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PURIFICATION AND PROPERTIES OF DIHYDROOROTASE FROM *ESCHERICHIA COLI* B

EUGENE G. SANDER* AND MARY JO HEEB
Department of Biochemistry, University of Florida, Gainesville, Fla. 32601 (U.S.A.)

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SUMMARY

Dihydroorotase (4,5-L-dihydroorotate amidohydrolase, EC 3.5.2.3) from *Escherichia coli* B was purified 145-fold. Following the terminal purification procedure, polyacrylamide gel electrophoresis showed only one protein band. In contrast to dihydroorotase from *Zymobacterium oroticum*, the enzyme is neither inhibited by EDTA nor activated by divalent metals. The enzyme is specific for its natural substrates L-ureidosuccinate and L-dihydroorotate and will not catalyze the hydrolysis of *p*-nitrophenylacetate. The *N*-carbamyl derivatives of glycine, DL-serine, and L-glutamic acid are competitive inhibitors. The enzyme is stabilized by dithiolthreitol and is reversibly inhibited at pH 5.8 by the mercaptide-forming reagents $AgNO_3$ and *p*-chloromercuribenzene sulfonate but not the alkylating agent iodoacetamide. Phosphate and arsenate buffers appear to specifically increase the initial rates of dihydroorotase catalyzed reaction. The molecular weight of *E. coli* dihydroorotase in 0.05 M sodium phosphate buffer (pH 7.0) is estimated from gel filtration to be 76 000 ± 10%.

INTRODUCTION

Dihydroorotase (4,5-L-dihydroorotate amidohydrolase, EC 3.5.2.3) has been identified in *E. coli*[1], *Z. oroticum*[2], rat liver[3,4], human erythrocytes and leucocytes[5–8], Novikoff ascites tumor cells[9–11], and pea seedlings[12]. The enzyme from *Z. oroticum* was found after partial purification by SANDER *et al.*[13] to require a divalent cation, most likely Zn^{2+}, for its activity, however, the partially purified pea seedling enzyme tested under approximately the same conditions was neither inhibited by EDTA nor stimulated by Zn^{2+} [12]. Evidence obtained with the mammalian and other bacterial sources of the enzyme would also indicate no specific requirement for divalent metals. Of the various tissues and cells in which dihydroorotase has been identified, *Z. oroticum* is unique in that it uses orotic acid as a metabolic carbon source. YATES AND PARDEE[1] showed that dihydrorotase activity was increased about 20-fold in

* To whom inquiries should be addressed.

extracts from orotate compared to glucose grown *Z. oroticum* indicating that dihydroorotase in the orotate grown cells was adaptively formed to metabolize orotate rather than synthesize pyrimidine bases. TAYLOR *et al.*[14] using an aerobically grown *Pseudomonad* have demonstrated the existence of two distinct types of dihydroorotic dehydrogenase which differ in both their cellular location and terminal electron acceptor and are formed in varying degrees depending upon the inclusion of either glucose or orotate in the culture medium. These latter two pieces of evidence might lead to the proposition that orotate induced metal requiring dihydroorotase from *Z. oroticum* is uniquely different than the constitutive enzyme from other sources. To help test this hypothesis and to obtain information on the enzymatic properties of dihydroorotase, we have purified and partially characterized the enzyme from *E. coli* B grown on minimal salts media since the function of the enzyme in this organism is the biosynthesis rather than degradation of the pyrimidine bases.

MATERIALS AND METHODS

All organic reagents were obtained from Sigma Chemical Company with the exception of 2-(*N*-morpholino)ethane sulfonic acid which was from Nutritional Biochemicals. L-Ureidosuccinate was prepared by the alkaline hydrolysis of L-dihydroorotate in 1.0 M NaOH followed by adjustment of pH to 5.8 [15]. Enzyme grade $(NH_4)_2SO_4$ was obtained from either Sigma or Nutritional Biochemicals. Reagent grade inorganic chemicals were used in all cases. DEAE-cellulose (Whatman DE-52, microgranular), diethyl-2-hydroxypropyl ammonium Sephadex (QAE-Sephadex, A-50), and hydroxylapatite (BioGel HT) were obtained from Reeve Angel, Pharmacia Fine Chemicals, and California Biochemicals, respectively. G-200 Sephadex and a column calibration kit of purified proteins were obtained from Pharmacia Fine Chemicals. *E. coli* B was commercially grown by General Biochemicals, Chagrin Falls, Ohio at 35° using the minimal salts media of YATES AND PARDEE[1]. The bacteria were harvested in the late logarithmic phase, frozen, and stored at −70° for periods up to 10 months with no measurable loss of dihydroorotase activity.

