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# PHYSICAL AND KINETIC DISTINCTION OF TWO ORNITHINE DECARBOXYLASE FORMS IN *PHYSARUM*

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## Summary

Two forms of ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17) can be isolated from crude plasmodial homogenates of *Physarum polycephalum*. Both forms catalyze the stoichiometric production of putrescine and $CO_2$ from ornithine, yet they are distinguished by (a) a large difference in their affinity for coenzyme (apparent $K_m$ values of 0.13 and 33 $\mu$M); (b) a differential stability to extended dialysis of crude homogenates at 4°C; and (c) the tendency of the low affinity form to polymerize when suspended in low ionic strength borate and phosphate buffers. These forms appear to be alternate states of a basic catalytic subunit in that (a) they both demonstrate monomer and dimer molecular forms of 80000 and 160000 daltons, respectively, depending on the buffer content; (b) they coelute from DEAE-cellulose ion-exchange columns; and (c) they vary in activity in approximately equivalent yet opposite directions in response to factors which alter this organism's growth or metabolism. These data suggest that ornithine decarboxylase activity may be modulated by the control of the transition of this enzyme between the active and the relatively less active form.

## Introduction

Very rapid changes in the activity of ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17) are thought to play a major role in the sensitive regulation of polyamine synthesis. Evidence based on inhibitor studies suggests that these activity fluctuations are produced by alterations in the rate of synthesis and degradation of this extremely labile enzyme [1—3]. Recently, however, we reported [4] that some fluctuations in ornithine decarboxylase activity in *Physarum* were accompanied by alterations in the ability of the enzyme to be activated by coenzyme, which suggested an alternate means of regulating this enzyme, i.e. through the modulation of its catalytic activity. Such a regulatory mechanism was supported by observations of non-linear Lineweaver-Burk

plots and multiple peaks of activity eluted from Sephadex G-200 columns indicating that several forms of this enzyme might exist, which differ in their activity with respect to the ability to bind the coenzyme, pyridoxal-5′-phosphate.

We now report that we have successfully separated two forms of ornithine decarboxylase from *Physarum* which differ considerably in their catalytic properties and we have begun a more detailed analysis of their characteristics, and their involvement in the control of polyamine synthesis. In this study we have attempted to determine (a) whether these are indeed distinct enzyme forms or merely artifacts of the isolation procedure; (b) whether these forms represent products of separate genes or post-translational modifications of one enzyme protein; and (c) whether these forms which are observed in vitro are of any significance in vivo, i.e. do they correlate with changes in the cell growth or biochemistry.

## Materials and Methods

*Chemicals.* Pyridoxal-5′-phosphate, L-ornithine, blue dextran, bovine serum albumin, DNAase, yeast alcohol dehydrogenase and cycloheximide were purchased from Sigma Chemical Co.; Sephadex G-200 (40—120 μm) from Pharmacia Fine Chemicals Inc.; DL-[1-$^{14}$C]ornithine · HCl (58 Ci/mol) from Amersham/Searle Corp.; and DL-[5-$^{14}$C]ornithine · HCl (3.29 Ci/mol) from New England Nuclear.

*Culture techniques.* Cultures of *Physarum polycephalum* were maintained and sampled as described earlier [5].

*Enzyme assay.* Preparation of enzyme extracts and enzyme assay have been described previously [4]. Components of assay mixtures are detailed in figure legends.

*Sephadex G-200 column chromatography.* Frozen samples were sonicated in 10 volumes of 0.05 M borate buffer (pH 7.8), containing 1.0 mM dithiothreitol, 0.5 mM EDTA and 2 μM pyridoxal-5′-phosphate. 1 ml of this sonicate was mixed with blue dextran and applied to a 100 × 1.6 cm Pharmacia column containing Sephadex G-200 which had been pre-equilibrated with this buffer, and eluted with the same buffer at 4.5 ml/h. All procedures were performed at 4° C. Where indicated in text the 0.05 M borate buffer was replaced by the stated concentrations of phosphate. Tris or *N*-2-hydroxyethylpiperazine-*N*′-20ethanesulfonic acid (HEPES) buffer.

