BBA 65755

STUDIES OF MONOAMINE OXIDASES

I. PURIFICATION AND PROPERTIES OF THE RABBIT LIVER MITOCHONDRIAL ENZYME

SABIT GABAY AND ALFRED J. VALCOURT*

*Biochemical Research Laboratory, Veterans Administration Hospital, Brockton, Mass.** and Department of Biochemistry, Boston University School of Medicine, Boston, Mass. (U.S.A.)*

(Received February 2nd, 1968)

SUMMARY

1. Rabbit liver mitochondrial monoamine oxidase (monoamine:O_2 oxidoreductase (deaminating), EC 1.4.3.4) was purified approx. 270 fold. The purification procedure included isolation of the mitochondrial fraction, solubilization by sonication in the presence of 0.4% isooctylphenoxypolyethoxyethanol, and centrifugation at 12 000 $\times$ g_{max} for 30 min. The supernatant was then fractionated successively on DEAE-cellulose, Biogel P-300, DEAE-Sephadex A-50, and hydroxylapatite columns.

2. Kynuramine was used both to measure enzyme activity in following the course of purification and to carry out kinetic studies. The K_m value of kynuramine was 77 μM at pH 7.4. The pH optimum of the enzyme was about 8.4 with kynuramine and about 9.2 with another substrate, *m*-iodobenzylamine.

3. Highly purified material was used to study some of its properties and the effect of chelating agents. The metal-chelating agents (*o*-phenanthroline and neocuproine) displayed a dual effect on the activity of purified monoamine oxidase after preincubation of the enzyme with chelating agent at 37°. At low concentrations of chelator (2–100 μM), the enzymic activity was greater than without chelators, whereas at higher concentrations of these agents (400 μM and greater), enzyme inhibition took place.

INTRODUCTION

Several integrative and critical reviews make it clear that much remains to be done in the isolation and characterization of homogeneous preparations of amine oxidases[1,2]. Although considerable progress has been made in the area of plasma amine oxidase[3,4], liver or brain monoamine oxidase (monoamine:O_2 oxidoreductase (deaminating) EC 1.4.3.4), being largely associated (70–80%) with the mitochondria[5,6],

* Present address: St. Joseph Hospital, Providence, Rhode Island (U.S.A.).
** Address to which correspondence should be sent.

has defied adequate solubilization necessary for its purification. Furthermore, the recognized multiplicity of this enzyme[7,8] undoubtedly presents a severe limitation which could only be lifted by further studies concerning the purification and physicochemical characterization. Procedures used in an attempt to solubilize the enzyme have achieved varying degrees of success[9–11] and some progress has been made with both beef[12] and rat liver[13] mitochondrial monoamine oxidase.

This report describes the development of a simple and reproducible procedure for the solubilization and purification of rabbit liver mitochondrial monamine oxidase. Partial characterization, consisting of kinetic studies and effect of chelating agents on this purified preparation, is also included. A preliminary communication of part of these findings was previously presented[14].

MATERIALS AND METHODS

Reagents

All reagents were prepared in doubly glass-distilled water. Kynuramine dihydrobromide was purchased from Regis Chemical Co., Chicago, and stored as 0.75 mM stock solution. Absorbancy measurements (scanned from 500 to 250 mμ) and other physical characteristics were found to be identical to those described by WEISSBACH *et al.*[15]. Similar checks at frequent intervals indicated that the stock solutions were very stable when kept at 2–4°. Tyramine · HCl and serotonin creatinine sulfate (sodium salt) were purchased from Mann Research Laboratories, New York; and isooctylphenoxypolyethoxyethanol (Cutscum) from Fisher Scientific Co., Boston. *m*-Iodobenzylamine · HCl was a gift from Dr. WILLIAM F. BRUCE of Wyeth Laboratories, Inc., Philadelphia, who synthesized it beginning with *m*-iodotoluene, following essentially the steps of ZELLER, RAMACHANDER AND ZELLER[16] and the reference cited therein. *m*-Iodobenzylamine was purified by recrystallization from absolute ethanol; m.p. 192–193°, literature[17] m.p. 193°. Parenthetically, we would like to draw attention to the fact that the commercially available products were found to be completely unsatisfactory. The K and K Labs (New York) sample melted at 145–147°, and those of Aldrich Chemical Co. (Milwaukee) and Sapon Labs (New York) HCl salt melted at 165–167°, and 161–163°, respectively. The former's ultraviolet absorption was not found in the right region (marked drop in absorbance at 253 mμ), and both the infrared and NMR analysis indicated a strong *N*-methyl group (F. BRUCE, private communication). As ZELLER[16,18] indicated, the high degradation rate of *m*-iodobenzylamine and the strong absorbance at 253 mμ of the *m*-iodobenzyaldehyde formed by the action of monoamine oxidase were found to be extremely sensitive indeed (unpublished data). DEAE-Sephadex A-50 was obtained from Pharmacia Fine Chemicals Inc., New Market, New Jersey; DEAE-cellulose, Biogel P-300 and Biogel HTP (hydroxylapatite powder) were procured from Bio-Rad Laboratories, Richmond, Calif. The metal-chelating agents: 1,10-phenanthroline monohydrate (*o*-phenanthroline), 2,9-dimethyl-1,10-phenanthroline (neocuproine) and bis-cyclohexanone oxaldihydrazone (cuprizone) were products of Frederic Smith Chemical Co., Columbus, Ohio.

