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# Biosynthesis *in vitro* of Tryptophanase by Polyribosomes from Induced Cultures of *Escherichia coli*

BY S. A. M. KHAIRUL BASHAR, J. H. PARISH AND MARJORIE BROWN
*Department of Biochemistry, University of Leeds, 9 Hyde Terrace, Leeds LS2 9LS, U.K.*

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1. Polyribosomes were isolated from *Escherichia coli* grown in media in which tryptophanase is induced and in which it is repressed. The polyribosomes from the induced bacteria had a small amount of tryptophanase activity associated with them. 2. A portion of the enzyme activity remained bound to polyribosomes during centrifuging in sucrose gradients. 3. Incubation of tryptophanase-containing polyribosomes with puromycin released enzyme activity. 4. The binding of the enzyme to the polyribosomes did not depend on the presence of DNA. 5. When the polyribosomes were incubated under conditions of protein synthesis with supernatant fraction obtained from repressed bacteria, a small but statistically significant increase in enzyme activity was produced. 6. When a radioactive amino acid was included in the incubation mixture for the tryptophanase system a radioactive protein was obtained whose chromatographic, electrophoretic and sedimentation properties were identical with those of tryptophanase. 7. The amount of incorporation was consistent with the amount of new enzyme synthesis predicted by the increase in enzyme activity. Both radioactive incorporation and increase in enzyme activity were shown to be energy-dependent and also negative controls were obtained by using zero-time incubations or polyribosomes isolated from either repressed cells or a mutant lacking the ability to produce tryptophanase. 8. The distribution of radioactive leucine in the carboxyl region of the newly labelled tryptophanase was examined by digesting the labelled protein with carboxypeptidases. It was shown that the radioactivity was more highly concentrated towards the carboxyl terminus when the incubation times for protein synthesis were shorter (implying that, with longer incubation times, longer lengths of polypeptide chain contained radioactive amino acid residues).

We are concerned with the identification of specific, non-viral messenger RNA molecules. An approach to identifying such molecules is to identify a protein in a particular population of polyribosomes. Several proteins have been found associated with mammalian polyribosomes and these polyribosomes have been shown to synthesize the protein *in vitro*. The biosynthesis of one mammalian enzyme, NADPH–cytochrome *c* oxidoreductase of rat liver, has been characterized in this way (Ragnotti, Lawford & Campbell, 1969). Bacterial enzymes are suitable proteins for such studies as they can be induced to high activities and controls (repressed cultures or mutant organisms) are readily available. Nevertheless the number of bacterial proteins whose biosynthesis has been characterized *in vitro* is unexpectedly small.

A bacterial enzyme that has been studied extensively in relation to polyribosome preparations is $\beta$-galactosidase from *Escherichia coli*. The presence of this enzyme in a fast-sedimenting fraction of polyribosomes from an induced culture of *E. coli* was demonstrated by Kiho & Rich (1964). By comparing the apparent size of these polyribosomes containing enzyme activity from a variety of deletion and point mutants they obtained data consistent with the polycistronic nature of the corresponding messenger RNA (Kiho & Rich, 1965). However, there are no published data on the protein-synthesizing capacity of these polyribosomes and in fact the successful biosynthesis *in vitro* of this enzyme requires a DNA-dependent coupled transcriptional and translational system (Zubay & Chambers, 1969). In *E. coli* the only well-established case in which isolated polyribosomes can be used to synthesize a specific protein is that of polyribosomes obtained from cells infected with bacteriophage, which will synthesize the head protein of bacteriophage T4 (Klagsbrun & Rich, 1970).

In this paper we report the properties of polyribosomes isolated from *E. coli* induced for the production of tryptophanase. (There is no Enzyme Commission number or recommended nomenclature for this enzyme. It is the enzyme responsible for production of indole in the Enterobacteriaceae; the substrate is L-tryptophan, the other stoicheiometric reactant is water, pyridoxal phosphate is cofactor and the products are indole, pyruvate and ammonia.) We have demonstrated that the enzyme activity is present in the polyribosomes and that the polyribosomes are capable of synthesizing tryptophanase *in vitro*.

We believe this to be the first enzyme in *E. coli* whose synthesis on isolated polyribosomes has been demonstrated *in vitro*.

A preliminary report of these results has already been published (Parish, Khairul Bashar & Brown, 1970).

## MATERIALS AND METHODS

*Chemicals.* Except for those stated below, chemicals were obtained from British Drug Houses Ltd., Poole, Dorset, U.K. and were of AnalaR grade wherever possible. Pyridoxal phosphate, bovine pancreatic ribonuclease A and spleen deoxyribonuclease (ribonuclease-free), phosphoenolpyruvate (trisodium salt) and pyruvate kinase from rabbit skeletal muscle, also carboxypeptidases A and B (treated with di-isopropyl phosphorofluoridate to remove endopeptidase activity) were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. Naphthol Black (Wool Black) was obtained from George T. Gurr, London S.W.6., U.K., and DEAE-cellulose (Whatman, grade DE23) from H. Reeve Angel and Co. Ltd., London E.C.4, U.K. Alumina (bacteriological grade A-305) was kindly supplied free of charge by Alcoa International S.A., Lausanne, Switzerland. Radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K., scintillation reagents from Nuclear Enterprises Ltd., Sighthill, Edinburgh, U.K., puromycin dihydrochloride from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., and chloramphenicol from Parke–Davis Ltd., Hounslow, Middx., U.K. Defined agar media were made up with Ionagar no. 2; this, nutrient agar and Soya Tryptone Broth were all obtained from Oxoid Ltd., London E.C.4, U.K.

