On the Purification of L-Ornithine Decarboxylase from Rat Prostate and Effects of Thiol Compounds on the Enzyme*

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SUMMARY

The activity and stability of L-ornithine decarboxylase from rat ventral prostate is markedly increased by certain thiol compounds, notably dithiothreitol. The enzyme was purified about 300-fold. Many properties of the purified enzyme are described. In the absence of added thiol compounds, the purified ornithine decarboxylase apparently undergoes polymerization, as evidenced by its behavior on sucrose density gradients and in molecular sieving experiments. The larger forms of the enzyme appear to be catalytically inert, but can be reactivated by a number of sulfhydryl compounds, certain dithiols being superior to a number of monothiols in this respect.

It has long been known that a variety of bacteria contain enzymes which catalyze the decarboxylation of L-ornithine, yielding putrescine and carbon dioxide, and which utilize pyridoxal 5-phosphate as coenzyme (1-4). Only quite recently, however, have comparable L-ornithine decarboxylases (EC 4.1.1.17, L-ornithine carboxy-lyase) been detected in higher animal tissues. This work was supported in part by Research Grant HD-04592 from the United States Public Health Service.

tion with ammonium sulfate, could be maintained for long periods at low temperatures.

The key role of ornithine decarboxylase in the biosynthesis of spermidine and spermine (7, 14, 21), the possibility that this enzyme regulates the disposition of L-ornithine in mammalian tissues vis-a-vis other metabolic pathways such as the formation of urea, glutamate, and proline (21), the question as to whether the enzyme is subject to regulation by small molecules that participate in neighboring metabolic reactions, and the desirability of checking the effects of thiol compounds on the physical behavior of purified rather than crude preparations of the enzyme were among the considerations which led us to embark on extensive purification of ornithine decarboxylase from rat ventral prostate and examination of its stability and properties.

MATERIALS AND METHODS

Animals—Adult male (weighing over 250 g) and female (weighing 200 g) Sprague Dawley rats were used in all experiments. Partial hepatectomy was performed under light ether anesthesia by the method of Higgins and Anderson (22).

Preparation of Tissue Extracts—The animals were killed by cervical dislocation. The ventral prostates were rapidly dissected free of fat and capsule in situ, excised, and placed in ice-cold homogenization medium. The livers were first perfused with cold homogenization medium via the hepatic vein. The cold homogenization medium, which is referred to as standard buffer, was 25 mM Tris·HCl buffer of pH 7.5, containing 0.1 mM disodium EDTA. The tissue samples were first minced with scissors and then homogenized with a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenates were centrifuged in a Sorvall RC2-B centrifuge at 20,000 g for 10 min at 2°C. The pellet was discarded, and the supernatant fluid was recentrifuged at 45,000 g for 90 min at 2°C. The resulting supernatant fraction was passed through a pad of glass wool in order to remove floating lipids and was used for assay of ornithine decarboxylase activity or, following dialysis, as the starting material for further purification of the enzyme.

Materials Used in Preparative Procedures—Ammonium sulfate fractionation was performed at 0° by slow addition of the solid salt. Enzyme grade ammonium sulfate (Mann) was used. Serva DEAE cellulose ion exchanger was purchased from Gallard-Schlesinger Chemical Manufacturing Corporation (Carle Place, New York) and previously cycled before use with 0.5 N HCl and 0.5 N NaOH, followed by washing with standard buffer until the starting pH value was attained. Elution was accomplished with the use of a peristaltic pump (Buchler Instruments Inc., Fort Lee, New Jersey) at a flow rate of 120 to 130 ml per hour.

Gel filtration was carried out on Bio-Gel P-200, 100 to 200 mesh from Bio-Rad, Richmond, California. Flow adaptors were used in the column with an upflow elution technique. The dry gel material was allowed to swell at least for 48 hours in water or starting buffer before packing the column. The fines were carefully removed by five to 10 subsequent decantations. The elution was accomplished by hydrostatic pressure only with a constant flow rate of 6 to 8 ml per hour. The columns were calibrated with blue dextran 2000, potassium dichromate, crystalline horse liver alcohol dehydrogenase, and lyophilized alcohol dehydrogenase from yeast (both enzymes purchased from Calbiochem) for determination of molecular Stokes radii by the method of Ackers and Steere (23).

