Catalytic Effect of *p*-Nitrosophenol on the Nitrosation of Diethylamine

Ernest Albert Walker,* Brigitte Pignatelli, and Marcel Castegnaro

In kinetic studies using a gas chromatographic method the nitrosation of diethylamine in water at 37 °C was shown to be second order with respect to nitrite, with a pH-independent rate constant of 0.87 $\times 10^5$ M^{-2} s^{-1}. The reaction catalyzed by *p*-nitrosophenol over a wide pH range was first order with respect to nitrite, amine, and *p*-nitrosophenol. A mechanism for this reaction is suggested. The possibility that *p*-nitrosophenol could influence in vivo formation of the nitrosamines is considered.

The carcinogenicity of *N*-nitroso compounds in many animal species is well established as is also their remarkable organotropic action (Magee et al., 1976). Investigation of the relatively high incidence of oesophageal cancer in Northern France, which has been associated with high alcohol consumption (Tuyns, 1970), has included a study of the occurrence of volatile nitrosamines in a wide variety of alcoholic drinks. The results (International Agency for Research on Cancer, 1977) show several nitrosamines including nitrosodiethylamine (NDEA), an oesophageal carcinogen, to be present in the types of alcoholic beverage consumed. However, the levels, which are mainly 1 μg/L or less, suggest that attention should also be given to consideration of factors which might promote in vivo formation of nitrosamines.

Various authors have demonstrated the inhibiting effect of ascorbic acid (Fan and Tannenbaum, 1973; Mirvish et al., 1972) and α-tocopherol (Mergens et al., 1978) on the formation of nitrosamines. Thiocyanate (Boyland et al., 1971) present in the saliva of smokers has been shown to have a catalytic effect, whereas phenolic compounds such as tannin have been shown to act both as catalysts (Challis and Bartlett, 1975) and inhibitors (Bogovski et al., 1972). Phenolic compounds occur widely in food and drinks in many forms, but as their structures are often complex, we elected to study the effect of the simplest, phenol, on the nitrosation of diethylamine.

Initial studies showed that although phenol promoted the formation of nitrosodiethylamine, this was dependent on the relative concentrations of nitrite and phenol. Reaction of nitrous acid with phenol, which leads to formation of *p*-nitrosophenol, is very rapid in comparison with its reaction with strongly basic secondary amines (Challis, 1973). It is, therefore, possible that the *p*-nitrosophenol derivative is the promoting agent rather than phenol itself. Comparison of the two catalyzed rates of NDEA formation, which were seven times higher in the presence of *p*-nitrosophenol, supports this view. During this work, Davies and McWeeny (1977) reported a similar catalytic effect from *p*-nitroso-*o*-cresol on the nitrosation of pyrrolidine.

EXPERIMENTAL SECTION

All materials employed were of analytical reagent grade. *p*-Nitrosophenol (Aldrich Chemicals) was used as purchased. Recrystallization was difficult but the product when recrystallized from water/acetone gave the same kinetics as the commercial product. *m*-Nitrosophenol was synthesized by the method of Alfonso and Kravtsov (1968). Fixanal citrate buffer solutions were purchased [Riedel de Haën, AG).

Kinetics were all carried out at 37 °C (body temperature) using solutions buffered with citrate at pH 4. Diethylamine solutions were made using diethylamine hydrochloride and nitrite solutions using sodium nitrite.

The kinetics of reaction were determined by the method of initial rates to minimize any effects due to decomposition of nitrous acid which was found to be stable under the conditions employed for the duration of the experiment. Solutions were gravimetrically prepared in the buffer and thermostated at 37 °C. Reaction was initiated by adding appropriate buffered reagent solutions to the nitrite buffered at the same pH. The concentrations were selected so that the volumes of reagents employed would give a final volume of 50 mL and levels of NDEA which could be conveniently extracted and measured. The solution was shaken vigorously for 15 s and returned to the bath. Ten-milliliter aliquots were withdrawn at intervals over a 30-min period and transferred to a separator containing 10 mL of 1 M sulfamic acid to destroy excess nitrite. After shaking for 1 min, the NDEA was extracted using three successive quantities of 15 mL of methylene chloride. The organic phase was then drawn off, dried over anhydrous sodium sulfate, and finally bulked to 50 mL in a graduated flask. Ten-microliter aliquots of solution were used to determine the NDEA by gas chromatography (GC) using an 8-m column of 10% Carbowax 20 M on Chromosorb W (80–100 mesh) and a Thermal Energy Analyzer (TEA) for detection. The nitrosophenols and diethylamine give no response to this nitrosamine-selective detector so the method is highly specific for the nitrosamine. As the *p*-nitrosophenol is not amenable to gas chromatography and tends to decompose on the column, a 5-cm precolumn containing glass wool, which is changed periodically, was used as a trap. This prolongs the life of the column but is not essential.

