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In Vitro Synthesis of Enzymes of the Tryptophan Operon of *Escherichia coli*

(anthranilate synthetase/tryptophan synthetase/DNA-dependent protein synthesis)

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ABSTRACT **Two active enzymes of the tryptophan operon of *E. coli*, anthranilate synthetase (product of *trp E* and *trp D*) and tryptophan synthetase (EC 4.2.1.20) (product of *trp B* and *trp A*), have been synthesized in a DNA-dependent preparation for protein synthesis. Anthranilate synthetase and the two components of tryptophan synthetase (tryptophan synthetase A and B) are made *in vitro* in equimolar amounts, suggesting that regulation of the translation is similar *in vivo* and *in vitro*. Kinetics of the synthesis of the two enzymes suggest that transcription and translation proceed *in vitro* in a correct temporal sequence.**

The recent development of a preparation for the *in vitro* synthesis of bacterial and phage-specified enzymes is important for the elucidation of the regulatory mechanisms involved in the transcription of genetic information and translation of the genetic message (1–7). Zubay, Chambers, and Cheong (4), for example, have shown that the synthesis *in vitro* of the inducible bacterial enzyme, β-galactosidase, is controlled by the repressor of the lactose operon. Their studies have also led to the discovery of a hitherto unknown mechanism of control for the synthesis of enzymes of the lactose operon.

We have used the cell-free system of Zubay, Chambers, and Cheong (4) to study the synthesis of enzymes of another bacterial operon, the tryptophan (*trp*) operon.

Yura and coworkers (8, 9) have demonstrated the *in vitro* synthesis of one of the enzymes of the tryptophan (*trp*) operon in a preparation that is independent of added DNA. The *trp* operon of *Escherichia coli* consists of five contiguous genes, *trp E, D, C, B,* and *A*, specifying five proteins, which are concerned with the synthesis of tryptophan (10). Transcription of the *trp* operon begins at a promotor located at the *E*-gene end of the operon (11, 12). Translation of the *trp* operon also starts at the *E*-gene end, and the five gene products are synthesized, in equimolar amounts, in the sequence corresponding to the gene order in the operon (10, 13).

We report here the DNA-dependent synthesis of two enzymes of the *trp* operon. Our preparation for *in vitro* protein synthesis consists of a bacterial extract that is programmed with purified DNA from a *trp* transducing phage. The extract, which serves as a source of enzymes and factors for transcription and translation, is prepared from a strain of *E. coli* with a deletion over the entire *trp* operon. Therefore, the extract is devoid of endogenous *trp* enzyme activity. The DNA used originates from phages that contain either the entire *trp* operon, including the *trp* promotor and operator, of only part of the *trp* operon (the promotor proximal or the promotor distal genes). Using this preparation we have studied the synthesis of the first enzyme, anthranilate synthetase (ASase), and of the last enzyme, tryptophan synthetase (TSase) (EC 4.2.1.20). ASase is composed of two pairs of nonidentical subunits encoded by genes *trp E* and *trp D* (14), and TSase also consists of nonidentical subunits encoded by genes *trp B* and *trp A* (15). The results of our experiments show that the two enzymes are made *in vitro* in equimolar amounts and in the correct sequence, which means that four, and probably all five, genes of the *trp* operon can be faithfully transcribed and translated *in vitro*.

Abbreviations: ASase, anthranilate synthetase; TSase, tryptophan synthetase.

MATERIALS AND METHODS

Bacterial Strains. All strains originate from *E. coli* 514 *lac*$^-$*trp*$^-$*Str*^{R} used by Zubay *et al.* (4) for the *in vitro* synthesis of β-galactosidase. Replacement of the *trp*$^-$ marker by a *trp* deletion (*AE*^{del}) and introduction of a mutation in the structural gene for the *trp* repressor (*trp R*$^-$) were performed by P1 transduction according to Yanofsky and Lennox (16).

