PURIFICATION AND CHARACTERIZATION OF BOVINE LIVER ORNITHINE DECARBOXYLASE

by

Mari Kristine Haddox

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF MICROBIOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY
WITH A MAJOR IN MOLECULAR BIOLOGY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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SIGNED: Mari Kristine Haddoy

To DHR and NDG, my mentors and my friends.

To my parents, for their love and encouragement.

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ABSTRACT

Ornithine decarboxylase has been purified to apparent homogeneity from thioacetamide-stimulated calf liver. The purification process, which has been developed to circumvent the lability of the enzyme, employs ion exchange chromatography, gel filtration, hydroxylapatite chromatography, non-denaturing gel electrophoresis, and sulfhydryl affinity chromatography. The enzyme is purified 71,500-fold to a final specific activity of 286,000 pmol/min/mg protein. Non-denaturing gel electrophoresis indicates a single protein present in the final preparation. The enzyme has a Stokes radius of 3.14 nm as indicated by gel filtration and a monomeric molecular weight of 52,000 daltons as indicated by denaturing gel electrophoresis. The K_m values for ornithine and pyridoxal phosphate are 0.16 mM and 2.5 μM, respectively. Putrescine inhibits the enzyme (K_i 10 mM). The existence of three ionic forms of ornithine decarboxylase is suggested by fractionation of the preparation by gradient sievorptive chromatography. Mammalian ornithine decarboxylase is apparently a metalloenzyme. A variety of structurally distinct metal chelators inhibit the enzyme. A non-chelating analog of the most potent chelator, 1,10-phenanthroline, is without effect. The order of efficacy of the chelators suggests the involvement of a metal from the transition series. Incubation of the enzyme with charcoal or Cibacron Blue-Agarose results in a loss of catalytic activity suggesting that the ornithine decarboxylase may also contain a bound nucleotide.

INTRODUCTION

Ornithine decarboxylase (EC 4.1.1.17), the initial and rate-limiting enzyme in the polyamine biosynthetic pathway, is a key regulatory enzyme in the growth process. Elevated levels of enzyme activity were first shown in 1968 to be a characteristic feature of rapidly growing eucaryotic cells (Russell and Snyder, 1968). Nongrowing cells contain virtually no ornithine decarboxylase and appear to be arrested in a nonmitotic state prior to the events required for the induction of the enzyme. Certain physiologic growth-arrest signals, such as vitamin A, appear to regulate proliferation by acting to limit expression at this site (Haddox and Russell, 1979; Haddox, Scott, and Russell, 1979). An increase in the measurable cellular activity of ornithine decarboxylase is a ubiquitous event in the cell's response to a growth stimulant (Russell, Byus, and Manen, 1976). Growth processes during which the enzyme has been shown to increase include the compensatory proliferation of rat liver after partial hepatectomy, tissue developmental changes during embryogenesis, the anabolic response of differentiated target tissues to trophic hormones, and the biosynthetic cascade of events constituting G_1 phase during cell cycle progression (reviewed by Jänne, Pösö, and Raina, 1978; Russell and Haddox, 1979; and discussed below). Furthermore, several lines of evidence suggest that alterations in the regulation of ornithine decarboxylase induction and expression in growing cells may be associated with the transformed state (Haddox, Magun, and Russell, 1980a).

Previous purifications of the enzyme have been reported (see below). However, tangible amounts of active enzyme suitable for definitive physical, chemical, and biological characterization have not been obtained. There is little doubt that the further elucidation of the molecular phenomena operating at the transcriptional, translational, and post-translational levels to control the cellular expression of ornithine decarboxylase, in addition to the resolution of the relevance of the enzyme to growth control mechanisms, is heavily dependent on progress in the purification and characterization of the molecule itself. The present investigation was initiated to contribute toward this goal and reports a method of purification of ornithine decarboxylase to apparent homogeneity (71,000-fold) by a procedure which circumvents the lability of the enzyme. This study also reports the results of a number of characterization studies performed on the purified protein.

Polyamines: Fundamental Constituents of Macromolecular Biosynthesis

The polyamines are aliphatic nonprotein nitrogenous bases. The naturally occurring polyamines, putrescine and spermidine, are found in all living cells including bacteria, plants, and animals (C. Tabor and H. Tabor, 1974; Cohen, 1971). Spermine, although it can be utilized by procaryotes, is synthesized only in eucaryotic cells (Cohen, 1971). Polyamines function as the organic cations of the cell; at physiological pH all of the primary and secondary amine groups are in the protonated form. By virtue of this polycationic nature, they form tight, noncovalent complexes with many proteins and nucleic acids and modify events involved in macromolecular biosynthesis. While the amount of Mg^{2+} or Ca^{2+} available to support cellular function is ultimately limited by the amount available to the cell in the circulation, the polyamines can be synthesized within the cell at the time of physiological need. Polyamines are found in highest concentration in those tissues which exhibit a high rate of macromolecular synthesis (H. Tabor and C. Tabor, 1964). These include: (1) organs increasing in size by virtue of an increase in cell number, such as in the regenerative response of the liver following partial hepatectomy (Dykstra and Herbst, 1965); (2) organs increasing in size by virtue of an increase in cell mass, as exemplified by the cardiac hypertrophy response to the increased stress of a higher workload after aortic constriction (Feldman and Russell, 1972); (3) organs which maintain a high rate of macromolecular synthesis to sustain the excretion of a particular cellular product, such as the prostate (Williams-Ashman, Pegg, and Lockwood, 1969); and (4) developmental systems such as chick, rat, sea urchin, and amphibian embryos (Caldarera, Barbiroli, and Moruzzi, 1965; Raina, 1963; Russell, 1971; Russell and McVicker, 1972; Manen and Russell, 1973; Russell, Snyder, and Medina, 1969). From the studies cited and a multitude of others (reviewed in Russell and Durie, 1978), it became apparent that the accumulation of polyamines, specifically spermidine, occurred in parallel with the increase in cellular RNA.

The first demonstration that polyamines were essential to growth was made by Herbst and Snell (1948) who showed that _Hemophilus parainfluenzae_ would only grow on synthetic medium when an exogenous polyamine source was provided. This apparent stringent relationship has proven to be ubiquitous such that it has been stated that "there is no unequivocal demonstration of a bacterium or any other cell that can grow or multiply without polyamines" (Cohen, 1971, p. 22).

Polyamines have been shown to have a variety of effects *in vitro*, both stimulatory and inhibitory, on many enzymatic activities. It is often difficult to determine which of these represent specific physiologic functions of these compounds and which are due to non-specific polycationic interaction. However, several detailed and careful studies, controlling for physiological salt concentrations, have revealed functions for the polyamines which are not satisfied by inorganic cations. Of these, three will be cited as examples.

1. It has been demonstrated that tRNA requires two spermine molecules, in addition to a Mg^{2+}, to stabilize the stem of the dihydrouridine arm which allows the molecule to assume the appropriate "activated" tertiary structure facilitating interaction with the ribosome (Pochon and Cohen, 1972; Sakai and Cohen, 1976a). These results obtained further confirmation when the crystallographers found that added polyamine was essential to obtain stable tRNA conformation in a crystal (Sigler, 1975).
2. Investigations regarding the mode of action of the antibiotic edeine have revealed that this small peptide, which contains a homologue of spermidine, inhibits protein synthesis. It does so by binding to and inactivating a spermidine binding site on the ribosome which is essential for translation to occur (Obrig et al., 1971; Wagenmann and Klingmuller, 1974).

3. Studies utilizing inhibitors of polyamine biosynthesis in fibroblasts in culture have demonstrated that polyamines are essential for cytokinesis to occur (Sunkara et al., 1979). The inhibitor-treated cells are blocked in the binucleate stage unless exogenous polyamines are added.

As the limiting factor in the polyamine biosynthetic pathway, the control and expression of ornithine decarboxylase activity plays a key role in implementing these and other normal cellular biosynthetic functions.

Ornithine Decarboxylase Expression During Normal Growth

Ornithine is an unusual amino acid in that it is not used for the biosynthesis of proteins. It is a substrate for the biosynthesis of putrescine, the precursor of the polyamines spermidine and spermine, and acts as an acceptor moiety in the urea cycle. Ornithine is provided to the cell from that supply circulating in the plasma and also by the action of arginase which catalyzes the hydrolysis of arginine into ornithine. Arginase activity is present in most cells, even in the many organs which do not conduct the detoxification of ammonia via the urea cycle (Greenberg, 1960; Knox and Greengard, 1965; Williams-Ashman and Canellakis, 1979). When there is an accentuation of growth function in a tissue, such as found in the liver after partial hepatectomy, ornithine is selectively utilized in the biosynthetic pathway and diverted from the urea cycle (Cohen and Sallach, 1961; Weber, Queener, and Morris, 1972). Regulation of flux through the polyamine biosynthetic pathway is unusual as it is not controlled by changes in the level of the precursor or by any known allosteric regulation of the decarboxylating enzyme. Rather, it is regulated by the rapdily fluctuating changes in the amount of the cellular pool of ornithine decarboxylase present.

In 1940 (Gale) an inducible enzyme catalyzing the conversion of L-ornithine into putrescine was discovered in E. coli. Morris and Pardee (1965, 1966) characterized this activity and demonstrated that E. coli contained two distinct species of ornithine decarboxylase, one "inducible" and one "biosynthetic," which displayed different regulatory and kinetic properties and were formed under different cellular conditions. Decarboxylation of ornithine also was reported to occur in higher plants (Hasse, Ratych, and Salnikow, 1967).

The first evidence that the decarboxylation of ornithine to putrescine might lead to polyamine biosynthesis in eucaryotic cells was suggested in 1956 (Tabor, Rosenthal, and Tabor). However, it was not until 1968 that ornithine decarboxylase was shown to exist in animal tissues and fluctuate in activity parallel with the growth state of the tissue (Russell and Snyder, 1968; Jänne, Raina, and Siimes, 1968; Jänne and Raina, 1968). These reports established that the activity of the enzyme increased markedly in rat liver after partial hepatectomy or growth hormone administration and in chick embryos at the time of most rapid protein synthesis. In the studies of liver regeneration, hepatic ornithine decarboxylase increased specific to the liver regenerative process and did not occur in other tissues in the body (Snyder and Russell, 1970). The rapid (i.e., detectable within 2 hours) and dramatic (i.e., achieving a 20-fold elevation within 16 hours) increase in ornithine decarboxylase activity was established as one of the earliest occurring enzymatic changes detectable in regenerating rat liver (Bucher, 1963).

These original findings have been expanded over the last decade to show that the induction of ornithine decarboxylase is a universal feature of the anabolic response of target tissues to trophic hormones (Russell et al., 1976). Tissues in the adult animal in the resting or non-stimulated state have little or no detectable enzyme activity. Calculations based on the specific activity of the purified enzyme indicate a basal enzyme concentration of at most 1×10^{-9}M (see Results section below). This is 1/100 to 1/1000 the concentration of most constitutive, metabolic enzymes. If, however, the tissue is stimulated to increase the rate of RNA and protein biosynthesis, a rapid and large increase in the amount of enzyme present occurs. Typically, within 2 hours of addition of a membrane-active trophic stimulus, an increase in tissue ornithine decarboxylase is detectable. By 4 to 6 hours, the time of usual, maximal response, the ornithine decarboxylase activity has increased 20- to 200-fold, attaining an enzyme concentration near 10^{-7}M.

This increase in measurable decarboxylase activity represents a true induction of new enzyme. Immunotitration studies utilizing specific antibodies to ornithine decarboxylase have shown a correlation between increased enzymatic activity and increased immunoreactive protein in several tissues after a variety of trophic stimuli. These include rat liver after partial hepatectomy or the administration of growth hormone or thioacetamide, cultured HTC hepatoma cells stimulated by dibutyryl cyclic AMP or dexamethasone, and rat thyroid activated by thyrotrophin in the presence of isobutylmethylxanthine (Canellakis and Theoharides, 1976; Höltta, 1975; Obenrader and Prouty, 1977b). Translation-inhibitor studies have shown that protein synthesis is required for the hormonally promoted elevation in ornithine decarboxylase to occur (Russell and Snyder, 1969; Fausto, 1969; Schrock, Oakman, and Bucher, 1970; Ono, Inoue, and Takeda, 1973; Jänne and Raina, 1969; Cohen, O'Malley, and Stastny, 1970; Hogan, 1971).

Finally, studies with actinomycin D, an inhibitor of DNA-dependent RNA synthesis, have shown that the increase in enzyme molecules after a trophic stimulus is dependent, at least in part, on the synthesis of new mRNA molecules (Russell and Snyder, 1969; Fausto, 1969, 1970; Brandt, Pierce, and Fausto, 1972; Kaye, Icekson, and Lindner, 1971; Russell, Snyder, and Medina, 1970; Beck, Bellantone, and Canellakis, 1973; Hogan, 1971). Time-dependence studies, which have shown that the administration of actinomycin D at the time of growth stimulation completely blocks the increase in ornithine decarboxylase while, if the blocker is delayed 1-2 hours, the increase occurs as usual or to a greater extent, support the proposal that new mRNA for the enzyme is synthesized as an early component of the growth response and make it unlikely that artifacts involving a possible effect of the drug on other cellular processes are involved (Russell and Snyder, 1969; Costa, 1978).

The increase in ornithine decarboxylase in a growth-stimulated tissue is usually transient, typically declining toward baseline within 8 to 10 hours. This is a reflection of the fact that ornithine decarboxylase has the shortest half-life of any enzyme delineated to date, 10 to 20 minutes (Russell and Snyder, 1969; Russell et al., 1970). Rapid turnover of the enzyme has been confirmed in the absence of any protein biosynthetic inhibitors by following the natural decline of enzyme activity in the rat liver following attainment of peak activity levels as the result of growth hormone administration (Russell et al., 1970). Although changes in the half-life of ornithine decarboxylase are an apparent possible site of regulation of enzyme activity based on studies conducted on cells in culture (Clark, 1974; Hogan and Murdin, 1974; Prouty, 1976), an examination of possible changes in turnover during a physiologically-controlled growth response in the whole animal revealed no variation in the ca. 15-minute half-life (Russell and Snyder, 1969). A rapid mechanism for degradation or turnover of an enzyme protein has been shown to be fundamental to the cellular ability to rapidly change the concentration of the enzyme (Berlin and Schimke, 1965; Schimke, 1964; Schimke, Sweeney, and Berlin, 1965). Although the governing principles regarding specific protein degradation within the cell are still largely unknown, the discovery of group-specific proteases, such as the ones directed toward pyridoxal phosphate-containing enzymes (Kominami et al., 1972), may contribute to the elucidation of the control mechanisms regulating ornithine decarboxylase expression.

In addition to regulation at the transcriptional level, the cellular expression of ornithine decarboxylase is subject to multiple regulatory processes. These include: (1) stimulation at the translational level of synthesis of new ornithine decarboxylase molecules from apparently preexisting stable mRNA (Beck, Bellantone, and Canellakis, 1972, 1973; Kay, Lindsay, and Cooke, 1972; Hogan, 1971); (2) alterations in the half-life of the enzyme prolonging the existence of active enzyme (Hogan, Murden, and Blackledge, 1973; Kay and Lindsay, 1973a; Kay et al., 1972); (3) direct feedback inhibition of the enzyme by the polyamines acting as competitive inhibitors (Pegg and Williams-Ashman, 1968); (4) indirect feedback inhibition by which high intracellular levels of spermidine cause an apparent inhibition of translation of new enzyme (Kay and Lindsay, 1973b; Kallio et al., 1977); (5) expression of antizyme, a protein which can be released from apparent membrane-storage sites or synthesized during changing cellular states which inhibits ornithine decarboxylase activity in a noncompetitive fashion by binding to the enzyme (Heller, Fong, and Canellakis, 1976; Heller et al., 1977); (6) sensitivity to the cellular level or availability of the enzymic cofactor, pyridoxal phosphate (Murray and Froscio, 1977); (7) changes in the K_m of the enzyme for the amino acid substrate (Lau and Slotkin, 1978); and (8) an as yet unknown molecular mechanism, the necessity for which is suggested by <u>in vitro</u> studies and which conceivably would change under different physiological conditions, which satisfies the enzyme's high requirement for thiols for optimal activity (Jänne and Williams-Ashman, 1971; and see Results section below).

Ornithine Decarboxylase Induction as a Universal Feature of Cell Cycle

Cells undergo three major processes in order to proliferate, i.e., growth, DNA replication, and cell division. The events that constitute these processes are integrated in an ordered sequence which progresses during the cell generation cycle. The cell cycle is defined by the universally occurring and readily measured events of chromosome replication, commonly known as S phase, cell division, identified as M for mitosis, and the less well-characterized periods before and after S phase termed the gaps, G_1 and G_2 (Howard and Pelc, 1953). Progress of the cell through the initial gap of time between the nongrowing state or a preceding mitosis and the onset of DNA synthesis is generally accepted as constituting the sequence of events required to capacitate the cell to initiate DNA replication. However, this period is not yet characterized or identified by any universal occurrence of specific and/or required events. In a recent monograph on the reproduction of eucaryotic cells, G_1 was identified as a "major gap in our understanding of the cause and effect continuity of the cell life cycle" (Prescott, 1976, p. 3). The physiologic restriction points for cell growth occur during this period and therefore the key to understanding and controlling growth awaits a full exposition of the events of this phase of the cell cycle.