The order of reaction for each reactant was determined using the method of isolation in which the rate is determined on a range of concentrations for a given reactant using the other reactants in excess. When carrying out reactions in aqueous solution in the presence of the excess *p*-nitrosophenol, difficulties were experienced due to inadequate solubility. Kinetic measurements were therefore also made in water to which a suitable solvent had been added. A dioxane–water mixture was tried but discarded as frequent contamination of the solvent with traces of peroxide affected the rates and caused poor reproducibility. Water containing 10% acetone was, however, found to be suitable. Where the diethylamine hydrochloride concentration used was 0.5 M in solutions buffered at pH 4, the final pH of the reaction mixture was 3.85 in aqueous solution and 4.0 in the aqueous acetone. pH remained constant throughout the reaction. In determination of the order of reaction with respect to amine in the presence of *p*-nitrosophenol, where low concentrations of amine hydrochloride were required in the range of 0.0005–0.002 M, the pH of the reaction in aqueous acetone was 4.2. As seen from Figure 1, this small difference of pH has no significant effect on the rate of the catalyzed reaction.

International Agency for Research on Cancer, 69008 Lyon, France.

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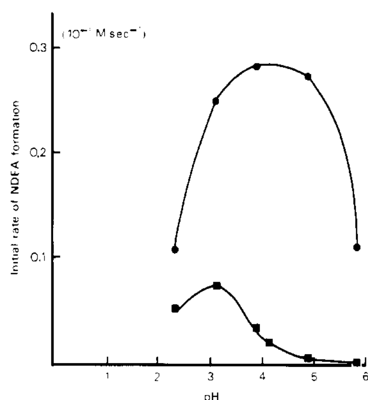


Figure 1. pH-dependent influence of *p*-nitrosophenol on the nitrosation of diethylamine: (■) in absence of *p*-nitrosophenol, (●) [*p*-nitrosophenol] = 0.002 M; temperature, 37 °C; [DEA,HCl], 0.5 M.

RESULTS

Figure 1 shows the variation with pH in rate of formation of NDEA under normal conditions and in the presence of 0.002 M *p*-nitrosophenol. A distinct promoting effect by the *p*-nitrosophenol on the rate of reaction over the range pH 2–6 is evident. Even at pH 6, where nitrosation is normally very slow, the rate still exceeds the optimum rate in the uncatalyzed reaction. It may also be noted that the optimum pH for the catalyzed reaction is slightly higher. *m*-Nitrosophenol appears to have no effect on the rates of reaction.

For the uncatalyzed reaction normal kinetics for the nitrosation of a secondary amine were found, i.e., first order with respect to amine and second order with respect to nitrite. Thus, the pH-dependent rate is given by the equation:

$$\text{rate} = k'[\text{total diethylamine}][\text{total nitrite}]^2 \quad (1)$$

in which the nitrosating series is N_2O_3 formed from two molecules of HNO_2 as confirmed by Mirvish (1970), $2HNO_2 = N_2O_3 + H_2O$. As this author has shown, the rate depends on the concentration of nonionized species and the pH-independent constant is given by:

$$k = k'\left[\frac{\text{total amine}}{\text{nonionized amine}}\right]\left[\frac{\text{total nitrite}}{\text{nonionized nitrite}}\right]^2$$

The ratios [total]/[nonionized species] are calculated using the Henderson equation, pH = $pK_a + \log [A^-]/[HA]$, as indicated by Fan and Tannenbaum (1973).

