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METAL ION REQUIREMENT AND TRYPTOPHAN INHIBITION OF NORMAL AND VARIANT ANTRHANILATE SYNTHASE-ANTHRANILATE 5-PHOSPHORIBOSYLPYROPHOSPHATE PHOSPHORIBOSYLTRANSFERASE COMPLEXES FROM *SALMONELLA TYPHIMURIUM*

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Summary

1. Both Mn^{2+} and Co^{2+} can replace Mg^{2+} as the required divalent cation for all activities of the enzyme complex between anthranilate synthase (chorismate pyruvate-lyase (amino-accepting), EC 4.1.3.27) and anthranilate-5-phosphoribosylpyrophosphate phosphoribosyltransferase (*N*-(5′-phosphoribosyl)-anthranilate:pyrophosphate phosphoribosyltransferase, EC 2.4.2.18) from *Salmonella typhimurium*. They have much lower apparent K_m values than Mg^{2+}, both for glutamine-dependent anthranilate synthase (Mn^{2+} = 1.1 μM, Co^{2+} = 2.6 μM, Mg^{2+} = 83 μM) and for phosphoribosyltransferase (Mn^{2+} = 16 μM, Co^{2+} = 14.6 μM, Mg^{2+} = 133 μM). The ratio of total Mg^{2+} to total Mn^{2+} found in a cell extract of *S. typhimurium trpE2*, the source of normal enzyme complex, was found to be 350, suggesting that Mg^{2+} is probably utilized by the enzyme complex in vivo under our growth conditions.

2. An enzyme complex has been isolated from a mutant strain of *S. typhimurium* (SO-515) that has a variation in the anthranilate synthase subunit which is thought to be a single amino acid substitution. This variation causes glutamine-dependent anthranilate synthase to be hypersensitive to feedback inhibition by tryptophan (K_i = 0.4 μM compared to K_i = 20 μM for normal enzyme complex). The phosphoribosyltransferase in the variant enzyme complex is also hypersensitive to tryptophan but the kinetics are complex and involve activation by tryptophan in the presence of low amounts of 5-phosphoribosyl 1-pyrophosphate.

3. In the variant enzyme complex the apparent K_m for Mg^{2+} is elevated to 360 μM for glutamine-linked anthranilate synthase but reduced to 75 μM for phosphoribosyltransferase.

4. These results suggest that the variant enzyme complex has altered terti-

ary and quaternary structures and that regulation of both activities is effected by tryptophan binding to only anthranilate synthase.

Introduction

Anthranilate synthase-anthranilate-5-phosphoribosylpyrophosphate phosphoribosyltransferase (chorismate pyruvate-lyase (amino-accepting), EC 4.1.3.27) · (*N*-(5′-phosphoribosyl)-anthranilate:pyrophosphate phosphoribosyltransferase, EC 2.4.2.18), is an enzyme complex from *Salmonella typhimurium* which consists of the first two enzymes in the tryptophan biosynthetic pathway [1]. When in the complex, anthranilate synthase (both ammonia- and glutamine-dependent) and phosphoribosyltransferase have been shown to require Mg^{2+} for activity [2—4]. Uncomplexed anthranilate synthase, which utilizes only ammonia, also requires Mg^{2+} for activity [2] but there are conflicting reports as to whether the uncomplexed phosphoribosyltransferase shows this requirement [4,5].

Both the anthranilate synthase [2,3] and phosphoribosyltransferase [4] activities of the complex are inhibited by tryptophan, the end-product of the pathway. Inhibition of anthranilate synthase can be complete whereas phosphoribosyltransferase shows a maximum inhibition of only 50—60% and this requires saturating concentrations of substrates [4]. While tryptophan inhibits uncomplexed anthranilate synthase [2], free phosphoribosyltransferase can only be inhibited about 15% at very high tryptophan concentrations [4] and the significance of this inhibition has been questioned [5,6]. Equilibrium binding studies showed one tryptophan binding site per anthranilate synthase subunit [7] but the question of such a site on phosphoribosyltransferase remains unresolved [6]. Tryptophan inhibition of the complex also involves Mg^{2+}; it antagonizes inhibition of anthranilate synthase [3] but is required for inhibition of phosphoribosyltransferase [4].

