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PURIFICATION AND PROPERTIES OF MONOAMINEOXIDASE[1]

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SUMMARY

Mitochondrial monoamineoxidase from beef liver has been purified twenty-fold. The enzyme was shown to be metal-dependent by using phenanthroline as an inhibitor. The Lineweaver–Burk treatment suggests a non-competitive inhibition. The same procedure applied to phenylcyclopropylamine shows a strictly competitive and irreversible inhibition, and the pH–activity curve suggests further the presence of two monoamineoxidases. The differential action of oxygen and potassium cyanide on iproniazid and phenylcyclopropylamine inhibition of the enzyme has been studied, and the phenylcyclopropylamine inhibition was shown to be independent of oxygen and potassium cyanide. The significance of these experiments is discussed.

INTRODUCTION

Attempts to solubilize mitochondrial monoamineoxidase[1,2] have until recently been unsuccessful. ZELLER *et al.*[3] extracted the enzyme by subjecting mitochondria to sonic oscillations and exposure to desoxycholic acid. The resultant supernatant, which was separated after centrifugation, contained 70% of the activity. An attempt to solubilize the enzyme[4] by treating mitochondria with snake venom was unsuccessful, since the lipoprotein which constitutes the enzymic unit of monoamineoxidase was impervious to the phosphoesterases of snake venoms. The purpose of the present work was to develop more efficient procedures for the purification of the enzyme in order to facilitate the study of its properties.

MATERIALS AND METHODS

Assay of monoamineoxidase activity was done either manometrically, using tyramine hydrochloride as substrate, or spectrophotometrically after the method of TABOR *et al.*[5]. In the manometric method, the incubation system contained a given amount of enzyme, 10 μg of catalase (which in general was not essential, since all preparations contained catalase activity), and 0.2 M phosphate buffer (pH 8.0), in a final volume of 3.0 ml. The reaction was started by tipping in from the side arm of the Warburg flask enough tyramine for a final concentration of $1.3 \cdot 10^{-2}$ M. Blanks which were

run without substrate did not utilize any oxygen during a 30-min incubation period. The enzyme units were expressed in terms of μl of oxygen absorbed during the first 30 min per ml of enzyme preparation.

In the spectrophotometric method two substrates were usually employed: benzylamine and kynuramine. Benzylamine ($6.6 \cdot 10^{-4}$ M final concentration) was added to the enzyme in the Beckman cell and brought to a final volume of 3.0 ml with 0.2 M potassium phosphate buffer (pH 8.0). The reaction was read against a blank containing everything but the substrate. The increase in absorbancy at 250 mμ measured during the first 2 min was taken as the number of enzyme units.

Kynuramine, recently introduced by WEISSBACH *et al.*[6] appears to be a better substrate, particularly when working with carbonyl reagents such as KCN, which can react with the benzaldehyde formed from benzylamine, thereby decreasing the absorbancy; or when working with phenanthroline whose extinction coefficient at 250 mμ is extremely high. With 10^{-4} M kynuramine as substrate, the absorbancy was measured at 360 mμ, since at this wavelength the absorption due to phenanthroline is negligible. Carbonyl reagents have no effect when kynuramine is used, evidently because the intramolecular reaction between the aldehyde group and the amino group of kynuramine is much more rapid than the reaction of the aldehyde group with the carbonyl reagents.

Protein content was determined on the residue obtained after precipitating with 10% trichloroacetic acid and washing with a little methanol in order to eliminate traces of digitonin or Triton X-100, according to the method of FOLIN–CIOCALTEU[7]. A standard albumin solution was used to calculate the amount of protein in the sample.

Procedure for purification of monoamineoxidase from liver mitochondria

Preparation of mitochondria: About 400 g of liver were homogenized in 3 vol. of 0.25 M sucrose, employing a Servall Omnimixer at maximum speed for 90 sec. The thick suspension was diluted with doubly distilled water to 2 l and the pH adjusted to 7.0. After sedimenting twice at 800 $\times$ *g* for 20 min to separate nuclei and cellular debris, the mitochondria were sedimented at 8000 $\times$ *g*, homogenized in doubly distilled water, and brought to a final volume of 300 ml. The suspension was immediately frozen, in which state the activity was stable for several weeks. All operations were performed at 0–5°.