To calculate the independent rate constant k, a value of $pK_a = 2.95$ for nitrous acid was calculated from the equation of Lumme et al. (1965). For diethylamine, the value of $pK_a = 10.489$ was taken from the tables of Perrin (1965). From these and the value of $k' = 2.5 \times 10^{-4}$ M^{-2} s^{-1} derived experimentally, a pH-independent rate constant of 0.87×10^5 M^{-2} s^{-1} was calculated. Gehlert and Rolle (1976) determined the independent rate constant, k, in the temperature range of 5–25 °C. This on extrapolation would give a value of $k = 2.05 \times 10^5$ M^{-2} s^{-1}, which is approximately double that reported here. It is interesting to observe that the independent rate constant found by these authors for the formation of nitrosodimethylamine (NDMA) at 25 °C was also approximately double that reported by Mirvish (1975). As they suggested this might be due to a catalytic effect by the phthalate buffer employed, we carried out some kinetics with a phthalate buffer but found rates appeared to be unaffected.

Rates determined in the aqueous acetone medium, both for catalyzed and uncatalyzed reactions, were slightly

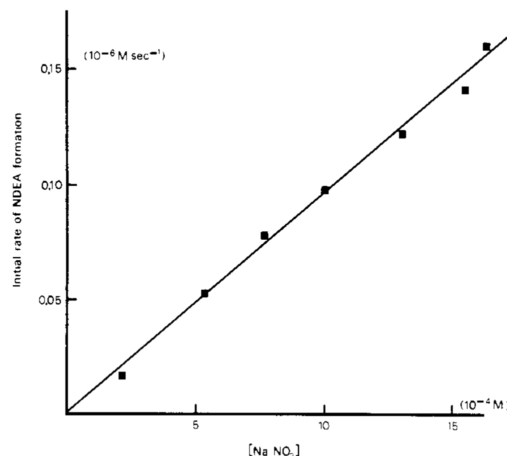


Figure 2. Plot showing first order of reaction with respect to nitrite in presence of *p*-nitrosophenol: temperature, 37 °C; pH 4; medium, 10% acetone in water; [DEA,HCl], 0.5 M; [*p*-nitrosophenol], 0.008 M.

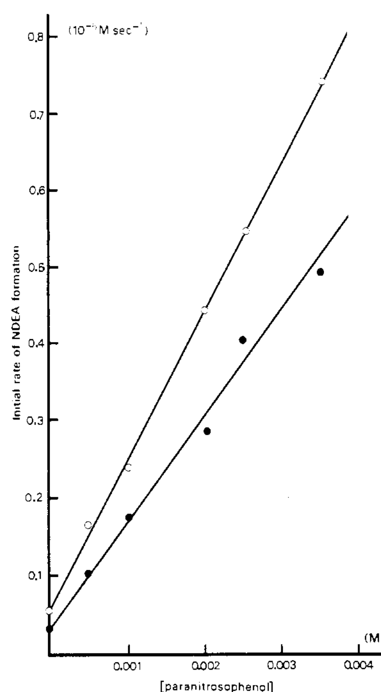


Figure 3. Plots indicating first order of reaction with respect to *p*-nitrosophenol in aqueous and aqueous/acetone media: temperature, 37 °C; (●) medium, water, pH 3.85; (○) medium, 10% acetone in water, pH 4; [DEA,HCl], 0.5 M; [$NaNO_2$], 0.016 M.

greater than those determined in aqueous solution. It is reasonable to suppose that this increase was due to a tendency on the part of the solvent to suppress ionization, thereby increasing the concentration of the nonionized species.

For the *p*-nitrosophenol-catalyzed reactions in water–acetone, the reaction was first order with respect to each reactant (*p*-nitrosophenol, amine, and nitrite). The reaction was also first order with respect to *p*-nitrosophenol in aqueous solution. Thus, in the presence of *p*-nitrosophenol there is a change from second to first order with respect to nitrite as shown by the linear plot of initial rate as a function of [$NaNO_2$] in Figure 2, which suggests some modification by *p*-nitrosophenol to the mechanism of reaction. In Figure 3, each plot of rate against [*p*-nitrosophenol] is linear and passes through the value for the uncatalyzed initial rate at zero concentration of *p*-

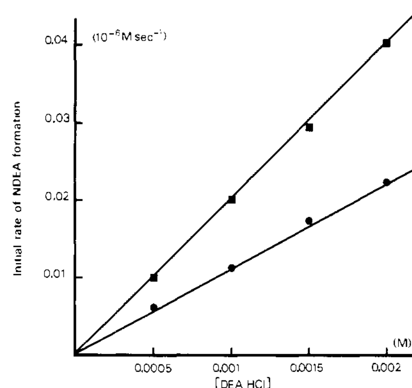


Figure 4. Plots showing first-order reaction with respect to amine in water containing 10% acetone: (■) at pH 4.2 in a solution of weak ionic strength, (●) at pH 3.9 in a solution of constant ionic strength ($[Cl^-]$ = 0.5 M); $[NaNO_2]$, 0.01 M; [p-nitrosophenol], 0.04 M.