Phage Strains and Isolation of Phage DNA. The following tryptophan transducing strains of bacteriophage φ80 were used: φ80h$^-$*ptAE190* isolated by Deeb *et al.* (17) and provided by C. Yanofsky; i$^\lambda$h$^+$$^{\phi 80}$*ptAEd62* and λ*ptEDd3*, both isolated by J. P. Gratia (18); φ80*ptED* isolated by Matsushiro and provided by J. P. Gratia; and i$^\lambda$h$^+$$^{\phi 80}$*pt5–2AB* isolated by N. Franklin. All phages except i$^\lambda$h$^+$$^{\phi 80}$*pt5–2AB* contain the *trp* promotor and operator (P. H. Pouwels, manuscript in preparation).

Mass lysates (1–2 × 10^{11} phages/ml) were prepared by infection of *E. coli* W 1485 with five phage per bacterium and cultivation for 2–20 hr. After lysis of the cells, the phage were recovered by precipitation with ammonium sulfate, followed by three cycles of high-speed–low-speed centrifugation. The final step in the purification was a CsCl density gradient centrifugation. Phage DNA was isolated by deproteinization of the phage with phenol. After removal of the phenol by dialysis, phage DNA was stored at 4° at a concentration of 0.5–2 mg/ml in 0.01 M Tris (pH 8.0)–1 mM EDTA. Phage DNA was routinely checked for the presence of single-strand breaks by sedimentation analysis in a neutral sucrose gradient after denaturation of the DNA with alkali. DNA preparations containing over 0.5 breaks per strand were rejected.

Preparation of the Cell-Free Extract. The growth of cells and the preparation of extracts were as described by Zubay *et al.* (4) with the following modification (suggested by Dr. R. Ehring, Cologne): Preincubation of the lysate was as

described by Nirenberg (19). Dialysis was as indicated by Zubay *et al.* (4) for 4 hr.

In Vitro Protein Synthesis; Effect of Polyethyleneglycol. Conditions for protein synthesis were as described by Zubay *et al.* (4) except for the following modifications: ϕ80 *dlac* DNA and the cell-free extract were replaced by components appropriate for the synthesis of *trp* enzymes; 3′:5′-cyclic AMP, pyridoxine·HCl, triphosphopyridine nucleotide, flavine adenine dinucleotide, and *p*-aminobenzoic acid had no measurable effect on the synthesis of *trp* enzymes and were, therefore, omitted from the incubation mixture. The concentration of amino acids was kept at 0.22 mM, except for tryptophan, which was used at a lower concentration (0.07 mM) because at the higher concentration tryptophan significantly interferes with the assay of ASase (20). The Mg^{++} concentration giving optimal results varied and has, therefore, been determined for each bacterial extract (see also Fig. 2). This variation in Mg^{++} dependency has also been observed in other preparations (5) and is not understood.

Since polyethyleneglycol stimulated the synthesis of tryptophan synthetase and anthranilate synthetase by a factor of 2–3, when added at a final concentration of 10–30 mg/ml, it has been included in all protein-synthesizing incubation mixtures at a concentration of 10 mg/ml.

Enzyme Assays. Anthranilate synthetase was measured according to Ito *et al.* (20). Incubation was for 20–30 min at 37°. Enzyme activity is expressed as units of activity per ml of protein-synthesizing incubation mixture.

Tryptophan synthetase component A (TSase A) and component B (TSase B) were measured separately after addition of either excess component B or excess component A, respectively, according to Smith and Yanofsky (21). Incubations were for 5–20 hr. Enzyme activity is expressed as units of activity per ml of protein-synthesizing incubation mixture.

One unit of each of the tryptophan biosynthetic enzymes is defined as that amount of enzyme that catalyzes the conversion of 0.1 μmol of substrate to product in 20 min at 37° under the conditions of the experiment.

TABLE 1. *Synthesis of tryptophan synthetase*

Preparation	TSase A (units/ml)	TSase B (units/ml)
Complete + ϕ80 h$^-$*ptAE190* DNA	0.84	0.88
Complete + i$^\lambda$h$^{+\phi 80}$*pt5–2AB* DNA	0.70	—
Complete + ϕ80 *ptED* DNA	0.04	—
Complete − phage DNA	0.04	0.03
Complete, at zero time for synthesis	0.01	—
Complete + 10 μg rifampicin/ml	0.03	—
Complete + 100 μg chloramphenicol/ml	0.08	—

Cell-free synthesis of TSase was performed under standard conditions (Mg^{++} concentration, 13 mM) with an extract prepared from *E. coli* strain 514 *trpR*$^-$*trpAE*^{del}. The concentration of phage DNA in this and all other experiments was 50 μg/ml. Samples of 0.1 ml were incubated at 37° for 60 min. Then, an excess of either partially purified component B or purified component A was added and the activity of TSase A or B, respectively, was assayed as described under *Methods;* the incubation was for 17 hr. Activity is presented as units of activity per ml of incubation mixture. Each value represents the average of two separate incubations.

