Developmental Regulation of Pyrimidine Biosynthesis

in *Dictyostelium discoideum*

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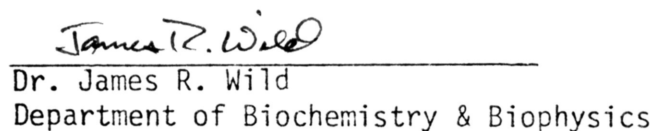

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ABSTRACT

The research reported in this thesis demonstrates that the initial steps for pyrimidine biosynthesis in the cellular slime mold *Dictyostelium discoideum* are catalyzed by the enzymes carbamoyl phosphate synthetase (CPSase, EC 2.7.2.9) and aspartate transcarbamoylase (ATCase, EC 2.1.3.1). Furthermore, it has been observed that the crude cell-free extract from *Dictyostelium discoideum* possesses a non-dialyzable inhibitory component of CPSase. It was striking to note that this inhibitory component interfered with CPSase activity from *Escherichia coli* as well.

ACKNOWLEDGEMENTS

I would like to express my gratitude toward the graduate lab staff. Their support and cooperation made my Undergraduate Fellows Program experience not only rewarding but enjoyable. I would especially like to thank Melinda Wales for sharing with me her skills and knowledge of Dictyostelium. And, of course, Dr. James Wild, without whom this study would not have been possible. (Thank you Jim for your guidance, understanding, and most importantly your friendship.)

INTRODUCTION[1]

Dictyostelium discoideum (Acrasieae) is a cellular slime mold, usually haploid (N=7), which elegantly lends itself to the study of eukaryotic regulatory behavior. The unique life cycle of *D. discoideum* involves three distinct types of cells and provides a simple model system for developmental studies (figure 1). The free living *amoebae* differentiate into *stalk cells* and *spore cells*, the intermediates have been termed pre-stalk cells and pre-spore cells. Actively growing cells are found in the form of uninuclear amoebae which feed on bacteria by phagocytosis and divide by mitotic cleavage. Upon nutritional starvation the amoebae will cease to grow and divide; they will aggregate to form a pseudoplasmodium ("grex") and undergo morphogenic and biochemical differentiation.

At all of these stages the slime mold is capable of thermotactic, phototactic, and chemotactic responses. The cellular attraction leading to aggregation is due to a positive chemotactic response to cAMP in nutritionally deprived amoebae. A period of migration follows the physical aggregation of 10^4 to 10^5 amoebae. During this time cellular differentiation occurs based on their position within the pseudoplasmodium. The anterior cells, those which first arrive at the aggregation center, develop into stalk cells. The posterior cells develop into spores which ascend the developing the developing stalk to form a spherical sorus or cap at the top of

[1]The format followed in this text is that of the Journal of Bacteriology, a publication of the American Society for Microbiology.