Enzyme purification

Unless otherwise stated, all operations were performed at 0–5°. All buffers used in the purification procedures were $1 \cdot 10^{-5}$ M in $ZnSO_4$ and following $(NH_4)_2SO_4$ precipitation contained $6.5 \cdot 10^{-4}$ M (0.1 mg/ml) reduced dithiothreitol.

(1) Cell disruption. Frozen *E. coli* cell paste was thawed, suspended in 6 ml of 0.05 M sodium phosphate (pH 7.0) per g of frozen cell paste, placed in a 250 ml glass Rosett cell, and subjected to sonic oscillation below 10° for 5 min using a Biosonik II sonifier equipped with a one-half inch probe and adjusted for maximum output. The disrupted cells were centrifuged at 24 000 × *g* for 20 min. The resulting supernatant fluid, designated the "Crude extract," was the starting material for the purification of dihydroorotase.

(2) Protamine sulfate precipitation. The crude extract was made 0.40 M NaCl by slowly adding the solid salt. To remove nucleic acids, 0.30 vol. of 0.5% protamine sulfate in 0.40 M NaCl were added with stirring. After 10 min, the turbid solution was centrifuged at 24 000 × *g* for 10 min and the pellet discarded. The $A_{280\ nm}/A_{260\ nm}$ ratio generally increased from about 0.5 for the crude extract to between 0.7–0.9 for

the protamine supernatant fraction. If this absorbance ratio was below 0.7, additional volumes of the protamine sulfate solution were added, however 0.40 vol. of protamine solution per vol. of crude extract was never exceeded, since beyond this point dihydroorotase activity was diminished.

(3) Ammonium sulfate precipitation. The protamine sulfate supernatant solution was slowly made 40% saturated with solid enzyme grade $(NH_4)_2SO_4$. After 10 min, the precipitate was removed by centrifugation at 24 000 × *g* for 10 min and discarded. The supernatant solution was then made 60% saturated in $(NH_4)_2SO_4$ and after standing 10 min the precipitate was collected by recentrifugation at 24 000 × *g*. The resulting pellet was dissolved in 0.05 M sodium phosphate buffer (pH 7.0) and stored at −17° until further purification steps could be initiated. The enzyme is fairly stable under these conditions, although in several cases as much as 20% of the original activity was lost in a two-week period of time.

(4) Heat precipitation. The frozen $(NH_4)_2SO_4$ fraction was thawed, diluted with 0.05 M sodium phosphate buffer (pH 7.0) to a final protein concentration of 10 mg/ml, and divided into 100-ml aliquots. Each aliquot was made 0.025 M in DL-ureidosuccinate and poured into a 600-ml beaker immersed in a 60° water bath.

TABLE I

PURIFICATION OF DIHYDROOROTASE FROM *E. coli* B

Fraction	*Protein (mg)*	*Dihydro-orotase (units)*	*Specific activity*	*Recovery (%)*
Crude extract	21 600	2 240 000	104	100
Protamine sulphate	19 100	2 390 000	125	107
$(NH_4)_2SO_4$	7 840	1 910 000	244	85
Heat	6 440	1 820 000	282	81
QAE-Sephadex I	781	813 000	1 130	36
DEAE-cellulose	280	342 000	1 220	15
Hydroxylapatite	49.2	208 000	4 240	9.3
Sephadex G-200	18.9	193 000	10 200	8.6
QAE-Sephadex II	3.03	44 900	14 800	2.0

Heating was continued for 5 min at 60° followed by rapid cooling and centrifugation to remove the heat denatured protein.

