Cell-Free Synthesis of Tryptophanase from *Escherichia coli*

USE OF RIBONUCLEIC ACID ISOLATED FROM INDUCED CELLS AND A COMPARISON OF THE PRODUCT FROM A SYSTEM EMPLOYING RIBOSOMES WITH THAT FROM ONE EMPLOYING RIBOSOMES AND EXOGENOUS RIBONUCLEIC ACID

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1. Polyribosomes and RNA were isolated from cultures in which tryptophanase (EC 4.2.1.-) was induced. The polyribosomes were incubated under conditions of protein synthesis, in the presence of a radioactive amino acid and a post-ribosomal supernatant fraction obtained from repressed cells. The RNA preparations were incubated under conditions of protein synthesis in the presence of a radioactive amino acid and a supernatant fraction containing ribosomes from repressed cells. 2. The system was characterized and the synthesis of a radioactive protein with the same chromatographic properties as tryptophanase was demonstrated. This synthesis was shown to be time-dependent and required the presence of RNA from induced cultures, ribosomes and an energy supply; it was inhibited by chloramphenicol. 3. The maximum activity for the synthesis of this protein was found to be associated with 23S rRNA isolated from sucrose gradients. 4. The *N*-terminal amino acid of tryptophanase was labelled in the protein synthesized in this system but not in the protein synthesized by polyribosomes (without added RNA). Conversely, the *C*-terminal amino acid of tryptophanase was labelled in the polyribosome system but not in the RNA-containing system. 5. Tryptic digests of protein labelled *in vitro* were compared with those of tryptophanase. No labelled tryptic peptides were identified other than tryptophanase tryptic peptides. An analysis of the results implied that in the polyribosome system almost the complete tryptophanase subunit chain was labelled but that in the RNA-containing system these chains were incompletely synthesized. 6. Sucrose-gradient analysis of protein synthesized in the RNA-containing system suggested that it cannot be converted into structures with the same sedimentation properties as native tryptophanase. 7. The significance of these results for the assay of tryptophanase mRNA and for an understanding of the control of the translation of this mRNA *in vivo* is discussed.

We have demonstrated the synthesis of a protein with the same chromatographic properties as tryptophanase [tryptophan hydro-lyase (deaminating), EC 4.2.1.-] by using polyribosomes isolated from induced cultures of *Escherichia coli* and the supernatant fraction from repressed (or mutant) cultures (Khairul Bashar, Parish & Brown, 1971). We have extended the above work to investigate whether synthesis of the protein could be demonstrated in a system in which the only component isolated from the induced cells was RNA. RNA-directed synthesis of a bacterial (as opposed to viral) protein of *E. coli* has been demonstrated previously for alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1). In this case, Dohan, Rubman & Torriani (1969) used a cell-free system containing super-

natant, ribosomes, some DNA and (presumably) some endogeneous mRNA present in polyribosomes (their system was not preincubated). The synthesis of a protein that co-chromatographed with alkaline phosphatase was found to be dependent on the addition to the system of RNA from a constitutive mutant for the enzyme.

We have employed a system that differs from that of Dohan *et al.* (1969) in the following respects. Ribosomes and supernatant were prepared by a procedure involving the use of deoxyribonuclease and were preincubated (to minimize the amount of endogeneous mRNA). Our procedure does not involve the use of labelled amino acid mixtures. This is a technical necessity in studies of tryptophanase, because several amino acids bind to the enzyme as substrates or products. The results

given in the present paper demonstrate that, although the tryptophanase subunits synthesized in the RNA-containing system are incomplete and incapable of assembly into the quarternary structure of the enzyme, the controls are sufficiently well established for tryptophanase mRNA to be assayed.

A preliminary account of the present work has been published (Parish, Khairul Bashar, Brown & Brown, 1971).

MATERIALS

Bacteria. *E. coli* M.R.E. 600 was used, except for the preparation of [³⁵S]methionine (see below). Conditions for growth in media in which tryptophanase is either induced or repressed were those used by Khairul Bashar *et al.* (1971).