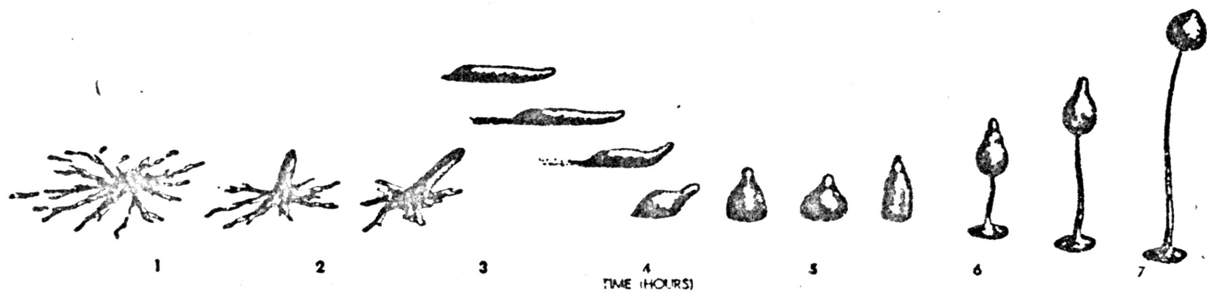


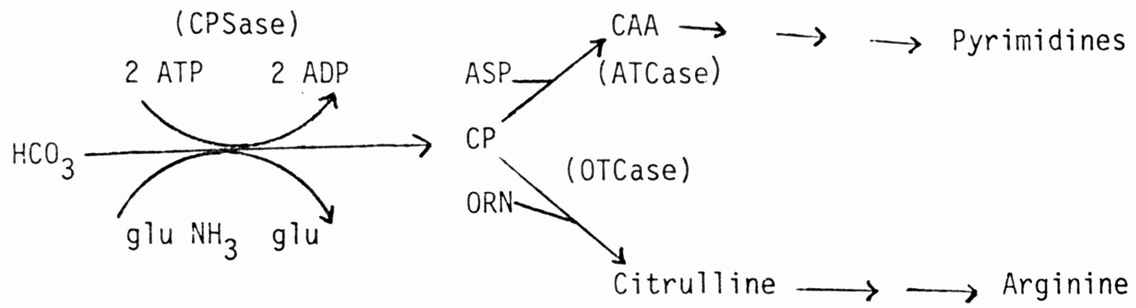
Figure 1. Life cycles of Dictyostelium discoideum.

a slender stalk. Mature stalk cells are no longer viable as they have differentiated into cellulose-ensheathed cells which have sloughed chromosomes in the process of reductive aneuploid segregation. However, the spores are genetically stable and can withstand unfavorable environmental conditions. Germination of the spores can be initiated in adequate nutritional environments and results in the release of small normal haploid amoebae.

D. discoideum can be grown on a variety of bacteria, on complex media, and on a totally defined medium (Franke and Kessin, 1977). This defined medium requires most of the amino acids, but does not require purines or pyrimidines. It appears, therefore, that nucleotides can be provided to the cell for nucleic acid synthesis by one of three routes: *de novo* synthesis, reutilization of salvaged nucleotides from RNA degradation, and/or from exogenous sources. The relative contributions of these has not yet been determined. *De novo* biosynthesis of pyrimidines appears to proceed in a manner similar to bacteria (O'Donovan and Neuhard, 1970) and other eukaryotic systems (Makoff and Radford, 1978). The role of *de novo* biosynthesis of purines and pyrimidines in *D. discoideum* is not known.

There are two proposed schemes for pyrimidine biosynthesis in *D. discoideum* (figure 2). Aspartate transcarbamoylase (ATCase, EC 2.1.3.2) and carbamoylphosphate synthetase (CPSase, EC 2.7.2.9) often provide the first two steps of pyrimidine biosynthesis. ATCase is often considered to be the first unique step in pyrimidine

A. Capable of De Novo Arginine Biosynthesis



B. Absolute Arginine Requirement

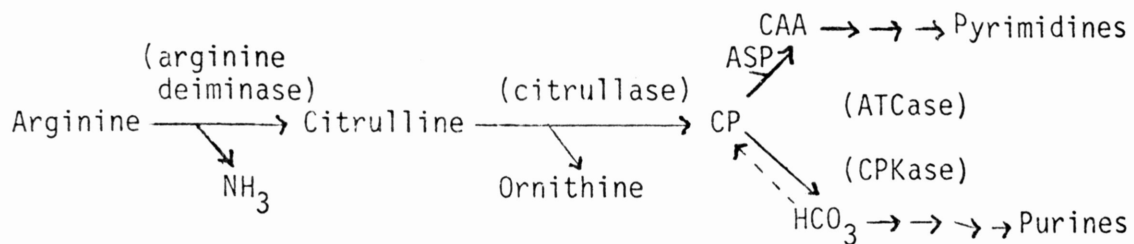


Figure 2. Schemes for Pyrimidine Biosynthesis.

biosynthesis, since carbamoyl phosphate is a common intermediate for pyrimidine and arginine biosynthesis. *D. discoideum* has an absolute requirement for arginine. It is relevant, therefore, to determine the presence of CPSase and other enzymes necessary for either of these pathways in *D. discoideum*.

