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N-Phenylglycolhydroxamate Production by the Action of Transketolase on Nitrosobenzene

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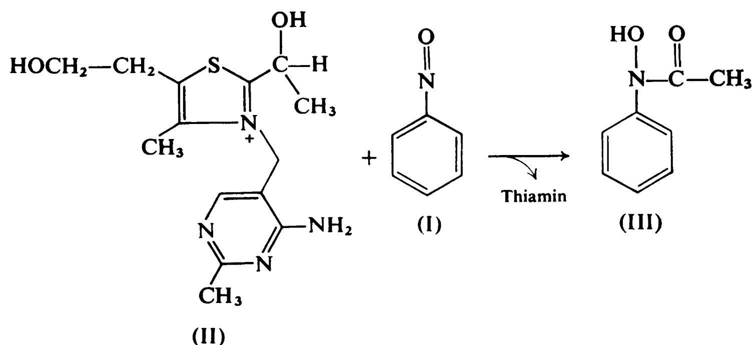
The incubation of nitrosobenzene with yeast transketolase and D-xylulose 5-phosphate resulted in the production of *N*-phenylglycolhydroxamic acid. The addition of D-ribose 5-phosphate decreased the amount of hydroxamic acid that was produced. This conversion of nitrosobenzene into the glycollic acid-derived hydroxamic acid was shown to be an enzymic process, and a chemical mechanism for the conversion was proposed.

The conversion of nitrosobenzene (I, Scheme 1) into *N*-phenylacetohydroxamic acid (III, Scheme 1) through reactions mediated by biochemically important thiamin derivatives has been previously reported by us (Corbett, 1974; Corbett *et al.*, 1975). This transformation, depicted in Scheme 1, occurs in non-enzymic model reactions with α-hydroxyethylthiamin (II, Scheme 1), a thiamin derivative generally agreed to be an intermediate for pyruvate decarboxylation (Breslow, 1958; Ullrich *et al.*, 1970). The process shown in Scheme 1 occurs even more readily in the presence of certain enzymes, including pyruvate decarboxylase (2-oxo acid carboxy-lyase, EC 4.1.1.1).

The occurrence of similar reactions *in vivo* has not yet been demonstrated, but in view of the ready interconversion of aromatic amines and nitro compounds with the nitroso functional group *in vivo* (Kiese & Taeger, 1976; Mitchard, 1972) this transformation requires further investigation. The toxicological significance of such conversions *in vivo* is that hydroxamic acids might be produced in tissues via pathways not currently recognized. Extensive research by other investigators has demonstrated that the metabolic activation of carcinogenic amides is initiated by *N*-oxidation to produce hydroxamic acids. It has always been assumed that hydroxamic acid production *in vivo* is mediated only by microsomal oxidases. Our results from experiments *in vitro* suggest that hydroxamic acid production might not be restricted to microsomal enzymes, but that thiamin-dependent enzymes could be of importance.

Our interest in the adventitious interaction of nitroso aromatics with thiamin-dependent enzymes prompted us to investigate this reaction in the presence of transketolase (sedoheptulose 7-phosphate–D-glyceraldehyde 3-phosphate glycoaldehyde-trans-



Scheme 1. *Production of hydroxamic acids from α-hydroxyethylthiamin and nitrosoaromatic compounds*
I, Nitrosobenzene; II, α-hydroxyethylthiamin; III, N-phenylacetohydroxamic acid.

ferase, EC 2.2.1.1). As predicted from Breslow's (1958) proposed mechanism for thiamin-catalysed reactions, we found that transketolase produces the glycollic acid derivative (VI, Scheme 2) of phenylhydroxylamine.

Materials and Methods

Yeast transketolase, D-xylulose 5-phosphate, D-ribose 5-phosphate and thiamin pyrophosphate were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Nitrosobenzene (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) was purified by repeated recrystallization from ethanol. *N*-Phenylglycolhydroxamic acid (VI) was prepared by the reaction of phenylhydroxylamine with glycollic acid and dicyclohexylcarbodi-imide (Smissman *et al.*, 1972). The u.v., i.r. and n.m.r. (nuclear-magnetic-resonance) spectra of the product were consistent with structure (VI).