Chemicals. Except for the materials listed below, the sources and purity of the chemicals were the same as those described by Khairul Bashar *et al.* (1971). Trypsin (crystalline), dithiothreitol, iodoacetic acid and 1-dimethylaminonaphthalene-5-sulphonyl chloride were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A., and guanidinium chloride (AnalaR) and polyamide-layer sheets for t.l.c. from BDH Chemicals Ltd., Poole, Dorset, U.K. Soluene was obtained from Packard Instrument Co., Downers Grove, Ill., U.S.A. Toluidine Blue was obtained from George T. Gurr, London S.W.6, U.K. Radioactive amino acids and inorganic sulphate were obtained from The Radiochemical Centre, Amersham, Bucks., U.K., except for [³⁵S]methionine which was prepared by the procedure given below.

E. coli B was grown in a medium containing 20 mM-NH₄Cl, 0.1 mM-Na₂SO₄, 1 mM-MgCl₂, 0.1 M-KH₂PO₄ and 4.3 mM-glucose. The pH was adjusted to 7.6 by the addition of 1 M-KOH. After overnight growth at 30°C, the $E_{550}^{1\text{cm}}$ was 0.5. A sample (0.1 ml) of culture was added aseptically to a flask containing 10 ml of the same medium in which the Na₂SO₄ was replaced by carrier-free inorganic [³⁵S]sulphate (10 mCi). The culture was swirled at 30°C until it had noticeably thickened (48 h). Trichloroacetic acid (10% w/v, 10 ml) was added and the mixture was incubated at 95°C until the faintly cloudy liquid had produced a granular precipitate (15 min). The precipitate was collected by centrifugation and washed six times with trichloroacetic acid (5% w/v). The precipitate was then dispersed in 6 M-HCl (0.2 ml) and was heated at 100°C for 16 h. The solution was cooled and streaked on Whatman 3MM paper. Markers (cysteine and methionine) were run as standards away from the radioactive streak. The paper was developed by ascending chromatography with butan-1-ol-acetic acid-water (12:5:3, by vol.) as solvent. The paper was dried, then the strips containing the marker compounds were cut off and stained with ninhydrin reagent. The positions of the standard compounds were marked on the radioactive paper, which was then radioautographed (30 min exposure to X-ray film). The band of radioactivity corresponding to methionine was eluted with water, freeze-dried and stored.

METHODS

Protein synthesis. Tryptophanase, polyribosomes, supernatant fraction and tRNA were isolated as described

(or referred to) by Khairul Bashar *et al.* (1971). RNA was isolated (from both repressed and induced cells) by the method of Parish (1968). RNA was fractionated by sucrose-density-gradient centrifugation and analysed by polyacrylamide-gel electrophoresis. Details are given in the legend to Fig. 1. The methods for the preparation of polyacrylamide gels were those described by Loening (1968).

The RNA-directed synthesis of protein was done by using preincubated S-30 fraction. The preparation of this fraction and the components of the incubation system are those described by Nathans (1968). The concentration of RNA employed in this method is 0.2 mg/ml. Repressed cells were invariably used for preparing the S-30 fraction.

In all experiments the incubation mixture contained 19 unlabelled amino acids and one radioactive amino acid. The final volume of incubation mixture was 1 ml and the radioactive amino acid was one of the following: DL-[4,5-³H]leucine (16 Ci/mmol; 0.2 mCi/ml), L-[G-¹⁴C]-leucine (62 mCi/mmol; 20 μ Ci/ml), [³⁵S]methionine (200 mCi/mmol; 20 μ Ci/ml) or DL-[G-³H]valine (250 mCi/mmol; 0.1 mCi/ml).

Protein synthesis was stopped by immersing the tube in ice. In most experiments, unlabelled tryptophanase was added immediately and the mixture was purified for tryptophanase and chromatographed on columns of DEAE-cellulose. The tryptophanase activity and radioactivity were assayed as described by Khairul Bashar *et al.* (1971). In one case, tryptophanase carrier was not added, the purification was done as far as obtaining the supernatant from the precipitation with (NH₄)₂SO₄ (40% saturation). This fraction was dialyzed against starting buffer at 4°C for 18 h and the dialysate was loaded on to a column of DEAE-cellulose and chromatographed as described by Khairul Bashar *et al.* (1971).