The sucrose density gradient centrifugations were carried out in a Spinco 65 B ultracentrifuge with SW 65 rotors. Linear 5 to 20% sucrose ( ultra pure preparation from Mann) gradients were made with a gradient former from Beckman Instruments. Polyacrylamide gel electrophoresis was carried out according to Davis (24) and Jaraabak, Seeds, and Talalay (25). The ornithine decarboxylase activity was recovered from gels sectioned prior to staining by eluting the gel pieces overnight in the standard incubation buffer.

Chemicals—DL-Ornithine-1-14C-hydrochloride (specific activity 26.4 mCi per mmole) was purchased from Amersham-Searle Corporation (Arlington Heights, Illinois). These as well as other commercial preparations were found to contain variable amounts of radioactive CO₂, which increased the blank values in the ornithine decarboxylase assays. The Dl-ornithine-1-14C was therefore treated as follows prior to its use in the ornithine decarboxylase determinations. The labeled amino acid was dissolved into 0.1 N HCl and evaporated to dryness in a Rotary-Mix evaporator (Buchler Instruments) at 50–60°C. The residue was then dissolved with 0.01 N HCl and stored at -20° until used. After this procedure the nonenzymic control incubations (i.e. the complete incubation mixture minus enzyme) gave about 100 cpm with the usual final specific radioactivity of added ornithine (0.4 μCi per pmole of DL-ornithine).

Thiol Compounds—Dithiothreitol, reduced and oxidized, was obtained from Calbiochem. Dihydrolipoic acid (reduced form of DL-6,8-thioctic acid) and glutathione were purchased from Sigma. 2-Mercaotepethanol was a product of Fastman, 2,3-Dimercaptopropanol was obtained from Mann, and coenzyme A was obtained from Pabst Laboratories (Milwaukee, Wisconsin).

Miscellaneous Compounds—(—)-S-Adenosyl-L-methionine iodide was purchased from Calbiochem and purified on a Dowex AG 50W-X2 column as described by Pegg and Williams-Ashman (26). Thiomethyladenosine was prepared from purified S-adenosylmethionine by the method of Schlenk and Ehninger (27). Cycloheximide was obtained from Sigma.

Analytical Methods—Ornithine decarboxylase activity was measured as described earlier (6, 8, 20). The standard incubation mixture contained in a final volume of 0.5 ml: 50 μmoles of glycylglycine buffer, pH 7.2; 0.5 μmole of L-ornithine; 0.4 μCi of DL-ornithine-1-14C (specific activity 26.4 mCi per mmole); 2.5 μmoles of dithiothreitol; and 0.1 μmole of pyridoxal 5-phosphate. The incubations were carried out in Corex centrifuge tubes (18 × 102 mm) with a disposable polypropylene center well attached to rubber stopper ( Kontes Glass Company, Vineland, New Jersey). The released 14CO₂ was trapped into 0.1 ml of 1 N Hyamine hydroxide (Amersham-Searle) in the center well. In all experiments, two or more nonenzymic controls were used; these contained the standard incubation mixture with appropriate additions, but enzyme was replaced by water. The incubation time was 60 min at 37°C. The reaction was halted by injecting 0.5 ml of 40% trichloracetic acid through the rubber stopper. The tubes were then incubated further for 20 min at 25°C to ensure that all the dissolved CO₂ was released from the acidified medium. The center well and its contents were directly plunged into a scintillation counting vial containing 10 ml of a dioxane-based scintillation fluid (98) and counted in a Nuclear-Chicago Liquid Scintillator at an efficiency of 55%.

Protein was measured by the method of Lowry et al. (29). However, because of the interference of this reaction by dithiothreitol, and also because the protein solutions at the later stages
of purification were very dilute, the proteins were first precipitated with 20% trichloroacetic acid, and residual trichloroacetic acid was extracted with diethyl ether. The precipitates were dissolved and dissolved in a small volume of 0.1 n NaOH and assayed with a microscaled Lowry procedure for their protein content.