*Assay of tryptophanase.* Tryptophanase was assayed by the method of Yanofsky (1955), which was originally designed for tryptophan synthetase, with certain modifications suggested to us by Dr D. G. Herries. The assay mixture consisted of 0.05M-L-tryptophan (0.2ml), pyridoxal phosphate (0.2mg/ml) and enzyme preparation in 1M-$KH_2PO_4$, adjusted to pH 7.4 by the addition of 4M-KOH, and additional 1M-phosphate buffer to give a final volume of 1.0ml. After incubation for a suitable time at 37°C the solution was extracted with 4.0ml of toluene. To 1.0ml of the toluene layer was added 3.0ml of a solution previously prepared by dissolving 1.40g of *p*-dimethylaminobenzaldehyde in ethanol, adding 7.0ml of conc. HCl and then adding more ethanol to a final volume of 100ml. This method detects indole produced in the assay mixture. The extinction at 570nm was recorded after a period of more than 20min but less than 2h. The assay was calibrated for each batch of reagent by using standard solutions of indole. Units of enzyme activity (U) refer to the number of $\mu$mol of indole produced/min. For the assay of the total amount of tryptophanase, a sample of culture was added to 2vol. of acetone previously cooled to −20°C. The resulting precipitate was collected by filtration under vacuum and washed with successive portions of acetone, ether–acetone (1:1, v/v) and ether and was finally dried *in vacuo*. The powdery residue was suspended in the 1M-potassium phosphate buffer, pH 7.4 (made up as before), for the assay.

*Bacteria. E. coli* strain M.R.E.600 was maintained on slants of nutrient agar and was inoculated into 100ml swirls, which were then transferred into larger vessels and grown with artificial aeration at 30°C to late exponential phase and harvested by the method of Parish (1969). Two media were employed: one in which tryptophanase is repressed is that described by Parish (1969); the enzyme was induced in the medium of Boezi & DeMoss (1961).

*E. coli* 54.23 is a mutant of M.R.E. 600 incapable of producing indole and in which tryptophanase is not induced. It was obtained, together with other strains with the same characteristics, by the following procedure. Strain M.R.E. 500, grown in broth, was suspended ($10^8$ cells/ml) in 0.05M-tris–0.05M-maleic acid–$(NH_4)_2SO_4$, (1g/l), pH 6.0, adjusted by addition of 1M-NaOH; *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was added (0.4mg/ml) and the suspension was incubated at 30°C for 30min. This treatment results in approx. 0.1% survival. The cells were removed by centrifugation, washed in buffer (above) and grown overnight in broth. After overnight growth the cells were harvested by centrifugation, washed in buffer and plated on 2% agar containing the medium of Boezi & DeMoss (1961). Small colonies were picked off and tested for their ability to grow on 2% agar containing 2.45mM-tryptophan, 0.1M-$KH_2PO_4$, adjusted to pH 7.4 with 1M-KOH, 0.05M-NaCl and 8.1mM-$MgSO_4,7H_2O$. Organisms capable of growing on this medium (and hence capable of using tryptophan as sole source of carbon and nitrogen) were rejected. The remaining mutants were grown on the original agar on which tryptophanase should be induced. After several days of growth, the individual colonies were tested for production of indole by dropping on (with a Pasteur pipette) a reagent made up by dissolving 0.4g of *p*-dimethylaminobenzaldehyde in 4.0ml of ethanol and then adding 0.8ml of conc. HCl. Wild-type colonies become magenta indicating the presence of indole. Mutants giving no colour reaction were grown in liquid medium (Boezi & DeMoss, 1961) and acetone-dried and assayed for the enzyme. Out of 25 colonies originally scored as 'small' in the original screening and incapable of growth on the tryptophan–agar, six were identified as tryptophanase$^-$ by the later tests. The one used in this work (54.23) was carefully checked by disrupting the cells by different methods (sonication and grinding with alumina) and assaying for tryptophanase at different stages of growth to ensure that it was tryptophanase$^-$. *E. coli* 54.23 was invariably grown in the medium of Boezi & DeMoss (1961) to ensure that it had not on any occasion reverted to an indole-producing type.

*Purification of tryptophanase.* Tryptophanase was purified by a procedure that combines parts of the methods of Gunsalus, Galeener & Stamer (1955) with those of Burns & DeMoss (1962). An induced culture of *E. coli* (10–100 litres) in late exponential or early stationary phase was harvested and resuspended in 0.1 M-potassium phosphate buffer, pH 7.0. These suspensions were disrupted either by sonication or freezing and crushing. With sonication the cell suspension was adjusted to a concentration of 0.2 g wet wt./ml and 50 ml portions were disrupted with the Soniprobe (Dawe Instruments, London, U.K.) at 20 kcyc./s for 7 min at 0°C. With crushing the suspension was washed several times in the phosphate buffer and finally a thick slurry of cells was frozen (−20°C) in 10 ml portions. These portions were then crushed in the Hughes press and then resuspended in 10 vol. of phosphate buffer. In both procedures the suspension was centrifuged at 17000g for 40 min at 4°C. The supernatant was removed and (with the temperature maintained at 0–4°C throughout) the pH was adjusted to 6.0 with ice-cold 1 M-acetic acid; the solution was diluted with water to give a protein concentration of 10 mg/ml as assayed by the procedure of Lowry, Rosebrough, Farr & Randall (1951) and by using bovine serum albumin as standard for calibration of the assay. The solution was carefully warmed to 14°C and an aqueous solution of protamine sulphate ('salmine sulphate', 2%, adjusted to pH 5.0 with acetic acid) was added (7.0 μl/mg of protein). The precipitate, which contains the nucleic acids, was removed by centrifugation at 4°C and 17000 $g_{av.}$ for 10 min. Solid $(NH_4)_2SO_4$ was added to the supernatant and the fraction precipitated between 40 and 75% saturation was isolated by centrifugation (17000 $g_{av.}$ for 15 min at 4°C). This precipitate was dissolved in 'dilution buffer' (0.1 M-$KH_2PO_4$–5 mM-β-mercaptoethanol–2 mM-EDTA–0.04 mM-pyridoxal phosphate–0.8 M-$(NH_4)_2SO_4$; adjusted to pH 7.0 with 1 M-KOH) to give a protein concentration of 10 mg/ml. Hydroxyapatite gel was prepared by the method of Tiselius, Hjerten & Levin (1962) and was added to the solution in dilution buffer (6 mg of gel/mg of protein) and left to equilibrate at 0°C for 10 min; the gel was then removed by centrifugation at 17000g for 30 min at 4°C. More gel was added to the supernatant (15 mg/mg of protein) and the mixture was equilibrated and the gel removed by centrifugation as before. These values are only a guide and are typical for most batches of gel. However, preliminary experiments must be performed on each batch of gel; the amount used for the second treatment should be just less (by 1–2 mg of gel/mg of protein) than that at which significant binding of tryptophanase to the gel will occur. The supernatant (from the centrifugation after the second treatment with hydroxyapatite) was loaded on to a column of DEAE-cellulose in the phosphate form. The column was pre-equilibrated with 'starting buffer' (5 mM-$KH_2PO_4$–1 mM-EDTA (sodium salt), adjusted to pH 6.8 with 0.1 M-KOH). The columns had a height/diameter ratio of 5:1 and a protein/total column volume ratio of 2 mg/ml or less. Starting buffer (1/10 column volume) was pumped through at a flow rate of 1 ml/min and the column was then developed with a linear gradient of 0–0.5 M-KCl in starting buffer. The total gradient volume was eight times (or more) the total column volume and 50–100 fractions were collected and assayed for tryptophanase. The fractions containing the peak of tryptophanase activity were pooled and the protein was collected by precipitation with $(NH_4)_2SO_4$ (80% saturation). Analysis of this material was by disc electrophoresis in polyacrylamide gels, according to the procedure of Ornstein (1964). Details are given in the legend to Fig. 5.

