BIOORGANIC CHEMISTRY 17, 13–27 (1989)

Investigation of Lysine: N-Hydroxylation in the Biosynthesis of the Siderophore Aerobactin

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Received December 14, 1987

N-Ethylmaleimide was found to inhibit both pyruvate oxidation and lysine: N^6-hydroxylation catalyzed by an enzyme system derived from *Aerobacter aerogenes* 62-1. Studies of pyruvate utilization in the presence and absence of lysine indicated a 1:1 stoichiometric relationship between consumption of pyruvate and production of N^6-hydroxylation. Similar studies with an *Escherichia coli* mutant enzyme system revealed a 2:1 stoichiometric relationship between the above-mentioned processes. The formation of a nitrone derivative by interaction of N^6-hydroxylysine with pyruvate has been suggested to provide the basis for the consumption of an additional mole of pyruvate in the *E. coli* enzyme system. Phenylhydrazine and 6-diazo-5-oxo-L-norleucine, the former after standing for appropriate periods, have been found to inhibit lysine: N^6-hydroxylation reaction. The implications of these observations to the mechanism(s) operative in lysine: N^6-hydroxylation are discussed.

INTRODUCTION

Aerobactin, a dihydroxamate siderophore produced by *Aerobacter aerogenes* 62-1 (*1*) and *Escherichia coli* (*2*) when grown under conditions of iron deprivation, contains N^6-acetyl N^6-hydroxylysine residues linked to the primary carboxyls of citric acid (Fig. 1). The biosynthetic pathway of this siderophore has been shown to involve three steps: (i) oxidation of lysine to its N^6-hydroxy derivative; (ii) acetylation of N^6-hydroxylysine leading to the formation of N^6-acetyl N^6-hydroxylysine; and (iii) condensation of N^6-acetyl N^6-hydroxylysine residues with the two distal carboxylic acid functions of citric acid. Studies with a cell-free system of *Aerobacter aerogenes* 62-1 have shown that the enzymes catalyzing the initial two reactions are located in the membrane component (P2) while those involved in the peptide bond formation reactions are present in the supernatant (S2) component (*3*). Recently, the genetic map of aerobactin operon in *E. coli* has been elucidated and mutants capable of performing partial reactions in the aerobactin biosynthetic pathway have been isolated (*4–8*). Previous studies in our laboratory had demonstrated the ability of pyruvate to stimulate P2-catalyzed N^6-hydroxylation of lysine (*9*). The presence, in P2, of enzymes capable of pyruvate oxidation has also been documented (*10*). Furthermore, the following observations, (i) concurrent abolition of P2 associated lysine: N^6-hydroxylation and pyru-

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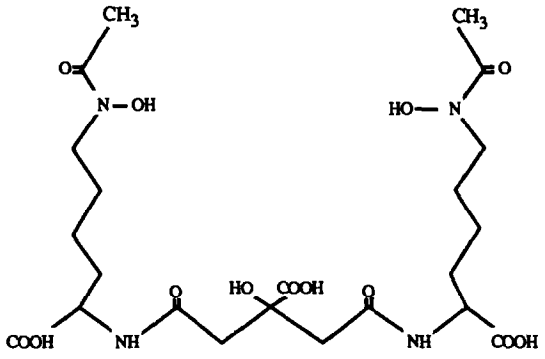


FIG. 1. Structure of aerobactin.

vate oxidation activities by inhibitors of thiamine pyrophosphate (TPP)[1] dependent enzymes; and (ii) ability of pyruvate to serve as a source of acetyl moiety in the P2-mediated conversion of N^6-hydroxylysine to its N^6-acetyl derivative, have been considered as evidence for the indispensability of pyruvate oxidation to the process of lysine: N^6-hydroxylation (*11*). The above-mentioned findings suggest that pyruvate not only serves as a precursor of the acetyl moiety but also as a source of reducing equivalents for the N^6-hydroxylation reaction. An essential element required for establishing unequivocally the involvement of pyruvate in the above mentioned processes concerns the stoichiometric relationship between pyruvate consumption and N^6-hydroxylysine production. Current studies address this particular aspect and the results obtained have provided additional insights into this stoichiometry as well as into enzymes operative in pyruvate oxidation. Lysine: N^6-hydroxylation is susceptible to inhibition by appropriately aged preparations of phenylhydrazine and by L-6-diazo-5-oxonorleucine (DON). These and other observations presented in this report form the basis for a tentative proposal concerning the mechanism operative in the transformation of lysine to its N^6-acetyl N^6-hydroxy derivative.

EXPERIMENTAL PROCEDURES

Materials. D- and L-6-diazo-5-oxo-norleucine, L-azaserine, 2-methyl-1,2-di-3-pyridyl-1-propanone (metyrapone), and *N*-ethylmaleimide (NEM) were purchased from Sigma Chemical Co. (St. Louis, MO). Deuterium oxide was obtained from MSD Isotopes (Montreal, Canada). Hydroxylamine hydrochloride and *N*-methylhydroxylamine hydrochloride were products of Aldrich Chemical Co. (Mil-

[1] Abbreviations used: TPP, thiamine pyrophosphate; DON, L-6-diazo-5-oxo-norleucine; NEM, *N*-ethylmaleimide.

waukee, WI). Phenylhydrazine, free base or the hydrochloride, analytical reagent grade, was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ). Ethanolic solution (1 M) or aqueous solution (100 mM) of phenylhydrazine or its hydrochloride salt, respectively, was allowed to stand in stoppered amber-colored bottles prior to its use in the experiments.

