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MOLECULAR SIZE OF THE ACTING SPECIES OF TRYPTOPHANASE

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SUMMARY

The tryptophanase enzyme from *Bacillus alvei* which carries out both α,β-elimination and β-replacement reactions was subjected to centrifugation at 37 °C on linear sucrose gradients containing complete reaction mixtures including substrate. The reaction proceeded during the course of sedimentation. After fractionation of the gradients the amount of product present was determined and revealed that the tetrameric form of the enzyme is responsible for both types of activities and that the dimer is inactive in both reactions, regardless of the substrate used. When tryptophan was used as the substrate, the presence of the three products indole, pyruvate, and ammonia was determined. It was found that during the course of the reaction indole, probably due to its greater affinity for tryptophanase, is released from the enzyme complex at a later time than are pyruvate and ammonia which apparently are released simultaneously. It was also found that cellulose nitrate tubes reversibly bind large amounts of indole.

INTRODUCTION

Many properties of the enzyme tryptophanase from *Bacillus alvei* have been characterized in this laboratory. The purification, physiological role, and some physicochemical attributes[1–3] as well as the physical properties and kinetics (refs 4, 5 and 16) have been extensively investigated. The enzyme from *B. alvei* exists normally as a tetramer which consists of four identical monomers[4,5]. However, the tetramer readily dissociates to dimers in the presence of Tris and in the absence of pyridoxal 5′-phosphate (PLP)[6,7]. As in the case of the tryptophanase from *Escherichia coli*[8,9] the enzyme from *B. alvei* has been demonstrated to carry out both α,β-elimination reactions and β-replacement reactions with a variety of substrates[16]. These reactions are as follows:

(1) α,β-elimination reaction:

$$RCH_2CHNH_2COOH + H_2O \rightarrow CH_3COCOOH + RH + NH_3$$

Abbreviation: PLP, pyridoxal 5′-phosphate.

(2) β-replacement reaction:

$$RCH_2CHNH_2COOH + R'H \rightarrow R'CH_2CHNH_2COOH + RH$$

The tryptophanase and dehydratase reactions are of the α, β-elimination type while the synthase reactions are of the β-replacement type.

At the time the present studies were initiated, kinetic studies of coenzyme activation by O'Neil[5] and Hoch and DeMoss[16] had suggested a difference between the two types of reactions. They found that the α, β-elimination reactions all have sigmoidal activation curves indicating cooperativity among the PLP sites, and they concluded that if the tetramer, or even the dimer, is the active form of the enzyme, the binding of PLP to each monomer unit may be required before catalysis can proceed. However, in the β-replacement reactions no such cooperativity in the PLP activation curves was observed. This indicated that the same degree of association of enzyme and coenzyme is not required for the β-replacement reaction as for the α, β-elimination reaction.

In light of this information it was of interest to determine whether the same aggregational state of the enzyme, *i.e.* dimer or tetramer, is responsible for both types of reactions or whether the two types of activities are carried out by different forms of the enzyme.

To answer this question an entirely new technique had to be developed. Although linear sucrose gradients were employed, they were used in a new manner, *i.e.* they contained the complete reaction mixture including substrate and were centrifuged at 35–37 °C.

The purpose of this new procedure was not to determine the ultimate point to which the various forms of the enzyme had migrated, but rather to determine where the enzyme had acted during its progress through the gradient. This could be done by fractionating the gradient after centrifugation and measuring the amount of product that had been formed by the *acting* enzyme as it moved through the gradient. The distribution of product throughout the gradient reflects the size of the acting enzyme and does not accurately reflect the location of the various forms of the enzyme after centrifugation.

EXPERIMENTAL PROCEDURE

Materials

Tryptophanase from *B. alvei* was purified by the procedure of Hoch *et al.*[3] and had a specific activity of approx. 13. Tryptophanase from *Aeromonas liquefaciens* was a gift from J. L. Cowell of this laboratory. All other enzymes and chemicals used in the study were obtained commercially and used without further purification.

Sucrose density gradient centrifugation

Sucrose density gradient centrifugation was carried out in essentially the same manner as described by Martin and Ames[10] with some variations as described below. Under the following conditions the enzyme retained approx. 93% of its original activity. The temperature of centrifugation was 35–37 °C unless otherwise specified. The sucrose gradients (5–20%) contained complete reaction mixtures and were prepared as follows:

Tryptophanase reaction mixture consisted of the following: 100 mM buffer

(Tris–HCl or potassium phosphate depending on the experiment), 40 μM PLP, 1.47 μM bovine serum albumin, 8 mM L-tryptophan, and sucrose.

Serine dehydratase reaction mixture consisted of the following: 100 mM buffer (Tris–HCl or potassium phosphate), 40 μM PLP, 1.47 μM albumin, 400 mM serine, and sucrose.

S-Methyl-L-cysteine dehydratase reaction mixture consisted of the following: 100 mM buffer (Tris–HCl or potassium phosphate), 40 μM PLP, 1.47 μM albumin, 30 mM *S*-methyl-L-cysteine, and sucrose.

Tryptophan synthase reaction mixture consisted of the following: 100 mM buffer (Tris–HCl or potassium phosphate), 40 μM PLP, 1.47 μM albumin, 400 mM serine, 1.5 mM indole, and sucrose.

Immediately prior to centrifugation, 30 pmoles of pure tryptophanase (in 100 μl of buffer) was layered on the top of each gradient. In addition, 10 μl of the appropriate internal marker protein was added to the top of each gradient. The proteins used as markers were lactate dehydrogenase (62.5 units/ml) and catalase (100 000 units/ml).

After centrifugation, approximately thirty 165-μl fractions were collected from each gradient and were immediately placed in an ice bath. Significant amounts of the products could not have been formed between the times centrifugation was stopped and the fractions collected and placed in the ice bath. Preliminary experiments in which samples from each fraction were incubated at 37 °C for 10 min showed no additional formation of products.

