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Aspartate Transcarbamylase from *Leishmania donovani*

A DISCRETE, NONREGULATORY ENZYME AS A POTENTIAL CHEMOTHERAPEUTIC SITE*

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***Leishmania donovani* is a protozoal pathogen that belongs to the kinetoplastida order. Unlike in other eucaryotic systems, the first three enzymes of the *de novo* pyrimidine biosynthetic pathway are not components of a multifunctional protein system. The three enzyme activities in the crude extract were separated on a Sephacryl S-200 column.**

Aspartate carbamoyltransferase (EC 2.1.3.2) has been purified to apparent homogeniety. The enzyme has an approximate molecular weight of 135,000 and seems to be a tetramer of equivalent subunits of molecular weight 35,000. The enzyme shows strictly hyperbolic kinetics with both the substrates under a variety of conditions and is not inhibited by nucleotide phosphates. K_m for carbamyl phosphate is 3.1×10^{-4} M and for aspartate is 7.6×10^{-3} M. Apparently, the enzyme has no regulatory role in pyrimidine biosynthesis.

***N*-(Phosphonoacetyl)-L-aspartic acid is a powerful competitive inhibitor ($K_i = 5 \times 10^{-7}$ M) for this enzyme with carbamyl phosphate as substrate. This inhibitor completely inhibits the growth of the vector form of organism at 60 μM and significantly affects the growth of the pathogenic form in a macrophage assay system. The potency of the inhibitor is comparable with allopurinol which is undergoing human clinical trial as an antileishmanial drug.**

The enzymatic and genomic organization of pyrimidine biosynthetic pathway in eucaryotes presents some unusual features of considerable interest for comparative biochemistry and molecular biology (1). The six enzyme activities required for the *de novo* biosynthesis of the pyrimidine ring are discrete proteins in procaryotic systems. In contrast, in higher eucaryotes, some of these enzyme activities are expressed in a single polypeptide chain. In mammals, as well as in *Drosophila*, the first three enzymes of the pathway, namely carbamoyl-phosphate synthetase (ammonia) (EC 6.3.4.16), aspartate carbamoyltransferase (EC 2.1.3.2), and dihydroorotase (EC 3.5.2.3) are encoded by a single structural gene that produced a single polypeptide of trimeric quarternary structure with all three enzymatic activities (1-3). The yeast and the *Neurospora* also express a multienzymic protein, but this has activity for only the first two enzymes of the pathway (4). Dihydroorotase is a discreet enzyme for these organisms. The situation in other types of lower eucaryotes that include the unicellular protozoal systems remains essentially unexplored.

Leishmania donovani is an important member of the kinetoplastida group of protozoal parasites. The organism has a digenic life cycle; a flagellated promastigote form in the sandfly vector and a nonflagellated amastigote or pathogenic form in macrophage systems of hosts. The organism is the causative agent for kala azar, a lethal form of visceral leishmaniasis, that is widely prevalent in many parts of the tropical world (5, 6). The kinetoplastida, including *L. donovani*, are unable to synthesize purine *de novo* and depend for their purine requirement on preformed purines of the host and its own salvage pathway (7, 8). In contrast, the kinetoplastida appears to have the enzymatic machinery necessary to synthesize pyrimidine *de novo*. All six enzymes of the pyrimidine pathway have been detected in the promastigote form of *L. mexicana*, in the trypromastigote form of *Trypanosoma brucei* and in the culture form and amastigote form of *T. cruzi* (9-11). Isotopic studies with *L. donovani* promastigotes indicate the presence of both the *de novo* and the salvage pathway in this organism (12). However, apart from these initial works, very few systematic studies have been reported on the characterization of the individual enzymes of the pyrimidine pathway in kinetoplastida. Important questions of possible existence of multifunctional single polypeptide enzyme systems, enzymatic regulatory sites, and potential chemotherapeutic sites etc. all remain unexplored at the moment. In this paper, we report that, in the cultural or the promastigote form of *Leishmania donovani*, the first three enzymes of the pyrimidine biosynthetic pathway remain as three discrete proteins with separable enzyme activities. Further, extensive purification and characterization of aspartate transcarbamylase show that unlike in many bacterial systems, this enzyme does not have any obvious regulatory role in this biosynthetic pathway. Finally, *N*-(phosphonoacetyl)-L-aspartic acid, a transition state analogue for this enzymatic reaction, has a powerful growth-inhibitory effect on both the promastigote form and on the model screening system for the pathogenic amastigote form. This inhibition by PALA[1] is fairly comparable with allopurinol that is undergoing clinical trial for visceral leishmaniasis at present (13).