Enzyme preparation. Cell-free extract of *A. aerogenes* 62-1 was obtained by a procedure previously described (*9*). P2 component, which served as the source of enzyme in these studies, was suspended in a solution of phosphate buffer (10 mM, pH 7.0) containing glutamine. Following centrifugation (12,000 *g*, 10 min) the pellet was resuspended in the above-mentioned buffer.

Cell-free extract of mutant *E. coli* was obtained by procedures identical to those used in the case of *A. aerogenes* except for the omission of $MgSO_4$ from the lysis medium. P2 component was washed and resuspended in phosphate buffer (10 mM, pH 7.0) containing glutamine (1 mM) as described above.

Freezing treatment. An aliquot of P2 (1.10 ml) was placed into each Eppendorf tube and capped. The tube was immersed into liquid nitrogen for the appropriate time period. The frozen content was thawed at room temperature and immediately placed into a 4°C ice bath. P2 preparations subjected to similar conditions of treatment except for freezing served as controls. Aliquots (1.0 ml) of such treated P2 were used in the assays for pyruvate oxidation and lysine: N^6-hydroxylation.

Assay for enzymatic activities. Lysine: N^6-hydroxylase activity was determined using assay conditions and analytical methods involving iodine oxidation of the product by procedures previously described (*9*). In inhibition experiments, the desired compound, except in the case of phenylhydrazine, was included in the assay at the concentrations indicated. In the case of phenylhydrazine, enzyme (P2) preparation was preincubated at the desired concentration of the compound for 1–2 h. Prior to the determination of enzymatic activity, P2 preparation was centrifuged and the pellet was resuspended in phosphate buffer (50 mM, pH 7.0) containing glutamine (1 mM). The suspension was centrifuged (12,000 *g*) and the pellet was subjected to two more successive washings by suspending in the buffer and centrifugation as described above. Such extensive washing of P2 preparation was essential to ensure complete removal of phenylhydrazine which, because of its susceptibility to oxidation by iodine, would interfere in the determination of N^6-hydroxylysine. The corresponding controls were handled similarly.

Pyruvate determination. Quantitative determination of pyruvate was achieved by HPLC procedures (*10*).

^{13}C NMR spectroscopy. Carbon-13 nuclear magnetic resonance spectra were obtained on a Bruker AM250 spectrometer at 62.5 MHz. All spectra were broadband decoupled and were obtained at room temperature. Samples were prepared by reacting equimolar amounts of pyruvate (100 mg) and hydroxylamine hydrochloride (63.2 mg) or *N*-methylhydroxylamine hydrochloride (75.9 mg) in D_2O (2.0 ml). The reaction was performed in 10-ml capped vials and at 37°C for 30 min. Pyruvate (100 mg) or *N*-methylhydroxylamine hydrochloride (75.9 mg) was dissolved in D_2O (2.0 ml) and was used as standard.

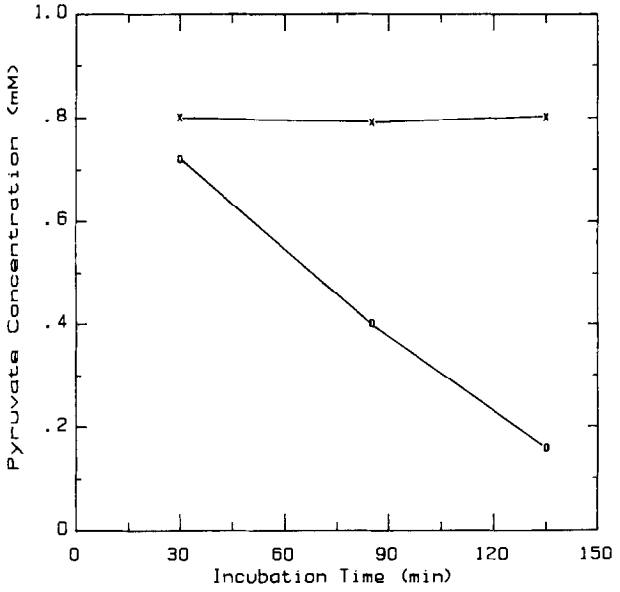


FIG. 2. Influence of *N*-ethylmaleimide on P2-mediated pyruvate oxidation. The ability of P2 preparation to catalyze the oxidation of pyruvate was determined in the absence and presence (0.2 mM) of *N*-ethylmaleimide by HPLC procedure (see text for details). Absence of *N*-ethylmaleimide, O; presence of *N*-ethylmaleimide, X. In the presence of *N*-ethylmaleimide, the ability of P2 to catalyze N^6-hydroxylation of lysine was completely inhibited.

RESULTS

Influence of NEM

Treatment with NEM (0.2 mM) resulted in a total abolition of both lysine : N^6-hydroxylating activity and pyruvate oxidizing activity present in P2 preparations (Fig. 2). These observations are analogous to those recorded in experiments with inhibitors of thiamine pyrophosphate dependent enzymes (*11*). The inactivation of *E. coli* pyruvate oxidase by NEM has been reported (*12*).