Incubation procedure

All incubations were conducted in triplicate at 30°C in a heated water bath. Incubation mixtures were prepared by adding 5.0ml of 0.05M-Tris/HCl buffer, pH7.6, containing 479μg (1.0μmol) of thiamin pyrophosphate, 3.7mg (15μmol) of $MgSO_4$,-$7H_2O$, 5.0mg (13.9μmol) of D-xylulose 5-phosphate (70% purity) and the amounts of D-ribose 5-phosphate indicated in Table 2. To each mixture was then added 5.0ml of 0.05M-Tris buffer containing 5 units of yeast transketolase. A unit of transketolase produces 1μmol of glyceraldehyde 3-phosphate/min from D-xylulose 5-phosphate (in the presence of D-ribose 5-phosphate) at pH7.7. Enzyme activity was determined as described by Saitou *et al.* (1974). The addition of enzyme was followed immediately by the indicated amount of nitrosobenzene (Table 2) as a 20mg/ml solution in ethanol. An appropriate quantity of ethanol was added to each incubation so that each received a total of 200μl of ethanol. A control was prepared containing heat-denatured transketolase in an incubation otherwise corresponding to no. 4 (Table 2). Single incubations were also prepared as for no. 4, but containing either phenylhydroxylamine or nitrobenzene in place of nitrosobenzene. Incubations were conducted for 60min, then the tubes were transferred to a −5°C ice/salt bath. Each incubation was extracted three times with 10ml of ethyl acetate, and the combined ethyl acetate extracts were dried over Na_2SO_4 and evaporated. The residues were dissolved in 1.0ml of methanol, treated with 3.0ml of 1.0% (w/v) $FeCl_3$ in methanol containing 0.12M-HCl and the A_{530} was determined immediately with a Beckman model 24 spectrophotometer. Pure synthetic *N*-phenylglycolhydroxamic acid was used to prepare a standard absorption curve, which was linear with respect to concentration for freshly prepared complexes. No hydroxamic acid was detected in the control, phenylhydroxylamine or nitrobenzene incubations.

Identification of products

Analysis was performed by t.l.c. on each of the methanolic solutions obtained from the first incubation series before the addition of $FeCl_3$ reagent. Samples (5μl) were spotted on commercial silica-gel plates (EM Silplate F-52, Brinkmann, Westbury, NY, U.S.A.) and developed in methanol/chloroform (1:19, v/v). Spots on the chromatograms were located with u.v. light, and with 1% (w/v) $FeCl_3$ in methanol sprayed on the lower half of the chromatogram to detect hydroxamic acids and 1% (w/v) pentacyanoamineferroate sprayed on the upper half of the chromatogram to detect phenylhydroxylamine and nitrosobenzene (Radomski & Brill, 1973). More extensive t.l.c. analysis was conducted on earlier incubation mixtures to conclusively identify the hydroxamic acid produced in the incubations. Authentic *N*-phenylacetohydroxamic acid (III, Scheme 1), *N*-phenylglycolhydroxamic acid (VI, Scheme 2) and azoxybenzene were co-chromatographed with the incubation products in a series of solvents that readily separated these marker compounds.

Four solvent systems were used (see Table 1). The R_F values for each of the authentic marker compounds in these solvent systems are also shown in Table 1.

Results

Analysis of the products resulting from the incubations of nitrosobenzene with transketolase clearly demonstrated the presence of a hydroxamic acid product, as indicated by the violet colour produced with $FeCl_3$ reagent. Examination of this product by means of extensive t.l.c. analysis proved that it was *N*-phenylglycolhydroxamic acid (VI). Each incubation with active transketolase and nitrosobenzene showed that this hydroxamic acid was present.

Table 1. *R_F values for authentic compounds in four solvent systems*

Key to solvent systems: 1, methanol/chloroform (1:19, v/v); 2, acetonitrile/chloroform (1:9, v/v); benzene/ethyl acetate (1:1, v/v); 4, butan-2-one/benzene (1:1, v/v).

	R_F			
Solvent system ...	1	2	3	4
N-Phenylacetohydroxamic acid (III)	0.31	0.15	0.25	0.38
N-Phenylglycolhydroxamic acid (VI)	0.18	0.07	0.15	0.27
Azoxybenzene	0.60	0.67	0.58	0.60

Similar incubations with boiled enzyme failed to produce the hydroxamic acid, suggesting that the acid is produced by an enzyme-catalysed reaction. In addition, incubations with nitrobenzene and phenylhydroxylamine did not yield the hydroxamic acid in detectable amounts. No unchanged nitrosobenzene was detected in any incubation except the control. The production of azoxybenzene was noted in all incubations, including the control. As evidenced by t.l.c. analysis, the amount of azoxybenzene production increased disproportionately with an increase in the amount of nitrosobenzene used in the incubation.