MATERIALS AND METHODS AND RESULTS[2]

Separation of Carbamyl Phosphate Synthetase, Aspartate Transcarbamylase and Dihydroorotase Activities—In crude

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[1] The abbreviations used are: PALA, *N*-(phosphonacetyl)-L-aspartic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; ATCase, aspartate transcarbamylase; DHOase, dihydrorotase.

[2] Portions of this paper (including "Materials and Methods," part of "Results," Figs. S1-S6, and additional references) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

extracts of *L. donovani*, carbamyl phosphate synthetase activity was found to be very unstable. The activity could be stabilized for subsequent operations only in the presence of glycerol and dimethyl sulfoxide. In a typical experiment, washed pelleted cell (3 g) was suspended in 3.0 ml of glass-distilled water containing 1 mM DTT, 5 mM benzamidine, 0.1 mM PMSF, 0.75 mg of soybean trypsin inhibitor and allowed to swell for 15 min. 1.75 ml of dimethyl sulfoxide/glycerol mixture (6:1) was added to the swelled cells and homogenized in a Potter-Elvehjem homogenizer. The extract was adjusted to 50 mM Tris-HCl buffer, pH 7.2, and centrifuged at 18,000 × *g* for 30 min. One ml of the supernatant was then applied on a Sephacryl S-200 column (57 × 1.25 cm), previously equilibrated with 50 mM Tris-HCl buffer, pH 7.5, 1 mM DTT, 30% dimethyl sulfoxide, and 5% glycerol. The column was eluted also with 50 mM Tris-HCl buffer containing 1 mM DTT, 30% dimethyl sulfoxide, and 5% glycerol at a flow rate of 6 ml/h.

The elution profile is shown in Fig. 1. It is evident that the three enzymatic activities were separated as distinct activity peaks on this column. In a separate experiment, using a partially purified enzyme fraction, we could demonstrate the separation of aspartate transcarbamylase and dihydroorotase activities on a Sephadex G-100 column (see Miniprint Section). In this case, however, we could not detect any activity for carbamyl phosphate synthetase which was presumably lost during processing.

Purification and Characterization of Aspartate Transcarbamylase—Results of a typical purification procedure are summarized in Table I. The approximate molecular weight of the purified homogenous protein was calculated to be 135,000, and analysis under denaturing conditions showed it to consist of a single subunit of molecular weight 35,000. The enzyme failed to show any sigmoidal kinetics under a variety of conditions and was not significantly inhibited or activated by a large number of related metabolites. PALA was found to be a strong inhibitor of pure aspartate transcarbamylase with a K_i of 0.5 μM. Experimental details of these results are presented in the Miniprint.

Effect of PALA on Growth of L. donovani Promastigotes—

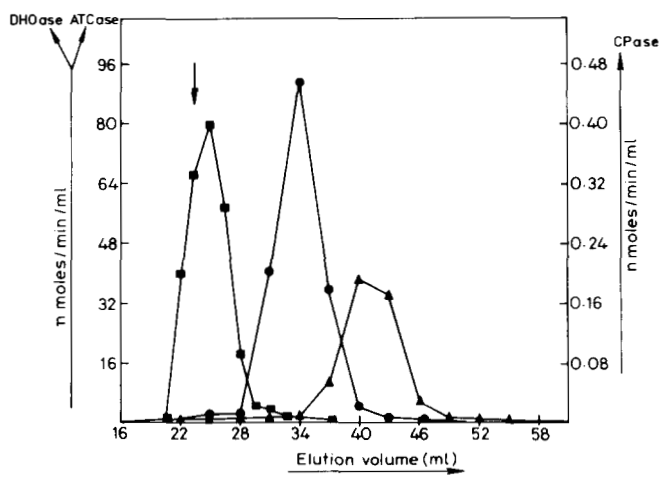

FIG. 1. **Profile of carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase activities, eluted from Sephacryl S-200 column.** The crude extract containing 12 mg of protein in 1 ml of crude extract was applied to a column (57 × 1.25 cm), previously equilibrated with 50 mM Tris-HCl buffer, pH 7.5, 1 mM DTT, 30% dimethyl sulfoxide, 5% glycerol. Elution rate was 6 ml/h and 1.5-ml fractions were collected. Carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase activity are represented by ■——■, ●——●, and ▲——▲, respectively. The void volume is indicated.

