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# Tryptophan synthetase * of *E. coli*: a multifunctional, multicomponent enzyme; a commentary by

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on 'A second reaction catalyzed by the tryptophan synthetase of *Escherichia coli*'
by C. Yanofsky
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*Escherichia coli* tryptophan synthetase, an $\alpha_2\beta_2$ enzyme complex, catalyzes the final two reactions in tryptophan biosynthesis. This article retraces the scientific issues and experimental findings leading to the conclusion of the accompanying BBA article, namely that the *E. coli* enzyme catalyzes these two sequential reactions.

The tryptophan biosynthetic pathway was one of the first to yield to biochemical and genetic analyses with *Neurospora crassa*. Observations with tryptophan-requiring mutants of this organism [1], buttressed by previous findings with natural tryptophan-requiring bacteria [2,3], suggested that at least two aromatic compounds, anthranilate and indole, served as intermediates in tryptophan formation. Investigations with *Neurospora* mutants established that two genes encode the enzymes that convert anthranilate to indole, while a single genetic locus specifies the pyridoxal phosphate-dependent enzyme that catalyzes the coupling of indole and serine to form tryptophan [1,4]. The latter enzyme was initially named tryptophan desmolase [5], hence its initial corresponding gene designation, the *td* locus. Biochemical analyses with tryptophan-requiring mutants of *Escherichia coli* and *Salmonella typhimurium*, performed soon after those with *Neurospora*, reinforced these conclusions [6,7]. However, the genetic procedures then available for bacteria did not permit fine structure analyses; hence, it was not known how many genes participated in tryptophan formation. This deficiency was particularly acute in this case because the tryptophan biosynthetic genes appeared to be within a single multigene cluster [6,7]. We now know that in these bacterial species there are five structural genes within a single transcriptional unit, the tryptophan operon [8].

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Continuing attempts to unravel the gene-enzyme relationships for this pathway in bacteria focused on enzymology as the major experimental approach. Evidence was obtained with *E. coli* extracts showing that a

* The name assigned to this enzyme by the International Union of Biochemistry is tryptophan synthase: L-serine hydro-lyase (adding indole), 4.2.1.20. The historically common name, tryptophan synthetase, is used throughout this article.

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phosphorylated compound, indole-3-glycerol phosphate, was an intermediate in the conversion of anthranilate to indole [9,10]. The studies leading to this conclusion exploited several observations. First, anthranilate-responding mutants, when grown on low concentrations of anthranilate, produced elevated levels of tryptophan desmolase and the other tryptophan pathway enzymes [11]. (The regulatory significance of this observation was not fully appreciated at the time.) Secondly, tryptophan desmolase was a pyridoxal-phosphate-dependent enzyme [5] and, as such, hydroxylamine inhibited its activity. Accordingly, addition of hydroxylamine to extracts or cells of an anthranilate-responding mutant incubated with anthranilate and any of several sugars or sugar phosphates led to the accumulation of indole [11,12]. These features provided the opportunity to investigate the intermediary reactions between anthranilate and indole and let to the discovery of indoleglycerol phosphate. With the postulation [9] and demonstration [13,14] of two additional intermediates between anthranilate and indoleglycerol phosphate, *N*-5′phosphoribosyl anthranilate and 1-(*o*-carboxyphenylamino)-1-deoxyribulose 5-phosphate, the pathway of tryptophan formation from anthranilate was complete. Subsequently, the Gibsons isolated and identified chorismate, the last intermediate common to the synthesis of aromatic compounds [15]. When they demonstrate its conversion to anthranilate by one of the enzymes encoded by the tryptophan pathway genes, all the chemical reactions of the pathway were identified [15].

Continuing investigations on the genes and enzymes of the tryptophan biosynthetic pathway proceeded in two directions. Extracts of *E. coli* and *N. crassa* were fractionated with the objective of separating, purifying and characterizing individual enzymes of the pathway. Concomitantly, tryptophan-requiring mutants of these organisms were scrutinized to determine if their growth and enzymatic characteristics were consistent with accumulating knowledge about the steps in the pathway [16]. In studies underway in my laboratory, we were startled by the outcome of a routine enzymological experiment. When comparing the rates of conversion of indoleglycerol phosphate to indole and indoleglycerol phosphate to tryptophan by tryptophan synthetase preparations of *Neurospora* [17] (the name of the enzyme had been changed from desmolase to synthetase), we were surprised to find that tryptophan was formed 50-fold faster than indole. How, then, could indole be the enzymatic intermediate between indoleglycerol phosphate and tryptophan? Was indole actually off the pathway, but convertible to the 'true' intermediate?