Determination of products in gradient fractions

The amount of each of the products formed by tryptophanase activity during centrifugation was measured by the following procedures: (1) The presence of indole was determined by the addition of 3 ml of color reagent (14.7 g of *p*-dimethylaminobenzaldehyde in 948 ml of 95% ethanol *plus* 52 ml of concentrated H_2SO_4) to a sample from each gradient fraction, and the color was read at 568 nm on a Gilford 300-N spectrophotometer after a 20-min incubation at room temperature. (2) The presence of pyruvate was determined by using lactate dehydrogenase. 3 ml of a reaction mixture containing 6.25 units of lactate dehydrogenase, 100 mM potassium phosphate buffer (pH 7.0), and 30–100 μM NADH was added to a sample of each fraction and was then incubated at 37 °C for 15 min. This reaction involves the conversion of pyruvate and NADH (which has a molar extinction coefficient of $6.22 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ at 340 nm) to lactate and NAD^+. After incubation the absorbance was measured at 340 nm on a Zeiss PMQII spectrophotometer. (3) The presence of ammonia was determined by adding sufficient water to a sample from each gradient fraction to bring it to a final volume of 1.0 ml, then 2.0 ml of Johnson's reagent[11] and 1.0 ml of 6 M NaOH were added to each. After a 15-min incubation at room temperature the color was read at 410 nm on a Gilford 300-N spectrophotometer.

The pyruvate and ammonia produced by the serine dehydratase and *S*-methyl-L-cysteine dehydratase activities were measured by the procedures described for tryptophanase activity.

The amount of indole converted to tryptophan by the tryptophan synthase activity was determined by measuring the indole present in each gradient fraction as described for the tryptophanase activity.

The internal reference proteins were assayed as follows:

(1) Catalase: 10-μl samples of each fraction were assayed in 3.0 ml of a reaction mixture containing 10 mM potassium phosphate buffer (pH 7.5) and 7 mM H_2O_2; the activity was monitored directly on a Gilford 2000 recording spectrophotometer at a wavelength of 240 nm.

(2) Lactate dehydrogenase: 10-μl samples of each fraction were incubated for 15 min at 37 °C in 3.0 ml of a reaction mixture containing 100 mM potassium phosphate buffer (pH 7.0), 130 μM NADH and 7 mM sodium pyruvate. The absorbance was measured at 340 nm.

Radioactivity was measured by placing a sample in 15 ml of the scintillation fluid of Bray[12] and counted in a Nuclear-Chicago Mark I Scintillation Computer, model 6860.

Determination of location of enzyme after centrifugation

After fractionation of the gradients, samples of each fraction were supplemented with additional tryptophan (1.2 μmoles/sample) and PLP (0.03 μmole/sample), layered with 3 drops of toluene, and allowed to incubate for 24 h at room temperature. The samples were then assayed for the presence of the various products.

RESULTS AND DISCUSSION

As mentioned above, the purpose of this study was to determine the acting size of tryptophanase from *B. alvei*. To analyze the experimental results it was necessary to determine the type of curve we should expect depending on whether the active species is the dimer, the tetramer, or both. This was accomplished by rearranging the Martin and Ames[10] equation and solving for dt to obtain the following equation:

$$dt = \left(\frac{A}{\omega^2 s_{20,w}}\right)\left(\frac{\eta_{T,m}}{\varrho_p - \varrho_{T,m}}\right) \cdot \frac{1}{x}\, dx$$

The numerical integration of this equation provided the residence time for the enzyme at each increment of the gradient during centrifugation. From the residence time and the total amount of enzyme activity it was possible to determine the amount of product which should be present in each fraction of the gradient after centrifugation. The theoretical curves obtained by this method for dimer alone, for tetramer alone, and for a mixture of equal amounts of dimer and tetramer (assuming no interconversion of one form to another) are presented in Fig. 1. Diffusion was ignored in developing the theoretical curves.

In the curve for the mixture of dimer and tetramer (Fig. 1) it is assumed that the same weight of each form is present, and that the specific activity is the same for each form. If the specific activity of the dimer were one half that of the tetramer, and if both forms were present in equal weight, the theoretical curve would slope from the initial value of 820 nmoles (at 5% sucrose) to about 760 nmoles (at 11% sucrose), and would be easily detectable.

In order to determine the type of curve we would expect to observe if there were an equilibrium between the dimeric and tetrameric forms of the enzyme, we developed a computer program based on the Martin and Ames equation. The program is written in such a manner that we can determine not only the distribution of product formed during centrifugation, but also the point to which both forms of the

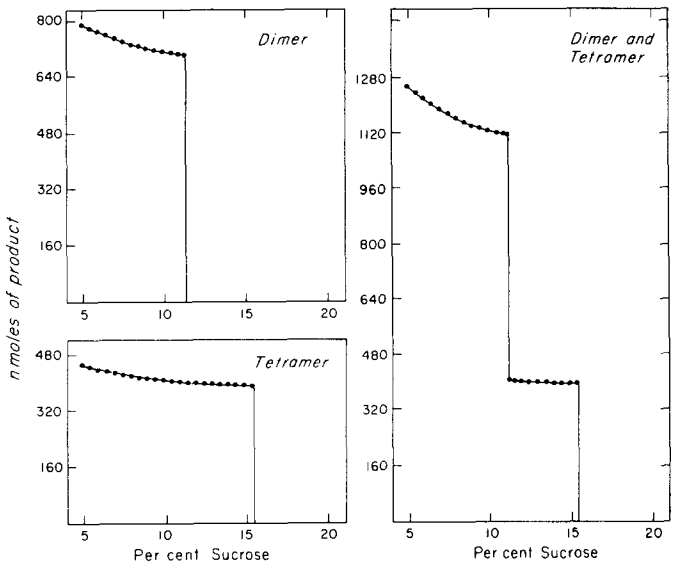


Fig. 1. Theoretical curves indicating the amount of product which should be present in each fraction of the gradient after centrifugation under conditions described in Experimental procedure.

enzyme would have migrated during sedimentation. These parameters can be determined for any desired equilibrium value between dimer and tetramer. It is also possible to generate theoretical curves in which different specific activities are assigned to the dimer and tetramer.

Due to the number of possible alternative theoretical curves which can be generated from the computer program just described, it would be impossible to present a meaningful sample. For that reason, it seems best to summarize the general trends evident from the hypothetical curves.

