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# Action of Sodium Nitrite on Folic Acid and Tetrahydrofolic Acid

Lynn S. Reed and Michael C. Archer*

Folic acid reacted with sodium nitrite in dilute aqueous solution at both pH 1.5 and 5.0 to yield exclusively $N^{10}$-nitrosofolic acid. At pH 1.5, the reaction was second order in nitrite, but at pH 5.0, the order in nitrite was one, and buffer anions participated in the nitrosation reaction. Tetrahydrofolic acid was rapidly oxidized by sodium nitrite at both pH 1.5 and 5.0 to produce *p*-aminobenzoylglutamate and several pterin products. Ascorbate had a protective effect on the oxidation of tetrahydrofolate by nitrite at pH 5.0, but even when the ascorbate was in 100-fold excess, tetrahydrofolate oxidation was not completely inhibited. 5-Methyltetrahydrofolate was oxidized by nitrite at pH 5.0 to produce several pterin products. 5-Formyltetrahydrofolate reacted with sodium nitrite at pH 5.0 to yield a single product which was probably the $N^{10}$-nitroso derivative.

The occurrence and formation of *N*-nitroso compounds, especially in foods, has received much attention recently (Scanlan, 1975; Gough et al., 1977). The widespread occurrence of both nitrite and folic acid and its derivatives in food material makes the interaction of these compounds of interest. We have shown that $N^{10}$-nitrosofolic acid (Figure 1) is a weak carcinogen for the rat (Wogan et al., 1975). $N^{10}$-Nitrosofolic acid is also a bacterial mutagen and is active in a mammalian cell transformation assay (Purchase et al., 1978).

In addition to being a nitrosating agent, nitrous acid is a potent oxidizing agent. For example, it readily oxidizes ascorbic acid (Bunton et al., 1959; Archer et al., 1975). The naturally occurring forms of folic acid are reduced derivatives (Stokstad et al., 1977) and interaction of these forms with nitrous acid may hence lead to a loss of vitamin activity.

Because of the complex chemistry of tetrahydrofolate oxidation reactions, the multiplicity of products and rapid reaction rates (Chippel and Scrimgeour, 1970; Blair and Pearson, 1974), kinetic analyses have been difficult to perform. To overcome these problems, we have developed methods for the rapid, efficient analysis of folate and pterin (2-amino-4-hydroxypteridine) derivatives using high-performance liquid chromatography (Reed and Archer, 1976; Archer and Reed, 1979). These separations include the reduced and the $N^5$- or $N^{10}$-substituted folates, the products of nitrosation of folic acid, and pterins that result from the oxidative breakdown of tetrahydrofolic acid (xanthopterin, pterin-6-carboxaldehyde, and pterin). Analysis of microgram samples of these derivatives which can be injected sequentially at 5–10-min intervals has thus become possible. We present here our work on the nitrosation of folic acid and oxidation of 5,6,7,8-tetrahydrofolic acid by nitrous acid using these methods. We also present some preliminary work on the reaction of nitrous acid with 5-methyl- and 5-formyltetrahydrofolate.

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## MATERIALS AND METHODS

**Important Safety Note.** *N*-Nitroso compounds have been shown to be carcinogenic in test animals and all experimental work should be done in an efficient fume hood. Safety gloves should be worn whenever these compounds are being handled. The gloves should not be re-used.

Folic acid, dihydrofolic acid, tetrahydrofolic acid, 5-methyltetrahydrofolic acid, *p*-aminobenzoylglutamate, xanthopterin, pterin, and pterin-6-carboxylic acid were purchased from Sigma (St. Louis, MO). 5-Formyltetrahydrofolic acid was purchased from Grand Island Biochemical Co. (Grand Island, NY). Pterin-6-carboxaldehyde was prepared by the method of Waller et al. (1950); $N^{10}$-nitrosofolic acid was prepared by the method of Cosulich and Smith (1949); 2-hydroxy-$N^{10}$-nitrosofolic acid and 2-hydroxyfolic acid were prepared by the method of Angier et al. (1952).

High-performance liquid chromatography of the various folic acid derivatives and pterins has been described previously (Reed and Archer, 1976; Archer and Reed, 1979).

