Biochem. Cytol. 7, 37.
Jansson, E. H., and Weaver, E. S. (1964), Abstracts, 147th National Meeting of the American Chemical Society, Philadelphia, Pa., April, p 6H.
Johnston, J. P., and Ogston, A. G. (1946), *Trans. Faraday Soc. 42*, 789.
Kenchington, A. W., and Ward, A. G. (1954), *Biochem. J. 58*, 202.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Steinhardt, J., and Beychok, S. (1964), *in* The Proteins Composition, Structure, and Function, Vol. II, Neurath, H., Ed., New York, N. Y., Academic, Chapter 8.
Stevens, F. S., and Tristram, G. R. (1962), *Biochem. J. 83*, 245.
Tanford (1955), *in* Electrochemistry in Biology and Medicine, Shedlovsky, T., Ed., New York, N. Y., Wiley, Chapter 13.
Tanford, L. (1962), *Advan. Protein Chem. 17*, 69.

Purification and Some Properties of Tryptophanase from *Bacillus alvei**

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ABSTRACT: Tryptophanase was purified from cell-free extracts of *Bacillus alvei*. The enzyme was homogeneous by sedimentation and electrophoretic criteria, and was 3400-fold purified over the crude extract. Potassium or ammonium ions are required for enzyme activity; sodium ions are inhibitory at concentrations >0.1 M. At 80°, the enzyme is only slightly denatured within 10 min but at 100°, 90% of the activity is lost in 10 min. Heat stability is enhanced by pyridoxal phosphate, but not by pyridoxal; cysteine decreases the heat stability.

The Michaelis constant for the enzyme–tryptophan complex was 0.27 mM. The dissociation constants, kinetically determined, for competitive inhibitors were 3.23 mM phenylalanine, 0.107 mM anthranilic acid, and 0.71 mM kynurenine. The enzyme is apparently specific for L-tryptophan, and does not catalyze any chemical change in the above inhibitors. The amino acid composition was reported and differs from the composition of *Escherichia coli* tryptophanase. The turnover number of the enzyme is 1400 moles of indole formed/min per 100,000 g of enzyme. Rabbit antiserum prepared *vs.* pure tryptophanase from *B. alvei* forms a single precipitin band in gel diffusion plates with either pure enzyme or crude extract, but forms no precipitin band with purified enzyme from *E. coli*.

The enzymatic fission of tryptophan to indole, pyruvate, and ammonia is catalyzed by the enzyme tryptophanase. The stoichiometry of this reaction was first shown by Wood *et al.* (1947) who partially purified the enzyme from *Escherichia coli*. They also showed that the catalysis was pyridoxal phosphate dependent. Their enzyme preparation was free of serine and alanine deaminase activity, thus ruling out the possibility that the reaction was analogous to a reversal of the tryptophan synthetase reaction (Tatum and Bonner, 1944) or that the products of the reaction were indole and alanine (Baker and Happold, 1940).

Burns and DeMoss (1962) first isolated the enzyme in pure form from a tryptophan auxotroph of *E. coli* K-12. Their enzyme preparations were pure as judged by sedimentation and electrophoretic criteria. The protein they obtained was of a high molecular weight. A sedimentation coefficient of 9.0 S was obtained and a molecular weight of 490,000 was calculated from sedimentation equilibrium data. The enzyme was found to be unstable in either pure or crude form. Recently Newton *et al.* (1965) have crystallized tryptophanase from a mutant of *E. coli* B. They reported the molecular weight to be 281,000, a value which is probably more nearly correct than that previously estimated by Burns and DeMoss (1962). The enzyme has been found by Newton and Snell (1964) to catalyze the formation of tryptophan from indole and serine. Serine could be replaced by either cysteine or *S*-methylcysteine. The enzyme will catalyze a number of other α,β-elimination reactions analogous to the tryptophanase reaction.

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The physiological significance of the reactions catalyzed by the enzyme has recently been discussed (Newton and Snell, 1965).

In our recent study of the physiological effect of tryptophanase on the biosynthesis of tryptophan in *Bacillus alvei*, the physiological state of the enzyme in this organism was shown to differ from that of *E. coli* (Hoch and DeMoss, 1965). In *B. alvei* the enzyme is not inducible by tryptophan but rather is constitutive and is not subject to catabolite repression. Since the enzyme differed physiologically from that of *E. coli*, it was of interest to examine some catalytic properties of the enzyme. The purification of the enzyme from cell-free extracts of *B. alvei* is reported here along with some general properties.

Materials and Methods

Bacteria and Growth. The parent strain of the organism used in this study was *B. alvei* ATCC 6348. This strain was difficult to disrupt by the usual methods and the yield of enzyme in cell-free extracts was about one-half of the enzyme present in the whole cells. A rough colony variant of this strain was isolated from nutrient agar plates and used for all subsequent experiments. This strain, designated strain F, gives much higher yields of enzyme upon disruption.

The medium used for the isolation of tryptophanase from this organism contained per liter of distilled water: trypticase (Baltimore Biological Laboratories) 20 g, and thiamine hydrochloride, 10 mg. The medium was adjusted to pH 7.0–7.1 before sterilization. The cells were grown at 37° with forced aeration in an American Sterilizer Co. Biogen. After 14–16 hr of growth, the cells were harvested by centrifugation in a Sharples centrifuge and frozen at −20° until preparation of the cell-free extract.

