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STUDIES ON THE FORMATION OF N^6-HYDROXYLYSINE IN CELL-FREE EXTRACTS OF *AEROBACTER AEROGENES* 62-1

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Summary

We have investigated conditions optimal for the conversion of L-lysine to its N^6-hydroxy derivative by partially purified cell-free extracts of *Aerobacter aerogenes* 62-1. The enzyme system was highly specific to L-lysine: the D-isomer and, the N^2- or N^6-derivatives of lysine, and α-amino acids were not hydroxylated. Most of the latter compounds had little effect on the hydroxylation of L-lysine. However, L-glutamic acid and L-glutamine enhanced the hydroxylation, with half-maximal activation achieved at 100 μM concentration of the effector. The K_m values for pyruvate and L-(+)-lactate (compounds known to stimulate *N*-hydroxylysine formation) were found to be approx. 100 μM. The data show that N-hydroxylation of the amino acid precedes acylation in the biosynthesis of hydroxamic acid in *A. aerogenes* 62-1.

Introduction

The production of a wide variety of hydroxamic acids in living systems has been well-documented [1—5]. However, despite extensive investigation, the mechanisms involved in the biosynthesis of such compounds have remained elusive.

The formation of a hydroxamic acid is believed to involve essentially two reactions: (i) generation of the hydroxylamino function, and (ii) acylation of the *N*-hydroxyamino acid. The majority of earlier investigations have been concerned with the identification of precursors and determination of the sequence of the above two reactions. In some cases, such as the biosynthesis of hadacidin [6] and ferrichrome [7], N-hydroxylation of the parent amino acid appears to precede acylation. Whereas for others, such as pulcherriminic acid [8], the

reaction sequence is reversed. Little information has been available as to the events involved in the formation of the hydroxylamino moiety of hydroxamic acids, primarily due to difficulty in obtaining active cell-free extracts from hydroxamate-producing organisms. Recently, this laboratory has provided the first demonstration of a cell-free system, from *Aeroacter aerogenes* 62-1, capable of converting L-lysine to its N^6-hydroxylamino derivative [9]. This cell-free system has been resolved into two components, particulate and supernatant, both of which appear to be required for the optimal formation of N^6-hydroxylysine. Pyruvate or L-(+)-lactate-induced stimulation of the reaction, especially the latter in the presence of lactate dehydrogenase and NAD, suggested a possible coupling of the N-hydroxylation process to pyruvate metabolism [9]. The current studies are an extension of the above investigations, performed in order to further elucidate the factors governing the formation of N^6-hydroxylysine in *A. aerogenes* 62-1.

Methods

Azaserine was obtained from Calbiochem, and L-lysine from Aldrich Chemical Company. All other biochemicals were obtained from Sigma. 2-Hydroxybutynoic acid was a generous gift from Dr. C.T. Walsh of the Massachusetts Institute of Technology (Cambridge, MA).

Preparation of enzyme. A. aerogenes 62-1 was grown and harvested as previously described [9]. The washed cells were suspended in 100 mM phosphate buffer (pH 7.0) containing 10 mM EDTA and lysozyme (1 mg/ml). The suspension was incubated at 37° C for 30 min and diluted ten-fold with a cold solution of 10 mM $MgSO_4$ containing 1.6 mM dithiothreitol, 1 mg deoxyribonuclease and 1 mg ribonuclease. This mixture was allowed to stand at room temperature for 1 h and centrifuged at 700 × *g* for 10 min. The resulting pellet (P_1), which consisted of intact cells and other debris, was discarded. The supernatant (S_1) was centrifuged at 12 000 × *g* for 10 min. The resulting clear supernatant (S_2) was retained and stored at 4° C. The pellet (P_2) was suspended in a solution of 10 mM phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 mM dithiothreitol and 30% (v/v) glycerol, and centrifuged at 12 000 × *g* for 20 min. The washed P_2 was resuspended in a volume of the same solution equal to one-half the volume of S_2, and stored at 4° C.