The simplest hypothesis consistent with our *Neurospora* data was that tryptophan synthetase catalyzes two reactions, hydrolysis of indoleglycerol phosphate to indole, and ligation of indole and serine to form tryptophan, with indole participating in these reactions as an enzyme-bound intermediate. In our first test of this hypothesis we performed a bizarre in vitro 'mixed species' experiment [16]. We asked – would the indole produced enzymatically by a preparation from *Neurospora* be converted to tryptophan rapidly by an enzyme preparation from *E. coli* when the two preparations were incubated together? The *N. crassa* enzyme preparation used was partially purified, hydroxylamine-treated tryptophan synthetase; this preparation efficiently catalyzed the conversion of indoleglycerol phosphate to indole, but it could not synthesize tryptophan from indole and serine. The second fraction was tryptophan synthetase prepared from an *E. coli* tryptophan auxotroph. This preparation was fully active in the conversion of indole to tryptophan but was incapable of forming indole from indoleglycerol phosphate. When these two preparations were mixed, indoleglycerol phosphate should be efficiently converted to tryptophan unless rapid formation of tryptophan was dependent on indole remaining enzyme-bound during catalysis of the second reaction. The result – only a modest rate of conversion of indoleglycerol phosphate to tryptophan [17]. Our interpretation was that the native *Neurospora* enzyme had both activities and that indole was indeed an enzyme-bound intermediate [17]. Noteworthy in this regard was the observation that indoleglycerol, rather than indole, was accumulated by many *Neurospora* tryptophan synthetase mutants [18,19]. This finding was consistent with the interpretation that in *Neurospora* one enzyme catalyzes both the indoleglycerol phosphate to indole and indole to tryptophan reactions.

While these investigations were underway, a totally unrelated experimental tool contributed to further developments on this project. In the mid-1950's, Sigmund Suskind, David Bonner and I were the first to use antiserum to an enzyme – anti-*N. crassa* tryptophan synthetase – to demonstrate that mutants lacking an enzyme nevertheless may form a cross-reacting material (CRM) that reacts with antibodies to that enzyme [20]. What we observed was that an antiserum prepared against partially purified tryptophan synthetase from *N. crassa* inhibited the enzyme's activity, and some mutant extracts contained a material we named CRM that blocked this antibody inhibition [20]. Appreciating the utility of this approach, we prepared antisera to partially purified *E. coli* tryptophan synthetase and performed similar CRM tests with extracts of *E. coli* tryptophan synthetase mutants [21]. Two classes of mutants were recognized, those with CRM and those without it, as we had found in *Neurospora*. *E. coli* mutants in the $CRM^-$ category had two additional characteristics that distinguished them from $CRM^+$ mutants: they accumulated indoleglycerol phosphate rather than indole, and they lacked both enzymatic activities [16,21,22]. Was the tryptophan synthetase of *E. coli*, like that of *N. crassa*, capable of catalyzing two

sequential reactions? Could this explain the finding that some *E. coli* mutants could not perform either the indoleglycerol phosphate → indole or the indole → tryptophan reaction? Why did only the latter category of tryptophan synthetase mutants lack the protein immunologically related to tryptophan synthetase?

In further enzymological probings we showed that *E. coli* extracts could be fractionated into two components, designated A and B, both of which were required for the conversion of indoleglycerol phosphate to indole [9]. We next asked – is tryptophan synthetase CRM the active component in one of these fractions? If it was, then it must be component B, because mutants that lacked CRM also lacked component B. Numerous comparisons were performed between the active component in fraction B and CRM, and the conclusion was reached – "that component B activity and CRM represent, respectively, the enzymatic and immunological properties of the same protein" [22].

At this point, two reasonable possibilities were imagined. First, that CRM (component B) and tryptophan synthetase were distinct but closely related proteins, and alternatively, that a single protein had both enzymatic activities but CRM was a mutational altered form of this protein that retained component B activity [22]. Similarly, there were alternative genetic interpretations. To explain the finding that one class of mutants lacked both component B and tryptophan synthetase activities we proposed that perhaps component B and tryptophan synthetase were encoded in overlapping genes with a common functional domain which was altered in these mutants [22]. Alternatively, the component B and tryptophan synthetase reactions could be performed by a single protein, formation of this protein could be under the control of a single gene, and mutations in this gene could affect *either* enzymatic activity. Following this line of reasoning, we expected that some mutations that altered component B would eliminate both CRM and tryptophan synthetase activity. What was clearly needed was closer scrutiny of the protein or proteins catalyzing the terminal steps in tryptophan formation.

The historical background summarized in the preceding pages brings us to the starting point for the studies described in the accompanying article reprinted from BBA [23]. The objective of that study was to distinguish between the alternative explanations for the relationship between CRM, component B, and tryptophan synthetase. The experimental approach used was to determine the ratios of the three activities of concern – component B enzymatic activity, tryptophan synthetase enzymatic activity, and CRM levels – in extracts of wild-type and many defective mutants, and in various preparations obtained by extract fractionation. Extracts of revertants and suppressed mutants derived from tryptophan synthetase mutants also were examined.

