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RECOVERY OF DIAMINE OXIDASE ACTIVITY INHIBITED BY ISONICOTINIC ACID HYDRAZIDE AND ITS DERIVATIVES*

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Abstract—Evidence is presented for the quantitative recovery of mammalian diamine oxidase (DO) activity originally inhibited by isonicotinic acid hydrazide or its derivatives. The inhibition is suppressed by the addition of ferricyanide which causes evolution of nitrogen equivalent to that contained in the hydrazide moiety of the inhibitor and prevents loss of enzyme activity. It is suggested that ferricyanide can be used as an assay for isonicotinic acid hydrazide and its derivatives. Ferricyanide did not prevent the inhibition of DO by semicarbazide or aminoguanidine.

THIS LABORATORY has been engaged in the purification of the enzyme, diamine oxidase. During the course of the study, ferricyanide was investigated as a possible cofactor for this enzyme because it is a possible hydrogen acceptor of amine dehydrogenase of bacteria.[1] The addition of ferricyanide was found to catalyze the oxygen uptake of diamine oxidase, and it also appeared to reverse the inhibition of this enzyme by isonicotinyl hydrazide (INH) or its derivatives. Other studies on the action of INH-type inhibitors have been concentrated on monoamine oxidase,[2] and their action was found to be irreversible because of their long-lasting effect; attempts to reverse their action have been unsuccessful.[3] Recent studies of the effects of INH-type inhibitors on diamine oxidase have given data similar to those obtained for monoamine oxidase.[4] This paper presents data supporting the contention that inhibition *in vitro* of diamine oxidase by INH or its derivatives can be relieved and the original enzyme activity fully restored.

MATERIALS AND METHODS

Diamine oxidase was prepared from hog intestines and kidneys.[5] Oxygen uptake was measured manometrically under the following conditions: diamine oxidase, containing approximately 6 mg N, was in a final volume of 2·8 ml in 0·1 M sodium phosphate buffer, pH 7·6. Five μmoles of cadaverine in 0·10 ml was tipped in and the oxygen uptake read at 10-min intervals; 0·2 ml of 25% sodium hydroxide was included in the center well. When used, ferricyanide was added with the cadaverine or from the second sidearm. All incubations were done in air at 37 °C. The following inhibitors of diamine oxidase were tested at the indicated final concentrations:

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aminoguanidine (AG) 10^{-5} M; semicarbazide 10^{-4} M; INH 5×10^{-4} M; 1-isonicotinyl-2-isopropyl-hydrazine (IPINH) 5×10^{-4} M; and 1-isonicotinyl-2-isobutyl-hydrazine (IBINH) 5×10^{-4}. These inhibitors were preincubated with enzyme 10 to 20 min at 38° before addition of substrate.[3] The theoretical uptake of 2·5 μmoles oxygen per 5 μmoles cadaverine was observed for the enzyme in the absence of inhibitor because of catalase activity in our preparations.[6]

^{14}C-putrescine and ^{14}C-histamine were assayed by isotope dilution as the picrate.[7] The enzymatic destruction of ^{14}C-putrescine was also determined by our extraction technique.[5] ^{14}C-putrescine dihydrochloride (New England Nuclear Corp.) had a specific activity of 4·7 mc/mmole and ^{14}C-histamine dihydrochloride (Nuclear-Chicago Corp.) had a specific activity of 4·75 mc/mmole.

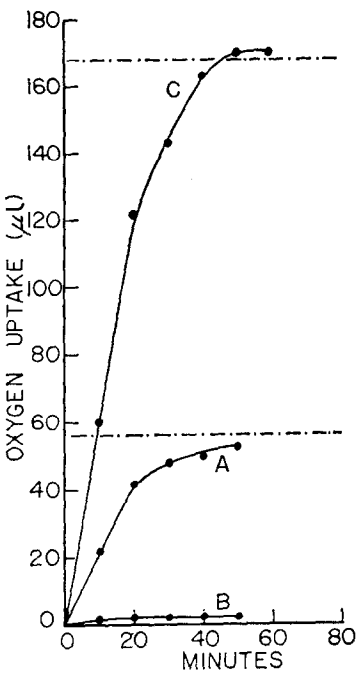

FIG. 1. Effect of inhibitors and ferricyanide on oxygen uptake by hog intestine diamine oxidase. A: Hog intestine diamine oxidase with 5 μmoles cadaverine. B: Hog intestine DO preincubated with inhibitors (see text) before addition of 5 μmoles cadaverine. C: Potassium ferricyanide, 10 μmoles, added with 5 μmoles cadverine to hog intestine DO.