Assay procedure. Unless otherwise specified, the assay consisted of the following components in a 10 ml volume: 50 mM phosphate buffer (pH 7.0), 1 mM lysine, 1 mM L-(+)-lactate or potassium pyruvate, P_2 (1 ml) and S_2 (2 ml). The mixture was incubated at 37°C with vigorous shaking for 0—4 h (2 h was the standard incubation time). The reaction was terminated by the addition of 5 ml of a thick slurry of Dowex 50W-X8 (H^+, 200—400 mesh).

The resin-assay mixture was heated in a water bath at 60—70° C for 1 h, cooled to room temperature and poured into a 1.2 × 25 cm glass column. The resin was washed with 10 ml water, 40 ml 0.2 M HCl, and 20 ml 6 M HCl. The latter eluate was evaporated under reduced pressure, and the residue was dissolved in 5 ml water. The amount of N^6-hydroxylysine was estimated by subjecting aliquots of the aqueous solution to the iodine oxidation procedure [10].

The use of Dowex resin enabled the separation of the reaction product from most of the other components of the mixture, resulting in highly reproducible determinations of the hydroxylamine content by the iodine oxidation procedure. Heating the resin-assay mixture resulted in higher yields of hydroxylamine than those noted in identical unheated samples, ostensibly by deacylating any hydroxamic acid formed during the reaction.

Results

Components of the N-hydroxylation system

In all the studies reported here, an appropriate mixture of the pellet (P_2) and supernatant (S_2), obtained from cell-free extracts of *A. aerogenes* 62-1 (see Methods), served as the source of enzyme. No appreciable N^6-hydroxylysine formation could be observed when P_2 was omitted from the assay. S_2 was devoid of enzymatic activity, but its inclusion in the assay resulted in a 2—4-fold enhancement of the activity normally exhibited by P_2 alone.

The *N*-hydroxylase activity of P_2 was found to be very labile, all activity being lost upon a short exposure to 100° C. Storage of P_2 at —20° C or at room temperature resulted in a complete loss of activity within 24 h. No activity was detectable upon reconstitution of lyophilized preparations. When stored at 4° C, P_2 preparations underwent a time-dependent loss of *N*-hydroxylase activity, with 80, 60, and 5% of initial activity remaining after 3, 4 and 8 days of storage, respectively. In contrast, the ability of S_2 to enhance *N*-hydroxylase activity in P_2 was extremely stable. No loss of stimulatory ability was noted even after exoposure of S_2 to 100° C for 10 min. S_2 preparations could be stored indefinitely, with no loss of stimulatory ability, at either 4° C or —20° C.

The extent of N^6-hydroxylysine formation was found to increase linearly over a four-fold increase in enzyme concentration (Fig. 1A). Varying the

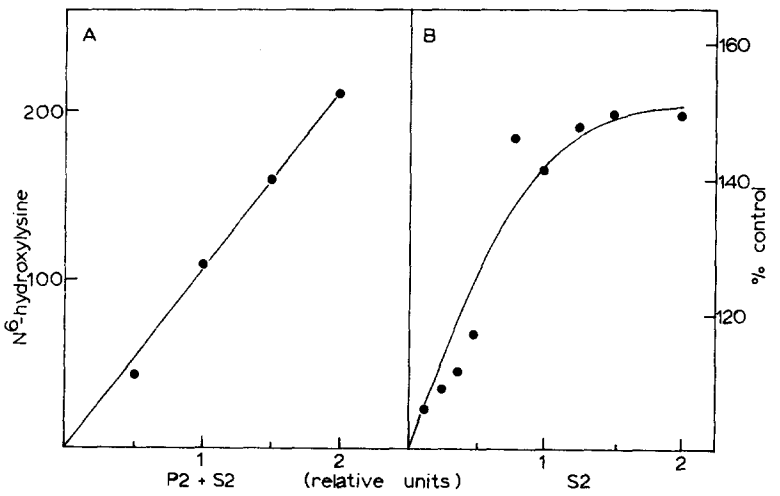

Fig. 1. Effect of enzyme concentration on the extent of N^6-hydroxylysine formation. (A) Both P_2 and S_2 concentrations varied. (B) Varying S_2 concentration; P_2 constant. Control assays contained P_2 only. Relative units refer to the proportion of enzyme concentration normally used (see Methods).