The component B/tryptophan synthetase ratio in extracts of *E. coli* wild-type strains and in a tryptophan auxotroph blocked before anthranilate was constant, regardless of the growth conditions [23]. Quantitative enzyme-antibody precipitation studies and enzyme activity inhibition analyses, with extracts of wild-type and indole-accumulating tryptophan synthetase mutants, revealed that the component B/tryptophan synthetase ratio and the component B/CRM ratio also was constant [23]. Likewise, partial purification of tryptophan synthetase and component B failed to separate the two. However, ammonium sulfate fractionation at pH 5.8 did yield a fraction that was enriched for component B activity. This observation obviously favored the interpretation that component B and tryptophan synthetase were separate proteins. Careful analysis of the data, however, revealed that although all the component B activity was recovered, much of the tryptophan synthetase activity was lost during acidification. Should this finding be ignored? Fortunately, it was not! The hypothesis we considered was that a single enzyme had both activities, and the effect of acidification was merely to inactivate the indole to tryptophan activity without affecting component B activity. To test this hypothesis we analyzed the pH 5.8 fraction enriched in component B activity with tryptophan synthetase antiserum. Our intent was to determine whether there was more CRM than would be expected on the basis of the residual tryptophan synthetase activity. What we observed was that there was a considerable excess of CRM. Further, our analyses indicated that the component B/CRM ratio was normal. We concluded that tryptophan synthetase and component B activities were in fact activities ascribable to the same protein.

We also showed that a mutant lacking tryptophan synthetase and component B activities regained both when it reverted or when it was suppressed by an unlinked suppressor. Furthermore, the tryptophan synthetase activity that was restored in both instances was normally sensitive to inhibition by tryptophan synthetase antiserum. By contrast, when suppressed mutants were isolated from a tryptophan synthetase mutant that had both component B activity and CRM, the component B/tryptophan synthetase ratio was 10-times higher than normal. Apparently, suppression of the latter mutants only partially restored tryptophan synthetase activity. Consistent with this interpretation, our tryptophan synthetase antiserum was largely ineffective in inhibiting the tryptophan synthetase activity of this strain, indicating that its extracts contained an excess of tryptophan synthetase CRM. We now know that the first mutant was a nonsense mutant, while the second had a missense change.

Our conclusion from these investigations was that in *E. coli* both component B activity and tryptophan synthetase activity were associated with the same protein,

and that CRM was a mutationally altered form of this protein with only component B activity. Thus, our observations suggested that in *E. coli*, as in *N. crassa*, the indoleglycerol phosphate to indole and indole to tryptophan reactions were catalyzed by the same enzyme.

This first chapter on tryptophan synthetase was completed only a few months later when Irving Crawford joined my group and convincingly demonstrated that tryptophan synthetase of *E. coli* was composed of two separable protein subunits [24]. When both were present, all three reactions, indoleglycerol phosphate → indole, indole plus serine → tryptophan, and indoleglycerol phosphate plus serine → tryptophan, were catalyzed efficiently. By themselves, the separated subunits catalyzed the first or second reaction inefficiently, but neither of the other two reactions. In the presence of an excess of the second subunit, however, each subunit was activated in its reaction, 50- to 100-fold, and the overall reaction was performed. This unusual activation/complementation was clearly responsible for the difficulties experienced in solving this enzymological puzzle.

The two protein components were soon shown to be the $\alpha$ chain, which does not dimerize spontaneously, and the $\beta$ chain, which does [25,26]. Although the $\alpha$ chain has the active site for the indoleglycerol phosphate to indole reaction, the 'component B' assayed in the reprinted paper was in fact the $\beta_2$ subunit, measured by its stimulation of the $\alpha$ subunit which was added in excess. The predominant antigenic sites for tryptophan synthetase are present on the $\beta_2$ subunit, accounting for the parallelism between CRM and 'component B' activity. Both missense mutations and low pH treatment can result in modified $\beta_2$ subunits still retaining full antigenic and $\alpha$ subunit stimulating activity but defective in the $\beta_2$ subunit's intrinsic enzymatic activity, the conversion of indole and serine to tryptophan.

Most recently, a three-dimensional structure has been reported for the closely related tryptophan synthetase $\alpha_2\beta_2$ complex of *Salmonella typhimurium* [27]. Of particular relevance to this article, the three-dimensional structure reveals the presence of a tunnel connecting the active sites catalyzing the indoleglycerol phosphate to indole and the indole to tryptophan reactions [27]. The tunnel presumably facilitates passage of indole from its site of synthesis in the $\alpha$ subunit to the active site of the $\beta$ subunit [27]. This structural feature elegantly explains the puzzling finding of unequal rates of the two reactions and the more rapid conversion of indoleglycerol phosphate to tryptophan than to indole. Indeed, it gives a precise meaning to the vague term 'enzyme-bound intermediate'.