TABLE I

Purification of ATCase from L. donovani promastigotes

Step	Total protein	Total activity	Specific activity	Purification
	mg	*units*	*units/mg*	*-fold*
(i) Crude	451	15.9	0.035	1.0
(ii) Protamine sulfate	276	15.2	0.055	1.6
(iii) First ammonium sulfate step	82	14.7	0.179	5.1
(iv) Heat treatment	25.5	11.7	0.458	13.0
(v) Second ammonium sulfate step	14.4	10.1	0.701	20.0
(vi) Sephadex G-100 column	2.6	5.1	1.96	56.0
(vii) DEAE-cellulose column	0.12	1.1	9.16	261.0

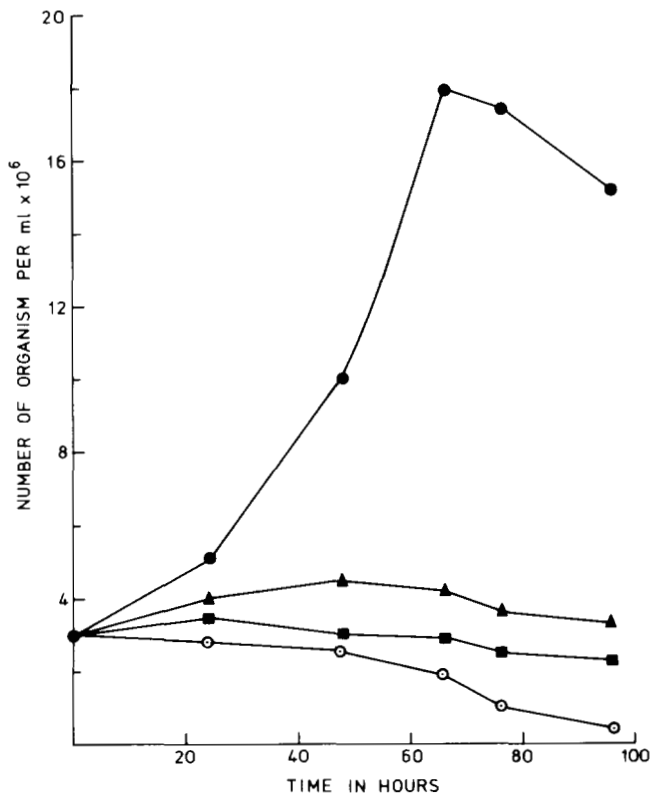

FIG. 2. **Inhibition of growth of *L. donovani* in the presence of PALA.** The control growth curve in the absence of PALA is represented by ●——●. ▲——▲, ■——■, and ⊙——⊙ indicate growth pattern in the presence of 40, 60, and 150 μM PALA, respectively.

PALA has been found to be a potent inhibitor for growth of several mammalian cell lines including transformed cells (14, 15). PALA either alone or in combination with other anti-cancer drugs is undergoing screening as a possible anti-tumor agent (16, 17). In view of the strong inhibition of PALA on *L. donovani* aspartate transcarbamylase, its possible inhibitory effect on the growth of *L. donovani* promastigotes was checked. Fig. 2 shows that PALA is a fairly powerful inhibitor of growth for the organism, and the minimum inhibitory concentration was calculated to be approximately 60 μM. The 50% effective dose is less than 20 μM (data not shown) and is quite comparable in its effect with allopurinol and other pyrazolopyrimidine analogues that are being developed as possible chemotherapeutic agents against the pathogenic kinetoplastida (13, 18). We could further demonstrate that addition of uracil or uridine (100 μM) in the growth medium

TABLE II

Effect of PALA and pentamidine on the multiplication of L. donovani amastigotes in hamster peritoneal macrophages in vitro

Experiment	Amastigotes/macrophage (mean ± S.D.)	Inhibition of amastigotes count/cell
		%
At 0 day	2.08 ± 0.14	
At 3rd day		
Infected control (untreated)	5.01 ± 0.22	
Pentamidine	2.46 ± 0.13[a]	51
PALA (25 μM)	3.80 ± 0.32[a]	24
PALA (50 μM)	3.70 ± 0.15[a]	26
At 6th day		
Infected control (untreated)	6.86 ± 0.10	
Pentamidine	1.70 ± 0.20[a]	75
PALA (25 μM)	3.43 ± 0.18[a]	50
PALA (50 μM)	3.36 ± 0.04[a]	49

[a] $p < 0.001$ ($N = 4$).

could substantially protect the organism (nearly 50%) against inhibition by PALA (data not shown).

