Ornithine Decarboxylase from Calf Liver. Purification and Properties†

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ABSTRACT: Ornithine decarboxylase (ODC) was purified 25 000-fold from calf liver to apparent homogeneity by methods developed to circumvent the lability of the enzyme. Appropriate ratios of sample protein applied to column size and/or gradient size were derived for each purification procedure (ion-exchange, gel filtration and hydroxylapatite chromatography, electrophoresis, and thiol affinity chromatography) to maintain enzymatic activity. The enzyme was labile to dilution at all steps of the purification; the inclusion of poly(ethylene glycol) or additional protein decreased but did not eliminate the activity loss. The purified enzyme had a Stokes radius of 3.14 and a molecular weight of 54 000. The K_m for ornithine was 0.12 mM, and pyridoxal phosphate was 2.0 μM; the pH optimum for the decarboxylation reaction was 7.0. Analysis by sievorptive ion-exchange chromatography indicated the presence of three ionic forms. In the presence of Tris–barbital buffer containing thioglycolic acid, the ODC preparation assumed an apparent molecular weight of 100 000 and a Stokes radius of 4.5 and retained full catalytic activity.

Ornithine decarboxylase (ODC, EC 4.1.1.17) catalyzes the decarboxylation of ornithine to putrescine, the initial and rate-limiting step in polyamine biosynthesis. The pathway is not controlled primarily by changing the precursor level or by any known allosteric regulation of the decarboxylating enzyme but rather by rapidly fluctuating changes in the amount of cellular ODC[1] [reviewed by Russell & Durie (1978); Williams-Ashman & Canellakis (1979)]. Elevated levels of ODC are characteristic of rapidly growing eucaryotic cells, and changes in enzyme activity occur in parallel with changes in the growth state of the tissue (Jänne et al., 1978; Russell & Haddox, 1979). The induction of ODC is a ubiquitous and early change in the anabolic response of target tissues to trophic hormones. The increase in ODC in a hormone-stimulated tissue is usually transient, a reflection of the 10–15-min half-life of ODC, the shortest of any enzyme studied to date (Russell & Snyder, 1969). Nongrowing cells contain small or nondetectable ODC activity and appear to be growth arrested prior to the events required for induction of the enzyme. Certain physiologic growth-arrest signals, such as vitamin A, appear to regulate proliferation by acting to limit expression at or before this site (Haddox & Russell, 1979; Haddox et al., 1979). Furthermore, alterations in the regulation of ODC induction and expression in growing cells are associated with the transformed state (Lembach, 1974; Bethell & Pegg, 1979; Isom & Backstrom, 1979; Haddox et al., 1980).

Although several reports dealing with the purification of ODC have appeared (Jänne & Williams-Ashman, 1971; Friedman et al., 1972; Ono et al., 1972; Boucek & Lembach, 1977; Obenrader & Prouty, 1977), the detailed physical, chemical, and biochemical characteristics of the enzyme are still not well-defined due to the difficulty of obtaining tangible amounts of active enzyme with sufficient purity for such studies. Even in the induced state, the ODC present constitutes a very small fraction of the eucaryotic cell protein. Furthermore, the enzymatic activity, even in a semipurified state, is extremely labile (Pegg & Williams-Ashman, 1968). In this paper, we report a method of purification of ODC from calf liver 25 000-fold to apparent homogeneity by a procedure developed to circumvent the lability of the enzyme as well as the results of a number of characterization studies performed on the purified protein.

Experimental Procedures

Animals. Calves (male, 40–60 kg) were obtained from the University of Arizona Dairy Farm and were injected (ip) with 150 mg/kg thioacetamide (in 50% ethanol, pH 7.0). Eighteen hours later they were sacrificed and the livers excised.

Chemicals. L-[1-^{14}C]Ornithine hydrochloride (54 mCi/mmol) was purchased from Amersham/Searle Corp. or New England Nuclear. DTT, PYP, PMSF, PEG (M_r 20 000), EDTA, thioglycolic acid, mercaptoethanol, glutathione, putrescine dichloride, monothioglycerol, BSA, thioacetamide, EGTA, ovalbumin, transferrin, and all the buffers, nucleotides, metals, and metal chelators employed in this study were purchased from Sigma. 4,7-Phenanthroline was obtained from K & K Laboratories. Reagents and chemicals for electrophoresis were obtained from Bio-Rad. Sodium dodecyl sulfate was purchased from Pierce, ultrapure sucrose and glycerol were from Bethesda Research Laboratory, and hemocyanin was from Calbiochem.

DEAE-Sephacel, activated thiol-Sepharose 4B, and DEAE-Sephadex A-25 were obtained from Pharmacia. Ultrogel AcA 44 (4% acrylamide, 4% agarose) and Ultrogel HA (hydroxylapatite) were obtained from LKB. Reference proteins used for Stokes' radius determination or molecular weight estimation were [*methyl*-^{14}C]albumin, -ovalbumin, -carbonic anhydrase and -cytochrome *c* from New England Nuclear. YM-20 Diaflow membranes were purchased from Amicon.

Buffers. All pH values were determined at 25 °C. The standard purification buffer used was 50 mM Na_2H–KH_2PO_4, pH 7.2, 5 mM NaF, 0.06 mM PYP, and 0.1% PEG containing

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[1] Abbreviations used: ODC, ornithine decarboxylase; DTT, dithiothreitol; PYP, pyridoxal phosphate; PMSF, phenylmethanesulfonyl fluoride; PEG, poly(ethylene glycol); EDTA, ethylenediaminetetraacetic acid disodium salt; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; barbital, 5,5′-diethylbarbituric acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; BSA, bovine serum albumin; DDC, diethyldithiocarbamic acid; Bis, *N,N′*-methylenebis(acrylamide); TEMED, *N,N,N′,N′*-tetramethylethylenediamine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid; DEAE, diethylaminoethyl; Mes, 2-(*N*-morpholino)ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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Table I: Purification of Calf Liver Ornithine Decarboxylase

	times performed	total protein (mg)	total act. (milliunits)	sp act.[a] (pmol min^{-1} mg^{-1})	rel purification (fold)	yield (%)
20000*g* supernatant	6	484800	1939	12		
(1) DEAE-Sephacel	6	3864	2094	542	45	108
(2) Ultrogel AcA 44	12	387	1260	3252	271	65
(3) hydroxylapatite	12	77	756	9756	813	39
(4) Tris-barbital electrophoresis	4	3.4	407	117072	9756	21
(5) thiol covalent	2	0.9	263	292680	24390	14

[a] Specific activities listed are the average values obtained from all the preparations.

DTT, EDTA, and PMSF at the concentrations noted. PYP and DTT were made fresh monthly, stored frozen as concentrated stock solutions (10 and 100 mM, respectively), and added to the buffer on the day of use. PMSF was stored at 4 °C (250 mM in ethanol) and was added to a final concentration of 1 mM immediately before use as indicated.