Treatment with Triton X-100: (a) The original mitochondrial suspension was diluted twice its volume with distilled water, and enough 10% Triton X-100 added to yield a final concentration of 0.5%. After adjusting the pH to 7.0 with 0.1 N sodium hydroxide, the suspension was kept in the cold for 30 min with occasional stirring, and then centrifuged at 80 000 $\times$ *g* for 20 min. The reddish opaque supernatant which contained little monoamineoxidase activity was discarded. (b) The residue was brought to the same volume as in (a) with 0.5% Triton X-100 and the pH adjusted to 7.5. After 30 min in the cold room, the suspension was centrifuged for 20 min at 8000 $\times$ *g* and the supernatant discarded. (c) The residue of the above centrifugation was treated exactly as described in (b), except that the pH was adjusted to 8.0. The residue was suspended in half of the volume used in (b) of 0.5% Triton X-100 and the pH brought to 8.5. After standing 30 min in the cold room, the suspension was centrifuged at 100 000 $\times$ *g* for 30 min. The pH of the super-

natant of this centrifugation was adjusted to 7.0 and the supernatant was again centrifuged for 2 h at 100 000 × *g* and the residue discarded.

Treatment with calcium phosphate gel: The clear, slightly yellow supernatant of the last centrifugation was adjusted to pH 8.5, and calcium phosphate gel[8] in an amount equal to 4 times the weight of protein was added. After standing at 0° for 20 min, the suspension was centrifuged at 5000 × *g* for 15 min. The supernatant was adjusted to pH 7.0 and calcium phosphate gel was added as before. The suspension was centrifuged at 5000 × *g* for 20 min, and the supernatant, which was devoid of protein, discarded. After the residue was washed with two 5.0-ml portions of 0.2 M potassium dihydrogen phosphate and centrifuged at 50 000 × *g*, it was extracted 3 times with 5.0-ml portions of 0.2 M potassium dibasic phosphate with or without 0.5% Triton. The extract without Triton X-100 was not clear and the monoamineoxidase activity could be sedimented at 2000 × *g*. Table I presents data

TABLE I

STEPS OF MONOAMINEOXIDASE PURIFICATION

Units per ml = 1.4/2 min/ml.

Step	*Units/ml*	*Total units*	*Protein (mg/ml)*	*Units/mg protein*
Original mitochondrial suspension (twice diluted)	1.3	818	5.0	0.26
Triton treatment pH 8.5	1.08	340	0.9	1.21
1st calcium phosphate gel treatment	0.57	206	0.23	2.47
2nd calcium phosphate gel treatment				
1st K_2HPO_4 elution	0.82	40	0.34	2.41
2nd K_2HPO_4 elution	0.42	21	0.14	3.00
3rd K_2HPO_4 elution	0.42	21	0.08	5.40

on the purification procedure. (An additional attempt was made to solubilize the enzyme by lyophylizing the mitochondria and extracting the powder with butyl alcohol at −25°. After 1 h, the suspension was homogenized and centrifuged. The powder was then washed with acetone at −25°, dried in air and extracted with phosphate buffer at pH 7.5. No activity was found in the buffer, while only a small percentage of the initial activity was present in the insoluble residue.)

RESULTS

Mitochondrial monoamineoxidase was already found to be a sulfhydryl enzyme insofar as it was inhibited by *p*-chloromercuribenzoate. It has been suggested by DAVISON[9] that a thiol group may be involved in the attachment of the substrate to the active site of the enzyme. The present experiments do not give any indication of a competitive-type inhibition of monoamineoxidase by *p*-chloromercuribenzoate. Since the concentration of inhibitor (Table II) required was appreciably lower than that used by FRIEDENWALD AND HERMANN[10], the sensitivity to *p*-chloromercuribenzoate might depend upon the degree of purification of the monoamineoxidase. Inhibition was apparent at 10^{-6} M and complete at 10^{-3} M.

TABLE II

INHIBITION OF ENZYME BY *p*-CHLOROMERCURIBENZOATE

Benzylamine concentration was $6.6 \cdot 10^{-4}$ M. Reaction was started by adding inhibitor and substrate together to the enzyme in 0.2 M phosphate buffer (pH 8). Total volume was 3 ml, and temperature was 27°.