amount of P_2 added to the assay while maintaining a constant S_2 concentration resulted in a similar linear relationship. Increasing the amount of S_2 added to the assay containing constant P_2 yielded an apparent saturation effect (Fig. 1B). This observation, as well as the heat stability of S_2, suggests that the component responsible for the stimulatory effect is possibly non-protein in nature.

Substrate specificity of the N-hydroxylation reaction

The enzyme preparations were found to exhibit a low level of N^6-hydroxylysine production even in the absence of added L-lysine. This activity was traced to the small amount of endogenous lysine (approximately 1 μM) associated with the preparations. However, the rate of N-hydroxylation (linear with time under the assay conditions employed) was strongly dependent upon the concentration of L-lysine added to the assay (Fig. 2). An apparent K_m value of 50 μM for L-lysine was obtained. In all experiments performed, however, an apparent discontinuity in the relationship between rate and substrate concentration was observed. This discontinuity was found to consistently occur between 100 and 150 μM L-lysine. Velocity increased rapidly with substrate concentrations up to 100 μM. Between 100 and 150 μM L-lysine, there was no noticeable increase in velocity, whereas above 150 μM L-lysine further increases in lysine concentration resulted in a steady increase in velocity which approached a contant maximum value.

The following compounds were unable to serve as substrates in the N-hydroxylation reaction: D-lysine, N^2-acetyl-L-lysine, N^6-acetyl-L-lysine, N^6-methyl-L-lysine and 6-aminocaproic acid. These compounds also failed to inhibit the N-hydroxylation of L-lysine, even when present at 10—100-fold molar excess over L-lysine. L-Isomers of ornithine, 2,4-diaminobutyric acid and 2,3-diaminopropionic acid were poorer substrates than L-lysine. The

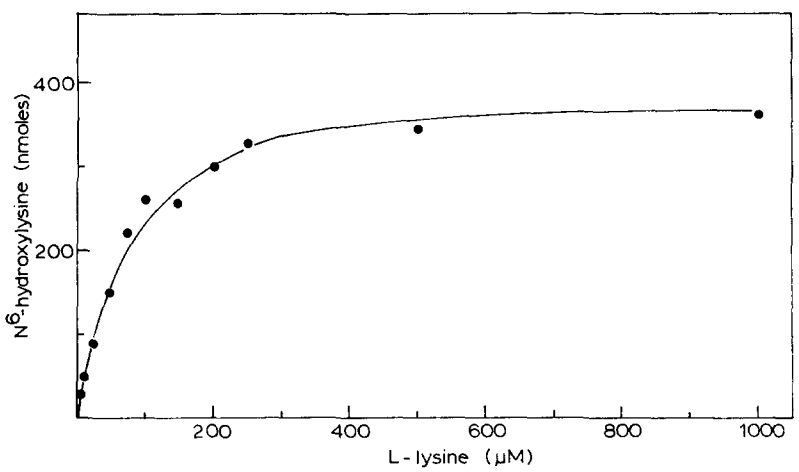

Fig. 2. Effect of added L-lysine concentration on the extent of N^6-hydroxylysine formation. The data have been corrected for the small amount of N^6-hydroxylysine (less than 5% of the maximum) formed in the absence of added L-lysine. All assays consisted of 50 mM phosphate buffer (pH 7.0), 1 mM L-(+)-lactate, P_2 + S_2, and L-lysine in the appropriate concentration. A least-squares fitting of the data to a double-reciprocal plots yielded an apparent K_m for L-lysine of 50 μM.

N-hydroxylation of these dibasic amino acids was concentration dependent. In general, at saturating levels of these substrates, the extent of N-hydroxylation was less than 50% of that noted with L-lysine under identical conditions.