During earlier genetic work examining the separability of the tryptophan operator and the anthranilate synthase gene, a mutation in this gene (*trpA574*) was induced which produced a variant enzyme complex [8]. Analysis of extracts prepared from cells carrying this mutation showed that the complex contained an anthranilate synthase which was hypersensitive, and a phosphoribosyl - transferase which was resistant to tryptophan inhibition as compared to normal enzyme complex [8,9]. From reversion studies of the mutational site, it was concluded that these effects were probably caused by a single amino acid substitution in the anthranilate synthase subunit [9]. The present study was initiated to order to examine this variant enzyme complex, which proved to have interesting differences not only in tryptophan inhibition but in the metal ion involvement in catalysis.

Materials and Methods

Enzyme complex was obtained from *Salmonella typhimurium trpE2* (ATCC 25566) (hereafter referred to as normal enzyme complex). An enzyme complex was also isolated from SO-515 (*trp0517 trpA574 trpC3*) (hereafter termed

TABLE I

PURIFICATION OF THE VARIANT ANTHRANILATE SYNTHASE-PHOSPHORIBOSYLTRANSFERASE COMPLEX FROM *S. TYPHIMURIUM* SO-515

Methods for the purification, protein determination and assays are in Grove and Levy [11].

Step	Volume (ml)	Protein		Glutamine-dependent anthranilate-synthase			Phosphoribosyltransferase			
		mg/ml	Total mg	Total units	Specific activity	% Yield	Total units	Specific activity	% Yield	Ratio*
Cell extract	138	15	2070	119 600	58	—	193 200	93	—	0.62
Ultracentrifuge supernatant	33.5	3.5	117	83 750	716	70	117 250	1000	61	0.71
Bio Gel A 1.5 m peak	280	0.08	22.4	46 620	2030	39	82 130	3580	43	0.57
DEAE Bio Gel A peak	53.5	0.34	18.2	36 560	2010	31	87 380	4800	45	0.42
50% $(NH_4)_2SO_4$ precipitation	2.1	7.16 **	15.0	24 500	1630	20	54 600	3630	28	0.45

* Ratio of glutamine-dependent anthranilate synthase to phosphorbosyltransferase.

** Determined by the Method of Warburg and Christian [12].

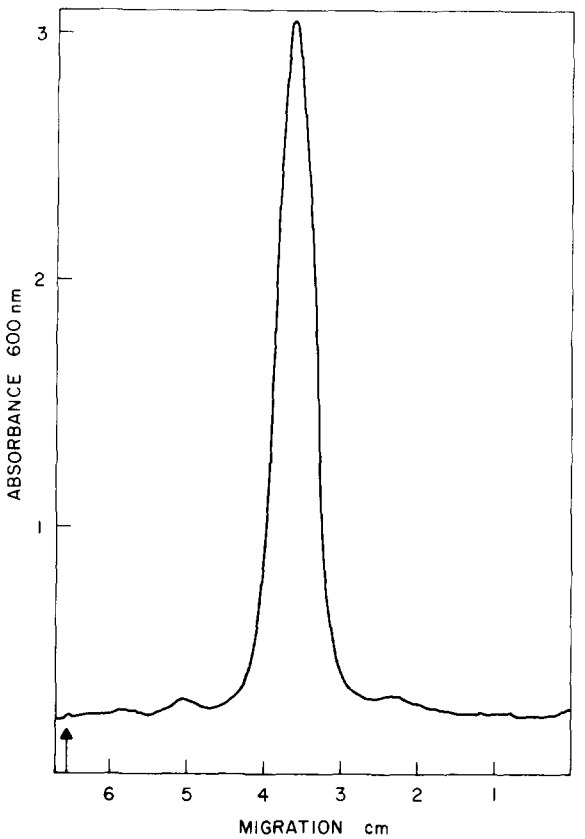

Fig. 1. Sodium dodecyl sulfate electrophoresis of the variant enzyme complex. Electrophoresis of the complex (50 μg) was from right to left. The gel was scanned with a Gilford scanner and the arrow indicates the position of the Bromophenol Blue tracking dye.