Ornithine Decarboxylase Assay. ODC activity was determined by measuring the liberation of $^{14}CO_2$ from L-[1-^{14}C]ornithine (Russell & Snyder, 1968). Incubations were conducted at 37 °C in the presence of 50 mM Na_2H–KH_2PO_4, pH 7.2, 0.1 mM EDTA, 2 mM DTT, 5 mM NaF, 0.06 mM PYP, and 0.1% PEG. Under these conditions, the reaction velocity was constant with respect to time (up to 60 min), although not always proportional to enzyme concentration (see below). The reaction was initiated by the addition of L-[1-^{14}C]ornithine (specific activity 5 mCi/mmol) to a final concentration of 0.5 mM. Pyridoxal phosphate independent release of $^{14}CO_2$ was determined in the presence of 4-bromo-3-hydroxybenzyloxyamine dihydrogen phosphate, and the amount of release was subtracted from the sample value. The reaction was terminated by addition of 0.6 mL of 1 M citric acid. The incubation was continued for another 15 min, and the CO_2 evolved was trapped by 20 μL of 2 N NaOH on a 3MM filter paper (Whatman) suspended above the reaction in a plastic well (Kontes). Filter papers were placed in 8 mL of toluene Omniflour (New England Nuclear), and the radioactivity was determined in a liquid scintillation spectrometer. One unit of ODC activity catalyzed the decarboxylation of 1 μmol of ornithine per min at 37 °C.

Concentration. Concentration of enzyme samples was carried out by filtration through Amicon YM-10 Diaflow membranes by using a stirred ultrafiltration cell at 4 °C under N_2 pressure at 60 psi. Buffer exchange was also conducted by using this apparatus by connecting a reservoir containing the indicated buffer.

Polyacrylamide Gel Electrophoresis under Nondenaturing Conditions. Disc gel electrophoresis was carried out in a stacking system. A 0.5 × 10 cm separating gel was cast containing 7.5% acrylamide, 0.2% Bis, 0.1% PEG, 0.07% ammonium persulfate in 70 mM Tris-HCl, pH 7.5, and 11.5 μL of TEMED per 10 mL of gel reaction. After gelification, a 0.5 × 1 cm stacking gel was layered on top containing 2.5% acrylamide, 0.625% Bis, 0.1% PEG, 5 μg/mL riboflavin 5′-phosphate, and 1 μL of TEMED/mL in 140 mM Tris–PO_4, pH 5.5. Polymerization was initiated by exposure to ultraviolet light. Upper and lower buffer chambers contained 30 mM diethylbarbituric acid, 8.2 mM Tris, 2 mM thioglycolic acid, and 0.06 mM PYP, pH 7.0. Samples (100 μL) containing 5% glycerol were layered on top of the stacking gel, and electrophoresis was carried out at 4 °C at 4 mA/gel until the bromophenol blue tracking dye exited the gel (~3 h).

Nondenaturing electrophoresis was also carried out in phosphate buffer. Gels (7.5%, 0.5 × 10 cm) were prepared and electrophoresed in 28 mM NaH_2PO_4, 72 mM Na_2HPO_4, 10 mM thioglycolic acid, and 0.06 mM PYP, pH 7.0.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Electrophoresis was carried out on 4–30% gradient gels in a buffer containing 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, 0.2% $NaDodSO_4$, adjusted to pH 7.4. Samples were denatured by incubation at 100 °C for 5 min in 10 mM Tris-HCl, 1 mM EDTA, 1% $NaDodSO_4$, and 2.5% mercaptoethanol. After denaturation, sucrose was added to 10% and bromophenol blue to 0.01%. Electrophoresis was conducted at 4 °C at 8 mA/tube gel or 150 V/slab gel until the tracking dye had been exited from the gel for 1 h. Gels were fixed in 25% methanol and 10% acetic acid and stained with Coomassie blue.

Preparation of Cytosol. All steps in the purification procedure were carried out at 4 °C. Liver (~800–1200 g wet weight) from a thioacetamide-stimulated calf was homogenized in 2 volumes of purification buffer containing 0.5 mM DTT, 0.1 mM EDTA, and 1 mM PMSF in four batches at high speed in a Waring blender for two 45-s periods intercalated by a 1-min cooling period. Cubes of frozen homogenization buffer were included during the tissue disruption to maintain low temperature. The homogenate was centrifuged at 40000*g* for 30 min and the supernatant fluid filtered through several layers of cheesecloth. A total of 1800 mL of cytosol was obtained.

First Purification Step: Ion-Exchange Chromatography Using DEAE-Sephacel. The supernatant fluid was mixed for 30 min at 4 °C with 600 mL of a 1:1 slurry of DEAE-Sephacel. The resin was preequilibrated with purification buffer containing 2 mM DTT, 0.1 mM EDTA, 1 mM PMSF, and 50 mM NaCl before use by stirring 4–6 times with 10 volumes of fresh buffer (15 min) until no change in the pH of the equilibrating buffer was detectable. The resin was separated from the liquid phase by filtration on a Büchner filter (coarse) and washed 5–7 times with a total of ~4 L of additional equilibration buffer by resuspension and stirring for 10 min prior to filtration. The resin was suspended in an equal volume of buffer, layered into a column on top of a presettled bed of 100 mL of fresh DEAE-Sephacel, and eluted with a linear gradient of 50–600 mM NaCl in purification buffer containing 2 mM DTT, 0.1 mM EDTA, and 1 mM PMSF (total volume 3 L), and 10-mL fractions were collected. ODC activity eluted between 175 and 250 mM NaCl (Figure 1A). The peak fractions (i.e., exhibiting enzymatic activity equal to or greater than 25% of the fraction containing maximal activity) were combined and stored at −80 °C. A 45-fold increase in specific activity was observed routinely (Table I).

Second Purification Step: Ultrogel AcA 44 Chromatography. Pooled ODC fractions containing 150–300 mg of protein were concentrated by ultrafiltration to a 5-mL volume. The concentrate was applied to a 2.5 × 90 cm column of Ultrogel AcA 44 which had been preequilibrated by passage of 10 volumes of purification buffer containing 2 mM DTT, 0.001 mM EDTA, 1 mM PMSF, 0.1% PEG, and 100 mM