Other amino acids were unable to serve as substrates. Furthermore, with the exception of L-glutamic acid, L-glutamine, L-aspartic acid and L-asparagine, all other amino acids had little influence on the N-hydroxylation of L-lysine.

Stimulation of N^6-hydroxylysine formation

As recorded in Table I, the addition of 1 mM L-glutamic acid or L-glutamine to the assay produced a considerable enhancement of N^6-hydroxylysine formation. *L*-Aspartic acid and L-asparagine also stimulated the reaction, although to a lesser extent than that observed with L-glutamic acid or L-glutamine. The stimulation appeared to require the L-configuration of the amino acid.

The stimulation of N^6-hydroxylysine formation was dependent upon the concentration of L-glutamine or L-glutamic acid added to the assay. Half-maximal activation of N-hydroxylation was obtained at 100 μM L-glutamine or L-glutamic acid, at both saturating and subsaturating concentrations of L-lysine. Experiments performed at a fixed concentration of L-glutamine (1 mM) but varying concentrations of L-lysine revealed no observable change in the apparent K_m for L-lysine (50 μM). Furthermore, inclusion of glutamine in the assay failed to eliminate the discontinuity in the velocity versus lysine concentration profile normally observed in the absence of the effector. The effect of glutamine (1 mM) was to approximately double the extent of N^6-hydroxylysine formation, regardless of the concentration of L-lysine added to the assay. The observed enhancement was due to the increased formation of N^6-hydroxylysine and not due to the hydroxylation of added effector since (i) glutamic acid or glutamine added to the assay mixture could be quantitatively recovered following completion of the reaction, and (ii) analysis for N^6-hydroxylysine by ion-exchange chromatography [11] revealed a greater amount of this product in assays containing glutamine than in those not supplemented with glutamine.

In addition to stimulating the activity of P_2 preparations, glutamine was found to stabilize the *N*-hydroxylase activity. No apparent loss of activity

TABLE I

EFFECT OF GLUTAMINE AND RELATED COMPOUNDS ON N^6-HYDROXYLYSINE FORMATION

Control assay mixtures consisted of 50 mM phosphate buffer (pH 7.0), 1 mM L-(+)-lactate, 1 mM L-lysine, P_2 + S_2. The effect of various compounds was assessed by their addition (1 mM final concentration) to the assay mixture.

Compound	Activity (% control)
L-Glutamine	200
L-Glutamic acid	190
L-Asparagine	160
L-Aspartic acid	160
2-Amino-L-adipic acid	100
D-Glutamic	100
D-glutamic acid	80
N^2-Acetyl-L-glutamine	100
2-Ketoglutaric acid	95

could be detected in P_2 preparations containing 1 mM glutamine, even after storage for 2 weeks at 4°C. The enhancement and stabilizing effects of glutamine and glutamic acid required the L-configuration of the amino acids, as well as the presence of an unmodified α-amino group (Table I). The stimulatory effect exerted by these amino acids was independent of, and additive to, that produced by S_2.

Effect of keto acids

The ability of pyruvate or L-(+)-lactate (the latter especially in the presence of NAD and lactate dehydrogenase) to stimulate the conversion of lysine to N^6-hydroxylysine had been previously reported [9]. Subsequent studies have shown that, at fixed saturating L-lysine (1 mM), the stimulation of N-hydroxylation by pyruvate or L-(+)-lactate was concentration dependent. This concentration dependence exhibited normal hyperbolic saturation kinetics. An apparent K_m of 100 μM for both pyruvate and L-(+)-lactate was observed.