Tryptophanase Assay. Tryptophanase activity was assayed according to Boezi and DeMoss (1961). The reaction mixture contained: pyridoxal 5-phosphate, 40 μg; potassium phosphate buffer, pH 8.0, 300 μmoles; 250 μg of bovine serum albumin; and enzyme solution. The volume was 1.5 ml. The reaction mixture was overlaid with 4 ml of toluene and shaken slowly at 37° for 5 min in a 50-ml erlenmeyer flask. The reaction was initiated by the addition of 0.5 ml of 0.02 M L-tryptophan and incubation was continued at 37° with shaking for 15 min. The reaction was terminated by the addition of 0.1 ml of 2 N NaOH and the shaking was continued for 5 min. The toluene layer was assayed for indole according to the method of Yanofsky (1955).

One unit of enzyme is defined as that amount of enzyme which forms 1.0 μmole of indole/min at 37°. Specific activity is expressed as units of enzyme per milligram of protein.

Protein. Protein was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as the standard.

Pyruvate. Pyruvate was determined by the method of Friedemann and Haugen (1943). Crystalline sodium pyruvate was the standard.

Ammonia. In any reaction mixture where ammonia was to be determined the reaction was terminated by addition of trichloracetic acid at a final concentration of 10%. After 15 min at 0° the precipitated protein was sedimented by centrifugation at 8700*g* for 15 min. The deproteinized supernatant was placed in the outside well of a Conway microdiffusion dish and the ammonia was released by the addition of 1 ml of saturated Na_2CO_3. The inner well contained 1 ml of 0.1 N HCl. After allowing 12 hr at room temperature for diffusion, the ammonia trapped in HCl was determined (Johnson, 1941).

Amino Acid Composition. Protein samples were hydrolyzed in 6 N HCl at 110° for 22, 48, and 72 hr. Amino acids in the hydrolysate were estimated in a Beckman/Spinco amino acid analyzer.

Gel Diffusion. Ouchterlony plates were prepared with 0.02 M potassium phosphate, pH 6.5, containing 0.85% (w/v) Oxoid Inoagar No. 2, 0.01% (w/v) merthiolate (Lilly), and 0.07 M KCl. Antiserum was prepared from rabbits which had received a course of subcutaneous inoculations of a mixture of pure enzyme and complete Freund's adjuvant (Difco). Reproductions of the plates were made with Type 55 P/N Polaroid film and a Crown Graphic Model 45 camera with film packet adaptor. Dark-field illumination was employed.

Purification of Tryptophanase. In the following purification procedure, suspension of DEAE-cellulose or cells was usually accomplished by stirring to a smooth slurry with a motor-driven plastic propeller. Unless otherwise indicated, the SS-34 head of the Servall RC-2 centrifuge was employed.

STEP 1. Frozen cells (400–500 g) were thawed and suspended in 2 volumes (ml/g) of 0.005 M KH_2PO_4 (no pH adjustment). The slurry was placed in an Eppenbach colloid mill (Gifford-Wood Co.) with a quantity of 100-μ glass beads (Minnesota Mining and Manufacturing Co.) equal to the weight of the cells in the suspension. The cells were disrupted in the mill for 40 min. The temperature of the contents rose from 20 to 25° during this period. The disrupted cell suspension was centrifuged at 20,000*g* for 15 min and the pellet was discarded. The supernatant was made 2.5 mM with respect to pyridoxal 5-phosphate by the addition of 0.05 M pyridoxal 5-phosphate solution. The pH was adjusted to 6.0 at 0° by the addition of cold 6 N acetic acid. The pH meter (Metrohm) previously had been calibrated at 22° (room temperature) with standard buffer.

STEP 2. Samples (100–150 ml) of the cell-free extract in 500-ml erlenmeyer flasks were swirled in a boiling water bath until the temperature of the contents reached 70°. At this time the flasks were transferred to a water bath held at 71–72°. After 10 min at this temperature with occasional swirling the flasks were rapidly cooled in a salt–ice bath to 5°. The heated suspension was centrifuged at 20,000*g* for 15 min to sediment the denatured protein. The pellets were extracted with 0.3 volume (of the cell-free extract) of distilled water and recentrifuged. The supernatant was combined with the original supernatant and the pellets were discarded.

The supernatant was frozen at this stage. The enzyme was stable for 2–3 weeks in this condition.

STEP 3. DEAE-cellulose was suspended in 10 volumes (ml/g) of 1 N NaOH, filtered on a sintered glass filter, and washed on the filter with 0.005 M KH_2PO_4, pH 6.0, supplemented with 2.0 mM EDTA, until the pH of the filtrate was 6.0. The DEAE-cellulose was suspended in 10 volumes (ml/g) of the same buffer and stirred for 1–2 hr. The DEAE-cellulose was again filtered on a sintered glass filter until most of the excess water was removed. The DEAE-cellulose was further dried in a 45° oven until no longer moist to the touch.