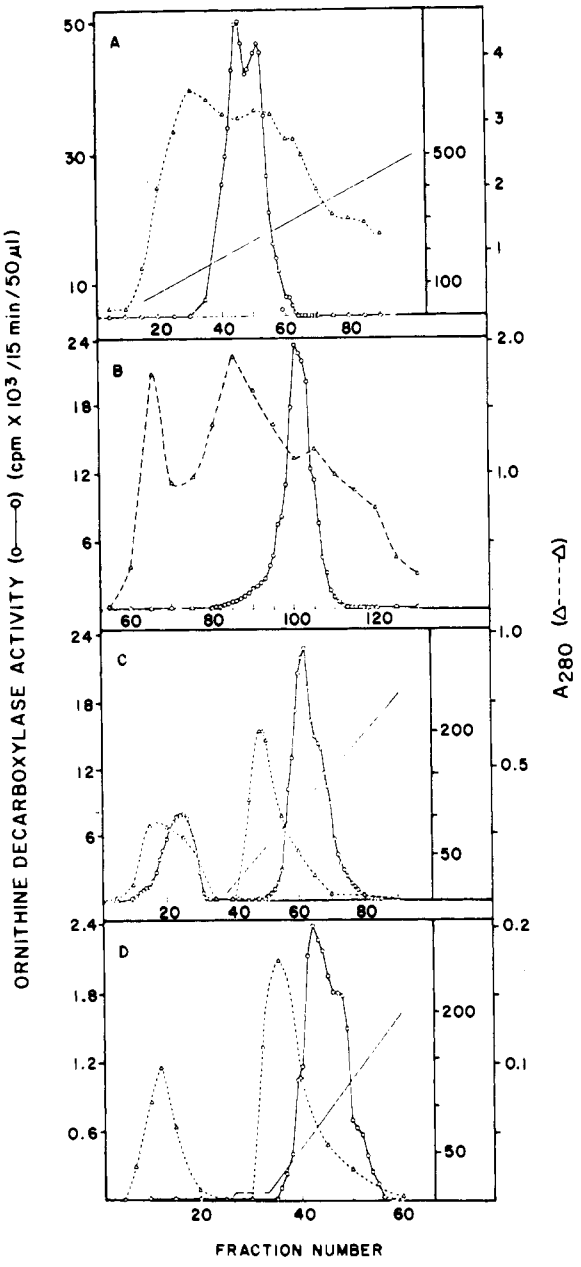

FIGURE 1: Ion-exchange, gel filtration, adsorption, and covalent chromatography of ornithine decarboxylase. Representative chromatographic profiles are shown of the elution of calf liver ODC activity (O—O) and sample protein (Δ---Δ) obtained from columns of (A) DEAE-Sephacel after the application of an increasing NaCl concentration gradient, (B) Ultrogel AcA 44, (C) Ultrogel HA during sample application and elution with an increasing potassium phosphate concentration gradient, and (D) activated thiol–Sepharose during sample application and elution with an increasing DTT concentration gradient. All chromatographic separations were performed as described under Experimental Procedures.

NaCl. Chromatography was carried out by upward flow generated by a peristaltic pump at a rate of 12 mL/h, and 2-mL fractions were collected. ODC eluted as a single peak of activity in the latter half of the eluted protein (Figure 1B). The peak fractions were pooled, concentrated to 2 mg of protein/mL, and stored at −80 °C. The gel filtration procedure resulted in a 6- to 7-fold increase in enzyme purity with ~60% recovery (Table I).

Third Purification Step: Hydroxylapatite Chromatography. Pooled ODC fractions containing 20–30 mg of protein were buffer exchanged by ultrafiltration with 10 volumes of 5 mM Na_2H–KH_2PO_4, pH 7.2, 5 mM NaF, 0.06 mM PYP, 0.1% PEG, 2 mM DTT, and 1 mM EDTA. The sample was applied to a 1.1 × 5 cm column containing Ultrogel HA. The column was washed with additional buffer until the 280-nm absorbance of the eluate equaled that of the buffer. The enzyme was then eluted with a linear gradient of 5–250 mM KPO_4 in the same buffer. ODC eluted as a single peak toward the back of the major eluted protein peak (Figure 1C). The peak fractions were pooled, concentrated to greater than 1 mg of protein/mL and stored at −80 °C. Specific activity of the enzyme in the peak fractions was increased 3-fold with a 60% recovery (Table I).

Fourth Purification Step: Nondenaturing Gel Electrophoresis. Disc gel electrophoresis was carried out in a stacking, Tris–barbital buffer system. Pooled fractions of ODC containing 10–20 mg of protein were concentrated to 1 mL, and the osmolarity of the buffer in the concentrate was reduced to one-third by ultrafiltration with 5 mM Na_2H–KH_2PO_4, pH 7.2. The sample was adjusted to 5% glycerol, and 200-µL aliquots were layered on top of the stacking gel. Electrophoresis was conducted for 3 h until the bromophenol blue tracking dye had exited the gel. The gels were cut into 2-cm slices and quartered, and the similar slices from each gel were pooled and allowed to diffuse overnight in 3 volumes of purification buffer containing 2 mM DTT, 0.1 mM EDTA, and 0.1% PEG. The liquid fractions from the slice incubations were assayed for ODC activity, the peak fractions were pooled, the gel slices were allowed to diffuse for 1 h in additional buffer and then were loaded into a syringe containing a glass wool plug, and pressure was applied to extrude as much buffer as possible from the gel slices. This procedure gave a 55% recovery of activity and a 12-fold purification (Table II). The total pooled fraction was concentrated to a protein concentration greater than 0.5 mg/mL and stored at −4 °C.

Fifth Purification Step: Thiol Covalent Chromatography. Pooled fractions containing ~2 mg of protein were buffer exchanged by ultrafiltration with 10 volumes of purification buffer containing 0.1% PEG and 0.1 mM EDTA. The sample was applied at 0.2 mL/min to a 0.6 × 3.5 cm column of activated thiol–Sepharose preequilibrated in purification buffer containing 0.1% PEG, 0.1 mM EDTA, an 100 mM NaCl. The column was washed with additional equilibration buffer until the 280 nm of the eluate equaled that of the buffer. A linear gradient of 2–250 mM DTT was applied in the same buffer (15 mL total), and 0.5-mL fractions were collected. ODC activity eluted at the latter portion of the eluted protein peak (Figure 1D). The specific activity was increased 2.5-fold by this step, and recovery was ~65% (Table I). Those fractions exhibiting peak activity were pooled, concentrated to 0.5 mL, and stored at −4 °C.

Results

Purification of Calf Liver Ornithine Decarboxylase. The purification is summarized in Table I. In order to ensure enough enzyme protein at the final purification steps of gel electrophoresis and activated thiol–Sepharose chromatography so that the enzyme can be recovered in active form, a total of six calf livers must be processed. Table I lists the number of times each procedure was performed as an individual step, in addition to the cumulative yield, recovery, and relative purification as each step in the process was completed. Fractionation of a total of 5 kg of liver typically yielded ~1 mg of enzyme protein with an average specific activity of 290 milliunits/mg of protein, although values as low as 150 and as high as 340 milliunits/mg of protein were occassionally observed. This represented a relative purification of ~ 25 000-fold and an overall recovery of 14% of the cytosol activity.

Table II: Enhancement of Ornithine Decarboxylase Recovery through Purification Procedures by Application of Samples of Increased Protein Content

purification step	sample applied (mg)	ODC act. recovered (%)
hydroxylapatite	8[a]	11
	18	42
	26	60
disc gel electrophoresis	0.2[b]	19
	0.4	32
	1.0	50
	2.0	55

[a] The sample applied was purified through Ultrogel AcA 44 gel filtration and had a specific activity of 2789 pmol min^{-1} (mg of protein)$^{-1}$. [b] The sample applied was purified through hydroxylapatite chromatography and had a specific activity of 11 240 pmol min^{-1} (mg of protein)$^{-1}$.