Buffers

Phosphate buffers were always prepared from K_2HPO_4–KH_2PO_4. In the majority of studies this buffer contained 0.4% Cutscum. For convenience, therefore, it

was designated as 'Buffer I'. The pH used throughout was 7.6, unless noted otherwise.

Isolation of mitochondrial fraction

Fresh liver was obtained from male New Zealand white rabbits (Camm Research Laboratories, Wayne, New Jersey) decapitated with a guillotine (Harvard Apparatus Co. Inc., Cambridge, Mass.). Livers were removed immediately after decapitation and washed several times in ice-cold 0.25 M sucrose (Merck and Co.). The livers were trimmed free of fat and adjoining tissues, cut into small pieces, weighed and homogenized with a motor-driven Teflon pestle for 2 min at approx. 600 rev./min in a chilled Potter–Elvehjem type glass homogenizer (Kontes: 0.005–0.007 inch clearance) with sufficient unbuffered ice-cold 0.25 M sucrose. The volume was adjusted to give a 10% (w/v) homogenate and centrifuged at 4° for 10 min at 600 × g* (International PR-2). The sediment was washed with 0.25 M sucrose in half the homogenizing volume and the suspension recentrifuged in a similar manner. All the supernatants were combined and centrifuged at 4° in a Spinco Model L-2 ultracentrifuge at 15 000 × g for 15 min. The sediment containing the particle-bound monoamine oxidase was washed with 0.25 M sucrose in one-fourth of the homogenizing volume and the suspension recentrifuged in the Model L-2 at 10 000 × g for 15 min. This step was repeated and the final pellet resuspended in sufficient 0.01 M Buffer I to make 1 ml equivalent to 2 g original tissue. This suspension was then stored in the frozen state (−20°), usually overnight.

Assay of monoamine oxidase activity

Quantitative assay methods have been described based on the uptake of O_2, or the liberation of NH_3, but none of these is entirely satisfactory for studies of the kinetics of the reaction. The manometric method is relatively insensitive and complicated by secondary reactions. Measurement of NH_3 liberated may provide a reliable and sensitive assay under certain conditions. Both of these methods, however, require a prohibitive amount of purified enzyme. Thus, we have adopted the kynuramine method of WEISSBACH *et al.*[15] which offers a very convenient means of measuring the enzyme activity and following the course of purification. Moreover, kynuramine was found[9] to be a better substrate, particularly when working with chelating agents such as phenanthroline whose absorbance coefficient at 250 mμ is extremely high (benzylamine and its analogues are measured at 250 mμ). The assay was carried out at 37° rather than 30°, unless otherwise indicated. Details of the reaction mixture are given in the legend to Fig. 3. The Beckman Model DB spectrophotometer, equipped with a thermospacer attached to a Haake water-circulating pump, was used to measure the disappearance of substrate. The Gilford Model 2000 multiple sample absorbance recorder with a Beckman Model DU monochromator equipped with thermospacer was used for spectrophotometric assays which required high concentrations of kynuramine.

Protein determination

Protein was determined by the method of LOWRY *et al.*[19] with crystalline bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as the standard. The standard was assayed in the presence of Cutscum and phosphate to compensate for interference

* Gravitational forces are given as g_{max}.

by these substances[20]. The unit of monoamine oxidase activity is defined as the amount which catalyzes the oxidative deamination of 1 μmole of kynuramine in 10 min at 37° (pH 7.4). Specific activity is expressed as the number of enzyme units per mg of protein.

RESULTS

Purification of mitochondrial monoamine oxidase

Unless otherwise specified, all operations were carried out in a cold room with an ambient temperature rigidly kept at 2–4°.