Addition of L-(+)-lactate to assays at the start of the incubation resulted in a considerable enhancement in the rate of N^6-hydroxylysine formation (Fig. 3). Addition of L-(+)-lactate, to assays not containing this compound, 1 h after initiation of incubation also resulted in a pronounced stimulation of N-hydroxylation. L-(+)-Lactate (or pyruvate) had little effect when added to assays not containing L-lysine. Addition of L-lysine to assays, not supplemented

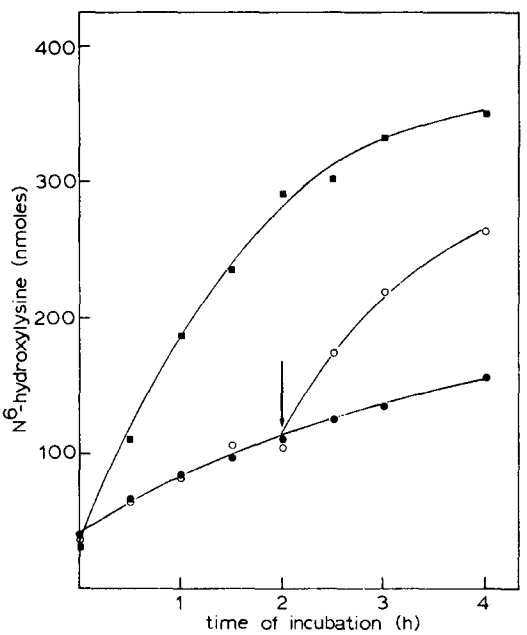

Fig. 3. Time course of N^6-hydroxylysine formation. ■, control assay: 50 mM phosphate buffer (pH 7.0), 1 mM L-(+)-lactate, 2.5 mM L-lysine, P_2 + S_2. ○, assay minus L-(+)-lactate. L-(+)-lactate (1 mM) was added to the assay after 2 h incubation. ●, assay minus L-lysine, L-lysine (2.5 mM) was added to the assay after 2 h incubation. The arrow indicates the point of addition of the appropriate metabolite.

with the amino acid, 1 h after initiation of incubation failed to affect the reaction.

The effect of a variety of keto and hydroxyacids on N^6-hydroxylysine formation was examined (Table II). These studies were performed in the presence and absence of pyruvate and L-(+)-lactate in order to assess their influence on the pyruvate or lactate-mediated stimulation of N-hydroxylation. None of the compounds tested were themselves able to enhance the N-hydroxylation of lysine. 2-Ketobutyric acid and 2-ketoisovaleric acid appeared to inhibit the reaction, whereas the corresponding 2-hydroxy acids had little effect.

Influence of metabolic inhibitors

The effect of a number of metabolic inhibitors on the N-hydroxylation reaction was investigated (Table III). Malonic acid, an inhibitor of the tricarboxylic acid cycle [12], and oxamic acid, a competitive inhibitor of lactate dehydrogenase, exerted little effect in the presence of L-(+)-lactate or pyruvate. 2-Hydroxybutynoic acid, a novel inhibitor of flavoprotein oxidases [13], appeared to inhibit N^6-hydroxylysine formation in the presence of L-(+)-lactate, but had no effect in the presence of pyruvate. Inclusion of arsenite in the assay resulted in an almost complete abolition of *N*-hydroxylase activity. This reagent inhibits pyruvate metabolism by reacting with dihydrolipoamide, an essential component of the pyruvate dehydrogenase complex [14]. Cyanide and rotenone, known inhibitors of electron transport, apparently stimulated N-hydroxylation.

Earlier studies had indicated the possible involvement of pyridoxal-5′-phosphate in the N-hydroxylation reaction [9]. Propargylglycine, an irreversible inactivator of pyridoxal-dependent enzymes [13], was able to inhibit N^6-hydroxylysine formation. In addition, 4-deoxypyridoxine, an antagonist of pyridoxal-requiring enzymes, exerted a slight inhibitory effect, although only at relatively high concentrations. Azaserine, an inhibitor of certain reactions involving glutamine [12], almost completely inhibited the N-hydroxylation process, even at concentrations as low as 0.2 mM (data not shown). This inhibition could not be reversed by addition of glutamine.

TABLE II

EFFECT OF KETO AND HYDROXY ACIDS ON N^6-HYDROXYLYSINE FORMATION

Control assays consisted of 50 mM phosphate buffer (pH 7.0), 1 mM L-lysine, P_2 + S_2.