Stability. The qualifying characteristic in the development of the purification procedure for calf liver ODC was the elucidation of the appropriate sample protein to column size and elution buffer volume ratios would allow maintenance of the enzyme activity. Many chromatographic procedures resulted in very poor or even no recovery of enzymatic activity. Increasing the amount of protein applied in successive trials (or, alternatively, decreasing column or gradient size or increasing the gradient slope) eventually yielded the appropriate ratio to obtain greater than 50% recovery for all purification steps described. For example, for the hydroxylapatite chromatographic step, it was necessary to overload the column by applying 20–30 mg of sample from the gel filtration step to completely saturate the binding sites on the adsorbent in order to retain greater than 50% of the applied enzyme activity upon elution (Table II). This approach was preferable to decreasing gradient volume or increasing gradient slope because it yielded a higher fold purification than the alternatives. For the disc gel electrophoresis procedure, application of 100–200 μg of hydroxylapatite-purified enzyme, an amount optimal for resolution of multiple protein bands by this technique, resulted in less than a 20% recovery of applied catalytic activity (Table II). Experiments in which the sample amount was increased showed that application of 1–2 mg of protein to the gels was required to maintain ODC activity.

The semipurified ODC (through the hydroxylapatite procedure) was stable to freeze–thawing, and the enzyme activity was best preserved by storage at −80 °C. After gel electrophoresis and thiol covalent chromatography, the enzyme was inactivated by freeze–thawing and was stored at 4 °C. Catalytic activity was retained for 1–2 weeks under these conditions.

Cytosol Preparation. The DTT concentration optimal for ODC recovery from the homogenization procedure was distinct from that optimal for the catalytic activity. Disruption of the liver in the presence of 0.5 mM DTT resulted in a 40% greater supernatant ODC activity than disruption of the same liver sample in 2 mM DTT. Both supernatant samples required the presence of 2 mM DTT in the assay for optimal catalytic activity. Other reported ODC purifications have utilized a batch precipitation procedure, either by acid or by ammonium sulfate treatment, as the initial purification step. Attempts to apply these to the supernatant from thioacetamide-stimulated calf liver resulted in very poor recoveries of enzyme activity (20–40%).

Gel Filtration. ODC activity was invariably recovered in poor yield from gel filtration, regardless of the amount of protein applied. Dialysis experiments were conducted to determine optimal buffer components for maintaining enzyme activity in a changing buffer system. Dialysis of an ODC preparation purified by DEAE-Sephacel chromatography for 18 h at 4 °C against 100 volumes (four changes) of purification buffer containing 2 mM DTT, 0.1 mM EDTA, and 1 mM PMSF (the concentrations used in the DEAE fractionation) resulted in a loss of 60% of the ODC activity. Substituting Hepes buffer, or varying the PO_4, PYP, or DTT concentration, had no effect on this loss, except that at thiol concentrations less than 0.2 mM, recovery was even less. Elimination of EDTA from the dialysis buffer resulted in a greater loss of ODC activity. Since ODC is sensitive to inhibition by heavy metals, which are often present as contaminants in buffer preparations, EDTA was added back to the dialysis buffer to a concentration of 1 μM to try and prevent this further decline. This minimal addition of chelator not only prevented the further decline but also resulted in the maintenance of more of the original enzyme activity than was preserved in the presence of 0.1 mM EDTA (72% vs. 40% recovery). Gel filtration in the presence of buffer containing 1 μM EDTA was subsequently found to yield good recoveries of enzyme activity.

ODC recovered after gel filtration in the presence of 1 μM EDTA exhibited a requirement for EDTA in the assay for optimal activity which was not seen with the enzyme before gel filtration. The enzyme preparation after gel filtration is stimulated 2-fold as the concentration of the chelator in the assay is raised from 1 to 100 μM while ODC activity recovered from the initial ion-exchange chromatography is insensitive to these concentrations of EDTA. Because of this requirement, the subsequent purification procedure, hydroxylapatite chromatography, was performed in the presence of 1 mM EDTA, after which the enzyme preparation no longer displayed any requirement for EDTA for optimal activity.

Thiol Covalent Chromatography. A previously reported ODC purification procedure (Obenrader & Prouty, 1977) indicated that the enzyme would bind to Sepharose to which glutathione was conjugated (activated thiol–Sepharose 4B). The calf liver enzyme also bound readily to the gel; however, the application of 5 mM DTT, which eluted the rat liver enzyme in the previous report, released less than 20% of the calf liver ODC activity. Increasing the temperature to 37 °C enhanced elution of the enzyme; however, very little purification of the enzyme was obtained by this procedure. In an attempt to take advantage of the theoretical high resolving power suggested by this tight interaction of the enzyme with the resin, various gradients of DTT were applied to test columns of the activated thiol–Sepharose. A linear gradient from 2 to 250 mM DTT, as described above, gave optimal enzyme recovery while it was resolved from the major portion of the applied protein (see Figure 1D).[2]

Protein Assay. Determination of protein content by the dye binding method described by Bradford (1976) in purification fractions of ODC obtained during the latter steps of the procedure often resulted in values inconsistent with the protein content suggested by increased absorbance (at 280 nm) of the enzyme-containing fractions above that of the chromatography buffer. Comparative Bradford and Lowry (Lowry et al., 1951) protein assays were done of purification samples precipitated by 10% trichloroacetic acid and resuspended in 0.1 N NaOH prior to assay. While the values obtained for the step 1 enzyme

[2] Another sulfhydryl derivative of Sepharose, thiopropyl-Sepharose 6B, also bound the ODC preparation; however, only 2% of the activity was recoverable after incubation (8 h at 37 °C) in the presence of 5 mM DTT.

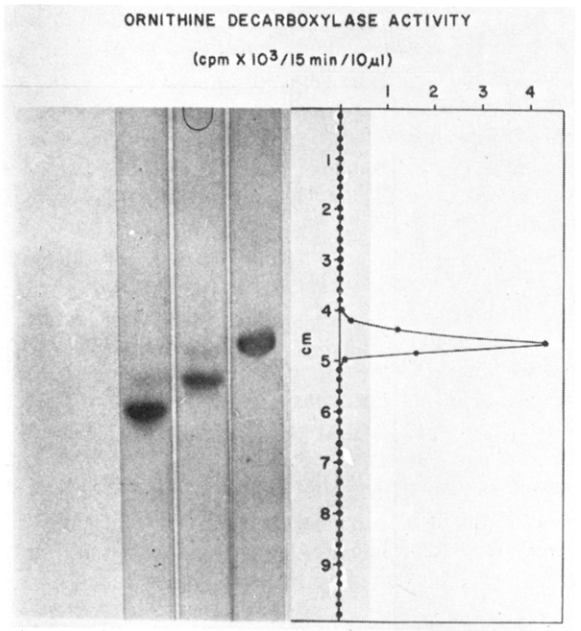

FIGURE 2: Nondenaturing gel electrophoresis of purified ornithine decarboxylase. Purified calf liver ODC (step 5, 75 μg) (right gel) or 10 μg of BSA (center gel) or ovalbumin (left gel) were subjected to electrophoresis in a stacking system utilizing Tris–barbital buffer as described under Experimental Procedures and stained with Coomassie blue. A companion gel to which the ODC preparation had been applied was sliced into 2-mm sections, allowed to diffuse for 2 h at 4 °C in assay buffer, and then assayed for ODC activity at 37 °C after the addition of [^{14}C]ornithine.

were very comparable, the protein content as determined by the Bradford method became progressively less compared to the Lowry method as the purification was carried further. By the last purification step, the determination by dye binding gave values equivalent to one-tenth those obtained with the Folin phenol reagent. Therefore, Bradford determinations were utilized only during the initial purification stages.