In the kinetic experiments, folic acid was dissolved in 0.01 M citrate/0.02 M phosphate buffer adjusted to either pH 5.0 with NaOH or pH 1.5 with perchloric acid. Some experiments were also performed in acetate buffer, pH 5.0, at concentrations in the range of 0.025 to 0.2 M. Sodium nitrite was added to start the reactions. Reaction mixtures were maintained in a constant temperature bath (25 or 30 °C) with stirring. In the reaction at pH 5, samples were removed at various time intervals and immediately analyzed by liquid chromatography. Dilution of reactants caused by the high eluant flow rate had the effect of

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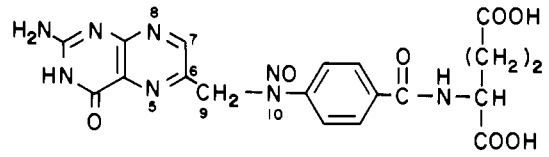

**Figure 1.** $N^{10}$-nitrosofolic acid.

stopping the reaction. Samples of the reactions at pH 1.5 were first diluted with base to pH 5. Because of the low concentrations of nitrite used in these experiments, this procedure essentially stopped the reaction. The samples were then chromatographed as soon as possible.

Tetrahydrofolate derivatives were dissolved in 0.01 M citrate/0.02 M phosphate buffer at either pH 1.5 or pH 5.0 which had previously been deaerated with nitrogen gas. Nitrite was added to start the reactions and they were maintained in an anaerobic condition with nitrogen gas. Control reactions showed that under these conditions, there was no measurable loss of tetrahydrofolate in the absence of nitrite.

Initial concentrations of folate or tetrahydrofolate were calculated from their chromatographic peak areas immediately prior to the start of the reactions. Products were identified by comparing their chromatographic retention times and UV spectra to authentic standards. Quantitation of chromatographic peak areas was performed using a computing integrator (Columbia Scientific, Austin, TX). Molar absorptivities for $N^{10}$-nitrosofolic acid and 2-hydroxy-$N^{10}$-nitrosofolic acid at pH 4.8 and 254 nm were determined to be 18540 $cm^{-1}$ and 20000 $cm^{-1}$, respectively. For other compounds, molar absorptivities at 254 nm and at the pH of the chromatographic eluant buffer were determined by comparison with their reported spectra (Blakley, 1969). Rate constants were calculated from initial rates for reactions at pH 1.5, and pseudo-first-order or pseudo-zero-order plots at pH 5.0, following the disappearance of either folic acid or tetrahydrofolic acid.

## RESULTS AND DISCUSSION

**Formation of $N^{10}$-Nitrosofolic Acid.** We have examined the kinetics of forming $N^{10}$-nitrosofolic acid from folic acid and nitrite in aqueous solution. The experiments were performed at pH 1.5 and pH 5.0 since folic acid is not significantly soluble at intermediate pH values.

At pH 1.5, we used nitrite in the range of 0.1 to 0.4 mM and folate in the range 50 to 60 $\mu$M. Under these conditions, the only reaction product we observed chromatographically was $N^{10}$-nitrosofolic acid. 2-Hydroxy-$N^{10}$-nitrosofolic acid has been shown to form by reaction of folate and nitrite in a solution of 6 N sulfuric acid and glacial acetic acid at 70 °C (Angier et al., 1952), but we did not see any of this product under our conditions of milder acidity. We found that the rate of N10-nitrosofolate formation was second order with respect to nitrite and hence the nitrosating agent under these conditions is nitrous anhydride. The reaction was first order with respect to folic acid. The pH-independent rate constant was determined to be $2.8 \times 10^7$ $M^{-2}$ $s^{-1}$. This compares with a rate constant of $1.8 \times 10^6$ $M^{-2}$ $s^{-1}$ for *N*-methylaniline (Mirvish, 1975) which is a simple analogue for the $N^{10}$ amino group of folic acid. The $pK_a$ of the $N^{10}$ position in folic acid is 0.2 (Poe, 1977) and hence at pH 1.5, the molecule is predominantly unionized. For methylurea, which has a similar $pK_a$ of 0.9 (Perrin, 1965), in the pH range 1.5 to 3.0, Mirvish found that the main nitrosating species is the nitrous acidium ion, though he points out that there is a contribution from the nitrous anhydride mechanism at relatively high nitrite concentrations and high pH values.