Step 1. Solubilization. Several attempts to extract sufficient amounts of particle-bound mitochondrial monoamine oxidase have been reported. Although no extensive comparative study was made, we have adopted the use of Cutscum, a non-ionic detergent, in the solubilization at a relative concentration of 0.4%. Its advantages will be discussed later.

The frozen mitochondrial preparation was thawed, gently homogenized by hand (5–10 passes of the pestle), and then sonicated in a Raytheon sonic oscillator (250 W, 10 kcycles/sec) cooled by circulating ice water. Optimum results were achieved by sonicating aliquots of 20 ml or less at maximum power (10 kcycles/sec) for 3 min. Too large a volume of sample and too long a sonication time resulted in greater losses of monoamine oxidase activity. The sonicated material was then centrifuged at 12 000 × *g* for 30 min. The resultant supernatant, designated as Prep. A, was found to contain 90% or more of the enzyme present in the mitochondrial fraction before sonication and was deemed satisfactory for purification by column chromatography. It is of interest to note that sonication in the presence of kynuramine followed by dialysis did not improve the recovery of the solubilized enzyme. Prep. A was found to be very stable over a period of at least 90 days when maintained at −20°.

Step 2. Chromatography on DEAE-cellulose. The adsorbent (6 g per 200 mg protein) was suspended in a 30-fold volume of 0.02 M phosphate buffer, allowed to settle for 15 min and the finer particles decanted. This treatment was repeated. The DEAE-cellulose slurry was then poured into a glass column (Kontes glass: 2.5 cm × 50 cm), and the adsorbent permitted to settle by gravity. Prep. A was applied to the column and 5-ml fractions of the effluent were collected with the aid of an automatic fraction collector (Gilson Medical Electronics).

The column was first washed with 0.02 M phosphate buffer, using a volume (in ml) numerically equivalent to 0.2 of the column volume. Elutions were started with a 0.3 volume of 0.02 M Buffer I and followed by a 0.5 volume of the same buffer containing increasing molarities of NaCl. The concentrations of NaCl used successively were: 0.01 M, 0.07 M, 0.1 M and 0.5 M. The elution rate was maintained at 1 ml/min up to the passage of 0.07 M NaCl, then adjusted to 0.5 ml/min.

The elution profile of the DEAE-cellulose column showed almost complete adsorption of monoamine oxidase. Although considerable protein was present in the fractions which followed the void volume, no monoamine oxidase was eluted until the passage of 0.02 M Buffer I containing 0.1 M NaCl. It was at this stage that the monoamine oxidase with the highest specific activity appeared. Fractions containing the major enzymic activity were combined and concentrated to nearly 3-fold (Step 2, concentrate) in the Amicon ultrafiltration cell equipped with a membrane having a 40 000 mol. wt. solute cutoff, as described by BLATT *et al.*[21]. We have found this method

very satisfactory for concentrating dilute protein solutions. Practically no loss of monoamine oxidase activity occurred during this procedure.

Step 3. Gel filtration with Biogel P-300. Sufficient Biogel P-300 was allowed to swell for 24 h in 0.02 M Buffer I. Excess buffer was decanted, then the gel poured all at once into a column (2.5 cm × 40 cm) and permitted to pack by gravity. Routinely, 3 g of gel powder resulted in a column-bed height of 20–25 cm, on which 15–20 ml of the "Step-2 concentrate" was applied. Elution was carried out with the same buffer, the flow rate was maintained at 10 ml/h, and the effluent was collected as 5-ml fractions. Eluates containing the highest specific activity were pooled. This step not only served to increase the purity of the preparation but also desalted it sufficiently for subsequent chromatography on DEAE-Sephadex.

Step 4. Chromatography on DEAE-Sephadex A-50. Prior to its use the gel was allowed to swell in water for at least 2 h, then washed successively with 100 ml of 0.5 M HCl, water, 0.5 M NaOH, water, 0.5 M HCl, and sufficient 0.02 M phosphate buffer. The slurry was then poured into a column (2.0 cm × 25.0 cm) and allowed to settle by gravity. In most of the purifications, an 11-cm column-bed height was used per 30 mg protein. The combined eluate obtained from Step 3 was applied, and elutions were initiated with 1 volume of both 0.02 M Buffer I and the same buffer containing 0.01 M NaCl. The column was next eluted with 0.02 M Buffer I containing 0.07 M and 0.1 M NaCl, respectively. In each case 1.5 column volumes were used. The final elution consisted of a passage of one column volume of the same buffer containing 0.5 M NaCl.