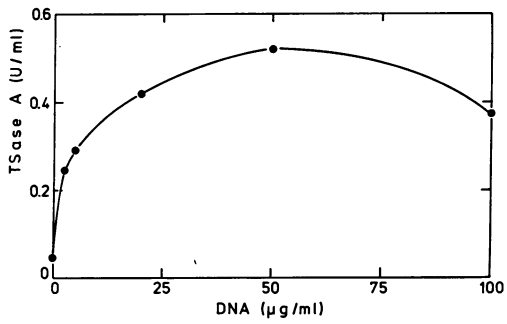

FIG. 1. Dependency of TSase synthesis on DNA concentration. The conditions for protein synthesis are the same as the ones given in Table 1 except for the concentration of DNA from phage Φ80 h$^-$*ptAE190*, which was varied. Conditions for TSase assay were as given in Table 1.

Reagents. Folinic acid (Leucovorin) was a product of Lederle, Brussels; rifampicin was generously donated by Prof. Dr. L. Silvestri (Lepetit); and chloramphenicol was obtained from the Koninklijke Nederlandse Gist—en Spiritus Fabriek. Chorismic acid was a gift of Dr. I. E. Mattern (Rijswijk).

RESULTS

Synthesis of tryptophan synthetase

The synthesis of the two individual subunits of TSase was followed by assay of the activity after addition of an excess of the other component.

When the bacterial extract prepared from *E. coli* 514 *trp AE*^{del} is programmed with purified DNA isolated from phage ϕ80 h$^-$*ptAE190*, containing the complete *trp* operon, the synthesis of both components of TSase is observed (Table 1). When the system was programmed with DNA from phage i$^\lambda$h$^{+\phi 80}$*pt5–2AB*, which lacks the *trp* promotor and the genes, *trp E*, *D*, and *C*, the same amount of TSase A is produced. This result suggests that despite the absence of the true *trp* promotor, the *trp* genes are efficiently transcribed and translated. The synthesis of active TSase is dependent on the presence of phage DNA carrying the genes for TSase, since in the absence of phage DNA or in the presence of DNA from a ϕ80 phage containing the genes, *trp E* and *D*, only, synthesis of active enzyme is not observed. Antibiotics that interfere with the transcription (rifampicin) or the translation (chloramphenicol) completely abolish the synthesis of active enzyme. The activities of TSase A and B (Table 1) are nearly the same, which means that the two proteins are made in equimolar amounts. This suggests that, *in vitro*, the translation of the region of the messenger encoded by the promotor distal part of the operon, takes place in a similar fashion as *in vivo*. Enzyme synthesis is dependent on the amount of phage DNA added (Fig. 1). Maximal activity is obtained at a concentration of 50 μg DNA per ml of incubation mixture.

To examine the reliability of the activities measured, we have determined the activities of a mixture of TSase A and B (of known activity) as a function of duration of incubation. The results of these experiments show that enzyme activity is proportional to the amount of enzyme and to the incubation time for at least 20 hr. Therefore, the enzyme activities from the *in vitro* synthesis experiments truly reflect the amount of enzyme synthesized.