In retrospect, it is apparent that difficult-to-explain experimental observations forced us to consider the existence of a molecular species that had not been recognized before – a multisubunit, multifunctional enzyme complex – an enzyme capable of catalyzing two sequential reactions [24]. Studies with tryptophan synthetase were the first to demonstrate the existence of an enzyme complex of this type. The legacy of these investigations is that enzyme complexes are now recognized as one of nature's solutions to the problem – how to perform sequential or related reactions efficiently.

## Dedication

I dedicate this article commemorating the 1000th volume of Biochimica et Biophysica Acta to Arthur Kornberg, the 'pied piper' of enzymology, in recognition of his 70th birthday and his many outstanding contributions to biochemistry and the way we practise science.

## Acknowledgements

I am greatly indebted to Irving Crawford for sharing his interest in the tryptophan synthetase problem, and for his valuable comments on this historical review. I also thank C. Craig Hyde et al. [27] for permission to cite their exciting structural findings prior to their publication.

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# A SECOND REACTION CATALYZED BY THE TRYPTOPHAN SYNTHETASE OF *ESCHERICHIA COLI*

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SUMMARY

Evidence is presented suggesting that the tryptophan synthetase of *Escherichia coli* also participates in the conversion of indoleglycerol phosphate to indole. The protein immunologically related to tryptophan synthetase which is formed by certain mutants lacking tryptophan synthetase appears to be an altered form of this enzyme which still retains the ability to participate in indole formation from indoleglycerol phosphate but cannot convert indole to tryptophan.

* Some of the experiments reported here were performed while the author was a member of the Department of Microbiology of Western Reserve University.

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## INTRODUCTION

Immunological studies with tryptophan auxotrophs of *Escherichia coli* and *Neurospora crassa* which lack the enzyme tryptophan synthetase (indole + L-serine → L-tryptophan) have shown that certain of these strains form a protein, designated CRM, which is immunologically similar to the enzyme[1,2,3]. Other tryptophan auxotrophs in both organisms do not form detectable amounts of tryptophan synthetase (TSase) or CRM, indicating that mutations affecting TSase may also affect CRM formation[1,2,3]. In *E. coli*, all auxotrophs lacking CRM and TSase also lack an enzyme designated component B which is required for the conversion of indoleglycerol phosphate to indole[4]. Mutants of this type have been shown to result from single mutational events. Studies performed to examine the relationship between component B and CRM have provided substantial evidence indicating that component B activity is associated with the protein CRM[4]. This finding led to the following two alternative explanations of the relationship between CRM, component B and TSase: 1. CRM is component B; it is involved in the conversion of indoleglycerol phosphate to indole; it is a protein immunologically related to TSase, and its formation is controlled in part by genic material which also participates in TSase formation; or 2. TSase is component B and participates in both the conversion of indoleglycerol phosphate to indole and the conversion of indole to tryptophan, and CRM represents a mutationally-altered form of TSase which can no longer catalyze tryptophan synthesis but can function in the conversion of indoleglycerol phosphate to indole.

To distinguish between these possibilities, experiments were performed to determine whether component B activity and TSase activity are normally associated with the same protein in *E. coli*. The results obtained suggest that the same protein is involved in both the conversion of indoleglycerol phosphate to indole and the conversion of indole to tryptophan; thus CRM is probably a mutationally-altered form of this protein which can participate in only one of these reactions.

## MATERIALS AND METHODS

The minimal medium of VOGEL AND BONNER[5] was used throughout and was supplemented with L-tryptophan where required. Wild type K-12 and a tryptophan auxotroph which responds to anthranilic acid or indole as well as tryptophan, strain T-3, were used as sources of TSase and component B in these experiments. As reported previously[6], strain T-3 forms approximately 15 times more TSase than wild type strains when it is grown on low levels of anthranilic acid. Other tryptophan auxotrophs examined include strains $td_2$, $td_3$, $td_4$ and T-41. Of these, the first 3 form CRM and component B but lack TSase, while strain T-41 lacks all these activities.

### *Tryptophan synthetase and component B assays*

TSase activity was determined as described previously[4,7]. One unit of TSase activity is defined as the amount of enzyme which will convert 0.1 μmole of indole to tryptophan in 20 minutes at 37° C in an incubation mixture containing tris buffer (pH 7.8), sodium chloride, pyridoxal phosphate, indole and L-serine. Specific activity is expressed as units of TSase activity per milligram protein. Protein was determined by the method of LOWRY *et al.*[8]. Component B assays were performed by determining

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the amount of indole formed from indoleglycerol phosphate in the presence of hydroxylamine and an excess of the second enzyme component required for this conversion, component A[4]. One unit of component B activity is defined as the amount of enzyme which will form 0.1 $\mu$mole of indole from indoleglycerol phosphate in 20 minutes at 37° C under standard assay conditions[4]. Specific activity is expressed as units of B per mg protein.