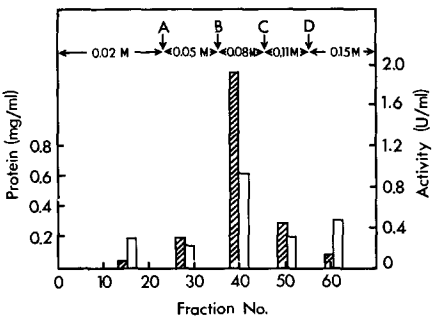
The elution pattern of this column showed a major peak of monoamine oxidase activity during the passage of the buffer which contained 0.07 M NaCl. Only those fractions possessing the highest specific activities were combined and immediately used in the subsequent step. It is noteworthy that at this stage the enzyme was less stable than at any other.

Step 5. Chromatography on hydroxylapatite. Biogel hydroxylapatite (0.5 g of dry powder per mg protein) was twice suspended in 10 volumes 0.02 M Buffer I, and allowed to settle for at least 15 min. The supernatant was decanted, the sediment resuspended in the same buffer, and the slurry poured into a column (2 cm × 25 cm). The column was then equilibrated by passing 2 column volumes of 0.02 M Buffer I, and subsequently Step-4 eluate was applied. Stepwise elutions were carried out with increasing molarities of Buffer I as described in the legend to Fig. 1. As seen in this figure, 0.08 M Buffer I eluted the highly purified monoamine oxidase preparation (designated as Prep. B) as an abrupt and narrow zone. This fact was observed repeatedly with the six preparations so far made. The results of a typical purification sequence are summarized in Table I.

Enzyme properties

Only Prep. B, that is the fractions emerging at 0.08 M Buffer I, which had an average specific activity of 20, was used throughout the studies herein reported. These final preparations were always clear and appeared slightly yellowish.

1. K_m and inhibition by high substrate concentrations. Apparent Michaelis constants (K_m) were graphically derived from LINEWEAVER–BURK plots[22]. As illustrated in Fig. 2, the K_m for kynuramine oxidation at 37°, pH 7.4, was routinely of the order of 80 μM. As is evident, at low concentrations of the substrate K_m obeys the Michaelis–Menten equations. At relatively high substrate concentrations, however,



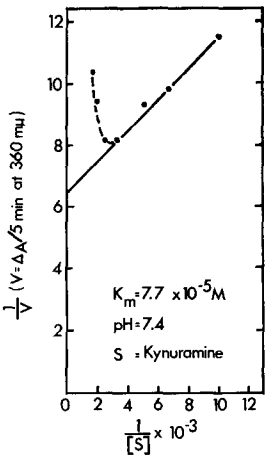


Fig. 1. Typical elution profile of enzyme on hydroxylapatite. Cross hatched bars show monoamine oxidase activity while other bars show protein concentration. Elution was initiated with 0.02 M phosphate buffer, then continued in stepwise fashion with 0.5 M (A), 0.08 M (B), 0.11 M (C), and 0.15 M (D) phosphate buffer. All buffers were adjusted to pH 7.6 and contained 0.4% Cutscum. Fractions of 3 ml (Fractions 22–63) and 5 ml (all others) were collected. Each bar represents the average of all fractions in the indicated elution step.

Fig. 2. Double reciprocal plots of activity *versus* substrate concentration for kynuramine at 37° (pH 7.4). Aliquots of the purified enzyme were added to 0.3 ml of 0.5 M phosphate buffer followed by the addition of various concentrations of kynuramine (as shown in the abscissa). The total volume of each reaction mixture was brought up to 3.0 ml with distilled water. The velocity is expressed as the change in absorbance at 360 mμ during a reaction time of 5 min. All measurements were made in the Gilford Model 2000 Multiple Sample Absorbance Recorder.

the velocity of the reaction declines. Thus, the enzyme is inhibited by high substrate concentrations. When the experiment was repeated at 25° (pH 7.4), high concentrations of kynuramine induced a similar inhibition. The K_m value was of the same order of magnitude. Although it would thus seem that the active center of the rabbit mito-

TABLE I

SUMMARY OF A TYPICAL PURIFICATION

Step	*Fraction*	*Volume (ml)*	*Units**	*Protein (mg/ml)*	*Specific activity (units/mg)*	*Purification (-fold)*
I	Mitochondrial fraction	77	855	23.4	0.49	4.9**
II	12 000 × g_{max} supernatant (Prep. A)	68	595	20.4	0.43	4.3
III	DEAE-cellulose	50	218	4.7	0.94	9.4
IV	Biogel P-300	27	153	2.7	2.18	21.5
V	DEAE-Sephadex	58	80	0.15	9.15	90.5
VI	Hydroxylapatite (Prep. B)	24	38	0.065	24.0	238