A convenient volume (usually 1200–1400 ml) of frozen supernatant from step 2 was thawed and adjusted to pH 6.0 by the addition of cold 6 N acetic acid. For each milligram of protein present, 5 mg of DEAE-cellulose was suspended in 10 volumes (ml/g) of 0.1 mM pyridoxal 5-phosphate–0.1 mM 2-mercaptoethanol[1] (0.1 M PLP-ME) and mixed to form a smooth slurry. The DEAE-cellulose was recovered by filtration on a sintered glass funnel. The moist pad was added to the thawed enzyme preparation and stirred for 5 min at 0°. The suspension was filtered on a sintered glass funnel and the pad of DEAE-cellulose rinsed on the funnel with a volume of 0.05 M potassium phosphate (pH 6.0, supplemented with 0.1 M PLP-ME) equal to the original volume of the thawed supernatant. The enzyme was eluted by suspending the moist pad in elution buffer (0.6 M potassium phosphate, pH 6.0, supplemented with 0.1 M PLP-ME) at the ratio of 70 mg of DEAE-cellulose/ml of buffer. After 5 min of stirring the suspension was filtered on a sintered glass funnel and the DEAE-cellulose pad was washed with a little additional elution buffer.

STEP 4. The eluate from the DEAE-cellulose was cooled to 0° in an ice bath and solid ammonium sulfate was added to 45% saturation (27.7 g of ammonium sulfate/100 ml of solution). After 30 min the suspension was centrifuged at 14,000*g* for 35 min (GSA head of Servall RC-2 centrifuge). The supernatant was discarded.

STEP 5. The pellet from the above step was suspended in a volume of cold 37% saturated ammonium sulfate in 0.1 M potassium phosphate (pH 7.0, supplemented with 0.1 M PLP-ME, the volume equal to one-tenth of the volume of the DEAE-cellulose eluate. In this and subsequent extractions, the buffer pH had been measured before solution of the ammonium sulfate. The suspension was kept at 0° for 15 min and centrifuged at 20,000*g* for 15 min. The supernatant was discarded.

The pellet from the 37% step was suspended in a volume of cold 30% saturated ammonium sulfate in 0.1 M potassium phosphate (pH 7.0, supplemented with 0.1 M PLP-ME), the volume equal to one-tenth of the volume of the DEAE-cellulose eluate. As in the previous step the resuspension was carried out as nearly as possible to 0°. The suspension was kept at 0° for 15 min and then was centrifuged at 20,000*g* for 15 min. The supernatant contained 5–10% of the original enzyme units but was discarded.

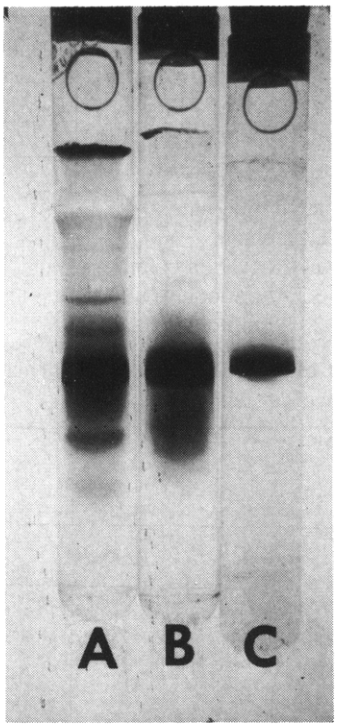

FIGURE 1: Disc electrophoresis of tryptophanase preparations at varying degrees of purity. Migrations were made in 7.5% gels. The enzyme samples (0.1 ml, containing 5% sucrose) were layered through the buffer solution onto the top of the upper gel. A, 265 μg of protein, sp act. 7.15; B, 110 μg of protein, sp act. 15.6; C, 70 μg of protein, sp act. 15.6. Sample B was passed through a column (2.5 × 80 cm) of Sephadex G-200; the tryptophanase activity peak samples were pooled to obtain sample C.

The pellet from the 30% step was suspended in a volume of cold 0.1 M potassium phosphate (pH 7.0, supplemented with 0.1 M PLP-ME), the volume equal to 0.05 volume of the DEAE-cellulose eluate. The suspension was centrifuged at 20,000*g* for 15 min. The pellet was discarded.

STEP 6. The supernatant from the above step was brought to 35% saturation by the addition of solid ammonium sulfate. After 20 min at 0°, the suspension was centrifuged at 20,000*g* for 15 min. The precipitate was dissolved in a volume of 0.1 M potassium phosphate (pH 7.0, supplemented with 0.1 M PLP-ME), the volume equal to one-half of the volume of the previous step.

The redissolved pellet was allowed to come to room temperature and solid ammonium sulfate was added to 25% saturation. This solution was stored overnight at 0°. The precipitate that formed was centrifuged at 20,000*g* for 15 min and resuspended in a volume of 0.1 M potassium phosphate (pH 7.0,

[1] Abbreviation used: PLP-ME, pyridoxal 5-phosphate–2-mercaptoethanol.

TABLE I: Purification of *B. alvei* Tryptophanase.[a]

Purifcn Step	Vol. (ml)	Protein (mg)	Total Units	Sp Act.	Purifcn Factor	Recov (%)
(1) Cell-free extract				0.0039		100
(2) Heated supernatant	1230	9000	676	0.075	19	100
(3) DEAE eluate	730	2197	576	0.26	67	85
(5) Back-extraction buffer	40	123	489	3.9	1000	72
(5) 0–35% ammonium sulfate	25	73	540	7.4	1900	80
(6) 0–25% ammonium sulfate	10	26.2	350	13.4	3400	52

[a] One unit of enzyme catalyzes the formation of 1.0 μmole of indole/min at 37°. Specific activity is units of enzyme per milligram of protein.

