 36 and 50% at pH 4.0 and 5.5, respectively. Table II shows that the best fitted regression model at different pH levels was of the form ln (rate) = a + b[NaCl] + c[NaCl]^2. These equations could be used to estimate rates of nitrosation in systems with known sodium chloride concentrations and pH levels. The effect of sodium chloride was statistically significant at the 95% level at all pH levels, and the quadratic effect was significant except at pH 5.5 (Table III). The small residual mean square terms show that the variability between replicates in the experiment was very low. The highest nitrosation rates of proline were at pH 2.5, which confirms earlier results by Mirvish et al. (1973).

DISCUSSION

In the absence of an anionic promotor the rate of nitrosation of amines in dilute acid is given by eq 1 (Hughes et al., 1968). As nitrous anhydride, formed from two molecules of nitrous acid, is the nitrosating species, the reaction is second order with respect to nitrous acid. In the presence of promoting anions the rate expression is described by eq 2 (Hughes and Ridd, 1958). With bromide or thiocyanate in the system, the anion promoting mechanism is prevalent at pH values below 2 (Fan and Tannenbaum, 1973). In addition, these investigators demonstrated that at pH values greater than 2, the nitrous anhydride mechanism also becomes operative. In the presence of chloride, it is likely that similar effects would be observed. However, as chloride is a weaker promotor than bromide or thiocyanate (Fan and Tannenbaum, 1973), the nitrous anhydride mechanism may start exerting a dominant influence at a pH level lower than 2.

$$\text{rate} = k_N[HNO_2]^2[R_2NH] \quad (1)$$

$$\text{rate} = k_X[HNO_2][H^+][X^-][R_2NH] \quad (2)$$

In this experiment, at pH 0.5, the rate of formation of nitrosoproline was strongly enhanced by increased sodium chloride concentrations (Figure 1), which was probably due to the formation of activating nitrosyl chloride. As the concentration of nitrosyl halide is proportional to the square of the hydrochloric acid concentration, the rate of nitrosation with nitrosyl chloride increases rapidly with acidity (Challis and Butler, 1968). The rate of nitrosation increased linearly with sodium chloride concentration, which agrees with eq 2 where the anion is a first-order participant.

At pH 2.5 the situation is more complex as both nitrosation mechanisms were probably operative. A slightly inhibiting effect of sodium chloride on the rate of nitrosation was observed at this pH. The activating effect of nitrosyl chloride would be less at pH 2.5 than at pH 0.5, and was apparently counterbalanced by the inhibiting effect of the high ionic strength caused by sodium and chloride ions in the medium.

By increasing the sodium chloride concentration at pH 4.0 and 5.5, moderately inhibiting effects were observed. The nitrous anhydride mechanism was predominant at these pH levels, and the promoting effect by nitrosyl chloride was negligible. Thus, the inhibition effects by high ionic strength at pH 4.0 and 5.5 became more evident than observed at pH 2.5.

The effects of the addition of a salt on the reaction rate can be at least of two different types. The increase in the ionic strength of the medium caused by salt changes the activity coefficients of neutral molecules, which can lead to changes in the reaction rates (Frost and Pearson, 1961). This effect is called the primary salt effect. The influence of salt on the activity coefficients of both the reactants and the activated complex must be considered when evaluating the primary salt effect.

For reactions involving catalysis by acids or bases there is a secondary salt effect, which has to do with the effect of ionic strength on the dissociation of weak acids or bases (Frost and Pearson, 1961). As the nitrosation of amines is an acid-catalyzed reaction, secondary salt effects are likely in the presence of sodium chloride. When nitrous anhydride is the nitrosating species, the concentration of this species in the system evidently affects the reaction rate. Nitrous anhydride, a neutral molecule, is formed from ionic nitrite through a series of equilibrium reactions (Noller, 1965):

$$ONO^- + H^+ \longrightarrow HONO \quad (3)$$

$$HONO + HONO \longrightarrow H_2\dot{O}NO + ONO^- \quad (4)$$

$$H_2\dot{O}NO + ONO^- \longrightarrow ONONO + H_2O \quad (5)$$

Increasing the ionic strength will favor the stability of charged species and should shift the reactions responsible for the formation of nitrous anhydride to the left thereby reducing the concentration of nitrous anhydride. Increased ionic strength will also tend to favor protonated rather than free amine and it is the free amine which is involved in the rate-limiting step (eq 1). It seems reasonable, therefore, that secondary salt effects were involved in the inhibition observed with sodium chloride.