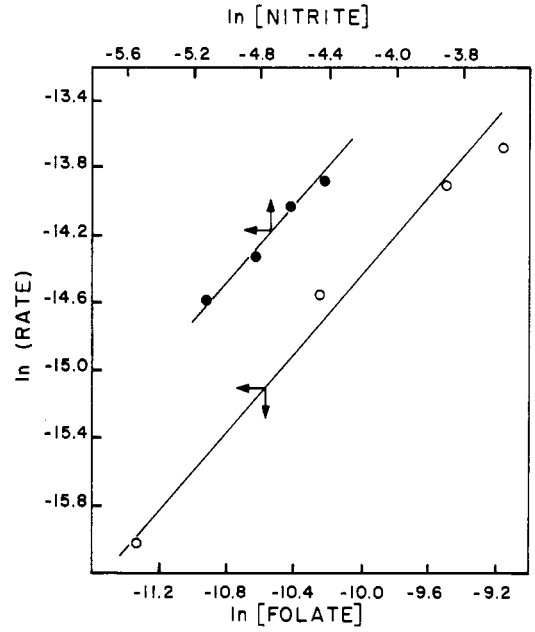


**Figure 2.** Dependence of the rate of $N^{10}$-nitrosofolate formation on the concentrations of nitrite and folate, in 0.01 M citrate/0.02 M phosphate buffer, pH 5.0, 30 °C: (○) nitrite concentration, $10^{-2}$ M; (●) folate concentration, $6.2 \times 10^{-5}$ M.

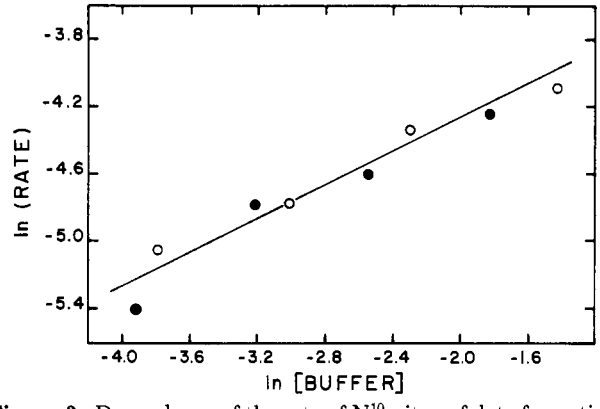


**Figure 3.** Dependence of the rate of $N^{10}$-nitrosofolate formation on concentrations of acetate (○) or citrate (●; phosphate held constant at 0.02 M) buffer, pH 5.0, 25 °C; ionic strength maintained constant by addition of sodium perchlorate; nitrite concentration, $10^{-2}$ M; folate concentration, $6.2 \times 10^{-5}$ M.

At pH 5.0, we used nitrite in the range 6 to 24 mM and folate in the range 10 to 100 $\mu$M. Again the exclusive reaction product was $N^{10}$-nitrosofolic acid. The reaction, however, was first order with respect to both folic acid and nitrite (Figure 2). The reaction rate was shown to decrease approximately tenfold for a 1-unit rise in pH from 5 to 6. The order was approximately 0.5 with respect to acetate or citrate buffer at pH 5.0 (Figure 3). (Care was taken to maintain constant ionic strength of reaction solutions by addition of sodium perchlorate. Cachaza et al. (1978) and Fan and Tannenbaum (1973) have shown that perchlorate has no catalytic effect on nitrosation reactions.) Thus a portion of the reaction is catalyzed by buffer anions in which the nitrosating agent is probably nitrosyl acetate or nitrosyl citrate. Nitrosyl acetate has been proposed as a species involved in the diazotization of aniline (Edwards et al., 1959), the azide–nitrite reaction (Stedman, 1960) and the nitrosation of dimethylamine at pH 4 (Masui et al., 1974).