MATERIALS AND METHODS

Culture of amoebae. The axenic strain AX2 of *Dictyostelium discoideum* was used in this study. Spores were isolated following development of *Escherischia coli* B/r and frozen. Spore germination was induced by incubating at 45°C for thirty minutes and inoculated into fresh axenic medium containing glucose, bacteriological peptone, and yeast extract from OXOID (ASOX, as described by Watts and Ashworth, 1970). Cultures growing in axenic broth were grown in an incubator at 22°C, shaking constantly to prevent development. Cultures were transferred every three days into fresh axenic medium to maintain proper cell concentration. Cells were inoculated at 5×10^5 cells/ml. Cell doubling time was approximately 8-9 hours. Amoebae were most active and viable from $1 - 10 \times 10^6$ cells/ml. Cells used for experimental purposes were taken from these conditions. Stock cultures were returned to growth on bacteria every two weeks, new spores picked from fruiting bodies and started back as ASOX medium. All inoculations were made in a laminar-flow transfer hood set up for *D. discoideum* manipulations in order to maintain sterile conditions and prevent contamination. Cell concentration was determined by use of a hemocytometer.

Preparation of cell-free enzyme extract. Amoebae were harvested directly from growth medium. The cells were then washed in buffer (.04mM KH_2Po_4, .02mM ZnAc)(pH 7.0) to prevent osmotic shock or lysis. The cells were then suspended in the phosphate buffer and 1mM β-mercaptoethanol. The cells were disrupted by sonication and

clarified by centrifugation for 90 minutes at 40,000xg.

Enzyme assay: carbamoyl phosphate synthetase (CPSase, EC 2.7.2.5). CPSase activity was assayed as described by Kempe, et al., (1976). (^{14}C-)$NaHCO_3$ was converted to carbamoyl phosphate by CPSase. Carbamoyl phosphate, being unstable, was then converted to hydroxyl urea by the addition of hydroxylamine (figure 3). The reaction was allowed to run for 30 minutes with 20,000cpm to 60,000cpm utilized in each sample as determined by a standard.

Column Chromatography. Prepared cell-free extract (5 mls) was chromatographed over a 74cm. Sephadex G-200 (separating ability: 300,000g/mole) column, with a flow rate of 18.9 ml/hr. The column was equilibrated with a standard phosphate buffer (.04mM KH_2PO_4, .02mM ZnAct) (pH 7.0). Fractions were collected in approximately 5 ml volumes (90 drops). Blue dextran was used to determine void volume (V_o).

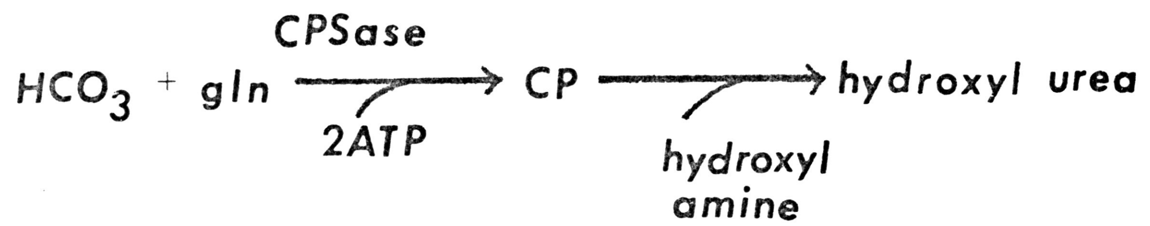


Figure 3. Assay reaction: CPSase.

RESULTS

Detection of CPSase activity in Dictyostelium discoideum. CPSase catalyzes the transamination of carbamoyl phosphate from bicarbonate, with either glutamine or ammonium chloride providing the amide moiety. It was not possible to obtain a linear time progression from *D. discoideum* amoebae when assaying for CPSase activity. It appeared that *D. discoideum*, being eukaryotic, had a variety of enzymes, inhibitors, and/or proteases, which could interfere with enzymatic activity and thus explain the assay results. Therefore, *D. discoideum* crude extract was chromatographed and the resulting fractions were assayed for enzymatic activity.

The chromatographic profile of CPSase revealed the presence of three distinct peaks (figure 4). These peaks were located in fractions numbered 30, 35, and 42. The same fractions were assayed for ATCase activity. ATCase was observed as a single peak in fraction 42 (figure 4). Standards of known molecular weights (ribonuclease - 13,700 daltons; chymotrypsin - 25,000 daltons; albumin - 67,000 dlatons; aldolase - 158,000 daltons; ferritin - 440,000 daltons) were used to calibrate the column (figure 5). Molecular weights of CPSase and ATCase were approximated by determining K_{av}[2]. The ATCase peak corresponded to a molecular weight of 122,500 and the three CPSase peaks corresponded to molecular weights of 122,500, 240,000, and over 400,000 daltons (figure 6).