variant enzyme complex). Cells were grown in a depression medium [10] which resulted in a hundred-fold increase of enzyme complex activity. Both the normal and variant enzyme complexes were purified according to the method described in the accompanying paper [11]. Table I summarizes the purification procedure for the variant enzyme complex; the final specific activity and activity ratio are very close to those of the normal enzyme complex [11]. The variant enzyme complex was virtually homogeneous by the criterion of sodium dodecyl sulfate electrophoresis (Fig. 1). The method of Weber and Osborn [13] was followed except that gels were 5% acrylamide and were run until the dye front reached the end of the gel. Glutamine-dependent anthranilate synthase activity was measured using the fluorescent assay procedure of Henderson et al. [3] substituting triethanolamine · HCl buffer for potassium phosphate, and ammonia-dependent activity by the method of Grove and Levy [16]. Phosphoribosyltransferase was assayed spectrophotometrically [11] at 250 nm. All assays using Co^{2+} were performed omitting 2-mercaptoethanol since this reagent reacts with Co^{2+}. All assays were performed at 25° C.

The K_m values are apparent Michaelis constants calculated from double re-

ciprocal plots varying the ligand from about 0.2 to 10 × K_m with the other substrate and/or metal ions at standard assay concentrations [3,10,11]. With the exception of glutamine, these concentrations are all greater than 10 times their respective K_m values. Lines were fitted to the double reciprocal plots using a continuous linear regression program which gave zero order correlation coefficients generally >0.99.

Metal ion concentrations were determined by atomic absorption spectroscopy from standard curves using a Perkin Elmer model 303 atomic absorption spectrophotometer with an acetylene compressed air mixture. Metal ion ratios in the cell were determined by measuring the atomic absorption (Mg^{2+} at 285 nm, Mn^{2+} at 279 nm) of a crude extract [11] of *trpE2*.

The following chemicals were obtained from Sigma Chemical Co.: 5-phosphoribosyl 1-pyrophosphate, L-tryptophan, L-glutamine, triethanolamine · HCl and barium chorismate. The latter was converted to potassium chorismate before use. All other chemicals were of reagent grade. The concentrations of 5-phosphoribosyl 1-pyrophosphate and chorismate were calculated from enzymatic assays using an anthranilate standard. The concentrations of tryptophan solutions were calculated using its molar extinction coefficient of 5500 at 279 nm [14].

Results

Metal ion requirement

Mn^{2+} and Co^{2+} will replace Mg^{2+} as the divalent cation required for anthranilate synthase (both ammonia- and glutamine-dependent) and phosphoribosyltransferase activities in the enzyme complex. Tables II and III show that Mn^{2+} and Co^{2+} have much lower apparent Michaelis constants than Mg^{2+} for glutamine- and ammonia-dependent anthranilate synthase activities for normal en-

TABLE II

KINETIC CONSTANTS FOR GLUTAMINE-DEPENDENT ANTHRANILATE SYNTHASE OF NORMAL AND VARIANT ENZYME COMPLEXES

Enzyme assay and calculation of apparent K_m values are described under Materials and Methods. The K_i values were determined from Dixon plots (Figs. 3 and 4).

Ligand	Normal enzyme complex		Variant enzyme complex, apparent K_m or K_i values (μM) *
	Apparent K_m or K_i values (μM) *	Maximum velocity: μmol anthranilate produced per minute **	
Mg^{2+}	83	1.65	360
Mn^{2+}	1.1	0.81	1.8
Co^{2+}	2.6	1.08	3.0
Tryptophan	20	—	0.4
Chorismate	2.5	—	3.8
Chorismate ***	1.9	—	2.5
Glutamine	850	—	580

* All values are apparent K_m values except those given for tryptophan which are K_i values.

** The concentration of each metal ion is 20 times its K_m value. Each assay cuvette contains 7.6 μg of normal enzyme complex.

*** Performed in the presence of 100 μM Mn^{2+} instead of Mg^{2+}.

TABLE III

APPARENT K_m VALUES FOR THE AMMONIA-DEPENDENT ANTHRANILATE SYNTHASE AND PHOSPHORIBOSYLTRANSFERASE OF NORMAL AND VARIANT ENZYME COMPLEXES.