In order to determine the approximate molecular weight of the eluted enzyme peaks, selected enzyme samples were mixed with the proteins DNAase, bovine serum albumin and yeast alcohol dehydrogenase (5 mg each). This mixture was applied to the Sephadex G-200 column and eluted in the usual fashion, and the eluant monitored by optical absorbance at 280 nm. The molecular weight of the enzyme forms were then approximated by comparison of the peaks of enzyme activity with the peaks of the eluted standards as detailed by Andrews [6].

Blue dextran was used in column separations to visualize the exclusion volume and was found not to affect the position of elution of any of the enzyme forms.

## Results

*Separation of ornithine decarboxylase forms*

The ornithine decarboxylase activity contained in crude *Physarum* homogenates eluted from Sephadex G-200 columns as a single peak of about 80000 daltons in the presence of 0.05 M HEPES, phosphate or Tris column buffer (pH 7.8). However, when 0.005 M phosphate or 0.05 M borate buffer (pH 7.8) was used as column buffer, ornithine decarboxylase activity was found to be associated with higher molecular weight forms. These forms are shown in Fig. 1A, where crude homogenates were prepared and eluted with 0.05 M borate buffer. Some activity was found in eluted fractions corresponding to a protein of molecular weight of about 160000, with a second, rather broad peak of activity near the exclusion volume of the column. These two peaks differ in

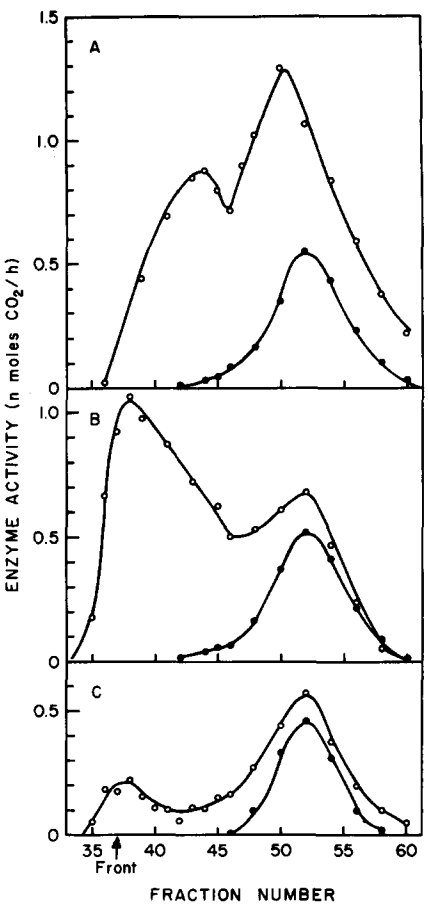


Fig. 1. Peaks of ornithine decarboxylase activity eluted from a Sephadex G-200 column. Identical microplasmodia samples were sonified in 0.05 M borate column buffer (pH 7.8) and applied directly to the Sephadex column (A), or dialyzed against column buffer for 24 h (B), or 48 h (C), and then eluted through the column as described in Materials and Methods. The exclusion volume is indicated at Fraction 37 as the Blue Dextran "Front". Fractions (1.7 ml) were assayed as described in Materials and Methods by placing 0.1 ml in 1.9 ml of 0.05 M borate buffer (pH 7.8), 0.5 mM dithiothreitol, 0.2 mM EDTA, 0.10 mM L-ornithine (0.10 $\mu$Ci) and either 1 (●) or 200 (○) $\mu$M pyridoxal-5'-phosphate.

their enzymatic ability in that both demonstrate activity when assayed in the presence of 200 μM pyridoxal-5′-phosphate, yet only the 160000 dalton peak is active in low (1 μM) coenzyme levels. As previously noted [5], neither shows activity in the absence of pyridoxal-5′-phosphate.

Dialysis of the crude homogenate before chromatography does not alter the amount of this more active enzyme form in the 160000 dalton peak, but this treatment does shift the activity observed in assays using high coenzyme levels to the faster running peak (Fig. 1B). Thus it appears that there are two forms of this enzyme: one which is stimulated equally by 1 and 200 μM pyridoxal-5′-phosphate (Form A); and a second which requires the higher coenzyme level for complete activation (Form B). Dialysis in 0.05 M borate buffer (pH 7.8) appears to promote either the polymerization of the B form or else its aggregation to another, very large molecule which is present in the crude homogenate. Extended dialysis of this crude fraction, however, causes a much more rapid loss of this B form than of the more active A form.