The events of G_1 and G_2 could be envisioned as constituting the growth phase of the cell cycle during which the prerequisite RNA and protein for the replication of the daughter chromosomes and cell are synthesized under the control of key regulatory enzymes signaled by the external growth factors. (This suggestion is not made to exclude these events from occurring, at least in part, temporally concurrent with DNA synthesis.) Just as induction of ornithine decarboxylase is a universal feature of the anabolic response of target tissues to trophic hormones, as discussed in the previous section, it is also a general event in the trophic process of cell cycle progression. Many cell types in culture have been shown to express an induction of ornithine decarboxylase as the cells progress through G_1 phase and enter S phase. This increase in enzyme activity has been shown to occur after fresh serum-containing media addition has stimulated the growth of Rat-1 and RSV-transformed Rat-1 cells (Haddox et al., 1980a), HTC cells (McCann et al., 1975; Hogan, 1971; Hogan, McIlhinney, and Murden, 1974), H35 Hepatoma cells (Fong, Heller, and Canellakis, 1976), HeLa cells (Hodgson and Williamson, 1975), BHK cells (Melvin, Thomson, and Hay, 1972; Hibasami et al., 1976), 3T3 cells (Lembach, 1974), neuroblastoma and glioma cells (Bachrach, 1976), WI-38 cells (Heby et al, 1975), L cells (Yamasaki and Ichibara, 1976), and cultured endothelial cells (D'Amore and Shepro, 1978). Increased ornithine decarboxylase activity is observed after specific growth factor stimulation of cell cycle progression (Clark, 1974; Stastny and Cohen, 1970). Furthermore, the activity of the enzyme is elevated in a phase-specific manner during G_1 progression of chemically synchronized cells (Fuller, Gerner, and Russell, 1977; Russell and Stambrook, 1975; Heby et al., 1976; Friedman, Bellantone, and Canellakis, 1972; Hibasami et al., 1977). The initial rise in ornithine decarboxylase activity generally occurs within 2-3 hours and peaks from 4-6 hours after the stimulus to initiate cell cycle, whether the progression is due to synchronous cell cycle traverse, the addition of serum, or the addition of growth factors. A second maximum in ornithine decarboxylase activity generally occurs after the cessation of DNA synthesis during G_2.

The G_1 progression of cells is governed by a complex mixture of extracellular components including both low molecular weight nutrients, such as amino acids and vitamins, and high molecular weight serum growth factors. It is not surprising, therefore, that the increase in ornithine decarboxylase activity is governed by at least several of the regulatory mechanisms discussed above. In all cases tested, the addition of cycloheximide or puromycin at the initiation of cell cycle traverse completely inhibited the increase in ornithine decarboxylase (Bachrach, 1975; Hogan, 1971; Fong et al., 1976; Hodgson and Williamson, 1975; Yamasaki and Ichihara, 1976; Clark, 1974; Stastny and Cohen, 1970; Friedman, Bellantone, and Canellakis, 1972). Therefore, the mechanisms regulating ornithine decarboxylase induction involve either transcriptional or translational events resulting in the synthesis of new enzyme molecules rather than activation of preexisting enzyme. Studies with actinomycin D indicate that a major pathway whereby ornithine decarboxylase is induced involves activation of genetic transcription. Actinomycin D administration results in complete inhibition of the ornithine decarboxylase increase which occurs in Don C (Friedman, Bellantone, and Canellakis, 1972) and Chinese hamster ovary (CHO) cells (unpublished data, Haddox, Scott, and Russell, 1979) during cell cycle and in neuroblastoma (Bachrach, 1975), CHO (Costa, 1978), and 3T3 (Clark, 1974) cells after the addition of serum or growth factors. Partial inhibition (40-70%) of the increase in enzyme activity also has been reported in HTC (Hogan, 1971; Hogan, McIlhinney, and Murden, 1974), H35 (Fong et al., 1976), and HeLa (Hodgson and Williamson, 1975) cells after the addition of serum or growth factors.

Time-dependent addition studies in 3T3 (Clark, 1974) and CHO (Costa, 1978) cells have shown that the actinomycin D-sensitive transcriptional event occurs during the first 2 hours after stimulation. In those cells in which the actinomycin D sensitivity is only partial, it appears to involve events which occur after the first 2 hours of cell cycle traverse (Hogan, McIlhinney, and Murden, 1974).

Cyclic AMP-Dependent Mechanism of Ornithine Decarboxylase Induction

Hutchings and Sato (1978) have defined a set of five components (epidermal growth factor, fibroblast growth factor, transferrin, insulin, and hydrocortisone) which can replace the serum in the culture medium of HeLa cells. They have concluded that the growth functions previously attributed to serum macromolecules are served mainly by hormones or hormone-like substances. The different pattern of hormonal requirements characteristic of different cell lines are presumed to reflect the differentiated state of the cell, although the same intracellular growth processes are, of course, thought to occur. Cyclic nucleotides are classically viewed as intracellular messengers for hormone function, such that different hormones interacting with membrane receptors on specific target cells can modify the same intracellular events (Robison, Butcher, and Sutherland, 1968, 1971). Therefore, it would seem reasonable for cyclic AMP to play a second messenger role in the hormonally-controlled processes of growth.

Sutherland and his coworkers defined a set of criteria for establishing that a hormonally-controlled event was mediated by a cyclic AMP-dependent mechanism (Sutherland, 1972). Most of these have now been met and establish cyclic AMP as the positive intracellular signal for the trophic polypeptide hormone-promoted induction of ornithine decarboxylase, the resulting increase in polyamines, and the consequent facilitation of increased RNA and protein biosynthesis. These include:

1. Trophic hormones which promote an increase in target tissue ornithine decarboxylase also stimulate an increase in cyclic AMP and/or an activation of cyclic AMP-dependent protein kinase which precedes the induction of the enzyme (reviewed by Russell and Haddox, 1979).
2. Inhibitors of phosphodiesterase, the enzyme which hydrolyzes the 3'-5' bond of cyclic AMP, display differential abilities to inhibit the hydrolytic enzyme and therefore elevate intracellular cyclic AMP and activate protein kinase. This relative potency is reflected in their ability to induce ornithine decarboxylase (Byus and Russell, 1975).
3. Analogs of cyclic AMP promote an increase in ornithine decarboxylase in several cell types (reviewed in Russell and Haddox, 1979). Furthermore the ability of a series of 8-substituted cyclic AMP analogs to activate protein kinase *in vitro* is directly correlated to their ability to induce ornithine decarboxylase *in vivo* (Byus, Wicks, and Russell, 1976).
4. Mutant subclones of adrenal tumor cells which lacked adenylate cyclase failed to induce ornithine decarboxylase in response to ACTH in contrast to the parent cell line in which the

activity of the enzyme increased 30-fold after administration of the adrenotrophic hormone. Mutant lines which displayed decreased cyclic AMP-dependent protein kinase also showed attenuated ornithine decarboxylase induction after ACTH (Kudlow et al., 1979).

The cyclic AMP-promoted increase in ornithine decarboxylase occurs generally 3 to 5 hours after the addition of the cyclic nucleotide, a time-dependence comparable to that for serum stimulation, so it is unlikely that the action of cyclic AMP is to directly modify the activity of existing enzyme molecules. This time-dependence is more consonant with a requirement for synthesis of new RNA and protein molecules. The addition of cyclic AMP to hepatoma cells results in an increase in [^{14}C]leucine incorporation into enzyme protein immunoprecipitable by antibody monospecific to ornithine decarboxylase, indicating that the cyclic nucleotide promoted the de novo synthesis of the enzyme protein (Canellakis and Theoharides, 1976). Furthermore, prevention of RNA synthesis by the presence of actinomycin D inhibits 70-100% of the cyclic AMP-induced increase in ornithine decarboxylase (Hogan, Shields, and Curtis, 1974; Costa, 1978; Canellakis and Theoharides, 1976), indicating that transcription is required to support the new enzyme synthesis. Therefore, it appears that at least part of the mechanism by which cyclic AMP increases ornithine decarboxylase is by promoting the activation of transcription of the ornithine decarboxylase gene.

If cyclic AMP regulates the synthesis of messenger RNA for ornithine decarboxylase during cell cycle, the level of the cyclic nucleotide or the activation state of the kinase it regulates would be expected to increase in cultured cells during G_1 prior to the increase in ornithine decarboxylase activity. This is, in fact, the case. In several cell systems, cyclic AMP levels have been shown to increase to a maximum in mid to late G_1 and then fall as cells enter S phase (Costa, Gerner, and Russell, 1976a, 1976b; Russell and Stambrook, 1975; Yamasaki and Ichihara, 1976; Hibasami et al., 1977; Zeilig et al., 1974; Sheppard and Prescott, 1972; Seifert and Rudland, 1974; Millis, Forrest, and Pious, 1974; MacManus et al., 1972; Zeilig and Goldberg, 1977; Wang, Sheppard, and Foker, 1978). In CHO (Costa et al., 1976a, 1976b) and glioma cells (Bachrach, Katz, and Hochman, 1978), this increase has been shown to be accompanied by an activation of the cyclic AMP-dependent protein kinase and the maximum is achieved prior to the peak increase in ornithine decarboxylase activity.

These studies suggest that the regulation of ornithine decarboxylase during growth in the cell cycle is very comparable to that discussed for trophic stimulation of tissues. Cells in culture, responding to the presence of serum growth factors (hormones), exhibit an elevated level of cyclic AMP, an increase in activity of cyclic AMP-dependent protein kinase, and a subsequent induction of ornithine decarboxylase. The increased ornithine decarboxylase activity results, at least in part, from activation of a transcriptional event leading to *de novo* synthesis of the enzyme and can be mimicked by the addition of exogenous cyclic AMP.

There are also cell types and/or cell growth states in which addition of cyclic AMP inhibits ornithine decarboxylase activity (Yamasaki and Ichihara, 1976; Insel and Fenno, 1978). This inhibition, which occurs after abnormally high cellular cyclic AMP levels are achieved by the addition of exogenous analogs of cyclic AMP and/or phosphodiesterase inhibitors, occurs in conjunction with an arrest of cellular growth (Burk, 1968; Ryan and Hedrick, 1968; Johnson and Pastan, 1971; Sheppard, 1971; Pardee, 1974).

The preceding paragraphs cited the evidence implicating cyclic AMP as a positive effector of the hypertrophy (i.e., increased RNA and protein) component of cellular growth. These data do not obviate the possibility that cyclic AMP can act as a negative effector of proliferation if the process is measured as a consumate event (i.e., cell doubling) since the hypertrophy component has to be coupled to hyperplasia (i.e., doubling of the chromosomes) in order for cell division to occur. In support of this notion, Wang and coworkers (1978) have shown in concanavalin A-stimulated lymphocytes that the G_1 phase increase in cyclic AMP and subsequent cyclic AMP-dependent protein kinase activation is absolutely required for the proliferative response. However, if the naturally-occurring, temporally-discrete elevation in the level of the cyclic nucleotide is prolonged by 8-bromo-cyclic AMP addition, it become inhibitory to progression into the S phase of growth and ultimate cell doubling.

Although the mechanism of this inhibition is not known, it may result from a different cyclic AMP-dependent kinase isozyme pattern in the inhibited cells as opposed to the positively responsive cells.

Fluctuations in the cellular content of both cyclic AMP and its specific protein kinases occur as a normal component of cell cycle traverse (Costa et al, 1976a, 1976b; Byus et al., 1977). In non-dividing cells, cyclic AMP has been implicated as the second messenger for trophic hormone action, mediating increases in general and/or specific protein and RNA synthesis (Costa et al., 1976a, 1976b; Byus et al., 1977; Guidotti and Costa, 1973; Richman et al., 1976; Levine et al., 1975; Byus, Haddox, and Russell, 1978; Cooke, Lindh, and Janszen, 1976; Dufau et al., 1977). The trophic process by which a cell increases its mass through increased RNA and protein synthesis is an essential aspect of the cell cycle in dividing cells constituting the major events related to G_1 progression of cell cycle. It appears, therefore, that there exists some major difference between the intracellular events promoted by high exogenously-derived cellular cyclic AMP content, which lead to growth arrest, and those that occur as the result of trophic hormone or growth factor triggered changes in cyclic AMP concentrations that fall within the normal physiological range, which are associated with a growth-stimulatory response.

Cyclic AMP-dependent protein kinase has been found in all mammalian tissues and most, if not all, of the events promoted by the cyclic nucleotide are believed to be a consequence of the activation of the enzyme (Kuo and Greengard, 1969). Two types of kinase exist (Corbin, Keely, and Park, 1975) which, although containing the same catalytic subunit, have been postulated to play different functional roles in the cell as a result of their different activation and regulatory characteristics and intracellular localizations which are a function of differences in the regulatory subunit (reviewed by Nimmo and Cohen, 1977). The two types are differentially activated by trophic hormones (Byus et al., 1977; Byus et al., 1979; Schwoch, 1978; Podesta et al., 1978) and differentially synthesized in specific tissues during changing developmental (Lee et al., 1976; Haddox, Roeske, and Russell, 1979) or endocrine (Fuller, Byus, and Russell, 1978) status. Therefore, in a recent study in our laboratory (Haddox, Magun, and Russell, 1980b), we examined the activation, synthesis, and turnover of protein kinase in proliferating and dibutyryl cyclic AMP growth-arrested fibroblasts to see if any major differences in these primary effectors for cyclic AMP action could be elucidated which could explain the apparently opposing consequences ascribed to the action of the cyclic nucleotide in proliferating cells.

This study provided evidence that abnormally high changes in the cellular cyclic AMP level may regulate the differential synthesis and turnover of type I and type II cyclic AMP-dependent protein kinase. The addition of a cyclic AMP analog to cultured fibroblasts results in a marked increase in the amount of type I and a simultaneous decrease in the amount of type II kinase in association with growth arrest. This phenomenon is not restricted to one cell type since it occurred in CHO cells, in Rat-1 cells, and in RSV-transformed Rat-1 cells.

The pattern of expression of type I and type II kinase in response to the addition of a cyclic AMP analog in G_1 is markedly different from that detected during normal cell cycle progression. Type I protein kinase is relatively constant in specific activity whereas type II increases rapidly in specific activity during G_1 with a greater than a 2-fold increase in activity expressed at the G_1/S border. When dibutyryl cyclic AMP is added to CHO cells after exit from mitosis, there is a rapid increase in the amount of type I kinase, more than doubling by 2 hours after mitosis and reacing a level at 9 hours comparable to the level of type II kinase during normal cell cycle progression. Further, type II protein kinase has dropped in amount of activity significantly and by 5 hours has only about 30% the amount of activity present after exit from mitosis. These cAMP-promoted changes result in the almost complete inversion of the expression of type I and type II kinases.

Thus, the resolution of the conflict between cyclic AMP functioning as a positive effector of trophic processes in proliferating cells and cyclic AMP functioning as a negative effector of progression of cells into and/or through the DNA synthetic phase apparently lies in the molecular mechanism for exerting two effector roles for cyclic AMP, which is the proper, temporally discrete activation of the appropriate type of protein kinase. In support of this, Byus et al. (1977) have shown that incubation of human lymphocytes with the mitogenic plant lectin concanavalin A results in the selective activation of only type I kinase during pre-S phase, even though the cells contain significant amounts of both type I and type II protein kinase and that this activation is followed by induction of ornithine decarboxylase and subsequent cell proliferation. In contrast, the prolonged activation of both type I and type II kinase in the concanavalin A-stimulated lymphocytes as the result of dibutyryl cyclic AMP addition was associated with an inhibition of ornithine decarboxylase induction and inhibition of DNA synthesis.

Ornithine Decarboxylase Expression During Transformed Growth

Several lines of evidence suggest that alterations in the regulation of ornithine decarboxylase induction and expression in growing cells may be associated with the transformed state: (1) increases in ornithine decarboxylase activity have been correlated with the tumor-promoting action of phorbol esters in mouse epidermis (O'Brien, Simsiman, and Boutwell, 1975); (2) ornithine decarboxylase activity increases in cultured cells after infection with Rous sarcoma (Don and Bachrach, 1975), murine sarcoma (Gazdar et al., 1976), polyoma (Goldstein, Heby, and Marton, 1976), or cytomegalo-viruses (Isom, 1979); (3) higher ornithine decarboxylase activity is detectable in the transformed state in comparisons between fast-growing versus slow-growing Morris hepatomas (Williams-Ashman, Coppoc, and Weber, 1972), in epidermal carcinoma vs. normal epidermis (O'Brien, 1976), and in chemically- (O'Brien and Diamond, 1977) and virally- (Lembach, 1974) transformed cells vs. their normal counterparts; and (4) feedback regulation of ornithine decarboxylase activity is altered in some transformed cells (Bethell and Pegg, 1979; Isom and Backstrom, 1979).

In general, the characteristics of polyamine biosynthesis in transformed cells reflect a diminished response to normal control mechanisms. For example, the chemically-transformed fibroblasts, as compared to their untransformed counterparts, exhibit larger, longer-lasting induction of ornithine decarboxylase in response to serum-containing medium (O'Brien and Diamond, 1977). Furthermore, much higher concentrations of polyamines added to the growth media are required to suppress ornithine decarboxylase activity in CMV-transformed cells (Bethell and Pegg, 1979) as compared to normal.

Recent studies in our own laboratory (Haddox et al., 1980a) have demonstrated three different characteristics of the induction of ornithine decarboxylase during G_1 progression in RSV-transformed fibroblasts as compared to their normal counterparts which suggest that the regulation of ornithine decarboxylase is altered in the transformed phenotype.

1. Ornithine decarboxylase activity is induced to a greater extent during G_1 phase and is maintained at a higher level during G_1/S phase transition in transformed cells.
2. Transcription-dependent ornithine decarboxylase induction can occur in transformed cells independent of exogenous growth factor addition.
3. Ornithine decarboxylase induction is less sensitive to putrescine repression in the Rat-1 (wt/RSV) cell line than in the parent Rat-1 cell.

These differences could be inherent to the transformation process promoted by the virus in these cells or could simply reflect a secondary consequence of some other virally affected event. Therefore, the pattern of ornithine decarboxylase induction in a cell line transformed by a thermosensitive mutant of RSV was determined. If the differences in G_1-dependent ornithine decarboxylase induction detected in the cells transformed by the wild-type virus are a consequence of the transforming function of the virus they should be a function of the incubation temperature in the cells transformed by the mutant virus in which the transforming function is thermosensitive. The Rat-1 (tsLA24/RSV) cell line transformed by the thermally labile RSV-LA24 was found to express a greater ornithine decarboxylase activity throughout G_1 phase and into S phase which was less sensitive to putrescine inhibition when incubated at 35°, the permissive temperature for the viral transforming function, after the addition of serum-containing media than when incubated at 39°, the temperature at which transformation was repressed. Furthermore, a shift of this cell line from 39° to 35° in the absence of any added serum growth factors resulted in the induction of the enzyme. Thus, the possibility that the altered regulation of ornithine decarboxylase is intrinsic to neoplastic transformation is strongly supported by these findings that it is suppressed at the nonpermissive temperature and expressed at the permissive temperature in cells transformed by the tsLA24/RSV. Whether the altered, apparently less stringent regulatory characteristics of this key event in the growth cycle, described here as occurring in RSV-transformed fibroblasts, confers on these cells a selective growth advantage, as has been suggested to occur during epidermal transformation (Boutwell, 1977; O'Brien, 1976), remains to be elucidated. However, these findings, taken together with the previous studies discussed above, do suggest that the alteration in ornithine decarboxylase regulation may be a universal feature of neoplastic expression.