Purity of the Ornithine Decarboxylase Preparation. So that the homogeneity of the purified ODC preparation could be tested, samples of the concentrate from the fifth purification step were analyzed by nondenaturing disc gel electrophoresis utilizing the Tris–barbital stacking system described under Experimental Procedures. Because the enzyme was refractory to interaction with Coomassie Blue (see above), 75 μg of the purified enzyme had to be subject to electrophoresis in order to obtain a detectable, stained protein band, in contrast to the well-defined bands obtained with 10 μg of the ovalbumin and BSA standards. As shown in Figure 2, even in these comparatively overloaded gels, there was only one observable protein band which corresponded to the ODC activity as assayed in a parallel gel. The results show that the ODC preparation appears homogeneous in that visible contaminants are absent.

Other Purification Procedures Evaluated. Because of the theoretical resolving power they should afford, substrate and cofactor affinity resins were evaluated as potential purification steps. A previous report of a purification procedure for ODC from fibroblasts (Boucek & Lembach, 1977) utilized a pyridoxamine 5′-phosphate affinity matrix with elution by PYP to obtain a single-step purification of 500-fold. This method was tested with semipurified calf liver enzyme, according to the procedure outlined by the authors. No significant binding of the enzyme to the matrix was observed. Various other affinity resins were synthesized in an effort to find one which would allow efficient recovery of enzyme. These included (listed in their order of capacity to adsorb the enzyme) (1) PYP conjugated via the hydroxyl group by an epoxy linkage to a 12-atom spacer arm on Sepharose 4B, (2) α-methylornithine conjugated via an amino group by a carbodiimide-catalyzed peptide linkage to a 6-carbon atom spacer arm (C_6) on Sepharose 4B, (3) PYP conjugated via the hydroxyl group by a *O*-bromoacetyl-*N*-hydroxysuccinimide-catalyzed linkage to a C_6-Sepharose 4B, (4) a borohydride-reduced Schiff base of ornithine with resin 2, (5) ornithine coupled via an amino group by a carbodiimide-catalyzed peptide linkage to C_6-Sepharose 4B, (6) ornithine coupled via the carboxyl group by a carbodiimide-catalyzed peptide linkage to C_6-Sepharose 4B, (7) a borohydride-reduced Schiff base of PYP with resin 5, and (8) a borohydride-reduced Schiff base of ornithine to resin 1. All of these resins displayed at least some affinity to bind ODC. However, an efficient method for recovering enzyme activity from any of them was not found. A similar result was found with an RNA polymerase I affinity column which had been found effective in the purification of ODC from rat liver (Manen & Russell, 1977).

Other chromatographic resins tested to which calf liver ODC did not adsorb included phosphocellulose, double- and single-stranded DNA cellulose, heparin–agarose, concanavalin A–agarose, and wheat germ agglutinin–Sepharose.

Lability of Ornithine Decarboxylase to Dilution. The enzyme, at all stages of the purification, was labile to dilution. Serial dilutions of the enzyme purified through the first step from the original protein content of 4 mg/mL, at which it had eluted from the ion-exchange column, resulted in progressively greater losses of activity. A dilution to 2 mg/mL resulted in 68% of the expected activity while further dilution to 0.2 mg/mL gave only 20% of the expected activity. This loss of activity appeared to be immediate and did not increase appreciably upon incubation of the diluted fraction before assay. Reconcentration of a diluted fraction did not restore the original enzyme activity.

Addition of exogenous protein to purified preparations of ODC stabilizes the catalytic activity (Boucek & Lembach, 1977; Jänne & Williams-Ashman, 1971). Therefore, various proteins and other known protein stabilizing agents were tested for their ability to stabilize the calf liver enzyme. Representative data from these experiments are reported in Table III. The dilution of the semipurified ODC fraction 10-fold resulted in a 50-fold decrease in enzyme activity. Inclusion of gelatin in the diluent had no effect on the loss in activity. The addition of glycerol or sucrose accelerated the loss, and thioglycerol completely inhibited the enzyme. These compounds also were found to inhibit the undiluted enzyme 33%, 24%, and 98%, respectively (data not shown). The inclusion of PEG (M_r 20 000) preserved 2.5 times more of the enzyme upon dilution, maximum protection being afforded by a 0.1% concentration of the polymer.

Inclusion of additional protein in the diluent also provided various degrees of protection to the ODC. Transferrin and hemocyanin almost totally prevented the loss of enzyme activity while BSA preserved 60% and ovalbumin offered little protection. Although the metalloproteins were found to be the best stabilizing agents, for the purposes of the purification of the enzyme, the less effective PEG was included in all the buffers to help maintain enzyme activity since it would not interfere with the resolution of the pure enzyme protein.

Evidence for Ornithine Decarboxylase as a Metalloenzyme. The sensitivity of ODC to the concentration of EDTA during dialysis suggested a possible involvement of metal with the enzyme. Since no metal chelator has been shown to meet the

Table III: Effect of Proteins and Other Stabilizing Agents on the Loss of Ornithine Decarboxylase Activity upon Dilution[a]

	ornithine decarboxylase (cpm/ 30 min)	expected activity (%)
undiluted	18090	
diluted 1:10	361	20
+1% gelatin	358	20
+30% glycerol	241	13
+30% thioglycerol	0	0
+20% sucrose	270	15
+0.1% PEG	938	52
+1.0% PEG	979	54
+1 mg/mL ovalbumin	472	26
+ 10 mg/mL ovalbumin	485	27
+1 mg/mL BSA	684	38
+10 mg/mL BSA	1069	59
+1 mg/mL hemocyanin	1267	70
+10 mg/mL hemocyanin	1545	86
+10 mg/mL transferrin	1617	90

[a] An ODC preparation purified through step 1, which contained 4 mg of protein/mL and had a specific activity of 485 pmol min^{-1} mg^{-1}, was assayed directly or after a 10-fold dilution in the presence of the indicated compounds in purification buffer containing 2 mM DTT, 0.01 mM EDTA, 1 mM PMSF, and the compounds listed.