Effect of PALA on the Growth of the Pathogenic Form of L. donovani—PALA was found to have a definite inhibitory effect on the multiplication of the pathogenic or amastigote form, in the *in vitro* macrophage assay system. This is evident from Table II. PALA (30 μM) could significantly retard the growth of *Leishmania* amastigotes when assayed in the hamster peritoneal macrophage system. The extent of inhibition observed with pentamidine which was used as the control drug agrees well with the recently reported value for this drug in the same assay system (19). Increasing the concentration of PALA to 50 μM did not result in further inhibition of growth.

DISCUSSION

Resolution of carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase activities on a Sephacryl S-200 column (Fig. 1) and of the latter two activities on Sephadex G-100 column (Fig. S1, Miniprint) clearly show that in *L. donovani* the three enzymes are not components of a multifunctional protein system. Partial separation of the three activities had earlier been achieved by density gradient technique in *Toxoplasma glondi* (11), a parasitic protozoa belonging to a different family. Apparently, in all protozoal systems, the three enzymes are expressed as three distinct proteins, and gene fusion for these activities may have started at the level of yeast and *Neurospora* (1). Interestingly, a multifunctional protein system has recently been detected in *Leishmania tropica* (20, 21). In this case, an overproduction of a bifunctional thymidylate synthetase-dihydrofolate reductase protein takes place because of gene amplification when the organism is gradually exposed to higher concentrations of methotrexate.

Leishmania aspartate transcarbamylase is probably a tetramer of four identical subunits (Figs. S1*B* and S2, Miniprint). The enzyme failed to show any regulatory property or cooperative kinetic phenomenon under a variety of conditions. Apparently, the flux of pyrimidine biosynthetic pathway is regulated at some other enzymatic step of this pathway. The quarternary structure of the enzyme, absence of a second subunit, and general lack of sensitivity to nucleotides suggest some resemblance with the enzymes from *Streptococcus faecalis* or *Bacillus subtilis* (22).

The moderately strong growth inhibitory property of PALA for both the vector (Fig. 2) and the host pathogenic form (Table II) may be of some chemotherapeutic value. Considering the extremely high affinity of PALA for leishmanial aspartate transcarbamylase, the extent of inhibition or the concentration needed for complete inhibition of growth is not remarkable. Inefficient uptake of PALA to build up an effective cellular concentration may be a possible cause for this discrepancy. In any case, when compared to allopurinol (18, 23) which is already undergoing clinical trial with some success (24), the concentration of PALA to get the desired effects is fairly encouraging. In view of these results, the possibility of combination therapy where the purine salvage pathway and the *de novo* pyrimidine pathway are simultaneously inhibited should be explored. This is particularly relevant in the context of increasing reports of resistance to the treatment of pentavalent antimonials in the case of kala azar (24).

Our present study with aspartate transcarbamylase from *L. donovani* indicates that sustained and intensive work on the enzymes of this pathway will be useful in several directions. PALA can possibly be exploited as a probe for studying the phenomenon of gene amplification and drug resistance as it is being done in mammalian systems (25). Its potential as a chemotherapeutic agent, either alone or in combination, after entrapment in liposomes or in suitable carriers should be further explored. In experimental models of leishmaniasis, passive targeting to liver macrophages of pentavalent antimonials encapsulated in liposomes has already shown considerable promise (26). Extensive knowledge on the regulatory and other properties of the *de novo* pyrimidine pathway is expected to contribute significantly to the comparative biochemistry of protozoal systems and to development of new avenues for chemotherapy.

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Additional references are found below.

ASPARTATE TRANSCARBAMYLASE FROM LEISHMANIA DONOVANI: A DISCRETE, NON-REGULATORY ENZYME AS A POTENTIAL CHEMOTHERAPEUTIC SITE.