Any significant contribution by the dimeric form of the enzyme (in rapid equilibrium with tetramer) would be recognizable as a steeper negative slope of the product distribution. In addition, upon analysis for enzyme location after centrifugation, depending upon the amount of dimer in the equilibrium, the ultimate distance travelled by the tetramer (in dynamic equilibrium) will be less than that observed when only tetramer is present or when dimer and tetramer are both present or when dimer and tetramer are both present but not interconvertible. Also, in the case of an equilibrium between dimer and tetramer, the two forms travel to essentially the same point in the gradient.

Tryptophanase was centrifuged through gradients containing potassium phosphate buffer (pH 7.8) with tryptophan as the substrate. Measurement of the three products (indole, pyruvate and ammonia) formed during centrifugation revealed that this particular activity is carried out by the tetramer (Fig. 2). Several points are evident from this figure: (1) The amount of each of the products is lower at the top of the gradient than had been predicted (Fig. 1). (2) The maximal amount of indole occurs further down the gradient (9.3 S) than does the maximal amount of either pyruvate or ammonia, both of which are maximal at 8.1 S. (3) In no case did the maximal amount of any of the products occur at a point in the gradient which would

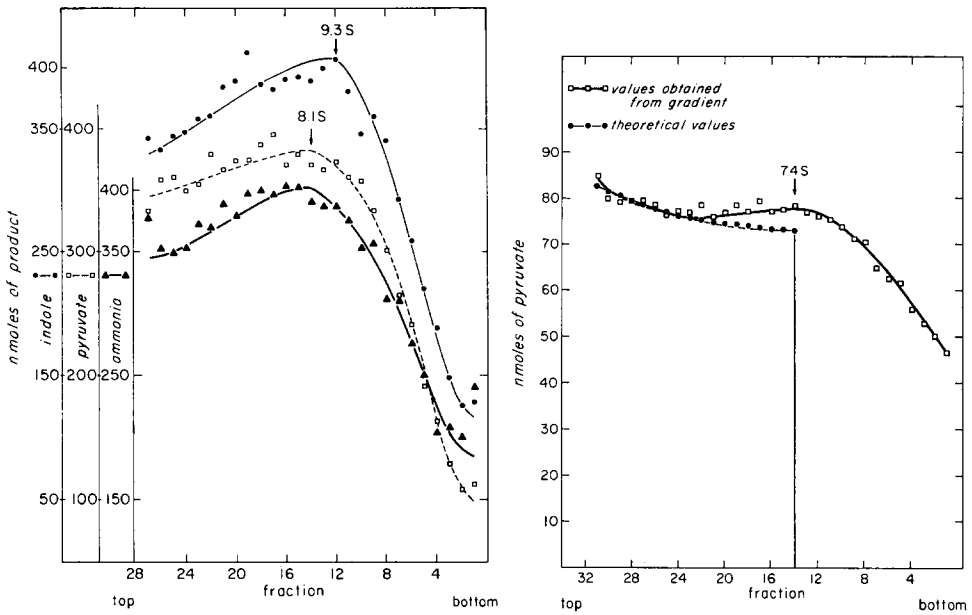


Fig. 2. Products resulting from tryptophanase activity in sucrose gradients made in potassium phosphate buffer, pH 7.8 (see Experimental procedure for details). Centrifugation was for 16·5 h at 65 000 × *g* at 37 °C in a Spinco Model L ultracentrifuge using an SW 39 rotor. Fractions collected from the gradient contained 165 μl. Immediately after collection, a 10-μl sample of each fraction was assayed for the presence of indole, a 10-μl sample for pyruvate, a 50-μl sample for ammonia, and a 10-μl sample for catalase activity. Catalase was assumed to have a sedimentation coefficient of 11.4 S. Each point represents an average from three gradients. Although the products were present in stoichiometric amounts, the ordinates were staggered so that the curves for each product would be distinguishable from that of the other products. The ordinate represents nmoles of product per fraction.

Fig. 3. Pyruvate converted to lactate during centrifugation of lactate dehydrogenase in sucrose gradients (see Results and Discussion for details) compared with the theoretical amount of conversion of pyruvate expected under the conditions employed in this study.

correspond to a protein with a sedimentation coefficient greater than 9.3 S and was usually considerably lower. This seemed to be inconsistent with the sedimentation coefficient of 9.4 to 9.6 S previously reported for the tryptophanase tetramer from *B. alvei*[6,7]. A variety of control experiments was designed in order to determine the cause of each of these observations.

The first hypothesis proposed that the unexpectedly low amounts of products at the top of the gradient constituted a phenomenon peculiar to tryptophanase but not to other enzymes. If it were true for all enzymes, this would indicate that the theoretical curve was incorrect for these conditions. To test this hypothesis, we applied 1.25 μg of pure lactate dehydrogenase to gradients containing pyruvate (0.63 mM) and NADH (1.4 mM) in 100 mM potassium phosphate buffer (pH 7.0), then centrifuged for 17 h at 23 °C at 85 000 × *g*. The data resulting from these gradients closely approximated the theoretical curve for a tetramer of lactate dehydrogenase as shown in Fig. 3, thus indicating that the phenomenon observed for tryptophanase is not necessarily a general phenomenon. The theoretical curve for lactate dehydrogenase was terminated at the point to which the majority of the tetramers should

have traveled. No attempt was made to account for diffusion which would obviously occur, particularly at 23 °C. The presence of product in the lower portion of the gradient may also be partially attributable to variability in the gradient preparation technique.