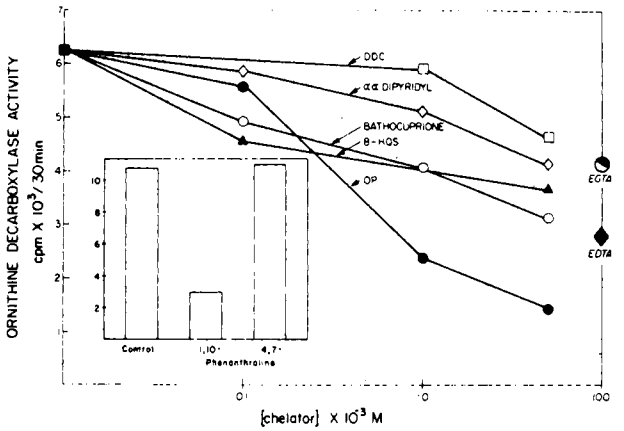

FIGURE 3: Inhibition of calf liver ornithine decarboxylase by metal chelators. An ODC preparation purified through step 4 (108 600 pmol min^{-1} mg^{-1}) was incubated for 10 min at 4 °C in the presence of the indicated concentrations of the metal chelators prior to assay for catalytic activity. (Inset) An ODC preparation (126 300 pmol min^{-1} mg^{-1}) was preincubated with 5 mM 1,10- or 4,7-phenanthroline prior to assay.

requirements of total specificity in studying the behavior of a particular metalloenzyme (Vallee & Wacker, 1970), a series of different complexing agents was employed. Figure 3 shows an experiment in which semipurified ODC (step 4) was incubated with increasing concentrations of several metal cheltors for 10 min at 4 °C prior to assay. All of the chelators displayed some ability to inhibit the enzyme. The most potent was 1,10-phenanthroline, a chelator of transition and group 11B metals (Vallee & Wacker, 1970). Since 1,10-phenanthroline is a planar molecule, it can possibly insert into hydrophobic areas of proteins and alter enzymatic activity. Therefore, the structurally similar but nonchelating analogue, 4,7-phenanthroline was tested. This compound had no effect on the activity of ODC (Figure 3, inset), suggesting that the metal chelator was not inhibiting due to the aromatic properties of its structure.

A number of metals were tested for the ability to reactivate ODC that had been inhibited by incubation with 1,10-phenanthroline. Zn^{2+}, Fe^{2+}, Cu^{2+}, Fe^{3+}, Cu^{+}, Ni^{2+}, Co^{2+}, and Mn^{2+} were unable to restore enzyme activity. In addition, all of the metals except Mn^{2+} were found to inhibit the untreated enzyme, their order of potency being that listed above.

Catalytic Activity of Ornithine Decarboxylase. The apparent affinity of purified ODC (step 5) for ornithine at 37 °C was calculated by assaying the enzyme in the presence of various substrate concentrations and 0.06 mM PYP. The enzyme displayed standard Michealis–Menton kinetics, giving a linear double-reciprocal plot which indicated a single K_m for ornithine of 0.16 mM. The enzyme also displayed a single affinity for PYP: the K_m of ODC for the cofactor was 2.5 μM when measured in the presence of 1 mM ornithine. Addition of the product of the enzymatic reaction, putrescine, inhibited the rate of ornithine decarboxylation. Putrescine (10 mM) inhibited the enzyme 50% when assayed in the presence of 0.5 mM ornithine.

pH Optimum of Ornithine Decarboxylase. The pH dependence of the ODC-catalyzed release of CO_2 was determined at 0.5-unit intervals from pH 5.5 to 9.5. The enzyme displayed greater than 50% of maximal activity over a broad range of 6.0 to 8.0. Enzyme activity was sensitive to the type of buffer employed. Imidazole and Mes supported almost twice the activity at pH 7.0, the optimum, as did PO_4 buffer.

Sensitivity of Ornithine Decarboxylase to Reducing Agents. Initial experiments conducted to characterize the sensitivity of ODC to the redox environment of the assay completely removed the enzyme from the presence of reductants by double precipitation with $(NH_4)_2SO_4$ and resuspension in buffer in the absence of reductants. The subsequent addition of DTT, mercaptoethanol, or glutathione all stimulated the residual (20%) enzyme activity, the order of efficacy being that listed. However, only 50% of the original enzyme activity was recoverable by this procedure, perhaps due to irreversible aggregation of the enzyme into a less active form (Jänne & Williams-Ashman, 1971). Therefore, a further experiment was conducted in which the ODC preparation was depleted of reductant by buffer exchange. The enzyme retained 53% of its activity when the DTT concentration was reduced from the standard 2 mM to 0.02 mM. Incremental addition of DTT back to the enzyme progressively increased the activity to 96% of the the original at an optimal concentration of the thiol of 2 mM. However, the addition of mercaptoethanol or glutathione had no stimulatory effect on the thiol-depleted enzyme. In fact, when either monothiol was raised to a concentration greater than 10-fold of the residual DTT concentration, the enzyme was inhibited. Only 18% of the original activity remained in the presence of 10 mM monothiol.

A further indication of the necessity of reduced sulfhydryl groups for the activity of ODC is the finding that preincubation of the enzyme for 5 min in the presence of 0.05 mM *p*-(chloromercuri)benzoic acid completely inhibited all release of CO_2 during the subsequent assay.

Dimeric Form of Ornithine Decarboxylase. The activity of purified ODC eluted as a single peak upon gel filtration. The volume of elution of the enzyme from an Ultrogel AcA 44 column which had previously been calibrated with proteins of known molecular weight and Stokes' radius was used to determine these values for the enzyme (Andrews, 1964, 1965; Siegel & Monty, 1966). The enzyme displayed an approximate molecular weight of 54 000 and a Stokes radius of 3.14 nm. The ODC preparation was also subjected to NaDodSO$_4$-denaturing electrophoresis on 4–30% gradient polyacrylamide gels. Previous experiments had shown that the enzyme was very refractive to dye binding. Application of 10 μg of sample, as determined by the Lowry protein assay,

Table IV: Comparison of Characteristics of Purified Ornithine Decarboxylase

reference; source	sp act. (pmol min^{-1} mg^{-1}) original	sp act. (pmol min^{-1} mg^{-1}) final	rel purification (fold)	M_r	Stokes' radius (nm)	K_m ornithine (mM)	K_m PYP (μM)	K_i putrescine (mM)
Jänne & Williams-Ashman (1971); rat ventral prostate	33	10 000	292	44 000–85 000	3.87	0.1		1.0
Friedman et al. (1972); regenerating rat liver	18	3 000	175	66 000				
Ono et al. (1972); thioacetamide-stimulated rat liver	36	193 000	5372	100 000		0.2		>10.0
Boucek & Lembach (1977); SV40-transformed 3T3 cells	281	1 660 000	5917	55 000–110 000		0.125	3.62	not inhibitory
Obenrader & Prouty (1977); thioacetamide-stimulated rat liver	33	233 000	6900	48 000–100 000		0.13	0.25	
Haddox & Russell; thioacetamide-stimulated calf liver	12	292 680	24390	54 000–110 000	3.14	0.16	2.5	10.0