Concentration (M)	*Inhibition (%)*
$1.2 \cdot 10^{-3}$	100
$1 \cdot 10^{-3}$	90
$1.2 \cdot 10^{-5}$	50
$1 \cdot 10^{-5}$	25
$5 \cdot 10^{-6}$	0

Different concentrations of phenanthroline were tested for their inhibitory action after 10 min preincubation with the enzyme. It was observed with the manometric technique, employing tyramine as substrate ($2.0 \cdot 10^{-3}$ M), that there is actually an activation when the concentration of phenanthroline was $1.0 \cdot 10^{-3}$ M. Since catalase was always present in the enzyme preparation and would be responsible for an evolution of oxygen, it was suspected that phenanthroline, by inhibiting catalase, could alter the manometric determination; but no inhibition of catalase was evident when the preparations were tested using hydrogen peroxide as substrate in the presence of phenanthroline.

With kynuramine as substrate and concentrations of phenanthroline ranging

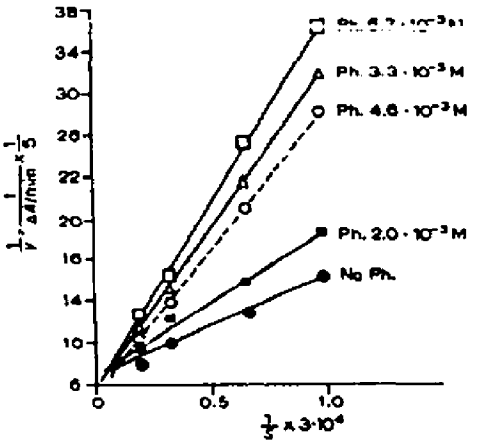


Fig. 1. Lineweaver–Burke plot with phenanthroline as inhibitor. V is expressed as Δ absorbancy/min. Kynuramine (10^{-4} M) was the substrate.

from 2.0 to $7.0 \cdot 10^{-3}$ M, a Lineweaver–Burke plot was obtained (Fig. 1). It can be seen that the inhibition produced by phenanthroline is neither non-competitive nor competitive, and the picture is further complicated by the lower inhibitory capacity of phenanthroline at $4.6 \cdot 10^{-4}$ M.

The effect of potassium cyanide on the inhibition of phenanthroline was deter-

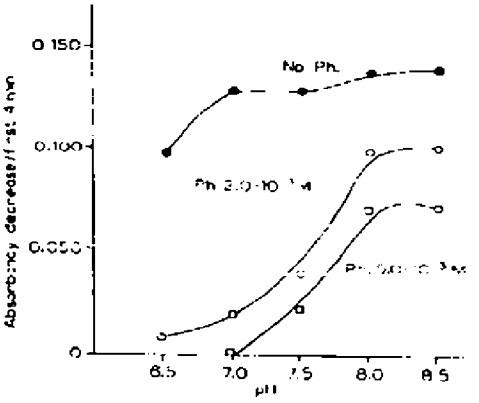


Fig. 2. pH–activity curve of enzyme alone and enzyme plus phenanthroline at different concentrations. Substrate is 10^{-4} M kynuramine.

mined using kynuramine as substrate. Identical curves were obtained with phenanthroline alone and with phenanthroline plus cyanide, indicating that potassium cyanide has no effect on the inhibitory action of phenanthroline. The pH–activity curve (Fig. 2) for monoamineoxidase alone differs markedly from that obtained in the presence of phenanthroline.

One of the most potent inhibitors of monoamineoxidase is phenylcyclopropylamine, effective at concentrations as low as 10^{-7} M. The Lineweaver Burke treatment was also applied to the inhibition of the enzyme by phenylcyclopropylamine with kynuramine as substrate. When substrate and inhibitor were added together,

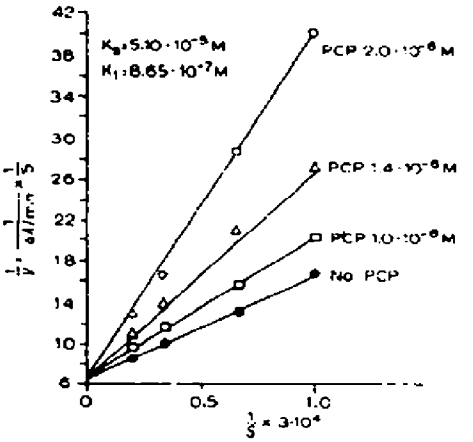
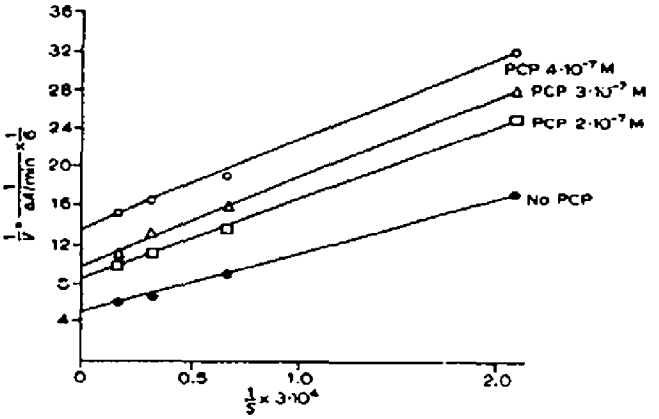