*Immunological tests with tryptophan synthetase and component B*

Antibodies to TSase were produced in rabbits in response to the injection of partially purified preparations of TSase[3]. The antisera obtained were heated for 60 minutes at 56°, fractionated with ammonium sulphate, and dialyzed against saline. These sera were then repeatedly treated with portions of extracts of K-12 mutants lacking both CRM and TSase to absorb antibodies to other *E. coli* antigens. The absorbed antisera were again heated, fractionated with ammonium sulfate, and dialyzed before use. As reported previously[4], antibodies to TSase both precipitate and neutralize (inhibit) TSase activity; however, a small portion of the enzyme combining with antibody usually remains unneutralized unless large excesses of antibody are employed. Component B is also precipitated by antibody to TSase, but much less of the component B precipitated is neutralized[4]. CRM was assayed immunologically as described previously[4]. The CRM unit is based on the TSase unit and is defined as the amount of cross-reacting material which combines with as much antibody as does one unit of TSase.

## RESULTS

*Component B/TSase ratios in various strains grown under different culture conditions*

Comparisons of component B activity and TSase activity were performed with extracts of a wild type strain and strain T-3 grown under conditions which inhibit or increase TSase activity. It can be seen from the activity data in Table I that the component B/TSase ratio is fairly constant in these strains, both under normal culture conditions and when inhibitory amounts of tryptophan are included in the growth medium. It can also be seen that the component B/TSase ratio is approximately the same in extracts of strain B of *E. coli*. In other tests performed under somewhat

TABLE I

COMPARISON OF THE COMPONENT B/TSASE RATIOS OF VARIOUS EXTRACTS

| *Extract* | *Specific activity* | | *B/TSase × 100* | *TSase units neutralized by 0.02 ml antiserum* |
|---|---|---|---|---|
| | *B* | *TSase* | | |
| Wild type – K-12 | 0.09 | 3.3 | 2.7 | 1.0 |
| Wild type – K-12 | 0.053 | 2.1 | 2.5 | — |
| T-3 | 1.1 | 42 | 2.6 | 1.2 |
| T-3 | 0.67 | 25 | 2.7 | — |
| K-12 (HT)* | 0.028 | 1.1 | 2.5 | 1.2 |
| T-3 (HT) | 0.06 | 2.6 | 2.3 | 1.1 |
| T-3 (HT) | 0.045 | 1.9 | 2.4 | — |
| Strain B | 0.067 | 2.3 | 2.9 | 1.3 |

* HT = extract of a culture grown in the presence of 200 $\mu$g L-tryptophan/ml.

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different conditions it was found that the component B/TSase ratio of *Shigella dysenteriae* extracts is approximately the same as the value obtained with extracts of *E. coli*. The neutralization data presented in the last column in Table I indicate that the same amount of antigenic material per unit of TSase is present in all the extracts examined.

*Comparison of the effect of TSase antibodies on component B and TSase*

Absorbed TSase antisera were employed in a comparison of the precipitation of the TSase and component B in extracts of strain T-3. The results of this comparison are presented in Fig. 1. It can be seen from the curves obtained that except in the region of large antigen excess, equivalent amounts of component B activity and TSase activity were precipitated by antiserum and the equivalence point was the same for both enzyme activities. In the region of antigen excess (low antiserum levels) somewhat more component B activity was detected in the supernatant solutions than TSase activity. As reported previously[4], component B activity is only slightly inhibited by antiserum levels which precipitate; thus in the region of large antigen excess the B activity of a soluble antigen–antibody complex is probably being detected.

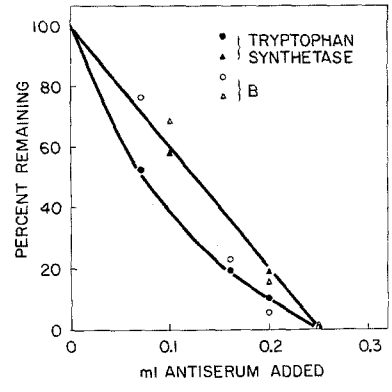


Fig. 1. Comparison of the precipitation of the component B and TSase in a T-3 extract by absorbed TSase antiserum. Varying amounts of antiserum were incubated with a constant amount of T-3 extract in the presence of pyridoxal phosphate and glutathione as activity stabilizing agents. After 2 days at 4° C, the mixtures were centrifuged and the supernatant solutions assayed for component B and TSase activity. The circles and triangles represent the results of two separate experiments.

*Comparison of B/TSase and B/CRM ratios*

If component B and TSase were distinct proteins but were immunologically similar, mutants which form component B but lack TSase might exhibit less immunologically reactive material per unit of component B activity than wild-type extracts exhibit. On the other hand, if component B activity and TSase activity are normally associated with the same protein, and if CRM is an altered form of this protein which exhibits only component B activity, the B/TSase ratio of wild-type extracts and the B/CRM ratio in mutant extracts might be identical. The data presented in Table I indicate that the B/TSase × 100 value is approximately 2.5. In previous studies with *E. coli* auxotrophs lacking TSase[4], a B/CRM × 100 value of approximately 2.5 was found for 4 of 5 mutants examined. A value of about 3.4 was observed for the fifth strain examined, mutant $td_3$. The similarity of the mutant and wild type ratios suggests that the same amount of antigenic material is associated with a given amount of component B activity in both mutant and wild-type extracts.