The activity of alcohol dehydrogenases was assayed, essentially as described by Racker (20).

**RESULTS**

**Effects of Thiol Compounds on Activity of Ornithine Decarboxylase in Crude Ventral Prostate Extracts**—It was shown previously (5) that most of the ornithine decarboxylase activity of rat ventral prostate homogenates remained in solution after prolonged centrifugation at high gravitational forces. Recent studies (20) revealed that if such soluble preparations were fractionated at 0° by addition of solid ammonium sulfate, rather than by use of neutralized saturated solutions of the salt as in earlier experiments (5), then most of the ornithine decarboxylase was precipitated by ammonium sulfate between 20% and 50% saturation. It was also found (20) that, even when ventral prostate soluble extracts were prepared with buffered solutions containing 10 mm 2-mercaptoethanol and then dialyzed and fractionated with ammonium sulfate in the presence of 2-mercaptoethanol, the further addition of thiols to the enzyme assay system markedly enhanced release of CO2 from l-ornithine. In the latter respect, a saturating concentration of 5 mm dithiothreitol was at least 3 times more active than 30 mm 2-mercaptoethanol.

Evidence was obtained that inactivation of ornithine decarboxylase can start very soon after demise of the rats and process of ventral prostate tissue. Table I amplifies previous statements (20) that inclusion of 5 mm dithiothreitol in the homogenization medium and all other solutions used for dialysis and ammonium sulfate fractionation yielded preparations whose activity was several-fold higher than that obtained in absence of dithiothreitol, even though all of the decarboxylase activities were determined in the presence of 5 mm dithiothreitol. It may be mentioned here that if soluble ventral prostate extracts were fractionated with ammonium sulfate between 20 and 50% saturation at 0° in the absence of exogenous thiol compounds, then the extent of activation of ornithine decarboxylase due to addition of 5 mm dithiothreitol to the reaction mixtures was about 50% greater if the enzyme preparations were incubated for 15 to 30 min at 37° with dithiothreitol in the absence of pyridoxal phosphate and labeled ornithine, and the decarboxylation reactions were then initiated by addition of the latter two components of the standard test system, as compared with measurements in which the reactions were started by addition of untreated enzyme to incubation media containing dithiothreitol and all other ingredients.

**Purification of Ornithine Decarboxylase from Rat Ventral Prostate**—The representative data in Table II summarize a procedure for extensive purification of rat ventral prostate ornithine decarboxylase. Many preliminary experiments were carried out to establish the validity of the prescriptions for each step of the purification. Essentially the same results were obtained on four separate occasions when the following recipe was applied to isolation of the enzyme, starting with fairly large amounts of ventral prostate tissue.

An ultracentrifuged supernatant fraction from homogenates of the prostates of 30 elderly rats was prepared in the presence of 5 mm dithiothreitol as described above. This and all subsequent operations were conducted at 0-3°. Solid ammonium

### Table I

**Effect of addition of thiol compounds during processing of tissue extracts on prostatic ornithine decarboxylase activity**

The prostates from 10 male rats were homogenized, dialyzed, and fractionated with ammonium sulfate in the absence of any thiol compound (A), in the presence of 10 mm 2-mercaptoethanol (B), or in the presence of 5 mm dithiothreitol (C). Ornithine decarboxylase assays were in every instance carried out in the presence of 5 mM dithiothreitol.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Ornithine decarboxylase activity (nmol CO₂/mg protein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Processed in the absence of thiols</td>
<td></td>
</tr>
<tr>
<td>45,000 X g supernatant fraction</td>
<td>0.162</td>
</tr>
<tr>
<td>Ammonium sulfate fraction (0.2-0.5 saturation)</td>
<td>1.210</td>
</tr>
<tr>
<td>B. Processed in the presence of 10 mm 2-mercaptoethanol</td>
<td></td>
</tr>
<tr>
<td>45,000 X g supernatant fraction</td>
<td>0.845</td>
</tr>
<tr>
<td>Ammonium sulfate fraction (0.2-0.5 saturation)</td>
<td>3.370</td>
</tr>
<tr>
<td>C. Processed in the presence of 5 mm dithiothreitol</td>
<td></td>
</tr>
<tr>
<td>45,000 X g supernatant fraction</td>
<td>1.400</td>
</tr>
<tr>
<td>Ammonium sulfate fraction (0.2-0.5 saturation)</td>
<td>5.220</td>
</tr>
</tbody>
</table>