In some experiments, the incubations were stopped with trichloroacetic acid and the incorporation of radioactivity into trichloroacetic acid-insoluble peptide was determined. The methods of Stahl, Lawford, Williams & Campbell (1968) were used.

Tryptic digestion and determination of terminal amino acids. Fractions (eluted from DEAE-cellulose) containing tryptophanase activity and radioactivity were pooled, dialysed against 5 mM-KH₂PO₄ (adjusted to pH 7.6 by the addition of 0.1 M-KOH) for 16 h at 4°C, freeze-dried and dissolved in 6 M-guanidinium chloride-50 mM-dithiothreitol-10 mM-EDTA (disodium salt)-20 mM-tris-HCl, pH 8.5. The protein was reduced and carboxymethylated by the method of Morino & Snell (1967b). The reduced, carboxymethylated product was used for all the procedures described below.

Protein, labelled either with [¹⁴C]leucine or [³⁵S]-methionine, was digested with trypsin then dialysed and freeze-dried by the method of Morino & Snell (1967b). The residue was resuspended in a small volume (approx. 0.02 ml) of water and was analysed by two-dimensional electrophoresis and chromatography by a procedure that is a modification of that described by Morino & Snell (1967b). A sheet (35 cm $\times$ 30 cm) of Whatman 3MM paper was wetted with pyridine-acetic acid-water (1:10:289, by vol.) and the tryptic digest was spotted on the origin (near a corner, 2 cm in from each of two sides) and was fractionated by electrophoresis in the longer dimension

(2500 V for 2 h) in a tank under white spirit. The paper was removed and dried at room temperature. Ascending chromatography in the second dimension was performed for 16 h with butan-1-ol-acetic acid-water (3:1:1, by vol.) as solvent. The paper was dried in air, sprayed with cadmium-ninhydrin reagent (Heilmann, Barollier & Watzke, 1957) and placed in the dark in an ammonia-free atmosphere for 16 h. The stained spots were marked in pencil. Radioactivity on the papers was assayed by two methods. Either the spots, together with randomly chosen, unstained areas, were cut out and counted for radioactivity, or alternatively the paper was cut into 2 cm squares and counted. In either case the radioactivity on the pieces of paper was determined by liquid scintillation counting (see below).

The *N*-terminal amino acid was determined by using protein labelled with [³⁵S]methionine. The protein was treated with DNS-chloride and the terminal DNS-amino acid was removed and analysed (by two dimensional t.l.c.) as described by Woods & Wang (1967).

The *C*-terminal amino acid was determined by using protein labelled with [³H]valine by digestion with carboxypeptidase B. The procedure used was that of Morino & Snell (1967b) except that the release of [³H]valine was measured by analysis of samples (removed from the incubation mixture at suitable times) by ascending chromatography on Whatman No. 1 paper, with butan-1-ol-pyridine-water (1:1:1, by vol.) as developing solvent.

Liquid scintillation counting. The methods employed were those of Khairul Bashir *et al.* (1971). Additionally, the radioactivity of pieces of paper (from two dimensional electrophoresis and chromatography) or pieces of polyamide layer sheets (cut from two-dimensional thin-layer chromatograms) was determined. The pieces were soaked for 24 h in 0.5 ml of Soluene and then 15 ml of toluene-based scintillation fluid (Khairul Bashir *et al.* 1971) was added. Usually the background radioactivity for such measurements (40–50 c.p.m.) was determined by separately counting the radioactivity of randomly chosen regions and has been subtracted from the results given in the present paper. However, when the radioactivity was measured after the paper had been cut systematically into squares the background radioactivity was not subtracted (see Table 3). The background was not subtracted in the results given for the labelling of *N*-terminal methionine (Table 1).