*Kinetic difference between these forms*

In order to test whether these two peaks represented catalytically distinct enzyme forms, peak activity tubes were collected from column separations similar to that shown in Fig. 1B, and each fraction was concentrated by membrane filtration (Amicon PM-10), and dialyzed against 0.05 M borate column buffer. Each fraction was then assayed in the presence of a wide range of pyridoxal-5′-phosphate concentrations to determine their comparative ease of activation by this coenzyme. In both cases normal hyperbolic saturation kinetics were observed, producing linear Lineweaver-Burk plots. These were extrapolated by linear regression to estimate $K_m$ values for pyridoxal-5′-phosphate of 0.13 and 33 μM for forms A and B, respectively. This very large difference between their affinities for coenzyme is consistant with the scans of enzyme activity shown in Fig. 1B. The enzyme form running with the column front showed no detectable activity at 1 μM pyridoxal-5′-phosphate because this coenzyme level was only 1/30 of the apparent $K_m$ for pyridoxal-5′-phosphate of this form. However, the low $K_m$ enzyme form which eluted in the 160000 dalton peak demonstrated almost complete activation by these assays in the presence of 1 μM pyridoxal-5′-phosphate. The fact that, in this peak, the 200 μM pyridoxal-5′-phosphate assays did not show much additional activity over that in the presence of 1 μM pyridoxal-5′-phosphate, suggests that there is very little of the B form of this enzyme remaining in this 160000 dalton peak after the 24 h dialysis described in the legend to Fig. 1. This method of separating the enzyme forms and testing their homogeneity by their relative activity in 1 and 200 μM pyridoxal-5′-phosphate, was used in subsequent experiments.

In these experiments we chose to use a level of substrate which was not saturating (0.1 mM) in order to minimize alterations in the level of coenzyme due to non-enzymatic Schiff base formation. Under these conditions, the $K_m$ for the formation of Schiff base between ornithine and pyridoxal-5′-phosphate is approx. 0.62 mM (Mitchell and Dillon, unpublished observations). Thus saturating levels of the substrate, ornithine, greatly alter the concentration of free coenzyme available for this enzyme, thereby complicating the kinetic studies. In order to test the possible effect of this reduced substrate level on these

enzyme forms, similar kinetic experiments were performed at a saturating substrate level (2.6 mM ornithine). When coenzyme and substrate concentrations were adjusted for loss due to Schiff base formation, the apparent $K_m$ values for pyridoxal-5′-phosphate agreed closely with those found at 0.1 mM ornithine.

*Evidence that these forms both represent ornithine decarboxylase*

In order to test whether or not these two peaks of enzyme activity both represented ornithine decarboxylase, identical enzyme fractions were assayed in the presence of [1-$^{14}$C]- and [5-$^{14}$C]ornithine and examined for stoichiometric production of $^{14}CO_2$ and [$^{14}$C]putrescine using techniques previously described [5]. Both forms were found to produce putrescine concentrations equivalent to the labeled $CO_2$ released, indicating that they both catalyze the same reaction.

*Physical characteristics*

Although two kinetically distinct ornithine decarboxylase forms can be demonstrated, the possibility remains that these differences in enzymatic ability merely reflect the state of polymerization of one basic subunit. To test this possibility, the enzyme forms were isolated as described above and subjected to buffer changes which altered their apparent molecular size, and thus their state of polymerization. Each form dissociated into an 80000 dalton monomer during chromatography in the presence of 0.01 M HEPES or 0.05 M phosphate buffer, yet when these monomer peaks were assayed in the normal 0.05 M borate buffer (pH 7.8), each enzyme form exhibited its characteristic coenzyme affinity. Concentrating and chromatographing the monomer A form in the 0.05 M borate (pH 7.8) assay buffer produced a single peak of activity in the 160000-dalton, dimer range. Similar chromatography of the monomer B form produced both this dimer and the polymer peaks. Thus it appears that these forms are kinetically distinct due to some stable difference in their 80000-dalton, monomer structure. Such a basic difference between these forms is consistent with our recent observations [7] that these forms demonstrate slightly different isoelectric points.