*Isolation and fractionation of polyribosomes.* Polyribosomes were isolated by the method of Parish (1969). In this procedure the polyribosomes are obtained as a pellet. In certain experiments the suspensions of polyribosomes were analysed after incubation with either ribonuclease (10 μg/ml at 37°C for 5 min) or deoxyribonuclease (10 μg/ml at 0°C for 10 min) before layering over the sucrose density gradients.

In experiments in which DNA was to be labelled, the cultures were labelled with [$^3$H]thymidine (17 Ci/mmol; 0.2 mCi/l) for 30 min before harvesting. Details of experiments in which the polyribosomes were isolated from cultures labelled with $^{14}$C-labelled amino acids are given in the legend to Table 1.

Incubation with puromycin was by the procedure of Cannon (1968). After incubation, the preparations were separated into pellet and supernatant fractions by centrifugation at 159000 $g_{av.}$ for 2 h at 4°C.

Analysis of polyribosomes by sucrose-density-gradient centrifugation was by the method of Parish (1969). After centrifugation the gradient was pumped through a flow cell of the Gilford model 2000 recording spectrophotometer at a flow rate of 1 ml/min. Fractions were taken and assayed for enzyme activity.

*Protein synthesis* in vitro. Polyribosomes were isolated by the method of Parish (1969) except that chloramphenicol was omitted from all stages in the procedure. It is essential to maintain the temperature at 0–2°C throughout and to keep all handling procedures as short as possible. Supernatant fraction was isolated either from repressed cells of *E. coli* M.R.E. 600 or, for certain experiments, from strain 54.23 grown in the medium of Boezi & DeMoss (1961). The cells were washed in a small quantity of buffer (54 mM-$NH_4Cl$–10 mM-magnesium acetate–6 mM-β-mercaptoethanol–0.05 M-tris–HCl, pH 7.4). The washed pellets were frozen and ground with half their weight of alumina. The solids were dispersed in the same buffer (3 ml/g of alumina) and, after centrifugation at 23000 $g_{av.}$ for 30 min at 4°C, the supernatant was centrifuged at 164000 $g_{av.}$ for 2 h at 4°C. The supernatant was dialysed against the same buffer overnight at 4°C.

Transfer RNA was prepared from *E. coli* M.R.E. 600 by following the method of Parish (1968) to obtain total RNA. After precipitation of ribosomal RNA with 4 M-NaCl, nucleic acid was precipitated from the supernatant by adding 1 vol. of 2-ethoxyethanol; tRNA was isolated from this precipitate by the method already described (Parish, 1968) for obtaining tRNA from rat liver. Amino acids were removed (from aminoacyl tRNA molecules) and polysaccharides were removed from the preparation by the methods of Zamecnik, Stephenson & Scott (1960).

The medium used for protein synthesis was based on that of Reinicke, van Reisen, Voorma & Bosch (1968). It contained, in addition to the components of the buffer used for the isolation of the supernatant fraction in the same final concentration, the following components (per ml): 20 μg of tRNA; 1 μmol of ATP; 0.12 μmol of GTP; 5 μmol of phosphoenolpyruvate; 20 μg of pyruvate kinase; 1.0 mg of polyribosomes; 0.2 ml of dialysed

supernatant fraction and 20nmol each of 20L-amino acids. In experiments in which the incorporation of radioactivity into protein was being studied, L-leucine was omitted from the reaction mixture and was replaced with either DL-[4,5-$^3$H]leucine (16Ci/mmol; 0.2mCi/ml) or with DL-[$^{14}$C]leucine (62mCi/mmol; 10$\mu$Ci/ml).

*Identification of proteins obtained after incubation with radioactive leucine.* Protein synthesis was stopped by cooling the reactions to 0°C and then tryptophanase, purified as far as the hydroxyapatite step (about 10mg of protein), was added and the mixture was purified for tryptophanase according to the earlier scheme, starting with the addition of acetic acid before precipitation with protamine sulphate. About 30–40 fractions were collected from the chromatography on DEAE-cellulose and samples of each fraction were assayed for tryptophanase and radioactivity. In separate experiments, radioactive material eluted in the region of the tryptophanase peak was analysed by sucrose-density-gradient centrifugation or polyacrylamide disc electrophoresis. Details are given in the legends to the appropriate figures. In one experiment, to determine the nature of the radioactive residues present in the labelled protein, a fraction was dialysed against water, freeze-dried and then dissolved in either 6M-HCl or saturated $Ba(OH)_2$. After repeated flushing with $N_2$ and evacuation, these samples were heated *in vacuo* at 105°C for 16h. The samples were then evaporated to dryness by rotatory evaporation (after first removing the $Ba^{2+}$ ions as $BaSO_4$ in the second case) and were redissolved in water and evaporated to dryness again. This procedure was repeated three times. The hydrolysates were analysed by ascending paper chromatography on Whatman no. 1 paper with butan-1-ol–acetic acid–water (12:3:5, by vol.) as solvent.