BY

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MATERIALS AND METHODS

All the biochemicals, unless otherwise mentioned were purchased from Sigma Chemical Co. U.S. Pentamidine was a gift from Research Division of May and Baker Co. PALA was obtained as a generous gift from Dr. G. R. Stark, then at Department of Biochemistry, Stanford University. [^{14}C]-NaHCO$_3$ (50 mci/mmol) was obtained from New England Nuclear.

The organism used for this work was Leishmania donovani UR-6, a strain obtained from Dr. D. K. Ghosh of this Institute. The organism was grown at 22°C on a solid blood-agar medium that has been described in detail recently (1). The cells were maintained by subculture made at intervals of 72 hours. For growth experiments and for inhibition studies on the promastigote form, a semisynthetic medium recently developed by Chowdhuri et al. (2) and partially modified by us (1) was used. Cells were counted in a Neubauer haemocytometer. 1 x 10^6 cells as counted by this method contained 0.8 mg of cell protein, when determined by biurate method. Only fully motile cell preparations were used for growth inhibition experiments. For some experiments, where suspended promastigote cells were needed, cells grown for 66 hours on solid medium were scraped into phosphate saline buffer pH 7.2 (1), washed by centrifugation and resuspended in the same buffer.

Amastigote or the host form of Leishmania donovani (strain AG-83) was maintained in golden hamster (Mesocricetus auretus). Amastigotes were isolated from infected spleen essentially by the method of Hart et al. (3) as modified by Looker et al. (4). Parasitic burdens of spleen were assessed from stained impression smears (5). The effect of PALA on the amastigote was examined by following the growth of parasites in an in vitro assay system based on hamster peritoneal macrophage culture following the method developed by Chang and Dweyer (6). The macrophages were cultured on coverslips in 4 ml RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum, L-glutamine (4 mM), Hepes (25 mM), Streptomycin (100 µg/ml) and penicillin (100 U/ml) for 48 hours at 37°C. The macrophage culture were then inoculated with amastigotes at a ratio of 5:1 and after inoculation for 3 hours, the non-phagocytized parasites were removed by washing with medium. Fresh medium was added along with drugs. After 3 days of incubation, medium including drug was replenished. At three and six days after treatment, the number of amastigotes per cell was determined according to the method of Berman and Wyler (7). Counts of at least 100 infected macrophages were made in triplicate assays.

Carbamyl phosphate synthetase was assayed according to Levine and Kretchmer (8). The assay mixture in 0.5 ml contained 25 µmole Hepes-KOH buffer pH 7.6, 5 µmole L-glutamine, 10 µmole ATP, 10 µmole Magnesium chloride, 10 µmole Potassium chloride, 5 µmole sodium bicarbonate, (2 x 10^6 CPM) and requisite amount of enzyme to initiate the reaction. Aspartate transcarbmylase was assayed colorimetrically, following essentially the method of Prescott and Jones (9). One ml of incubation medium contained 50 µmole Tris-HCl buffer pH 8.5, 5 µmole carbamylphosphate, 10 µmole of L-aspartate and the requisite amount of enzyme. The reaction was started by addition of carbamyl phosphate followed by aspartate. After preincubation for 20 mins at 37°C the reaction was terminated by addition of 0.1 ml of 10% trichloroacetic acid. After centrifugation, carbamyl aspartate in the supernatant was estimated colorimetrically following the method of Prescott & Jones (9) as modified by Savage et al.(10) one unit of enzyme was defined as the amount of enzyme needed to generate 1 µmole of carbamylaspartate per min at 37°C. Dihydroorotase was assayed according to the method of Kempe et al. (11). In this case, the formation of carbamyl aspartate from dihydroorotic acid was colorimetrically estimated following procedure of Prescott and Jones (9). The standard assay mixture in 1 ml contained 100 µmole Tris-HCl buffer pH 8.8, 2.5 µmole L-dihydroorotic acid and the requisite amount of enzyme. The reaction was initiated with the substrate. After incubation of 20 mins. at 37°C the reaction was terminated by addition of 1 ml antipyrin-monoxime solution (2:1) and carbamyl aspartate formed was measured as described above.

RESULTS

Purification of ATCase : All operations unless otherwise stated were carried out at 2-4°C.

The cells grown on solid blood-agar medium for 66 hrs were scraped in phosphate-saline buffer and washed twice by centrifugation.