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[2] $K_{av} = \frac{Ve-Vo}{Vt-Vo}$ is the partition coefficient between the liquid phase and gel phase; where (Ve) is the elution volume, (Vo) is the void volume, and (Vt) is the total volume of the gel bed.

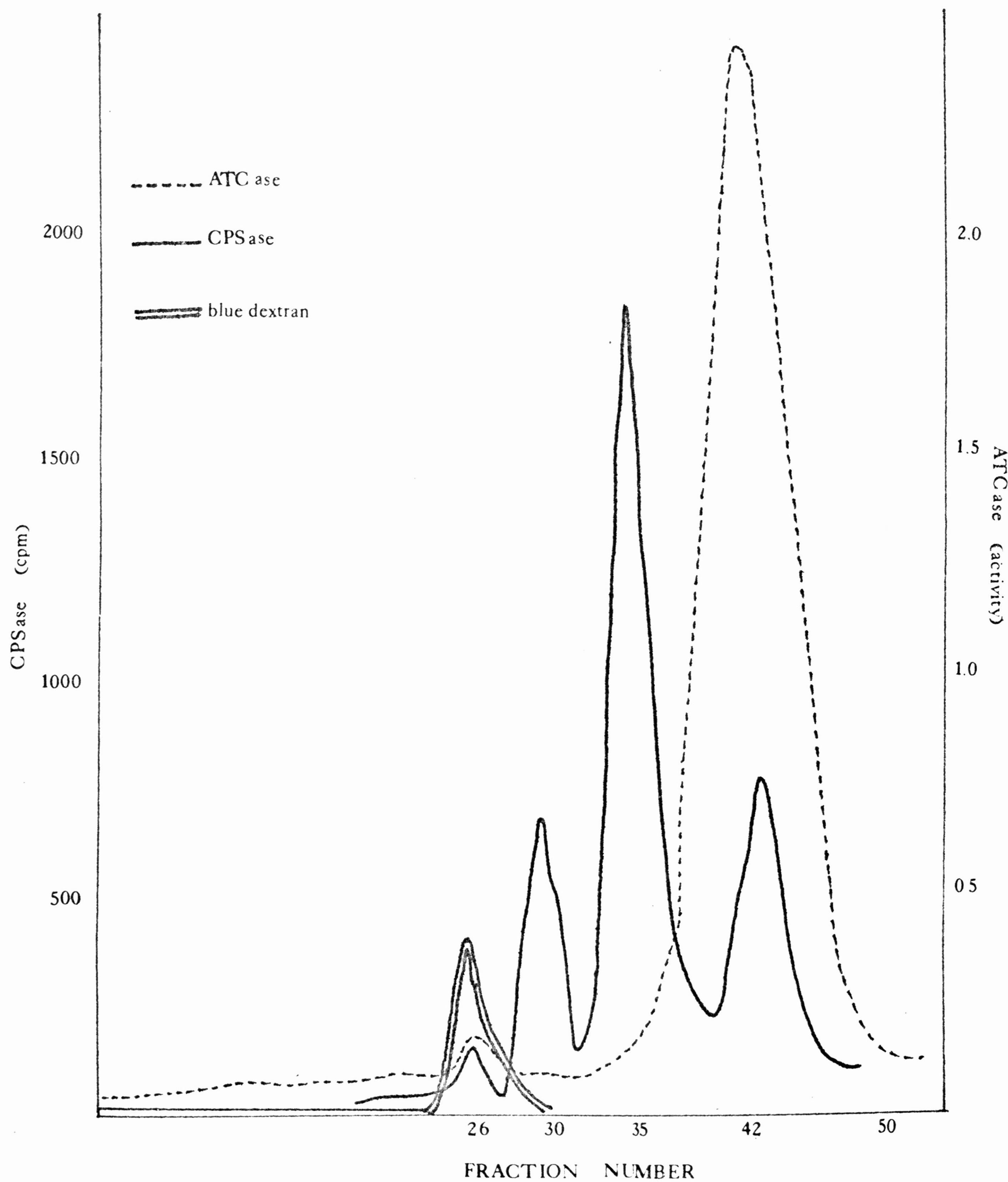

Figure 4. Assay results: Fraction Number vs. CPM (CPSase and ATCase).