RESULTS

Protein synthesis by ribosomes and exogenous RNA. RNA was isolated from whole cells from induced cultures of *E. coli* and this total RNA was fractionated by sucrose-density-gradient centrifugation. Pooled fractions consisting of (1) all the RNA sedimenting more slowly than the 16S zone, (2) the 16S zone, (3) the region between 16S and 23S RNA, (4) the 23S zone and (5) all the RNA sedimenting faster than the 23S zone were collected. The density-gradient fractions associated with 23S rRNA (fraction 4) directed the synthesis of a protein which, when purified for tryptophanase activity (by protamine-sulphate precipitation,

Fig. 1. Fractionation and characterization of RNA employed for studies on protein biosynthesis. (a) A sample of RNA, isolated from induced culture of *E. coli* M.R.E. 600 was centrifuged through a linear sucrose gradient (7.5–30% w/v, of sucrose in 0.15M-sodium acetate, pH was not adjusted) in a tube for the SW 25.1 rotor of the Beckman model L 1 ultracentrifuge. The gradient was centrifuged for 15 h at 4°C and 24000 rev./min. After centrifugation, the gradient was pumped through a flow-through cell of the Gilford 2000 system attached to a Unicam SP.500 spectrophotometer. Fractions (0.8 ml) were collected and those containing the 23S (largest) peak were pooled; 2 vol. of ethanol was added and the precipitate was recovered by centrifugation. (b) A sample of the precipitate (approx. 0.1 mg) was fractionated by electrophoresis for 15 h at 4°C in the 2.6% polyacrylamide gel described by Loening (1968). After electrophoresis (8 V/cm; 2 h) the gel was removed from the tube and stained in Toluidine Blue (0.1% dissolved in aq. 40%, v/v, 2-ethoxyethanol) for 1 h at room temperature. The gel was de-stained overnight in aq. 2-ethoxyethanol (40%, v/v) and scanned in the Joyce-Loebel Chromoscan with the 0.045 orange filter. The extinction scale is in arbitrary units.

ammonium sulphate precipitation and hydroxy-apatite-gel adsorption) was indistinguishable from tryptophanase by DEAE-cellulose chromatography.

An example of the fractionation of the *E. coli* RNA by sucrose-density-gradient centrifugation is shown in Fig. 1(a). The components of the 23S peak (employed for subsequent experiments on protein synthesis) were analysed by gel electrophoresis (Fig. 1b). The '23S RNA' is a heterogeneous preparation. Some of the minor components undoubtedly arise because it is not possible to isolate completely pure components from a zone in a sucrose

gradient. However, it is likely that some aggregation of minor components (and 16S rRNA) occurs in sucrose gradients and that such aggregates occur in the 23S region (Parish & Kirby, 1966; Hastings, Parish, Kirby & Cook, 1968). The conclusion that is made in the present paper is that tryptophanase mRNA sediments with the 23S rRNA in sucrose gradients; we do not claim that there is evidence that it is necessarily of the same molecular weight as 23S rRNA.

The results of the fractionation of protein synthesized *in vitro* in the RNA-directed system are summarized in Fig. 2. They demonstrate that

Fig. 2. Characterization of protein synthesized in the RNA-directed, cell-free system with [^3H]leucine as the labelled amino acid. Radioactivity (—) and tryptophanase activity (----) were measured in fractions (10 ml) eluted from DEAE-cellulose columns. With the exception of (i) (see below) tryptophanase was added to the incubation mixture after protein synthesis and the mixture was purified and then chromatographed by the methods of Khairul Bashir *et al.* (1971). In (a)–(f) results of parallel experiments in which RNA and S-30 fraction were taken from the same batches (see the Methods section) are shown. In (a)–(c) the incubation mixture was complete and incubation times were (a) 0 min, (b) 5 min and (c) 40 min. In (d)–(f) the incubation times were 40 min and in (d) the S-30 fraction was replaced with buffer, in (e) the GTP and ATP were replaced with buffer; (f) was a complete system but contained additionally chloramphenicol (0.1 mg/ml). In (g) the conditions were the same as in (c) except that 23S RNA from repressed cells was employed; in (h) the same conditions as in (c) were employed except that the 23S RNA was replaced with buffer. In (i), no tryptophanase was added to the preparation and the modified work-up procedure (see the Methods section) was employed; the absence of radioactivity eluted at the start of the chromatography is caused by the prolonged dialysis of this sample.