Fig. 3. A. Lineweaver–Burke plot for phenylcyclopropylamine, using 10^{-4} M kynuramine. V is expressed as absorbancy decrease per minute. Inhibitor and substrate were added together.

the inhibition was of the competitive type (Fig. 3A); but when the enzyme was incubated with the inhibitor for 10 min at 27° prior to the addition of substrate, the nature of the inhibition was both competitive and irreversible (Fig. 3B).



B. Same as A, except that enzyme is incubated with the inhibitor for 10 min at 27° prior to starting the reaction by addition of substrate.

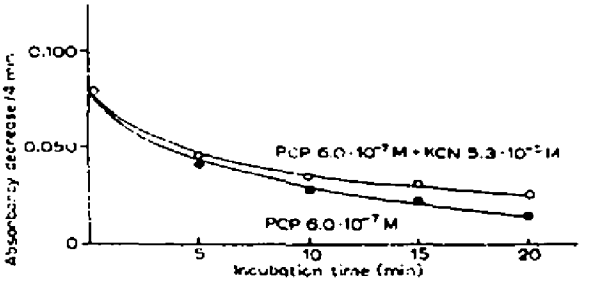


Fig. 4. Effect of potassium on the inhibition by phenylcyclopropylamine. Lower curve is obtained when enzyme is incubated with $6 \cdot 10^{-7}$ M phenylcyclopropylamine. Reaction was started by adding 10^{-4} M kynuramine. Upper curve is the same, except $5.3 \cdot 10^{-5}$ M potassium cyanide is added together with phenylcyclopropylamine.

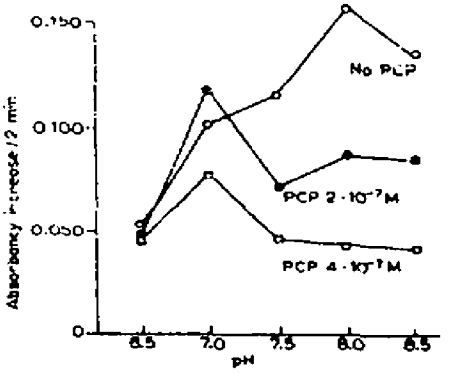


Fig. 5. pH-activity curve of enzyme with and without phenylcyclopropylamine. Ordinates are absorbancy increases during first 2 min, using $6.6 \cdot 10^{-4}$ M benzylamine. Upper curve is obtained with enzyme plus substrate; middle curve, enzyme plus substrate plus $2 \cdot 10^{-7}$ M phenylcyclopropylamine; lower curve, $4 \cdot 10^{-7}$ M phenylcyclopropylamine.

Potassium cyanide was tested on the inhibition of monoamineoxidase by phenylcyclopropylamine, and found to be essentially without effect (Fig. 4).

The pH–activity curve was determined for the enzyme alone and in the presence of phenylcyclopropylamine, using benzylamine as substrate (Fig. 5). In the absence of inhibitor, the curve shows an optimum at pH 8.0, while in the presence of phenylcyclopropylamine there is a sharp pH optimum at pH 7.0. As the concentration of phenylcyclopropylamine increased from 2.0 to $4.0 \cdot 10^{-7}$ M, a secondary optimum at pH 8.0 entirely disappears. The pH–activity curve of monoamineoxidase in the presence of iproniazid was similar to that of the enzyme alone, and had an optimum at pH 8.0.

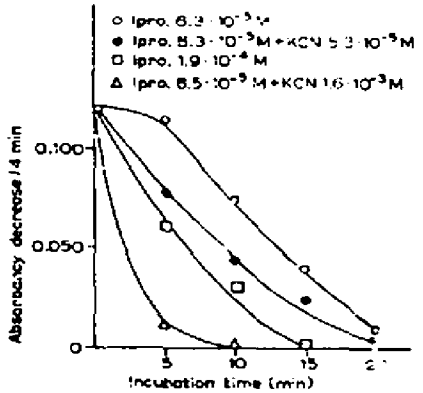


Fig. 6. Inhibition of enzyme by iproniazid plus potassium cyanide with 10^{-4} M benzylamine as substrate. Ordinates are absorbancy decreases during first 4 min, abscissae are incubation times.