(5) Chromatography. The several chromatographic methods utilized in dihydroorotase purification are indicated in the order employed in Table I. For the first QAE-Sephadex column, 5 mg of protein were applied for each ml of packed column bed volume, but for all subsequent ion exchange columns 2 mg of protein were applied per ml of packed column bed volume. Linear ionic strength elution gradients of the following composition were employed: QAE-Sephadex, 0.10 to 0.40 M NaCl in 0.05 M Tris–HCl buffer (pH 8.0); DEAE-cellulose, 0.10 to 0.25 M NaCl in 0.05 M sodium phosphate buffer (pH 7.0); hydroxylapatite, 0.01 to 0.15 M sodium phosphate (pH 7.0). A total volume of 6 ml of each of these elution gradients per ml of packed column bed volume was employed. The Sephadex G-200 column (2.5 cm × 88 cm) was eluted by upward flow with 0.05 M sodium phosphate buffer (pH 7.0). Before each chromatography step, the dihydroorotase containing fractions were pooled,

concentrated by membrane filtration at 40 lb/inch^2 pressure using a Diaflo ultrafiltration cell equipped with a PM-30 membrane, and dialyzed against starting buffer.

Determination of dihydroorotase activity and protein concentration

Units of dihydroorotase activity were spectrophotometrically measured at 35° [13]. Either a Gilford 2000 or a Zeiss PMQ II spectrophotometer was used to measure the initial rate of L-dihydroorotate synthesis at 230 nm. Unless indicated, each reaction mixture was 0.10 M sodium phosphate buffer (pH 5.8) and 0.0167 M DL-ureidosuccinate in a final volume of either 1.0 or 3.0 ml. The enzyme was preincubated for 5 min prior to initiation of the reaction by the addition of DL-ureidosuccinate.

Protein concentration was measured by the microbiuret method using crystalline bovine serum albumin as a standard[16]. After hydroxylapatite chromatography and in all subsequent steps, protein concentration was determined by the spectrophotometric method of WARBURG AND CHRISTIAN[17].

Polyacrylamide gel electrophoresis

As a criterion of homogeneity, enzyme preparations were electrophoresed on 6% polyacrylamide gels at room temperature in 0.083 M Tris, 0.0033 M sodium EDTA, 0.012 M boric acid buffer (pH 9.1) for 45 min at 4 mA per disc gel. The gels were then stained with amido black. Excess stain was removed by washing with water–methanol–acetic acid–glycerol (20:8:2:1, by vol.).

Determination of the equilibrium constant

The equilibrium constant for the dihydroorotase catalyzed reaction was measured at 35° in 0.10 M sodium phosphate buffers at both pH 5.80 and 7.0. For each pH value, the approach to equilibrium was measured at 230 nm starting with both DL-ureidosuccinate and L-dihydroorotate. Each reaction mixture contained 450 units of dihydroorotase in a final volume of 3.0 ml. Absorbance readings were continued until there was no longer any change as a function of time. After a short extrapolation to time zero, $\Delta A_{230\,nm}$ was determined by the difference in the absorbance at the end of the reaction and at time zero. Values of the ratio [L-dihydroorotate]/[L-ureidosuccinate] were then calculated for the reaction measured in both directions using $\Delta A_{230\,nm}$, E_{max} (230 nm) L-dihydroorotate $= 1.17 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ (ref. 13), and the initial concentrations of each of the substrates. K_{eq} was then defined as equal to [L-dihydroorotate]/[L-ureidosuccinate] $\times$ activity of $H_3{}^+O$) considering water concentration to be unity.

Sulphydryl reagents on dihydroorotase activity

In addition to either iodoacetamide, *p*-chloromercuribenzene sulfonate or $AgNO_3$ which were employed at final concentrations of 0.206 mM, 0.109 mM and 0.0124 mM, respectively, reaction mixtures were 0.10 M sodium phosphate buffer (pH 5.8); 10 mM DL-ureidosuccinic acid and contained 10 units of dihydroorotase, specific activity 7500 in a final volume of 1 ml. Reactions were initiated with substrate preceded by 5 min preincubation of the inhibitor and the enzyme. In the case of *p*-chloromercuribenzene sulfonate and $AgNO_3$ experiments were performed in which the inhibitors were added 2 min after the initiation of the reaction with substrate.