Since the phenomenon appears to be peculiar to tryptophanase, a number of alternative hypotheses for explaining the lower amount of products present at the top of the gradient after centrifugation were considered and tested: (1) The products formed at the top of the gradient were binding to some other component of the gradient and migrating down the gradient, rather than remaining at the position where they were formed. This was ruled out by forming gradients containing complete reaction mixture (*minus* substrate) and enzyme to which the products were added at a constant level throughout the gradient. After centrifugation the products were evenly distributed and had not migrated. (2) The albumin in the gradient migrates and alters the density and viscosity in the lower portion of the gradient, thus slowing the migration of the enzyme. This was ruled out by preparing gradients without albumin. After centrifugation, the amounts of the products were again lower at the top of the gradient than predicted. (3) The enzyme activity is stimulated increasingly by greater concentrations of sucrose as the enzyme moves down the tube. This hypothesis was tested by incubating the enzyme for 24 h at 37 °C in reaction mixtures containing various concentrations of sucrose. The concentration of sucrose had no stimulatory effect on the enzyme activity. (4) Sucrose is sedimented, thus altering the gradient making it more dense at the lower portion of the tube. This possibility appears untenable since the centrifugation of lactate dehydrogenase, as described in the preceding paragraph, approximated the theoretical curve. (5) The enzyme is partially in an inactive dimeric form when placed on the gradient, but the dimeric forms associate to form the tetramer, thus resulting in more enzyme of an active size as it moves down the gradient. Although this hypothesis has not been tested directly, we can approximate a test by summing the area under the product curve and comparing the sum to the total product expected if all of the added enzyme were fully active for the entire period of centrifugation. The sum of the activity under the enzyme curve of Fig. 4 shows clearly that nearly all of the applied activity (*i.e.* 93%) is recovered after 40 h. This result provides convincing evidence that relatively small amounts of free dimer were exposed to the centrifugal field during the course of the experiment, and that the tetramer is indeed enzymatically active. This conclusion is further supported by the results obtained when the location of the enzyme was determined after centrifugation (Fig. 4). It was found that the two forms of the enzyme had migrated to the predicted points. This would not have occurred if there were an active tetramerization of the dimer during centrifugation. In the latter case, theory showed that the tetramer could not have moved as far down the gradient as would a tetramer which had existed throughout the entire period of centrifugation. (6) A final hypothesis is that the products are volatile and evaporate from the upper portion of the gradient. This was tested by forming gradients with tryptophan (^{14}C-labeled indole ring). Three gradients were run: (1) a control with no enzyme, (2) 12 pmoles of enzyme, and (3) 64 pmoles of enzyme. After centrifugation and fractionation samples of each fraction were assayed for the presence of indole and for radioactivity. Since the tryptophan was ring-labeled, the product indole should be labeled upon cleavage. Thus, if there were no vaporization of product, the radioactivity should

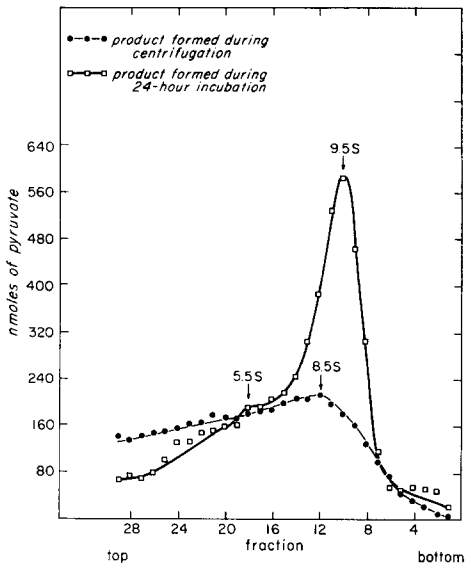


Fig. 4. Pyruvate produced by tryptophanase activity in sucrose gradients made in potassium phosphate buffer (pH 7.8) (see Experimental procedure for details). Centrifugation and collection of fractions was as in Fig. 2. Immediately after collection, a 10-μl sample of each fraction was assayed for the presence of pyruvate and a 10-μl sample was assayed for catalase activity. A second 10-μl sample was incubated with additional substrate and PLP for 24 h at room temperature, then assayed for pyruvate. Each point represents an average from three gradients.

remain constant throughout the gradient regardless of enzymatic activity. The results are shown in Fig. 5 and indicate that some product disappears. In the control gradient the radioactivity remained uniformly distributed, whereas in the gradients containing enzyme, radioactivity was decreased at the top of both gradients. More radioactivity disappeared from the gradient containing high enzyme concentration, an expected result because more indole was produced, and thus more indole volatilized, leading to a greater disappearance of radioactivity. In the gradient containing 12 pmoles of enzyme, the standard deviation at the top of the gradient was 1421 cpm as opposed to 161 cpm at the bottom of the gradient. In the gradient with 64 pmoles of enzyme, the standard deviation at the top was 2258 cpm and only 342 cpm at the bottom.

As mentioned above, a second point evident in Fig. 2 is the occurrence of the maximal indole concentration further down the gradient (9.3 S) than the maximal amount of either pyruvate or ammonia, both of which are maximal at 8.1 S. This indicates that during the reaction indole is released from the enzyme complex at a later time than are either of the other two products which appear to be released simultaneously.

The observation is consistent with the fact that indole is a strong non-competitive inhibitor ($K_i = 8 \cdot 10^{-6}$ M) of tryptophanase and readily binds to the enzyme. The indole apparently remains bound to the enzyme longer than do pyruvate and ammonia, neither of which binds to the enzyme as avidly as does indole. Pyruvate acts as a competitive inhibitor [$K_i = 1.6 \cdot 10^{-2}$ M for the *B. alvei* enzyme (Isom, H. C.,