The effect of potassium cyanide on the inhibition of monoamineoxidase by iproniazid was determined under the same conditions used in studying the effect of phenylcyclopropylamine (Fig. 6). In the presence of cyanide, the residual activity decreased with increasing time of preincubation of the enzyme with the inhibitor. Increasing the concentration of cyanide to $1.6 \cdot 10^{-3}$ M resulted in almost complete inhibition of activity.

It had been reported[8] that iproniazid is dependent upon oxygen for its in-

TABLE III

DEPENDENCE ON OXYGEN OF MONOAMINEOXIDASE INHIBITION BY IPRONIAZID

Activities expressed as absorbancy increase during first 2 min with benzylamine ($6.60 \cdot 10^{-4}$ M) as substrate. All reactions were carried out at 27°.

	Inhibition (%)
A. Enzyme + iproniazid ($2.28 \cdot 10^{-4}$ M final concentration) diluted to 5 ml with phosphate buffer (pH 8); 2.5 ml of this are immediately transferred to a cuvette, plus 0.3 ml of buffer, and reaction started by adding 0.2 ml of benzylamine.	0
B. Same as A, except enzyme and inhibitor are pre-incubated for 20 min.	83
C. Enzyme + buffer is evacuated and passed with argon. Iproniazid ($2.28 \cdot 10^{-4}$ M final concentration) is tipped in mixture, and kept for 20 min at room temperature under argon. Residual activity is measured as in A.	34

hibitory effect on monoamineoxidase. After gassing with nitrogen for 5 min, the inhibitory action of iproniazid was unaltered (Table III). Since this technique did not eliminate the possibility of trace amounts of oxygen, the following procedure was adopted to insure its complete removal. The reaction was carried out in a modified Thunberg tube to which was added another ground-glass lateral connection for evacuation purposes. The enzyme and the buffer were placed in the main compartment and the inhibitor in the side arm. After the enzyme and the inhibitor were frozen with dry ice, the tube was evacuated for a few minutes, an operation which was repeated four times by alternate freeze-thawing and evacuation. The vacuum connection was closed and argon was introduced through the other connection, this procedure also being repeated four times. After sealing off both connections, the inhibitor was tipped in from the side arm and the tube incubated at 27° for 20 min. In such an anaerobic system there was still a 34% inhibition by iproniazid. When phenylcyclopropylamine was used as the monoamineoxidase inhibitor, using the same technique, the inhibition in the absence of oxygen was 80%, in air 40%, and in oxygen 46% (Table IV).

TABLE IV

DEPENDENCE ON OXYGEN OF MONOAMINEOXIDASE INHIBITION BY PHENYLCYCLOPROPYLAMINE

Activities expressed as absorbancy increase during first 2 min with benzylamine ($6.60 \cdot 10^{-4}$ M) as substrate. All reactions were carried out at 27°.

	Inhibition (%)
A. Enzyme + buffer + $3 \cdot 10^{-7}$ M phenylcyclopropylamine to final volume of 5 ml. Activity measured immediately after adding 0.3 ml buffer and 0.2 ml benzylamine to 2.5 ml of mixture.	0
B. Same as A, except pre-incubated at 27° for 20 min. Residual activity measured as in A.	40
C. Same as A, except enzyme is now evacuated and gassed with argon. Phenylcyclopropylamine is tipped in, and mixture kept for 20 min. Residual activity is measured as in A.	80
D. Same as C, except that, prior to starting the reaction by addition of substrate, oxygen is bubbled through the enzyme solution.	46

DISCUSSION

The purification of monoamineoxidase continues to remain a problem because of the difficulty in solubilizing the enzyme. Although the procedure described in the present study has not succeeded in solubilizing the enzyme, it has yielded a relatively pure preparation suitable for spectrophotometric studies. The inhibition of monoamineoxidase by phenanthroline would indicate that the enzyme is metal-dependent, although nothing is known concerning the nature of the metal. On the basis of the Lineweaver–Burke plot, and the pH–activity curve, the inhibitory action of phenanthroline would appear to be of the non-competitive type. Since inhibitory chelating agents at low concentrations are known to have an activating effect on enzymes, it is not surprising that phenanthroline was stimulatory at low concentration.