To compare more accurately the B activity to antigen ratio in mutant and wild-type preparations, two types of competition experiments were performed. In the first series of experiments, varying amounts of extracts of mutants which form

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component B but not TSase (td extracts) were mixed with a constant amount of an extract containing TSase and component B. Antibody was then added to each mixture, and the amount of TSase neutralized plotted against the ratio of the B activities of the two extracts. If the B:antigen ratios were the same in the two extracts, and if CRM and TSase compete equally well for antibody, then a td B to T-3 B ratio of 1 should be the point at which 50 % of the neutralization in the control tube is obtained. It can be seen from the data plotted in Fig. 2 that this was observed for extracts of strains $td_2$ and $td_4$ while a value of 55 % was observed with an extract of strain $td_3$. Thus it appears from these experiments that, per unit of antigenic material, there are approximately equivalent amounts of component B activity in wild-type and mutant extracts.

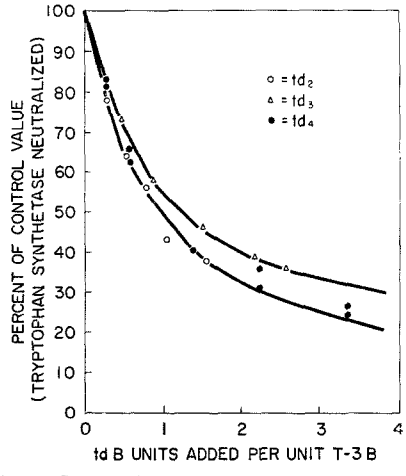


Fig. 2. Competition studies with component B from strains $td_2$, $td_3$, $td_4$ and T-3. Varying amounts of td extracts were added to a constant amount of an extract of strain T-3 to establish various td B to T-3 B ratios. Antiserum was then added and the extent of the neutralization of the TSase in the various mixtures determined. The control value represents the extent of neutralization of the TSase in the T-3 extract alone. The lower curve is the theoretical curve expected if the component B/CRM and component B/TSase ratios were identical.

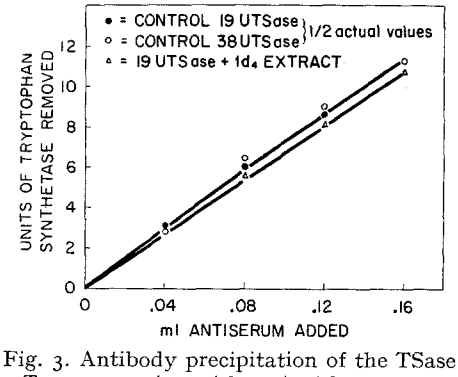


Fig. 3. Antibody precipitation of the TSase in a T-3 preparation with and without the addition of a $td_4$ extract containing an equivalent amount of component B. Tubes containing TSase, and a mixture of TSase and a $td_4$ extract (with equivalent amounts of component B), were incubated overnight at 4° C with various amounts of TSase antiserum. The incubation mixtures were centrifuged and the supernatant solutions were assayed for TSase activity.

A second method employed in comparing the B: antigen ratios of mutant and normal extracts is illustrated in Fig. 3. Here it can be seen that the addition of an equal amount of $td_4$ component B to T-3 component B resulted in a 50 % reduction in the precipitation of the TSase in the T-3 preparation. Thus, per unit of component B activity, the $td_4$ extract had as much immunologically active material as the TSase preparation.

*Purification of TSase*

It was mentioned previously that under various conditions of growth TSase activity and component B activity increase or decrease proportionately (see Table I). Fractionation experiments have also been performed to examine the association of

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the two enzymic activities. The results of a typical experiment are summarized in Table II. It can be seen that all the fractions obtained have approximately the same B/TSase ratio. The purification achieved was not great; 8-fold over the starting material and 100-fold over the TSase specific activity of extracts of wild-type strains of *E. coli*.