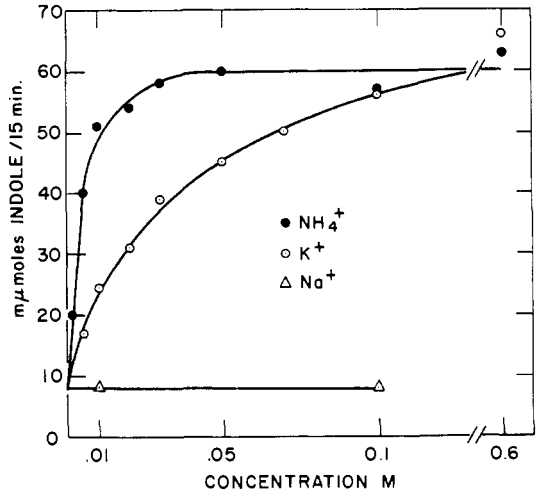

FIGURE 2: Stimulation of tryptophanase activity by monovalent cations. Purified enzyme (sp act. 6.66) was freed of cations by filtration through a 1 × 9 cm column of Sephadex G-25 equilibrated with 0.05 M Tris–Cl containing 0.1 mM pyridoxal phosphate. The reaction mixture contained: 200 μmoles of Tris–Cl, pH 8.0, 40 μg of pyridoxal phosphate, and 3 μg of enzyme. The monovalent cations were added as the corresponding chlorides. The reaction was initiated by the addition of 10 μmoles of L-tryptophan, and was incubated at 37° for 15 min. The reaction was terminated with 0.1 ml of 2 N NaOH; indole was determined on the toluene layer.

supplemented with 0.1 M PLP-ME), the volume equal to one-half of the volume of the previous step.

The purification procedure presented above yields an enzyme preparation that is pure by ultracentrifugation (J. A. Hoch and R. D. DeMoss, submitted for publication, 1966) and disc electrophoretic (Figure 1) criteria. The purification of tryptophanase from *B. alvei* was accomplished because of two important properties of the enzyme. The enzyme is stable to heat treatment under the proper conditions and is insoluble in ammonium sulfate solutions. The insolubility of the enzyme is most pronounced at 0° and a slight rise in temperature will render the enzyme soluble. It is important during the back-extraction steps to keep the enzyme solutions as near to 0° as possible to prevent loss of enzyme. Tryptophanase is unstable when adsorbed to DEAE-cellulose. The batchwise DEAE-cellulose step was adopted to shorten the time that the enzyme was in contact with the DEAE-cellulose. This step of the procedure must be carried out in the shortest time possible to prevent loss of enzyme.

A summary of a typical purification is shown in Table I. The values for the cell-free extract are missing because the extracts are made, heated, and frozen from large batches of cells and the rest of the purification procedure is not representative of any one particular cell-free extract. The heated supernatant reflects the activity of the cell-free extract since no loss in total units occurs upon heating. The volume of the heated supernatant in the protocol is roughly equivalent to that obtained from 1 lb (wet weight) of cells.

Results

Optimum pH for Tryptophanase Activity. Tryptophanase shows maximal activity at pH 9.0 in cell-free extracts but upon purification the optimum shifts to the range pH 7.5–8.4. The activity of the enzyme falls to almost zero in Tris buffer without the addition of ammonium or potassium ions. Even with the addition of these ions the activity is less than one-half of that in potassium phosphate buffer.

Cation Requirement for Tryptophanase Activity. The enzyme shows an absolute requirement for either ammonium or potassium ions. Although both ions are effective, ammonium ions, at concentrations <0.1 M, give the greater stimulation of tryptophanase activity (Figure 2). Sodium is inactive and at concentrations >0.1 M shows a progressive inhibition of the reaction.

Stoichiometry of the Tryptophanase Reaction. The

TABLE II: Stoichiometry of the Tryptophanase Reaction.[a]

Expt No.	Indole (μmoles/ml)	Pyruvate (μmoles/ml)	Ammonia (μmoles/ml)
1	0.101	0.106	...
2	0.097	0.107	0.116
3	0.100	0.108	0.124
4	0.098	0.094	...
5	0.111	0.120	0.108
6	0.076	0.078	0.067
Molar ratio	1.00	1.05	1.07

[a] Conditions: 50 μg of tryptophanase, 200 μg of pyridoxal 5-phosphate, 1500 μmoles of potassium phosphate, pH 8.0, and 50 μmoles of L-tryptophan in a total volume of 10 ml. Reaction mixture incubated 20 min at 37°. Reaction terminated with 1 ml of 100% TCA. After 15 min at 0° the mixture was centrifuged at 8700*g* and the products were determined on the supernatant as described in the Methods.

TABLE III: The Effect of Various Compounds on the Heat Stability of Crude Preparations of Tryptophanase.[a]

Addn	Time (min) at 60°	% of Tryptophanase Act. in Nonheated Control
Tryptophan	10	99
	20	75
Tris	10	64
	20	48
Pyridoxal	10	64
	20	47
Pyridoxal phosphate	10	95
	20	95
EDTA	10	93
	20	65
Cysteine	10	40
	20	26
None	10	84
	20	53

[a] Conditions: A crude extract of *B. alvei* in 0.1 M acetate–phosphate–borate buffer, pH 6.0, contained the above compounds, 0.01 M, and was heated at 60° for the specified times, prior to estimation of tryptophanase activity. The solutions of the various compounds were adjusted to pH 6.0 before use. The reaction mixture contained 300 μmoles of potassium phosphate, pH 8.0, 40 μg of pyridoxal phosphate, 250 μg of bovine serum albumin, 1 mM L-tryptophan, and enzyme solution. The enzyme assay mixture was incubated for 15 min at 37°.