### Table II

**Purification of ornithine decarboxylase from rat ventral prostate**

About 12 g of rat ventral prostates were obtained from 30 male rats (weighing over 250 g). The ultracentrifuged supernatant fraction, prepared as described under "Materials and Methods," was treated after dialysis as described in the text. The activities are expressed as nanomoles of CO₂ released during 60 min under normal incubation conditions.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total enzyme activity (nmol CO₂/mg protein/hr)</th>
<th>Recovery (%)</th>
<th>Enzyme activity (nmol CO₂/mg protein/hr)</th>
<th>Purification 1000 fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Ultracentrifuged supernatant fraction</td>
<td>590</td>
<td>1216</td>
<td>100</td>
<td>2.06</td>
</tr>
<tr>
<td>2. Ammonium sulfate fraction, 0.2-0.5 saturation</td>
<td>192</td>
<td>600</td>
<td>91</td>
<td>5.78</td>
</tr>
<tr>
<td>3. Pooled DEAE-cellulose fraction</td>
<td>11.4</td>
<td>596</td>
<td>49</td>
<td>52.2</td>
</tr>
<tr>
<td>4. Ammonium sulfate fraction, 0.55 saturation</td>
<td>5.9</td>
<td>690</td>
<td>56</td>
<td>117</td>
</tr>
<tr>
<td>5. Pooled Bio-Gel P-200 fraction</td>
<td>0.61</td>
<td>354</td>
<td>30</td>
<td>601</td>
</tr>
</tbody>
</table>

*If only one aliquot of previous preparation was used for further purification, the total proteins and activities are corrected to the original volume.*
Tris-HCl of pH 7.5 containing 0.1 mM disodium EDTA and 2.5 mM dithiothreitol. After application of the protein, the column was equilibrated against 25 mM Tris- HCl of pH 7.5 containing 0.1 mM disodium EDTA and 2.5 mM dithiothreitol. After application of the protein, the column was washed with 150 ml of the latter buffer, which was sufficient to remove all unadsorbed proteins. Ornithine decarboxylase was then eluted with a linear gradient of 0.1 to 0.4 mM dithiothreitol. In this and all subsequent steps, 0.1 mM L-ornithine was included in the solutions because this substrate helped to stabilize the enzyme (with the aliquots used, the amounts of L-ornithine from this source that were added with the enzyme to the assay systems had hardly any influence on the final specific radioactivity of the labeled L-ornithine substrate). The NaCl gradient was generated by means of a mixing chamber of 200-ml volume containing 0.1 mM NaCl solution connected to a reservoir containing 0.4 mM NaCl in Tris- EDTA-dithiothreitol buffer. Fractions of 10 ml were collected. Ornithine decarboxylase was eluted by NaCl in concentrations about the range of 0.20 to 0.25 M, clustered around tube 20. (It must be emphasized that this purification step was highly reproducible with Serva DEAE-cellulose but that the behavior of the enzyme was not exactly the same on other types of commercial preparation of the DEAE-cellulose ion exchanger.) The three to four most active fractions were pooled (Fraction 3), and solid ammonium sulfate was added to 55% saturation (33 g/100 ml). After stirring for 30 min, the precipitate was collected by centrifugation of 25,000 × g for 10 min and dissolved in 2 ml of standard buffer containing 5 mM dithiothreitol and 0.1 mM L-ornithine (Fraction 4). An aliquot of the latter fraction containing 5 to 6 mg of protein was immediately subjected to gel filtration on a column of Bio-Gel P-200 (2.5 × 32 cm; treated as described under “Materials and Methods”) which had been equilibrated against 25 mM Tris- HCl of pH 7.5 containing 0.1 mM disodium EDTA, 5 mM dithiothreitol, and 0.1 mM L-ornithine. Ornithine decarboxylase was eluted at approximately 1.8 times the void volume, whereas the bulk of the proteins applied to the column was eluted much closer to the void volume.