To separate the reaction into acetate-catalyzed and uncatalyzed components, we used a kinetic treatment similar to that described by Masui et al. (1974) in their

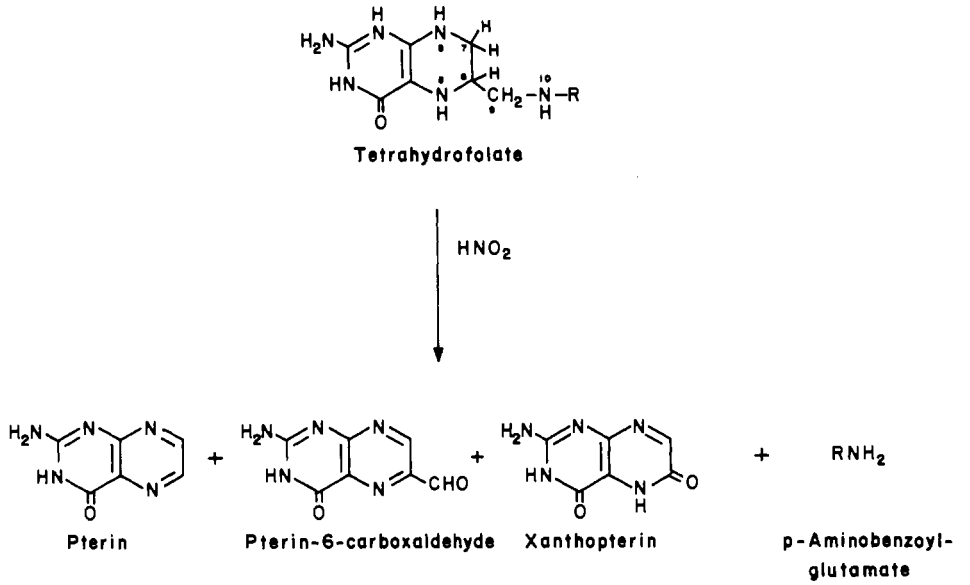


**Figure 4.** Products formed by reaction of tetrahydrofolate ($9.5 \times 10^{-5}$ M) with sodium nitrite ($4 \times 10^{-4}$ M) at pH 1.5 for 1 h at 30 °C.

study of the nitrosation of dimethylamine in acetate buffers. The rate of the overall nitrosation of folate in acetate buffer is described by the following equation:

$$\text{rate} = k_3[\text{folate}][HNO_2][OAc^-] + k_2[\text{folate}][HNO_2] \quad (1)$$

Thus,

$$k_{2obs} = k_3[OAc^-] + k_2 \quad (2)$$

where $k_{2obs}$ is the measured second-order rate constant at constant nitrite and folate concentrations. A plot of $k_{2obs}$ vs. $[OAc^-]$ yielded values of 6.3 $M^{-2}$ $s^{-1}$ for $k_3$ and 0.4 $M^{-1}$ $s^{-1}$ for $k_2$. The percentage of the reaction catalyzed by acetate rose from 25 to 66% as the concentration of acetate rose from 0.25 to 0.2 M.

At this stage we do not know the nitrosation mechanism for that portion of the reaction which does not depend on buffer concentration. It is known, however, that at pH 6.5, bisulfite reacts with folate to form an unstable nucleophilic adduct at C7 in the pyrazine ring (Vonderschmitt et al., 1967). Folate may suffer a similar nucleophilic attack by free nitrite, which is abundant at pH 5.0, the adduct undergoing intramolecular rearrangement to yield the $N^{10}$-nitroso derivative.

During the course of our experiments on the nitrosation of folate, we found that $N^{10}$-nitrosofolic acid is rather heat-labile. At pH 5 and pH 10, the rate of degradation of nitrosofolate was slow, less than 10% being lost in 1 h at 70 °C. At pH 1.5, however, 65% was lost at 70 °C in the same time period. The degradation products under these conditions are *p*-aminobenzoylglutamate and pterin-6-carboxaldehyde, which are probably formed by loss of nitroxyl and subsequent hydrolysis of the azomethine. The pH dependency of the thermal decomposition of $N^{10}$-nitrosofolic acid is similar to that observed for *N*-nitrososarcosine at high temperatures (Fan and Tannenbaum, 1972).

**Oxidation of Tetrahydrofolic Acid by Nitrous Acid.** Tetrahydrofolate is readily oxidized by a variety of oxidizing agents (Chippel and Scrimgeour, 1970; Blair and Pearson, 1974) to yield primarily *p*-aminobenzoylglutamate and several pterin products. We have found that nitrous acid leads to rapid oxidative degradation of tetrahydrofolate. Interaction of nitrous acid with tetrahydrofolic acid was studied under anaerobic conditions by monitoring the disappearance of tetrahydrofolate by high-performance anion-exchange chromatography and the appearance of pterin products by high-performance cation-exchange chromatography, as previously described (Reed and Archer, 1976; Archer and Reed, 1979).