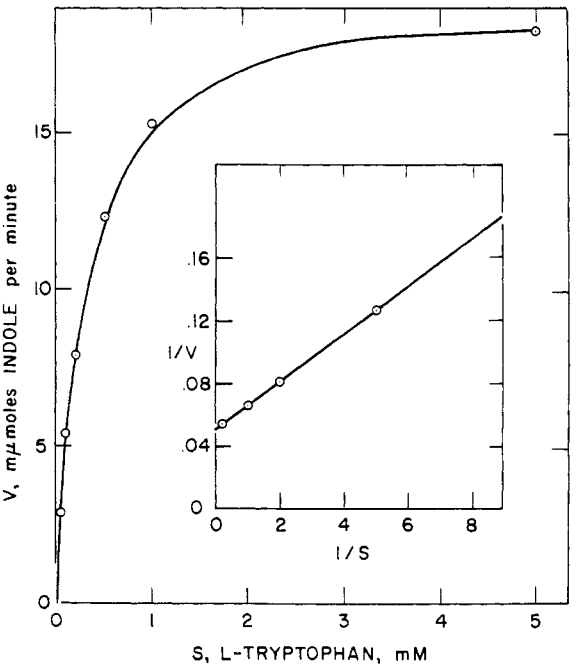

FIGURE 3: Affinity of tryptophanase for tryptophan. Each reaction flask contained: 300 μmoles of potassium phosphate, pH 8.0, 40 μg of pyridoxal phosphate, 250 μg of bovine serum albumin, and various levels of L-tryptophan. The reaction was initiated with the addition of enzyme (360 μg, sp act. 2.0) and incubated for 15 min at 37°.

results of several determinations of the products of the tryptophanase reaction are shown in Table II.

Stability of Tryptophanase. The enzyme was unstable in crude preparations but a high degree of stability was gained after the heat step of the purification procedure. The enzyme was most stable at pH 6.0, and could be heated to 80° for 10 min with slight loss of activity, although exposure to 100° for 10 min led to irreversible loss of 90% of the activity. Table III shows the effect of additions on the heat stability of the enzyme. The protective effect of pyridoxal phosphate and the lack of a protective effect by pyridoxal may be noted. Enhanced stability was found later to be effective at much lower concentrations (*e.g.*, 0.1 mM of pyridoxal phosphate). The addition of the substrate did not protect as well as did addition of the coenzyme.

Purified preparations of the enzyme are not stable at pH 6.0 and show progressive inactivation at this pH even when frozen. Raising the pH to 7.0 prevented inactivation but repeated freezing and thawing slowly inactivated the enzyme.

Affinity of Tryptophanase for Tryptophan. The saturation curve for the tryptophanase–L-tryptophan complex is shown in Figure 3. The K_m for L-tryptophan calculated from these data is 0.272 mM. D-Tryptophan was inert as a substrate.

Inhibition of Tryptophanase by Other Amino Acids.

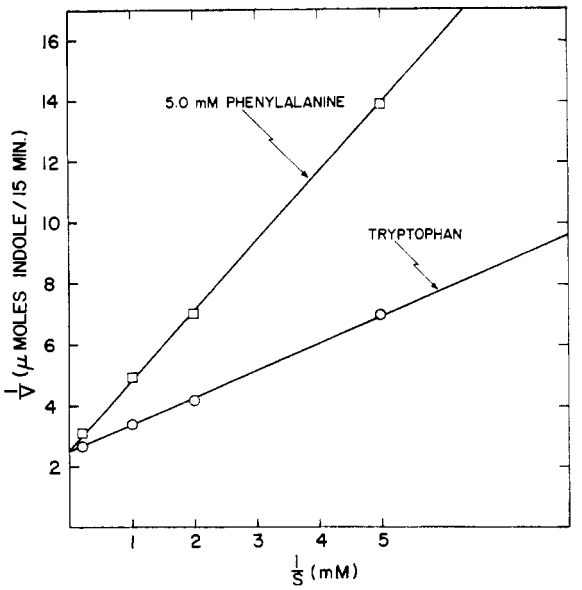

FIGURE 4: Inhibition of tryptophanase activity by phenylalanine. Each reaction flask contained: 300 μmoles of potassium phosphate, pH 8.0, 40 μg of pyridoxal phosphate, 250 μg of bovine serum albumin, and various levels of L-tryptophan. L-Phenylalanine was present at 5 mM. The reaction was initiated by the addition of enzyme (4 μg, sp act. 13.3). After 15 min of incubation at 37° the reaction was terminated by the addition of 0.1 ml of 2 N NaOH; indole was determined on the toluene layer.

Table IV shows the effect of various amino acids on tryptophanase activity. Enzyme activity was not inhibited by serine or threonine, amino acids which usually undergo degradation by an α,β elimination. The strong inhibition by cysteine and phenylalanine was further investigated. Phenylalanine showed competitive inhibition kinetics (Figure 4) and a K_i of 3.23 mM was calculated. Cysteine is not effective as an inhibitor at 1 mM (Table V).