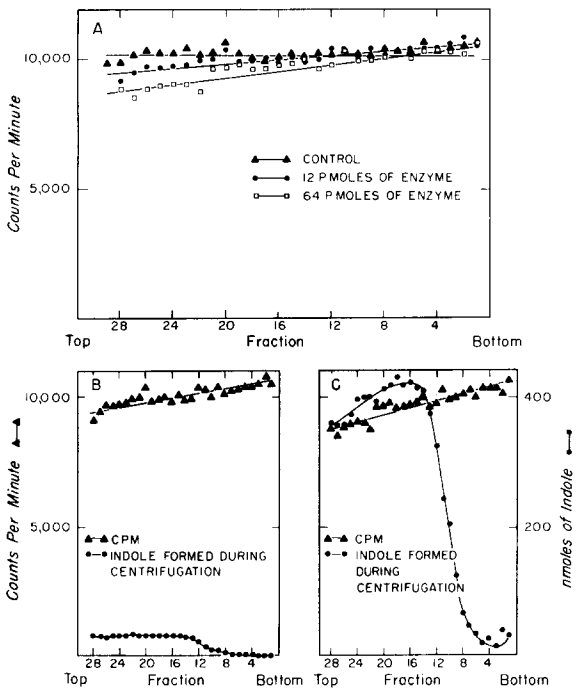


Fig. 5. (A) Distribution of radioactivity in a control gradient and in gradients containing two concentrations of enzyme. (B) Distribution of radioactivity and amount of indole formed during centrifugation of 12 pmoles of tryptophanase. (C) Distribution of radioactivity and amount of indole formed during centrifugation of 64 pmoles of tryptophanase. Centrifugation and collection of fractions was as in Fig. 2. 0.2 μmole of ^{14}C-labeled L-tryptophan (0.995 Ci/mole) uniformly indole-ring-labeled *plus* 40 μmoles of L-tryptophan was incorporated into each gradient.

personal communication), $K_m = 5.5 \cdot 10^{-2}$ M for the reverse reaction catalyzed by the *E. coli* enzyme[13]] of tryptophan degradation.

The *E. coli* enzyme exhibits a weak affinity for ammonia ($K_m = 125$ mM)[13]. Watanable and Snell[13] have proposed a reaction mechanism for tryptophanase from *E. coli*, involving the sequential formation, first, of indole, and subsequently of pyruvate and ammonia. Although indole is formed earlier in the reaction sequence, the relative values of the product binding constants suggest that indole may not be released from the enzyme–product complex as quickly as the other products. In fact, we expected the reaction rate to be so rapid that all three products would be released in such a short period of time that it would be impossible to determine which product is released first. However, determination of the position of the maximal amounts of the products as well as the position of the maximal amount of enzyme shows that the enzyme migrated farther in the gradient than did the products (see Fig. 4). This would suggest that although the enzyme had reacted, the products had not been released immediately in a detectable form.

The third phenomenon observed in Fig. 2, *i.e.* that the maximal amount of none of the products was observed to occur at a point in the gradient corresponding to a sedimentation coefficient greater than 9.3 S, was examined in the following manner. In order to determine how far down the gradient the enzyme itself had

migrated, gradient fractions were divided into two samples. One sample was assayed immediately after centrifugation, and the other sample was allowed to incubate for 24 h at room temperature with additional substrate and PLP. After the 24-h incubation, the maximal amount of each of the three products occurred in the same tube, as would be expected. The peak occurred at a position corresponding to 9.5 S, the point to which the tetramer should have migrated. The amount of one of the products, pyruvate, formed during centrifugation and after a 24-h incubation is shown in Fig. 4. Similar data were obtained for the other products.

In Fig. 4 there is also an indication of some activity at 5.5 S after the 24-h incubation, corresponding to the sedimentation coefficient of the dimeric form of the enzyme. About 25% (as determined by graphical integration) of the total enzyme migrated as dimer, but appears not to have contributed to product formation during centrifugation. The product formed by this amount of dimer (assuming that it were active) would be readily detectable as indicated from the theoretical curve (Fig. 1). The 5.5-S peak first appears after the additional 24-h incubation since the dimer presumably associates to active tetramer during this period. From these and other data we conclude that the dimer is catalytically inactive in the tryptophanase reaction.

To verify this conclusion, a series of gradients was formed using Tris–HCl as buffer. This buffer was used because Tris has been shown to cause dissociation of the tryptophanase tetramer[6,7]. Buffers at three different pH values (7.5, 8.0 and 8.5) were used. This pH range should cause the enzyme to consist of 50% to 95% dimers[7]. After centrifugation at 35–37 °C, fractions were collected and divided into two samples and assayed as described for the previous gradients. As in the case of the potassium phosphate buffer there was no activity at the position of the dimer when the products were measured immediately after centrifugation. After a 24-h incubation there was still very little activity at the position of the dimer, although in Tris buffer at the higher pH values most of the enzyme should have been in the dimeric form. On the basis of the assumption that the dissociation might be temperature-dependent, identical gradients in Tris buffer were centrifuged at 10 °C. Although there was a slight increase in the amount of dimer present at the higher pH, as determined by measuring the products after a 24-h incubation, the enzyme was still primarily in the tetrameric form.

To test further the hypothesis that the enzyme remained primarily in the tetrameric form at higher temperatures, the enzyme was centrifuged in 5–20% sucrose gradients containing 100 mM Tris buffer (pH 8.5). No other reaction components were included. After centrifugation at 37 °C the gradients were fractionated and 50-μl samples were assayed for tryptophanase activity in a reaction mixture containing 100 mM potassium phosphate buffer (pH 7.8), 400 μM PLP, 1.47 μM albumin and 8 mM L-tryptophan. It was found that the enzyme was primarily in the tetrameric form. Identical gradients centrifuged at 0 °C and assayed in the same manner revealed that the enzyme was primarily in the dimeric form. These data support the hypothesis that dissociation of the enzyme into the dimeric form is temperature dependent.

The aggregation of the tryptophanase apoenzyme from *E. coli* has also been shown to be temperature dependent[14] although the holoenzyme remained in the tetrameric form regardless of the temperature.

To rule out the possibility that the tetramer was protected against dissociation by one of the other gradient components, the following control gradients were run at 0 °C: (1) 100 mM Tris buffer (pH 8.5) + 1.47 μM albumin; (2) 100 mM Tris buffer (pH 8.5) + 1.47 μM albumin + 8 mM L-tryptophan; and (3) 100 mM Tris buffer (pH 8.5) alone. In all cases the enzyme was primarily in the dimeric form.