Purification of Ornithine Decarboxylase

Although many studies have appeared on the cellular regulation of ornithine decarboxylase, only five reports on attempts to purify and characterize the enzyme have appeared (Jänne and Williams-Ashman, 1971; Friedman, Halpern, and Canellakis, 1972; Ono et al., 1972; Obenrader and Prouty, 1977a; Boucek and Lembach, 1977). Detailed studies of the chemical, physical, and kinetic properties of ornithine decarboxylase, and its postulated role in transcription (Manen and Russell, 1977a, 1977b), have been delayed by the difficulty of obtaining milligram amounts of active enzyme with satisfactory purity. This is apparently because the great lability of the enzyme, even in a semi-purified state in the presence of protease inhibitors, has thwarted attempts at further purification (Pegg and Williams-Ashman, 1968). The final goal of the present study was to devise a purification scheme which would make available purified ornithine decarboxylase in amounts that would allow for definitive characterization of this important regulatory molecule.

MATERIALS AND METHODS

Animals

Male calves (1 month-old, 40-60 kg) were obtained from the University of Arizona Dairy Farm. Sprague-Dawley rats (100-125 g) were obtained from the Division of Animal Resources, University of Arizona, or purchased from Charles River, Boston, MA. The rats were maintained on an 0700-1900 hr photoperiod and fed *ad libitum*. Animals were given an intraperitoneal injection of 0.75 ml/kg body weight of 200 mg/ml thioacetamide (in 50% ethanol, pH 7.0) to achieve a final drug dosage of 150 mg/kg. Eighteen hours later they were sacrificed and the livers excised.

Chemicals

L-[1-^{14}C]Ornithine hydrochloride was purchased from Amersham/Searle Corporation or New England Nuclear. Dithiothreitol (DTT), pyridoxal phosphate (PYP), phenylmethylsulfonyl fluoride (PMSF) poly(ethylene glycol) (M_r20,000) (PEG), disodium ethylenediaminetetraacetate (Na_2 EDTA), Trizma base (2-amino-2-hydroxymethyl-1,3-propanediol), thioglycolic acid, mercaptoethanol, glutathione, putrescine dichloride, imidazole, monothioglycerol, 5,5'-diethyl barbituric acid (Barbital), Blue Dextran, thioacetamide, ethylene-glycol-bis (β-amino-ethyl ether)-N,N'-tetraacetic acid (EGTA), bovine serum albumin (BSA), ovalbumin, transferrin, 1-10 phenanthroline (O-phenanthroline, OP), diethyldithiocarbamic acid (DDC),

α,α'-dipyridyl, 8-hydroxyquinoline, bathocuproine disulfonate, cyanocobalamin, coenzyme B_{12}, hydroxocobalamine, and all the nucleotides, metals, and other enzyme cofactors employed in this study were purchased from Sigma. 4-7 Phenanthroline (m-phenanthroline) was from K and K Laboratories. Acrylamide, N,N'-methylenebis(acrylamide) (Bis), N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulfate, riboflavin-5'-phosphate, Coomassie brilliant blue, and Affi-Gel Blue resin were from Bio-Rad. Sodium dodecyl sulfate ($NaDodSO_4$) was from Pierce and Charcoal Norit A was from Matheson Coleman and Bell. Ultrapure sucrose and glycerol were purchased from Bethesda Research Laboratory. Hemocyanin and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes) were from Calbiochem. 3MM filter discs were purchased from Whatman.

DEAE (diethylaminoethyl)-Sephacel, Activated Thiol-Sepharose 4B, Thiopropyl-Sepharose 6B, Sephacryl S-200 Superfine, DEAE-Sephadex A-25, and polyacrylamide gradient gels PAA4/30 were obtained from Pharmacia. Ultrogel AcA 44 (4% acrylamide, 4% agarose) and Ultrogel HA (hydroxylapatite) were from LKB. Reference proteins used for calibration of Sephacryl S-200 and Ultrogel AcA 44 columns for Stokes radius determination or molecular weight estimation were [methyl-^{14}C]-albumin, -ovalbumin, -carbonic anhydrase, and -cytochrome C from New England Nuclear. Molecular weight calibration of $NaDodSO_4$-polyacrylamide gel electrophoresis was performed using phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and α-lactalbumin from Pharmacia. YM-20 Diaflow membranes were purchased from Amicon. All remaining chemicals were reagent grade and were obtained from J. T. Baker or Fisher Scientific.

Buffers

All pH values quoted were determined at 25°C. The standard purification buffer used throughout the procedures was 50 mM $Na_2H\text{-}KH_2PO_4$ pH 7.2, 5 mM NaF, 0.06 mM PYP, and 0.1% poly(ethylene glycol) (Mr 20,000) containing DTT, EDTA, and PMSF at the concentrations noted. PYP and DTT were made fresh monthly and were stored frozen as concentrated stock solutions (10 and 100 mM, respective) 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.

Protein Assay

Determination of protein content in liver supernatants and purified fractions after initial DEAE chromatography was by the dye-binding method of Bradford (1976) using BSA as the standard. Analysis of protein at later purification stages was by the method of Lowry et al. (1951). If fractions contained high concentrations of thiols, which react in the assay, or were very dilute, they were precipitated by the addition of trichloroacetic acid to 10% and centrifugation for 10 min at 10,000 x g and were resuspended in 100 μl of 1 N NaOH prior to assay. Standards of known amounts of BSA were also carried through the precipitation procedure. If fractions were assayed directly an equivalent volume of purification buffer containing the same concentration of DTT was also assayed, to correct for true protein concentration. Protein in the column effluent during purification procedures was monitored by absorbance at 280 nm (Uvicord, LKB).

Ornithine Decarboxylase Assay

Ornithine decarboxylase activity was determined by measuring the liberation of $^{14}CO_2$ from L-[1-^{14}C]ornithine as described by Russell and Snyder (1968) with minor modifications. 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, 0.1% PEG for the times indicated. Since the enzymatic activity was found to be labile to dilution (see below), the total reaction volume of the assay varied depending on the activity of the experimental fractions. Typically, a sample ranging from 10 to 100 µl was assayed in the presence of an equal volume of reaction mixture to attain the concentrations described above. Whenever possible, protein concentrations in the assay were kept above 0.2 mg/ml. Under these conditions, the reaction velocity was constant with respect to time although not always proportional to enzyme concentration. The reaction was initiated by the addition of L-[^{14}C]ornithine (specific activity, 5 mCi/mmole) to a final concentration of 0.5 mM. Pyridoxal phosphate-independent release of $^{14}CO_2$ was determined by incubating an equivalent amount of experimental fraction in the presence of 4-bromo-3-hydroxylbenzyloxyamine 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 suspended above the reaction mixture in a plastic well (Kontes). The filter paper was then placed in a toluene-Omnifluor (New England Nuclear) and radioactivity was determined in a liquid scintillation spectrometer. Specific activity of the enzyme is expressed as pmoles of CO_2 liberated per minute per mg of protein. One unit of ornithine decarboxylase activity was defined as that amount of enzyme necessary to catalyze the decarboxylation of 1 μmole of ornithine per min at 37°C.

Concentration

Concentration of samples to appropriate volumes for chromatographic procedures and of pooled fractions after all purification steps except the initial ion exchange fractionation was carried out by filtration through Amicon YM-10 Diaflow membranes by using the Amicon Model 202 Stirred Ultrafiltration Cell. Filtration was conducted at 4°C by using N_2 at 60 psi. Dialysis and buffer exchange was also conducted using this apparatus by connecting an Amicon Reservoir containing the indicated buffer.

Gel Filtration

Gel filtration was performed by using Ultrogel AcA 44 and a LKB column equipped with an upward flow adaptor. The upward flow was generated by a peristaltic pump. Columns were calibrated under standard working conditions by using Blue Dextran 2000 to assess V_o and proteins of known molecular weight and Stokes radius. Molecular weight was estimated from a plot of elution data according to Andrews (1964, 1965) and the Stokes radius was computed on a calibration curve plotted according to Siegel and Monty (1966).

Polyacrylamide Gel Electrophoresis Under Nondenaturing Conditions

Disc gel electrophoresis was carried out in a stacking system. A 0.5 x 10 cm separating gel was cast containing 7.5% acrylamide, 0.2% Bis, 0.1% PEG, 0.07% ammonium persulphate in 70 mM Tris·HCl pH 7.5. The polymerization reaction was catalyzed by the addition of 11.5 μl TEMED per 10 ml gel reaction. After gelification, a 0.5 x 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 TEMED/ml in 140 mM Tris·PO_4, pH 5.5. Polymerization was initiated by exposure to ultraviolet light. Upper and lower chambers were both filled with precooled reservoir buffer pH 7.0 containing 30 mM diethylbarbituric acid, 8.2 mM Tris, 2 mM thioglycolic acid, and 0.06 mM PYP. Hundred-μl samples containing 5% glycerol were layered on top of the stacking gel and electrophoresis carried out at 4°C at 4 mA/gel until the Bromophenol blue tracking dye exited the gel (ca. 3 hour). The enzyme was removed from the gel by diffusion. The gels were sliced into 2 mm sections, quartered, and incubated overnight in 3 volumes of standard buffer containing 2 mM DTT, 0.1 mM EDTA, and 0.06 mM PYP.

Non-denaturing electrophoresis was also carried out in a phosphate buffer system. Seven and one-half per cent gels (0.5 x 10 cm) were pre-electrophoresed using, in both compartments, a pH 7.0 buffer containing 28 mM NaH_2PO_4, 72 mM Na_2HPO_4, 10 mM thioglycolic acid, and 0.06 mM PYP. Electrophoresis of the sample was carried out in this same buffer as described above.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

Electrophoresis was carried out on 4 to 30% gradient gels in a buffer containing 40 mM Tris, 20 mM NaAcetate, 2 mM EDTA, 0.2% $NaDodSO_4$, adjusted to pH 7.4. All 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% mercapto-ethanol. After denaturation sucrose was added to 10% and bromphenol blue to 0.01%. Electrophoresis was conducted at 4°C at 8 mA/tube gel or 150 volt/slab gel until the tracking dye had been exited from the gel for 1 hour. All gels were fixed in 25% methanol, 10% acetic acid, and stained with Coomassie blue.

RESULTS

Purification

Induction of Ornithine Decarboxylase in Calf Liver

Estimates of the amount of ornithine decarboxylase in tissue indicated that it constituted from 0.001 to 0.01% of the protein. This low concentration coupled with the well-known lability of the enzyme suggested that a large amount of starting material was necessary to sustain the experiments required to develop the purification protocol. Therefore, we explored the possibility of using a large mammal as a source of liver tissue. In the initial experiments, newborn calves were sacrificed in order to determine if the neonatal liver might retain some of the high ornithine decarboxylase activity which is typical of embryonic tissues. However, the basal enzyme activity was found to be less than 0.2 pmol/min/mg protein, a low level comparable to that found in adult animal liver. Attempts to induce the enzyme with classic stimulants such as isobutylmethylxanthine or thioacetamide did not increase this activity to any appreciable extent. Therefore, older animals were obtained. A protocol established to elicit maximal enzyme activity in rats (Fausto, 1969; Ono et al., 1972) was employed with 4- to 6-week-old (40 to 60 kg) calves. Administration of 150 mg/kg thioacetamide resulted in an increase in liver ornithine decarboxylase in these animals to a specific activity after 18 hours ranging from 5 to 20 pmol/min/mg protein.

Preparation of Cytosol

Unless otherwise indicated, all steps in the purification procedure were carried out at 4°C. In the typical preparation to be described here, a liver (ca. 800 gm wet weight) from a thioacetamide-stimulated calf was rapidly homogenized in 2 volumes of purification buffer containing 0.5 mM DTT, 0.1 mM EDTA, and 1 mM PMSF. Homogenization was carried out in 4 batches at high speed in a Waring blender for 2 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 then centrifuged at 40,000 x g for 30 min and the supernatant fluid collected and 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 from the homogenization of one liver was mixed for 30 min at 4°C with 600 ml of a 1:1 slurry of DEAE-Sephacel that had been equilibrated with purification buffer containing 2 mM DTT, 0.001 mM EDTA, 1 mM PMSF, and 50 mM NaCl. At the end of the incubation the liquid phase was separated from the resin by filtration on a Buchner filter (coarse). The filtrate was routinely assessed for ornithine decarboxylase activity to ensure that all of the enzyme had been absorbed by the resin. The resin was washed 7 times with a total of ca. 4 liters of additional buffer by resuspension and stirring for 10 min prior to filtration. The resin was then suspended in an equal volume of buffer and layered into a column on top of a pre-settled bed of 100 ml of fresh DEAE-Sephacel. The column was washed with additional buffer until no further absorbing material at 280 nm could be detected in the eluate. The column was eluted with a linear gradient of 50 to 600 mM NaCl in purification buffer containing 2 mM DTT, 0.001 mM EDTA, and 1 mM PMSF (total volume 3 liters) and 10-ml fractions were collected. The ornithine decarboxylase eluted as a single peak of enzymatic activity between 175 and 250 mM NaCl (Fig. 1). The peak fractions were combined and stored at -80°C. This ion exchange chromatographic procedure resulted in a 45-fold increase in the specific activity of the enzyme as compared to the original supernatant and a 108% recovery.

Second Purification Step: Ion Exchange
Chromatography After Cyclic AMP Treatment

Initial studies showed that a chief contaminant of the ornithine decarboxylase fractions at later purification steps was a cyclic AMP-dependent protein kinase which in the holoenzyme form elutes off of DEAE resins at a similar salt concentration as the decarboxylase. To remove the phosphotransferase activity, pooled fractions from the first step (containing 1 gm of protein) were incubated with 50 μM dibutyryl cyclic AMP for 30 minutes to dissociate the cyclic nucleotide dependent kinase into its catalytic and regulatory subunits which elute at different salt concentrations. At the end of the incubation the sample was diluted 1:1 with purification buffer containing 2 mM DTT, 0.001 mM EDTA, and 1 mM PMSF to lower the NaCl concentration so that the ornithine decarboxylase would adsorb to the ion exchange resin. The sample was then mixed for 30 min with 60 ml of 1:1 slurry of DEAE-Sephacel pre-equilibrated in the same buffer

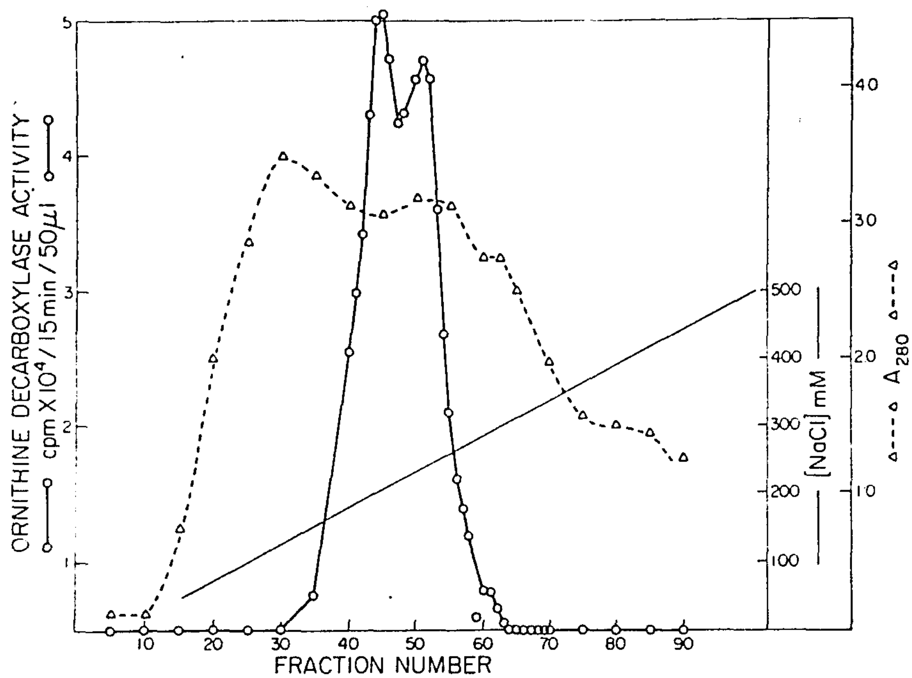

Figure 1. DEAE-Sephacel chromatography of ornithine decarboxylase from calf liver.

containing 50 mM NaCl, washed by filtration on a Buchner filter as described above, and layered into a column containing 70 ml of settled resin. The column was washed with additional buffer until no 280 nm absorbing material could be detected in the eluate and then eluted with a linear gradient of 50 to 500 mM NaCl in the same buffer (total volume 600 ml). Five-ml fractions were collected and the major ornithine decarboxylase containing fractions pooled and stored at -80°C. This procedure increased the specific activity of the preparation 2- to 3-fold with a 75% recovery. The treatment with cyclic AMP was found to have no detectable effect on either the catalytic activity or the elution position of the enzyme.

Third Purification Step: Ultrogel AcA 44 Chromatography

Pooled ornithine decarboxylase fractions containing 100-200 mg of protein were concentrated to a 5-ml volume by ultrafiltration under N_2 pressure. The concentrate was applied to a LKB column containing 400 ml of Ultrogel AcA 44 in purification buffer containing 2 mM DTT, 0.001 mM EDTA, 1 mM ornithine, and 100 mM NaCl. Chromatography was carried out by upward flow at a rate of 12 ml/hr and 2-ml fractions were collected. The ornithine decarboxylase eluted as a single peak of activity in the later half of the eluted protein (Fig. 2). The pooled fractions were concentrated to a protein concentration greater than 2 mg/ml and stored at -80°C. The gel filtration routinely resulted in a 6- to 7-fold increase in enzyme purity with ca. 70% recovery.