RESULTS

When cadaverine is incubated with diamine oxidase containing catalase activity, oxygen is consumed in a ratio of an atom of oxygen per molecule of substrate,[6] as shown in curve A, Fig. 1. Inclusion of diamine oxidase inhibitors, as described under Methods, almost completely depressed the oxygen uptake of the enzyme (curve B, Fig. 1). In the absence of any inhibitor, the addition of 10 μmoles ferricyanide to

5 μmoles cadaverine resulted in uptake of approximately 170 μl of oxygen which is about three times the expected value (curve C, Fig. 1). It was previously determined that ferricyanide added to either the enzyme or cadaverine alone resulted in no oxygen uptake. The catalytic effect of ferricyanide was observed only when cadaverine or other aliphatic diamines were used as substrates, and not when histamine was used.

These conditions were duplicated in another series of flasks, except for the addition of 10 μmoles potassium ferricyanide to the cadaverine in the sidearm. The flasks containing INH, IPINH, and IBINH, which were inhibited without ferricyanide, now absorbed oxygen. This finding indicated that enzyme activity was maintained (curves B and C, Fig. 2). The evolution of gas at the initial phase of the reaction was presumed to be nitrogen from the hydrazine portion of the inhibitors. The evidence for this will be presented later. The ferricyanide did not reverse the inhibition of diamine oxidase by either semicarbazide or aminoguanidine.

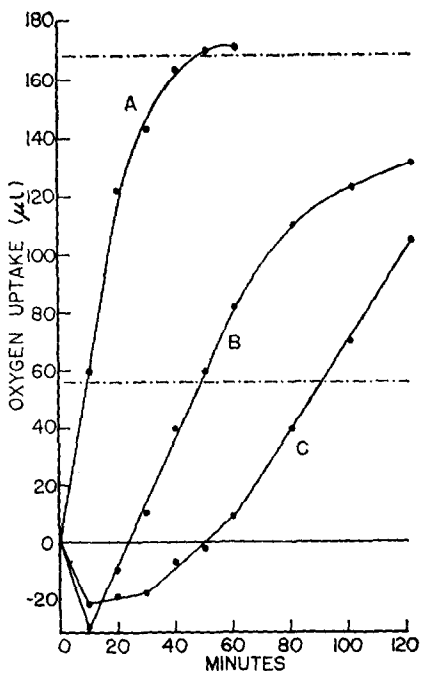

FIG. 2. Recovery of INH-, IPINH-, and IBINH-inhibited hog intestine diamine oxidase activity by ferricyanide. A: Same as Fig. 1, C. B: Same as Fig. 1, B. Inhibitors were INH or IPINH; 10 μmoles ferricyanide added with 5 μmoles cadaverine. C: Same as Fig. 2, B but inhibitor was IBINH.

The following experiment was done to determine the nature of the action of ferricyanide. Two series of Warburg vessels were prepared. In the first series, diamine oxidase was preincubated 20 min with INH and 5 μmoles cadaverine contained in one sidearm and 20 μmoles ferricyanide in the other. The second series was identical with the first except that INH was omitted. The cadaverine was tipped in at zero time and the manometers read at 10-min intervals. The ferricyanide was tipped in at varying

times (Fig. 3). Addition of ferricyanide to flasks containing INH resulted in an immediate production of gas followed by a rapid uptake of oxygen, indicating recovery of diamine oxidase activity (Fig. 3, curves C and D). Addition of ferricyanide to flasks containing no INH after the oxygen uptake had reached a plateau resulted in a rapid uptake of more oxygen until a second plateau was reached (Fig. 3, curves A and B). These data show that the end product of diamine oxidase action on cadaverine is further oxidized by the ferricyanide. However, in these experiments, only a twofold increase in total oxygen uptake was observed rather than the threefold increase recorded in earlier experiments.

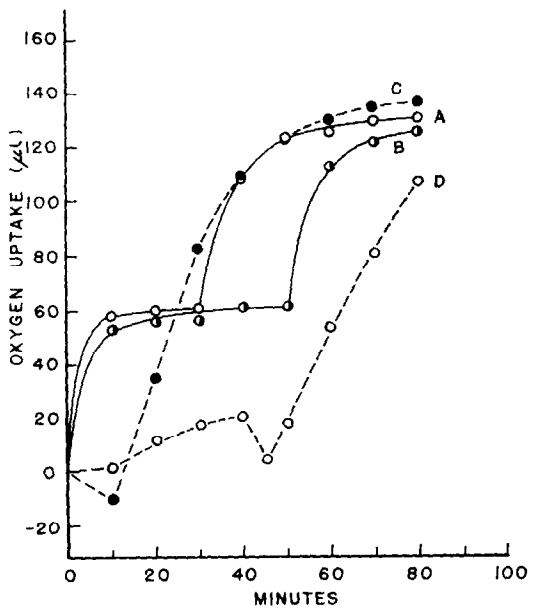

FIG. 3. The effect of adding ferricyanide at various time intervals to hog kidney diamine oxidase inhibited by isonicotinyl hydrazide. A: Hog kidney DO with 5 μmoles cadaverine; ferricyanide added at 30 min. B: Hog kidney DO with 5 μmoles cadaverine; ferricyanide added at 50 min. C: Potassium ferricyanide, 20 μmoles, added with 5 μmoles cadaverine at zero time to hog kidney DO preincubated 20 min with 1·5 μmoles INH. D: Same as C, except ferricyanide added at 40 min.