To this point we have not concerned ourselves with diffusion of either the substrate or the products of the reaction. However it seemed necessary to determine experimentally the extent of diffusion at 35–37 °C. Gradients containing all of the reaction components except substrate were formed. On these gradients we placed 100 μl samples of (1) indole (0.5 μmole or 4 μmoles), (2) indole (0.5 μmole or 4 μmoles) containing 60 pmoles of tryptophanase, (3) tryptophan (0.5 μmole or 4 μmoles), and (4) tryptophan (0.5 μmole or 4 μmoles) containing 60 pmoles of tryptophanase. The gradients were then centrifuged for 16½ h at 35 °C as described for the other gradients. After fractionation, the gradients which contained indole, indole + enzyme, and

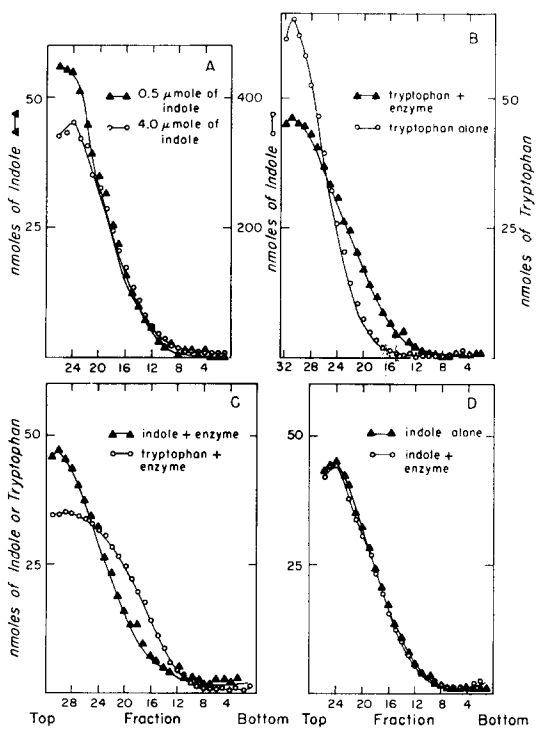


Fig. 6. Diffusion of indole and tryptophan during centrifugation. (A) Distribution of two concentrations of indole after centrifugation through gradients containing all reaction components. There was no enzyme in the sample centrifuged. (B) Distribution of tryptophan after centrifugation through a gradient containing all reaction components except tryptophan, and distribution of indole formed during centrifugation of a sample containing tryptophan and tryptophanase through a gradient containing all reaction components except tryptophan. (C) Distribution of indole after centrifugation of samples containing indole and tryptophanase or tryptophan and tryptophanase through gradients containing all reaction components except substrate. (D) Distribution of indole after centrifugation of samples containing indole or indole and tryptophanase through gradients containing all reaction components. In all cases centrifugation and collection of fractions was as described in Fig. 2.

tryptophan + enzyme were analyzed by measuring the amount of indole present in each fraction. The gradients in which the sample had contained only tryptophan were analyzed after fractionation by incubating a sample of each fraction with excess enzyme to allow complete conversion of the tryptophan to indole. The indole was then measured. The results, shown in Fig. 6, indicate that there is diffusion of both substrate and product. The diffusion was not significantly dependent upon the concentration of the substrate or product initially placed on the gradient. However, it is significant that the indole is found farther down the gradient in those gradients which contained tryptophan + enzyme. Two interpretations are possible; either the enzyme binds tryptophan and carries it farther down the gradient before converting it to indole, or the indole formed by the enzyme during reaction is carried farther down the gradient before it is released. These hypotheses are being tested independently (Fenske, J. D., personal communication).

The utility of this analytical method is partly dependent upon the successful demonstration that more than one acting enzyme form is detectable. After the major portion of the work contained in this report had been completed, J. L. Cowell, working with the tryptophanase from *Aerobacter liquefaciens*, found that this enzyme exists primarily as a tetramer, but the tetramers are capable of associating with each other to form larger aggregates (*e.g.* octamer, dodecamer, *etc.*), all of which apparently have tryptophanase activity (Cowell, J. L. and DeMoss, R. D., unpublished). We were thus able to test the experimental technique using a system in which we knew that more than one form was active.

The tetramer and the dodecamer forms of the *A. liquefaciens* tryptophanase were separated by gel electrophoresis, eluted from the gels and applied to individual gradients. A mixture of the two forms, 0.05 unit of dodecamer *plus* 0.025 unit of tetramer, was applied to a third gradient.

Fig. 7 shows the curves obtained for tetramer alone, dodecamer alone, a mixture of tetramer and dodecamer, as well as a composite curve obtained by adding the results from the tetramer alone and the dodecamer alone. It is evident from the components of this figure that the presence of more than one acting form is readily detectable.

From the data presented, we conclude that the acting species of the enzyme from *B. alvei*, using tryptophan as the substrate, is the tetramer and that the dimer is inactive.

When serine was used as the substrate (serine dehydratase activity) for the enzyme from *B. alvei* in the gradients, pyruvate and ammonia formation were determined. In Fig. 8 are shown the curves obtained when the products were measured immediately after centrifugation in potassium phosphate buffer at 35–37 °C. As in the case of tryptophanase the maximal amounts of the two products were located at approx. 8.2 S. In this particular experiment the amount of ammonia formed was lower than expected. However, in other experiments equimolar amounts of ammonia and pyruvate were formed. This particular set of curves was chosen for presentation because there were three replicates of the data and little variability. When samples of each fraction were allowed to incubate an additional 24 h (after the addition of excess serine) and then assayed for pyruvate, the peak of activity occurred at 9.4 S with some activity at 4.1 S which may represent activity contributed by a mixture of dimeric and monomeric forms of the enzyme. The amount of pyruvate formed