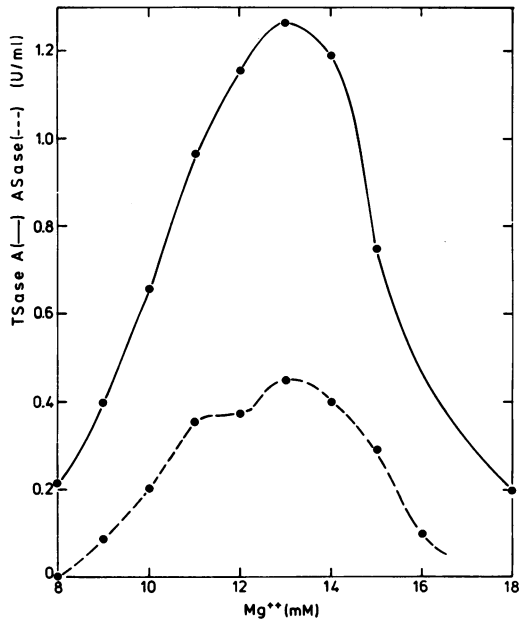

FIG. 2. Dependency of the synthesis of ASase and TSase on the concentration of Mg^{++} ions. Cell-free protein synthesis was performed under standard conditions with a bacterial extract from *E. coli* 514 *trpR*$^-$*trpAE*^{del} and DNA from phage Φ80 h$^-$*ptAE190* at various Mg^{++} concentrations. Synthesis was continued for 60 min at 37° in a total volume of 0.2 ml. After synthesis was completed, ASase and TSase activity were determined in 0.1-ml samples taken from the same incubation mixture. The results are plotted as units of enzyme activity per ml of protein synthesizing incubation mixture. Each value represents the average of two separate incubations. ●——●, TSase; ●- - -●, ASase.

Synthesis of anthranilate synthetase

Anthranilate synthetase (ASase) can be assayed by the formation of anthranilic acid from chorismic acid and glutamine. Under standard assay (20) conditions the presence of both components E and D is required for enzymatic activity. When an extract from *E. coli* 514 *trpAE*^{del} is incubated in the presence of DNA from phage ϕ80 h$^-$*ptAE190*, the synthesis of ASase can be demonstrated (Table 2). DNA from phages i$^{\lambda}$h$^{+\phi 80}$*ptAEd62*, ϕ80 *ptED*, and λ*ptEDd3* can replace this DNA, although at a lower efficiency, but DNA from phage i$^{\lambda}$h$^{+\phi 80}$*pt5-2AB*, which lacks the genes for ASase, does not induce the synthesis of this enzyme. Table 2 also shows that ASase activity is not found if protein synthesis is stopped at zero time, or if transcription or translation are inhibited by rifampicin or chloramphenicol. Since component E alone shows some activity when assayed in the presence of NH_4^+ ions (20), and since NH_4^+ ions are present in our assay mixture, it could be argued that the activity observed is due to the presence of component E only. However, no difference in ASase activity was found when the NH_4^+ ion concentration was varied between 1 and 10 mM, although the activity of component E strongly depends on the NH_4^+ ion concentration (20). Therefore, we conclude that both component E and D have been synthesized *in vitro*.

Effect of Mg^{++} concentration on protein synthesis

The dependency of the synthesis of ASase and TSase on the concentration of Mg^{++} ions in the incubation mixture is shown in Fig. 2. The profiles for the activity of the two enzymes are not significantly different. Since transcription starts at a single promotor, a difference in Mg^{++} dependency would reflect a difference in translation mechanism. Such a difference apparently does not exist.

Rate of synthesis of ASase and TSase

In vivo the enzymes of the *trp* operon are synthesized in an ordered fashion, starting at the E-gene end. Translation closely follows the transcription process since the first active TSase molecules are found at a time (5–6 min at 37°) when the transcription of the operon has just been completed (13, 22). When we measured ASase or TSase activity as a function of the time of protein synthesis in the *in vitro* preparation, we initially observed an unexpected long lag period (Fig. 3*A*). Even more surprising was the observation that the first active TSase molecules appeared before ASase activity could be detected; very little, if any, difference was found whether DNA containing the complete operon, or DNA containing the genes A and B only, was used as a template. In these experiments the reaction was started by addition of bacterial extract. However, if the reaction was started by the addition of UTP, after incubation of the complete protein-synthesizing incubation mixture in the presence of the other three triphosphates, we observed a much more rapid increase of TSase activity, that started sooner after the initiation of the reaction. When DNA containing genes *E* through *A* was used, the first enzyme molecules appeared 6–7 min after addition of UTP; with DNA containing genes *B* and *A*, this period was 2–3 min (Fig. 3*B*). Synthesis proceeded linearly for 5–10 min and reached a plateau between 20 and 50 min after initiation of synthesis.