Compound	Activity (% control)		
		+ 1 mM L-(+)-lactate	+ 1 mM pyruvate
None	100	120	180
2-Ketobutyrate (1 mM)	78	86	100
2-Hydroxybutyrate (1 mM)	90	89	146
2-Ketoisovalerate (1 mM)	65	79	114
2-Hydroxyisovalerate (1 mM)	97	103	170
3-Hydroxybutyrate (0.5 mM)	99	120	155
3-Phenylacetate (0.5 mM)	112	96	120

TABLE III

INHIBITION OF N^6-HYDROXYLYSINE FORMATION

Control assays consisted of 50 mM phosphate buffer (pH 7.0), 1 mM L-lysine, P_2 + S_2, and 1 mM L-(+)-lactate or 1 mM pyruvate as indicated. n.d., not determined.

Compound	Activity (% control)	
	+ 1 mM L-(+)-lactate	+ 1 mM pyruvate
(i) Inhibitors of pyruvate and lactate metabolism		
Malonic acid (1 mM)	105	100
Oxamic acid (1 mM)	100	100
2-Hydroxybutynoic acid (1 mM)	65	95
Sodium arsenite (1 mM)	20	15
(ii) Respiratory inhibitors		
Sodium cyanide (50 μM)	135	145
Sodium azide (0.5 mM)	100	100
Rotenone (5 μM)	165	110
(iii) Substrate-like compounds		
Norleucine (1 mM)	60	n.d.
Glutamic-γ-hydrazide (1 mM)	15	n.d.
Azaserine (1 mM)	10	n.d.
Azaserine (1 mM) + glutamine (1 mM)	10	n.d.
(iv) Miscellaneous		
Catalase (2000 U)	100	108
Superoxide dismutase (3000 U)	95	100
4-Deoxypyridoxine (10 mM)	80	n.d.
Propargylglycine (0.5 mM)	80	n.d.
Propargylglycine (1 mM)	65	n.d.
Propargylglycine (2 mM)	35	n.d.

Discussion

A. aerogenes 62-1, when grown in iron-deficient medium, produces large amounts of aerobactin, a dihydroxyamic acid consisting of one residue of citric acid and two residues of N^6-acetyl-N^6-hydroxylysine [15]. Isolation and purification of the enzyme system responsible for the production of N^6-hydroxylysine should prove advantageous for the elucidation of the mechanism of hydroxamate biosynthesis. Despite the initial success in the preparation and partial fractionation of cell-free extracts capable of converting lysine to its N^6-hydroxy derivative, repeated efforts to further purify the system have thus far been unsuccessful. Consequently, detailed mechanistic interpretation cannot yet be realized. The present studies were directed towards the elucidation of factors influencing the formation of N^6-hydroxylysine in cell-free extracts.

The specificity of the N-hydroxylation process for the L-configuration of lysine has been established. The inability of lysine modified at either the N^2- or N^6-amino groups indicates that N-hydroxylation probably precedes acylation in aerobactin biosynthesis. A similar reaction sequence has been proposed to occur in the biosynthesis of hadacidin [6] and ferrichrome [7]. In addition, it is likely that assembly of the aerobactin molecule occurs subsequent to the formation of N^6-hydroxylysine, due to the failure of N^2-acylated lysine to serve as substrate. Thus one might propose that the initial step in aerobactin biosynthesis is the N-hydroxylation of L-lysine.

The discontinuity observed in the lysine saturation curve has been observed in many experiments. It is not clear whether this discontinuity is an artifact of the preparation procedure or a true reflection of the presence of two forms of the *N*-hydroxylase, one of which possesses high affinity for substrate. Alternatively, the possibility of the substrate, at high concentrations, serving as a positive effector of the enzyme may provide the basis for these observations.