*Measurement of radioactivity.* Radioactivity present in protein or polypeptide residues was assayed by the method of Ragnotti *et al.* (1969). Radioactivity present in DNA was assayed by the method of Kirby (1968), except that the scintillation fluid consisted of a solution of naphthalene (10%, w/v), 2,5-diphenyloxazole (0.5%) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.0125%) in dioxan. This scintillant was also used for assay of radioactivity in the fractions from chromatography on columns of DEAE-cellulose. In this case 10ml of scintillant was added to 0.5ml samples of eluate. Samples were counted in a Beckman model LS 200B liquid-scintillation spectrophotometer to a standard error of 2%.

## RESULTS

*Induction of tryptophanase. E. coli* M.R.E. 600, when grown in a medium in which amino acids form the only carbon source and which is supplemented with tryptophan (Boezi & DeMoss, 1961), produces high activities of tryptophanase. We harvested the bacteria in late exponential phase ($E_{1cm}$ 1.5 at 550nm) when the amount of enzyme corresponds to 100U/ml of culture. In the presence of glucose (or any catabolite or carbohydrate) a corresponding culture contains less than 1U/ml. Cultures of *E. coli* 54.23 contain no detectable tryptophanase activity.

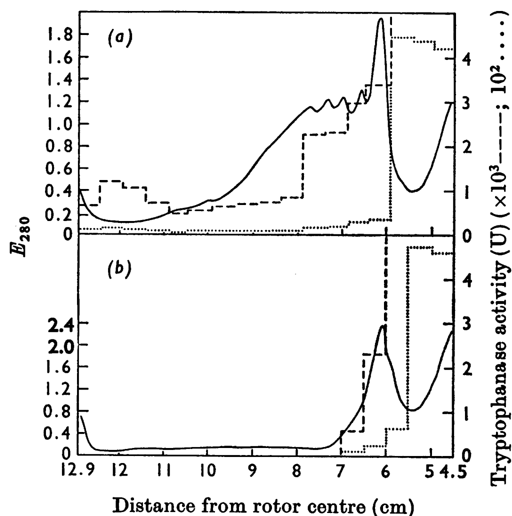


Fig. 1. Distribution of tryptophanase activity and polyribosomes in sucrose density gradients. Linear sucrose gradients (15–60% sucrose, w/v, in 0.05M-$NH_4Cl$–0.01M-$MgSO_4$ – 6mM-$\beta$-mercaptoethanol – 0.01M-tris–HCl, pH 7.4) were established in tubes for the SW 25.1 rotor of the Beckman model L ultracentrifuge. A suspension of polyribosomes from *E. coli* induced for tryptophanase was layered over the gradient and centrifugation was for 2h at 22500rev./min at 4°C. The gradients were analysed as described in the Materials and Methods section. The same preparation was either untreated (*a*) or was treated with ribonuclease (details in the Materials and Methods section) before layering on the gradients (*b*).

*Properties of polyribosomes.* Polyribosome preparations (obtained as a pellet) from induced cultures of *E. coli* M.R.E. 600 contain tryptophanase activity. No such activity was found associated with the polyribosomes from repressed cultures of *E. coli* M.R.E. 600 nor with those from strain 54.23.

Polyribosomes isolated from the induced culture were analysed by sucrose-density-gradient centrifugation. Fractions were analysed for the presence of enzyme activity. The results showed that considerable enzyme activity was present in the supernatant fraction and had presumably become associated with polyribosomes during the production of polyribosome pellets. However, there was a certain amount of enzyme activity associated with fast-sedimenting material in the gradients (Fig. 1*a*).

The nature of the particles carrying this enzyme activity could in principle be (i) large membrane fragments or other aggregate material not containing ribonucleoprotein, (ii) part of a DNA–mRNA–ribosome complex of the type envisaged to be present in a coupled transcriptional–translational

Table 1. *Effect of puromycin on tryptophanase associated with polyribosomes*

*E. coli* induced for tryptophanase was labelled *in vivo* for 3 min with $^{14}C$-labelled amino acids (algal hydrolysate, 50 mCi/mg-atom of carbon; 20 $\mu$Ci/l). Polyribosomes were isolated and suspended in buffer for treatment with puromycin (Cannon, 1968). After incubation (either control or with puromycin) the pellet and supernatant fractions were isolated as described in the Materials and Methods section. The pellets were suspended in water so that, from each incubation, 1 ml of supernatant and 1 ml of suspended pellet were obtained. After assay of tryptophanase, the radioactivity present in protein was measured by the method of Stahl, Lawford, Williams & Campbell (1968).

| | Control (incubation without puromycin) | | Incubation with puromycin | |
|---|---|---|---|---|
| | Tryptophanase (U) | Radioactivity (c.p.m.) | Tryptophanase (U) | Radioactivity (c.p.m.) |
| Supernatant | 0.00719 | 87 | 0.01945 | 304 |
| Pellet | 0.00856 | 860 | 0.00233 | 534 |
| Total | 0.01575 | 947 | 0.02178 | 838 |

system, (iii) enzyme molecules artifactually bound to ribosomes or (iv) enzyme molecules attached to ribosomes as nascent polypeptide chains.

Possibility (i) was examined by incubating the polyribosomes with ribonuclease to such an extent that the polyribosomes were degraded to 70 S ribosomes. The analysis of the products from such an incubation (Fig. 1*b*) showed no fast-sedimenting activity; rather the activity was found at the top of the gradient between the meniscus and the 70 S particles. We therefore conclude that ribonucleoprotein is in some way necessary for the maintenance of the structure of the fast-sedimenting, enzyme-containing, particles.