The amount of TSase that was produced after 20 min of protein synthesis under these conditions was comparable to that obtained with the original procedure.

When the synthesis of ASase was followed in a reaction started by the addition of UTP, the first active enzyme molecules were observed between 10 and 15 min after synthesis was started. The long lag period for the appearance of ASase

TABLE 2. *Synthesis of anthranilate synthetase*

Preparation	ASase (units/ml)
Complete + ϕ80 h$^-$*ptAE190* DNA	0.30
Complete + ϕ80 *ptED* DNA	0.06
Complete + λ*pt* ED*d3* DNA	0.09
Complete + i$^{\lambda}$h$^{+\phi 80}$*ptAEd62* DNA	0.18
Complete + i$^{\lambda}$h$^{+\phi 80}$*pt5-2AB* DNA	<0.01
Complete − phage DNA	<0.01
Complete + ϕ80 h$^-$*ptAE190* DNA + 10 μg rifampicin/ml	<0.01
Complete + ϕ80 h$^-$*ptAE190* DNA + 100 μg chloramphenicol/ml	<0.01
Complete + ϕ80 h$^-$*ptAE190* DNA, but at zero time for synthesis	<0.01

Synthesis was performed under standard conditions (Mg^{++} concentration, 12 mM) with a bacterial extract prepared from *E. coli* 514 *trpR*$^-$*trpAE*^{del}. After synthesis proceeded for 60 min at 37° in a volume of 0.1 ml, ASase activity was determined. The tryptophan concentration in this assay mixture (3.5 μM) is too low to interfere with the determination of ASase activity (20). Incubation was for 30 min at 37°.