the synthesis of the protein that co-chromatographed with tryptophanase was time-dependent (Figs. 2*a* and 2*c*); that it was dependent on the presence of the S-30 fraction (Fig. 2*d*) and on the presence of high-energy phosphates (Fig. 2*e*) and that it was inhibited by chloramphenicol (Fig. 2*f*). These results together confirm that this radioactive protein accumulates as a result of chain elongation and not as a result of adsorption of the labelled amino acid or transpeptidation. The protein is not made if the 23S RNA is obtained from repressed cells (Fig. 2*g*). To check that the effect of repressed RNA was not to inhibit in some way RNA-independent synthesis in the S-30 system, we showed (Fig. 2*h*) that the protein was not synthesized in the system in the absence of any added RNA (apart from tRNA, which is added to all these systems; Nathans, 1968). Fig. 2(*i*) shows that radioactive material with the same chromatographic properties as tryptophanase can be detected in extracts from the protein-synthesis mixtures, even when no carrier tryptophanase has been added. The profile of radioactivity in this case is more diffuse, implying that heterogeneous material is eluted from the column. This is not surprising, as the hydroxyapatite steps in the purification of tryptophanase (Khairul Bashar *et al.* 1971) were omitted from the preparation of this sample (see the Methods section). The hydroxyapatite steps

had to be omitted, as the efficiency of the hydroxyapatite fractionations depends critically upon the protein concentrations and, with no carrier tryptophanase present, the amount of protein remaining after the ammonium sulphate precipitation was only sufficient to allow dialysis and chromatography of the preparation.

The control in which high-energy phosphates are omitted (Fig. 2*e*) gave a much clearer demonstration of energy dependence than that found in work on the polyribosome-directed synthesis of this protein (Khairul Bashar *et al.* 1971). Presumably there is less adsorbed ATP and GTP contained in the S-30 preparation than the polyribosome preparations.

We separately confirmed that the 23S RNA fraction stimulated overall protein synthesis by demonstrating that under incubation conditions identical with those in Figs. 2(*c*) and 2(*h*), the radioactivity incorporated into total trichloroacetic acid-insoluble polypeptide was 129 c.p.m. in the absence of the RNA and 1774 c.p.m. in the presence of 23S RNA.

Characterization of protein synthesized in vitro. We have described the centrifugation of tryptophanase through sucrose gradients and have shown that the protein synthesized in the polyribosome-directed system (and purified for tryptophanase activity) sediments as a zone superimposable with

Fig. 3. Analysis of protein synthesized *in vivo* and fractionated by chromatography on DEAE-cellulose (see Fig. 2) by sucrose-gradient centrifugation. Linear sucrose gradients [5–20% (w/v) sucrose in 5 mM-KH₂PO₄–1 mM-EDTA (sodium salt), pH adjusted to 6.8 with 0.1M-KOH] were prepared in tubes for the SW 25.1 rotor for the Beckman model L ultracentrifuge. In (a) a fraction eluted from DEAE-cellulose in the absence of added tryptophanase (as in Fig. 2*i*) was layered directly over the gradient. In (b) a fraction containing both radioactivity and tryptophanase was isolated from DEAE-cellulose (as in Fig. 2*c*). It was incubated in the presence of pyridoxal phosphate (0.1 mM) for 15 min at 30°C and was then layered over the gradient. Centrifugation was for 15 h and 4°C at 24000 rev./min. The gradients were sampled by using a peristaltic pump and timer and fractions were assayed for radioactivity (—) and tryptophanase activity (----).

the zone of tryptophanase activity (Khairul Bashar *et al.* 1971). We therefore studied the properties of the protein eluted from the DEAE-cellulose in the absence of added tryptophanase carrier. The protein isolated in this way did not sediment in this position (Fig. 3a). As pyridoxal phosphate is required for the assembly of the quaternary structure of tryptophanase (Morino & Snell, 1967a), the preparation was incubated with the cofactor before centrifugation. Again, no significant amount of radioactivity was found in the region of the gradient characteristic of tryptophanase. There are two possible explanations for the failure to obtain assembly of structures with the same hydrodynamic properties as those of the native enzyme. Either the subunit chains are present in such low concentration that such association is very unlikely, or alternatively the radioactive protein synthesized in the RNA-directed system consists of incomplete subunit chains. The results of an experiment in which the protein was centrifuged through a gradient in the presence of tryptophanase (Fig. 3b) suggest that the second alternative is probably correct.