At the final stage (Fraction 5), the enzyme was stable for many days when stored at 0–2°C in the presence of buffer 5 mM dithiothreitol and 0.1 mM L-ornithine. Freezing and thawing at room temperature invariably resulted in appreciable losses of enzyme activity. Glycerol (20%, v/v) had, if anything, a negative influence on the stability of the enzyme on storage at low temperatures, and depressed decarboxylating activity when determined at 37°C under standard conditions. The addition of pyridoxal 5-phosphate (0.1 mM) to the purified enzyme in the presence of 5 mM dithiothreitol did not retard the gradual inactivation of the enzyme resulting from prolonged storage at low temperatures, although, in the absence of dithiothreitol, pyridoxal 5-phosphate affords some stabilization of crude preparations of prostatic ornithine decarboxylase (5).

The material designated as Fraction 5, obtained in four separate purifications, catalyzed the release of 370 to 770 nanomoles of CO₂ per mg of protein in 60 min. The fraction appeared to consist predominantly of a single protein component in the presence of 5 mM dithiothreitol. Insufficient quantities of the purified protein were available for accurate determination of its ultraviolet absorption spectrum. The Fraction 5 material, when subjected to gel electrophoresis in the presence of dithiothreitol at pH 8.3, exhibited only one major protein band that was stained with Amido black. A single band of protein material was also manifest after gel electrophoresis in the presence of dithiothreitol at pH 7.5. However, the material from Fraction 3 (pooled material eluted from a DEAE-cellulose column) contained at least three major bands of migrating protein in addition to some stainable material at the origin. Although recovery of active ornithine decarboxylase activity from unstained gels was poor, Fig. 1 shows that virtually all of the enzyme activity that could be eluted was closely associated with the single major band of protein material that was obtained after gel electrophoresis of Fraction 5 material in the presence of dithiothreitol at pH 8.3.

**Some Properties of Purified Prostatic Ornithine Decarboxylase**—Earlier studies (5) established that fairly crude ammonium sulfate fractions from rat ventral prostate homogenates catalyzed a release of CO₂ from L-ornithine that was completely stoichiometric with the formation of putrescine. Neither EDTA, Mg++; nor a variety of other divalent metal ions influenced the decarboxylation of ornithine. Formation of CO₂ from L-ornithine by the much more highly refined Fraction 5 enzyme preparations described above was linear with respect to time over an initial 60-min period when the substrate concentration was either suboptimal (0.1 mM) or in the excess (1 mM) normally present in the standard incubation mixture, provided that the amounts of enzyme added did not decarboxylate more than 5% of the added L-ornithine substrate. The reactions were also strictly proportional to the amount of enzyme protein added over a several-fold range. Fig. 2 shows that the apparent $K_m$ for L-ornithine was about 0.1 mM at pH 7.2 in the presence of the ingredients of the standard test system containing dithiothreitol. This value is slightly lower than the previously reported value (0.2 mM) for the $K_m$ for L-ornithine measured in the absence of dithiothreitol with crude ammonium sulfate fractions from rat ventral prostate (5) or regenerating liver (8). Reduction of the concentration of dithiothreitol from the standard level of 5 mM to one of 0.5 mM decreased the $V_{max}$ but had little influence on the $K_m$ for L-ornithine at pH 7.2 in experiments with the purified prostatic enzyme.