Packed cells (12 g) were chilled and then swelled in 48 ml of 2 mM Hepes buffer pH 7.8 containing 1 mM EDTA. The swelled cells were homoginized in a Potter-Elvjhem homoginizer and another 48 ml of a buffer containing 100 mM TRIS-HCl pH 8.0, 1 mM EDTA and 5 mM 2-marcaptoethanol, was added to it. The homogenate was centrifuged at 15,000 g for 30 mins and the supernatant solution was designated as crude extract (95 ml). 1% (w/v) protamine sulphate solution, was slowly added to the crude extract until 8% (v/v) with continuous stirring. The precipitate was removed by centrifugation at 18,000 g for 30 mins (98 ml).

Solid ammonium sulphate (20.48 g) was added to supernatant with gentle stirring to obtain approximately 35% ammonium sulphate saturation. The precipitate was discarded after centrifugation and the concentration of ammonium sulphate was raised to 50% saturation by further addition of solid ammonium sulphate (10.8 g). The precipitated protein, after centrifugation was dissolved in 5 ml of Buffer A containing 50 mM TRIS-HCl pH 8.0, 1 mM EDTA, and 2.5 mM 2-mercaptoethanol. This fraction was designated as the first ammonium sulphate fraction. This fraction was heated to 60°C in a constant water bath for 1 min and then immediately chilled in ice. The precipitated protein was removed by centrifugation at 18,000 g for 30 mins and the heat-treated clear supernatant (5 ml) was further processed by addition of solid ammonium sulphate (1.76 g) to 55% saturation. The precipitate obtained after centrifugation was redissolved in 1 ml of Buffer A.

The solution after this second ammonium sulphate treatment was passed through a column of Sephadex G-100 (1.7 cm x 61 cm) previously equilibrated with Buffer A. The enzyme came out in the void volume.

Active fractions were pooled and were carefully loaded on to a DEAE-cellulose column (1.3 cm x 12 cm) which was previously equilibrated with Buffer A. The column was washed with 40 ml of Buffer A. Batchwise elutions were carried out with 40 ml each of the same buffer containing 1 mM EDTA, 2.5 mM 2-mercaptoethanol and 100 mM, 150 mM, and 200 mM TRIS-HCl pH 8.0 respectively. Fractions of 1 ml were collected and protein as well as aspartate transcarbamylase activity were determined in all fractions. ATCase activity invariably came out in the final elution buffer, generally in 3-4 tubes. Fractions containing ATCase activity were pooled and stored at -20°C. The enzyme remained stable for 3 weeks.

Molecular weight and subunit structure of the enzyme: The polyacrylamide gel electrophoresis of the enzyme fraction after the final purification step revealed a single band of protein (Fig. 1A). The approximate molecular weight of the purified enzyme was determined to be 1,35,000 on a calibrated Sephadex G-200 column using marker proteins (Fig. 2). Sodium-dodecyl-sulphate-polyacrylamide gel electrophoresis in presence of 2 -mercaptoethanol was carried out according to the procedure of Laemmli (12) and this revealed the presence of one single polypeptide band for the enzyme (Fig. 1B). Approximate molecular weight was calculated to be 35,000 using marker proteins (Fig. 3).

Kinetic properties of the enzyme: ATCase exhibited strictly hyperbolic kinetics both with aspartate (Fig. 4) and with carbamylphosphate (Fig. 5) as substrates. The Km for aspartate and for carbamylphosphate were calculated to be 7.6 mM and 0.31 mM respectively. Since the purification procedure involved a heat-step and this often results in desensitization of potential allosteric sites, the kinetics with both the substrates were carried out with the partially purified dyalyzed enzyme before the heat-step was introduced. In this case also no sigmoidal kinetics could be observed at low concentrations of either of the substrate (data not shown). The optimum pH of the enzyme was determined to be 8.6.

A large number of nucleotides, mono, di and triphosphates were tested for their potential modulatory role on the kinetic properties of the enzyme. Amongst all the compounds tested only ATP (3 mM) showed a moderate inhibition of 25% with 8 mM aspartate and 0.5 mM carbamylphosphate as substrates. Lowering of aspartate concentration in presence of the same concentration of ATP failed to reveal any shift from normal hyperbolic kinetics.