This study describes the relationship between sodium

chloride concentration and rate of nitrosation of proline at different pH levels. Strong activation of the nitrosation of proline was seen at high acidities (pH 0.5) and inhibiting effects were observed at lower acidities. In recent publications (Boyland, 1971; Sander, 1973) the point has been made that halide ions are catalytic agents in nitrosation reactions. As shown by our work, one must consider the pH of the system to determine whether a catalytic or inhibiting effect from chloride ions would be predicted. Whereas chloride might activate nitrosations in strongly acidic media, the same ion would be expected to produce a moderately inhibiting effect on nitrosation in mildly acidic or neutral food systems.

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Biosynthesis of Averufin from Acetate by *Aspergillus parasiticus*

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Carbon-13 nuclear magnetic resonance analysis shows that ^{13}C-labeled averufin synthesized from [1-^{13}C]acetate by *Aspergillus parasiticus* was labeled at alternating positions in the molecule. This pattern indicates the polyketide origin of the compound and its biogenetic relationship to the bisfuranoids, sterigmatocystin and aflatoxin B_1.

The hypothesis that averufin is a biosynthetic intermediate of aflatoxin B_1 (Thomas, 1965; Moss, 1972) has been experimentally supported by: (1) the accumulation of averufin in the mycelium of a nitrosoguanidine-induced deficient mutant of *Aspergillus parasiticus* ATCC15517, an aflatoxin producing fungus (Donkersloot et al., 1972), and (2) the in vivo conversion of averufin into aflatoxin by the wild-type strain (Lin et al., 1973; Yao and Hsieh, 1974). Although evidence now indicates that aflatoxin is derived from acetyl CoA via the polyketide pathway (Donkersloot et al., 1968; Biollaz et al., 1970; Hsieh and Mateles, 1970, 1971), the precursor-product relationship between acetate and averufin has not yet been demonstrated.

In this report the distribution of ^{13}C labels in averufin derived from [1-^{13}C]acetate as determined by ^{13}C nuclear magnetic resonance is presented to substantiate the proposed synthesis of averufin from acetate via a polyketide chain and biogenetic relationship between averufin and aflatoxin as well as other related metabolites.

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EXPERIMENTAL SECTION

Materials. 1-^{13}C-Labeled acetate 68% enriched in ^{13}C was synthesized via a Grignard reaction of methylmagnesium iodide and $^{13}CO_2$ generated from $Ba^{13}CO_3$ which was purchased from Mallinckrodt Chemical Works, St. Louis, Mo. (Fitzell et al., 1975; Murray, 1958). Unlabeled averufin was produced from the culture of *Aspergillus parasiticus* ATCC24551 maintained in a medium containing sucrose, asparagine, and ammonium sulfate as described by Yao and Hsieh (1974). ^{13}C-Labeled averufin was prepared in a similar manner with [1-^{13}C]acetate added to the cultures.

Carbon-13 Magnetic Resonance Measurements. A JEOL PS-100 Fourier transform NMR, operating at 25.2 MHz in the ^{13}C mode, with a Digilab NMR-3 data system was used. It was operated with the probe at an ambient temperature of ~24° with Me_2SO-d_6, the solvent, used as the deuterium lock source and as an internal reference. All chemical shifts are reported as δ (ppm) downfield from Me_4Si, based on Me_2SO (δ 40.3). Concentrations are noted in Figures 1 and 2.

RESULTS

Assignment of the resonances for the [1-^{13}C]acetate-derived averufin spectrum (Figure 1) was aided by a natural abundance ^{13}C spectrum (Figure 2) and an off-resonance