Stoichiometry of Pyruvate Utilization and Lysine : N^6-Hydroxylation

Initial attempts in the determination of this stoichiometry pertained to an assessment of pyruvate consumption during P2 mediated lysine : N^6-hydroxylation. Assay mixtures containing the enzyme (P2 preparation of *A. aerogenes* 62-1), pyruvate, and lysine were incubated for desired intervals and pyruvate consumption and formation of N^6-hydroxylysine were determined by HPLC (*10*) and I_2 oxidation (*9*) procedures, respectively. Examination of the data indicated a stoichiometry value (moles of pyruvate consumed per mole N^6-hydroxylysine formed) of 6 or higher depending on the duration of incubation. The value higher than 6 was usually observed in experiments involving incubation periods of 30 min

or longer. When assays were performed in the presence or absence of lysine, a greater consumption of pyruvate was noted in the former instance than that recorded in the latter. The difference in the amount of pyruvate consumed in the two types of experiments indicated a stoichiometry of approximately 1 mol of pyruvate consumed (actual value, 1.3 mol) per mole of N^6-acetyl-N^6-hydroxylysine formed (Table 1). In order to determine unambiguously the involvement of pyruvate in lysine: N^6-hydroxylation, studies were performed with enzyme preparation from an *E. coli* mutant which, in view of the defect and/or deletion of genetic elements in the aerobactin operon (*4–8*), is capable of performing only the initial step in aerobactin biosynthesis, namely, N^6-hydroxylation of lysine. Pyruvate was found to stimulate lysine: N^6-hydroxylation catalyzed by the P2 component of the cell-free system of this *E. coli* mutant. In the determination of the stoichiometric relationship between pyruvate consumption and lysine: N^6-hydroxylation, advantage was taken of the lability of *N*-hydroxylase activity to freezing (*9*). The ability of native (not subjected to freezing) and frozen (as described under methods) P2 preparations obtained from the cell-free system of *E. coli* mutant to catalyze pyruvate oxidation and N^6-hydroxylation of lysine was determined. Native P2 preparations were found to catalyze both the reactions. Freezing of P2 preparations by exposure to liquid N_2 for 1 min resulted in an almost complete loss of the ability to catalyze lysine: N^6-hydroxylation. The small amount of N^6-hydroxylysine observed in the assay mixture containing frozen P2 would appear to be a reflection of endogenous *N*-hydroxy amino acid associated with the preparation. In contrast, such frozen P2 preparations were still capable of pyruvate oxidation;

TABLE 1

Stoichiometry between Pyruvate Utilization and N^6-Hydroxylysine Production

Source of P2	Treatment		Pyruvate consumed[a] (μM)	N^6-Hydroxylysine formed (μM)	Pyruvate / N^6-hydroxylysine
A. aerogenes[b]	Control	(+1 mM Lysine)	790.6	28.17	1.3
		(no lysine)	760.1	5.56	
E. coli mutant[c]	Control	(0 min)	244.4	40.22	2.6
	Liq-N_2	(1 min)	147.6	3.51	
	Liq-N_2	(3 min)	158.2	3.88	2.4
	Liq-N_2	(5 min)	156.0	3.65	2.4
	Liq-N_2	(10 min)	166.3	3.65	2.1

[a] The possibility of lysine reacting with pyruvate under the experimental conditions would appear unlikely since the latter compound was quantitatively recoverable from assay mixtures treated with inhibitors of pyruvate oxidation.

[b] P2 preparations were incubated with pyruvate (1 mM) in the presence or absence of lysine for 15 min at 37°C.

[c] P2 preparations, control or exposed to freezing for durations shown, were incubated with pyruvate (1 mM) and lysine (1 mM) for 35 min at 37°C. In both experiments, pyruvate consumption and N^6-hydroxylysine production were monitored by HPLC and iodine oxidation procedures, respectively (see text).

however, under identical experimental conditions, the amount of pyruvate consumed was lower than that observed in experiments containing native P2 preparation. Prolonged freezing (exposure to liquid N_2 for 10 min) did not lead to any further significant reduction in pyruvate utilization than that noted in preparations exposed to freezing for one minute. An assessment of pyruvate utilized during lysine : N^6-hydroxylation can be made on the assumption that the excess of pyruvate consumed in an assay mixture containing native P2 over that utilized in an incubation mixture with frozen P2 would reflect the amount of pyruvate used in the process of lysine : N^6-hydroxylation since purified pyruvate oxidase is freeze-insensitive and can be stored at −20°C for several months without loss of activity (*13*). Such calculations revealed a stoichiometry of approximately 2 mol (actual value, 2.4) of pyruvate consumed per mole of N^6-hydroxylysine production. These results are presented in Table 1.

As mentioned above, in experiments with the *A. aerogenes* enzyme system which contains catalytic activities for both lysine : N^6-hydroxylation and acetylation of N^6-hydroxylysine (*3*), a 1 : 1 stoichiometry was observed between pyruvate utilization and N-hydroxylation process (or formation of N^6-acetyl N^6-hydroxylysine since previous studies (*11*) have shown that incubation of lysine and pyruvate with P2 leads to the formation of this product). In contrast, experiments with mutant, the *E. coli* enzyme preparation (shown to possess lysine : N^6-hydroxylase activity but lacking in acetyl transferase activity) indicate a 2 : 1 stoichiometry with respect to pyruvate consumption and N^6-hydroxylysine production. A possible explanation for this paradoxical situation could be that N^6-hydroxylysine (in the absence of the enzyme for its conversion to its N^6-acetyl derivative) may form an adduct with pyruvate present in the assay mixture. This possibility was explored by a ^{13}C NMR study of reactions of model compounds, NH_2OH and CH_3NHOH, with pyruvate. Results are shown in Figs. 3A and 3B. Figure 3A shows the ^{13}C NMR spectra of CH_3NHOH (upper trace) and pyruvate (lower trace). The spectrum of CH_3NHOH essentially shows a single peak (δ = 28 ppm) arising from the carbon atom of the methyl group. Four ^{13}C resonance peaks were observed for pyruvate. In aqueous solution, pyruvic acid exists as a mixture of four species: the hydrated and nonhydrated undissociated acids and anions. Equilibration among the four species is fast on the NMR time scale relative to the ^{13}C chemical shifts. The ^{13}C chemical shift is pH dependent and with increasing pH, the shift of the hydration equilibrium is toward the keto form (*14*). Fischer *et al.* (*14*) have demonstrated that the ^{13}C chemical shift for the methyl groups of pyruvate and 2,2-dihydroxypropanoate, under acidic condition, differs by about 1 ppm. These findings have enabled us to unambiguously assign the peak at δ = 16 ppm to the methyl group of the equilibrating species, 2,2-dihydroxypropanate. The remaining three peaks strictly arise from the keto form of pyruvate and are assigned as follows; methyl group (δ = 17.6 ppm), α-keto-carbon (δ = 196 ppm) and carboxylate carbon (δ = 161 ppm). The weak resonance peaks of the α- and the carboxylate carbon of 2,2-dihydroxypropanoic acid are not observed in the spectrum due to relaxation time differences (as compared to its methyl carbon) and are lost as noise of the base line. NH_2OH reacts with pyruvate to generate solely the oximine adduct, antipyruvate-2-oxime (Fig. 3B, upper trace), corre-