Molecular weight estimation

The mol. wt. of *E. coli* B dihydroorotase was estimated in 0.05 M sodium phosphate buffer (pH 7.0) by gel filtration on a G-200 Sephadex column with a packed bed volume (V_t) of 181.6 ml (column dimensions 2.5 cm × 37 cm). After packing and equilibration the column was calibrated according to instructions supplied by Pharmacia Fine Chemicals, using ribonuclease A (mol. wt. 13 700), chymotrypsinogen A (mol. wt. 25 000), ovalbumin (mol. wt. 45 000) and aldolase (mol. wt. 158 000). The void volume (V_0) was determined with blue dextran. Values of K_{av}, the fraction of the gel phase available to a particular protein, were determined from V_o, V_e and V_t; the void volume, the elution volume, and the total bed volume, respectively[18]. Elution patterns were monitored by uv absorption and enzyme activity. A linear semilogarithmic plot of K_{av} *versus* molecular weight was used to estimate the molecular weight of dihydroorotase.

RESULTS AND DISCUSSION

Enzyme purification

E. coli dihydroorotase was purified about 145-fold 5 different times using either QAE Sephadex or hydroxylapatite as the terminal procedure. The results shown in Table I are typical and quite reproducible through chromatography on the first QAE-Sephadex column. After this stage of purification, the specific activity showed more variability as indicated by the fact that in the preparation reported in Table I, DEAE cellulose chromatography resulted atypically in a large loss in total units of enzyme and hence a small increase in specific activity. The enzyme appears fairly unstable following the terminal purification step as actual decreases in enzyme specific activity were twice observed, and yet the elution profiles of these columns showed that nonspecific protein was being separated from dihydroorotase. Polyacrylamide gel electrophoresis of concentrated fractions after the terminal steps of enzyme purification showed only one protein band, although the lack of complete symmetry between the enzyme activity and the protein elution profiles makes it difficult to conclude that the preparations were completely homogeneous.

Divalent metal requirement

The lack of EDTA inhibition and either Co^{2+} or Zn^{2+} stimulation of *E. coli* dihydroorotase under conditions that strongly affect the activity of the enzyme from *Z. oroticum*[13] are shown in Table II. Additional studies in which the enzyme was dialyzed against 0.50 M EDTA in 0.05 M sodium phosphate buffer (pH 6.0) for up to 8 days, poured over Sephadex G-25 to remove the excess EDTA and assayed separately in the presence of Zn^{2+}, Co^{2+} and Mn^{2+} also showed no decrease in enzyme activity which could be restored by adding divalent metals to the assay medium. These data indicate that *E. coli* dihydroorotase does not have an easily dissociable divalent cation required for its activity, however, the fact that EDTA cannot specifically inhibit the enzyme may merely reflect the fact that at pH 5.8–6.0 the metal-enzyme interaction is much stronger than is the metal–EDTA interaction. These results agree with the data of MAZUS AND BUCHOWICZ[12] who found no evidence for a metal requirement in pea seedling dihydroorotase using essentially the same methods. Studies are currently in progress with glucose grown *Z. oroticum* to see

TABLE II

EFFECTS OF EDTA AND DIVALENT CATIONS IN PRESENCE OF EDTA ON DIHYDROOROTASE

Reaction mixtures contained 72 units of enzyme, specific activity 4900 units/mg protein. Final concentrations of phosphate buffer (pH 5.8); EDTA; $ZnSO_4$; $CoCl_2$ and DL-ureidosuccinate were 0.1 M, $1 \cdot 10^{-5}$ M, $1 \cdot 10^{-4}$ M, $1 \cdot 10^{-4}$ M and 0.02 M, respectively, in a final volume of 1.0 ml. Enzyme and all reaction components were preincubated 15 min prior to initiation of the reaction by the addition of DL-ureidosuccinate.

Addition	$\Delta A_{230\ nm}$/*10 min*
None	0.630
Zn^{2+}	0.623
Co^{2+}	0.630
EDTA	0.630
EDTA + Zn^{2+}	0.638
EDTA + Co^{2+}	0.630

whether the constitutive enzyme in this organism is different in its metal ion requirements than the enzyme induced by the inclusion of orotic acid in the growth medium.

Equilibria of the dihydroorotase reaction

The equilibrium constants for the dihydroorotase catalyzed reaction expressed as [L-dihydroorotate]/([L-ureidosuccinate] × activity of $H_3{}^+O$) were $0.74 \cdot 10^6\ M^{-1}$ and $1.2 \cdot 10^6\ M^{-1}$ at pH 5.8 and 7.0, respectively. The reciprocal of K_{eq} at pH 5.8, $1.35 \cdot 10^{-6}$ M, agrees well with the value of $1.4 \cdot 10^{-6}$ M which was obtained using isotopic methods and crude cell free systems as does the tendency of K_{eq} to increase with increasing pH[1].