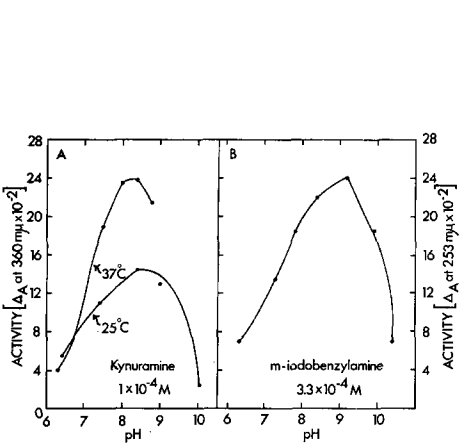
* Defined as the amount of monoamine oxidase which catalyzes the oxidative deamination of 1 μmole of kynuramine in 10 min at 37° (pH 7.4).

** It represents approximately 5-fold purification over the original starting material (10% w/v homogenate had a specific activity of 0.10).

chondrial enzyme consists of more than one site for the interaction with substrate[23], these findings should be further evaluated as to their bearing on the issue of single *versus* multiple monoamine oxidases[24].

2. Activity as function of pH. The effect of pH on the reaction velocity at 37° was investigated over the pH range of 6.4 to 9.0; a maximum activity was observed in the range of 8.0 to 8.4. Since the reaction rate at this temperature, in the pH range above 7.4, was too rapid to permit convenient measurement (the reaction appeared to be approaching completion as judged by a departure from linearity after 2 min), these experiments were repeated at 25° where the reaction rate was sufficiently slow to permit measurement up to 20 min. The results are depicted in Fig. 3A. As it can be seen, the enzyme exhibited a maximum activity in the same range (around 8.4). Similar results were obtained when these studies were carried out with *m*-iodobenzylamine, a substrate structurally dissimilar to kynuramine (pH optimum was around 9.2: see Fig. 3B).

3. Stability of the enzyme. No significant loss in enzyme activity was detected when preparations were kept in the frozen state (−15°) for 30 days. Similar storage of the dilute preparations, however, resulted in an appreciable loss of activity. Preincubation of the enzyme at pH 7.4 in the absence of substrate resulted in a rapid loss of activity with the temperature fixed at both 37° and 25°. As is shown in Fig. 4, this temperature-dependent property was found to be a function of preincubation time. When depicted in this manner the data fit the curve for a first order reaction, thus permitting calculation of the following rate constants (k): (1) no preincubation,



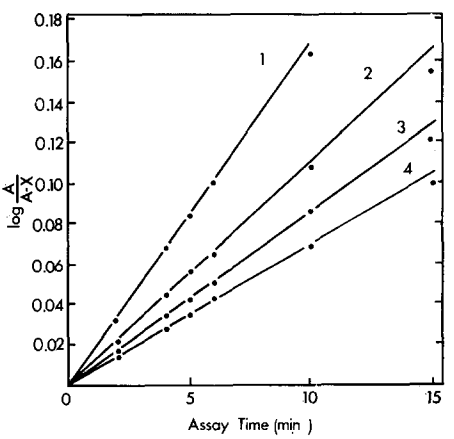


Fig. 3. pH–activity curves of highly purified monoamine oxidase. A. The standard reaction mixture consisted of 0.3 ml of 0.5 M phosphate buffer (pH 7.4), an aliquot of enzyme (0.1–0.5 ml) and 0.4 ml of 750 μM kynuramine (final concentration, 100 μM) in a total volume of 3.0 ml adjusted with water. In this experiment the pH of buffer was varied as shown, and the enzymatic activity was determined at 25° and 37°. B. The reaction mixture consisted of 0.2 ml of enzyme solution and 2.8 ml of 350 μM *m*-iodobenzylamine (final concentration, 330 μM) in 0.067 M phosphate buffer. The pH of the buffer was varied as shown, and the enzymic activity measured at 25°.

Fig. 4. Effect of preincubation on monoamine oxidase. The incubation mixture and assay procedure were identical with that described for Fig. 3, except that the enzyme (6 μg protein, specific activity 19.0) was preincubated for 0 (curve 1), 5 (curve 2), 10 (curve 3) and 15-min (curve 4) periods prior to the addition of the substrate which was added always last to initiate the reaction. The calculated values of log $a/(a - x)$ have been plotted against assay time (min).

0.040 min^{-1}; (2) 5-min preincubation, 0.025 min^{-1}; (3) 10-min preincubation, 0.020 min^{-1}; and (4) 15-min preincubation, 0.016 min^{-1}.