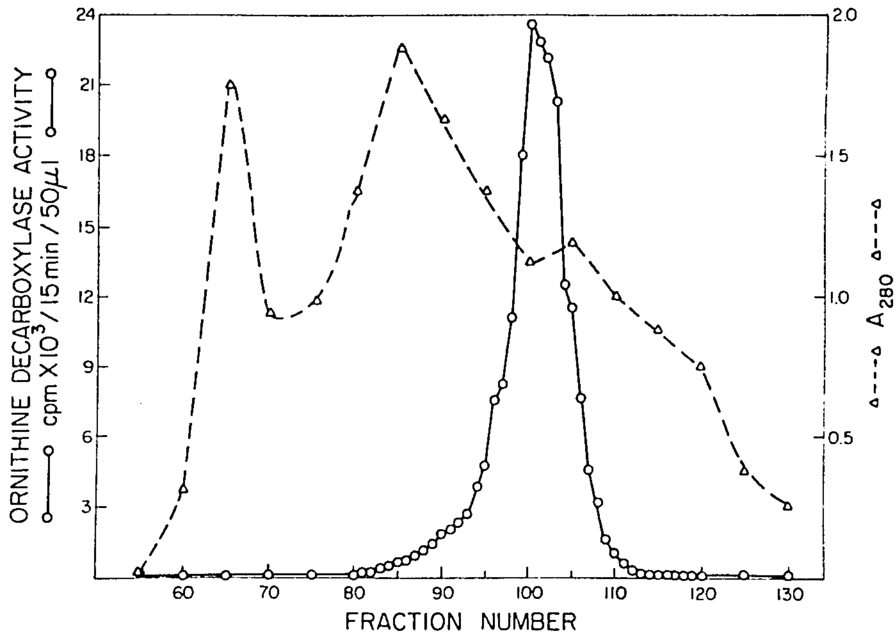

Figure 2. Ultrogel AcA 44 filtration of ornithine decarboxylase.

Fourth Purification Step: Hydroxylapatite Chromatography

Pooled fractions of ornithine decarboxylase containing 20-20 mg protein were filtered using the Amicon ultrafiltration device 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 to lower the PO_4 concentration so the enzyme would adsorb to the resin. The sample was applied to a column containing 5 ml of Ultrogel HA. The column was washed with additional buffer until no 280 nm absorbing material was detected in the eluate. The enzyme was then eluted with a linear gradient of 5 to 250 mM KPO_4 in the same buffer. The ornithine decarboxylase eluted as a single peak toward the back of the major eluted protein peak (Fig. 3). The pooled fractions were concentrated to a protein concentration greater than 1 mg/ml and stored at -80°C. The specific activity of the enzyme in the peak fractions was increased 3-fold with a 60% recovery. There was also enzyme activity eluting with the protein which did not adsorb to the column. Most of this was due to "overloading" of the resin which was found to be necessary to ensure recovery of enzyme activity. Reapplication of the flow-through to another hydroxylapatite column resulted in a major portion of the enzyme adsorbing to the resin. There was, however, found to be a small percentage of the activity in any preparation (4 to 10%) which would not adsorb, even after repeated reapplication.

Fifth and Sixth Purification Steps: Nondenaturing Gel Electrophoresis

Pooled fractions containing 10-20 mg protein were concentrated to 1 ml and the osmolarity of the buffer in the concentrate reduced to 1/3 by ultrafiltration with 5 mM NaH-KH_2PO_4 pH 7.2. The sample was

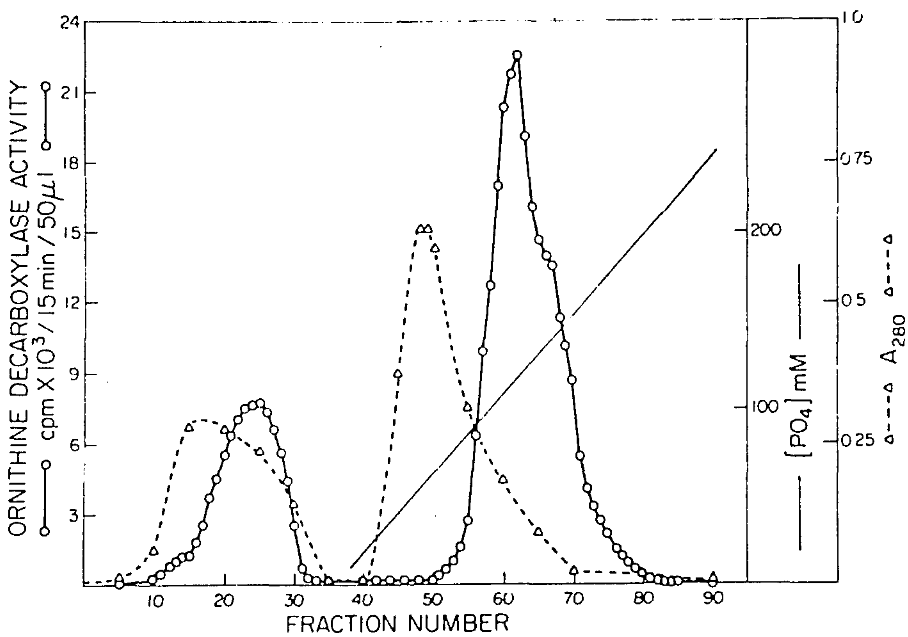

Figure 3. Hydroxylapatite chromatography of ornithine decarboxylase.

adjusted by the addition of glycerol to a final concentration of 5% and 50-100 µl aliquots layered on top of 7.5% polyacrylamide gels pre-electrophoresed in the phosphate system described in the methods section. Electrophoresis was conducted for 6 to 7 hours until the bromophenol blue tracking dye had exited the gels. The gels were cut into 2-mm slices and the similar slices from each gel were pooled and allowed to diffuse overnight as previously described. The ornithine decarboxylase moved as a single peak of activity and was localized in those fractions in an intermediate position between those containing standards of BSA and ovalbumin, as determined by marker bands on separate gels which were fixed and stained to detect the protein (Fig. 4a). The liquid fraction from the slice incubations containing peak activity were pooled, the gel slices were allowed to diffuse for 1 hr in additional buffer, and then were loaded into a syringe containing a glass wool plug and pressure applied to extrude as much buffer as possible from the gel slices. This procedure gave a 78% recovery of activity and a 2.8-fold purification. This total pooled fraction was concentrated to a protein concentration greater than 0.5 mg/ml and stored at 4°C.

A second disc gel electrophoresis was then carried out in the stacking, Tris-barbital buffer system described in the methods section. Pooled fractions containing 10 mg of protein were concentrated to 1 ml and reduced in osmolarity as described above. The sample was adjusted by the addition of glycerol to 5% and 200-µl aliquots layered on top of the stacking gel. Electrophoresis was conducted for 3 hours until the bromphenol blue tracking dye had exited the gel and the ornithine decarboxylase was diffused from 2 mm gel slices into the buffer as previously described. The enzyme moved as a single peak of activity

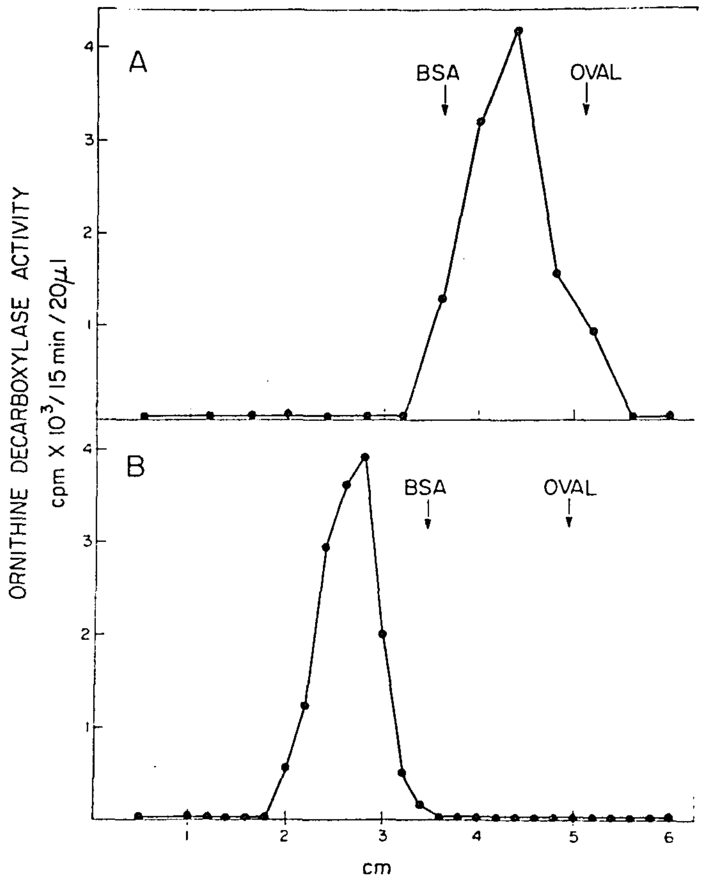

Figure 4. Non-denaturing gel electrophoresis of ornithine decarboxylase in phosphate buffer and stacking Tris-barbital buffer systems -- Ornithine decarboxylase purified through the fourth step was applied, electrophoresed and eluted as described in the Results in the phosphate buffer (upper panel) and Tris-barbital buffer (lower panel) systems. Arrows indicate the position in concomitantly run gels of marker bands of BSA and ovalbumin (oval) as detected by Coomasie Blue staining.

and was localized in those fractions behind both BSA and ovalbumin (Fig. 4b). The specific activity of the enzyme was increased 5-fold and recovery was 65%. The total pooled activity was concentrated to 0.5 ml and stored at 4°C.

Seventh Purification Step: Glutathione Affinity Chromatography

Pooled fractions containing ca. 2 mg of protein were filtered using the Amicon ultrafiltration device with 10 volumes of purification buffer containing 0.1 mM EDTA to lower the DTT concentration so the enzyme would adsrob to the resin. The sample was applied at 0.2 ml/min to a column containing 1 ml of Activated Thiol resin pre-equilibrated in purification buffer containing 0.1 mM EDTA and 100 mM NaCl. The column was washed with additional buffer until no 280 nm absorbing material was detectable in the eluate. A linear gradient was then applied of 2 to 250 mM DTT in the same buffer (15 ml total) and 0.5-ml fractions were collected. The ornithine decarboxylase eluted as a single peak of activity at the latter portion of the eluted protein peak (Fig. 5). The specific activity was increased 2.5-fold by this step and recovery was ca. 70%. Those fractions exhibiting peak activity were pooled and concentrated to 0.5 ml and stored at 4°C.

Comments on the Purification

Clearly, the qualifying characteristic in the development of the above described procedure of purification of ornithine decarboxylase was the attainment of appropriate protein to resin and elution buffer volume ratios optimal for each procedure. These were arrived at by trial and error during which many chromatographic procedures were run

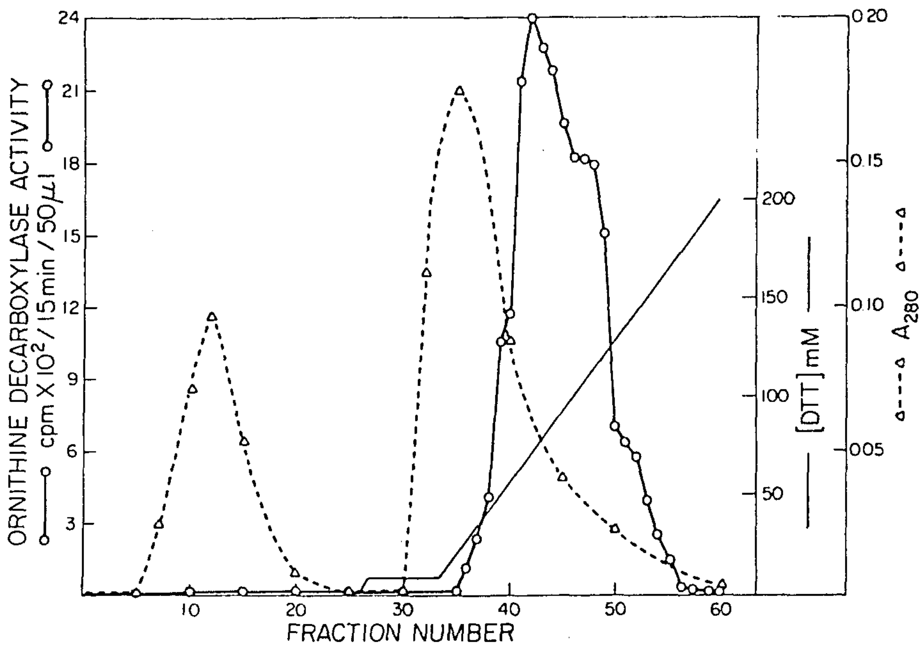

Figure 5. Glutathione affinity chromatography of ornithine decarboxylase.

according to standardly accepted ratios of sample size/column size and elution volume for obtaining optimal resolution. Many of these resulted in very poor or even no recovery of enzymatic activity. Increasing the protein applied in successive trial runs (or, alternatively, decreasing the column or gradient size or increasing the gradient slope) eventually yielded a ratio to obtain good recovery (i.e., > 50%) for all the purification steps described here. Whenever possible, conditions that resulted in a protein concentration $\geq$ 0.2 absorbance at 280 nm were used. The development of these conditions for three of the procedures will be discussed as examples.

1. The initial attempt at a second ion-exchange chromatography of the first step-purified ornithine decarboxylase on DEAE-Sephacel resulted in only a 7% recovery of enzymatic activity. A sample of 520 mg had been applied to a 400-ml bed volume column of resin and was eluted with a 2 L gradient. Eventual adjustment to the application of a 1000-mg sample to a 100-ml bed volume and elution with a 600-ml total volume gradient resulting in the recovery of 80% of the ornithine decarboxylase activity applied.
2. As mentioned under the description of the hydroxylapatite procedure, the column is "overloaded" so that not all of the applied ornithine decarboxylase is able to be adsorbed. This saturation of the resin was found to be necessary to ensure that the ornithine decarboxylase subsequently eluted retained its catalytic activity. If, as in the original trials, 8 to 10 mg of step 3 purified enzyme was applied, in which case no

decarboxylating activity was detectable in the sample flow-through, the subsequent gradient elution resulted in the recovery of only 11% of the applied ornithine decarboxylase. Maintaining all other conditions the same, but increasing the sample size 3- to 4-fold, so that the resin was apparently "saturated," resulted in consistent recoveries of 60-70%.

3. The recovery of ornithine decarboxylase activity after non-denaturing gel electrophoresis was also found to benefit from the application of high sample content. Application of 200 or 400 or 1000 μg of an ornithine decarboxylase fraction (in this test case, step 3 enzyme was used) to a 7.5% gel and electrophoresis in the PO_4 buffer system described above resulted in 19 vs. 32 vs. 60% recovery, respectively, of the applied activity. Subsequently the optimal application of 2 mg/gel was adopted for routine use.

Of course, what is sacrificed in all of these procedures in order to maintain catalytic activity is the optimal resolution of protein species obtainable with each procedure. Consequently, it was found necessary to employ all 7 steps described to obtain pure enzyme

Cytosol Preparation

The concentration of DTT included in the homogenization buffer was found to affect the ornithine decarboxylase activity recoverable in the supernatant. This effect appeared to be distinct from that of the dithiol on enzymatic activity; in fact, it was of an opposite nature. If high DTT concentrations, which had previously been shown to be optimal for catalytic activity with semi-purified preparations of the enzyme, were included in the homogenization buffer the consequent supernatant ornithine decarboxylase activity was reduced. Curiously, this reduction was only apparent if the enzyme was assayed in the presence of DTT. The results from an experiment examining this phenomenon are given in Table 1. If a homogenate is prepared in the presence of 2 mM DTT and the recovered supernatant is assayed with varying concentrations of the reductant, a maximal specific activity of 9.7 pmol/min/mg is observed at a concentration of 2 mM. The second supernatant from a homogenate prepared at the same time from the same liver but with a lower DTT concentration, 0.5 mM, appears to have a similar ornithine decarboxylase level when assayed at low reductant concentration. However, when the DTT concentration is elevated to the optimal 2 mM, this supernatant fraction exhibits a 40% greater specific activity of the enzyme, 13.6 pmol/min/mg. The protein concentration was found to be the same in the two supernatants. Therefore, in all following preparations, the DTT concentration in the homogenization buffer was reduced to 0.5 mM.

Despite the fact that care was taken to maintain low temperature and the liver was homogenized in the presence of protease inhibitors and enzyme cofactor, the activity of the enzyme was found to be rapidly decreasing in the supernatant fraction, even after removal of the particulate fraction. A minimal $T_{1/2}$ for disappearance of the enzyme during preparation of the supernatant was calculated by following the decline of activity in the cytosol subsequently. From the plot of this decline, as shown in Figure 6, it can be estimated that the

Table 1. The Effect of Dithiothreitol Concentration on the Recovery of Ornithine Decarboxylase Activity After Liver Homogenization

	[DTT]mM in homogenization	[DTT]mM in assay	Ornithine Decarboxylase (pmol/min/mg)
Supernatant #1	2	0.2	8.0
		2.0	9.7
		5.0	8.9
Supernatant #2	0.5	0.5	8.2
		2.0	13.6
		5.0	10.9

Cytosolic fractions of a liver from a thioacetamide-stimulated calf were prepared as described in the "Results" except that the concentration of DTT in the homogenization buffer and assay reaction were varied as indicated.