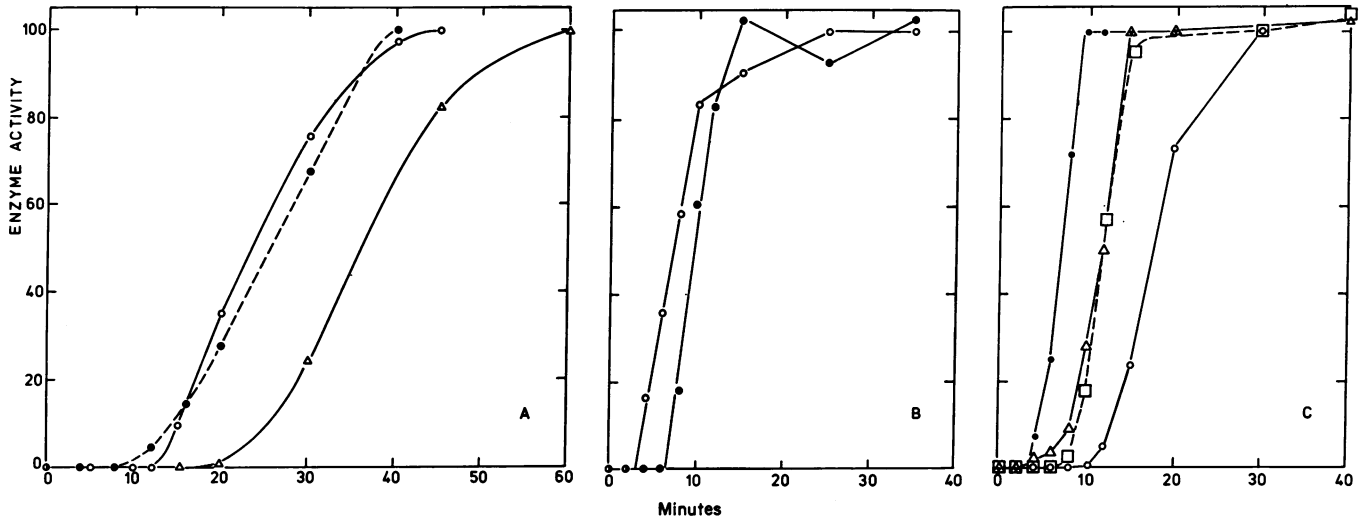

FIG. 3. Rate of synthesis of ASase and TSase A. (*A*) Synthesis of ASase (Δ) and TSase A (○ and ●) was performed under standard conditions (Mg^{++} concentration, 14 mM) with DNA from phage Φ80 h$^-$*ptAE*190 (——) or i$^\lambda$h$^{+\Phi 80}$*pt5-2AB* (– – –). The DNA–salt mixture was first incubated for 3 min at 37°, and then the reaction was started by the addition of warmed bacterial extract. At the times indicated, samples (0.1 ml) were taken for the determination of ASase and TSase A activity. Both assay media contained chloramphenicol at a concentration of 400 μg/ml. Enzyme activity is presented as the percentage of the maximal activity obtained. Each value represents the average of two separate protein synthesis incubations. The values of ASase activity after 0, 4, 8, and 12 min of synthesis were all zero and have been omitted from the figure. (*B*) Synthesis of TSase. The reaction was performed with DNA from phage Φ80 h$^-$*ptAE190* (●) or i$^\lambda$h$^{+\Phi 80}$*pt5-2AB* (○). All components for protein synthesis, including DNA and the bacterial extract, were first incubated for 7 min at 37° in the presence of three triphosphates but without UTP. The reaction was started by addition of UTP. Conditions for protein synthesis and enzyme assay are as given in Fig. 3*A*. Enzyme activity is presented as the percentage of the activity obtained after 30 min of synthesis. Each value represents the average of two separate protein synthesis incubations. (*C*) Synthesis of ASase. The reaction was performed with DNA from phage Φ80 h$^-$*ptAE190*. Conditions for protein synthesis are as given in Fig. 3*B*. Samples (0.1 ml) were taken at the times indicated, and protein synthesis was arrested by addition of 20 μl of chloramphenicol (1.2 mg/ml). ASase activity was determined either immediately thereafter (0 min; ○) or after incubation for 60 min (Δ) and 180 min (●) at 37° (in the presence of chloramphenicol). TSase A activity was determined after incubation for 5.5 hr at 37° (□). Enzyme activity is presented as the percentage of the activity after 30 min of synthesis.

activity might be caused by a slow reaction after protein synthesis to render the protein molecules enzymatically active. Such a reaction conceivably might be the formation of an active complex ED from the individual subunits. In order to test this hypothesis we performed the following experiment.

After incubation of the complete protein-synthesizing mixture without UTP, synthesis was started by addition of UTP and was arrested at various times afterwards by addition of chloramphenicol (200 μg/ml). The samples were then incubated for 0, 60, or 180 min before enzyme activity was assayed. The results (Fig. 3*C*) show that the ASase activity rises significantly during prolonged incubation periods. This rise in activity is probably not due to residual protein synthesis in chloramphenicol, since the same rate of TSase A synthesis was found either with or without 5.5 hr of incubation in the presence of chloramphenicol (see Figs. 3*B* and *C*). Since the activity of the samples could not be increased by longer incubation periods (6 hr) and since the complex ED is not freely dissociable (20) we may assume that after 3 hr the formation of the complex ED is completed.

DISCUSSION

The experiments presented in this paper show that ASase and TSase can be synthesized *in vitro* in a bacterial extract when DNA from a *trp* transducing phage is added. We will first discuss experiments in which phage DNA was used that contained the *trp* genes *E* through *A*, including the *trp* promotor and operator, and subsequently some experiments with DNA from a phage that lacks the promotor, the operator, and the segment *EDC*.

From the activities measured (Table 1) and the specific activity of purified TSase (15), 3.5×10^{12} molecules of TSase are made per hr per ml, or 3.5 enzyme molecules per DNA molecule (at 50 μg DNA/ml). A more meaningful value can be obtained by comparison with the rate of enzyme synthesis *in vivo* in a *trp* R$^-$ strain (23). Then the rate of synthesis of TSase (see Fig. 3*B*) in the cell-free preparation is 2–10% of that obtained *in vivo*. The possibility that the disappearance of indole from the reaction mixture during the enzyme assay is due to tryptophanase activity instead of TSase activity is highly unlikely, because one would have to assume that only in those experiments in which DNA from phages carrying genes *trp A* and *B* was used was tryptophanase synthesized.