In order to demonstrate the fact that cadaverine was being catabolized by diamine oxidase in the presence of INH derivatives and ferricyanide, the destruction of ^{14}C–putrescine in this system was studied by the isotope dilution method. ^{14}C–putrescine was used in place of ^{14}C–cadaverine, since the former is available commercially whereas the latter must be synthesized. As can be seen by the data in Table 1, the enzyme was completely inhibited by the concentration of inhibitor used; the inhibition was relieved by the inclusion of ferricyanide. The data showing the time course of the recovered enzyme activity are presented in Table 2. No loss of putrescine occurred in the presence of either INH or ferricyanide, singly or together, in the absence of enzyme at the longest incubation time used.

It has been demonstrated previously that the end product of diamine oxidase action on putrescine is a toluene-soluble heterocycle (either piperidine or its polymer).

This observation is the basis of an assay technique developed in this laboratory.[5] This extraction technique was used to measure the amount of end product formed by diamine oxidase from 5 μmoles of ^{14}C–putrescine in 1 hr at 37° in the presence and absence of 20 μmoles of ferricyanide. Without ferricyanide, approximately 44,000 cpm were found, whereas, with ferricyanide, only 3,800 cpm were found. The isotope dilution data in Table 1 indicate that almost complete metabolism of putrescine occurs with or without ferricyanide in this time period. The extraction and manometric data, therefore, suggest that the cyclic end products of putrescine and cadaverine formed by diamine oxidase were further oxidized by ferricyanide to acidic products which were not extractable into toluene from an aqueous bicarbonate medium.

TABLE 1. ^{14}C–PUTRESCINE METABOLISM BY HOG KIDNEY DIAMINE OXIDASE

	Putrescine catabolized (%)
Complete system* (CS)	99
CS + $K_3Fe(CN)_6$ (24 μmoles)	99
CS + INH (8 μmoles)	0
CS + $K_3Fe(CN)_6$ + INH	60

* Complete system: enzyme and 5 μmoles ^{14}C–putrescine in 2·0 ml final volume of 0·1 M phosphate buffer, pH 7·6. Incubation time was 2 hr at 37 °C in air. Putrescine determined by isotope dilution as the picrate.

TABLE 2. METABOLISM OF ^{14}C–PUTRESCINE BY HOG KIDNEY DIAMINE OXIDASE IN THE PRESENCE OF ISONICOTINYL HYDRAZIDE AND FERRICYANIDE

	Catabolism of Putrescine %				
Incubation time (hr)	1	2	3	4	5
Complete system	41	61	76	87	90
No $K_3Fe(CN)_6$					2
No enzyme					0
No enzyme; no $K_3Fe(CN)_6$					0
No enzyme; no INH					0

Complete system: enzyme, 5 μmoles ^{14}C–putrescine, 24 μmoles $K_3Fe(CN)_6$, 8 μmoles INH in 0·1 M phosphate buffer, pH 7·6, to a final volume of 2·0 ml. incubated in air at 37 °C with the time as indicated. ^{14}C–putrescine assayed by isotope dilution as the picrate.

When 5 μmoles of histamine was substituted for cadaverine in the manometric experiments, no increase in oxygen uptake over the theoretical value was observed with the inclusion of ferricyanide. When diamine oxidase was inhibited 75 per cent by INH, the inhibition, as measured by the rate of oxygen uptake, was not relieved by the inclusion of ferricyanide. In order to check this observation, the experiment was done by isotope dilution using only 1 μg ^{14}C–histamine as substrate (Table 3). The data show that inhibition of diamine oxidase by INH was relieved by ferricyanide. An interesting observation was that, in the absence of enzyme, 46 per cent

of the histamine was degraded by a combination of INH and ferricyanide. However, in the presence of enzyme, 96 per cent was degraded, suggesting the recovery of diamine oxidase activity.