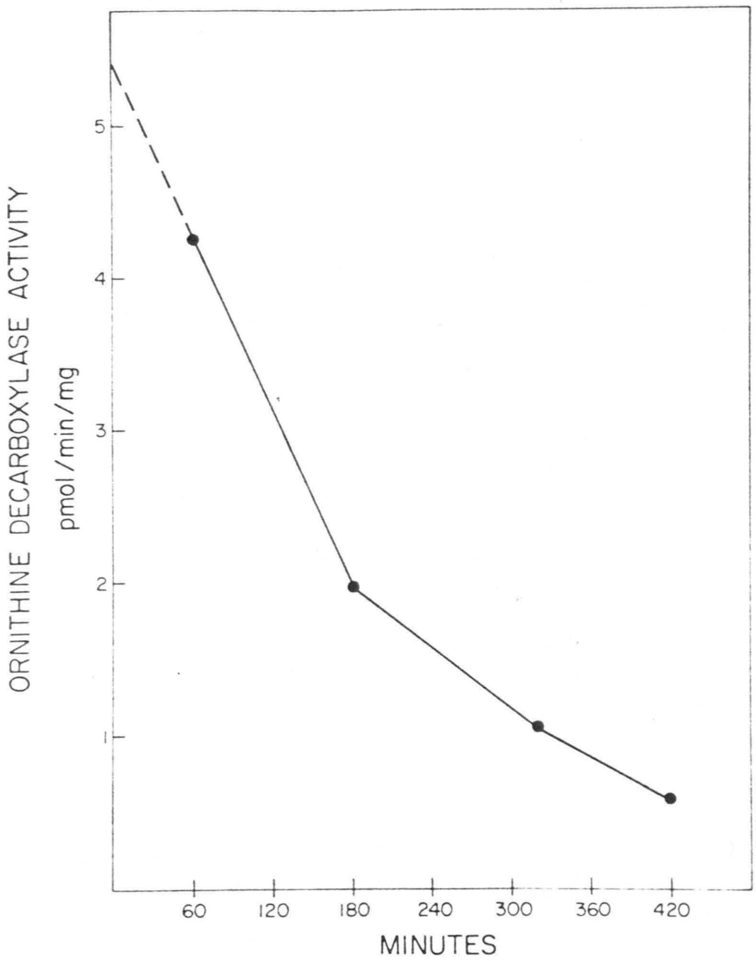

Figure 6. Decline in ornithine decarboxylase activity in liver supernatant maintained at 4° -- The cytosol from a thioacetamide-stimulated calf liver prepared under standard conditions was allowed to sit at 4° for the times indicated prior to assay for ornithine decarboxylase activity.

original activity in the tissue was at least 25% higher than the specific activity determined in the supernatant during a large-scale preparation. Varying the DTT concentration from 0.5 to 5 mM in the supernatant after centrifugation had no effect on the rate of decline. Since the activity of ornithine decarboxylase was stable at 4°C for at least 8 hours after the initial purification step, it appears that the decline in activity in the supernatant (estimated $T_{1/2}$ 2 hours at 4°C) was due to residual proteolytic activity directed toward the enzyme.

Initial Ion Exchange Chromatography

Other reported purifications of ornithine decarboxylase have commonly utilized a batch preparation procedure, either by acid or 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 to 40%). Since the enzyme could be recovered in good yield after application of either precipitation method to fractions obtained after the first ion-exchange chromatography, the initial poor yield may reflect the inability of these methods to separate ornithine decarboxylase from the proteolytic activity suggested by the data in Figure 6.

To circumvent this decline, it was found necessary to apply the supernatant immediately after centrifugation to the DEAE-Sephacel in a batch fashion. Under these conditions, all the enzyme was adsorbed by the resin within 15 min and was apparently resistant to further degradation.

The appearance of the peak of ornithine decarboxylase eluted from the ion exchange resin often suggested heterogeneity of enzyme forms (see Figure 1). For the purposes of the purification the major activity fractions were combined in one pool. However, the possibility of distinct ionic forms of the enzymes was explored in later studies using a more powerful ion exchange fractionation technique (see below, "Evidence for multiple enzyme forms").

Gel Filtration

The conditions for optimal recovery of ornithine decarboxylase activity from gel filtration were arrived at with some difficulty since the enzyme was invariably recovered in poor yield regardless of the amount of protein applied. The filtration procedure results in a continually changing buffer environment for the enzyme since its partial exclusion from the bead interior confers on it a different rate of movement than the totally included buffer. In an attempt to circumvent lengthy trial and error by successive filtrations, dialysis experiments of enzyme fractions were conducted to gather information on the optimal buffer components for maintaining enzyme activity in a changing buffer system. Dialysis of the enzyme for 18 hours at 4°C against 100 volumes (4 changes) of purification buffer containing 2 mM DTT, 0.1 mM EDTA, and 1 mM PMSF resulted in a loss of 50% of the ornithine decarboxylase activity. Varying the PO_4 buffer concentration, substituting HEPES buffer, and varying the PYP concentration had no effect on this loss. Similarly, varying the DTT concentration had no effect, except that at the lowest dithiol concentrations (i.e., $<$ 0.2 mM) the loss was even greater. Other experiments (see below, "Evidence for ornithine decarboxylase as a metalloenzyme") had suggested that a transition metal might be involved in maintaining the catalytic capacity of ornithine decarboxylase. Therefore, the optimal EDTA concentration was examined. Complete elimination of the chelator from the dialysis buffer resulted in even greater losses of activity. However, the enzyme was known to be sensitive to inhibition by heavy metals (see below) which are often present as contaminants in buffer preparations. Furthermore, heavy metals are known to form mercaptides and to catalyze thiol oxidation. Therefore, a concentration of 1 μM EDTA was added to the dialysis buffer to try and prevent this further decline. This minimal addition of chelator was found not only to prevent the further decline but also resulted in the maintenance of most of the original enzyme activity. Gel filtration in the presence of buffer containing 1 μM EDTA was subsequently also found to yield good recoveries of enzyme activity.

Curiously, the ornithine decarboxylase recovered after gel filtration exhibited a requirement for high EDTA concentrations in the assay for optimal activity which was not seen with the enzyme before the filtration. Figure 7 shows that the ornithine decarboxylase activity recovered from the second ion exchange chromatography is insensitive to the concentration of EDTA in the assay reaction until concentrations of 10 mM or greater are achieved at which inhibition is apparent. In contrast, the same enzyme preparation after gel filtration is stimulated almost 2-fold as the concentration of the chelator is raised from 1 to 100 μM. This stimulation may reflect the inability of the low EDTA concentration included in the filtration buffer to completely prevent interaction of heavy metals with enzyme free sulfhydryl groups necessary for maximal catalytic activity. Because of

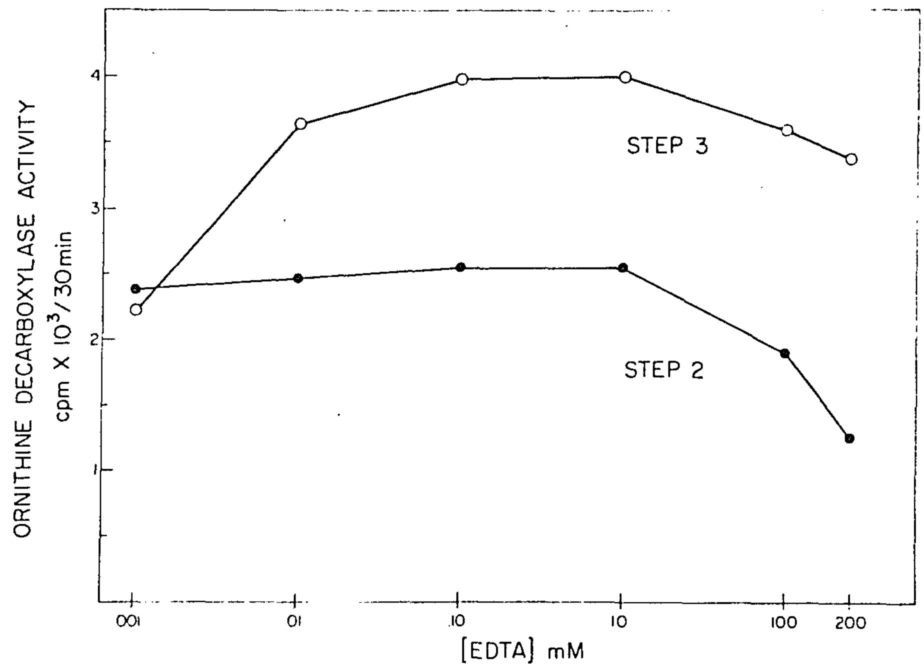

Figure 7. Stimulation by EDTA of ornithine decarboxylase recovered from gel filtration -- Samples of ornithine decarboxylase purified through the second step (closed circles) or the third step (open circles) were assayed in the presence of the indicated concentration of EDTA.

this requirement for elevated EDTA concentration, the subsequent purification procedure, hydroxylapatite chromatography, which is conducted fairly rapidly, was done in the presence of 1 mM EDTA, after which the enzyme preparation no longer displayed the requirement for high EDTA concentration in the assay.

Glutathione Affinity Chromatography

A previously reported purification procedure for ornithine decarboxylase (Obenrader and Prouty, 1977a) indicated that the enzyme would adsorb to Sepharose to which glutathione was conjugated (Activated Thiol-Sepharose 4B). Therefore, this resin and another derivative of Sepharose, thiopropyl-Sepharose 6B, with similar capability of forming mixed disulphides with sulfydryl containing proteins, was evaluated for use as a purification step. The enzyme bound readily to both gels, the thiopropyl derivative displaying a greater capacity. However, the application of 5 mM DTT, which eluted the rat liver enzyme in the previous report, was found to release very little of the applied activity. Figure 8 illustrates the time and temperature dependence for removal of calf liver ornithine decarboxylase from the thiol-containing resins. Incubation of the enzyme bound Activated-Thiol resin for 8 hours in the presence of 5 mM DTT at 4°C eventually resulted in the release of 26% of the applied ornithine decarboxylase. Increasing the temperature of the incubation to 37°C accelerated the elution of the enzyme so that 98% of the applied activity was recovered in 4 hours. However, very little purification of the enzyme was obtained by this procedure. No ornithine decarboxylase activity was ever released in

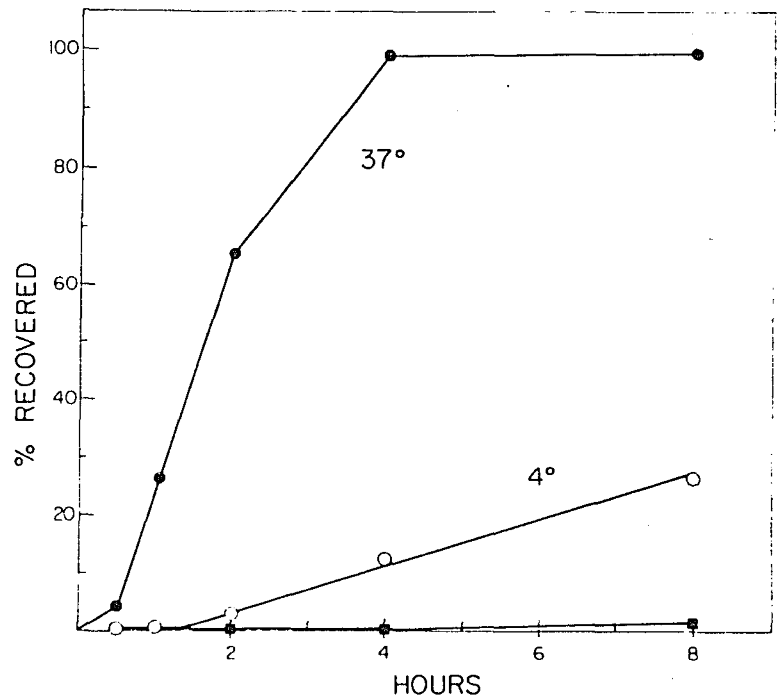

Figure 8. Time and temperature dependence of the elution by DTT of ornithine decarboxylase from Activated-Thiol Sepharose -- 0.5 ml of Activated Thiol- (circles) or thiopropyl- (squares) Sepharose, to which 1 ml of ornithine decarboxylase (step 3) had been bound, was incubated for the indicated time at 4° (open symbols) or 37° (closed symbols) in the presence of 2 ml of purification buffer containing 5 mM DTT. At the end of the incubation time, the gel was allowed to settle and the supernatant assayed for ornithine decarboxylase. The activity detectable is expressed as a percentage of the activity originally allowed to react with the gel.

the thiopropyl-Sepharose at 4°C in the presence of 5 mM DTT, even after 24 hours. Increasing the temperature to 37°C had very little effect, 1.5% of the activity being recovered after 8 hours of incubation at the elevated temperature. This lack of recovery was apparently not due to inactivation of the enzyme by the resin since direct assay of the enzyme-bound gel revealed 30% of the applied activity. Apparently, the covalent bond between the enzyme and the resin does not involve sulfhydryl groups at the active site of the enzyme.

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, was found to give optimal recovery of the enzyme while resolving it from the major portion of the applied protein (see Figure 5).

Protein Assay

The determination of protein content by the Bradford (1976) method in purification fractions of ornithine decarboxylase obtained during the later steps of the procedure often resulted in values which were inconsistent with the 280 nm absorbance of the fractions as they eluted from the column. Therefore, comparative Bradford and Lowry protein assays were done on purification samples taken from throughout the procedure. While the values obtained for the step 1 and 2 enzyme were very comparable, the protein content as determined by the Bradford method became progressively less as compared to the Lowry method as the purification was carried further. By the attainment of the last purification step, the determination by dye-binding gave values equivalent to 1/10 those obtained by the Folin phenol reagent. Therefore, Bradford determinations were utilized only during the initial purification stages.

Summary of Large-Scale Purification Procedure

Table 2 lists each individual purification procedure and describes its characteristics of efficiency of purification and yield when performed as previously described. The conditions for each step have been adjusted according to the rationale described above so that for any procedure, recovery of ornithine decarboxylase is greater than 50%.

Table 3 is a summary of the total purification process required to result in a yield of ca. 1 mg of pure, active protein. In order to ensure enough material 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 6 calf livers must be processed. The table lists the number of times each procedure was performed as an individual step as described in Table 2 to process the total material summarized in Table 3, in addition to the cumulative recovery and relative purification as each step in the process was completed. The final purification of enzyme activity was 71,500-fold with a recovery of 12%.

Table 2. Efficiency and Yield of the Individual Purification Procedures Employed in the Purification of Ornithine Decarboxylase

Step	mUnits applied (nmol/min)	Protein applied (mg)	mUnits recovered (nmol/min)	Protein recovered (mg)	Specific activity (pmol/min/mg)	Recovery (%)	Purification (-fold)
1. DEAE-Sephacel	323	80,800	349	1939	180	108	45
2. Cyclic AMP, DEAE-Sephacel	175	969	131	322	407	75	2.3
3. Ultrogel AcA 44	65	160	44	17	2,600	68	6.4
4. Hydroxylapatite	78	30	47	5,7	8,250	60	3.2
5. Gel Electrophoresis in PO_4 (6 gels)	89	10,8	69	3.0	23,100	78	2.8
6. Gel Electrophoresis in Tris-Barbital (6 gels)	205	8.9	134	1,2	115,200	65	5
7. Activated-Thiol	265	2,3	191	0,67	286,000	72	2.5

The data represent the combined average values obtained from no less than 10 preparations (as described under "Results").

Table 3. Summary of the Purification of Calf Liver Ornithine Decarboxylase

Step	Times performed	Total protein (mg)	Total activity (mUnits)	Specific activity (pmol/min/mg)	Relative purification (-fold)	Yield (%)
20,000 x g supernatant	6	484,800	1939	4		
1. DEAE-Sephacel	6	11,634	2094	180	45	108
2. Cyclic AMP, DEAE-Sephacel	12	3,864	1572	407	102	81
3. Ultrogel AcA 44	24	408	1061	2,600	650	55
4. Hydroxylapatite	12	78	640	8,250	2,062	33
5. Gel Electrophoresis in PO_4	6	22	504	23,100	5,775	26
6. Gel Electrophoresis in Tris-Barbital	2	2.9	330	115,200	28,800	17
7. Activated-Thiol	1	0.81	233	286,000	71,500	12

The data represent the combined average values obtained from 5 large-scale preparations (as described under "Results").

Purity of Ornithine Decarboxylase Preparation

Samples from the concentrate from the 7th purification step utilizing the glutathione-Sepharose were submitted to disc gel electrophoresis at 4°C under nondenaturing conditions utilizing the Tris-barbital stacking system described in "Materials and Methods."

The stained gel is shown in Figure 9. A single protein band is observed which migrates slower than either the BSA or ovalbumin markers. The activity of ornithine decarboxylase, as detected by direct assay of 2 mm gel slices, comigrates with the protein band. The results show that contaminants are absent.

Other Procedures Evaluated

It should also be mentioned that several other fractionation procedures were tried during the initial studies which, although exhibiting good adsorbing ability for the enzyme, resulted in little or no recovery of enzyme activity. Chief among these, because of the theoretical resolving power they should afford, were the substrate and cofactor affinity resins. A previous report of a purification procedure for ornithine decarboxylase from fibroblasts (Boucek and Lembach, 1977) had in fact utilized a pyridoxamine 5'-phosphate affinity matrix with elution by pyridoxal 5'-phosphate to obtain a single step purification of 500-fold. This method was tested with semi-purified calf liver enzyme, according to the procedure outlined by the authors. No adsorption of the enzyme by the resin was observed. Since the coupling through the NH (4) may have sterically hindered enzyme interaction with the cofactor 5'-PO_4 group another affinity resin was prepared.