Inhibition by PALA : PALA was introduced by Collins and Stark (13) as a transition state analogue for E.coli ATCase that powerfully inhibited its activity. PALA was found to be a very potent inhibitor for aspartate transcarbamylase from Leishmania donovani promastigotes. Kinetic analysis showed that it acts as a strictly competitive inhibitor with carbamylphosphate as varying substrate with Ki of 0.5 µM (Fig. 5). In contrast, PALA was found to be a strictly non-competitive inhibitor when aspartate was used as the varying substrate (Fig. 4).

Separation of ATCase and DHOase activities: Cells were suspended in 10 ml of phosphate-saline buffer with protease inhibitors, benzamidine (2.5 mM) and soyabean trypsin inhibitor (0.25 mg/ml). The cells were transferred to a centrifuge tube and pelleted by spinning for 5 mins at 3000 g. Same procedure was repeated again. The packed cell pellet (3 g) was resuspended in 12 ml of hypotonic buffer containing 5 mM sodiumphosphate buffer pH 7.4, 2 mM MgCl, 2 mM CaCl$_2$, 1 mM DTT, 2.5 mM benzamidine, 0.1 mM PMSF, 0.25 mg/ml soyabean trypsin inhibitor. The cells were allowed to swell for 10 mins prior to lysis in potter Elvjhem homogenizer. After homogenization another 12 ml of 200 mM sodiumphosphate buffer pH 7.4 was added to the lysate. It was centrifuged at 18,000 g for 30 mins. The supernatant solution was designated as crude extract (23 ml). Protamine sulphate and ammonium sulphate steps were carried out according to purification procedure of ATCase described earlier. Length of time between cell lysis and ammonium sulphate precipitation was reduced to as minimum as possible. The cell pellet (from 35 to 50% ammonium sulphate saturation) was redissolved in 1 ml of buffer B containing 50 mM Na-phosphate buffer pH 7.4, 0.6 DTT, 25 µM $ZnSO_4$. This solution was passed through a column of Sephadex G-100 (1.5 cm x 61 cm), previously equilibrated with buffer B. The elution pattern is shown in Fig. 6.

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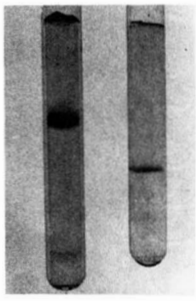

Figs. 1A & 1B : Polyacrylamide gel electrophoresis and sodium-dodecyl sulphate-polyacrylamide gel electrophoresis of ATCase : First tube (Fig. 1A) polyacrylamide gel electrophoresis. Second tube (Fig. 1B) sodium-dodecyl sulphate-polyacrylamide gel electrophoresis. The amount of protein in first tube was 40 µg and in the second tube was 35 µg. The stain used was coomassie brilliant blue. The migration was from top (cathode) to bottom (anode). In first tube (Fig. 1A) lower band represents dye front.

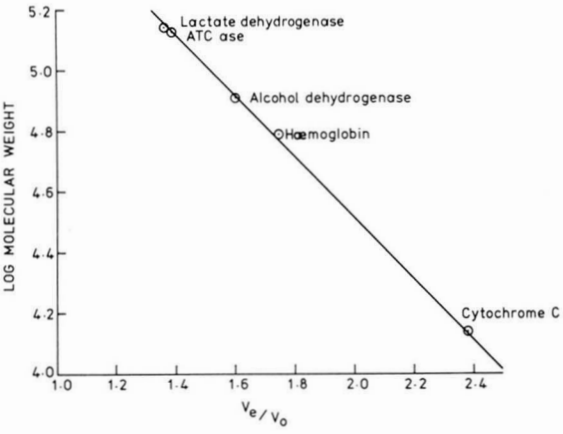

Fig. 2 : Determination of molecular weight of ATCase by gel filtration on a calibrated sephadex G-200 column: 'V_e' is the elution volume of protein and 'V_o' is the void volume of the column.

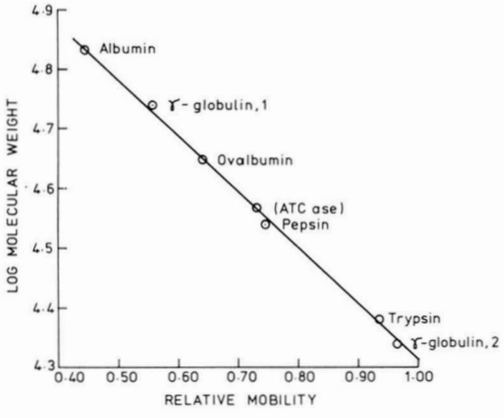

Fig. 3 : Determination of subunit molecular weight of ATCase by sodium dodecyl sulphate polyacrylamide gel electrophoresis.