Enzyme assay and apparent K_m calculations are described under Materials and Methods.

Ligand	Apparent K_m (μM)			
	Ammonia-dependent anthranilate synthase		Phosphoribosyltransferase	
	Normal enzyme complex	Variant enzyme complex	Normal enzyme complex	Variant enzyme complex
Mg^{2+}	100	940	133	75
Mn^{2+}	1.5	not determined	16	4.3
Co^{2+}	9.1	not determined	14.6	not determined
5-phosphoribosyl-1-pyrophospate	—	—	16	10.5

zyme complex. Substituting Mn^{2+} for Mg^{2+} reduces the apparent K_m for chorismate slightly (Table II). The values for normal phosphoribosyltransferase (Table III) also show lower apparent K_m values for Mn^{2+} and Co^{2+} than for Mg^{2+}. Thus Mn^{2+} and Co^{2+} apparently bind much more tightly than Mg^{2+} to the enzyme complex. The anthranilate synthase apparent K_m values also indicate that the enzyme's affinity is slightly greater for Mn^{2+} than for Co^{2+}. The maximum velocity for glutamine-dependent anthranilate synthase utilizing Mn^{2+} is approximately one half that when Mg^{2+} is the cation used; with Co^{2+} the V value is intermediate between the other two (Table II).

Mn^{2+}, like Mg^{2+} [3,4] antagonizes tryptophan inhibition of glutamine-dependent anthranilate synthase and fulfills the metal ion requirement for tryptophan inhibition of phosphoribosyltransferase (Table IV). Mn^{2+} performs these functions at much lower concentrations than Mg^{2+}, i.e. at concentrations approximating its apparent K_m. Whether Co^{2+} can also replace Mg^{2+} is these functions was not tested.

TABLE IV

RELATIONSHIP OF Mg^{2+} AND Mn^{2+} TO TRYPTOPHAN INHIBITION

Assays were performed as stated in Materials and Methods except for amounts of metal ions and tryptophan. Normal enzyme complex was used.

Enzyme activity	Mg^{2+} (μM)	% Inhibition by 100 μM tryptophan	Mn^{2+} (μM)	% Inhibition by 100 μM tryptophan
Glutamine-dependent anthranilate synthase				
	5000	17	60	6
	1000	39	10	17
	100	60	1	67
Phosphoribosyltransferase				
	400	48	45	44
	150	48	20	12
	75	30	10	11
	40	27	5	5

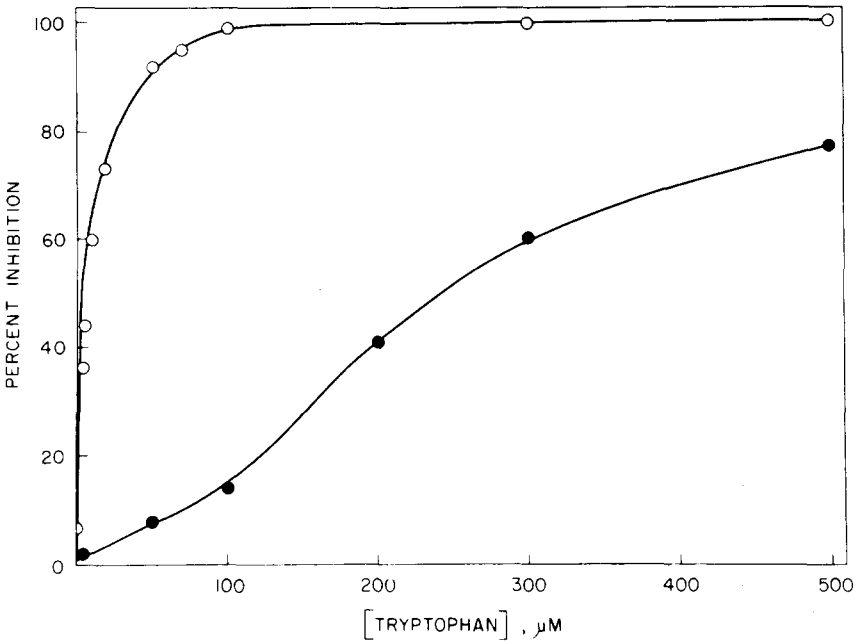

Fig. 2. Tryptophan inhibition of glutamine-dependent anthranilate synthase of normal and variant enzyme complexes. Enzyme assay is as described under Materials and Methods except for the addition of tryptophan. ●, normal complex; ○, variant enzyme complex.