Possibility (ii) (a possible role for DNA in the association of enzyme with ribosomes) was examined carefully as the polyribosomes had been isolated by a method (Parish, 1969) in which no deoxyribonuclease was employed. Induced bacteria were labelled with [$^3$H]thymidine as described in the Materials and Methods section. A certain amount of radioactivity was present in the gradient in the 'polyribosome region' (between 7 cm and 12.5 cm from rotor centre; see Fig. 1), which was destroyed after incubation of the preparation with deoxyribonuclease. The effects on polyribosome profile and distribution of enzyme activity were not significant. Actual values for the total radioactivity and enzyme activity in the polyribosome region were as follows (values for deoxyribonuclease-treated polyribosomes are in parentheses): radioactivity, 7200 c.p.m. (320 c.p.m.); enzyme activity 9.5 mU (9.9 mU).

To attempt to distinguish between possibilities (iii) and (iv) we studied the effect of incubating the polyribosomes with puromycin. In this case the polyribosomes were obtained from cells which had been labelled for a short time *in vivo* with radioactive amino acids so that the nascent polypeptide chains were radioactive. In this way it was possible to compare the release of any enzyme with the overall release of nascent polypeptide chains by the drug. The results are summarized in Table 1. If the results are considered from the point of view of the amount of enzyme activity remaining in the polyribosome material (isolated by centrifugation of the incubation mixture), the efficiency of release of enzyme activity is significantly greater than the efficiency of overall release of polypeptide, as assessed from the radioactivity released from these particles which had been isolated from bacteria pulsed-labelled with radioactive amino acids *in vivo*. However, the amount of enzyme activity appearing in the supernatant was in excess of that predicted. In other words, after incubation with puromycin, there is an overall increase in activity of tryptophanase. The control experiment consisted of a parallel incubation in which the only difference was the absence of puromycin because the incubation of a preparation containing tryptophanase subunits could result in a reassociation of subunits to form active molecules. The implication is that there are polypeptide chains of tryptophanase attached to the ribosomes that do not form active enzyme molecules on the ribosomes but which do associate to form active enzyme molecules after their release with puromycin.

*Biosynthesis of tryptophanase* in vitro. Two types of experiment were attempted. In the first instance we examined whether the activity of tryptophanase associated with the polyribosomes obtained from bacteria induced for the synthesis of this enzyme could be increased by incubating the polyribosomes under the conditions of protein synthesis. Secondly we examined whether a radioactive amino acid was incorporated into a protein with the same properties as tryptophanase under these conditions. An analysis of data from one set of incubations for the former type of experiment (Table 2) established that the increase in activity

Table 2. *Effect of incubation of polyribosomes under conditions of protein synthesis* in vitro *on the activity of tryptophanase associated with them*

Results from two separate experiments are recorded. In each a large incubation mixture for protein synthesis (10 or 20 ml) was kept at 0°C while it was divided into 1 ml samples, which were then incubated in parallel. Sets of tubes (three in Expt. 1, four in Expt. 2) were withdrawn at the times indicated and tryptophanase was assayed. In Expt. 2 one set of incubation tubes contained chloramphenicol (+CAM) at a concentration of 0.1 mg/ml.

| Expt. no. | Incubation time (min) | Units of tryptophanase ($\mu$mol of indole/min) ± S.E.M. |
|---|---|---|
| 1 | 0 | 0.0249 ± 0.0018 |
| 1 | 20 | 0.0270 ± 0.0008 |
| 1 | 40 | 0.0340 ± 0.0026 |
| 2 | 0 | 0.0084 ± 0.0009 |
| 2 | 40 | 0.0143 ± 0.0006 |
| 2 (+CAM) | 40 | 0.00865 ± 0.0007 |

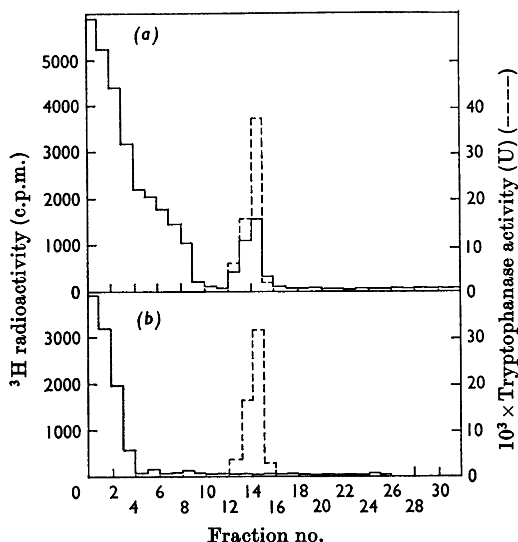


Fig. 2. Tryptophanase, labelled *in vitro* and chromatographed on a column of DEAE-cellulose (details in the Materials and Methods section), with polyribosomes from (*a*) induced and (*b*) repressed bacteria and supernatant fraction from repressed cells in both was incubated for 40 min under the conditions of protein synthesis in the presence of [$^3$H]leucine. The assays refer to 0.2 ml samples taken from each fraction but the radioactivity is corrected for total fraction size. The radioactivity was assayed on 0.5 ml samples and the background radioactivity (45 c.p.m.) has been subtracted.

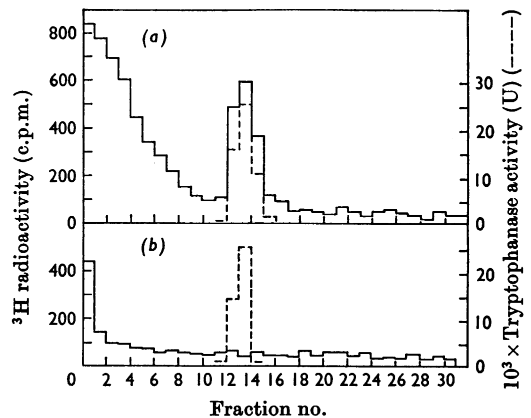


Fig. 3. Chromatography (as in Fig. 2) of tryptophanase labelled *in vitro* after incubation of induced polyribosomes and repressed supernatant for 40 min (*a*) and at zero time (*b*).

was time-dependent and was inhibited completely by chloramphenicol.

