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Formation in Aqueous Solution of N-nitroso Curzate and the Catalytic Effect of Some Anions

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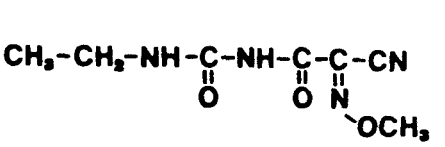
In recent years much information has become available on the occurrence of N-nitroso compounds in meat products, malt and beer, cigarette smoke, cosmetics, pesticides and other commodities. So far, little is known about the formation of N-nitroso compounds in human body as regards all these different substrates. Since residues of such compounds may be present in foodstuffs together with nitrite, their N-nitrosation in the gastro-intestinal tract is therefore possible.

For this reason we focused on curzate, a widely used fungicide, effective against grape downy mildew, as well as tomato and potato late blight (Klopping and Delp 1980). Its metabolism has already been studied in the rat (Barlasco and Baude 1981), where its major metabolite was characterized as glycine, both free and conjugated, as hippuric acid or phenylaceturic acid. Nevertheless, the presence of an N-nitrosable nitrogen in the structure of this compound possibly makes curzate to undergo, in suitable conditions, an N-nitrosation reaction. The occurrence of a similar reaction has already been proved for a chemically similar compound, the Benzthiazuron (Eisenbrand et al. 1975).

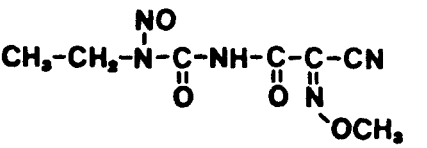
On the other hand the possibility that such compounds could exert an influence on the in vivo formation of carcinogenic N-nitroso intermediates deserves careful attention. At first we synthetized, by an original method, the N-nitroso curzate, then we studied if the N-nitrosation of curzate (Fig.1) occurs at least in vitro.

MATERIALS AND METHODS

N-nitroso curzate was obtained by treating curzate [1-(2-cyano-2-methoxyiminoacetyl)3-ethylurea] (courtesy of Du Pont, Italy) with an equimolar amount of nitrosonium tetrafluoroborate ($NOBF_4$) in anhydrous dichloromethane at 0°C; the mixture was evaporated and the residue was chromatographed on Silica Gel (230-400 mesh) (Merck) at medium pressure, with hexane/ethyl acetate 8:2. N-nitroso curzate was obtained as a pale yellow solid, yield 19%, melting point 64°C; ^{1}H-nmr ($CDCl_3$, 80 Mhz) δ : 1.06 (3H, t, J = 7.5 CH_3, 3.90 (2H, q, J = 7.5 CH_2, 4.30 (1H, s, NH), 4.33 (3H, s, OCH_3); mass, m/z (%): 227 (0.1), 199 (0.7), 197 (0.8), 195 (0.8),



A



B

Figure 1 - Structures of curzate (A) and N-nitroso curzate (B)

182 (1.6), 170 (2), 154 (2.9), 127 (4.2), 115 (3.0), 111 (15.4), 83 (10.7), 44 (100). (Fig. 2). A Finnigan model 4000 quadruple mass spectrometer, interfaced via an all glass transfer line and jet separator to a Finnigan model 9500 gas chromatograph, was used.

The nitrosation reaction in vitro was carried out as follows: the reaction mixture (final volume 5 ml) was constituted of curzate, dissolved in acetone and added in order to obtain a final concentration of 2 mM, 10 mM sodium nitrite ($NaNO_2$) (Merck), different concentrations of potassium thiocyanate (KSCN) (Merck) (10, 20 and 40 mM in the final volume) and acetic acid (Merck) (17.5, 35 and 350 mM in the final volume). Diluted HCl was used to obtain the desired values of pH. The resulting concentrations of nitrite and thiocyanate were very close to those actually found in human saliva.
The mixtures were then incubated overnight (about 18 hours) at 37°C without stirring. Each sample was then extracted with 10 ml chloroform and the organic layer was separated and dried under gentle stream of nitrogen. Curzate and N-nitroso curzate were detected by TLC (Kieselgel plates Merck 60 F-254 with fluorescence indicator) with hexane/ethyl acetate 8:2 and quantitated through comparison between the areas by means of a Camag TLC-scanner II. All the solvents used were analytical grade, Carlo Erba, Italy.

RESULTS AND DISCUSSION

The figures 3 A and 3 B represent the experimental data on the N-nitrosation of curzate; they point out how thiocyanate as well as acetate increase the yield of the N-nitrosation reaction, which is maximized by a particular pH value. There is evidence that both these anions are to be present simultaneously; on the contrary the reaction is not allowed to proceed. The effect of different concentrations of acetate and thiocyanate was studied too: evidence suggest that the catalytic action increases with the rise of the concentrations of both anions.
In particular, figure 3 A shows the percentage of N-nitrosation of curzate in presence of various thiocyanate concentrations and at different pH values. The percentage of conversion remains low: at pH 1.5 it is possible to detect only a small amount of