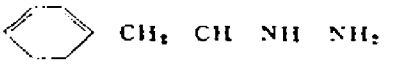
The inhibitory action of both phenylcyclopropylamine and iproniazid is com-

petitive and irreversible. According to SARKAR *et al.*[11], the nature of the interaction of the inhibitor and the substrate with monoamineoxidase is comparable: one hydrogen alpha to the amino group of either the substrate or the inhibitor attaches itself to a common receptor site on the enzyme, and a second hydrogen, also alpha, participates in the process of dehydrogenation.

In the class of hydrazine inhibitors, iproniazid

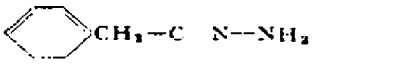
(a)

and benzylhydrazine,



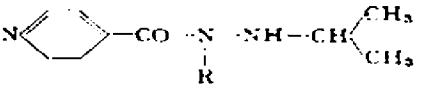
(b)

are strong inhibitors, while compound



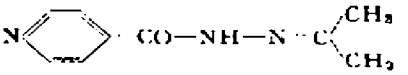
(c)

is not. Substitution in iproniazid of the hydrogen of the N nearest the acyl group makes the compound



(d)

inactive, as does dehydrogenation



(e)

It appears, therefore, that in the case of the hydrazines, the group NH—NH is necessary for inhibition.

BIEL *et al.*[12] reported that a compound of the general formula:

phenanthroline CH_3 CH (CH_3)—NH—N $(CH_3)_2$

is a more potent inhibitor *in vivo* than the corresponding monomethyl derivative,

phenanthroline CH_3 (CH_3) NH—NH—CH_3

If the reactive part of the hydrazine-type inhibitors is associated with the group N–N–C, the results obtained by BIEL are not compatible with those of others, which show that compound (e) is inactive. The ineffectiveness of symmetrical acylhydrazines as inhibitors of monoamineoxidase also points out the need for a hydrogen on the carbon atom of the group N-N-C.

Since the inhibitory action of iproniazid and isopropylhydrazine is oxygen-dependent[9], it was inferred that the NH–CH group underwent dehydrogenation with the resultant formation of an irreversible complex between the dehydrogenated compound and the active site of the enzyme. It is probable, by an extension of this

concept, that it is the NH–NH group which is dehydrogenated, with the formation of an irreversible complex. Since no one of the two possible dehydrogenated compounds is inhibitory in itself, it can be inferred that the activation energy for the formation of the complex would be too high for the unsaturated compound and only after dehydrogenation has taken place at the active center can the complex be formed.

The failure to abolish the inhibitory action of iproniazid in the absence of oxygen might be due to the presence in mitochondria of endogenous hydrogen acceptors capable of replacing oxygen. ZELLER[13] has shown that the inhibition takes place if hydrogen acceptors are added to the enzyme after elimination of oxygen. Since phenylcyclopropylamine is an amine and has a hydrogen available for direct attachment to the enzyme, it can exert an inhibitory action without first undergoing dehydrogenation. It is not possible to entirely explain the increased inhibitory action of phenylcyclopropylamine in the absence of oxygen unless a reduced form of the enzyme is considered more favorable for the binding of the inhibitor. DAVISON[9] concluded that since glutathione diminished the inhibitory effect of iproniazid on monoamineoxidase and that this effect could in part be prevented by cyanide, an –SH group may be involved in the attachment of the inhibitor to the active site of the enzyme. Since *p*-chloromercuribenzoate is not a competitive inhibitor, factors other than –SH groups must be involved at the active site.

The differential action of cyanide on the inhibitory effect of phenylcyclopropylamine and iproniazid would seem to indicate that cyanide was not directly attacking the active center[9]. It is more likely that cyanide, which has an activating effect on the degradation of substrates by monoamineoxidase[4], exerts the same effect on the dehydrogenation of iproniazid by monoamineoxidase, thereby increasing the number of iproniazid molecules in the inhibitory forms. Since phenylcyclopropylamine does not need to be dehydrogenated, cyanide would not alter its inhibitory action.

Notwithstanding that phenylcyclopropylamine and iproniazid are strictly competitive inhibitors, the pH activity curve of the enzyme alone and the enzyme plus either inhibitor are strikingly different. While the curve of monoamineoxidase plus iproniazid is parallel to that of the enzyme alone, this is not the case with phenylcyclopropylamine. A second maximum at pH 7.0 appears with phenylcyclopropylamine, suggesting the presence of two monoamineoxidases not equally sensitive to phenylcyclopropylamine and iproniazid.

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