Specificity of dihydroorotase for substrates

The ability of dihydroorotase to catalyze either ring closure of DL-ureidosuccinate, *N*-carbamyl-β-alanine and *N*-carbamyl-L-asparagine or ring opening of L-dihydroorotate, dihydrouracil and dihydrothymine was measured. These data indicate that the enzyme is specific for either L-dihydroorotate or L-ureidosuccinate. A small but measurable rate was observed using *N*-carbamyl-L-asparagine. This most probably reflects an L-ureidosuccinate contaminant although paper chromatography using a solvent system of ethanol–methanol–formic acid–water (6:6:3:5, by vol.) lacked sufficient sensitivity to detect ureidosuccinate in the concentrated stock solution of *N*-carbamyl-L-asparagine. The ability of dihydroorotase to promote the hydrolysis of *p*-nitrophenylacetate in the pH range of 7.0 and 9.0 was examined by spectrophotometrically measuring *p*-nitrophenolate production at 400 nm. When the rate of ester hydrolysis in the presence of 30 units of boiled dihydroorotase was subtracted from the rate in the presence of the same amount of active enzyme, it could be concluded that dihydroorotase does not catalyze *p*-nitrophenylacetate hydrolysis. This coupled with the data using substrate analogs leads to the conclusion that *E. coli* dihydroorotase is specific for its natural substrates.

Inhibition of dihydroorotase by substrate analogues

The ability of dihydrouracil, dihydrothymine and *N*-carbamyl derivatives of

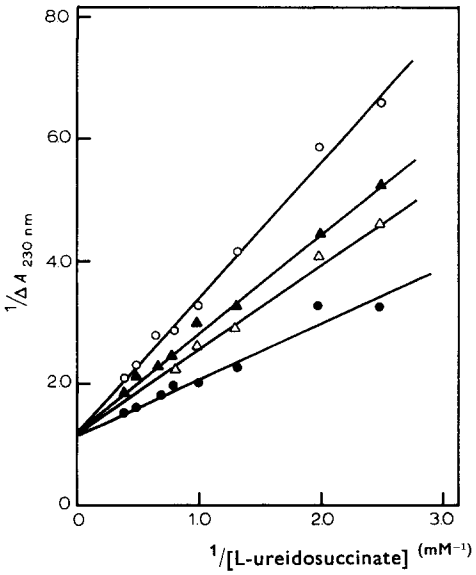

Fig. 1. Competitive inhibition of dihydroorotase by 0.05 mM concentrations of the *N*-carbamyl derivatives of glycine, L-glutamate and DL-serine. In addition to the inhibitors, reaction mixtures were 0.10 M sodium phosphate buffer (pH 5.8) and contained 39 units of dihydroorotase, specific activity 7500 in a final volume of 1.0 ml. ●—●, control, K_m = 0.76 mM; △—△, *N*-carbamyl-DL-serine, K_i = 9.3 mM; ▲—▲, *N*-carbamyl-L-glutamate, K_i = 6.0 mM; ○—○, *N*-carbamyl-glycine, K_i = 3.3 mM.

DL-serine, DL-threonine, L-glutamic acid, L-asparagine, beta alanine and glycine all at a final concentration of 5.0 mM to inhibit dihydroorotase was measured in 0.10 M sodium phosphate buffer (pH 5.8) using 5.0 mM L-ureidosuccinate as substrate. When the initial rate of the reaction in the absence of potential inhibitor was expressed as 0% inhibition, then only the *N*-carbamyl derivatives of DL-serine, glycine and L-glutamic acid exhibited significant degrees of inhibition (11, 15 and 17%, respectively). The effect of varying L-ureidosuccinate concentration on the initial rates of L-dihydroorotate synthesis in the presence of these three inhibitors is illustrated in double reciprocal form in Fig. 1. These results clearly indicate that *N*-carbamyl-DL-serine, -glycine, and -L-glutamic acid are competitive inhibitors of dihydroorotase with inhibition constants (K_i) of 9.3, 3.3 and 6.0 mM, respectively.