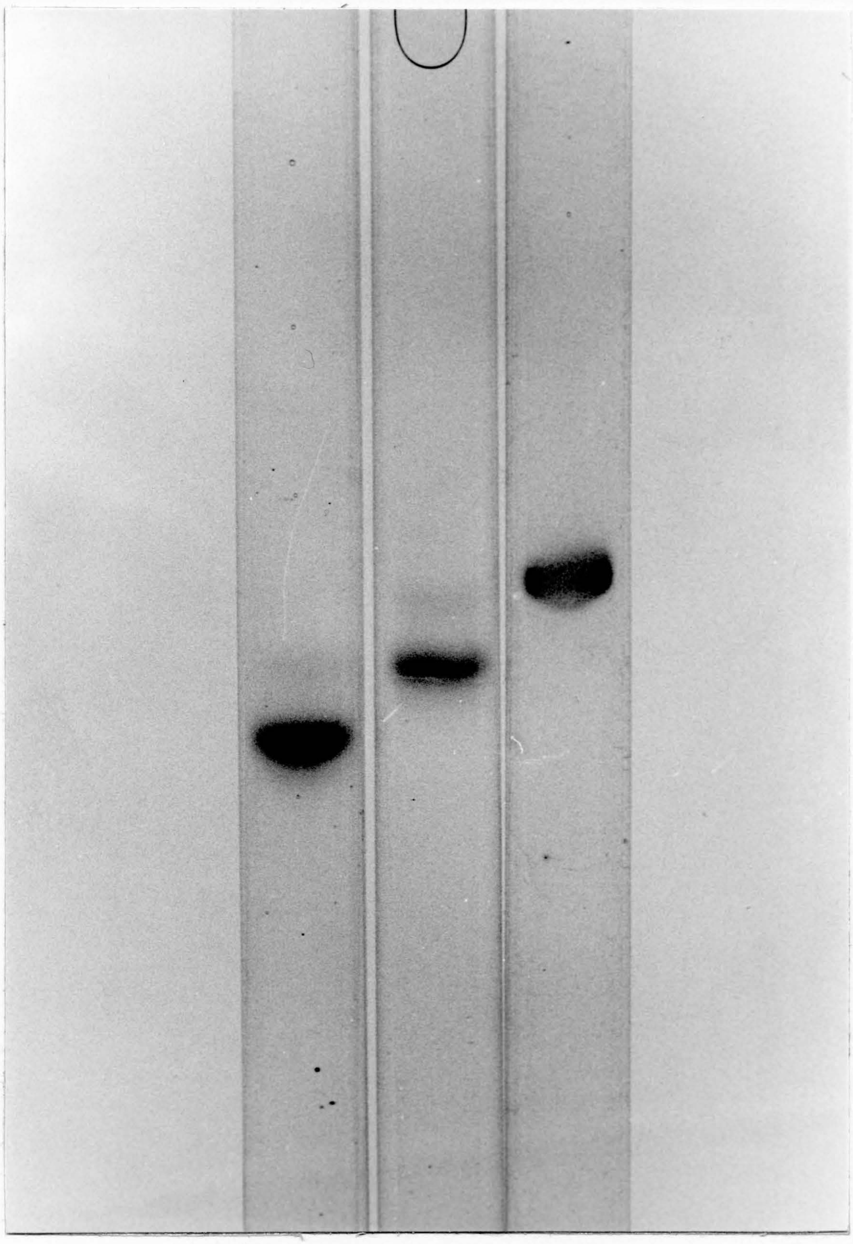

Figure 9. Non-denaturing gel electrophoresis of purified ornithine decarboxylase -- Seventy-five μg of step 7 purified enzyme was electrophoresed in the Tris-barbital disc gel system (right). Ten μg of ovalbumin (left) or BSA (middle) were run in parallel.

Pyridoxal-5'-phosphate was conjugated to a 12-atom spacer group linked to Sepharose by an epoxy linkage via the cofactor hydroxyl (3). This left the ring N (1) and the PO_4 (5), which have been shown to be most important in binding to other B_6-dependent enzymes, maximally exposed. This resin bound the ornithine decarboxylase with high capacity; however, at most 18% of the enzyme could be recovered.

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) α-methyl-ornithine conjugated via the amino group by a carbodiimide catalyzed peptide linkage to a 6-carbon atom spacer arm (C_6) on Sepharose 4B; (2) pyridoxal 5'-phosphate conjugated via the hydroxyl group by a O-bromoacetyl-N-hydroxysuccinimide catalyzed linkage to a C_6-Sepharose 4B; (3) a borohydride-reduced Schiff base of ornithine with resin #2; (4) ornithine coupled via the amino group by a carbodiimide catalized peptide linkage to C_6-Sepharose 4B; (5) ornithine coupled via the carboxyl group by a carbodiimide catalyzed peptide linkage to C_6-Sepharose 4B; (6) a borohydride-reduced Schiff base of pyridoxal 5'-phosphate with resin #5; and (7) pyridoxal 5'-phosphate coupled via the hydroxyl group by an epoxy linkage to a 12-atom spacer group linked to agarose, reacted with ornithine to form the Schiff base, and reduced with borohydride.

All of these resins displayed at least some affinity to bind ornithine decarboxylase. However, an efficient method for recovering enzyme activity was not found, although protocols of varying time, temperature, salt concentration, resin dilution, and competitive ligand concentrations were employed. It is possible, based on the subsequent experience with the more classical purification procedures described above, that the enzyme was simply being purified "too well" by these methods and was inactivating upon elution at dilute protein concentration. Alternatively, it is possible that the structure of the enzyme molecule is such that disruption of the bonds formed using the affinity technique is just not efficiently possible without the use of chaotropic or denaturing agents, as has been described for the steroid receptor.

Other chromatographic resins which were tested which calf liver ornithine decarboxylase was found not to adsorb to included phosphocellulose, double- and single-stranded DNA cellulose, heparin-agarose, concanavalin A-agarose, and wheat germ agglutinin-Sepharose. The enzyme was found to adsorb to alumina C_γ gel; however, to date no efficient method for recovery has been developed.

Lability of Ornithine Decarboxylase and Evidence for the Existence of Further Cofactors and Activators of the Enzyme

The difficulty in maintaining the activity of ornithine decarboxylase during the purification procedures and the sensitivity of the enzyme to dialysis, even in the presence of PYP and reductant, suggested the possible existence of other, hitherto unknown factors, the loss of which decreased optimal catalytic activity. Therefore, studies were undertaken to try and characterize the nature of the enzymatic instability.

Lability Of Ornithine Decarboxylase to Dilution

The enzyme, at all stages of the purification, was found to be labile to dilution. This is illustrated in Figure 10 with an enzyme fraction purified through the second step. Serial dilutions of the enzyme 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 only 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. Clearly, this sensitivity to being diluted could account for many of the recovery problems encountered in developing the purification procedures.

It had previously been reported that addition of exogenous protein to purified preparations of ornithine decarboxylase would stabilize the catalytic activity (Boucek and Lembach, 1977; Jänne and Williams-Ashman, 1972). 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 4. The dilution of the semi-purified ornithine decarboxylase fraction ten-fold resulted in a fifty-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 obliterated the enzyme. These compounds were also found to inhibit the undiluted enzyme 33%, 24%, and

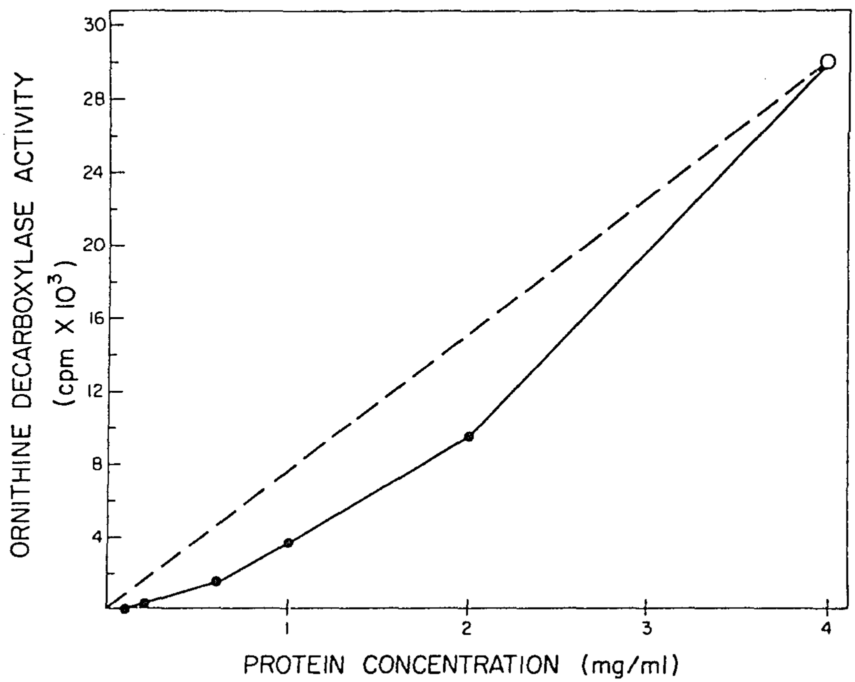

Figure 10. Loss of ornithine decarboxylase upon dilution -- Enzyme purified through the second step was diluted to the protein concentration indicated with purification buffer containing 2 mM DTT and 0.1 mM EDTA prior to assay.

Table 4. The Effect of Proteins and Other Stabilizing Agents on the Loss of Ornithine Decarboxylase Activity Upon Dilution

	Ornithine Decarboxylase (cpm/30 min)	% of Expected activity
Undiluted	18,090	
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	1,069	59
+ 1 mg/ml Hemocyanin	1,267	70
+ 10 mg/ml Hemocyanin	1,545	86
+ 10 mg/ml Transferrin	1,617	90

An ornithine decarboxylase fraction purified through step 2, containing 4 mg/ml, was assayed directly, or after a 10-fold dilution, in assay reaction buffer containing the compounds listed.

98%, respectively. The inclusion of polyethylene glycol (M_R 20,000) preserved 2-1/2 times more of the enzyme upon dilution, maximum protection being afforded by a 0.1% concentration of the polymer. Further characterization studies of the PEG effect showed that the fold stimulation of the enzyme by the PEG became greater as the dilution was increased and the magnitude of enzyme loss became greater. Enzyme diluted 1:4 retained only 42% of the expected activity and the inclusion of PEG increased that measurable activity 1.4-fold. Enzyme diluted 1:100 retained only 4% of the expected activity and the inclusion of PEG increased that measurable activity 4.5-fold.

Inclusion of additional protein in the diluent also provided various degrees of protection to the ornithine decarboxylase. Transferrin and hemocyanin almost totally prevented the loss of enzyme activity while BSA preserved 60% and ovalbumin offered little protection. It is of interest in view of the latter studies involving metal chelators (see below) that the proteins which provided the greatest protection were those which contain transition metals. 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.

Lability of Ornithine Decarboxylase to Charcoal Extraction

During the course of purification procedures to isolate isotopically-labeled ornithine decarboxylase from tissue culture cells which had been grown in the presence of a radioactive nucleotide and amino acid, it was discovered that charcoal treatment of semi-purified enzyme would totally inactivate the preparation. As shown in Table 5, the addition of 50 mg of charcoal to 1 ml of step 2 ornithine decarboxylase from Rat-1 fibroblasts resulted in the loss of all catalytic activity. Subsequently, the calf liver enzyme was also found to be labile to charcoal treatment (Table 5, Exp. B). Experiments using dextran-coated charcoal, in which only small molecules would have access to the charcoal binding sites, showed similar inactivation indicating that the reduction in measurable enzyme was not due to precipitation of the protein by the charcoal.

Charcoal displays a high capacity to bind aromatic compounds. Therefore, the possibility was examined that the inactivation resulted from the removal of PYP from the enzyme, which the concentration in the present assay (60 μM) was unable to restore effectively. However, the addition of ten-fold more PYP did not restore any of the enzymatic activity. To determine if the removal of PYP from the enzyme could result in the irreversible denaturation of the molecule with respect to catalysis, the ornithine decarboxylase was incubated with excess ornithine (10 mM), adjusted to pH 5 in the presence of high PO_4 (0.5 M), and then precipitated by adjustment to 50% $(NH_4)_2SO_4$. This procedure has been reported to promote the isolation of apoenzyme from other PYP-dependent enzymes. An 88% decrease in decarboxylase activity resulted from this treatment (Table 5, Exp. C). However, full catalytic activity was restored by the addition of 0.06 mM PYP indicating that the enzyme was not damaged by removal of the cofactor.

Table 5. The Loss of Ornithine Decarboxylase Activity Upon Charcoal Extraction, Pyridoxal Phosphate Removal, or Cibacron Blue Treatment

Enzyme Source	Treatment	Ornithine Decarboxylase (cpm/30 min/50 μl)
Exp. A:		
Rat-1 Fibroblast		4500
	50 mg/ml charcoal	0
Exp. B:		
Calf liver		4722
	30 mg/ml charcoal	1749
	60 mg/ml charcoal	74
	60 mg/ml charcoal + 600 μM PYP	80
Exp. C:		
Calf liver		5240
	$(NH_4)_2SO_4$ ppt (resusp. -PYP)	604
	$(NH_4)_2SO_4$ ppt (resusp. +PYP)	5704
Exp. D:		
Calf liver		4382
	Cibacron Blue-Agarose	172

Semi-purified (step 2) 1-ml fractions of ornithine decarboxylase from fibroblasts (0.5 mg/ml) or liver (3.5 mg/ml) were treated as follows: Exp. A and B, by the addition of the indicated amount of charcoal, incubation for 10 min at 4°C, and centrifugation to remove the charcoal; Exp. C, by incubation for 15 min in the presence of 10 mM ornithine, followed by 30 min in the presence of 0.5 M KPO_4 pH 5 and subsequent precipitation with 50% $(NH_4)_2SO_4$ and resuspension in the presence or absence of 0.06 mM PYP; Exp. D, by the addition of 0.4 ml settled volume Affigel Blue, incubation for 10 min at 4°C and centrifugation to remove the agarose.

Charcoal treatment has traditionally been used to remove contaminating nucleotides from enzyme preparations. Therefore, the possibility was considered that ornithine decarboxylase might contain a bound nucleotide required for stability of the active site which the charcoal was removing. Since there was no feasible way to directly resolve this question, the correlative possibility that the enzyme possessed a nucleotide binding site was considered. Affigel Blue is an agarose to which the reactive blue dye, Cibacron Blue F3GA, has been coupled. This dye is a biospecific probe for enzymes which contain binding sites for nucleotides (Thompson, Cass, and Stellwagen, 1975; Wilson, 1976), probably due to the structural similarities between the blue chromophore and nucleotide cofactors. Incubation of the dye-coupled agarose with the calf liver ornithine decarboxylase and subsequent removal by centrifugation resulted in the disappearance of more than 95% of the measurable activity indicating that the enzyme had bound to the blue dye. Attempts to release the enzymatic activity by incubation of the agarose with a variety of nucleotides (ATP, dATP, GTP, Adenosine, NAD, NADH, NADP) were unsuccessful. However, because of the lability of the enzyme to purification procedures, discussed above, it is impossible to conclude that any one of these did not result in release of the enzyme protein. These results with the Cibacron Blue conjugate support the possibility that ornithine decarboxylase has a nucleotide binding site. It cannot, however, be taken as more than that since other proteins, notably BSA, have also been recently reported to strongly interact with the dye by an as-yet uncharacterized mechanism. Resolution of the importance of a nucleotide to ornithine decarboxylase awaits the identification of such a component in a purified preparation of the enzyme.

A variety of metals and nucleotides, alone and in combination, were tested for their ability to restore activity to the charcoal-treated ornithine decarboxylase. None of them was found to be effective. During these experiments to identify the putative activator, a variety of physiologically occurring cofactors for other enzymatic activities were tested. In the course of these, it was discovered that Coenzyme B_{12} had a profound effect to inhibit the enzyme. The addition of 10 μM 5'-deoxyadenosylcobalamine resulted in 50% inhibition of the enzyme (Table 6). Cyanocobalamin had little effect on the enzyme. Coenzyme B_{12} has only previously been shown to play a positive role in enzyme catalysis, acting as a cofactor for a variety of enzymatic reactions (Hogenkamp, 1968). No examples of a negative regulatory role for the active vitamin are known. Therefore, this effect was examined further to try and elucidate the molecular mechanism. As part of its biological function, Coenzyme B_{12} serves to transfer H^+ from thiols to acceptor substrates. Since the activity of ornithine decarboxylase is known to be sensitive to the redox state of the reaction environment, the possibility was considered that the observed inhibition was due to an alteration involving auto-oxidation. The hydroxyl derivative of cobalamin can interact with thiols to form a disulfide; the resulting free radical state of the cobalt then reacts with O_2 to restore the hydroxyl derivative. This reaction, under appropriate conditions, can cycle indefinitely as long as O_2 is present, depleting the environment of reduced sulfhydryls. Therefore, the hydroxyl derivative was tested

Table 6. The Effect of Cobalamin Derivatives to Inhibit Ornithine Decarboxylase Activity

	Cobalamin Derivative Added	Ornithine Decarboxylase (cpm/30 min)
Exp. A:		2655
	1×10^{-6} M 5'deoxyadenosyl-	2090
	1×10^{-5} M 5'deoxyadenosyl	1270
	1×10^{-5} M cyano-	2580
Exp. B:		2380
	1×10^{-5} 5'deoxyadenosyl-	1200
	1×10^{-6} hydroxyl-	1075
	1×10^{-5} hydroxyl-	580
Exp. C: N_2 flush		2700
	1×10^{-5} 5'deoxyadenosyl-	2300
	1×10^{-4} 5'deoxyadenosyl-	1900
	1×10^{-5} hydroxyl-	2100
	1×10^{-4} hydroxyl-	500

Semi-purified ornithine decarboxylase (step 3) was incubated for 10 min at room temperature in the presence of the indicated concentration of Cobalamin derivative prior to assay.

for its ability to inhibit ornithine decarboxylase. As shown in Table 6, Exp. B, this derivative of cobalamin is more potent than the deoxy-adenosyl derivative to inhibit the enzyme. To further test this possibility of inhibition occurring as the result of artifactual redox cycling, the reaction was carried out in N_2-flushed buffers. As pointed out above, the auto-oxidation reaction promoted by hydroxylcobalamin required the participation of molecular oxygen. As shown in Exp. C (Table 6), the removal of O_2 from the reaction dramatically decreased the potency of both the Coenzyme- and hydroxyl-B_{12} forms so that a 10-fold greater concentration was required to inhibit the enzyme. Therefore, it appears that the effect of the vitamin forms on ornithine decarboxylase activity most probably reflects their ability to catalyze an auto-oxidation reaction rather than a regulatory function with regard to control of the decarboxylating enzyme.