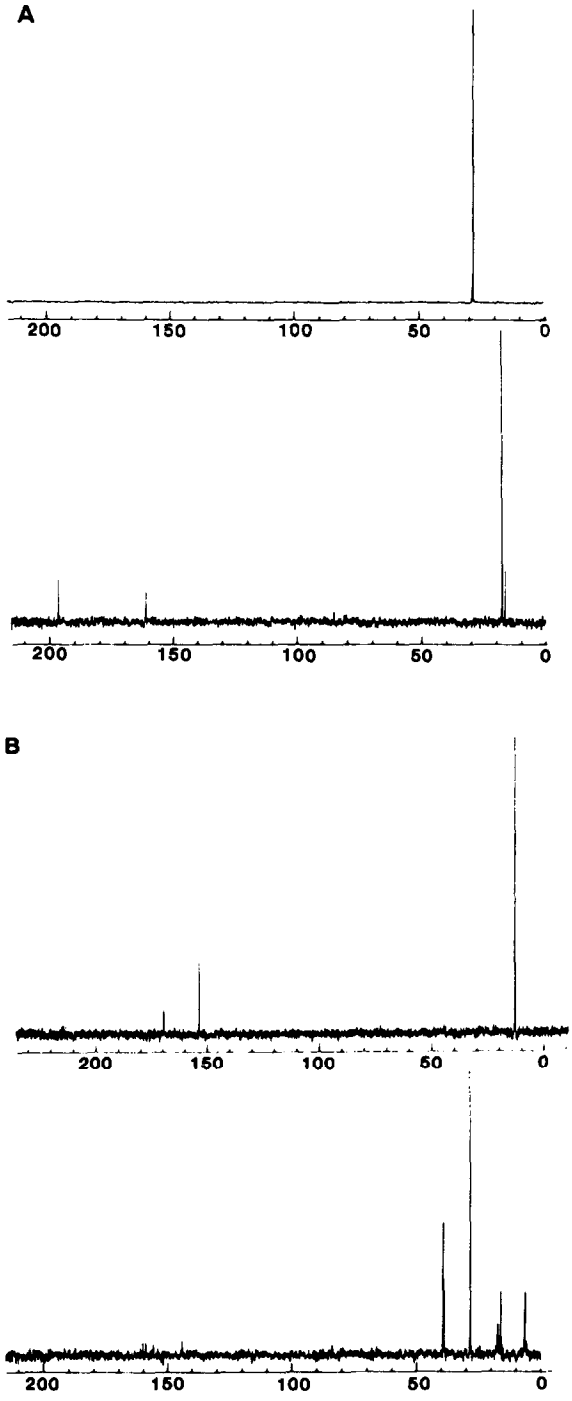
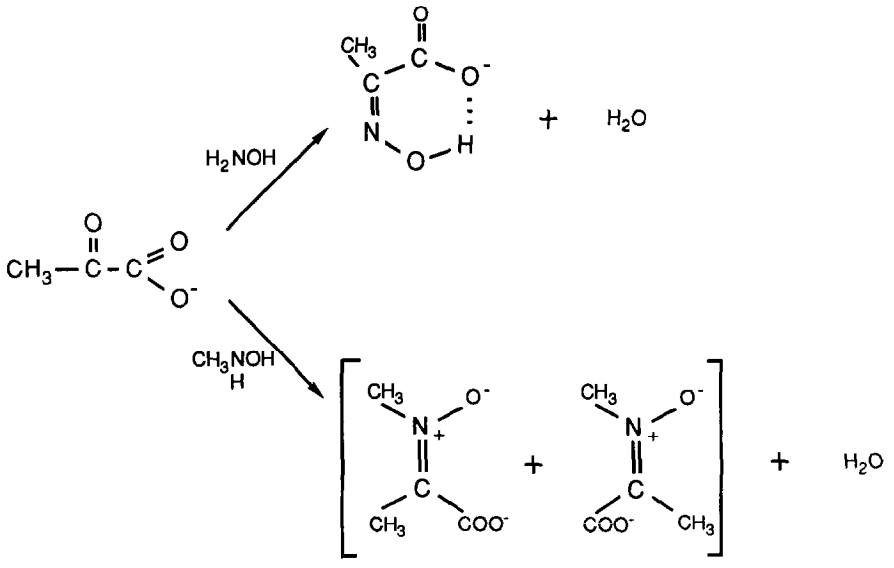


FIG. 3. ^{13}C NMR of pyruvate, *N*-methylhydroxylamine, and reaction products. (A) Top, *N*-methylhydroxylamine. Bottom, pyruvate. (B) Top, pyruvate–NH_2OH; Bottom, pyruvate–*N*-methylhydroxylamine adduct. See text for further explanation.