yielded no detectable bands after being stained with either Coomassie Blue or Amido Black. Electrophoresis of larger size samples of protein markers resulted in smearing. Therefore, the sample was iodinated with Bolton Hunter Reagent ^{125}I (New England Nuclear) according to the procedure detailed in the product bulletin, electrophoresis was conducted, and the gel was sliced into 1-mm pieces and counted in 3% NCS–toluene Omniflour. A single band of radioactivity was detected at a position indicating a molecular weight of 52 000 as compared to the movement of protein standards of known molecular weight. The migration of purified ODC in the Tris–barbital electrophoresis system did not correspond to the molecular weight as determined by gel filtration; in the electrophoretic system, the enzyme migrated behind both ovalbumin and BSA (Figure 2), while upon gel filtration, ODC eluted between the two marker proteins. The electrophoretic experiment was repeated in PO_4 buffer, as employed in the gel filtration column. In the PO_4 buffer, ODC migrated between the BSA and ovalbumin marker proteins, indicating that the anomalous migration in the presence of Tris–barbital buffer was not due to the electrophoretic technique per se and suggesting that the enzyme had assumed a larger molecular weight. However, since a protein's movement in an electrophoretic system is based on both the charge and size of the molecule, a gel filtration study was conducted to examine the effect of Tris–barbital buffer on enzyme size. The elution volume of ODC was much less, the enzyme preceding both ovalbumin and BSA, at an approximate molecular weight of 100 000 and a Stokes radius of 4.5.

Multiple Forms of Ornithine Decarboxylase. Previous reports have indicated that multiple forms of ODC may exist on the basis of differential elution patterns from activated thiol–Sepharose (Obenrader & Prouty, 1977) or on multiple kinetic parameters with respect to substrate (Lau & Slotkin, 1979) or cofactor (Clark & Fuller, 1976). Application of these analyses to the calf liver enzyme indicated a single species of the enzyme; however, the elution profiles of the enzyme off of the initial DEAE fractionation sometimes suggested heterogeneity of the enzyme (see Figure 1A). Sievorptive chromatography (Kirkegaard, 1973), which actively combines molecular sieve (gel filtration) and ion-exchange adsorption chromatography, was employed to try and resolve possible ionic forms of the enzyme.

Figure 4 shows the pattern of ODC activity elution from a Sephadex DE 25 column to which a 75–200 mM NaCl gradient had been preapplied. Three distinct peaks of enzyme activity were resolved. Since the enzyme binds PYP in a

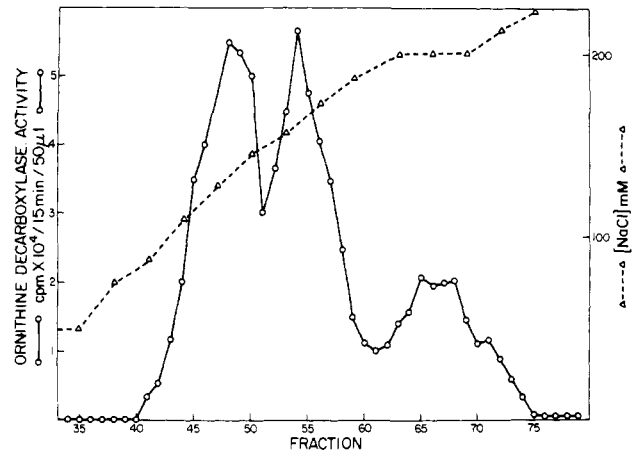

FIGURE 4: Resolution of multiple forms of ornithine decarboxylase by sievorptive chromatography. A linear gradient of 75–200 mM NaCl in purification buffer containing 2 mM DTT, 0.1 mM EDTA, and 1.0 mM phenylmethanesulfonyl fluoride (total volume 25 mL) was applied to a 2 × 20 cm column of DEAE-Sephadex A-25, which had been preequilibrated in buffer containing 50 mM NaCl. A concentrated sample (4 mL) containing 100 mg of protein of an ODC preparation purified by DEAE-Sephacel was then applied, followed by buffer containing 300 mM NaCl. A constant flow rate (0.5 mL/min) was maintained by peristaltic pumping, and 0.5-mL fractions were collected and assayed for ODC activity (○) and NaCl concentration (△) by comparison to standards using a conductivity meter.

reversible manner (see above), it was possible that the differences were due to differential binding of cofactor. Therefore, another chromatographic procedure was conducted in the absence of PYP. The three different peaks of activity were again observed in similar proportion to the pattern shown. Since no heterogeneity of the enzyme was observable when fractionation was carried out on the basis of molecular weight, it appears that the differences observed by this technique result from small modifications of the enzyme which alter the protein's charge. All three peaks of activity displayed similar affinities for ornithine and PYP as reported above for the unfractionated purified ODC preparation.

Discussion

Progress in defining the molecular characteristics of ODC has been hindered both by the small quantities of enzyme present in tissue, even in a highly stimulated growth state, and by the extreme instability of the enzyme when subjected to standard purification procedures. In the purification protocol described in the present report, the lability of ODC from calf liver has been circumvented by the maintenance of high protein

concentrations at each step in the procedure.

Table IV presents a comparison of the characteristics of purified ODC that have been reported. The greatest divergence lies in the ultimate specific activities of the purified enzyme. The specific activity determined in the studies detailed here for the enzyme purified from thioacetamide-stimulated calf liver is comparable to that reported for the enzymes purified from thioacetamide-stimulated rat liver, ~250 000 pmol min^{-1} mg^{-1}. However, it is almost 100 times greater than that obtained with enzyme purified from regenerating rat liver. This variation may reflect the catalytic instability noted in the present report.

The molecular weight determinations are all comparable, indicating a monomeric protein of M_r ~60 000. Several studies have reported the existence of a dimeric form of the enzyme, a possibility supported in this study by the shift in mobility of the enzyme with regard to BSA and ovalbumin in the Tris–barbital electrophoresis system. Gel filtration in the same buffer confirms that this shift is the result of an increased molecular size rather than a change in protein charge. A notable finding is that the dimeric form of ODC obtained under these conditions is just as active as the monomer. Previous reports had suggested that the dimeric form of ODC was inactive (Jänne & Williams-Ashman, 1971). However, the inactive dimer was obtained by removal of reductant, suggesting that free thiols essential for catalytic activity may have been complexed into intermolecular disulfide bridges. The physiologic control mechanism for governing monomer to dimer transition is unknown. Other enzymes have been shown to undergo association under the control of the enzymatic substrate. However, inclusion of ornithine in the standard purification buffer system was found to have no effect on the elution volume of the enzyme from the Ultrogel AcA 44 column (data not shown).

The K_m values determined for ornithine and PYP for calf liver ODC are very similar to those reported for other purified enzyme preparations (Table IV). No evidence for multiple forms of the enzyme with regard to kinetic values was found. Some reports have indicated that ODC was relatively insensitive to product inhibition. However, the calf liver enzyme was inhibited 50% by 10 mM putrescine.