All the experiments described in this paper were performed with bacterial extracts prepared from a strain of *E. coli* with a mutation in the structural gene for the *trp* repressor (*trp R*$^-$). If, however, extracts from *E. coli* 514 *trpR*$^+$*trpAE*^{del} were used for protein synthesis, the amount of ASase or TSase synthesized was not significantly different. This is not unexpected, since the concentration of *trp* DNA in our extracts exceeds the concentration of *trp* repressor present (24). Therefore, most of the DNA molecules will be accessible for the synthesis of *trp* enzymes, unhindered by repressor molecules.

A direct comparison of the amounts of TSase A, TSase B, and ASase that are synthesized in the cell-free extract can

be made from the experiments recorded in Table 1 and Fig. 2. The ratio of TSase A activity to TSase B activity is nearly unity, and the ratio of TSase A activity to ASase activity is 2.75. *In vivo*, the ratios of the enzyme activities are 1.0 and 3.9–4.5, respectively (10, 20, 25). The fact that our results are in fairly good agreement with the *in vivo* data suggests that *in vitro*, as *in vivo*, all enzymes of the *trp* operon are produced in equimolar amounts.

The rate of synthesis of TSase shows a rapid increase of enzyme activity if the reaction is initiated by addition of UTP after incubation of the complete protein-synthesizing incubation mixture in the presence of the other three triphosphates. Extrapolation of the curve leads to a value of 6–7 min for the time of appearance of the enzyme. The rate of synthesis of ASase is more complex. When protein synthesis is started with UTP, after incubation in the presence of the other three triphosphates, the first enzyme molecules are found after 10–15 min. However, ASase activity could be detected in samples taken after a much shorter period of protein synthesis (2–3 min), provided that these samples were incubated at 37° in the presence of chloramphenicol.

The simplest explanation for this shift in the appearance of ASase activity towards earlier times is that the formation of an active enzyme is a slow reaction that follows the more rapid synthesis of components E and D. This slow reaction might be the formation of the active complex ED from the individual subunits; this notion is supported by the following observations:

(*i*) The activity of the protein synthesis incubation mixtures increases only during the first 3 hr of incubation in chloramphenicol, but then remains constant.

(*ii*) When the incubation mixtures are diluted 3-fold after protein synthesis and then incubated for 6 hr in chloramphenicol, no significant increase in enzyme activity is observed.

(*iii*) Maximal increase in enzyme activity is already observed after 15 min of incubation in chloramphenicol when an excess of component D is added. In this case the first ASase activity (a measure for the presence of component E) was found in samples taken about 1 min after synthesis was started. The formation of the active complex from components E and D apparently occurs much more rapidly *in vivo* than *in vitro*, since no delay in the appearance of activity of the ED complex is found after derepression (26).

The times of appearance of the *trp* enzymes *in vitro* thus are in excellent agreement with the results from *in vivo* experiments [ASase 2.5 min; TSase 5.5 min (13)] suggesting that transcription and translation are occurring with the same speed *in vivo* and *in vitro*.

When DNA from phage $i^{\lambda}h^{+\phi 80}$*pt5-2AB*, in which the promotor, the operator, and the genes *EDC* are lacking, is used to direct enzyme synthesis instead of DNA from phage $\phi 80\ h^{-}$*ptAE190*, nearly the same amount of TSase A is found. The formation of *trp* enzymes in the absence of a *trp* promotor could be explained if, *in vitro*, the transcription machinery does not recognize termination signals ahead of the *trp* genes or if such stop signals are deleted from the phage by the insertion of bacterial genes. An alternative hypothesis would be that an artificial promotor has been formed just ahead of the *trp B* gene. Recent experiments suggest that *in vivo* transcription of the *trp* genes is initiated at the *trp* promotor on the DNA of phage $\phi 80\ h^{-}$*ptAE190*, but commences at a site near *trp B* on the DNA of phage $i^{\lambda}h^{+\phi 80}$*pt5-2AB* (P. H. Pouwels, manuscript in preparation). The rate of synthesis of TSase *in vitro* also agrees with such a hypothesis. The difference in time of appearance of TSase for the two DNA templates is 4 min (Fig. 3*B*), which corresponds to the time required for transcription and translation of the *trp EDC* segment (13).

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