sponding to the following signals: carboxylate carbon (δ = 169 ppm), imino carbon (δ = 153 ppm), and methyl carbon (δ = 12.5 ppm). The preference for the formation of the anti-oximine isomer is due to the stabilization effect achieved by hydrogen bonding between the electronegative oxygen atom of the carboxylate group and the H-atom of the hydroxyl group. On the other hand, CH_3NHOH reacts with pyruvate to generate both the syn and anti-nitrone isomers (Scheme 1). Furthermore, each of the isomers can undergo dimerization readily (*15*). These phenomena are reflected in the complexity of the ^{13}C NMR spectrum as shown in Fig. 3B (lower trace). However, the most significant feature is the disappearance of the α-carbon peak (δ = 196 ppm) of pyruvate upon interaction with either NH_2OH or CH_3NHOH. Thus, these observations indicated that of the 2 mol of pyruvate consumed during the *E. coli* enzyme-mediated lysine : N^6-hydroxylation, one would appear to be involved in the N-hydroxylation process while the other is utilized in the formation of an adduct with the *N*-hydroxy amino acid.

Affinity for Pyruvate

Previous studies on the enzyme system from *A. aerogenes* had indicated a K_m value of approximately 100 μM for pyruvate (*9*). This determination was based on the ability of pyruvate to stimulate P2-mediated lysine : N^6-hydroxylation. Consequently, experimental design involved an assessment of the initial rates of lysine : N^6-hydroxylation at a fixed concentration of lysine (1 mM) over a wide range of pyruvate concentration. Thus the K_m value of 100 μM for pyruvate was based on the quantitation of N^6-hydroxylysine formed in the reaction. In the current study, the K_m for pyruvate was assessed from initial rates of its disappearance (as



SCHEME 1. Reaction of hydroxylamine and *N*-methylhydroxylamine with pyruvate.

a consequence of its oxidation to acetate) at various concentrations. The double reciprocal plot (*16*) of the data yielded a K_m value for pyruvate of 400 μM or greater.

Effect of Phenylhydrazine, DON, and Other Compounds on Lysine : N^6-Hydroxylation

The influence of phenylhydrazine on both P2 (from *A. aerogenes*)-mediated lysine N^6-hydroxylation and pyruvate oxidation was examined since this compound has been shown to effect inactivation of trimethylamine oxidase by covalent modification of its flavin cofactor (*17*). The effect of phenylhydrazine on P2-mediated reactions was found to be strongly influenced by the age of the preparation. Freshly prepared solutions of phenylhydrazine were found to have no effect on either the N^6-hydroxylation or pyruvate oxidation mediated by P2. However, upon standing at room temperature, the same solutions of phenylhydrazine gained the ability to serve as a potent inhibitor of N^6-hydroxylation. Maximum inhibitory potency was achieved after standing for 24–48 h. Further storage, however, resulted in diminution of the ability to inhibit the N^6-hydroxylation reaction mediated by P2. Thus after 6 days of standing, the solution of phenylhydrazine was virtually devoid of any ability to inactivate P2-catalyzed N^6-hydroxylation reactions, while appropriately aged (standing for 24–48 h) phenylhydrazine solution caused pronounced inhibition (85–90%) of P2-catalyzed N^6-hydroxylation of lysine (Table 2). Furthermore, preincubation of the enzyme (P2) preparation with phenylhydrazine (prior to introduction of substrates for analysis of enzymatic activities) was found to be essential for the demonstration of its inhibitory activity. Thus the presence of lysine during the incubation of enzyme with phenylhydrazine appeared to lower the extent of inhibition exerted by the compound (Table 3). The inhibition of the enzyme was found to be progressive with the period of incubation with phenylhydrazine (Fig. 4). While phenylhydrazine exerted marked inhibition of lysine : N^6-hydroxylase, its influence on pyruvate oxidation was considerably less as indicated by the retention of approximately 85–90% of original activity. L-DON, but not the D-isomer, served as a potent irreversible inhibitor of lysine N-hydroxylation but not of pyruvate oxidation catalyzed by P2. Studies performed with varying concentrations of lysine at several fixed concentrations of DON revealed the inhibition exerted by the compound to be uncompetitive (data not shown). In keeping with this observation lysine failed to protect the enzyme from L-DON-induced inhibition, thus excluding the possibility of L-DON serving as a substrate analog. Furthermore, glutamine, which has been shown to serve as an allosteric activator of the lysine : N^6-hydroxylation reaction catalyzed by the enzyme, also failed to overcome the inhibition exerted by L-DON. Hence it would appear that adverse effects produced by L-DON are a consequence of its interaction at an enzyme site other than those involved in the binding of lysine and glutamine.

The involvement of either a cytochrome *P*-450 system (*18*) or of a metalloprotein (e.g., Fe^{2+}) was explored by experiments described below. Pretreatment of enzyme with carbon monoxide or incubation with metyrapone, known inhibi-

TABLE 2

Inhibition of N-Hydroxylation by Phenylhydrazine and Other Compounds

Compound	Nanomoles of N^6-hydroxylysine produced per hour
None	150
Phenylhydrazine (0.5 mM)	
(a) Freshly prepared	150
(b) 2–4 days old	30–50
(c) 6 days old	140
N,*N*-dimethylhydrazine (0.5 mM)	
(a) Freshly prepared	150
(b) 2–4 days old	150
L-DON (0.25 mM)	110
L-DON (0.5 mM)	75
D-DON (0.5 mM)	140
NEM (0.2 mM)	<5
Thiourea (2.0 mM)	105
Azaserine (0.5 mM)	21
Metyrapone (0.5 mM)	135
Carbon monoxide	145
EDTA (2.0 mM)	135–140

Note. The typical assay mixture comprised P2 (1 ml), lysine (1 mM), pyruvate (1 mM), phosphate (50 mM), pH 7.0 in a final volume of 10 ml. The amounts of the desired compounds were used at the concentrations indicated and were present in the assay mixture except for phenylhydrazine. In the case of the last-mentioned compound, P2 was preincubated with phenylhydrazine for 2 h at 25°C. Following removal of phenylhydrazine by washing with buffer (see Experimental Procedures), the P2 preparation was used in the assay.