TABLE II

PARTIAL PURIFICATION OF TSASE AND COMPONENT B

| *Fraction* | *Specific activity* | | *B/TSase × 100* |
|---|---|---|---|
| | *B* | *TSase* | |
| Starting material | 0.8 | 30 | 2.7 |
| pH 6.0 0–30% fraction | 1.6 | 54 | 3.0 |
| pH 6.0 30–45% fraction | 1.1 | 43 | 2.6 |
| 30% fraction, heat at 59° C | 3.6 | 150 | 2.4 |
| Heat supernatant, pH 7.8 | | | |
| 0–27% fraction | 3.4 | 114 | 3.0 |
| 27–37% fraction | 5.7 | 236 | 2.4 |

A sonic extract of strain T-3 was treated with protamine sulfate (15 mg/100 mg extract protein) and the supernatant solution obtained following centrifugation was adjusted to pH 6.0 with 1 *N* acetic acid. Solid $(NH_4)_2SO_4$ was added to 30% of saturation. The precipitate which formed was collected by centrifugation and the supernatant solution was further fractionated with $(NH_4)_2SO_4$ at 45% of saturation. 5 ml aliquots of the 30% fraction were heated for 2 minutes in a 59° C water bath, and the precipitates which formed were removed by centrifugation. The combined supernatant solutions were refractionated with $(NH_4)_2SO_4$ at pH 7.8, at 27% and at 37% of saturation.

When the same extracts were fractionated at lower pH values, evidence suggesting separability of component B activity and TSase activity was obtained. As can be seen from the results in Table III, the B/TSase ratios of the 25 % ammonium sulfate fractions are appreciably higher than the ratio observed with the starting material. Inspection of the recovery data reveals, however, that although almost all the component B activity was recovered in the various fractions, there was considerable loss of TSase activity. This fact raised the possibility that the apparent separation of TSase activity and component B activity might be due to inactivation of some of

TABLE III

LOW pH FRACTIONATION OF AN EXTRACT CONTAINING TSASE AND COMPONENT B

| *Fraction* | *Total units* | | *B/TSase × 100* |
|---|---|---|---|
| | *TSase* | *B* | |
| Starting material | 16,800 | 460 | 2.7 |
| pH 5.8, 25–45% fraction | 8,160 | 264 | 3.2 |
| pH 5.8, 0–25% fraction | 2,160 | 180 | 8.3 |
| pH 6.0, 0–25% fraction | 390 | 150 | 38 |
| pH 6.0, 25–45% fraction | 1,020 | 24 | 2.4 |

A sonic extract of strain T-3 was treated with protamine sulfate and the supernatant solution obtained following centrifugation was adjusted to pH 5.8 with acetic acid. Solid $(NH_4)_2SO_4$ was added to 25% of saturation. The precipitate which formed was collected by centrifugation. The supernatant solution was fractionated further at 45% of saturation. The 25% fraction was then refractionated at pH 6.0, with $(NH_4)_2SO_4$ at 25% and 45% of saturation. No appreciable activity is present in fractions above 45% of saturation.

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the TSase with respect to its ability to catalyze the conversion of indole to tryptophan, without similarly affecting its ability to convert indoleglycerol phosphate to indole. To examine this possibility immunological experiments were performed to determine whether the pH 5.8 fraction with a B/TSase × 100 value of 8.3 contained antigenic material, inactive in the TSase reaction, which if taken into account would reduce the B/TSase × 100 value of the fraction to a normal value. The results of the comparison of the neutralization of the TSase in this fraction with the neutralization of the TSase in the untreated extract are presented in Fig. 4. It can be seen that the antiserum is much less effective in inhibiting the TSase activity of the 25 % fraction, indicating that antigenic material is present in excess of the TSase activity which was detected. From the data obtained it can be calculated that per unit of TSase in the 25 % fraction there is approximately a one and one-half-fold excess of antigenic material capable of combining with TSase antibodies. Taking this figure into consideration in calculating the B/total antigen × 100 value for the 25 % fraction, a value of 3.3 is obtained, which is close to the B/TSase × 100 value of the original extract. Thus the apparent separation of component B activity and TSase activity by low pH fractionation can be accounted for as inactivation of the TSase activity but not the antigenic activity or component B activity of a single protein. Similar neutralization experiments were not performed with the second 25 % fraction (pH 6.0) since TSase antibodies were almost completely ineffective in inhibiting the TSase in this fraction.

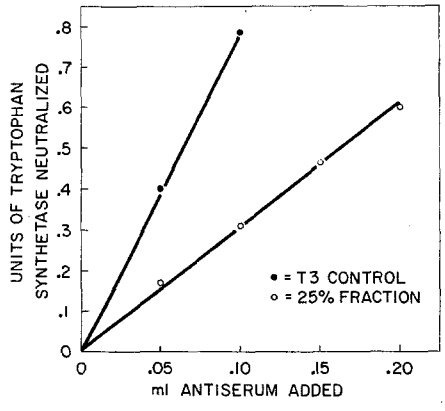


Fig. 4. Neutralization of the TSase activity of a 25 % ammonium sulfate fraction. Increasing amounts of antibody were added to a control extract (T-3), and to a pH 5.8–25 % ammonium sulfate fraction.

*Experiments with suppressed mutants and revertants*

It was mentioned previously that one group of mutants lacking TSase also lacks component B and the antigen or antigens associated with these activities. As a further test of whether component B activity is associated with TSase, mutational studies were performed with one representative of this group of mutants, strain T-41. Several suppressed mutants and revertants were isolated following ultraviolet irradiation of this strain; these stocks were examined with regard to their component B/TSase ratio and the sensitivity of their TSase to TSase antiserum.