From these results, we assumed that the polyribosome-directed system (Khairul Bashar *et al.* 1971) results in the essentially correct completion of tryptophanase subunit chains, whereas the RNA-directed system results in the initiation of, but incomplete or partial termination of synthesis of, these chains. To examine this possibility, we studied the labelling of the *N*- and *C*-terminal amino acids of the tryptophanase subunit in the two systems and also compared the tryptic peptide

Table 1. *Determination of whether the N-terminal amino acid of protein synthesized in vitro is [³⁵S]-methionine*

Protein synthesis (40 min incubations) was performed by incubating either the polyribosome (Khairul Bashar *et al.* 1971) or the RNA system described in the present paper with [³⁵S]methionine as labelled amino acid. The products were processed in the presence of tryptophanase carrier, and radioactivity (associated with tryptophanase activity) was eluted from DEAE-cellulose and converted into the reduced, carboxymethylated product. The *N*-terminal DNS-amino acid was removed and the resulting digest was analysed by two-dimensional t.l.c. The DNS-methionine spot was cut out and the radioactivity counted. Other pieces of the chromatogram were also cut out to measure the background radioactivity (see the Methods section). The background radioactivity has not been subtracted in these results. The error was $\pm 2\%$.

Protein synthesis system	Radioactivity (c.p.m.)		
	DNS-Met	Background	
Polyribosome system	70	61	54
RNA system	186	78	80

'maps' of the protein labelled *in vitro* with the 'map' of tryptophanase.

The *N*-terminal amino acid of the tryptophanase subunit is methionine (Morino & Snell, 1967b). Protein synthesis in each system was performed with [³⁵S]methionine as label. The results (Table 1) are consistent with the *N*-terminal amino acid of protein synthesized by preincubated ribosomes and 23S RNA being radioactive methionine; they imply that the *N*-terminal amino acid synthesized by the polyribosomes is probably not methionine.

The *C*-terminal amino acid of the tryptophanase subunit is valine (Morino & Snell, 1967b). Protein synthesis in each system was performed with [³H]valine as label. It was found that the *C*-

Fig. 4. Determination of whether the *C*-terminal amino acid of protein synthesized *in vitro* is [³H]valine. Protein synthesis and isolation of product was performed as described in the legend to Table 1 except that the labelled amino acid was [³H]valine. After reduction and carboxymethylation, the product was digested with carboxypeptidase B and the reaction was analysed for the presence of [³H]valine (see the Methods section). Results are plotted for the products of RNA-directed (○) and polyribosome-directed (●) synthesis.

Fig. 5. Distribution of tryptic peptides from tryptophanase in the two dimensional fractionation system described in the Methods section. The numbers are those used to identify the peptides in Table 2, Fig. 6 and the accompanying discussion.

terminal amino acid of the protein synthesized by the polyribosomes was radioactive valine; that of the RNA-directed protein was apparently not (Fig. 4). These experiments suggest that tryptophanase synthesis is not initiated in the polyribosome system. They also support our supposition that the subunits were erroneously terminated in the RNA-dependent synthesis.

The tryptic peptide 'map' of tryptophanase (Fig. 5) is similar to (but not identical with) that obtained by Morino & Snell (1967*b*) and we find one less peptide than they do. Part of the difference is undoubtedly caused by the differences in the methods of two-dimensional fractionation used (see the Methods section). Additionally we are studying a different strain of *E. coli*.

Labelled protein (containing tryptophanase as carrier), eluted from the DEAE-cellulose columns was digested with trypsin and the peptides were analysed by the two-dimensional system of electrophoresis and chromatography. In one experiment, protein labelled with [^{14}C]leucine from the polyribosome-directed and from the RNA-directed systems was digested with trypsin and run in parallel in the system for the production of the tryptic peptide 'maps' (Fig. 5 is a tracing of the ninhydrin-positive spots on one of these 'maps'). The 'maps' of ninhydrin positive material were essentially