In addition to this basic difference between these enzyme forms there appears to be a correlation between catalytic activity and the state of aggregation of these enzymes. The B form polymer, for example, is readily dissociated into the dimeric state by the addition of sufficient coenzyme (200 $\mu$M pyridoxal-5′-phosphate) to saturate this form (Fig. 2A). Subsequent dialysis of the B form dimer, to reduce the coenzyme concentration allows this form to repolymerize (Fig. 2B). In a similar fashion, the monomeric A and B forms are readily converted to the dimer by the elevation of coenzyme to this level.

*Variations in these forms in response to cycloheximide*

Quantitative physical separation of these forms did not appear to be a practical method of monitoring their changes during enzyme activity fluctuations associated with inhibitor treatments, growth cycles or states of differentiation. We therefore attempted to calculate the $V$ of each of the ornithine decarboxylase forms, as they were assayed simultaneously in crude fractions, using a computer program which was recently designed by Osmundsen [8] to estimate

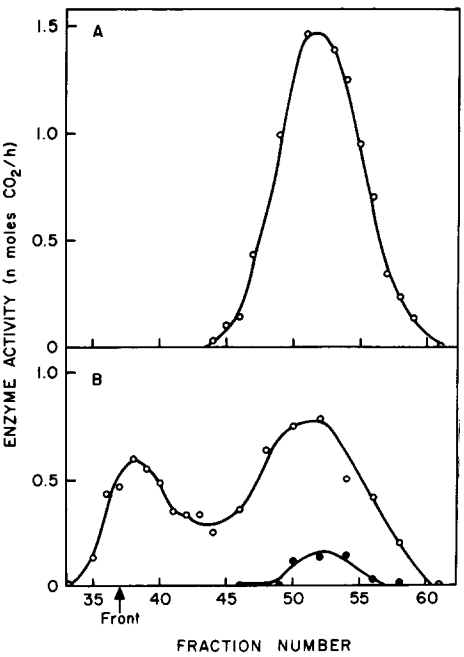


Fig. 2. Effect of pyridoxal-5′-phosphate concentration on elution of enzyme activity from a Sephadex column. (A) Microplasmodial sample sonified and eluted from column using buffer containing 200 μM pyridoxal-5′-phosphate. (B) Fractions 48—55 from A were combined and concentrated using an Amicon Diaflo filter, dialyzed against column buffer without pyridoxal-5′-phosphate for 24 h and eluted with this same buffer. Assay procedure and figure symbols are the same as in Fig. 1 except that the samples in A were assayed only at 200 μM pyridoxal-5′-phosphate.

the kinetic parameters of two enzymes acting simultaneously on the same substrate. An enzyme sample containing both the A and B forms produces a downward curving Lineweaver-Burk plot when assayed over a range of coenzyme concentrations (Fig. 3). This curve is iteratively approximated by the computer to be the sum of the activity of two enzymes with different $K_m$ values for coenzyme. In the experiment illustrated in Fig. 3, the $K_m$ values were computed to be 22.8 and 0.18 μM pyridoxal-5′-phosphate, which are in close approximation to the $K_m$ values of 33 and 0.13 μM calculated for these forms when assayed individually. The $V$ values calculated for the high and low $K_m$ forms in this sample are estimated at 57.2 and 5.04 nmol $CO_2$/h per mg protein, respectively. Thus an accurate approximation of the $V$ of each form can be rapidly obtained from assays on crude enzyme homogenates.

We utilized this technique to analyze the variations of the two ornithine decarboxylase forms in response to the inhibitor cycloheximide, which induces rapid ornithine decarboxylase inactivation in most tissues. We have previously shown that in *Physarum*, however, cycloheximide does not cause a rapid loss in ornithine decarboxylase activity, but rather it induces a sharp change in the ability of this enzyme to bind pyridoxal-5′-phosphate [4]. After the addition of cycloheximide, enzyme activity assayed in low (1 μM) pyridoxal-5′-phosphate concentrations is seen to increase abruptly, and then rapidly decrease below the starting activity level. Assays at saturating (200 μM) pyridoxal-5′-