4. Effect of metal-chelating agents. In all, five separate experiments were carried out. Each set of conditions was run at least in duplicate. The results of various experiments are summarized in Table II. As it can readily be seen *o*-phenanthroline and neocuproine display a dual effect. At higher concentrations, *o*-phenanthroline (1.0–5.0 mM final concentration) inhibits the oxidation of kynuramine, whereas at lower concentrations (23–330 μM) this agent enhances, or "activates", the enzyme activity. For instance, a concentration of 23 μM induces a net percent change as high as 77%,

TABLE II

EFFECT OF METAL-CHELATING AGENTS ON MONOAMINE OXIDASE ACTIVITY

The chelating agent was preincubated for 10 min at 37° with the enzyme (specific activity 19.0) prior to the addition of substrate under standard assay conditions. Percent change values were calculated on the basis of residual activity after preincubation of the enzyme without these agents.

Chelating agent	*Final concn. (μM)*	*% change*
o-Phenanthroline	23.3	+77
	100	+66
	330	+17
	1000	−23
	2000	−55
	5000	−72
Neocuproine	2	+45
	20	+31
	100	+2.7
	800	−31.5

calculated on the basis of residual activity after preincubation of the enzyme without phenanthroline. Although neocuproine yields similar results, an inhibitory effect can be achieved with lower concentrations than those used with *o*-phenanthroline. It is interesting to note that when the enzyme was assayed in the presence of lower concentrations of either chelating agent without preincubation, there was little or no enhancement of enzymic activity.

In addition, cuprizone, a highly specific Cu^{2+} chelator[25], was used to provide indirect evidence for the valency of the copper ion. This reagent at a final concentration of 700 μM produced 32% inhibition, whereas at a concentration of 10 μM no significant inhibition could be observed. On the other hand, EDTA at various concentrations (1–1000 μM) neither increased nor decreased the residual enzyme activity.

5. Miscellaneous properties. (a) Effect of sulfhydryl inhibitor: It was found that in the presence of 10 μM (final concentration) of *p*-chloromercuribenzoate the deamination of kynuramine was inhibited nearly 100%. (b) Absorption spectrum: When the enzyme (0.610 mg protein per ml) was scanned from 550 mμ to 300 mμ in a DB spectrophotometer, a maximum was present at 412 mμ (absorbancy 0.520). Although the detergent, at the concentration used, did not affect the kinetic behavior of the enzyme under the various conditions studied, its presence, nevertheless, precluded the scanning

of the enzyme in the ultraviolet range (Cutscum exhibits a very high absorbancy below 300 mμ).

DISCUSSION

The purification and determination of the properties of highly purified rabbit liver mitochondrial monoamine oxidase, as described in the present study, were the result of numerous attempts to devise the most effective and reproducible operational sequence for its preparation. Generally, the overall yield did not exceed 5%. This compares favorably with the yields obtained by NARA, GOMES AND YASUNOBU[12] and MCEWEN[4] in their beef-liver mitochondria and human plasma preparations, respectively.

Previous attempts to separate monoamine oxidase from insoluble structures have either failed or yielded only partially purified preparations of a very low specific activity[9–11,26,27]. Thus, the use of detergents has been generally accepted as an indispensable tool in order to bring the enzyme into solution[10–12]. The choice of Cutscum as a solubilizing agent was dictated by both its ability to release 90%, or better, of the particle-bound enzyme (when sonicated as described in RESULTS) and its non-interference with the enzyme activity when used at a relatively low concentration of 0.4%. Higher concentrations (*e.g.* 2%) of this non-ionic detergent, as used by other workers[26,28], followed by high-speed centrifugation, were found to extract from 20% to 70% of the total enzymic activity into the supernatant. The values for "physical release" (solubilization) afforded by treatment with Triton X-100 (refs. 9, 12) were not satisfactory in this case.

Since all enzyme measurements during the enzyme purification procedures were made at pH 7.4, it is likely that the specific activity reported in Table I for the most highly purified enzyme, possessing a pH optimum in the range of 8.4, is higher than the degree of purification stated. We realize that when only a single substrate is used for estimating monoamine oxidase activity, a great deal of valuable information may be overlooked. Preliminary studies (unpublished observations) have been carried out on substrate specificity with the limited amount of purified material available and with selected fractions from the purification sequence (as a method of measurement, we employed the microdiffusion technique of CONWAY[29] for determining NH_3 production). Oxidation of tyramine was demonstrated in practically all fractions tested, including the highly purified material, when precautions were taken to minimize discrepancies arising from the use of various methods in comparing results. The validity of the methods used in substrate oxidation was particularly instructive, for we interpret as indicative that the choice of substrates may have to be modified whenever enzymic activity is tested under significantly different conditions. From a methodological standpoint, however, it would appear that kynuramine constitutes an ideal substrate for assay of general monoamine oxidase activity and kinetic studies.