It has been found that 50 μmoles potassium ferricyanide will react rapidly with 3 μmoles of either INH or IPINH in 0·1 M phosphate buffer, pH 7·6, liberating 3 μmoles of gas, presumed to be nitrogen, in less than 5 min at 37°. However, when 2 and 4 μmoles of semicarbazide or 3 μmoles of 2,4-dinitrophenylhydrazine were

TABLE 3. ^{14}C-HISTAMINE CATABOLISM BY HOG KIDNEY DIAMINE OXIDASE

	Flask contents (final volume 2·0 ml, pH 7·6)							
Flask number	1	2	3	4	5	6	7	8
Enzyme	+	+	+	+	−	−	−	−
^{14}C-histamine, 1 μg	+	+	+	+	+	+	+	+
8 μmoles INH	−	+	−	+	+	+	−	−
24 μmoles $K_3Fe(CN)_6$	−	−	+	−	−	−	+	−
Histamine destroyed (%)	98	26	98	96	46	0	0	0

Conditions: incubation time was 2 hr in air at 37 °C. Histamine was determined by isotope dilution as the picrate.

reacted with 30–50 μmoles of ferricyanide, the liberation of gas was relatively sluggish and not quantitative even after 40-min incubation. This reaction, therefore, may be useful for the quantitative estimation of only INH and its derivatives.

All the data reported here were applicable to diamine oxidase prepared from either hog kidneys or intestines.

DISCUSSION

It has been well established that the diamine oxidase system will consume an atom of oxygen per molecule of diamine catabolized in the presence of catalase. The data presented show that this ratio can be altered by the inclusion of potassium ferricyanide. The increased oxygen uptake is probably due to the further oxidation of the cyclic end products from the catabolism of either putrescine or cadaverine by diamine oxidase. This was indicated by two observations: the change in solubility of the end products when ferricyanide was included with the enzyme; and the fact that ferricyanide, added to a flask containing 5 μmoles of cadaverine with diamine oxidase after 5 μatoms of oxygen was consumed, resulted in further uptake of between 5 and 10 μatoms of oxygen. Although the usual oxygen uptake of diamine oxidase in the presence of ferricyanide was 3 atoms of oxygen per molecule of diamine (Fig. 1), lesser ratios sometimes were observed (Fig. 3). The reason for this variation is unknown. The exact nature of the oxidation products was not determined.

The catalytic action of potassium ferricyanide on diamine oxidase was studied earlier by Stephenson[8] with negative results when histamine was used as substrate. We also have found that ferricyanide will not accelerate the oxygen uptake or increase the oxygen consumption beyond the expected ratio for diamine oxidase when histamine is the substrate. A puzzling observation was that the inhibition of diamine oxidase

by INH, measured manometrically, using histamine was not influenced by the inclusion of ferricyanide. Since, in these latter experiments, the only change was the substitution of 5 μmoles histamine for 5 μmoles cadaverine, one would expect the recovery of enzyme activity as we observed for cadaverine if one enzyme were oxidizing both substrates. Contrary to the manometric data, the inhibition by INH of histamine metabolism by diamine oxidase and the recovery of enzyme activity by the addition of ferricyanide could be demonstrated by the use of ^{14}C-histamine (Table 3). An interesting observation was the chemical destruction of ^{14}C-histamine in the presence of both INH and ferricyanide. The formation of a compound more basic than histamine was indicated by preliminary data from paper chromatography.

The catalytic oxidation by copper ions of the histamine end product, imidazole-4-acetaldehyde, was observed by Born,[9] but these ions were found inactive against the diamine oxidase end products of cadaverine or putrescine. Since the redox potential of ferri- to ferrocyanide is greater than that of cupric to cuprous ion, one would expect the former to be active in oxidizing the aldehyde of histamine. The data, however, show that this was not the case and that, conversely, the putrescine and cadaverine end products were oxidized by ferricyanide but that imidazole-4-acetaldehyde was not. These differences in activity, therefore, must be associated with the ability of each reagent to overcome the activation potential required for the occurrence of the oxidation in question.

The use of ferricyanide for the microdetermination of INH and derivatives in blood has been described.[10, 11] This colorimetric method is dependent on the reduction of ferricyanide in acid solution at 80 °C by INH with the subsequent formation of prussian or Thurnbull's blue. The method is empirical in nature and requires close adherence to the detailed procedure. Our data indicate that ferricyanide can be used to estimate in micromolar quantities the presence of INH and its derivatives by means of manometric techniques. The reaction has proven to be rapid and quantitative at physiological pH at 37 °C in air and, therefore, may be useful as another method for this group of compounds.

Our data show that it is possible to release diamine oxidase activity that has been inhibited by the apparent combination of INH or its derivatives with the enzyme. The data also show that this class of inhibitors does not cause the degeneration of the enzyme because full activity is observed after the addition of ferricyanide. The reactivation of diamine oxidase by ferricyanide represents the chemical reversal of enzyme inhibition as opposed to the usual methods employed for this purpose, such as dialysis and the use of concentration gradients.

Further work is anticipated to identify the nature of the end products resulting from the action of diamine oxidase and ferricyanide on diamines.

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