TABLE 3

Influence of Lysine on Phenylhydrazine-Induced Inactivation of Lysine: N^6-Hydroxylase

	Percentage of remaining lysine: N^6-hydroxylase activity	
No.	+Phenylhydrazine	+Phenylhydrazine + lysine
1	30	47[a] ± 9
2	30	53[b] ± 9
3	30	67[c] ± 9.5

Note. P2 preparations were incubated with 0.5 mM phenylhydrazine at 25°C for 2 h in either the presence or absence of lysine. Lysine concentrations were [a]0.25 mM; [b]2.5 mM; and [c]10 mM. Following washing of the preparation after incubation, enzymatic activities of the preparations were determined as described in Table 1.

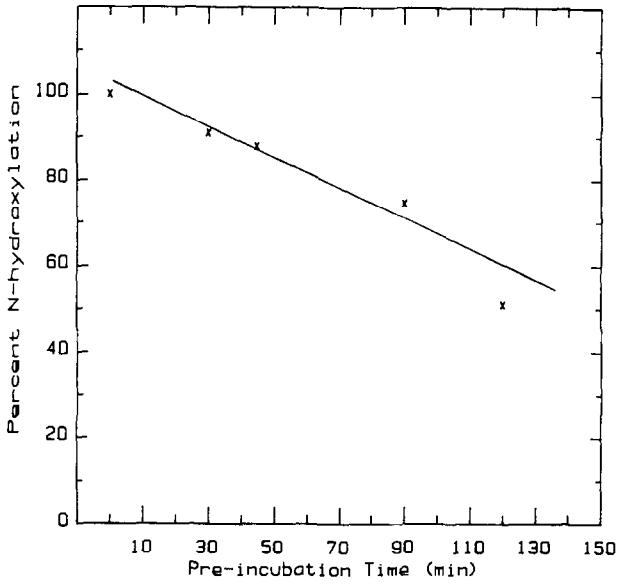


FIG. 4. Lysine: N^6-hydroxylase activity as a function of time of incubation with phenylhydrazine. P2 was incubated with 2- to 4-day-old preparation of phenylhydrazine for periods indicated. Following removal of phenylhydrazine by extensive washing, N^6-hydroxylase activity of P2 preparation was determined (see text for details).

tors of cytochrome *P*-450-mediated oxidative processes (*19*), failed to have any adverse effect on the lysine: N^6-hydroxylation catalyzed by P2 (Table 2). Likewise, incubation with EDTA (1–10 mM) in the assay had no detectable effect on the enzyme-mediated N^6-hydroxylation of lysine.