nitrosophenol. As for the uncatalyzed reaction, the rate is slightly higher in the presence of the solvent. Since at any given value of pH, both catalyzed and uncatalyzed reactions can be assumed to take place simultaneously, the overall rate equation will be given by:

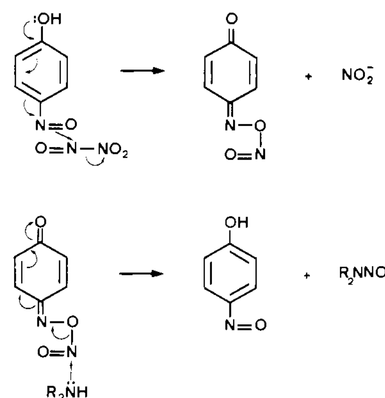
$$\frac{d(\text{NDEA})}{dt} = k'[\text{amine}][\text{nitrite}]^2 + k''[\text{amine}][\text{nitrite}][p\text{-nitrosophenol}] \quad (2)$$

In aqueous solution, when k'' is calculated from the slope of the plot for varying p-nitrosophenol concentrations, the value k''_p is 0.025 $M^{-2}\ s^{-1}$, and when calculated from the slope against nitrite, the value k''_n = 0.024 $M^{-2}\ s^{-1}$, which are in very good agreement. However, when determined from the plot for variation of amine concentration, the value k''_a was 0.05 $M^{-2}\ s^{-1}$. At the low concentration of amine employed, the chloride concentration, and therefore total ionic strength, is considerably lower than in either of the other two cases which, as also suggested by Mirvish (1970), would result in an increase in the rate of reaction. The plot of initial rate against amine concentration was therefore redetermined after addition of the requisite amount of sodium chloride to give a constant concentration of 0.5 M with respect to total chloride (Figure 4). Under these conditions, the pH of the reaction medium was 3.90, the rate of reaction was reduced, and the calculated value for k''_a was then 0.028 $M^{-2}\ s^{-1}$. This, within the limits of experimental error, then corresponds reasonably well with the values for k''_p and k''_n. No attempt was made to calculate the pH-independent rate value as the pK_a values under the experimental conditions are not available. However, under optimum conditions in the region of pH 4, the average value for the rate of about 0.026 $M^{-2}\ s^{-1}$ is about 60 times that (0.0004 $M^{-2}\ s^{-1}$) for the uncatalyzed reaction in the same solvent. As $k''_p \approx k''_a \approx k''_n$ in the acetone–water medium, assuming the mechanism of reaction is the same in both solvents, the measurable value k''_p in water can be taken as the value for k'' in eq 2. From Figure 4, k''_p = 0.018 $M^{-2}\ s^{-1}$ is about 70 times the rate constant of $2.5 \times 10^{-4}\ M^{-2}\ s^{-1}$ determined for the uncatalyzed reaction at the same pH.

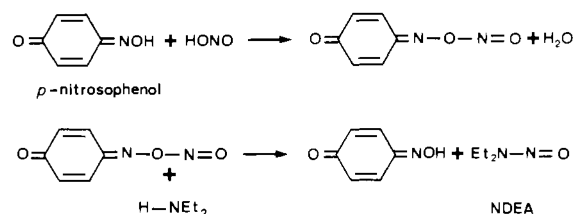
DISCUSSION

In a recent paper, Davies et al. (1978) examined the catalytic effect of several other nitrosophenols and nitrosoarylamines on the nitrosation of pyrrolidine and concluded that, for the nitroso derivative to function as a catalyst, it is essential that it should be capable of existing in a tautomeric quinone form and suggested the type

Scheme I



Scheme II



of mechanism shown in Scheme I. They do not, however, report the order of reaction with respect to nitrite. Their plot of initial rates against [p-nitroso-o-cresol] for formation of nitrosopyrrolidine, which is linear, indicates a first-order reaction with respect to the catalyst as in the present case, so that it seems reasonable to assume that the reaction mechanism would be similar. It seems unlikely to us that the first step would involve N_2O_3, which implies second-order kinetics. A second, but minor, point, their mechanism does not account for the phenolic hydrogen, although this could be accounted for by the formation of HNO_2 rather than NO_2^-. There appear to be good grounds for assuming that the nitrosophenol is involved in the quinone form as this is also supported by our own investigation in which m-nitrosophenol, which cannot exist in the quinone form, showed no influence on the rate of reaction. A mechanism which would better fit the kinetics would involve nitrous acid itself and the reacting species is given in Scheme II.