It can be seen from the data in Table IV, that the ratios of component B activity to TSase activity in these strains are approximately the same and identical with

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the ratio observed with extracts of wild-type strains. One revertant obtained, strain T-41 R-5, exhibits approximately 10 to 20 times more TSase activity than the wild-type strain. Nevertheless, both the component B activity and the TSase activity in this strain were increased to the same level of activity. It can also be seen (Table IV) that the TSase activity which was restored by reversion and suppression showed normal behavior with respect to neutralization by TSase antibodies.

TABLE IV

CHARACTERISTICS OF SEVERAL SUPPRESSED MUTANTS AND REVERTANTS

| *Strain* | *Mutant type* | *TSase specific activity* | *B/TSase × 100* | *TSase units neutralized by 0.02 ml antiserum* |
|---|---|---|---|---|
| Wild type | — | 2.8 | 2.6 | 1.24 |
| T41-R-2 | Suppressed mutant | 1.5 | 2.3 | 1.08 |
| T41-R-3 | Suppressed mutant | 1.5 | 2.4 | 1.38 |
| T41-R-4 | Suppressed mutant | 1.0 | 2.2 | 1.28 |
| T41-R-5 | Revertant | 40 | 3 | 1.31 |
| T41-R-6 | Revertant | 2.7 | 2.4 | 1.41 |
| T41-R-7 | Revertant | 3.3 | 2.7 | 1.3 |
| T41-R-8 | Revertant | 3.0 | 2.5 | 1.15 |

Numerous reversion experiments have been performed, both with ultra-violet treated and untreated populations of strain T-41 and similar *E. coli* auxotrophs, employing a plating medium containing indole. If it were possible to restore TSase activity to these strains without simultaneously restoring component B activity, some strains appearing on an indole-containing medium should be indole-requirers. All experiments performed in the search for such a mutant have given negative results, *i.e.*, whenever reversions or suppressor mutations occur, both component B activity and TSase activity are restored together.

Suppressor studies have also been performed with one mutant strain which lacks TSase activity but forms CRM and component B. Several suppressed mutants were obtained in ultraviolet irradiation experiments and two of these were examined for component B and TSase. It can be seen from the data presented in Table V that in contrast to the results obtained with the suppressed stocks of strain T-41, the B/TSase ratio in these suppressed mutants differs considerably from the ratio observed in wild-type strains. It would appear that in these strains TSase activity is only partially

TABLE V

TSASE AND COMPONENT B CONTENT OF SUPPRESSED $td_2$ STOCKS

| *Strain* | *Type* | *Specific activity* | | *B/TSase × 100* |
|---|---|---|---|---|
| | | *B* | *TSase* | |
| K-12 | Wild type | 0.07 | 2.5 | 2.8 |
| $td_2$suB* | Suppressed mutant | 0.12 | 0.42 | 29 |
| $td_2$suC* | Suppressed mutant | 0.1 | 0.25 | 40 |

* TSase antiserum was practically ineffective in inhibiting the TSase activity of extracts of these strains.

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restored by suppressor mutations and that there is a lower than normal TSase/antigen ratio. Similar observations have been reported in studies with the corresponding suppressed mutants in *Neurospora*[1]. Whether this excess antigenic material represents a mixture of normal TSase and mutant CRM, or TSase with a lower turnover number, is not known.

## DISCUSSION

The results of the various types of experiments presented in this paper suggest that component B activity and TSase activity are normally associated with the same protein. It appears probable, therefore, that CRM is a mutationally-altered form of this protein which still exhibits component B activity but cannot participate in the conversion of indole to tryptophan. This interpretation readily accounts for the peculiar class of mutants which lack component B activity, TSase activity, and CRM as a result of single mutational events.

Recent studies with TSase preparations from *Neurospora crassa*[9] indicate that this enzyme catalyzes the following reactions:

1. indoleglycerol phosphate $\longrightarrow$ indole + triose phosphate
2. indole + L-serine $\longrightarrow$ L-tryptophan
3. indoleglycerol phosphate + L-serine $\longrightarrow$ L-tryptophan + triosephosphate

These studies have also shown that free indole does not participate as an intermediate in reaction 3. Similar experiments with extracts of *E. coli* suggest that free indole may not be an intermediate in tryptophan synthesis in this organism either. If the conversions of indoleglycerol phosphate to indole and indole to tryptophan actually represent partial reactions of the terminal step in tryptophan synthesis in *E. coli*, it is perhaps not surprising that in this organism one enzyme appears to participate in both partial reactions*.

## ACKNOWLEDGEMENT

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* Preliminary experiments (CRAWFORD AND YANOFSKY, unpublished) have provided evidence indicating that component A may also be an essential participant in tryptophan formation from indole as well as in indole formation from indoleglycerol phosphate.