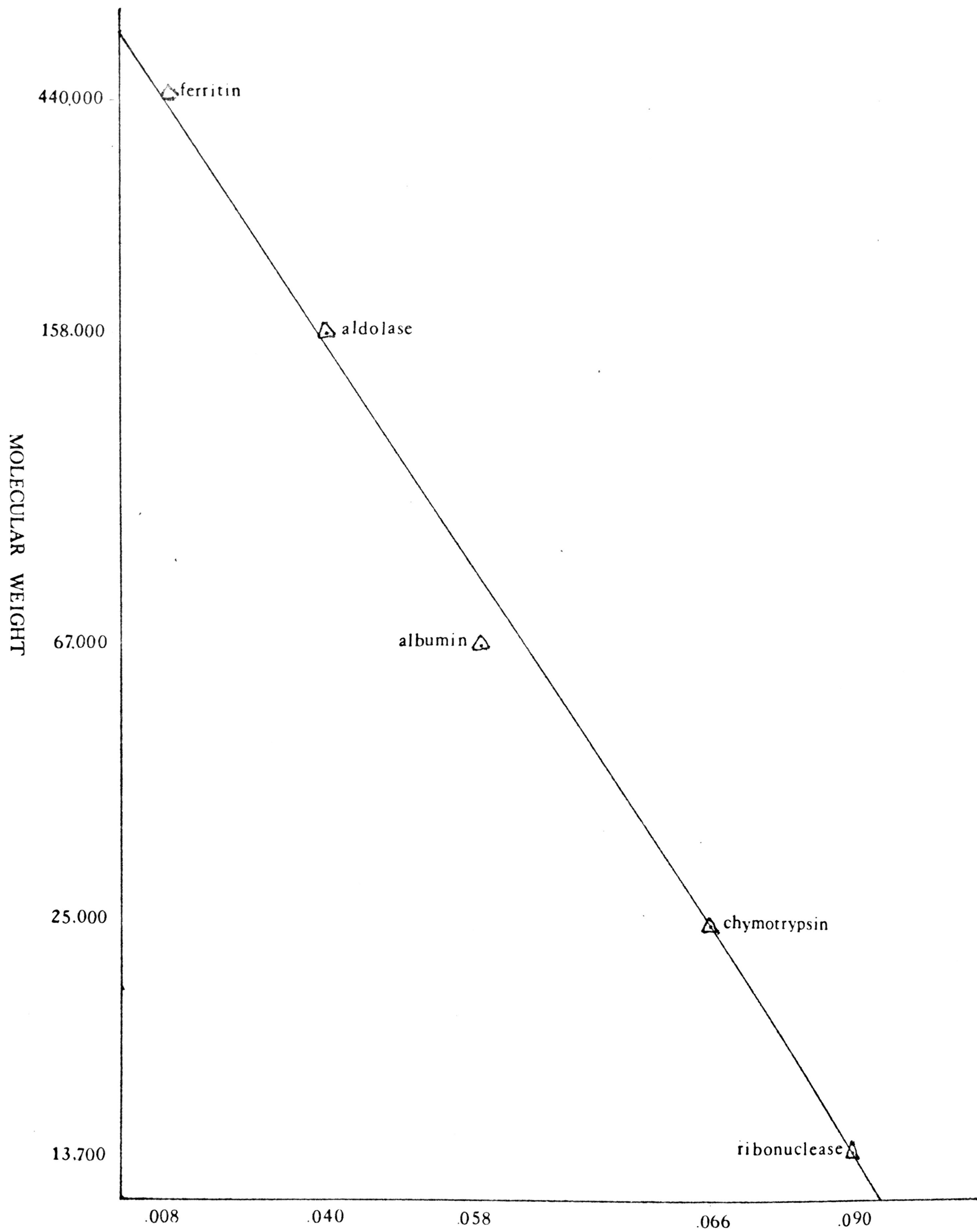

Figure 5. Protein standards.

CPSase

	Ve/Vo	Kav	MW
1	1.62	.032	122,000
2	1.35	018	240,000
3	1.15	.008	400,000

ATCase

Ve/Vo	Kav	MW
1.62	.032	122,000

Figure 6. Molecular weights of ATCase and CPSase.

Arguments for the presence of an inhibitor of CPSase in crude extracts of Dictyostelium discoideum.