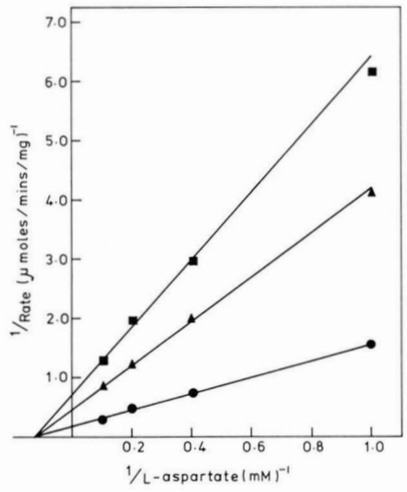

Fig. 4 : Effect of L-aspartate on inhibition of ATCase by PALA: Enzyme activity was assayed as described in Materials and Methods Section. Requisite amount of the purified enzyme was used per assay with fixed concentration of carbamyl phsophate (1 mM). The concentration of PALA were ●—●, zero: ▲—▲, 0.01 mM: ■—■, 0.02 mM.

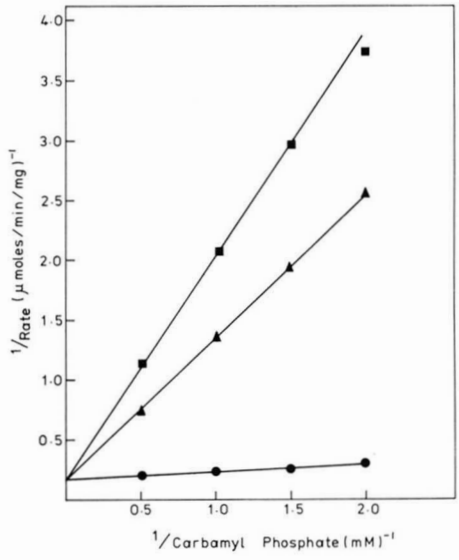

Fig. 5 : Competition of PALA with carbamyl phosphate for ATCase: Enzyme activity was assayed as described in Materials and Methods Section. Fixed concentration of L-aspartate (10 mM) was used in each assay. Requisite amount of the purified enzyme was used for the assays. The concentrations of PALA were ●—●, zero: ▲—▲, 0.01 mM: ■—■, 0.02 mM.

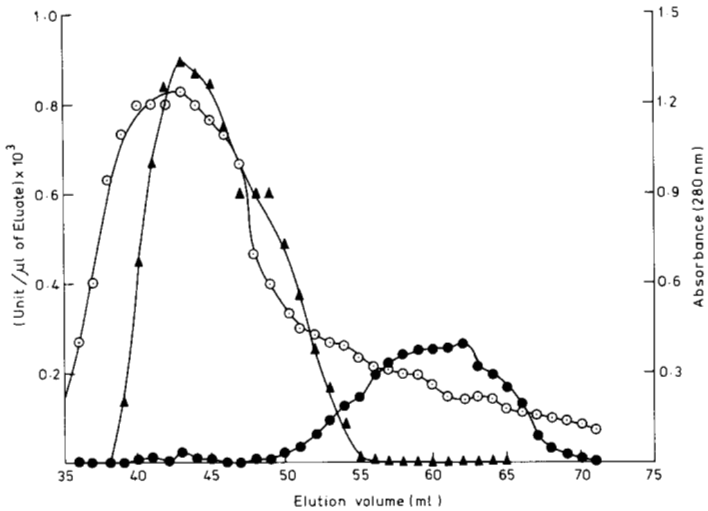

Fig. 6 : Profile of ATCase, DHOase activities and protein eluted from Sephadex G-100 column: The ammonium sulphate precipitate containing 22 mg of protein in 1 ml of buffer B was applied to a column (1.5 x 61)cm. Elution rate was 5 ml/hr and 1 ml fractions were collected. Protein content, ATCase activity and DHOase activity are represented by ○—○ , ▲—▲ , ●—● , respectively.