At pH 1.5, the rate of tetrahydrofolate oxidation was too fast to measure by our chromatographic techniques. After reaction of tetrahydrofolate with a 3–4 M excess of nitrous acid for 1 h, the yields of the products determined chromatographically were *p*-aminobenzoylglutamate (close to 100%), pterin (36%), pterin-6-carboxaldehyde (24%), and xanthopterin (8%; Figure 4). (We did not analyze the reaction mixtures for formaldehyde, which was detected by Chippel and Scrimgeour (1970) in the oxidation of tetrahydrofolate by ferricyanide.) Blair and Pearson (1974) reported that pterin was also the major pteridine product of tetrahydrofolate oxidation by air at acidic pH, as observed by thin-layer chromatography. This observation has been verified in our own experiments with air as oxidizing agent using high-performance liquid chromatography for analysis (unpublished results).

At pH 5.0, the rate of the reaction of tetrahydrofolate with nitrite was zero order with respect to tetrahydrofolate (Figure 5). The reaction order with respect to nitrite was one. The reaction also depended on the buffer anion concentration as was the case for folic acid nitrosation. For tetrahydrofolate oxidation, however, the order with respect to acetate was close to one. The rate constant defined by

$$\text{rate} = k_2[HNO_2][OAc^-] \quad (3)$$

was $2.5 \times 10^{-3}$ $M^{-1}$ $s^{-1}$. The oxidation of ascorbate by nitrite has also been shown to be zero order in reductone (Bunton et al., 1959), which indicates that formation of the oxidizing agent is rate limiting in these reactions. In the case of ascorbate, the reaction at pH 4 was second order in nitrite, showing that the oxidizing agent is nitrous anhydride. For tetrahydrofolate, however, since the oxidation reaction was first order with respect to both nitrite and acetate at pH 5.0, the oxidizing agent is nitrosyl acetate.

*p*-Aminobenzoylglutamate and several pterins were the products of tetrahydrofolate oxidation by an excess of nitrite at pH 5. Figure 5 shows the time course of a typical reaction at pH 5. The initial products of the reaction appear to be *p*-aminobenzoylglutamate and several di-

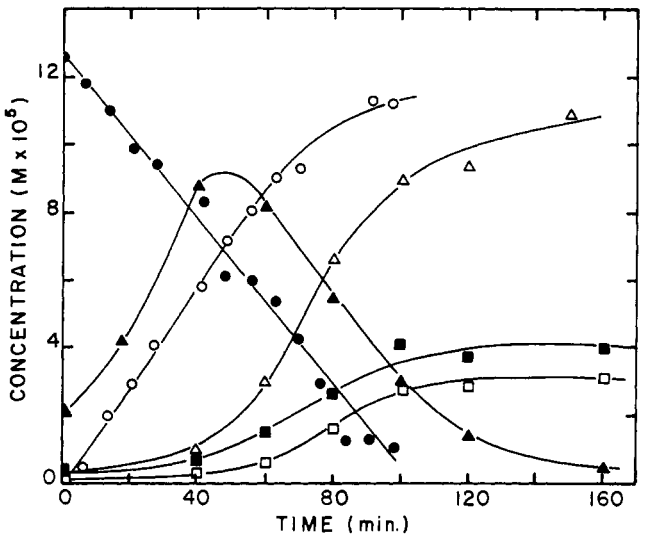


**Figure 5.** Time course of product formation by reaction of tetrahydrofolic acid ($12.6 \times 10^{-5}$ M) and sodium nitrite ($6 \times 10^{-3}$ M) at pH 5.0, 30 °C; (●) tetrahydrofolate, (○) *p*-aminobenzoylglutamate, (▲) dihydropterins (measured) as dihydroxanthopterin, (△) xanthopterin, (■) pterin, (□) pterin-6-carboxaldehyde. (The final concentrations of products in this reaction are somewhat greater than the initial concentration of tetrahydrofolate because there was some degradation of tetrahydrofolate to *p*-aminobenzoylglutamate, dihydropterins, and possibly undetected tetrahydropterins or quinonoid dihydropterins prior to the start of the experiment. The initial concentration of *p*-aminobenzoylglutamate was subtracted from the concentrations shown in this figure. The final concentrations of *p*-aminobenzoylglutamate equalled the sum of the final concentrations of the pterins.)