A series of incubations containing various amounts of nitrosobenzene showed that the total yield of hydroxamic acid (VI) increased as the nitrosobenzene concentration was increased (Table 2); however, the percentage conversion of nitrosobenzene into compound (VI) decreased as the concentration of nitrosobenzene increased. The maximum observed conversion into compound (VI) was 56%, which occurred at the lowest nitrosobenzene concentration in the absence of added ribose 5-phosphate. The minimum conversion into compound (VI) of 14% was observed when the amounts of nitrosobenzene and D-ribose 5-phosphate were highest.

Fig. 1 relates the percentage conversion of D-xylulose 5-phosphate into the hydroxamic acid (VI). D-Xylulose 5-phosphate is the limiting reagent for most of the incubations (Table 2). An expected increase in the incorporation of xylulose into compound (VI) was observed as the nitrosobenzene concentration increased. The effect of added D-ribose 5-phosphate on hydroxamic acid production is indicated in curves (*b*) and (*c*) of Fig. 1. The decreased production of compound (VI) in the presence of D-ribose 5-phosphate was expected.

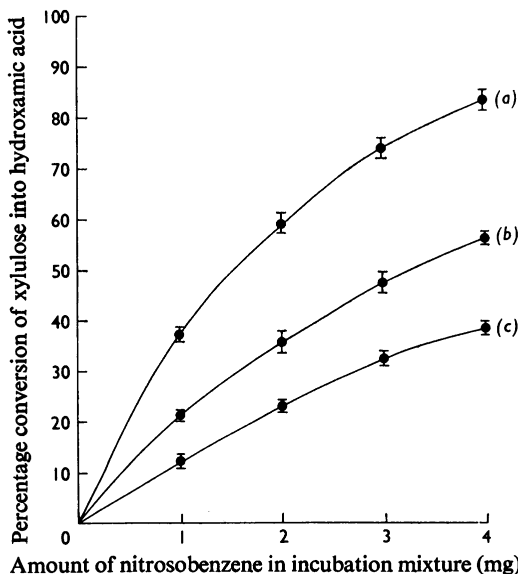


Fig. 1. *Percentage conversion of* D-*xylulose 5-phosphate into hydroxamic acid*

(*a*) In the absence of added D-ribose 5-phosphate; (*b*) in the presence of 9.1 μmol of D-ribose 5-phosphate; (*c*) in the presence of 18.2 μmol of D-ribose 5-phosphate. The conditions of incubation are described in the Materials and Methods section under 'Incubation procedure'.

Table 2. *Composition of incubations and hydroxamic acid production*

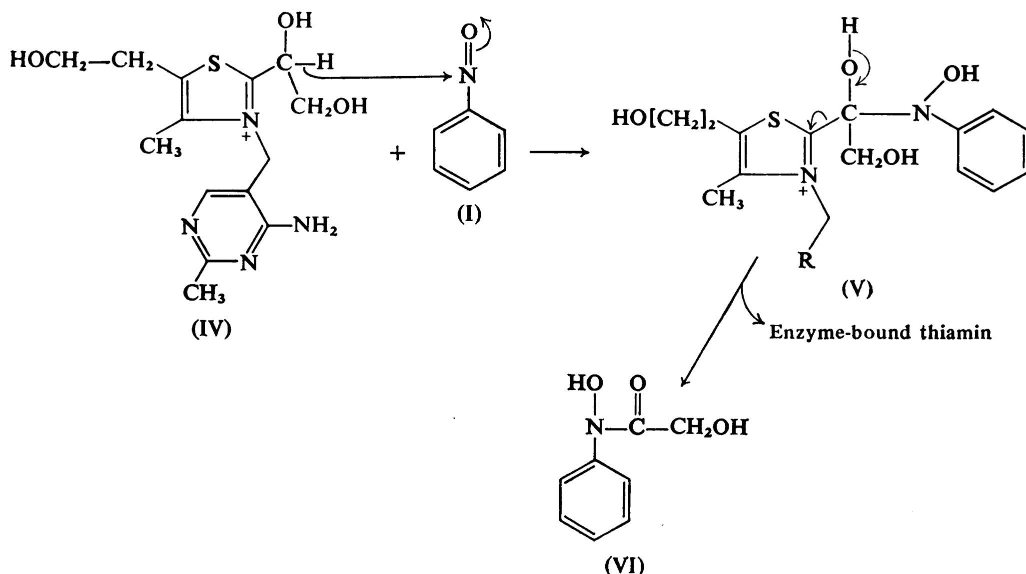
Each incubation also contained 13.9 μmol of D-xylulose 5-phosphate, 1.0 μmol of thiamin pyrophosphate and 15 μmol of $MgSO_4$, in a total volume of 10.0 ml of 0.05 M-Tris/HCl buffer, pH 7.6.