Although no definite explanation can be offered at this stage, the slight shift in optimum pH for the catalyzed nitrosation could be interpreted as support for the principle of a radical change from the normal mechanism of nitrosation. This can be only speculation until further work clarifies this point. From the practical point of view, it is important to note that in the presence of the p-nitrosophenol formation of NDEA still proceeds effectively under relatively high pH conditions which normally inhibit nitrosamine formation.

Using GC/mass spectrometry (MS), a number of nitrosophenols have been shown by Knowles et al. (1974) to be present in smoked bacon treated with nitrite. Although p-nitrosophenol was not detected since it is nonvolatile and not amenable to GC, its presence can be expected as p-nitrosophenol is formed very readily and the same authors (1975) have shown that phenol itself can constitute about 13% of the total phenols in smoked bacon. Lustre and Issenberg (1970) have shown that heavily smoked bacon may contain up to 280 ppm of phenols. Salival nitrite values are normally in the order of about 7 ppm (Spiegelhalder et al., 1976; Tannenbaum et al., 1974), and Klein et al. (1978) have found stomach levels of a similar order. Nitrosation of phenol is about 10^4 times faster than

nitrosation of strongly basic secondary amines (Challis, 1973). p-Nitrosophenol could also be formed in the digestive tract by reaction of phenol with nitrite ingested in the saliva.

Comparison of eq 1 and 2 gives a measure of the relative rates of formation in the catalyzed and uncatalyzed reactions at about pH 4 which would apply to anacid stomach conditions during digestion:

$$\frac{\text{rate (catalyzed)}}{\text{rate (uncatalyzed)}} = 1 + \frac{k'[p\text{-nitrosophenol}]}{k[\text{nitrite}]} = 1 + \frac{70[p\text{-nitrosophenol}]}{[\text{nitrite}]} \quad (3)$$

Although there is no information on the levels of p-nitrosophenol present in bacon nor the proportion of phenol nitrosated in the stomach, if we assumed complete nitrosation of the phenol by the nitrite which is continually ingested in the saliva using the values given above, this would indicate a maximum potential increase in rate of formation of NDEA under in vivo conditions by a factor of about 140. Whilst such a calculation based on a model system is obviously an oversimplification since there would be inhibiting factors as well as possible additional catalytic effects by other nitrosophenols, it illustrates a potential promoting effect on in vivo formation of nitrosamines due to constituents in food itself. This could be important to the evaluation of environmental exposure to these compounds.

Work is currently in progress to extend this study to some polyhydric and other phenols which constitute an essential part of the structure of naturally occurring polyphenols.

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Comparative Embryotoxicity of Dimethylnitrosamine in the Chick Embryo

Emmanuel N. Maduagwu* and Olumbe Bassir

The LD_{50} values of dimethylnitrosamine (DMN) for the chick embryo were estimated in four strains of the domestic fowl (*Gallus domesticus*), namely, White Leghorn, White Rock, Rhode Island red, and a local breed. The White Leghorn embryo was most susceptible to DMN poisoning, with an LD_{50} of 3.3 μg of DMN/50 g egg weight, when compared with the others which had LD_{50} values of 14.8, 5.7, and 8.4 μg of DMN/50 g egg weight, respectively. The main liver lesion observed in moribund embryos was a massive congestion of the lobule. No centrilobular cell necrosis of the tissue, characteristic of DMN acute poisoning, was evident. It would appear from the results that eggs from the White Leghorn flock are suitable for the bioassay of nitrosamines.

There is relatively very little information on the toxicological effects of N-nitrosamines on chick embryos. Aleksandrov (1967) showed that dimethylnitrosamine is lethal to and inhibits growth of the chick embryo. No teratogenic effect due to the compound was, however, observed.

The fertile avian egg is very sensitive to minute changes affecting its milieu interieur. Such minute changes, like

Department of Biochemistry, University of Ibadan, Ibadan, Nigeria.

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