1. The absence of CPSase activity in D. discoideum crude extract. CPSase activity could not be found in *D. discoideum* crude extract. However, when the extract was chromatographed, even after a ten-fold dilution over the column, CPSase activity was found (as reported earlier).

2. The inhibition of Escherichia coli CPSase by crude extract from Dictyostelium discoideum. A time course assay for CPSase in *E. coli* (using 50μl of cell-free extract per tube) demonstrated a linear time dependence (figure 7). In contrast, CPSase activity in *D. discoideum* could not be found (50μl of cell-free extract per tube). When the same cell-free extract of *E. coli* was assayed for CPSase activity in the presence of *D. discoideum* (50μl *D. discoideum* cell-free extract per tube + 50μl *E. coli* cell-free extract per tube) CPSase activity decreased significantly. This unexpected result indicated that some competing molecule(s) was present in the *D. discoideum* extract. Since the volume of *E. coli* extract was the same in both cases (+/- *D. discoideum* extract) the diminution clearly suggests the presence of an inhibitor. The *D. discoideum* extract was exhaustively dialyzed to remove samll molecules (retaining

only molecules greater than 16,000 daltons). Thus the presumed inhibitor would be larger than that size.

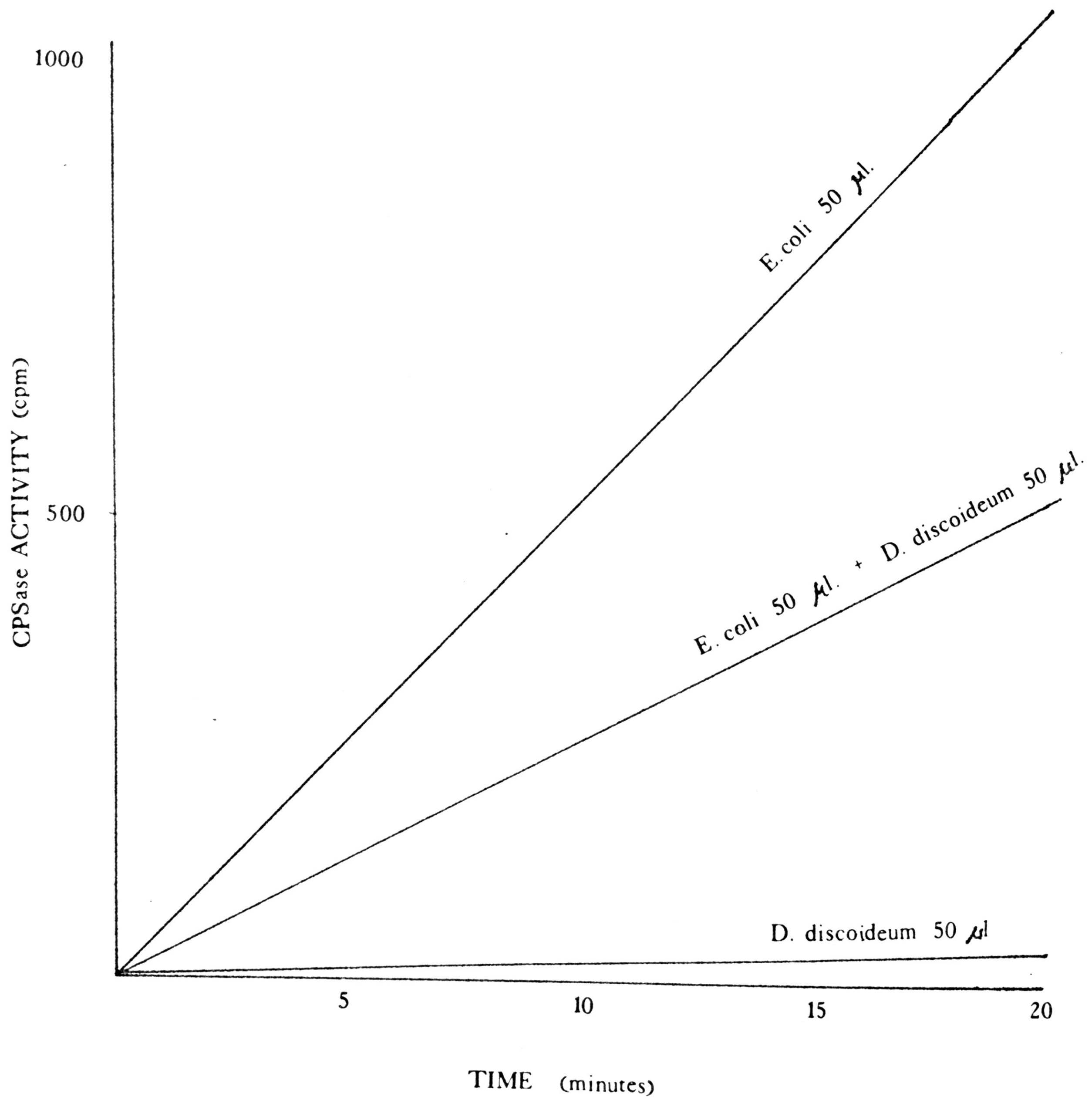

Figure 7. Inhibition of CPSase in E. coli.

DISCUSSION

The goals of this research are to determine the biosynthetic pathway of pyrimidine biosynthesis in the amoeba of *Dictyostelium discoideum*, and to identify any reorientation which may occur during development. The initial work sought evidence for the presence of arginine deiminase, the enzyme responsible for the conversion of arginine to citrulline producing carbamoyl phosphate for pyrimidine biosynthesis (figure 2). It was not possible to demonstrate arginine deiminase activity as the assay proved to be unstable in crude cell extract. (The assay procedure as described by E. L. Oginsky, 1955.) Perhaps the enzyme was not even present. (The assay was reproducible with extract derived similarly from *Streptococcus faecaelis*).