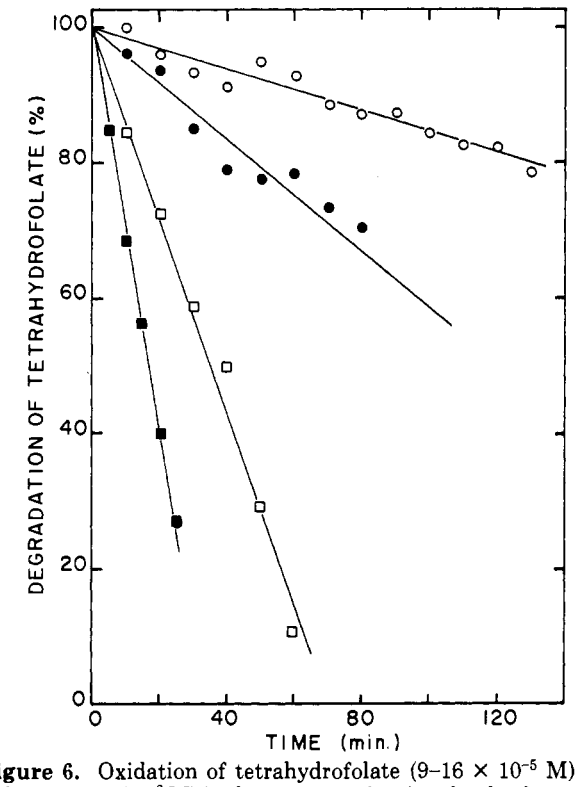


**Figure 6.** Oxidation of tetrahydrofolate ($9–16 \times 10^{-5}$ M) by sodium nitrite ($10^{-2}$ M) in the presence of various levels of ascorbic acid at pH 5.0, 25 °C. Molar ratios of ascorbate/tetrahydrofolate were (○) 100:1, (●) 10:1, (□) 1:1, (■) 0:1.

hydropterins. (Under the chromatographic conditions used, the dihydropterins coeluted, and quantitation of the dihydropterin products is based only on the absorbance of dihydroxanthopterin at 254 nm.) Figure 5 illustrates that the dihydropterins are further oxidized by nitrous acid to yield ultimately a mixture of pterin, pterin-6-carboxaldehyde and xanthopterin. No folate, dihydrofolate, pterin-6-carboxylic acid, or *p*-hydroxybenzoylglutamate were detected.

Chippel and Scrimgeour (1970) and Blair and Pearson (1974) suggested that pterin products form by rapid reaction of tetrahydrofolate with 2 equiv of oxidizing agent to yield a quinonoid dihydrofolate. This kinetically favored product is thermodynamically unstable, however, and it rearranges either by loss of a proton from the C7 position to yield 7,8-dihydrofolate, or by loss of the side chain to form dihydropterin. 7,8-Dihydrofolate is then the probable precursor of xanthopterin and pterin-6-carboxaldehyde, while dihydropterin is further oxidized to pterin. Although we did not detect 7,8-dihydrofolate in any of our experiments, it may have existed as a transient intermediate in the presence of an excess of oxidizing agent. 7,8-Dihydrofolate reacted with an excess of nitrite at pH 5.0 to yield both xanthopterin and pterin-6-carboxaldehyde in roughly equal amounts, although xanthopterin only appeared toward the end of the reaction. An explanation for formation of pterin-6-carboxaldehyde is direct attack of the oxidizing agent on the side chain of 7,8-dihydrofolate to form the Schiff base at the C9–N10 position. This intermediate would then be expected to hydrolyze to pterin-6-carboxaldehyde. Xanthopterin probably forms by covalent hydration of the N5–C6 double bond in 7,8-dihydrofolate prior to oxidation (Chippel and Scrimgeour, 1970).

Figure 6 illustrates the protective effect of ascorbic acid on tetrahydrofolate oxidation by nitrous acid at pH 5. Both tetrahydrofolate and ascorbate are oxidized under these conditions, but the oxidation of tetrahydrofolate is not completely inhibited even when the ascorbate is in a 100-fold molar excess over tetrahydrofolate. Ascorbic acid has previously been shown to protect tetrahydrofolate from air oxidation (Ramasastri and Blakley, 1964).