The more rigorous examination of the protein-synthetic system employed radioactive leucine to label the proteins made under the conditions *in vitro*. For these experiments repressed supernatant fraction was employed throughout. In this case unlabelled carrier tryptophanase was added to the system at the end of the incubation. The mixture was then purified for tryptophanase. The final stage in the purification is chromatography on columns of DEAE-cellulose. A peak of enzyme activity eluted from the column. Two controls were employed; in one repressed polyribosomes were incubated in the same way (Fig. 2) and in the other induced polyribosomes were included in the system and for a zero-time incubation (Fig. 3). Both controls were negative; the former ruled out the association of newly synthesized protein, other than tryptophanase, with the added tryptophanase and the latter the binding of radioactive leucine other than by the incorporation via leucyl-tRNA into the tryptophanase molecule.

The results of further controls are summarized in Table 3. For these experiments incubations with

Table 3. *Measurement of incorporation of* [14C]*leucine into tryptophanase* (*as assessed by DEAE-cellulose chromatography*) *and concomitant increase in enzyme activity after incubation* (45 min)

The same batch of polyribosomes isolated from 4 g (wet wt.) of cells was used for incubations 1–4. Conditions are described in the Materials and Methods section. Samples were removed at 0 and 45 min and units of enzyme activity were assayed. The remaining preparations were enriched and purified for tryptophanase as before.

| Incubation | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Origin of polyribosomes | M.R.E. 600 (induced) | M.R.E. 600 (induced) | M.R.E. 600 (induced) | M.R.E. 600 (induced) | 54.23 (induced) |
| Origin of supernatant fraction | M.R.E. 600 (repressed) | M.R.E. 600 (repressed) | M.R.E. 600 (repressed) | 54.23 (induced) | 54.23 (induced) |
| Incubation system | Complete | −GTP, ATP | Complete | Complete | Complete |
| Increase in tryptophanase (U) | 0.039 | 0.029 | 0.001 | 0.027 | 0.001 |
| Radioactive leucine incorporated into protein co-chromatographing with tryptophanase (pmol) | 2.3 | 1.4 | 0.05* | 1.8 | 0.1* |

* No peak corresponding to enzyme activity (see Figs. 1*b* and 2*b*).

[14C]leucine were assayed both for an increase in the tryptophanase activity and for the amount of radioactivity associated with the peak of tryptophanase isolated from the column of DEAE-cellulose. The experiments confirmed that the two criteria for synthesis of tryptophanase are complementary; controls shown to be negative when the incorporation into protein was considered were also negative when increased enzyme activity was assessed. Moreover the amounts of incorporation were found to be of the correct order of magnitude to account for the number of new polypeptide chains required to assemble the active enzyme molecules implied by the magnitude of the increase in enzyme activity. In an analysis of the results for incubation no. 1 (Table 3) the increased enzyme activity implies the assembly of 1.99 pmol of enzyme by using the value of 19 600 mol of indole produced/min by 1 mol of enzyme (Burns & DeMoss, 1962). As leucine constitutes 9.6% of the amino acid residues in the protein (Newton & Snell, 1964) we conclude that the amount of leucine incorporated into these new molecules is 0.19 pmol. This is approximately one-tenth of the amount of leucine incorporated into radioactive tryptophanase. The apparent excess of radioactive protein could be due to either the assembly of incomplete peptide quaternary structures that are then less active enzymically or different catalytic centre activities in these extremely dilute enzyme solutions.

*Further characterization of tryptophanase labelled* in vitro. Further characterization of the radioactive protein eluted with tryptophanase from the DEAE-cellulose was undertaken. First it is necessary to establish whether the radioactivity present in the protein is still present as leucine residues. The importance of this enquiry was seen when the amounts of incorporation into various fractions during the purification were calculated. In the positive result with induced polyribosomes it can be calculated that the proportion of the radioactivity originally included in the protein-synthesizing system, that is finally recovered as protein associated with the tryptophanase peak in the eluate from the DEAE-cellulose chromatography, is about 0.04%. This is significantly smaller than the amounts of impurities to be expected in the original sample of [3H]leucine. In practice, as only the middle tubes from such a peak are pooled for further characterization, less than this 0.04% was incorporated into the protein used for subsequent experiments. With tryptophanase, it is particularly important to establish that the radioactivity is not present in tryptophan residues, as it is possible that tryptophan might become bound to the enzyme as substrate. Fig. 4 shows the results of chromatograms of hydrolysates of protein, labelled *in vitro* with [3H]leucine and eluted (with tryptophanase) from DEAE-cellulose. The chromatography of the acid hydrolysate (Fig. 4*a*) establishes that most of the radioactivity is present either as leucine or isoleucine (these two amino acids are not resolved in this system). Radioactive material present in the alkaline digest (Fig. 4*b*) is clearly not present as tryptophan although the radioactive leucine present in the hydrolysate is present in other fragments (presumably peptides) produced during treatment with barium hydroxide.

Tryptophanase, associated with protein labelled *in vitro* and isolated from eluates of DEAE-cellulose columns (as in Figs. 2*a* and 3*a*), was further characterized by two independent procedures. In each case it was found that the radioactivity remained associated with tryptophanase.

Tryptophanase has a sedimentation coefficient ($s^0_{20,w}$) of 9.0 S (Burns & DeMoss, 1962). It follows

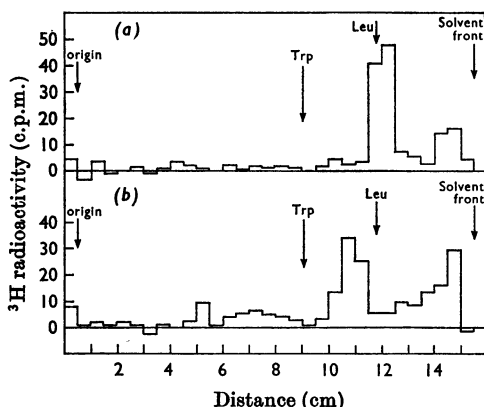


Fig. 4. Paper chromatography of (*a*) acid and (*b*) alkaline hydrolysates of material isolated from the region of tryptophanase activity in the eluate from a DEAE-cellulose column as in Fig. 1(*a*). Details are given in the Materials and Methods section. After development, the chromatograms were cut into 0.5 cm strips and the radioactivity was counted. The positions of the tryptophan and leucine markers were established by running markers in parallel on the same paper and locating them with the ninhydrin reagent.