In addition to the requirement for molecular oxygen [9], the N-hydroxylation process is influenced by the presence of pyruvate (or L-(+)-lactate) and L-glutamic acid (or L-glutamine). The ability of these compounds to enhance the reaction is distinct from and additive to that exerted by S_2. However, pyruvate appears to have no significant effect when added to a system supplemented with both S_2 and glutamine (data not shown). Citrate, 2-ketoglutarate and succinate fail to influence the extent of N^6-hydroxylysine formation.

A survey of the effects of a variety of metabolites reveals that the ability to enhance N-hydroxylation is restricted to those compounds which are intimately involved in the biosynthesis of components comprising aerobactin. Pyruvate, glutamic acid and aspartic acid are involved in the formation of lysine. In addition, pyruvate serves as the source of acetyl-CoA and citric acid, necessary components for the formation of hydroxyamate from N^6-hydroxylysine. The beneficial effects of pyruvate, glutamic acid and aspartic acid may reflect the tight coordination prevailing between the generation of precursors and their subsequent utilization in secondary metabolism.

Aerobactin, as implied above, is a product of secondary metabolism, formed under conditions when the activities of many enzymes of the primary metabolic pathways are depressed. The failture of malonate, a potent inhibitor of succinate dehydrogenase and consequently the tricarboxylic acid cycle, to adversely effect N-hydroxylation might suggest that the combustion of pyruvate by this cycle is minimal during hydroxamate biosynthesis. A reduction in the oxidation of pyruvate would prove beneficial since it would allow the channelling of pyruvate towards lysine biosynthesis and ensure greater availability of molecular oxygen for the N-hydroxylation of this lysine. Furthermore, iron deficiency, which normally leads to a diminution in the activities of certain enzymes in the tricarboxylic acid cycle [16], has been shown to be essential for microbial hydroxamate production [17]. The stimulatory effects of cyanide and rotenone might be due to their ability to minimize oxygen utilization by respiration, thus making it more available to the N-hydroxylation system.

The inhibition of N^6-hydroxylysine formation by propargylglycine and glutamic-γ-hydrazide provides additional support for the possible involvement of pyridoxal-5′-phosphate in the N-hydroxylation reaction. However, in view of the complexity of the enzyme preparation, the possibility of the inhibitory effects arising due to causes other than interaction with pyridoxal-5′-phosphate cannot be eliminated.

Acknowledgement

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References

1 Neilands, J.B. (1967) Science 156, 1443—1447
2 Snow, G.A. (1970) Bacteriol. Rev. 34, 99—125
3 Maehr, H. (1971) Pure Appl. Chem. 28, 603—636
4 Emery, T.F. (1971) Adv. Enzymol. 35, 135—185
5 Neilands, J.B. (1973) in Inorganic Biochemistry (Eichhorn, G., ed.), pp. 167—202, Elsevier, Amsterdam
6 Stevens, R.L. and Emery, T.F. (1966) Biochemistry 5, 74—81
7 Emery, T.F. (1966) Biochemistry 5, 3694—3701
8 MacDonald, J.C. (1965) Biochem. J. 96, 533—538
9 Murray, G.J., Clark, G.E.D., Parniak, M.A. and Viswanatha, T. (1977) Can. J. Biochem. 55, 625—629
10 Tomlinson, G., Cruickshank, W.H. and Viswanatha, T. (1971) Anal. Biochem. 44, 670—679
11 Spackman, D.H., Stein, W.H. and Moore, S. (1958) Anal. Chem. 30, 1190—1206
12 Webb, J.L. (1966) Enzymes and Metabolic Inhibitors, Vol. 2, Academic Press, New York
13 Abeles, R.H. and Maycock, A.L. (1976) Acc. Chem. Res. 9, 313—319
14 Koike, M. and Hayakawa, T. (1970) Methods Enzymol. 18A, 298—307
15 Gibson, F. and Magrath, D.I. (1969) Biochim. Biophys. Acta 192, 175—184
16 Lankford, C.E. (1973) CRC Crit. Rev. Microbiol. 2, 273—331
17 Emery, T.F. (1974) in Microbial Iron Metabolism (Neilands, J.B., ed.), pp. 107—123, Academic Press, New York