Inhibition of Tryptophanase by Anthranilic Acid and Kynurenine. The affinity of tryptophanase from *B. alvei* for anthranilic acid was found to be higher than the affinity for tryptophan.[2] The K_i for anthranilic acid from Figure 5 was computed to be 0.107 mM. A compound related to anthranilic acid, kynurenine, was found also to inhibit competitively. The K_i for kynurenine was 0.71 mM. The possibility that kynurenine could be cleaved by tryptophanase was entertained but the expected products, *o*-aminobenzaldehyde and pyruvate, were not found in the single experiment attempted.

Other Catalytic Properties of Tryptophanase. Attempts to demonstrate tryptophan synthesis with tryptophanase from *B. alvei* have been uniformly unsuccessful. In addition, added radioactive indole did not exchange with the indole moiety of tryptophan in the single experiment attempted. The dehydration of serine to pyruvate occurs but is a linear function of the serine supplied, and the rate of the reaction at the highest serine concentration (250 mM) was only 5% of the rate of the tryptophanase reaction.

Antigenic Properties. Antiserum prepared *vs.* purified tryptophanase formed a single precipitin band with pure tryptophanase and with crude extracts of *B. alvei* (Figure 6). No cross-reaction was observed with partially purified tryptophanase from *E. coli*. Antiserum prepared *vs.* tryptophanase from *E. coli* did not precipitate tryptophanase from *B. alvei*.

Amino Acid Composition. Table VI gives the amino acid composition of tryptophanase from *B. alvei* and for comparison the amino acid composition of tryptophanase from *E. coli* reported by Burns and DeMoss (1962). The values for serine, threonine and tyrosine

TABLE IV: Inhibition of Tryptophanase Activity by Amino Acids.[a]

Amino Acid	μMoles of Indole Formed	% Inhibition
L-Alanine	0.212	15
L-Cysteine	0.045	82
L-Asparagine	0.195	22
Glycine	0.230	8
L-Isoleucine	0.203	19
L-Leucine	0.227	9
L-Phenylalanine	0.141	44
L-Serine	0.233	7
L-Threonine	0.223	11
L-Valine	0.205	18
None	0.249	..

[a] Each reaction flask contained: 300 μmoles of potassium phosphate, pH 8.0, 40 μg of pyridoxal phosphate, 250 μg of bovine serum albumin, and 1 mM L-tryptophan. The inhibitors were present at 10 mM. The reaction was initiated by the addition of enzyme (4 μg, sp act. 13.3) and incubated at 37° for 15 min.

TABLE V: Lack of Effect of Cysteine on the Tryptophanase Reaction.[a]

L-Tryptophan (mM)	Cysteine (mM)	Indole Formed (μmoles)
5.0	0	0.197
5.0	1	0.194
0.2	0	0.071
0.2	1	0.075

[a] For experimental conditions, see Table IV.

[2] This comparison is made on the assumption that K_m represents a true dissociation constant.

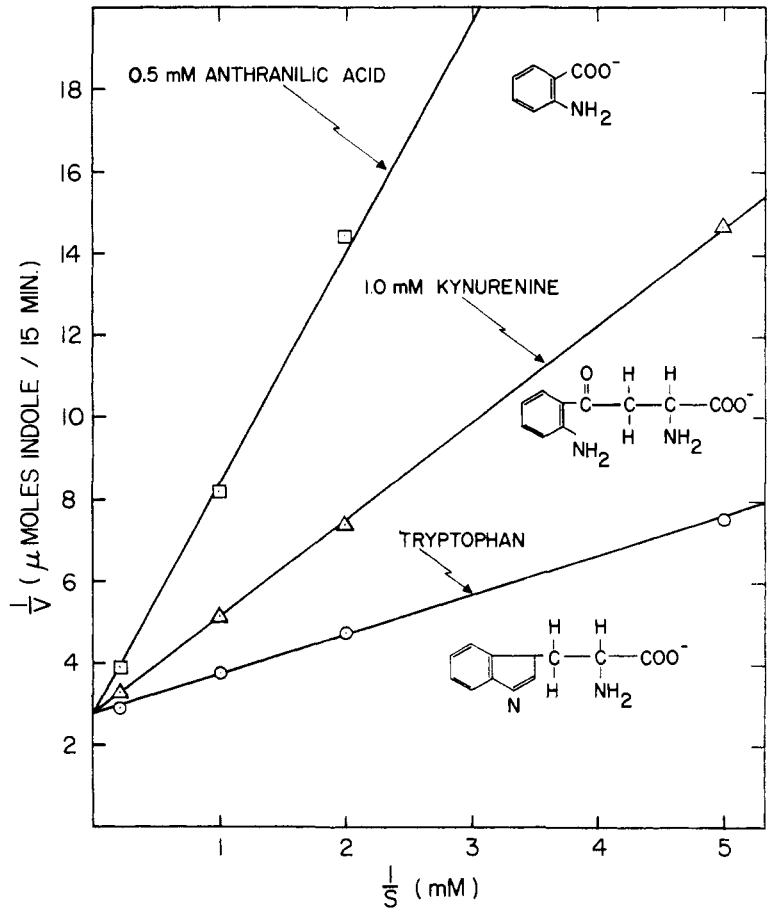

FIGURE 5: Inhibition of tryptophanase by anthranilic acid and kynurenine. Each reaction flask contained: 300 μmoles of potassium phosphate, pH 8.0, 40 μg of pyridoxal phosphate, 250 μg of bovine serum albumin, and various levels of L-tryptophan. Anthranilic acid was present at 0.5 mM. Kynurenine was present at 1.0 mM. The reaction was initiated with the addition of enzyme (5 μg, sp act. 13.3) and incubated for 15 min at 37°.

were obtained by extrapolation, to zero time, of the values for 22, 48, and 72 hr of hydrolysis times. Cysteine and tryptophan were not determined.