Evidence for Ornithine Decarboxylase as a Metalloenzyme

Nucleotides often interact with proteins in concert with a divalent metal. Furthermore, the sensitivity of ornithine decarboxylase to the concentration of EDTA during dialysis suggested a possible metal involvement with the enzyme. Therefore, experiments employing metal chelators were undertaken. Since no complexing agent has been shown to meet the requirements of total specificity in studying the behavior of a particular metalloenzyme, a series of different complexing agents was employed. Figure 11 shows an experiment in which semi-purified ornithine decarboxylase (step 3) was incubated with increasing concentrations of a variety of metal chelators for 10 min at 4°C prior to

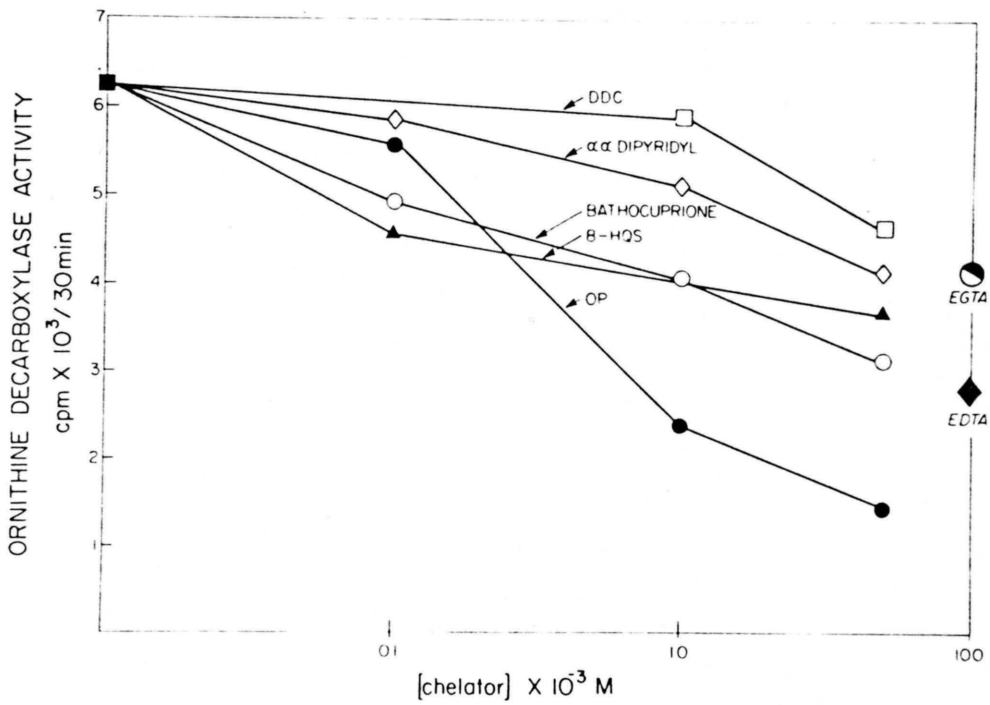

Figure 11. Inhibition of ornithine decarboxylase by metal chelators -- Step 3 purified ornithine decarboxylase was preincubated for 10 min at 4° with the indicated concentrations of metal chelator prior to assay.

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 and Wacker, 1970). The potency of this chelator (K_i < 1 mM), which displays only slight affinity for alkaline earth metals, in comparison to the relatively high concentrations of EDTA required to inhibit (10 mM), suggested that an element in the transition series is involved in the maintenance of ornithine decarboxylase activity.

Since 1,10-phenanthroline is a planar molecule it can possibly insert into hydrophobic areas of proteins; it has also exhibited the property under certain conditions to oxidize sulfhydryl groups. Therefore, the structurally similar but non-chelating analog, 4,7-phenanthroline, was tested. As shown in Figure 12, this compound had no effect on the activity of ornithine decarboxylase suggesting that the metal chelator was not inhibiting due to the aromatic properties of its structure.

A number of metals were tested for their ability to reactivate ornithine decarboxylase that had been inhibited by incubation with 1,10-phenanthroline. Zn^{2+}, Fe^{2+}, Cu^{2+}, Fe^{3+}, Cu^{1+}, 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.

A Macromolecular Activator for Ornithine Decarboxylase

The lability of ornithine decarboxylase to purification procedures suggested the possibility that the fractionation techniques

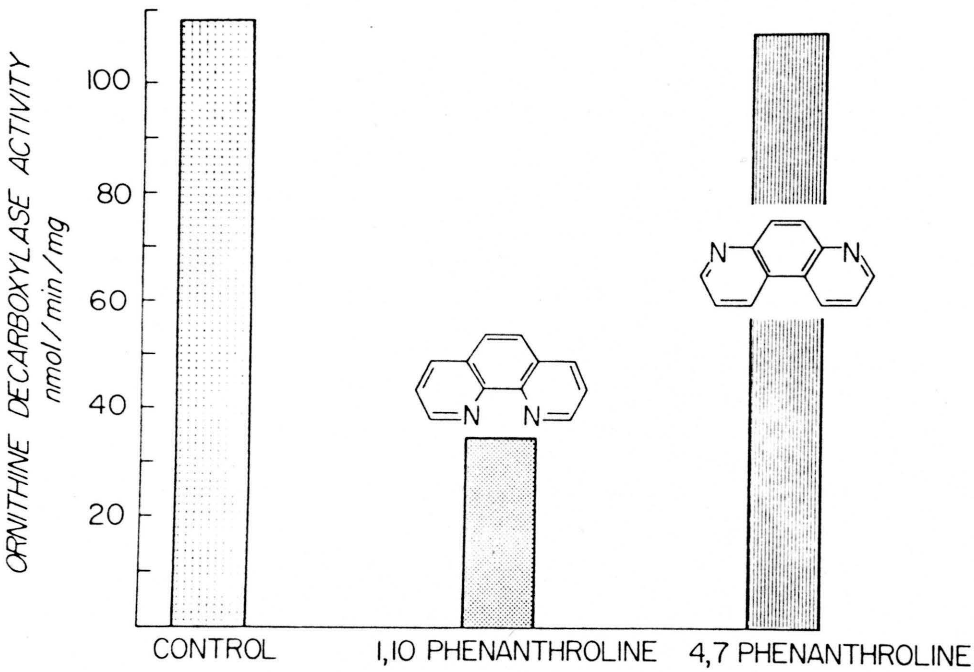

Figure 12. Inability of 4,7-phenanthroline to inhibit ornithine decarboxylase -- Step 3 purified ornithine decarboxylase was preincubated for 10 min at 4° with 1 mM 1,10-phenanthroline or 4,7-phenanthroline prior to assay.

employed were separating the enzyme from another proteinaceous molecule required to maintain stability and/or activity. Therefore, the protein-containing fractions eluting from the Ultrogel column were assayed for their ability to maintain the activity of a diluted ornithine decarboxylase fraction. It was found that the fractions in the area of the eluting ornithine decarboxylase peak itself were able to preserve as much as 200% of the enzyme being inactivated by dilution. Although this stabilizer/activator was eluting concomitantly with the enzyme, it appeared that it might be slightly preceding the enzyme off the column, indicating a somewhat greater molecular weight.

This observation was pursued further in the gel electrophoresis fractionations during steps 5 and 6 of the purification. The slices from a gel electrophoresed in the PO_4 buffer system, after diffusion overnight into assay buffer, were tested both for their ornithine decarboxylase activity and for their ability to maintain the activity of a fraction of previously electrophoresed ornithine decarboxylase upon dilution. The difference in cpm CO_2 released between the fractions assayed with the diluted sample minus the fractions assayed alone was then plotted vs. the activity of the fractions alone to see if any evidence for a stimulator of enzyme activity different than the enzyme itself could be obtained. As shown in Figure 13A, the gel slices containing the ornithine decarboxylase activity also displayed the ability to maintain the activity of a sample of enzyme upon dilution. However, in this system again, the stabilizing/activating activity was slightly offset from the enzyme activity toward the BSA marker band, indicative of a greater molecular weight. When this same type of experiment was

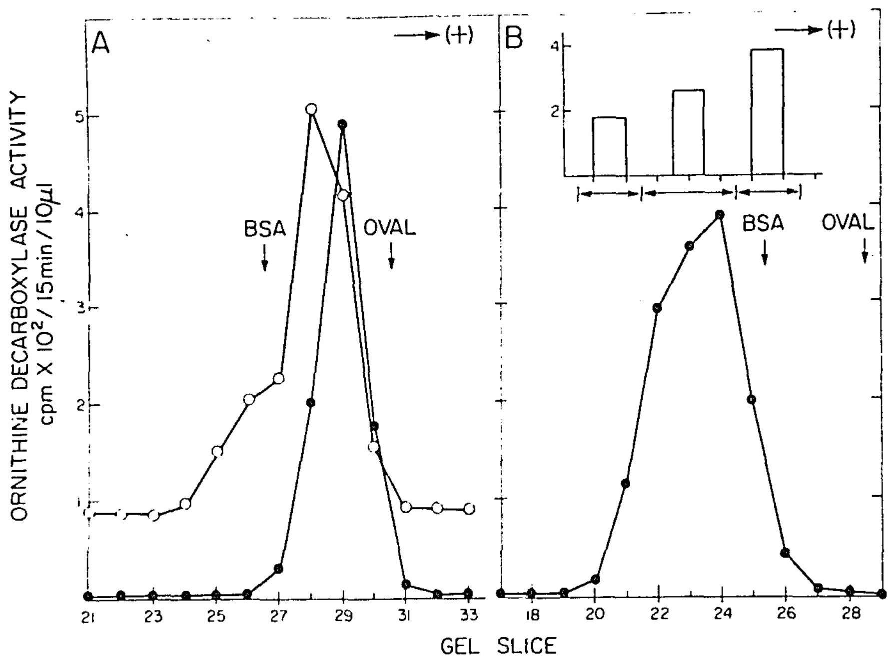

Figure 13. Electrophoretic resolution of an ornithine decarboxylase activator -- Slices from gels on which step 4 enzyme had been electrophoresed in the PO_4 buffer (panel A) or the Tris-barbital buffer (panel B) were assayed for ornithine decarboxylase activity (closed circles). Equivalent size samples from each slice (panel A) or from pooled slices (panel B) were then reassayed in the presence of a diluted sample of enzyme which had previously been shown to have lost 80% of the expected activity upon dilution. The cpm CO_2 released as detected in the first assay was subtracted from the value obtained in the second assay and the difference plotted to assess for possible stimulation (open circles, panel A; inset, panel B).

conducted after electrophoresis in the Tris-barbital system, the activating activity was again found in the area of the BSA marker band. However, in this buffer system the mobility of the ornithine decarboxylase is shifted to that of an apparently greater molecular weight protein. Although these experiments do not resolve whether the stabilizing principle for diluted enzyme found to be copurifying with the ornithine decarboxylase activity is a species of the enzyme itself or another unique protein, they do suggest the existence of a specific macromolecule which is distinct from the majority of the catalytic activity in its resistance to the mobility shift in the Tris-barbital buffer.

Biochemical Characterization of Ornithine Decarboxylase

Catalytic Activity of Ornithine Decarboxylase

The apparent affinity of ornithine decarboxylase for ornithine at 37°C was calculated by varying the substrate concentration in the presence of 0.06 mM PYP. As shown in Figure 14, 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. As shown in Figure 15, the K_m of ornithine decarboxylase for the cofactor is 2.5 μM when measured in the presence of 1 mM ornithine.

The addition of the product of the enzymatic reaction, putrescine, inhibited the rate of ornithine decarboxylation (Figure 16).

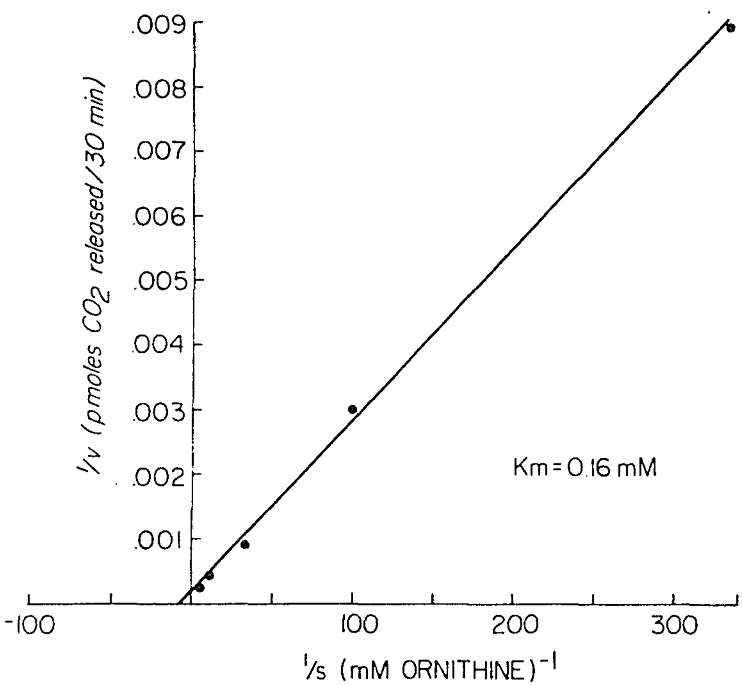

Figure 14. Double-reciprocal plot of ornithine decarboxylase activity with respect to ornithine concentration.

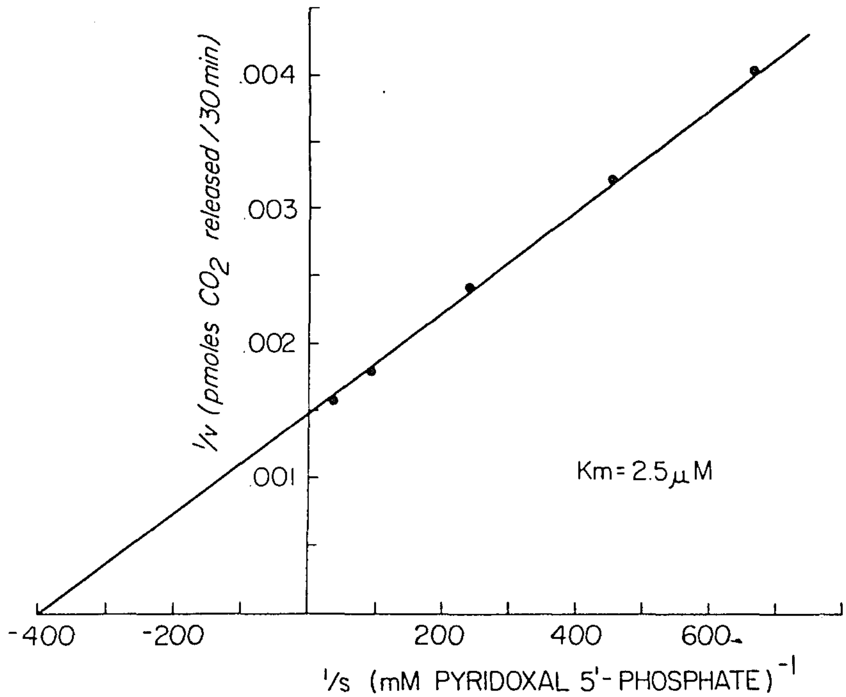

Figure 15. Double-reciprocal plot of ornithine decarboxylase activity with respect to pyridoxal-5'-phosphate concentration.

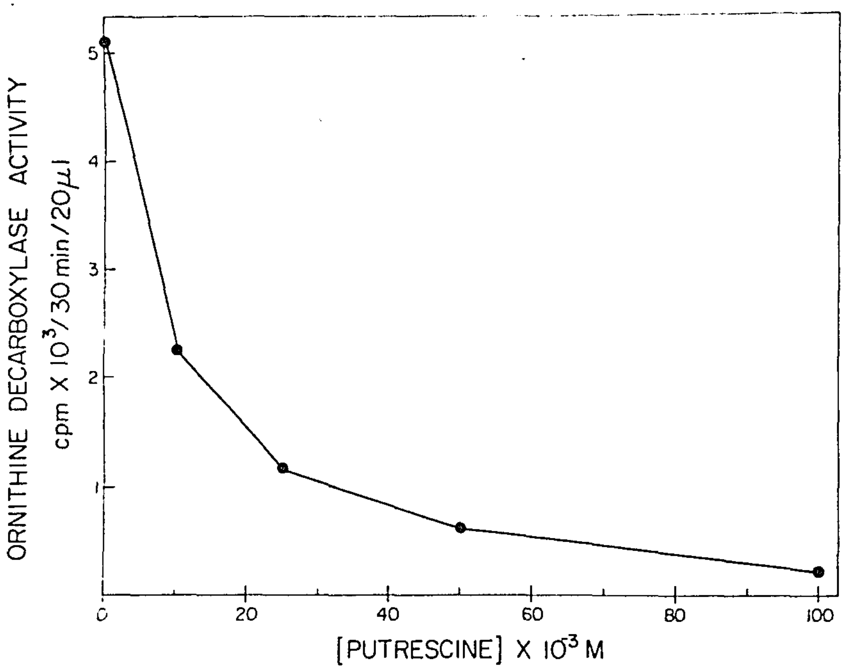

Figure 16. Concentration-dependent inhibition of ornithine decarboxylase by putrescine.

Putrescine (10 mM) inhibited the enzyme 50% when assayed in the presence of 0.5 mM ornithine.