The initial stages of pyrimidine biosynthesis vary depending upon the organization of arginine metabolism in various cells (figure 2). In those organisms which are capable of *de novo* biosynthesis of arginine and pyrimidines, pyrimidine biosynthesis is initiated through the transamination of bicarbonate by glutamine (Anderson & Meister, 1965). In many cells, a single carbamoyl phosphate synthetase produces a common pool of carbamoyl phosphate for both arginine and pyrimidine biosynthesis (e.g., enteric bacteria and higher plants) (Pierard, A. et al., 1965; O'Donovan and Neuhard, 1970; Makoff and Radford, 1978; O'Neal and Naylor, 1976). In other systems (most vertebrates, invertebrates, fungi, including yeast, and *Bacillus subtilis*) (Potvin and Gooder, 1975; Lacroute et al., 1965; Makoff and Radford, 1978) there are two carbamoyl phosphate

synthetases, producing distinct pools of carbamoyl phosphate for arginine and pyrimidine biosynthesis. In organisms which have an absolute requirement for arginine, carbamoyl phosphate is provided through the arginine dihydrolase system (Oginsky, 1955). This system may be the only source for carbamoyl phosphate (Hutson and Downing, 1968; Schich and Reddy, 1973; Kidder et al., 1976) or it may be induced under anaerobic conditions in lieu of CPSase (Broman et al., 1975; Stanier et al., 1966).

It has been possible to demonstrate the presence of three separate molecular weight forms of CPSase after separation on Sephadex G-200. These molecular weight forms could simply indicate enzyme aggregation or, perhaps, the presence of more than one structural gene product in *D. discoideum*. In other organisms which display distinct CPSases for both arginine and pyrimidine biosynthesis, it is common to find one CPSase associated with pyrimidine biosynthesis and ATCase. The other is usually mitochondrial and involved with arginine biosynthesis. The presence of overlapping peaks of ATCase and CPSase activity could indicate a multimeric association, or the two activities might reside in a single multifunctional polypeptide.

An interesting sideline to this latter possibility involves some additional work done by Melinda Wales. During development the pyrimidine biosynthetic scheme is reoriented by the loss of ATCase following nutritional starvation. If CPSase in *D. discoideum* is aggregated to, or a part of a multifunctional protein with ATCase,

it too would be expected to disappear during development.

The presence of a non-dialyzable CPSase inhibitor in *D. discoideum* has been suggested by the inhibition of CPSase in *E. coli* and the appearance of activity following chromatographic separation The presence of a large molecular weight CPSase inhibitory component has been demonstrated in the protozoan parasite, *Toxoplasma gondii* (O'Sullivan, W., et al., 1978).

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