The effect of pH on the standard reaction was investigated at 37° with both kynuramine and *m*-iodobenzylamine. The reaction rate of kynuramine, at this temperature, was quite rapid especially near the pH optimum. Owing to the observed inhibition induced by high concentrations of kynuramine (*cf.* Fig. 2), and to the fact that a substrate concentration which is sufficient to saturate an enzyme at one pH, will not necessarily do so at another[30], these experiments were repeated at 25°. Since the pH–

activity profile (*cf.* Fig. 4) appears to be unaffected by temperature, the validity of these experiments was thus confirmed. It is noteworthy that our findings resemble closely those reported with a highly purified beef liver monoamine oxidase preparation[12] and differ sharply from those obtained by BARBATO AND ABOOD[9] for 10-fold purified enzyme of the same species.

Our highly purified monoamine oxidase was very sensitive to the action of *p*-chloromercuribenzoic acid (nearly 100% inhibition with 10 μM). This is in marked contrast to the preparation of NARA, GOMES AND YASUNOBU[12] which was inhibited only 37.5% by 5 mM *p*-chloromercuribenzoic acid. This may be because our preparation was subjected to multiple column chromatography. Such treatment, according to GORKIN[31], causes a distinct increase in sensitivity of highly purified monoamine oxidase preparations.

The present study does not establish whether monoamine oxidase activity is due to a single enzyme with multiple active sites, or to a family of isoenzymes. The most unequivocal proof of such isoenzymes is their fractionation and the demonstration of a narrower substrate specificity or a wider difference in the enzyme/substrate ratio. The behavior of the enzyme on Biogel P-300 may be a clue to the magnitude of its molecular weight. Its appearance in the eluate immediately after the void volume indicates a molecular weight of 200 000–300 000.

The results obtained with metal-chelating agents indicate the involvement of a metal ion in the enzyme function. The available data are consistent with a role of Cu^{2+} or other metal ions. However, it is not necessary to conclude with certitude that a divalent metal ion is a functional component of the catalytically active center, since the metal ion, as is the case of many oxidative enzymes[32,33], may be involved in the maintenance of an effective structure of the enzyme. The dual effect of *o*-phenanthroline and neocuproine on the highly purified enzyme has never been described in studies with plasma[4], beef liver[12], or pea seedling[34] monoamine oxidase preparations. Thus, in this respect rabbit liver mitochondrial monoamine oxidase differs from those preparations. Obviously, the "activation" of the enzyme by chelators is subject to many factors, so that any one simple mechanism is almost certain to prove insufficient to depict the phenomenon in general. The "activation" by *o*-phenanthroline and neocuproine observed by us bears a certain resemblance to that observed by VAN EYS, CIOTTI AND KAPLAN[35]. Alternatively, the resultant inhibition at relatively higher concentrations of these agents implicates metals in enzymic catalysis, since they diminish the catalytic activity by binding to the metal. In this sense, the metal would be firmly incorporated into the enzyme structure and is therefore enzymically active as a native label of an active site[36]. That the addition of low concentrations of certain agents may increase activity due to the removal of traces of inhibiting or contaminating metals by chelating agents has been reported[37]. This is certainly not the case with our enzyme, for when concentrations much higher were used to exert this effect, the activity remained unchanged. For instance, *o*-phenanthroline and neocuproine at concentrations of 400 μM and 90 μM, respectively, were devoid of the ability to exert any effect when preincubated with the reaction mixture. It would thus appear, as VALLEE[38] has emphasized, that generally when the metal–chelate complex is in equilibrium with metal ions and chelating agents no change in enzymic activity should be observed. In the absence of studies concerning the metal composition (qualitative and quantitative), and the effect of Cu^{2+} and other metal ions, in order to enhance the

significance of this phenomenon, as well as detailed kinetic studies, an interpretation *a priori* is obviously very difficult. These points will have to be dealt with in future work.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. N. N. VALANJU, a member of this laboratory, for carrying out the experiments with metal-chelating agents. Thanks are also due to Mr. D. L. MACKEEN for his skilful technical assistance at the preliminary phases of this study.