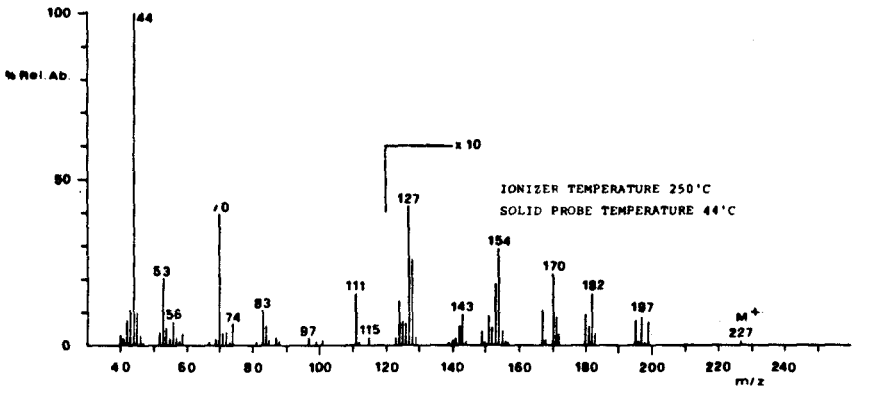


Figure 2 - Mass spectrum of N-nitroso curzate

N-nitroso curzate. The N-nitrosation of curzate appears to be maximum at pH 2.5, reaching a 10% of conversion; as the pH rises above pH 3 the amount of product formed decreases considerably. Fig 3 B shows the N-nitrosation curves in the presence of varying concentrations of acetate: the pattern of conversion is very similar to that of thiocyanate.
Both figures 3 A and 3 B show that the yield of N-nitroso curzate, even in the most favourable conditions, i.e. 350 mM acetic acid and 40 mM thiocyanate and at pH 2.5, is never higher than 10%.

Our results give prominence to the importance of the presence of some anions and of the pH value in the incubation medium for the N-nitrosation of curzate. The pronounced catalytic effect of thiocyanate on the reaction of nitrite has been widely accepted; in fact, it is known that thiocyanate greatly enhances the rate of this reaction at pH values between 0.5 and 3.0 (Boyland et al. 1971, Boyland and Walker 1974).

More interesting is the proved catalytic effect of acetate on the N-nitrosation reaction, which could be related to a particular mechanism. The nitrosation of secondary amides, such as curzate, (including ureas, guanidines and carbamates) is, in general, less easy than amine nitrosation. In contrast to the nitrosation of primary amines, it is known that the major nitrosating agent, in this case, is not the nitrous anhydride N_2O_3, but the more active nitrous acidium ion (Mirvish 1975). Consequently, the catalytic action of acetate, which has already been studied by Masui et al. (1974), could be explained by the production of a directly active agent, the nitrosonium acetate CH_3COONO.
However, it is known that the use of vinegar, which contains up to 5% acetic acid, in combination with nitrite in preparation of food similarly increases the rate of nitrosation (Boyland 1972). The acetate ion seems to be necessary also for the N-nitrosation of Dodine, another widely used fungicide; for this compound the yield of the N-nitrosation is low too, about 12% (Lascialfari, 1981). Finally, the results show that the N-nitrosation of curzate is extremely dependent on pH and is restricted to a narrow range in the region of pH 2.5.

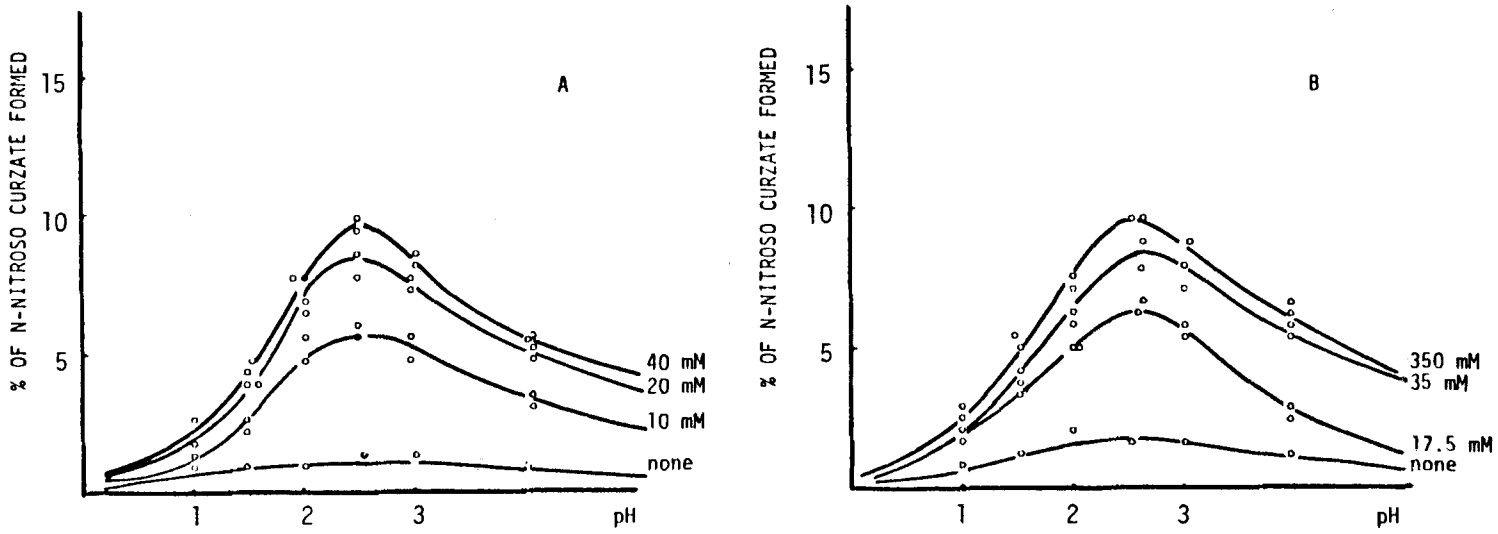


Figure 3 A - Catalytic action of different concentrations of thiocyanate in presence of 350 mM acetic acid on the formation of N-nitroso curzate

Figure 3 B - Catalytic action of different concentrations of acetate in presence of 40 mM thiocyanate on the formation of N-nitroso curzate.

Studies such as the present, even if performed in vitro, may provide evidence concerning the importance of N-nitrosation reactions and demonstrate the usefulness for the assessment of the formation in vivo of these N-nitroso intermediates.

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