Maximal rates of ornithine decarboxylation occurred near pH 7. In the presence of dithiothreitol, however, the pH activity profile exhibited a distinct and reproducible shoulder around pH 8 (Fig. 3). This alkaline shoulder of the pH activity curve disappeared when the assays were conducted without addition of dithiothreitol to the reaction mixture, and when the final concentration of dithiothreitol therein (because of that added with the enzyme preparation) was only 0.5 mM. It was previously reported that, in the absence of thiols, a single peak of maximal prostatic ornithine decarboxylase activity was observed at pH 7.0 with crude prostatic ammonium sulfate fractions (5).
Electrophoresis was carried out by the method of Davis (24) as modified by Jarabak et al. (25) but with only 10% (w/v) glycerol in the gels, and with 25 mM Tris-HCl of pH 8.3 containing 0.1 mM EDTA and 5 mM dithiothreitol as buffer. Fraction 5 protein (18 µg) was applied to the gel. Electrophoresis was conducted at 25° with the use of cold buffers and cold running water cooling, with a current of 2.5 mA per gel and for a total time of 3.5 hours. One gel was carried through the procedure and then fixed and stained with Amido black, followed by electrophoretic destaining. The upper part of the graph presents the location of the single protein band that stained with the dye. Another gel subject to electrophoresis in parallel was not fixed, but sectioned into 4-mm slices. The individual pieces of gel were eluted with the standard incubation mixture (but with ornithine and pyridoxal 5-phosphate omitted) for 15 hours at 2°. Ornithine decarboxylase activity was then assayed under conditions of the standard test system. The arrow shows the direction of migration of protein and enzyme activity toward the anode.

Even crude extracts (Fraction 1) showed a requirement for pyridoxal 5-phosphate under conditions of the standard test system, and this necessity for the coenzyme was absolute with all of the more purified preparations (Fractions 2 to 5). The concentration of pyridoxal 5-phosphate in the standard assay system (0.2 mM) represented a large excess of the coenzyme at pH 7.2. The standard glycyglycine buffer proved to be the most superior of the various buffers examined at pH 7.2. Substitution of Tris-HCl of the same concentration and pH gave lower rates of CO₂ formation, and at pH 7.2 sodium phosphate (100 mM) and Tris-maleate (100 mM) buffers were strongly inhibitory.

**Effects of Various Thiol Compounds on the Purified Enzyme**

In order to examine further the aforementioned effects of thiols on prostatic ornithine decarboxylase, portions of Fraction 5 enzyme were dialyzed against large volumes of standard buffer to which no thiols were added. Table III illustrates the effects of addition to the assay system of a variety of thiols on the decarboxylation of ornithine by such dialyzed preparations. There was virtually no formation of CO₂ in the absence of any sulfhydryl compound. Reactivation of about 60% of the enzyme activity present before dialysis was achieved by addition of 5 mM dithiothreitol. Oxidized dithiothreitol was totally inactive. Considerable reactivation of enzyme activity was, however, observed with other thiols; in general, thiols seemed to be superior to monothiol compounds. Thus, Table III shows that, at 5 mM concentrations, a marked enhancement of enzyme activity was seen, in decreasing order, with dithiothreitol, dihydrodipicolinic acid, and 2,3-dimercapto-1-propanol. In concentrations of 10 mM, the monothiols glutathione and 2-mercaptoethanol were considerably less effective. CoA in the reduced form at 5 mM neither activated the purified L-ornithine decar-
Fraction 5 enzyme was dialyzed against 500 volumes of 25 mM Tris-HCl of pH 7.5, 0.1 mM EDTA in the absence or presence of 5 mM dithiothreitol. An aliquot (8.4 µg of protein) of the appropriately dialyzed preparation was then layered over 5 to 30% linear sucrose gradient in 25 mM Tris-HCl of pH 7.5, 0.1 mM EDTA with (●) or without (○) 5 mM dithiothreitol (DTT) and centrifuged at 60,000 rpm for 13.5 hours at 2°C. The fractions (0.2 ml) were collected from the bottom of the tube and assayed for ornithine decarboxylase activity under standard conditions in the presence of 5 mM dithiothreitol. The arrow indicates the banding of 0.2 µg of bovine serum albumin (BSA). The recovery of the total enzyme activity was about 80%.