Incubation no.	Nitrosobenzene added (μmol)	D-Ribose 5-phosphate added (μmol)	Total hydroxamic acid produced* (mg)
1	9.3	0	0.87 ± 0.04
2	18.7	0	1.34 ± 0.04
3	28.0	0	1.69 ± 0.04
4	37.4	0	1.88 ± 0.05
5	9.3	9.1	0.50 ± 0.02
6	18.7	9.1	0.81 ± 0.03
7	28.0	9.1	1.09 ± 0.04
8	37.4	9.1	1.30 ± 0.03
9	9.3	18.2	0.28 ± 0.03
10	18.7	18.2	0.52 ± 0.02
11	28.0	18.2	0.74 ± 0.02
12	37.4	18.2	0.87 ± 0.02

* Average of three determinations ± S.D.

We propose that the mechanism for the formation of compound (VI) is that presented in Scheme 2. The 'active glycolaldehyde' (IV), which results from a C_2 transfer from D-xylulose 5-phosphate to transketolase-bound thiamin, condenses by nucleophilic attack on to nitrosobenzene (I) to produce the intermediate (V). This intermediate then decomposes to produce the hydroxamic acid (VI) and enzyme-bound thiamin. Such a mechanism is consistent both with Breslow's mechanisms for thiamin activity (Breslow, 1958; Ullrich *et al.*, 1970) and with the known chemistry of the nitroso group (Smith, 1966; Feuer, 1969).

The experimental design in the present study of transketolase was such that the expected reaction had gone to completion at the time of analysis. Thus the total amount of hydroxamic acid (VI) produced in each incubation is the maximum possible under the given conditions. No kinetic data for the production of compound (VI) could be derived, owing to the



Scheme 2. *Proposed mechanism for the production of glycollic acid-derived hydroxamic acids by transketolase*
I, Nitrosobenzene; IV, 'active glycolaldehyde'; V, 'intermediate' compound; VI, hydroxamic acid.

presence of nitrosobenzene in the early part of the incubation period, and the competing non-enzymic conversion of nitrosobenzene into azoxybenzene.

Curves (*b*) and (*c*) in Fig. 1 illustrate the inhibitory effect of D-ribose 5-phosphate on the production of hydroxamic acid (VI). Such an effect is to be expected, since D-ribose 5-phosphate is the normal acceptor for the 'active glycolaldehyde' of transketolase (Ullrich *et al.*, 1970). The most significant finding of the present study is that nitrosobenzene can effectively compete with D-ribose 5-phosphate for 'active glycolaldehyde'. Incubations 5 and 10 (Table 2) were conducted with nearly equimolar amounts of nitrosobenzene and D-ribose 5-phosphate, yet 23% of the available D-xylulose 5-phosphate was incorporated into the hydroxamic acid (Fig. 1). This successful competition by nitrosobenzene is a prerequisite if any significance is to be given to this reaction under conditions *in vivo*.

The non-linear increase in total hydroxamic acid production as the nitrosobenzene concentration was increased, along with the decreased percentage conversion of nitrosobenzene into hydroxamic acid at higher concentrations of nitrosobenzene, is the result of competing non-enzymic reactions of nitrosobenzene. Most evident in the present study was the production of azoxybenzene, a well-documented reaction of nitrosobenzene (Smith, 1966; Feuer, 1969; Knight & Saville, 1973). Azoxybenzene production is facilitated in the presence of protein thiol groups, by which some of the nitroso compound is reduced to the hydroxylamine stage (Kadlubar *et al.*, 1976). This interaction of nitrosobenzene with thiol groups, and the possibility of covalent binding of nitrosobenzene to protein (King *et al.*, 1976), are further complications to any attempted kinetic analysis of the present enzymic reaction.

An estimate cannot yet be made of the extent to which transketolase and other thiamin-dependent enzymes might contribute to the conversion of aromatic nitrogenous compounds into proximate carcinogenic hydroxamic acids. Further, nothing is known about the toxicological properties of glycollic acid-derived hydroxamic acids, such as compound (VI). The results of the present study on purified transketolase strongly indicate that this process might occur *in vivo*, and that glycollic acid-derived hydroxamic acids are possible metabolites of aromatic nitrogenous compounds.

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