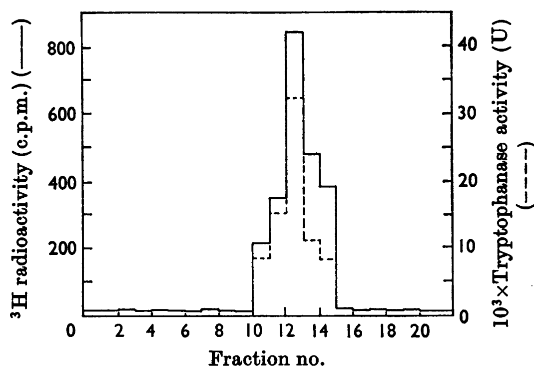


Fig. 5. Analysis by sucrose-density-gradient centrifugation of tryptophanase labelled *in vitro* by using induced polyribosomes and [$^3$H]leucine. The tryptophanase was purified as described before and a fraction from the tryptophanase region of the DEAE-cellulose chromatography was layered directly over a linear gradient of 5–20% (w/v) sucrose in 'starting buffer' (see the Materials and Methods section) established in a tube for the SW 25.1 rotor of the Beckman model L ultracentrifuge. Centrifugation was for 15 h at 24000 rev./min at 4°C. The gradients were sampled by using a peristaltic pump and timer and fractions were assayed for radioactivity and tryptophanase. Sedimentation was from right to left.

that the enzyme can be centrifuged as a zone through sucrose density gradients. Fig. 5 shows that the two zones (tryptophanase activity and radioactivity) are essentially coincident.

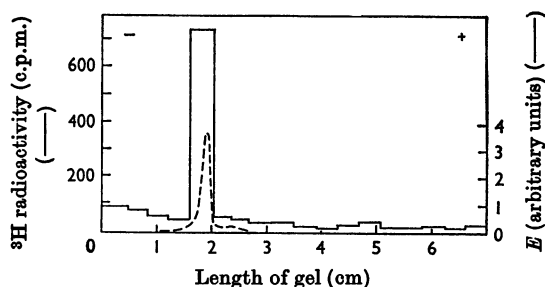


Fig. 6. Electrophoresis of tryptophanase labelled *in vitro* and isolated as before. After isolation of the labelled tryptophanase from the DEAE-cellulose column, the preparation was enriched with independently purified tryptophanase (see the text) and analysed by disc electrophoresis in gels of 4% polyacrylamide (Ornstein, 1964) with 0.06% (w/v) tris–0.285% (w/v) glycine, pH 8.3, as buffer. The potential gradient was 8 V/cm and the running gel was 7 cm long in tubes with an internal diameter of 0.5 cm. After 90 min (when a marker of Bromophenol Blue had just reached the bottom of the gel), the current was switched off, the gel was removed and stained for 2 h at room temperature with 1% Naphthol Black in 7% (v/v) acetic acid. The gel was destained in acetic acid (7%) in the presence of Dowex 1 anion-exchange resin at about 40°C for 24 h. The absorption was recorded in a Joyce–Loebel chromoscan with the 514 nm filter. The gel was frozen (liquid air) and sliced with a razor blade by using a piece of graph paper to identify distances. The slices were placed in counting vials, re-frozen, freeze-dried and the radioactivity was counted by liquid-scintillation counting. The abscissa is a linear representaton of the gel.

It was also demonstrated that the electrophoretic mobilities of the tryptophanase and radioactive material were identical. The detection of tryptophanase on the basis of its enzymic activity in polyacrylamide gels is difficult. The problem arises from the very high diffusion coefficients of the products of the reaction. (If such gels are incubated in the presence of phosphate buffer, pyridoxal phosphate and tryptophan and are then treated with a solution of *p*-dimethylaminobenzaldehyde, a very diffuse zone of colour is produced.) We therefore used a stain for protein to locate the tryptophanase band more exactly. The experiment was designed to establish that the protein eluted from the columns after incubations *in vitro* has the same electrophoretic properties as independently purified enzyme. An incubation (with [$^3$H]leucine) was stopped, partially purified tryptophanase was added and the preparation purified for tryptophanase as before. An equal amount of independently purified tryptophanase, which had already been shown to produce only one zone of detectable protein on polyacrylamide gels, was added to the

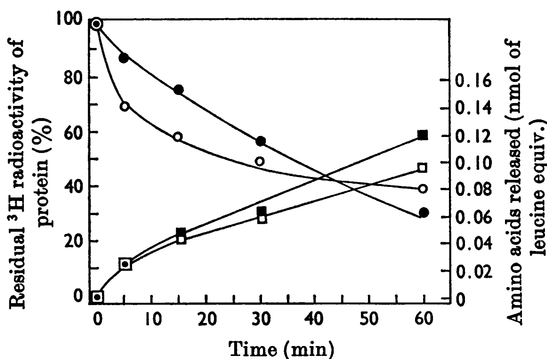


Fig. 7. Carboxypeptidase digestion of tryptophanase labelled *in vitro*. Induced polyribosomes were incubated under the conditions of protein synthesis with [$^3$H]leucine for 5 min (○, □) and 40 min (●, ■). The labelled protein was purified for tryptophanase as before. The yields of radioactivity (at the end of the DEAE-cellulose chromatography) were in the approximate ratio of 1:5. Conditions for the incubations with carboxypeptidases were those of Davies (1969). Samples were removed at various times. The two ninhydrin readings at 5 min gave almost the same value (equivalent to 0.024 and 0.025 nmol of leucine equivalent). Further readings (not plotted here) at periods of up to 24 h show plateaux for amino acid released (0.13 nmol of leucine equivalent), □, ■, and residual radioactivity (20%), ○, ●.

sample. This mixture was then analysed by gel electrophoresis and again only one band of protein stain could be seen. The radioactivity was found to be associated with this zone (Fig. 6).