Variant enzyme complex: metal ion differences

Besides differences in tryptophan inhibition, the variant enzyme complex shows differences in its interaction with metal ions. The apparent K_m for Mg^{2+} for glutamine-dependent anthranilate synthase of the variant enzyme complex (Table II) is approximately 4 times greater than that for the normal enzyme complex. The apparent K_m values for Mn^{2+} and Co^{2+} are also slightly higher than those for the normal enzyme complex (Table II).

There are also small but significant differences in the apparent K_m values for chorismate and glutamine in the variant enzyme complex (Table II). The apparent K_m for Mg^{2+} in the ammonia-dependent anthranilate synthase reaction for the variant enzyme complex is almost 10 times higher than that for the normal enzyme complex (Table III). The variation has no effect on the apparent K_m for NH_3. There are smaller differences in metal ion apparent K_m values for the phosphoribosyltransferase (Table III), but here the variant enzyme complex has lower values than the normal enzyme complex. The apparent K_m for 5-phosphoribosyl 1-pyrophosphate was similarly affected (Table III).

Variant enzyme complex: tryptophan inhibition

The differences between tryptophan inhibition of the variant and normal enzyme complexes observed in cell extracts [8] were re-examined using purified enzyme complexes. The hypersensitivity of the variant enzyme complex's glutamine-dependent anthranilate synthase was confirmed (Fig. 2). A K_i of 0.4 μM from a Dixon Plot [15] in the presence of 5 mM Mg^{2+} was determined for

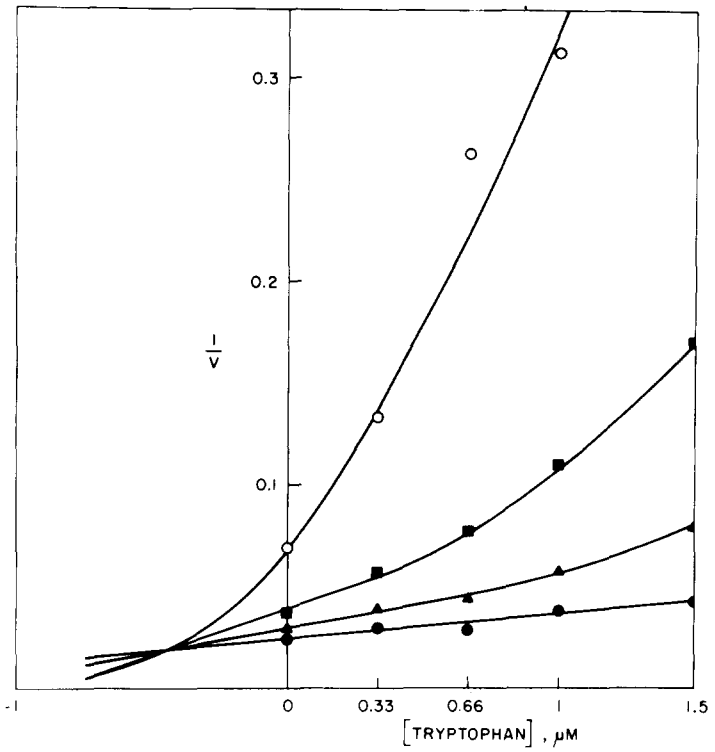

Fig. 3. Dixon plot of tryptophan inhibition of glutamine-dependent anthranilate synthase from the variant enzyme complex. Enzyme assay is as described in Materials and Methods except for the amounts of chorismate and the addition of tryptophan. ○, 2.7 μM chorismate; ■, 6.7 μM chorismate; ▲, 13.3 μM chorismate; ●, 26.7 μM chorismate.

the variant enzyme complex (Fig. 3) where the same plot for the normal enzyme complex gave a K_i of 20 μM (Fig. 4). Both these plots show upward curvature resulting from positive cooperativity in the presence of tryptophan [3].