REFERENCES

1 H. BLASCHKO, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 8, 2nd ed., Academic Press, New York, 1963, p. 337.
2 E. A. ZELLER AND J. R. FOUTS, *Ann. Rev. Pharmacol.*, 3 (1963) 13.
3 H. YAMADA AND K. T. YASUNOBU, *J. Biol. Chem.*, 237 (1962) 1511.
4 C. M. MCEWEN, JR., *J. Biol. Chem.*, 240 (1965) 2003.
5 P. BAUDHUIN, H. BEAUFAY, Y. RAHMAN-LI, O. Z. SELLINGER, R. WATTIAUX, P. JACQUES AND C. DE DUVE, *Biochem. J.*, 92 (1964) 179.
6 E. O. OSWALD AND C. F. STRITTMATTER, *Proc. Soc. Exptl. Biol. Med.*, 114 (1963) 668.
7 W. HARDEGG AND E. HEILBRONN, *Biochim. Biophys. Acta*, 51 (1961) 553.
8 V. Z. GORKIN, *Abstr. 6th Intern. Congr. Biochem. New York, 1964*, IUB Vol. 32, p. 309.
9 L. M. BARBATO AND L. G. ABOOD, *Biochim. Biophys. Acta*, 67 (1963) 531.
10 V. Z. GORKIN, *Nature*, 200 (1963) 77.
11 S. R. GUHA AND C. R. KRISHNA MURTI, *Biochem. Biophys. Res. Commun.*, 18 (1965) 351.
12 S. NARA, B. GOMES AND K. T. YASUNOBU, *J. Biol. Chem.*, 241 (1966) 2774.
13 M. B. H. YOUDIM AND T. L. SOURKES, *Can. J. Biochem.*, 44 (1966) 139.
14 S. GABAY, A. J. VALCOURT AND D. MACKEEN, *Federation Proc.*, 25 (1966) 319.
15 H. WEISSBACH, T. E. SMITH, J. W. DALY, B. WITKOP AND S. UDENFRIEND, *J. Biol. Chem.*, 235 (1960) 1160.
16 V. ZELLER, G. RAMACHANDER AND E. A. ZELLER, *J. Med. Chem.*, 8 (1965) 440.
17 S. L. SHAPIRO, E. S. ISAACS, V. BANDURCO AND L. FREEDMAN, *J. Med. Pharm. Chem.*, 5 (1962) 793.
18 E. A. ZELLER, *Ann. N.Y. Acad. Sci.*, 107 (1963) 811.
19 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
20 E. G. BRUNNGRABER AND V. AGUILAR, *J. Neurochem.*, 9 (1962) 451.
21 W. F. BLATT, M. P. FEINBERG, H. B. HOPFENBERG AND C. A. SARAVIS, *Science*, 150 (1965) 224.
22 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
23 M. DIXON AND E. C. WEBB, *Enzymes*, Academic Press, New York, 1964, p. 75.
24 J. L. WEBB, *Enzymes and Metabolic Inhibitors*, Academic Press, New York, 1963, p. 112.
25 R. PETERSON AND M. BOLLIER, *Anal. Chem.*, 27 (1955) 1195.
26 G. C. COTZIAS, I. SERLIN AND J. J. GREENOUGH, *Science*, 120 (1954) 144.
27 T. E. SMITH, H. WEISSBACH AND S. UDENFRIEND, *Biochemistry*, 1 (1962) 137.
28 E. A. ZELLER, J. BARSKY, E. R. BERMAN, M. S. CHERKAS AND J. R. FOUTS, *J. Pharmacol. Exptl. Therap.*, 124 (1958) 282.
29 E. J. CONWAY, *Microdiffusion Analysis of Volumetric Analysis and Volumetric Error*, 4th ed., Crosby, Lockwood and Sons, London, 1957.
30 J. M. REINER, *Behavior of Enzyme Systems*, Burgess, Minnesota, 1959, p. 57.
31 V. Z. GORKIN, *Pharmacol. Rev.*, 18 (1966) 115.
32 E. FRIEDEN, in M. KASHA AND B. PULLMAN, *Horizons in Biochemistry*, Academic Press, New York, 1962, p. 461.
33 G. R. STARK AND C. R. DAWSON, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 8, Academic Press, New York, 1963, p. 297.
34 P. J. G. MANN, *Biochem. J.*, 79 (1961) 623.
35 J. VAN EYS, M. M. CIOTTI AND N. O. KAPLAN, *J. Biol. Chem.*, 231 (1958) 571.
36 B. L. VALLEE, *Advan. Protein Chem.*, 10 (1955) 317.
37 H. E. REDETZKI AND W. W. NOWINSKI, *Nature*, 179 (1957) 1018.
38 B. L. VALLEE, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 3, Academic Press, New York, 1960, p. 225.