Sulphydryl reagents on dihydroorotase activity

The activity of dihydroorotase was diminished to 0% and 34% of the control by preincubating the enzyme at pH 5.8 with either 0.0124 mM $AgNO_3$ or 0.109 mM *p*-chloromercuribenzene sulfonate. Under these conditions 0.206 mM iodoacetamide had no effect. The addition of either *p*-chloromercuribenzene sulfonate or $AgNO_3$ 2 min after the reaction was initiated elicited the same degree of inhibition, indicating that the substrate does not prevent inhibition under these conditions. Mercaptide formation appears to be reversible because the addition of a 2-fold molar excess of dithiolthreitol completely restored activity to an aliquot of enzyme which was 70% inhibited by the addition of 0.109 mM *p*-chloromercuribenzene sulfonate. This is not

conclusive evidence that a sulphydryl group is essential for dihydroorotase activity for reasons which are pointed out in several reviews[19,20]; however this evidence coupled with the fact that dithiothreitol stabilizes the enzyme during chromatography and storage makes this hypothesis worthy of continued investigation.

pH stability of dihydroorotase

The stability of dihydroorotase as a function of pH was measured at 35° and ionic strength 0.30 M by preincubation for 20 min in either 0.10 M acetate, phosphate or Tris–HCl buffers followed by enzyme assay in 0.25 M phosphate buffer, (pH 5.6) and 17 mM DL-ureidosuccinate. Values of pH were determined both after preincubation and assay. A plot of initial rate, against preincubation pH showed a maximum in enzyme stability at pH 6.5. If the initial rate at pH 6.5 is assigned an arbitrary value of 100, then the relative rates at pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 are 0, 19, 84, 89, 50 and 31, respectively.

Effect of H_3^+O, phosphate and arsenate on dihydroorotase activity

The effect of H_3^+O activity on both L-dihydroorotate and L-ureidosuccinate synthesis was measured at 35° using 0.10 M acetate, phosphate, 2-(*N*-morpholino)-ethane sulfonate and Tris–HCl buffers. The substrate concentration was maintained several times greater than K_m and the ionic strength was maintained at 0.30 M by the addition of NaCl.

The results of these experiments (Fig. 2) indicate that phosphate buffers

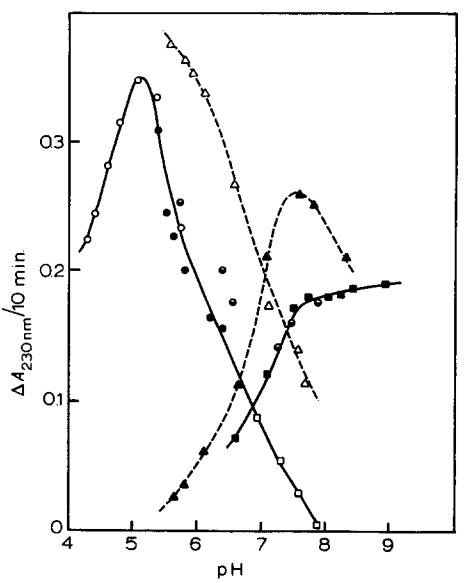

Fig. 2. Effect of H_3^+O on dihydroorotase activity. Each reaction mixture contained substrate at a final concentration equal to 3 to 5 times K_m, 0.10 M buffer and dihydroorotase (39 units for forward reaction and 200 units for the reverse reaction). Ionic strength was maintained at 0.30 M by the addition of NaCl. The following buffers were employed: L-Dihydroorotate synthesis: ○, acetate: ●, 2-(*N*-morpholino)ethane sulfonate; □, Tris–HCl; △, phosphate; and ◒, intercepts at zero phosphate concentration. L-Ureidosuccinate synthesis; ▲, phosphate; ■, Tris–HCl; and ◒, intercepts at zero phosphate concentration (see Fig. 3).

stimulate the rate of the reaction in both directions. This finding was verified by measuring the rates of both L-ureidosuccinate and L-dihydroorotate synthesis as a function of increasing phosphate buffer concentration at constant pH and ionic strength equal to 0.30 M. The results for L-dihydroorotate synthesis (Fig. 3) clearly indicate that between 0.017 M and 0.07 M total buffer, both phosphate and arsenate buffers promote the rate of L-dihydroorotate synthesis. Similar results were obtained for L-ureidosuccinate synthesis. The reasons for this stimulation in enzyme activity by phosphate and arsenate are not clear; however, increasing divalent anion concentration can be eliminated because the slopes of the phosphate data plotted in Fig. 3 increase slightly with decreasing pH and the results of using Na_2SO_4 to maintain constant ionic strength (Fig. 3) were the same as when NaCl was used for this purpose. Results similar to those shown in Fig. 3 were obtained in the presence of 0.10 mM EDTA; consequently, the stimulatory effect of removing a heavy metal inhibitor of dihydroorotase by either phosphate or arsenate can also be eliminated.