The two enzymes differ most significantly in the relative proportions of alanine and glutamic acid. Tryptophanase from *B. alvei* contains fewer basic amino acids and more acidic amino acids than does tryptophanase from *E. coli*.

Discussion

The optimum pH for activity of tryptophanase from *B. alvei* is similar to that observed for the enzyme from *E. coli* (Burns and DeMoss, 1962). The apparent shift in optimum pH from 9.0 to the range 7.5–8.4 as a consequence of purification is possibly correlated with the first (heat) step of purification. At the present time, the heat step cannot reasonably be eliminated from the procedure. Activity of the enzyme from *B. alvei* is inhibited by Tris, as is that of the enzyme from *E. coli* (Burns, 1962).

The tryptophanase of *E. coli* shows identical monovalent cation stimulation kinetics for the reaction (Newton and Snell, 1964). Wada *et al.* (1958) have postulated that the ammonium ion is an integral part of the substrate–cofactor–enzyme complex, although no definite role for the ammonium ion could be assigned. A reciprocal plot of velocity *vs.* metal ion concentration is frequently linear (Malmström and Rosenberg, 1959) and indicates a metal–enzyme complex. A plot of this type for the tryptophanase of *B. alvei* and the ammonium ion gives an apparent K_m of 2.2 mM.

Wood *et al.* (1947) found that the products of the *E. coli* tryptophanase reaction were indole, pyruvate, and ammonia in equimolar amounts. As expected, in the present analyses the products were equimolar amounts of indole, pyruvate, and ammonia. The value for indole is always low in an experiment of this type since the reaction must be stopped with acid to prevent the release of ammonia. Under acid conditions some of the indole and pyridoxal form a complex that cannot be detected with the indole reagent (Scott, 1961).

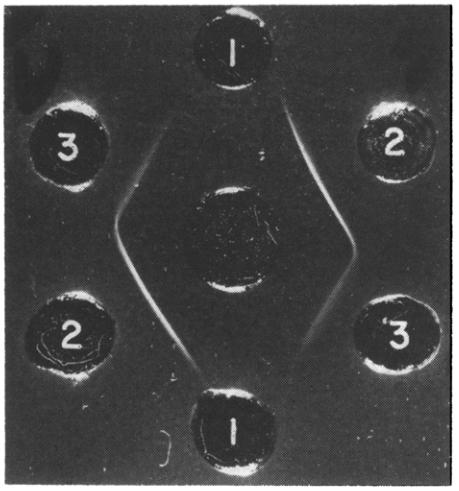

FIGURE 6: Gel diffusion analysis of antigenic properties of tryptophanase. The center well contained antiserum prepared in response to pure tryptophanase of *B. alvei*. The outer wells contained: (1) purified preparation of tryptophanase from *E. coli*, (2) pure tryptophanase from *B. alvei*, and (3) crude cell-free extract from *B. alvei*.

TABLE VI: A Comparison of the Amino Acid Composition of *B. alvei* and *E. coli* Tryptophanases.[a]

Amino Acid	Moles % in *B. alvei* Tryptophanase	Moles % in *E. coli* Tryptophanase[b]
Lysine	4.3	8.0
Histidine	2.1	1.8
Arginine	6.8	5.8
Aspartic	8.9	7.6
Threonine	6.1	4.9
Serine	4.1	1.2
Glutamic acid	12.1	4.9
Proline	4.5	5.0
Glycine	9.2	9.6
Alanine	9.2	13.1
Valine	5.4	7.2
Methionine	2.4	3.7
Isoleucine	6.4	7.1
Leucine	9.4	9.6
Tyrosine	4.4	5.4
Phenylalanine	4.7	4.8

[a] The enzyme used for amino acid analysis was homogeneous by ultracentrifugation and disc electrophoresis criteria. Samples (2.84 mg each) were hydrolyzed in 6 N HCl for 22, 48, and 72 hr at 110°. The amino acids obtained were chromatographed in the Beckman amino acid analyzer. The values for *B. alvei* represent the average for the three hydrolysis times except for serine, threonine, and tyrosine which were extrapolated to zero-time hydrolysis. [b] From Burns and DeMoss (1962).

Burns and DeMoss (1962) found that the tryptophanase of *E. coli* was unstable in both crude and pure preparations. The crystalline enzyme obtained by Newton and Snell (1964) was also relatively unstable under all conditions tested. In contrast, the enzyme from *B. alvei*, though unstable in crude extract, was very stable in purified form, possibly due to the inactivation of proteolytic enzymes during the heat step of purification.

From the data in Figure 3, the apparent K_m for L-tryptophan was calculated to be 0.272 mM. This value corresponds closely to the value of 0.33 mM reported for tryptophanase from *E. coli* (Newton *et al.*, 1965).