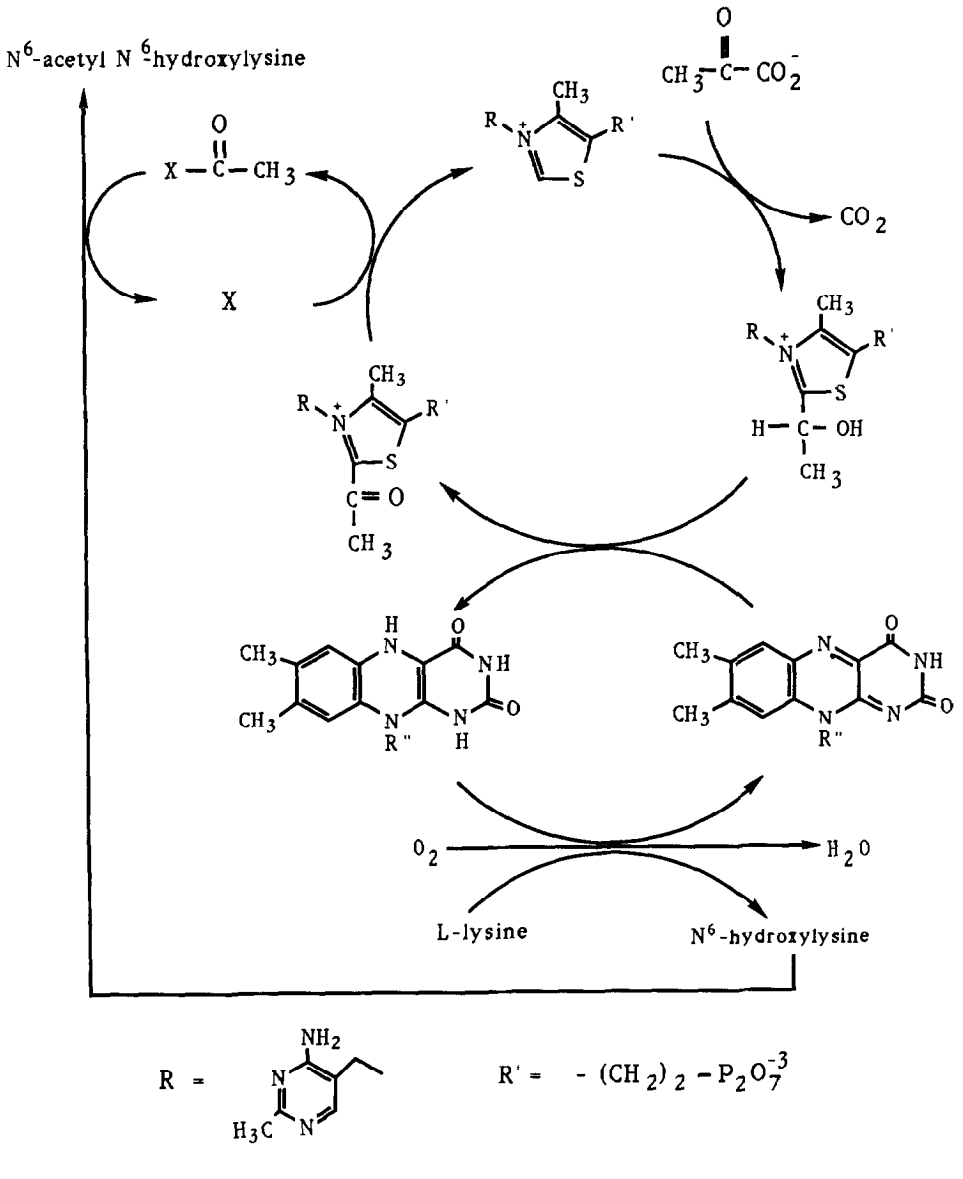
DISCUSSION

The inhibition of both P2-mediated pyruvate oxidation and lysine: N^6-hydroxylation reactions by NEM offer further support to the earlier proposals (*11*) concerning the interrelationship between the two processes. In the case of enzyme from *A. aerogenes,* studies of pyruvate consumption in the presence and absence of N-hydroxylation (achieved by inclusion and omission of lysine in the assay, respectively) suggested a stoichiometry of approximately 1 mol of pyruvate utilization per mole of N^6-acetyl-N^6-hydroxylysine produced. With the *E. coli* enzyme, such determinations were performed using P2 preparations with and without lysine: N^6-hydroxylase activity. These experiments indicated that native (unfrozen) P2 preparations which are capable of lysine: N^6-hydroxylation consumed more pyruvate than that observed in assays containing frozen P2 samples which are devoid of lysine: N^6-hydroxylase activity. Assessment of these data revealed a stoichiometry of approximately 2 mol of pyruvate utilized per mole of N^6-hydroxylysine produced by the enzyme. The cause for a twofold increase in

stoichiometry observed in the case of the *E. coli* enzyme would appear to be due to the facile reaction of the product, N^6-hydroxylysine, with pyruvate to yield the nitrone adduct. This view derives support from the studies of the interaction of hydroxylamine and methyl hydroxylamine with pyruvate. Thus, in the case of *A. aerogenes*, P2 preparations which contain both lysine : N^6-hydroxylase and acetyl transferase activities, the facile acetylation of N^6-hydroxylysine would preclude its interaction with pyruvate. With the *E. coli* mutant enzyme preparation which is devoid of acetyl transferase activity, N^6-hydroxylysine (formed by the action of lysine : N^6-hydroxylase) is channelled toward reaction with pyruvate present in the assay medium. The above-mentioned observations provide strong support for the participation of pyruvate in lysine : N^6-hydroxylation as well as in the subsequent step pertaining to the acetylation of N^6-hydroxylysine.

The second important finding of these studies concerns the demonstration of the presence of two distinct types of pyruvate oxidizing activities in both *A. aerogenes* and the *E. coli* mutant P2 preparations. Of these, one is freeze-sensitive while the other is freeze-insensitive. The former type of activity appears to be linked to lysine : N^6-hydroxylation and constitutes a minor component of the bulk pyruvate oxidizing activities present in P2. The latter, the freeze-insensitive type, could probably represent pyruvate oxidase (EC 1.2.2.2) which has been shown to be present in *E. coli* and other related organisms (*13*). The activity of the enzyme isolated from *E. coli* has been shown to be unaffected by exposure to subzero temperatures (*13*). Additional support for the presence of two distinct types of pyruvate oxidizing activities in P2 preparations pertains to the dependence of K_m value for pyruvate on analytical methods employed for its estimation. As reported earlier, determinations based on the ability to promote lysine : N^6-hydroxylation indicate a K_m value of approximately 100 μM for pyruvate (*9*). However, determinations, in the current investigations, based on direct measurement of substrate disappearance have yielded a K_m value of 400 μM for pyruvate. This latter measurement may represent a composite value derived from the action of two pyruvate oxidizing enzymes, one with low and the other with high K_m for its substrate. It is pertinent to note that *E. coli* pyruvate oxidase has been reported to have a relatively high K_m ($\approx$10 mM) for substrate (*20*) and the proton gradient generated during the reaction catalyzed by this enzyme is used by the organism to accomplish transport of solutes and/or ATP synthesis (*21*). Thus, although the freeze-insensitive pyruvate oxidase present in P2 is not directly linked to lysine : N^6-hydroxylation, the possibility of it playing an indirect role by facilitating transport of substrates cannot be excluded.

Concerning the nature of the enzyme involved in pyruvate dependent lysine : N^6-hydroxylation, the system is not inhibited by such agents as CO, metyrapone, and EDTA. These observations would appear to exclude the involvement of a cytochrome *P*-450 and metalloenzyme system in P2-mediated lysine : N^6-hydroxylation. Both DON and appropriately aged phenylhydrazine have been found to exert adverse effects primarily on P2-mediated lysine : N^6-hydroxylation. Such preferential inhibition by this process is possibly related to this particular enzyme system being a minor component of the total pyruvate oxidizing activities present in P2 preparations. In other words, these two compounds appear to have no



SCHEME 2. A hypothetical model for P2-mediated lysine: N^6-hydroxylation.

adverse influence on the pyruvate oxidase that is not directly linked to lysine: N^6-hydroxylation. Although DON exerts a strereospecific and uncompetitive type inhibition of lysine: N^6-hydroxylation, the precise mechanism of its action still remains to be elucidated.