For the Fraction 5 preparation was dialyzed against 500 volumes of 25 mM Tris-HCl of pH 7.5, 0.1 mM EDTA containing 5 mM dithiothreitol. About 90 µg of protein were applied to a Bio-Gel P-200 column (2.5 x 32 cm) equilibrated with 25 mM Tris-HCl of pH 7.5, 0.1 mM EDTA containing 5 mM dithiothreitol (●) and eluted with the same solution. The same column was again equilibrated with the Tris-EDTA buffer without dithiothreitol. Protein (135 µg) dialyzed in the absence of dithiothreitol (○) was then applied to the column and eluted with Tris-EDTA buffer. Fractions of 3 ml were collected and assayed for ornithine decarboxylase activity in the presence of 5 mM dithiothreitol in each case. The arrows indicate the elution position of yeast alcohol dehydrogenase (9) and the elution position of horse liver alcohol dehydrogenase (8).

The enzyme preparation was first dialyzed against standard buffer without dithiothreitol and then centrifuged in the same type of sucrose density gradient with no dithiothreitol added, most of the decarboxylase activity clearly sedimented more quickly (Fig. 4). Similarly, in the presence of dithiothreitol, prostatic ornithine decarboxylase was retained to a greater extent on passage through a Bio-Gel P-200 column than when dialyzed preparations of Fraction 5 enzyme were subjected to gel filtration in the absence of added thiols (Fig. 5). From the Bio-Gel P-200 gel filtration data it was calculated that the molecular Stokes radius of the apparently smaller form of the enzyme (manifest in the presence of 5 mM dithiothreitol) was 3.87 nm, whereas the molecular Stokes radius of the bigger form of the enzyme (obtained by gel filtration without added thiol) was 4.48 nm. These values are in excellent agreement with those previously obtained (19) from gel filtration measurements of relatively cruder preparations of prostatic ornithine decarboxylase. It therefore appears that the alterations in physical behavior of prostatic ornithine decarboxylase due to dithiothreitol reflect true changes in the size of the enzyme molecules (presumably a result of protomer-polymer equilibria), rather than being due to nonspecific protein-protein interactions involving the enzyme and other unrelated soluble prostatic proteins. If it is assumed that the partial specific volumes of both major forms of the enzyme are the same, it can be reckoned from the combined data obtained from sucrose density gradient and molecular sieve measurements that the molecular weight of the larger form of the enzyme is about 1.9 times greater than that of the smaller species (in the range of 65,000 to 85,000). The data in Table III hint that the apparently dimeric form of the enzyme, formed in the absence of thiols, is totally inactive catalytically and can be reactivated during the incubation to a considerable extent by dithiothreitol and some other sulfhydryl compounds. In addition to this apparent dimeric form of the enzyme, it appeared that even higher degrees of polymerization occurred as shown by the sucrose density gradient and gel filtration experiments (Figs. 4 and 5).

Raising the ionic strength of the standard incubation system for assay of decarboxylase activity by addition of sodium sulfate over a concentration range of 0.1 to 0.5 M caused a marked and progressive decrease in CO₂ release in the presence of 5 mM dithiothreitol. At none of the concentrations of sodium sulfate tested was there any enhancement of the very low rate of decarboxylation observed in the absence of dithiothreitol.

Search for Low Molecular Weight Effectors—It was previously found (5) from measurements in the absence of thiols that putrescine, and to a lesser extent spermidine and spermine, served as relatively feeble competitive inhibitors with respect to the L-ornithine substrate of the activity of crude preparations of rat ventral prostate ornithine decarboxylase (5). The competitive nature of the inhibition due to putrescine was confirmed with the use of purified preparations of the prostatic enzyme when the assays were conducted with addition of 5 mM dithiothreitol. The Kᵢ value for putrescine obtained under these conditions (1 mM) was close to that obtained earlier (5).

In light of the known metabolic relationships of ornithine decarboxylase to polyamine biosynthesis and also other biosynthetic pathways such as the urea cycle, various metabolic intermediates were tested for their action on the decarboxylase activity of prostatic Fraction 5 preparations under standard assay conditions in the presence of 5 mM dithiothreitol. At a
final concentration of 1 mM, none of the following compounds altered the rate of decarboxylation by more than 20%: S-adenosyl-L-methionine, L-methionine, L-citrulline, carbamylphosphate, ATP, ADP, 5'-AMP, adenosine 3',5'-cyclic monophosphate, 5'-GMP, and 5'-CMP. At a concentration of 2 mM, D,L-ureidosuccinate was similarly ineffective.  