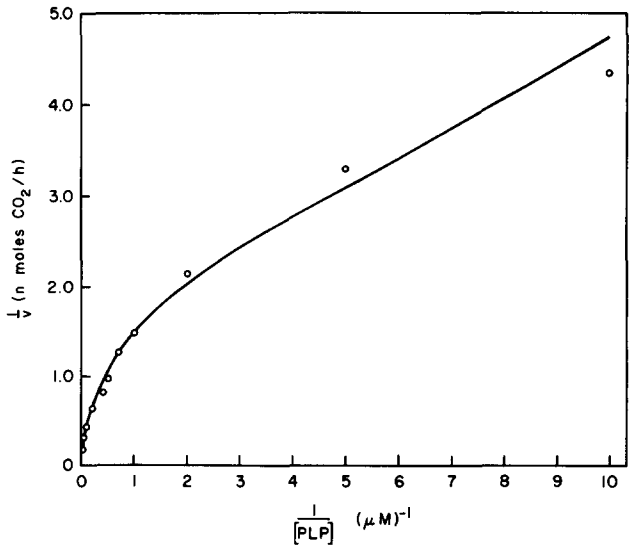


Fig. 3. Non-linear, double reciprocal plot of coenzyme activation of a sample containing two ornithine decarboxylase forms. Crude enzyme sample was assayed as in Fig. 1 in the presence of the indicated levels of pyridoxal-5′-phosphate (PLP). Data were analyzed by a computer program designed by Osmundsen [8], using the nominal inflexional substrate concentration of 1.5 μM. The curve indicated is computed to result from the sum of the activities of two enzymes with apparent $K_m$ values of 22.8 and 0.18 μM pyridoxal-5′-phosphate and $V$ values of 57 and 5.0 nmol $CO_2$/h per mg protein, respectively.

phosphate concentrations, however, only show minor fluctuations in activity to be induced by cycloheximide [4]. The explanation for this unusual response is readily apparent through the type of experiment illustrated in Fig. 4, where the

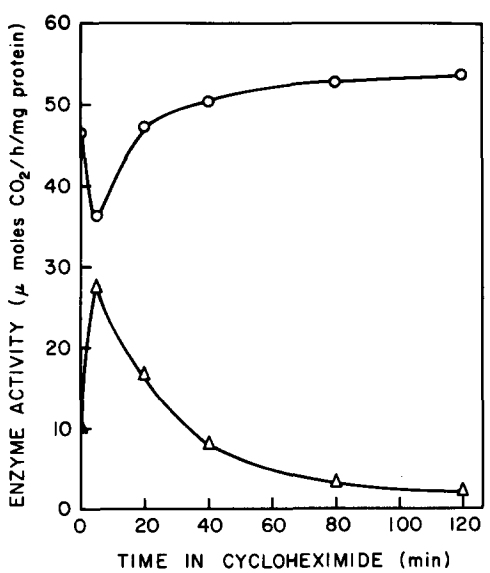


Fig. 4. Computed variations in $V$ of high and low $K_m$ forms of ornithine decarboxylase during cycloheximide treatment. Enzyme samples were extracted from microplasmodial cultures at the indicated times following addition of 25 μg/ml cycloheximide. Each sample was assayed, and data analyzed as described in Fig. 3. The computed $V$ values are plotted for the high (○) and low (△) $K_m$ enzymes.

amount of each ornithine decarboxylase form is individually monitored in response to cycloheximide addition. The A form of this enzyme increases rapidly to a peak 5—10 min after inhibitor addition, and subsequently decreases, while the B form closely mirrors these changes. As before, the sum of these two enzyme forms (activity measured in saturating pyridoxal-5′-phosphate concentrations) is found not to change sharply in response to this inhibitor.

Similar rapid variations in the relative amounts of the A and B enzyme forms have been found to occur during the progression through the *Physarum* mitotic cycle. In this instance the total of these forms exhibits a smooth, step-pattern doubling during the S period [9].

## Discussion

The observation that crude homogenates of *Physarum* contain two distinct forms of ornithine decarboxylase suggests the following possibilities: (1) these merely represent alternate conformational states of a common protein; (2) these are distinct proteins produced by two separate genes; or (3) these forms consist of a basic catalytic subunit which is modified by chemical alteration or the addition of a regulatory subunit. Consistent with the first possibility, several pyridoxal-5′-phosphate-requiring enzymes demonstrate a close correlation between coenzyme affinity and the degree of enzyme polymerization, suggesting that such structural changes may control enzyme function in vivo [10—12]. The variable size of this enzyme, depending on the nature and strength of the buffer, and coenzyme level, is quite suggestive of such a structural control mechanism. The constant kinetic difference observed between these forms in all polymeric conformations, however, diminishes the importance of this mechanism in the regulation of ornithine decarboxylase.