Examination of tryptophan inhibition of phorphosribosyltransferase revealed that the variant enzyme complex was also hypersensitive (Fig. 5). The earlier report of this enzyme's resistance to tryptophan inhibition probably resulted from the use of crude preparations of the variant enzyme complex and a different assay [9]. As previously observed [4], maximum tryptophan inhibition of phosphoribosyltransferase requires saturating concentrations of 5-phosphoribosyl 1-pyrophosphate. Whereas the variant enzyme complex is inhibited more than the normal enzyme complex (Fig. 5), the difference is not as great as with glutamine-dependent anthranilate synthase (Fig. 2). Fig. 5 also shows an activation of phosphoribosyltransferase activity by tryptophan in the presence of low concentrations of 5-phosphoribosyl 1-pyrophosphate.

The greatly enhanced sensitivity of anthranilate synthase to tryptophan inhibition suggests that the structural change in the variant enzyme complex facilitates tryptophan binding. The simplest, but not the only explanation is that an amino acid substitution has taken place at the anthranilate synthase tryptophan binding site. The altered K_m for Mg^{2+} and smaller changes seen in K_m

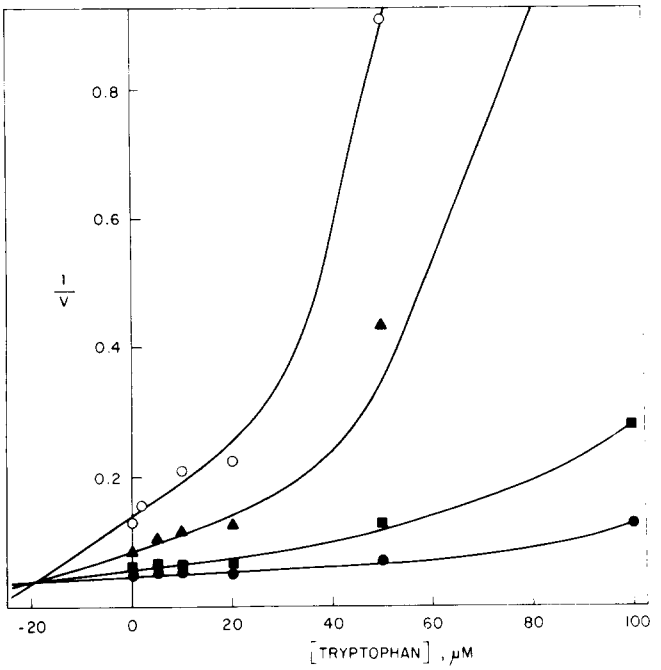

Fig. 4. Dixon plot of tryptophan inhibition of glutamine-dependent anthranilate synthase from the normal enzyme complex. Enzyme assay is described in Materials and Methods except for the amounts of chorismate and the addition of tryptophan. ○, 1.7 μM chorismate; ▲, 3.3 μM chorismate; ■, 8.3 μM chorismate; ●, 16.7 μM chorismate. Additional points for the two lowest chorismate concentration curves are not shown.

values for other ligands binding to anthranilate synthase in the variant enzyme complex would result from secondary changes accompanying small intra-subunit conformational alterations engendered by the structural change. The differences in kinetic constants of various ligands would reflect the extent to which these conformational alterations are sensed at the respective binding sites. Since Mg^{2+} antagonizes tryptophan binding [14] the binding sites for tryptophan and the metal ion may be close together. These possibilities can now be examined directly utilizing the paramagnetic properties of Mn^{2+}.

Our results bear on the question of whether there are binding sites for tryptophan on the phosphoribosyltransferase subunits in the enzyme complex [5,6]. An alteration in the anthranilate synthase subunit which causes a large increase in tryptophan inhibition for anthranilate synthase and a smaller increase in inhibition of phosphoribosyltransferase suggests that a single site controls both activities. Pabst et al. [16] have shown that regulation of the enzyme complex from *Escherichia coli* involves conformational changes upon tryptophan binding. It is possible that the variation we are examining here modifies those conformational changes and thus increases phosphoribosyltransferase sensitivity to tryptophan inhibition.