Since the tryptophanase of *E. coli* is capable of carrying out a number of α,β-elimination reactions it was of interest to determine if the tryptophanase of *B. alvei* could cleave any other amino acids. The inhibition of tryptophanase by amino acids should give a clue as to which ones are effective in binding at the active site and perhaps capable of being cleaved. Serine and threonine, ordinarily degraded by α,β elimination, were not effective as inhibitors. Cysteine, which may also undergo α,β elimination, effected inhibition when present at a concentration of 10 mM, but was not effective at 1 mM. It is suggested that the inhibition at 10 mM is due to a reaction other than competition for the active site of the enzyme. Evidence for this conclusion can be adduced from the increased heat lability of the enzyme caused by cysteine addition (Table III).

Burns (1962) found that tryptophanase from *E. coli* was inhibited by anthranilic acid. The affinity of the enzyme for this compound was high (0.63 mM) and the inhibition was competitive. Although anthranilic acid does not closely resemble tryptophan, a more detailed examination reveals that the presence of a benzene ring and a nitrogen with a lone pair of electrons confers a similarity to indole. For tryptophanase from *B. alvei*, K_i for anthranilic acid was calculated to be 0.107 mM. Thus, the enzyme exhibits a greater affinity for anthranilate than for its substrate, L-tryptophan.[2] The K_i for indole was not determined, but the strong inhibition of the tryptophanase reaction by indole may explain the lack of tryptophan synthesis by the enzyme.

Newton and Snell (1964) have shown that tryptophanase of *E. coli* will catalyze the formation of tryptophan from indole and serine or cysteine. In addition, the enzyme catalyzes dehydration of serine to pyruvate and desulfhydration of cysteine to pyruvate. The enzyme thus appears to act as a general catalyst for α,β elimination, and the specificity is presumed to depend on the ability of the substrate to bind at the active site rather than specificity in the reaction mecha-

nism. The enzyme from *B. alvei* appears to exhibit a narrower specificity than that from *E. coli*. Neither tryptophan synthesis nor indole exchange into tryptophan was observed. The kinetics of L-serine deamination by the enzyme from *B. alvei* differed from those observed by Newton and Snell (1964) with tryptophanase from *E. coli*. Furthermore, the data were consistent with the conclusion that the enzyme affinity for L-serine was at least twice as great in *E. coli* as in *B. alvei*.

The instability of tryptophanase from *E. coli* precluded determination of the turnover number from the purified enzyme. Burns and DeMoss (1962) determined the turnover number of the enzyme from the activity of a crude extract and the percentage composition of tryptophanase in the crude extract, the latter having been determined by an independent method. The recalculated turnover number was 4000 moles of indole formed/min per 100,000 g of protein. A similar calculation of the data of Newton *et al.* (1965) assuming a percentage composition of tryptophanase in the crude extract of 10% (Newton and Snell, 1964) yields a value of 3000 moles of indole formed/min per 100,000 g of protein. The latter may be a low estimate, due to the instability of the enzyme. Since tryptophanase from *B. alvei* is stable, the turnover number can be determined accurately from the purified enzyme. The value calculated from experiments with purified enzyme was 1400 moles of indole formed/min per 100,000 g of protein. This value is much lower than that of tryptophanase from *E. coli* and may reflect a difference in the reaction mechanisms and in the structures of the enzymes. The latter point is supported by the differences in amino acid composition, substrate specificity, and antigenic structure of the two enzymes.

References

Baker, J. W., and Happold, F. C. (1940), *Biochem. J. 29*, 1918.

Boezi, J. A., and DeMoss, R. D. (1961), *Biochim. Biophys. Acta 49*, 471.

Burns, R. O. (1962), Ph.D. Thesis, University of Illinois, Urbana, Ill.

Burns, R. O., and DeMoss, R. D. (1962), *Biochim. Biophys. Acta 65*, 233.

Friedemann, T. C., and Haugen, G. E. (1943), *J. Biol. Chem. 147*, 415.

Hoch, J. A., and DeMoss, R. D. (1965), *J. Bacteriol. 90*, 604.

Johnson, M. J. (1941), *J. Biol. Chem. 137*, 575.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Malmström, B. G., and Rosenberg, A. (1959), *Advan. Enzymol. 1*, 131.

Newton, W. A., Morino, Y., and Snell, E. E. (1965), *J. Biol. Chem. 240*, 1211.

Newton, W. A., and Snell, E. E. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 382.

Newton, W. A., and Snell, E. E. (1965), *J. Bacteriol. 89*, 355.

Scott, T. A. (1961), *Biochem. J. 80*, 462.

Tatum, E. L., and Bonner, D. (1944), *Proc. Natl. Acad. Sci. U. S. 30*, 30.

Wada, H., Yoshimatsu, H., Loizuma, T., Inoue, F., Ito, K., Morisue, T., Nasu, H., Ito, H., Sakamoto, Y., and Ichihara, K. (1958), Proceedings of the International Symposium on Enzyme Chemistry, Tokyo and Kyoto, 1957, p 148.

Wood, W. A., Gunsalus, I. C., and Umbreit, W. W. (1947), *J. Biol. Chem. 170*, 313.

Yanofsky, C. (1955), *Methods Enzymol. 2*, 233.