A previous study of rat liver enzyme (Obenrader & Prouty, 1977) indicated the existence of multiple forms of ODC on the basis of heterogeneous elution of the enzyme off activated thiol–Sepharose and gel electrophoresis. When these techniques with the calf liver enzyme were utilized, only single peaks of decarboxylase activity were detectable. However, the broad elution profile of the enzyme from the DEAE-Sephacel column suggested that several forms of activity may be separable by ion exchange. Application of the high-resolution technique of gradient sievorptive chromatography to the calf liver ODC resulted in fractionation of three forms of the enzyme. Currently, experiments are being conducted to determine the catalytic and physical properties of the three forms to obtain information on possible molecular differences.

Perhaps the most striking new finding contained in the present report is the studies indicating the existence of a possible metal cofactor for the mammalian enzyme. The effect of a variety of structurally unrelated metal chelators to inhibit the calf liver ODC suggests that an enzyme-bound metal is required for preservation of catalytic capacity. The finding that 4,7-phenanthroline is inactive strengthens the assumption of a metal involvement since this analogue possesses all of the attributes of the inhibitory 1,10-phenanthroline, except the ability to form a coordination complex with metals. The potency of this transition metal chelator, which displays only slight affinity for the alkaline earth metals, in comparison to the relative ineffectiveness of the broad-spectrum chelator EDTA, suggests that an element in the transition series is involved in the maintenance of ODC activity. The inability to regenerate enzyme activity upon metal readdition suggests the chelators are removing the constituent metal rather than forming a mixed complex in situ on the apoenzyme. Metal removal may then result in irreversible changes in the enzyme molecule.

The requirement for an enzyme-bound metal to maintain catalytic activity may also provide an explanation for the instability for ODC during purification procedures. Most metals bound to protein obey an equilibrium reaction governed by a stability constant (Vallee & Wacker, 1970). This results in there being some small amount of free metal in solution at any time, and as this is removed by differential movement of the protein and the surrounding buffer phase, a condition created in all purification processes, the maintenance of equilibrium results in the release of more metal from the protein.

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Simian Liver Alcohol Dehydrogenase: Isolation and Characterization of Isoenzymes from *Macaca mulatta*†

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ABSTRACT: Like human liver alcohol dehydrogenase, that of *Macaca mulatta* can be purified and separated into anodic and cathodic pyrazole-insensitive and cathodic pyrazole-sensitive enzyme forms. Their inhibition by 4-methylpyrazole and their substrate specificities are analogous to those observed for the corresponding isoenzymes of human liver. However, on the basis of data available so far, the physicochemical and compositional characteristics, i.e., molecular weight, zinc content, and dimeric structure, of all simian alcohol dehydrogenase forms are virtually identical with those of other mammalian alcohol dehydrogenases studied up to now. Zinc is essential for their enzymatic function, as demonstrated by inhibition with chelating agents.

Knowledge about the biochemical features of alcohol dehydrogenase (ADH),[1] the principle enzyme catalyzing the oxidation of ethanol, bears importantly on an understanding of alcohol metabolism and alcohol-related pathology. The presence of multiple molecular forms of the enzyme in human liver was recognized early (Blair & Vallee, 1966), and their identities have been the subject of intensive studies which have now identified isoenzymes π-ADH (Bosron et al., 1979), χ-ADH (Parés & Vallee, 1981), and $ADH_{Indianapolis}$ (Bosron et al., 1980), whose electrophoretic and kinetic properties differ strikingly from those of the other isoenzymes. π-ADH, initially identified by electrophoresis on starch gels as the "anodic band" (Li & Magnes, 1975) in human liver homogenates, is the isoenzyme with the lowest cathodic electrophoretic mobility, which stains in the presence of 4-methylpyrazole. In contrast, χ-ADH, a temporary designation of the new forms of human ADH, migrates toward the anode between pH 7.7 and 8.6. These isoenzymes are not inhibited by 4-methylpyrazole and stain only with long-chain monohydric alcohols, i.e., 1-pentanol or 1-octanol, as substrates. Until now, π-ADH and χ-ADH forms have been detected only in human livers, and they have been postulated to have an important role in ethanol metabolism (Li et al., 1977; Parés & Vallee, 1981). The recent isolation and characterization of pyrazole-sensitive and pyrazole-insensitive[2] ADH isoenzymes from squirrel monkey liver provided the first evidence of the existence of similar molecular forms in another species (Dafeldecker et al., 1981).

Excessive alcohol ingestion is a problem peculiar to the human, and studies of its pertinent biochemical consequences would ideally be performed in that species, though this is clearly not feasible. Hence, it would seem prudent to identify another species whose cellular biochemistry resembles that of the human as closely as possible. Toward this end, we are examining a number of primates by using the hepatic distribution of ADH isoenzymes as the criterion of selection. Among the species examined by starch gel electrophoresis displaying isoenzyme patterns similar to that of the human, the rhesus monkey was chosen for further investigation. The liver of *Macaca mulatta* contains the same three classes of ADH isoenzymes, i.e., anodic and cathodic pyrazole-insensitive and cathodic pyrazole-sensitive forms, as those found in human liver. The kinetic and physical properties of these three simian isoenzyme fractions are similar to the corresponding human liver ADH variants, suggesting that studies of the liver ADH of this species may serve to advance understanding of human alcohol metabolism and its pathological consequences.

Materials and Methods

NAD^+ (grade III), NADH (grade III), 12-hydroxydodecanoic acid, and 16-hydroxyhexadecanoic acid were obtained from Sigma Chemical Co., St. Louis, MO; alcohols, aldehydes, 4-methylpyrazole, EDTA, and 1,10-phenanthroline were from Aldrich Chemical Co., Milwaukee, WI; 4-bromopyrazole was from Research Plus Laboratories, Denville, NJ; 2,2′-bipyridine was from G. Frederic Smith Chemical Co., Columbus, OH; DEAE-cellulose (DE-52) was from Whatman Inc., Clifton, NJ; and agarose–hexane–AMP, type 2, was from P-L Biochemicals, Inc., Milwaukee, WI.

Enzymatic Assay. Alcohol dehydrogenase activity was determined in 0.1 M glycine, pH 10.0, at 25 °C by measuring the production of NADH in the presence of alcohol. The initial velocity at pH 10 in the presence of 2.4 mM NAD^+ and 33 mM ethanol for the pyrazole-sensitive fraction or 0.5 M ethanol for both pyrazole-insensitive isoenzyme fractions was

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[1] Abbreviations used: ADH, alcohol:NAD^+ oxidoreductase (EC 1.1.1.1); CapGapp, 4-[3-[(6-aminocaproyl)amino]propyl]pyrazole; DEAE, diethylaminoethyl; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; HQSA, 8-hydroxyquinoline-5-sulfonic acid; MCD, magnetic circular dichroism; CD, circular dichroism.

[2] Simian liver pyrazole-insensitive ADH is defined operationally as that form of the enzyme that does not bind to the CapGapp–Sepharose affinity resin at pH 7.5.

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