Kane [17] presented evidence that in *Bacillus subtilis* (where anthranilate synthase is not complexed with phosphoribosyltransferase), Mn^{2+} and not Mg^{2+} is utilized by anthranilate synthase. In the enzyme complex from *E. coli*, which

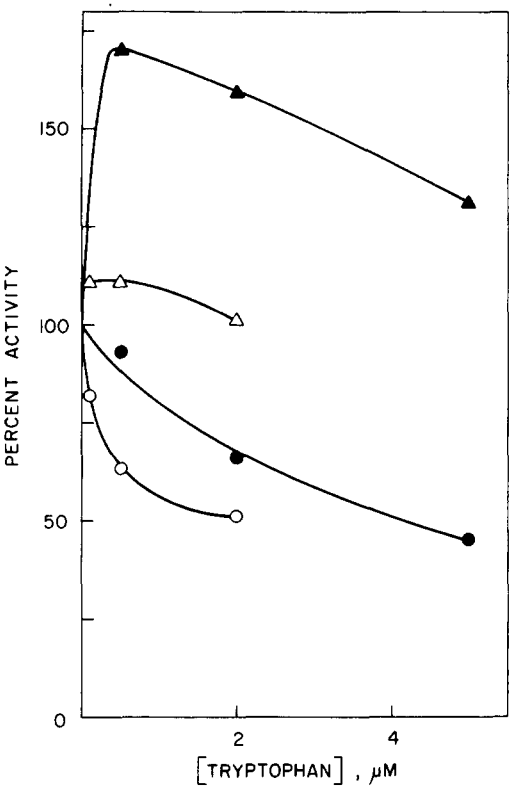

Fig. 5. The effect of tryptophan on phosphoribosyltransferase activity of variant and normal enzyme complexes. Enzyme assay is as described in Materials and Methods except for the amount of 5-phosphoribosyl 1-pyrophosphate. ▲, normal enzyme complex using 4.9 μM 5-phosphoribosyl 1-pyrophosphate; ●, normal enzyme complex using 123 μM 5-phosphoribosyl 1-pyrophosphate; △, variant enzyme complex using 4.9 μM 5-phosphoribosyl 1-pyrophosphate; ○, variant enzyme complex using 123 μM 5-phosphoribosyl 1-pyrophosphate. The experiment was also performed at the following concentrations of 5-phosphoribosyl 1-pyrophosphate: 1.23 μM, 1.9 μM, 2.5 μM, 12.3 μM, 24.7 μM and 49.3 μM. At the 3 lower concentrations the results resembled, qualitatively, those shown here with 4.9 μM substrate; at the 3 higher concentrations the results resembled, qualitatively, those shown for 123 μM 5-phosphoribosyl 1-pyrophosphate. These additional data are omitted for the sake of clarity.

closely resembles that from *S. typhimurium*, Co^{2+} was found to support ammonia-dependent anthranilate synthase activity at lower concentrations than Mg^{2+} but Mn^{2+} had no effect [18]. We have examined the utilization of metal ions by measuring the amounts of Mn^{2+} and Mg^{2+} by atomic absorption spectroscopy in the crude extract of *trpE2*, our source of normal enzyme complex. The ratio of total Mg^{2+}/Mn^{2+} in the extract was found to be approximately 350. Since the ratio of the apparent K_m values of Mg^{2+} to Mn^{2+} for the three activities varies from 8.3 to 75 (Tables II, III), there appears to be an excess of Mg^{2+} over Mn^{2+} available for the enzyme complex. This is not surprising since the derepression media in which the cells are grown contain 0.82 mM Mg^{2+} [19] and Mn^{2+} is present only as a contaminant.

Dixon et al. [20] have recently suggested that anthranilate synthase may contain a tightly bound metal ion which aids formation of an "enzyme-ammo-

nia" intermediate by complexing with ammonia. Since the metal ion effects in this study are all dependent on the addition of extraneous metal ions and the apparent K_m for ammonia in the variant enzyme complex is not changed, the alteration in our variant enzyme complex probably affects metal ion sites other than this "tight site".

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