**DISCUSSION**

The very considerable degree of purification of rat ventral prostate cytosol ornithine decarboxylase achieved in these studies was made possible by the finding that dithiols like dithiothreitol not only directly enhance the activity of the enzyme in both the crude and purified state, but also prevent the enzyme from inactivation on storage at low temperatures. It is evident that previously reported values for the activity of ornithine decarboxylase in the soluble fraction of homogenates from rat ventral prostate (9, 10) are many-fold lower than those obtained when dithiothreitol is added to both preparative and assay solutions. In the presence of dithiothreitol, it appears that a single discrete protein is very largely, if not exclusively, responsible for the ornithine decarboxylase activity of rat ventral prostate cytosol. In the absence of thiols, however, this protein apparently undergoes polymerizations to form larger molecules (mainly species which seem to be dimers) that are separable by sucrose density ultracentrifugation or molecular sieving. These larger forms of the enzyme are, in all probability, quite inert catalytically in the absence of thiols such as dithiothreitol.

Many obvious and pressing questions regarding the changes in physical state and catalytic functions of the prostatic ornithine decarboxylase described here could be easily scrutinized experimentally if reasonably large amounts of the purified enzyme were available. On the scale of isolation used in the present experiments, less than milligram amounts of the most purified material could be obtained, even though the over-all recovery of activity through a reproducible five-step procedure (between 15 and 30% in various large scale preparations) was pretty good. When prepared and tested in the presence of dithiothreitol, crude soluble fractions of rat ventral prostate homogenates exhibit considerably higher ornithine decarboxylase activity than most other rat tissues that we have examined, including regenerating liver. It can be calculated that, by the present procedure, only about 10 mg of the purified protein could be obtained from the ventral prostates of close to 1000 rats. These considerations have unfortunately precluded more thorough studies on the physical properties of ornithine decarboxylase from rat tissues.

The unavailability of large amounts of purified enzyme made it impossible to study in detail the stringent requirement for certain thiols with respect to both the catalytic activity and stabilization of the protein. The specificity toward various thiols, and particularly dithiols such as dithiothreitol, is striking, and some monothiols (e.g., CoA) are completely inert in this regard. This situation is comparable to the report of Nakashima, Pontremoli, and Horecker (31) that the catalytic activity of rabbit liver fructose diphosphatase is enhanced by only certain sulphydryl compounds; in this instance, however, CoA or acetyl carrier protein was highly active, whereas glutathione and cysteine were inert, although the latter monothiols reversed the activation by CoA or acetyl carrier protein, suggesting that the fructose diphosphatase became linked to the activators by disulfide bridges. No evidence is available to decide whether the activation and apparent depolymerization of large forms of rat ventral prostate ornithine decarboxylase by dithiothreitol occurs via reduction of inter- or intramolecular disulfide bridges, or possibly via oxidation or disulfide exchange reactions, as may take place at neutral or alkaline pH values, respectively, in the activation of rabbit liver fructose diphosphatase by CoA (31).

Nothing is known about the number and reactivity of sulphydryl groups (32) in the various forms of rat ventral prostate ornithine decarboxylase that we have purified.

Today there are known many examples of enzymes whose activity may be enhanced by dissociation into protomeric forms or individual subunits, or, on the contrary, by the formation of larger, polymeric species of the proteins. Jarabak et al. (25) have pointed out that some cold-sensitive enzymes may become inactivated concomitantly with dissociation into protomeric forms by exposure to low temperatures, whereas other enzymes of this class, including a highly purified 17β-hydroxysteroid dehydrogenase from human placenta, may in the cold polymerize to a series of high molecular weight forms that are catalytically inert. The rat ventral prostate ornithine decarboxylase appears to be inactivated when it undergoes polymerization in the absence of appropriate exogenous thiols. However, this enzyme is not inactivated by cooling to temperatures between 0° and 10°, and it is not stabilized by high concentrations of polyols such as glycerol, which are well known to preserve the activity of a number of cold-labile enzymes (29).
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