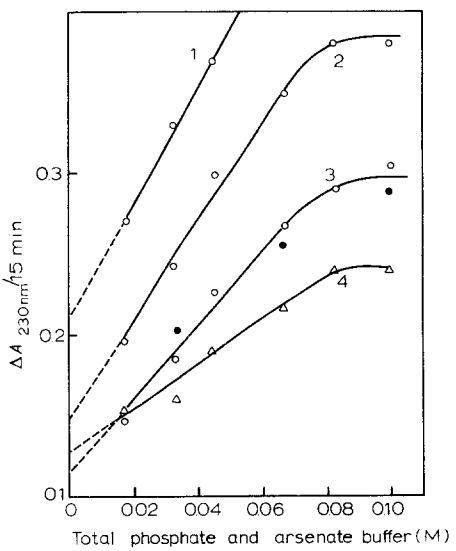

Fig. 3. Effect of phosphate and arsenate buffers on dihydroorotase activity. In addition to the buffers, each reaction mixture was 17 mM DL-ureidosuccinate and contained 114 units of dihydroorotase, specific activity 8250. Ionic strength was maintained at 0.30 M by the addition of NaCl except in the case of three points (●) in which Na_2SO_4 was used. Buffers were prepared by careful neutralization of stock NaH_2PO_4 solutions with standardized NaOH. pH was determined immediately following each determination of initial velocity. 1, phosphate, pH 5.70; 2, phosphate, pH 6.00; 3, phosphate, pH 6.60; 4, arsenate, pH 6.45.

Phosphate buffer stimulation of dihydroorotase activity complicates the interpretation of the pH-rate data for this reaction. When data similar to those reported in Fig. 3 are extrapolated to zero phosphate concentration, the initial rates obtained agree reasonably well with data obtained with other buffer systems. The pH optimum obtained for dihydroorotate synthesis at about pH 5.0 is about one pH unit below the previously reported value[1]. This difference may be due to phosphate stimulation of enzyme activity which was not previously recognized. The data for ureidosuccinate synthesis in Tris–HCl buffers also differs from previous reports[1] in that the rate does

TABLE III

ESTIMATION OF *E. coli* DIHYDROOROTASE MOLECULAR WEIGHT ON SEPHADEX G-200

Experimental conditions and the method used to calculate K_{av} are given in MATERIALS AND METHODS. Enzyme preparations with specific activities of 245 and 14 800 were after $(NH_4)_2SO_4$ fractionation and QAE-Sephadex chromatography, respectively. Numbers in parentheses indicate the number of determinations. Where indicated dithiothreitol was added at a final concentration of $6.5 \cdot 10^{-4}$ M*.

Protein	K_{av}	*Mol. wt.*
Aldolase (2)	0.35	158 000
Ovalbumin (3)	0.62–0.63	45 000
Chymotrypsinogen A (2)	0.76	25 000
Ribonuclease A (3)	0.86–0.87	13 700
Dihydroorotase (2) (specific activity 14 800)	1/0.50*–0.52	72 000
Dihydroorotase (2) (specific activity 245)	1/0.50*–0.51	79 000

not decrease with increasing pH above pH 8.0 but is instead essentially in a plateau region to about pH 9.0. The reason for this difference is not readily apparent; however non-enzymatic L-dihydroorotate hydrolysis was eliminated by showing that no change in absorbance at 230 nm occurred in a 20 min time period when the reactions were run in the absence of enzyme at both pH 8.5 and 9.0.

Estimation of molecular weight

The molecular weight of *E. coli* dihydroorotase was estimated to be 76 000 using Sephadex G-200 gel filtration. The data used to construct the linear protein selectivity curve as well as the enzyme data are presented in Table III. K_{av} values obtained for dihydroorotase varied between 0.50 and 0.52 which result in molecular weight values of 72 000 and 79 000, respectively. This would indicate that the method can estimate the enzyme's molecular weight to the nearest 10%.

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