Sensitivity of Ornithine Decarboxylase to Reducing Agents

As discussed previously, the activity of ornithine decarboxylase is sensitive to the redox state of the assay environment. The initial experiments conducted to characterize this effect completely removed the enzyme from the presence of thiols 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 were all found to stimulate 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 and Williams-Ashman, 1971). Therefore, a further experiment was conducted in which the ornithine decarboxylase was depleted of reductant by buffer exchange using the Amicon ultrafiltration device. As shown in Figure 17, 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 original at an optimal concentration of the dithiol 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

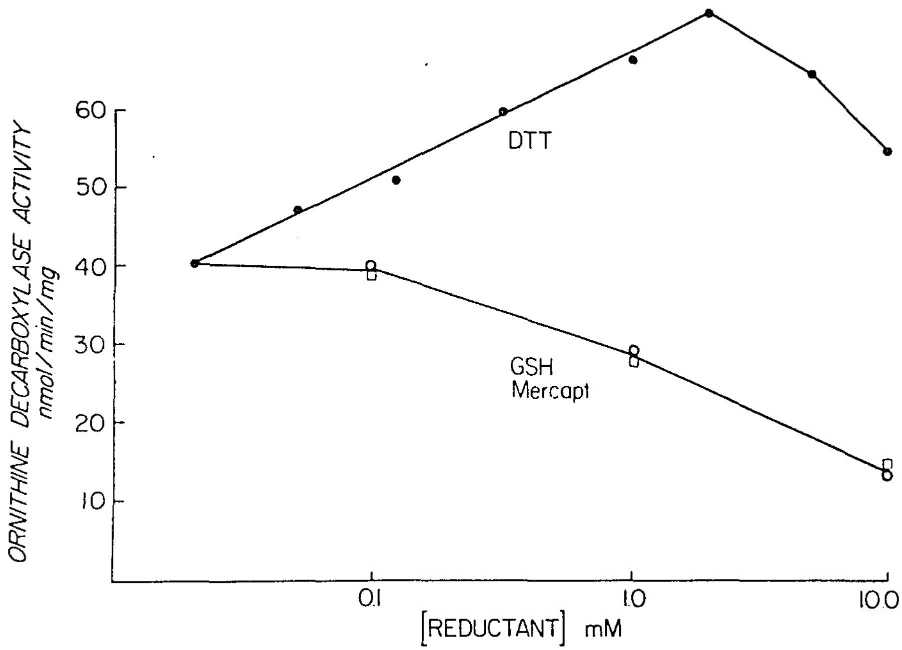

Figure 17. Effect of reducing agents on ornithine decarboxylase activity -- Buffer exchange with 100 volumes of purification buffer containing no reductant was conducted with the Amicon ultrafiltration device. Enzyme activity was then determined in the presence of the indicated concentration of reductant.

indication of the necessity of free sulfhydryl groups for the activity of ornithine decarboxylase is the finding that preincubation of the enzyme for 5 min in the presence of 0.05 mM p-chloromercuribenzoate completely inhibited all release of CO_2 during the subsequent assay.

Physical Characteristics of Ornithine Decarboxylase

The activity of ornithine decarboxylase 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. As shown in the calibration plots in Figure 18, the enzyme displayed an approximate molecular weight of 54,000 and Stokes radius of 3.14 nm.

Since gel filtration more accurately reflects molecular size and only yields an approximation of molecular weight, the ornithine decarboxylase preparation was subjected to $NaDodSO_4$-denaturing electrophoresis on 4 to 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, yielded no detectable bands after staining 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 as described in "Materials and Methods" and the gel was sliced into 1-mm pieces and counted in 3% NCS-Toluene Omnifluor. A single band

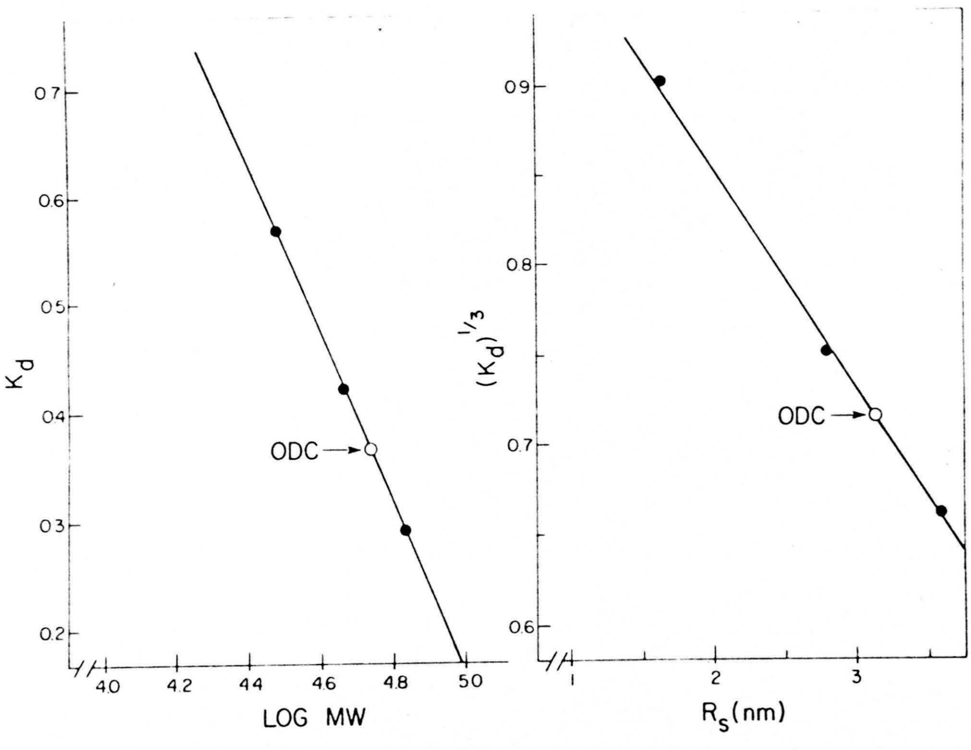

Figure 18. Molecular weight and Stokes radius of ornithine decarboxylase -- The Ultrogel AcA 44 column was calibrated with BSA (69,000; 3.6 nm), ovalbumin (46,000; 2.8 nm), carbonic anhydrase (30,000), and cytochrome C (12,300; 1.64 nm) prior to the application of a sample of ornithine decarboxylase.

of radioactivity was detected at a position indicating a molecular weight of 52,000 daltons as compared to the movement of protein standards of known molecular weight.

Multiple Forms of Ornithine Decarboxylase

Previous reports had indicated that multiple forms of ornithine decarboxylase may exist based on differential elution pattern from Activated-Thiol Sepharose (Obenrader and Prouty, 1977a) or based on multiple kinetic parameters with respect to substrate (Lau and Slotkin, 1979) or cofactor (Clark and Fuller, 1976). Application of these analyses to the calf liver enzyme indicated a single species of the enzyme. However, two other of the fractionation techniques indicated possible heterogeneity of the enzyme. First, a fraction of the enzyme could be isolated which would not adsorb to hydroxylapatite. This fraction amounted to only a small per cent of the total derived from gel filtration and was usually obscured by the larger amount of enzyme "flowing-through" the column due to the saturation procedure employed. However, repeated application of the pooled flow-through fractions to purify the enzyme contained in them eventually resolved a sample of the enzyme which would not adsorb to the resin. Second, the elution patterns of the enzyme activity from the first ion-exchange chromatography often indicated a double peak of activity, an example of which was shown in Figure 1.

To develop a reliable procedure for maximal resolution of possible multiple forms of the enzyme, the technique of gradient sievorptive chromatography was employed (Kirkegaard, 1973).

Sievorptive chromatography actively combines molecular sieve (gel filtration) and ion-exchange adsorption chromatography. When a gradient is preapplied to fill ca. 1/2 of the column volume, a focusing system is formed which concentrates macromolecules as they move through the column at positions determined by the adsorptive properties of the molecules. Since the gradient is composed of small molecules, it will move through the entire liquid phase of the column at the same rate as the buffer. The subsequently applied protein sample, however, is excluded from the gel matrix and until they reach a low enough salt concentration for adsorption to occur they will move through the column two to three times faster than the buffer. Once the adsorption point is reached, the individual protein will slow down and eventually travel at the same rate as the gradient.

Figure 19 shows the pattern of ornithine decarboxylase activity elution from a DE25-Sephadex column to which a 75 to 200 mM NaCl gradient had been preapplied. Three distinct peaks of enzyme activity are resolved. Since the enzyme binds pyridoxal-5'-phosphate in a fairly readily reversible manner (see above), it was possible that the different adsorption properties of the three species of enzyme on the cationically charged resin was due to differential binding of cofactor. Therefore, another chromatographic procedure was conduced 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 is observable when fractionation is carried out based on molecular weight, it appears that the differences observed by

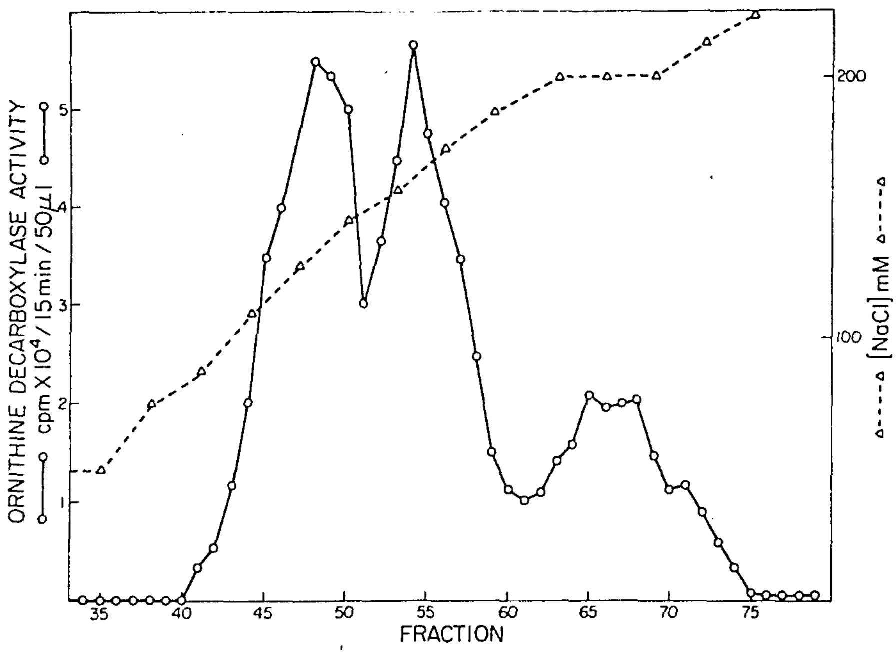

Figure 19. Resolution of multiple forms of ornithine decarboxylase by sievorptive chromatography.

this technique result from small, covalent modifications of the enzyme which alter the protein's charge.

Comparison of Ornithine Decarboxylase from Rat and Calf Liver

The purification procedure described in this report is now being applied to the isolation of ornithine decarboxylase from rat liver. The following comparisons between the two enzymes can be made at this time:

1. The rat liver enzyme is also labile to dilution showing a progressively greater loss of activity upon linear dilution of the enzyme. However, enzyme fractions during the initial stages of the purification also contain an inhibitor (finally removed by step 4) which makes the assay of the enzyme at high protein concentrations inaccurate.
2. The rat liver enzyme elutes as a single peak upon Ultrogel AcA 44 filtration at a volume indicating a slightly greater molecular weight, 61,000 and Stokes radius, 3.35 nm, than the calf liver enzyme.
3. Gradient sievorptive chromatography of the rat liver enzyme also indicates the existence of three species of the enzyme, the relative proportions of which are very similar to that found with the calf liver enzyme.
4. No substantial differences have been found between the behavior of the two enzymes during any of the purification procedures described.

DISCUSSION

Progress in defining the molecular characteristics of ornithine decarboxylase 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 ornithine decarboxylase from calf liver has been circumvented by the maintenance of high protein concentrations at each step in the procedure. The mechanism or mechanisms regulating the instability of the enzyme are still not known, although the inclusion of various proteins or PEG have been shown to provide partial protection. The data obtained from various of the studies presented do, however, suggest that at least a partial explanation may involve loss of a previously unknown metal cofactor. Most metals that are bound to proteins obey an equilibrium reaction governed by a stability constant (Vallee and 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.

Another possible underlying cause of the loss of ornithine decarboxylase activity upon extensive purification is the removal of a macromolecular cofactor. The existence of such an activity is suggested by the experiments showing that the fractions just offset from the enzyme peak itself after both gel filtration and gel electrophoresis contain an activating/stabilizing principle. It is not possible from these results to determine if this activity represents a form of the enzyme itself or another unique protein. Support for the notion that such a protein cofactor may be required for efficient catalysis by the enzyme is given by the observation that the homogeneous enzyme is relatively unstable to storage ($T_{1/2} \cong 24$ hours at 4°C) even in a concentrated form as compared to crude fractions which are stable for several months. Under these conditions no further dissociation of an enzyme-bound metal would be expected to occur. However, it is possible that the putative protein cofactor functions to present and/or preserve the required metal in a suitable form, a situation shown to exist for phosphoenolpyruvate carboxykinase which requires addition of a ferro-activator protein to allow demonstration of Fe^{2+} stimulation of enzyme activity (Bentle, Snoke, and Lardy, 1976).

Table 7 presents a comparison of the characteristics of purified ornithine decarboxylase that have been reported. Perhaps 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 very comparable to that reported for the enzymes purified from thioacetamide-stimulated rat liver, ca. 250,000 pmol/min/mg. However, it is almost 100 times greater than that obtained with enzyme from regenerating rat liver. This variation may reflect the catalytic instability noted in the present report.

Table 7. Comparison of Characteristics of Purified Ornithine Decarboxylase

Reference--source	Relative purification (-fold)	Specific activity (pmol/min/mg)	Molecular weight	Stokes radius (nm)	K_m Ornithine (mM)	K_m PYP (μM)	K_i Putrescine (mM)
1. Jänne and Williams-Ashman, 1971 Rat ventral prostate	292	10,000	44,000 → 85,000	3.87	0.1		1.0
2. Friedman, Halpern, and Canellakis, 1972 Regenerating rat liver	175	3,000	66,000				
3. Ono et al., 1972 Thioacetamide-stimulated rat liver	5372	193,000	100,000		0.2		>10.0
4. Boucek and Lembach, 1977 SV40-transformed 3T3 cells	5917	1,660,000	55,000 → 110,000		0.125	3.62	not inhibitory
5. Obenrader and Prouty, 1977a Thioacetamide-stimulated rat liver	6900	233,000	48,000 → 100,000		0,13	0.25	
6. This work Thioacetamide-stimulated calf liver	71500	286,000	54,000 → 110,000	3.14	0.16	2,5	10.0

The molecular weight determinations are all comparable indicating a monomeric protein of ca. 50,000 daltons. 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. A recent experiment employing 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 ornithine decarboxylase obtained under these conditions is just as active as the monomer. Previous reports had suggested that the dimer was inactive (Jänne and 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.

The K_m values determined for ornithine and PYP for the calf liver ornithine decarboxylase are very similar to those reported for other enzyme preparations (Table 7). No evidence for multiple forms of the enzyme with regard to kinetic values was found. Some reports have indicated that ornithine decarboxylase 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 and Prouty, 1977a) indicated the existence of multiple forms of ornithine decarboxylase based on heterogeneous elution of the enzyme on Activated-Thiol Sepharose and gel electrophoresis. Utilizing these techniques with the calf liver enzyme, 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 ornithine decarboxylase resulted in the 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 possible metal and/or nucleotide cofactors for the mammalian enzyme. The activity of bacterial ornithine decarboxylase is known to be affected by nucleotides (Höltä, Jänne, and Pispa, 1974; Sakai and Cohen, 1976b). GTP activates the procaryotic enzyme and ppGpp inhibits. However, no effect of added nucleotides upon mammalian ornithine decarboxylase has been detectable (Pegg and Williams-Ashman, 1968; Jänne and Williams-Ashman, 1971; Friedman et al., 1972; Boucek and Lembach, 1977). Although the present studies have also failed to find any effect of nucleotide added in vitro on enzyme activity, the inactivating effect of both charcoal and Cibacron Blue are suggestive of an involvement of a nucleotide in enzyme function. A further suggestion is the finding that under certain growth conditions the addition of hydroxyurea to cultured fibroblasts blocks the increase in detectable ornithine decarboxylase activity (Cress and Gerner, 1979). Hydroxyurea inhibits deoxynucleotide synthesis by virtue of its ability to complex with and inactivate an iron free radical in ribonucleotide reductase (Hogenkamp, 1968). Whether the action of the inhibitor on ornithine decarboxylase activity is the result of its effect on deoxynucleotide biosyntheis, its metal chelating capacity, or some other yet undefined mechanism remains to be determined.

The effect of a variety of metal chelators to inhibit the calf liver ornithine decarboxylase suggests that an enzyme-bound metal is required for preservation of catalytic capacity. This interpretation assumes that the complexing agents induce inhibition solely through the coordination of an essential metal. However, all of the agents employed are also capable of participating in non-specific interactions with various other protein reactive groups. The finding that 4,7-phenanthroline is inactive strengthens the assumption of a metal involvement since this analog possesses all of the attributes of the chelating 1,10-phenanthroline, except the ability to form a coordination complex with metals. Furthermore, the fact that several structurally unrelated chelators are all inhibitory also supports this interpretation. It is not possible to tell from the chelator data which metal is involved. However, the relative ineffectiveness of the broad spectrum chelator, EDTA, versus the potency of the transition metal chelator, 1-10-phenanthroline, suggests a metal from this series. The inability to regenerate enzyme activity upon metal readdition suggests that 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. An alternative possibility, to be considered in future experiments, is that the optimal binding reaction of the metal to the apoenzyme occurs with a redox state different from that involved in maintaining catalytic activity, in which case manipulating the DTT concentration of the enzyme reaction could profoundly influence the restoration process.

In conclusion, this dissertation presents a method which allows the purification of mammalian ornithine decarboxylase in milligram quantities. Furthermore, the initial characterizations which are reported herein suggest multiple regulatory processes which may serve to control the expression of the enzyme in vivo. These may also control its enzymatic versus initiation factor activity (Mannen and Russell, 1977a, 1977b). These regulatory processes include the control of enzymatic monomer to dimer transition, the provision of an appropriate metal to maintain enzymatic activity, the possible covalent modifications (e.g., phosphorylation, adenylation, ribosylation) underlying the three ionic enzyme forms, and the as yet unknown mechanism which maintains the free thiols required for optimal catalysis. Further progress in characterizing these phenomena should aid in the elucidation of the molecular mechanisms which operate at the cellular level to control the expression of ornithine decarboxylase and the trophic process of which it is a fundamental component.

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