A further experiment was performed to characterize the radioactive tryptophanase synthesized *in vitro*. If we assume that the new molecules (synthesized *in vitro*), which are isolated with tryptophanase, are essentially completed tryptophanase chains, then the number of residues labelled from the carboxyl terminus should be a function of incubation time, provided that the protein-synthesizing system is efficient and that chain extension can proceed from more or less any point in the mRNA. A confirmation of these predictions was obtained by digesting the tryptophanase synthesized *in vitro* with carboxypeptidase. This procedure has been used for the characterization of polypeptides synthesized *in vitro* by Davies (1969). In view of the fact that the carboxypeptidases (a mixture of enzymes A and B was employed) cannot be guaranteed to erode the protein chains uniformly, we have only considered the initial rates of release of amino acids and radioactive protein. In both incubations (Fig. 7) the initial rate of release of amino acids is approx. 6 pmol of leucine equivalents/min; the radioactive protein from the 5 min incubation mixture is about 10%/min whereas that from the 40 min mixture is about 2%/min. This comparison implies that in the former, a higher proportion of the [$^3$H]leucine residues are concentrated towards the carboxyl terminus of the chain.

## DISCUSSION

Apart from the studies on polyribosomes and protein synthesis, we believe that this paper covers an important technical point, namely a simple procedure for the isolation of mutants of *E. coli* which are tryptophanase$^-$. Such mutants are important in any studies on tryptophan metabolism in this organism. For several years there was confusion over the possible metabolic relationship of tryptophanase and tryptophan synthetase. Some of the early work has been reviewed by Happold & Scott (1963). Newton & Snell (1962) were the first to establish unambiguously that these two enzymes were completely different by characterizing tryptophanase in a tryptophan synthetase deletion mutant (*E. coli* B/It7). They also identified a source of confusion in the earlier literature by showing that tryptophanase itself had tryptophan synthetase activity although proceeding through different intermediates (Newton & Snell, 1964). In the current linkage map of *E. coli* (Taylor, 1970) the tryptophanase gene is represented as *tna*; both a structural gene (*tna*A) and regulatory gene (*tna*R) are recognized. These map in the region of *aro*I and *asn* at between 73 and 74 min, remote from the tryptophan synthetase (*trp*) genes at 25 min. The *tna* genes have been used as markers for studying the co-transduction of markers by generalized transducing phage (Pittard, 1965). Our procedure for isolating these mutants is based on the assumption that, as tryptophanase can represent up to 7% of the protein in induced bacteria (Newton & Snell, 1964), an organism lacking this enzyme is presumably at some sort of disadvantage in the medium in which such induction normally occurs, although the physiological role of the enzyme is obscure. The other property of tryptophanase$^-$ mutants (their inability to grow on tryptophan as sole carbon and nitrogen source) is not useful in the preliminary screening as it results in large numbers of auxotrophs being scored.

Our methodology for protein synthesis has consisted of identifying tryptophanase activity in preparations of polyribosomes, examining the nature of the binding of enzyme to polyribosome and demonstrating its biosynthesis *in vitro*. Apart from the use of characterizing such systems for subsequent analysis of mRNA, tryptophanase had the added interest of being a multi-chain protein. The subunit structure is well established (Morino & Snell, 1967*a*). The enzyme consists of a tetramer (mol.wt. 220000), which can be dissociated to form

monomers (mol.wt. 55000) that will only reassociate to form tetramers in the presence of the cofactor (pyridoxal phosphate). There is one binding site for pyridoxal phosphate per monomer.

The monomers themselves each contain two, apparently identical, polypeptide chains (Morino & Snell, 1967*b*). These two chains are linked by a single disulphide bond. The pyridoxal phosphate is linked via an azomethine bond to a lysine residue in one chain; the corresponding residue in the other chain has a free amino group. It is possible that nearly completed peptide chains released by puromycin are capable of forming subunits, which in the presence of the pyridoxal phosphate, required for enzyme assay, are able to re-form active tryptophanase. What appears to be less likely is that nascent polypeptide chains themselves should have enzyme activity. However, our results suggest that there is affinity between tryptophanase with the polyribosomes obtained from cells in which they are synthesized. We tentatively suggest that, as puromycin positively released significant amounts of enzyme, this association may be specific, although we have not shown that a nascent polypeptide chain is associated with another chain to form the type of subunit structure present in the intact active enzyme molecule.

The technique we have used for characterization of specific protein molecules newly synthesized *in vitro* is basically that which has been employed for several mammalian proteins and reviewed by Campbell (1970). After protein synthesis, the preparation is purified for the protein in question (after enriching it with carrier if necessary). For proteins containing more than one polypeptide chain, such as tryptophanase, it is important that the preparation and purification will facilitate the formation of active quaternary structures from the subunits. The conditions required for this association are well established. Pyridoxal phosphate is essential for the formation of the active molecule from the four subunits, although the 'half molecules' (dimers) will reassociate in the absence of the cofactor (Morino & Snell, 1967*a*). We therefore designed a purification procedure that involves treating the preparation with a large excess of pyridoxal phosphate (see the Materials and Methods section) rather than one which gives rise to apotryptophanase, which lacks the pyridoxal phosphate groups (Morino & Snell, 1967*a*). As each of the four tryptophanase subunits contains two probably identical polypeptide chains linked via a single cystine bridge (Morino & Snell, 1967*b*), our success with the biosynthesis of this enzyme implies that, under the conditions of protein synthesis *in vitro*, this cystine bridge is readily formed.

We present detailed evidence that the biosynthesis of the enzyme can be demonstrated *in vitro*. We have characterized the radioactive product of the protein synthesis by three independent methods: DEAE-cellulose chromatography, gel electrophoresis and sucrose-density-gradient centrifugation. These results, when considered with the controls, the concomitant increase in tryptophanase activity and the rates of release of [$^3$H]leucine with carboxypeptidases showing a difference in the distribution of label with incubation time, represent good evidence for the synthesis of tryptophanase molecules *in vitro* and indicate a satisfactory method for identifying polyribosomes involved in the synthesis of this enzyme.

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