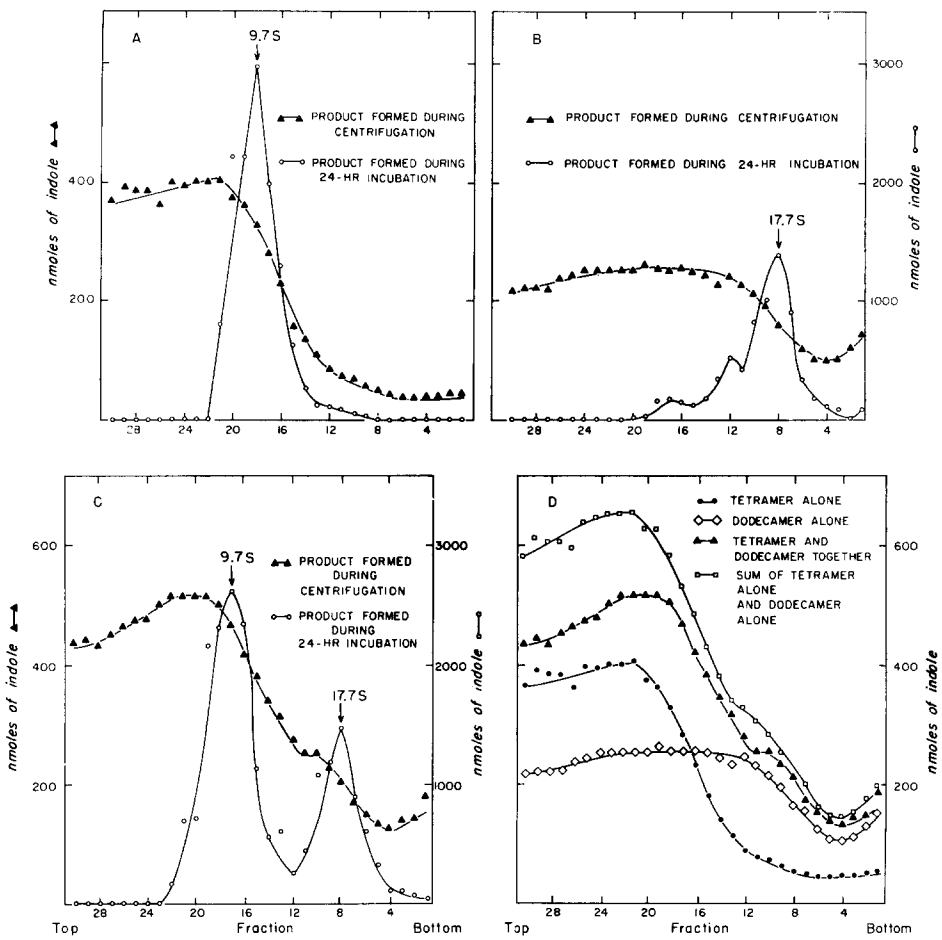


Fig. 7. (A) Indole produced by tryptophanase tetramer of *Aeromonas liquefaciens* in sucrose gradients made in potassium phosphate buffer (pH 8.0) (see Experimental procedure for details). Centrifugation and collection of fractions was as in Fig. 2. Immediately after collection, a 10-μl sample of each fraction was assayed for the presence of indole and a 10-μl sample was assayed for catalase activity. An additional 10-μl sample was incubated with additional substrate and PLP for 24 h at room temperature, then assayed for indole. (B) Indole produced by tryptophanase dodecamer of *A. liquefaciens* in sucrose gradients. Conditions of centrifugation, collection of fractions, and assay conditions were as described above for the tetramer. (C) Indole produced by a mixture of the tetrameric and dodecameric forms of tryptophanase from *A. liquefaciens* in sucrose gradients. Conditions of centrifugation, collection of fractions, and assay conditions were as described above for tetramer. (D) Distribution of indole formed during centrifugation by tryptophanase from *A. liquefaciens*. Curves shown are for tetramer alone, dodecamer alone, a mixture of tetramer and dodecamer, and the sum of the data from tetramer alone (A) and dodecamer alone (B).

during centrifugation and after a 24-h incubation is shown in Fig. 9. From these data it is apparent that the tetrameric form of the enzyme is responsible for the serine dehydratase activity, as well as the tryptophanase activity, and that the dimer is inactive.

When *S*-methylcysteine was used as substrate the presence of ammonia and pyruvate were determined. The results were essentially identical to those obtained

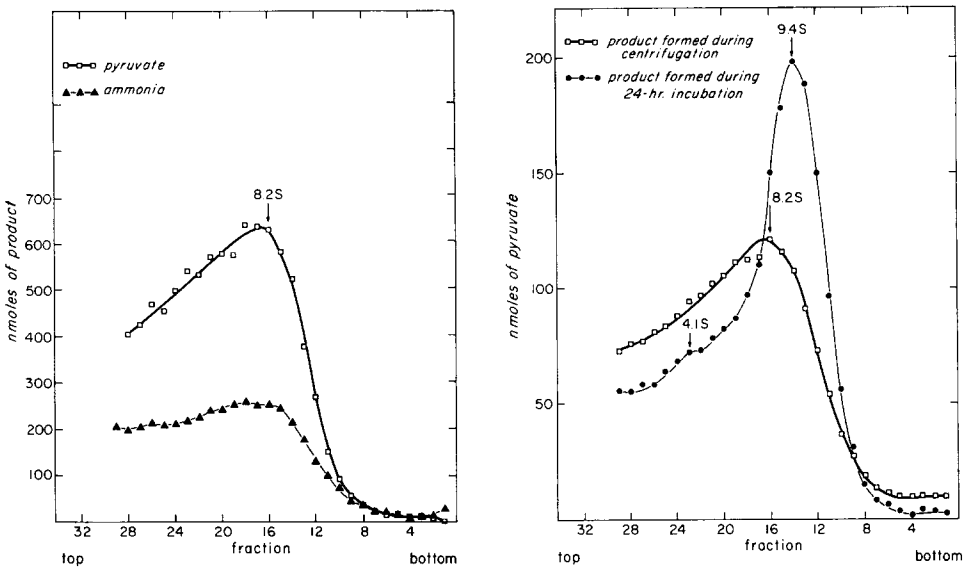


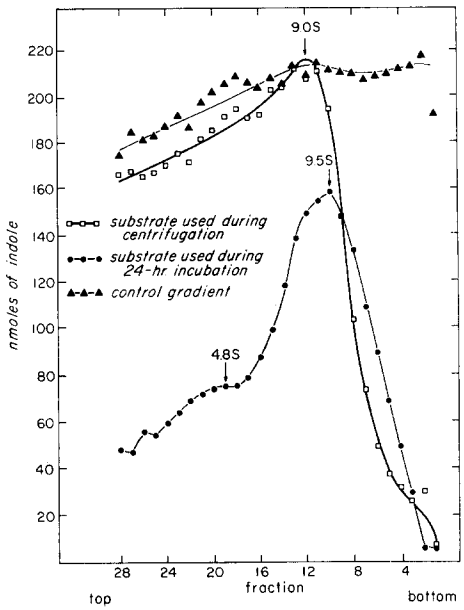
Fig. 8. Products of serine dehydratase activity in sucrose gradients made in potassium phosphate buffer, pH 7.8. Centrifugation and collection of fractions was as in Fig. 2. Immediately after centrifugation a 10-μl sample of each fraction was assayed for pyruvate, a 50-μl sample for ammonia, and a 10-μl sample for catalase activity. Each point represents an average from three gradients