As regards phenylhydrazine, inhibition of lysine: N^6-hydroxylation was dependent on appropriate aging of solutions of this reagent. It is well known that

hydrazines, including phenylhydrazine, can react with molecular O_2 in solution to form diazenes and H_2O_2. H_2O_2 has been shown by us not to promote or inhibit the N^6-hydroxylation process (*22*). Hence, the requirement for aging of phenylhydrazine to function as an inhibitor of P2-mediated N^6-hydroxylation would be consistent with the generation of a diazene intermediate, which could serve as the source of phenylanion capable of forming a covalent adduct at the electrophilic site C4a of oxidized flavin. In the case of trimethylamine oxidase which is susceptible to inhibition by phenylhydrazine the enzyme apparently catalyzes the formation of the reactive diazene intermediate (*17*). In the current studies, the requirement for prior formation of diazene (by aging) for achieving inhibition may be due to either the inability of the enzyme to mediate the dehydrogenation of phenyl hydrazine in view of its stringent specificity with respect to its substrates (*9*) and/or to the inaccessibility of phenylhydrazine (protonated species expected to prevail under assay conditions) to the active site of the enzyme due to permeability constraints imposed by the membrane. In contrast, preformed diazene, because of its permeability, may interact at or in the vicinity of the site of lysine : N^6-hydroxylation, a process which is dependent on pyruvate oxidation. Protection offered by lysine against phenylhydrazine-induced inactivation of the enzyme would appear to be compatible with this view. Since pyruvate oxidation coupled to lysine : N^6-hydroxylation has been shown to be a flavin dependent pyruvate oxidase (*10*), diazene bound at or near the lysine binding site may exert inhibition of the process by serving as the source of phenylanion capable of irreversible modification of the flavin cofactor. These considerations form the basis for a *tentative* model (Scheme 2) for P2-mediated conversion of lysine to its N^6-acetyl N^6-hydroxy derivative. Experiments are currently under progress to test the validity of this hypothesis.

ACKNOWLEDGMENTS

The authors express their sincere thanks to Professor J. B. Neilands, University of California, and to Professor V. Braun, Mikrobiologie, Universität Tubingen, for providing us with the *E. coli* mutants and for continued interest in the mechanistic studies pertaining to the biosynthesis of aerobactin. These studies were supported by a research grant from the Natural Sciences and Engineering Research Council of Canada.

REFERENCES

1. GIBSON, F., AND MAGARTH, D. T. (1969) *Biochim. Biophys. Acta* **192,** 175–184.
2. WILLIAMS, P. H. (1979) *Infect. Immunol.* **26,** 925–932.
3. VISWANATHA, T., SZCZEPAN, E. W., AND MURRAY, G. J. (1986) *in* Iron Transport (Neilands, J. B., Helm, V., and Winkelmann, Eds.), pp. 117–132, Springer-Verlag, New York.
4. GROSS, R., ENGELBRECHT, F., AND BRAUN, V. (1984) *Mol. Gen. Genet.* **196,** 74–80.
5. GROSS, R., ENGELBRECHT, F., AND BRAUN, V. (1985) *Mol. Gen. Genet.* **201,** 204–212.
6. DE LORENZO, V., AND NEILANDS, J. B. (1986) *J. Bacteriol.* **167,** 350–355.
7. BAGG, A., AND NEILANDS, J. B. (1987) *Microb. Rev.* **51,** 509–518.
8. HERRERO. M., DE LORENZO, V., AND NEILANDS, J. B. (1988) *J. Bacteriol.* **170,** 56–64.

9. PARNIAK, M. A., JACKSON, G. E. D., MURRAY, G. J., AND VISWANATHA, T. (1979) *Biochim. Biophys. Acta* **569,** 99–108.
10. SZCZEPAN, E. W., JACKSON, G. E. D., GRUNDY, B. J., AND VISWANATHA, T. (1986) *FEMS Microbiol. Lett.* **34,** 27–30.
11. SZCZEPAN, E. W., KALLER, D., HONEK, J. F., AND VISWANATHA, T. (1987) *FEBS Lett.* **211,** 239–242.
12. KOLAND, J. G., AND GENNIS, R. B. (1982) *J. Biol. Chem.* **257,** 6023–6027.
13. O'BRIEN, T. A. SCHROCK, H. L., RUSSELL, P., BLAKE R. II, AND GENNIS, R. B. (1976) *Biochim. Biophys. Acta* **452,** 13–29.
14. FISCHER, G., FLATAU, S., SCHELLENBERGER, A., AND ZSCHUNKE, A. (1988) *J. Org. Chem.* **53,** 214–216.
15. AURICH, H. G., SCHMIDT, M., AND SCHWERZEL, T. (1985) *Chem. Ber.* **118,** 1105–1114.
16. LINEWEAVER, H., AND BURK, D. (1934) *J. Amer. Chem. Soc.* **56,** 658–666.
17. NAGY, J., KENNEY, W. C., AND SINGER, T. P. (1979) *J. Biol. Chem.* **254,** 2684–2688.
18. GUNSALUS, I. C., PEDERSON, T. C., AND SLIGAR, S. G. (1975) *Annu. Rev. Biochem.* **44,** 377–407.
19. PETERSON, J. A., ULLRICH, V., AND HILDEBRANDT, A. G. (1971) *Arch. Biochem. Biophys.* **145,** 531–542.
20. WILLIAMS, F. R., AND HAGER, L. P. (1966) *Arch. Biochem. Biophys.* **116,** 168–176.
21. SHAW-GOLDSTEIN, L. A., GENNIS, R. B., AND WALSH, C. (1978) *Biochemistry* **17,** 5605–5613.
22. SZCZEPAN, E. W. (1986) Ph.D. Thesis, University of Waterloo.