**Reaction of Other Reduced Folates with Nitrous Acid.** 5-Methyltetrahydrofolate was oxidized by nitrite at pH 5 to produce products that eluted on the cation column in a similar manner to xanthopterin, pterin-6-carboxaldehyde, and pterin. The products in this case are probably the respective 5-methyl derivatives of the pterins. 5-Formyltetrahydrofolate reacted with sodium nitrite at pH 5 and yielded a single product observable by liquid chromatography that gave a positive reaction with the Griess reagent (Daiber and Preussmann, 1964) and was most probably the $N^{10}$-nitroso derivative. This product was not further characterized. In contrast to 5-methyltetrahydrofolate, 5-formyltetrahydrofolate is extremely resistant toward oxidation (Roth et al., 1952).

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# A New Isochroman Mycotoxin Isolated from *Penicillium steckii*

Richard H. Cox,* Oscar Hernandez, Joe W. Dorner, Richard J. Cole, and Dorothy I. Fennell[1]

*Penicillium steckii* (NRRL 6336) was isolated from moldy millet hay suspected of causing deaths in cattle. The mold produced a toxic metabolite identified by spectroscopic means as 3,7-dimethyl-8-hydroxy-6-methoxyisochroman. The median lethal dose of the toxin in 1-day-old chickens was 800 mg/kg.

We have isolated a toxigenic strain of *Penicillium steckii* Zalecki (NRRL 6336) as a result of our continuing efforts to identify the etiological agents responsible for naturally occurring toxicoses associated with ingestion of molded feed. The fungus was one of several toxigenic fungi isolated from moldy millet hay suspected of causing deaths in cattle. Saito et al. (1971) have reported the isolation of a strain of *P. steckii* that was toxic to cultured cells and animals. Davis et al. (1975) have also reported the isolation of a strain of *P. steckii* from chocolate syrup that was toxic in the brine shrimp and chicken embryo bioassays. In neither of these cases were the toxin(s) identified. Some strains of *P. steckii* are capable of producing the nephrotoxin citrinin (Krogh, 1974).

## EXPERIMENTAL SECTION

The toxigenic fungus was isolated from the suspect hay on potato dextrose agar (PDA) plates and was maintained at 5 °C after 7–10 days growth at 27 °C. The fungus was mass cultured in fifty 2.8-L Fernbach flasks containing 100 g of shredded wheat supplemented with 200 mL of mycological broth (pH 4.8), 15% sucrose, and 2% yeast extract. The cultures were incubated at 28 °C for 3 weeks prior to extraction. Extraction of the fungal cultures with hot chloroform yielded an oily extract that was highly toxic to day-old chickens.

The crude extract was systematically fractionated, and toxicity of the fractions was monitored with day-old chickens. The fractions were administered via crop intubation using 1 $cm^3$ of corn oil as the inert carrier at a rate of 1 $cm^3$ or less per chicken (Kirksey and Cole, 1974; Cole, 1978).

The crude chloroform extract was fractionated on a silica gel 60 column (9 × 18 cm) eluted sequentially with three column volumes each of benzene, diethyl ether, ethyl acetate, and acetone. The diethyl ether eluate was the most toxic. This fraction was evaporated to dryness and applied to a second silica gel 60 column (3.5 × 40 cm). The column was packed as a benzene slurry and the sample was applied to the column in benzene solution. The residual oil was eluted from the column with 3 L of benzene. The column was then eluted with a linear gradient from benzene to diethyl ether (240/17 mL fractions). Bioassay of every tenth tube revealed that toxicity was between the 40th and 60th tubes. These fractions were combined and thin-layer chromatography (silica gel 60F-254, 5 × 10 cm; solvent systems, chloroform/acetone, 93:7, v/v and toluene/ethyl acetate/formic acid, 5:4:1, v/v/v) showed the combined fractions to contain predominantly one metabolite. The toxic fractions were reduced in volume and placed at 5 °C overnight. Approximately 3 g of very fine microcrystals were collected. TLC showed there to be only one metabolite present. The crystals were recrystallized from acetone solution.

Infrared spectra (IR) of the metabolite were taken with a Perkin-Elmer 257 IR spectrometer equipped with a 3X condenser. Samples were coated on KBr blocks as a thin film. Ultraviolet spectra (UV) were taken with a Beckman

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