Although the second possibility has not been tested directly, we have been able to purify this enzyme 500-fold by ammonium sulfate fractionation, ion-exchange chromatography and molecular sieving, without a separation of these two forms. It is unlikely that isozymes produced by separate genes would be so similar in size and charge.

That these forms represent stable changes in a common enzyme protein (the third possibility) is strongly supported by our observations that changes in the $V$ of one form almost always correspond to equal and opposite changes in the $V$ of the other form while the total remains unchanged. This correlation is demonstrated in this paper, in response to cycloheximide, and elsewhere [9] during growth rate changes and progression through the synchronous mitotic cycle. Similar unpublished observations have been made in this laboratory with respect to the response to other inhibitors, and alterations in external cation and polyamine concentrations. Thus it appears that these forms are two distinct states, relatively active and less active, of a common enzyme protein. This is consistent with a model for the sensitive modulation of ornithine decarboxylase activity by the regulation of the reaction which interconverts these two forms.

Although conversion of the A to the B form of this enzyme would reduce the activity of this enzyme severely at physiological pyridoxal-5′-phosphate

levels, this kinetic change may be only a passive consequence of a change in structure which allows polymerization or storage of this enzyme, or which may be the first step in preparing the enzyme for subsequent destruction by proteases. In all cases, the critical control point of this enzyme appears to be in the regulation of the interconversion between these two forms.

Since the nature of the chemical difference between these forms has not yet been elucidated, many mechanisms of this regulation must be considered. Fong et al. [13,14], and Icekson and Kaye [15], for example, have presented evidence that ornithine decarboxylase activity is regulated by the physical association of a regulatory protein. As yet, we have not found any evidence for such an "ornithine decarboxylase-antizyme" in *Physarum*.

A second possibility exists in which the active and less active ornithine decarboxylase forms differ by a covalent modification. The activities of many enzymes are regulated by phosphorylation-dephosphorylation reactions catalyzed by protein kinases and phosphoprotein phosphatases whose activity is frequently controlled by cyclic nucleotides. Although ornithine decarboxylase has not as yet been shown to be a phosphoprotein, recent reports [16—18] of the regulation of this enzyme by cyclic nucleotides suggests that this possible regulatory mechanism should be thoroughly investigated.

The mechanism of control of ornithine decarboxylase activity in *Physarum*, by the alteration of the equilibrium between active and inactive enzyme forms, enables the synthesis of the polyamines to be regulated with great speed and sensitivity. Since the level of active ornithine decarboxylase is also found to change rapidly in mammalian tissues, it may be likely that a similar mechanism is involved in the regulation of this enzyme in these evolutionarily more advanced cells. Rapid variations in mammalian ornithine decarboxylase, however, appear to be independent of coenzyme concentration, suggesting that these cells contain only one enzyme form. This conclusion is supported by immunochemical evidence indicating that there is only one immunoreactive ornithine decarboxylase enzyme protein, which varies in concentration in concert with observed changes in enzyme activity [19,20]. On the contrary, two separable ornithine decarboxylase forms have been shown in rat liver [21], 3T3 [22], and CHO cells (Anderson, S.N. and Mitchell, J.L.A., in preparation). In the last two reports, the difference between the forms has been shown, as in *Physarum*, to be in the ability of the enzymes to be activated by pyridoxal-5′-phosphate. At present, however, insufficient amounts of the less active (B) form of this enzyme have been found in these mammalian systems to account for the active enzyme not observed during periods of decreased ornithine decarboxylase activity. This suggests that either this less active enzyme form in mammalian cells is extremely unstable (perhaps an intermediate step in the degradation of this enzyme), or else this form is destroyed or not extracted during the normal preparation of enzyme fractions for immunological and catalytic assays. We feel the latter possibility to be most likely, through analogy with the *Physarum* system, and in view of the clear demonstration of the critical role of enzyme preparation techniques in the visualization of qualitative and quantitative differences in multiple enzyme forms [23]. In either case the interrelationship between these two forms of ornithine decarboxylase appears to be important in the regulation of this enzyme and deserves further study.

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