Fig. 9. Pyruvate produced by serine dehydratase activity in sucrose gradients made in potassium phosphate buffer, pH 7.8 (see Experimental procedure for details). Centrifugation and collection of fractions was as in Fig. 2. Immediately after collection, a 10-μl sample of each fraction was assayed for the presence of pyruvate and a 10-μl sample was assayed for catalase activity. A second 10-μl sample was incubated with additional substrate for 24 h at room temperature then assayed for pyruvate. Each point represents an average from three gradients.

from gradients in which serine was used as the substrate. This lends further support to the contention that the α, β-elimination reaction is carried out only by the tetramer and not the dimer.

Results of studies of a β-replacement reaction, in this case tryptophan synthase (in which serine and indole are condensed to form tryptophan), indicate that this type of reaction is also carried out by the tetrameric form of the tryptophanase enzyme. The curves obtained when indole was measured immediately after collection of the gradient fractions and after 24-h incubation with additional substrates are presented in Fig. 10. As in the case of tryptophanase activity, the peak amount of product when measured immediately after collection occurs at 9.0 S and then after a 24-h incubation shifts to 9.5 S with some activity at 4.8 S. This indicates that the β-replacement type of reaction is also carried out only by the tetramer and not the dimer.

Also presented in Fig. 10 is a curve for a control gradient. Unlike the other substrates used by this enzyme, the indole (at the concentration required for this activity) does not remain evenly distributed throughout the gradient but appears to migrate slightly to the lower portion of the gradient, an observation supported by the diffusion experiments presented in Fig. 6. The control values were used as a correction factor in determining the amount of indole converted to tryptophan by the enzyme present in the gradient fractions.



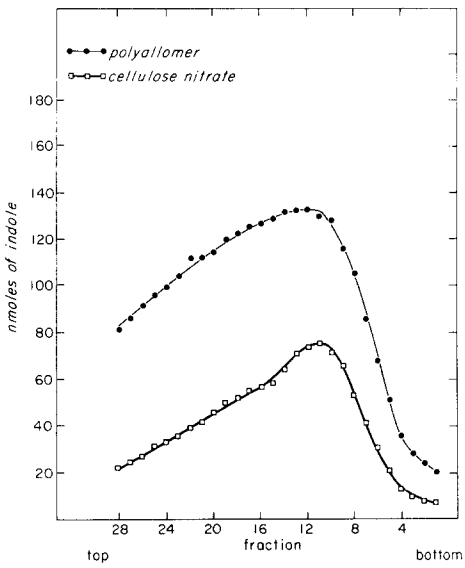


Fig. 10. Indole used by tryptophan synthase activity in sucrose gradients made in potassium phosphate buffer, pH 7.8. Centrifugation and collection of fractions was as in Fig. 2. Immediately after collection a 10-μl sample was assayed for indole, and a 10-μl sample for catalase activity. An additional 50-μl sample was incubated 24 h at room temperature then assayed for the presence of indole. Each point represents an average from two gradients.

Fig. 11. Indole produced by tryptophanase activity in sucrose gradients formed in cellulose nitrate and polyallomer tubes. All conditions as described in Fig. 2.

Since this study was initiated further investigations of the kinetics of coenzyme activation have revealed that there is apparently no cooperativity in the PLP activation curves for either the α, β-elimination or the β-replacement reactions (Isom, H. C., personal communication). This is consistent with our observations that the same aggregational state of the enzyme carries out both types of reactions.

When this study was initiated, the gradients were formed in cellulose nitrate tubes. We found that the amount of indole formed was much lower than the other products when tryptophan was used as substrate, and that the level at the top of the gradient was only slightly above background. It was also necessary to use much greater concentrations of indole in the tryptophan synthase gradients in order to detect any remaining substrate after centrifugation. Investigation of these phenomena revealed that the cellulose nitrate reversibly binds indole and removes it from the gradient. Indole can be recovered in significant quantities from the empty tubes by extraction with 95% ethanol. After this discovery, all subsequent gradients were formed in polyallomer tubes which do not absorb indole. Results from identical gradients in the two types of tubes are shown in Fig. 11. Approx. 60% of the indole was removed by the cellulose nitrate.

After the work presented in this paper had been completed, a similar technique was reported by other workers[15]. Their technique differed from the one reported here in that they used analytical centrifugation rather than preparative centrifugation.

Their method allows them to observe either the appearance of product or the disappearance of substrate as the enzyme migrates through a centrifugal field. However, the technique reported here has one distinct advantage, *i.e.* after the completion of centrifugation not only are we able to determine the amount of product formed during sedimentation, we are able to determine the point to which the enzyme sedimented and whether it existed in more than one polymeric state, a critical point for determination of the acting size of a polymeric enzyme system.

The experimental results presented in this report support the conclusion that the catalytically acting form of tryptophanase is the tetramer. This conclusion cannot be established by the usual techniques of enzyme study. Treatments effecting enzyme dissociation are often severe enough to inhibit catalytic activity. Consequently, when appropriate assay conditions are applied to a dissociated enzyme preparation, it cannot properly be assumed that the dissociated form is either active or inactive, because the possibility of obligatory association of subunits under assay conditions can be neither excluded nor established.

Introduction of the present technique allows direct analysis of the size of the acting enzyme and should be applicable to a wide range of enzyme activities. All enzymes which are reasonably stable, consume a stable substrate, form a stable product, or form a product which can be coupled to another enzyme, should be susceptible to analysis by this technique.

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