identical. The spots were cut out and counted for radioactivity. The results, together with those from an experiment in which protein was labelled with [^{35}S]methionine in the RNA-dependent system, are summarized in Table 2. Two extra ninhydrin-positive spots (13A and 19A) were found in the preparation obtained from the experiment with [^{35}S]methionine-labelling; these spots were not found in other preparations and are believed to be derived from a contaminant in this particular experiment. The results of Table 2 confirm that most of the chains are labelled in both cases. In some cases the amount of labelling was so small that significant conclusions cannot be drawn. However, peptides 1 and 30 are labelled in the RNA-dependent system but not in the polyribosome-dependent system and we suggest that these peptides occur at the *N*-terminal end of the chain. Several peptides (for example 20, 24 and 25) are labelled in the polyribosome-directed but not in the RNA-directed system. We therefore conclude that these peptides are present towards the *C*-terminus of the chain beyond a point at which synthesis in the RNA system is either prematurely terminated or (possibly) is caused by erroneous translation. The radioactivity of certain peptides is puzzling and may indicate some heterogeneity. For example, in the RNA-directed system, peptide

Table 2. *Radioactivity measured in tryptic peptides from tryptic digests of tryptophanase containing protein labelled in vitro*

The peptides were obtained as described in the Methods section, and are numbered as shown in Fig. 5. Background radioactivity has been subtracted. Spots 13A and 19A are discussed in the text. Spot 13A was just to the left of spot 19A and spot 19A was just above spot 19 (in the orientation of the paper illustrated in Fig. 5).

Peptide no.		Radioactivity in peptide (c.p.m.)		
Protein synthesis system	...	Polyribosomes [¹⁴ C]leucine	RNA-S-30 [¹⁴ C]leucine	RNA-S-30 [³⁵ S]methionine
1		-30	65	120
2		840	830	230
3		1000	80	100
4		680	80	130
5		125	20	75
6		100	20	80
7		40	20	30
8		80	0	30
9		700	25	55
10		200	15	25
11		490	70	10
12		110	25	5
13		110	-5	55
14		220	110	15
15		670	100	75
16		510	110	10
17		480	50	50
18		360	20	60
19		1260	0	25
20		340	5	10
21		670	0	0
22		350	0	45
23		170	5	45
24		50	5	5
25		5	0	10
26		270	0	180
27		305	140	300
28		500	150	180
29		1720	130	45
30		-40	20	45
31		270	130	600
32		180	225	565
13A				5
19A				10

26 is labelled with methionine but not with leucine. This might be taken to imply that it contained no leucine residues. However, in the polyribosome-directed system, this peptide is significantly labelled with leucine. One possibility is that the spot 26 contains two peptides, one (lacking leucine residues) labelled in the RNA-dependent system; the other (containing leucine residues) labelled in the polyribosome-directed system. We are also uncertain about the significance of peptide 2, which contains anomalously large amounts of radioactivity in both systems. It is probable that this region of the chromatogram contains low-molecular-weight radioactive contaminants.

We performed another series of experiments to establish that the apparent coincidence of the ninhydrin-stained and the radioactive 'maps' was genuine. The amounts of radioactivity in many of the spots was so small that reliable radioautographs could not be obtained. We therefore adopted a different technique to count the radioactivity of the papers that did not involve any biased choice of regions to count. Papers were stained with ninhydrin and dried. The position of the spots was traced on to a blank piece of paper. Identical grids 2cm × 2cm were ruled both on the chromatogram and on its copy. The chromatogram was cut into these 2cm × 2cm squares which

Table 3. *Example of the results obtained when the tryptic-peptide 'map' is cut into squares with no bias given to ninhydrin-positive spots*

The results tabulated (c.p.m., background not subtracted) were obtained from an experiment in which the polyribosome-directed system was labelled with [³⁵S]methionine. Squares (2 cm × 2 cm) were cut from a dried stained chromatogram as described in the Methods section. The presence of ninhydrin-positive material (not necessarily from only one spot) is indicated by the presence of plus signs above the c.p.m. Thus +++ indicates that half or more of the square was stained; ++ that between one-quarter and one-half was stained; + that one-quarter or less of the square was stained. The c.p.m. values are laid out in the patterns in which they appeared on the chromatogram, oriented as in Fig. 5 with the origin (a) in the bottom left-hand corner.

54	++ 98	++ 64	55	53	54	59	53	53	++ 93	+++ 100
++ 97	+++ 127	++ 89	+++ 88	++ 105	++ 103	+ 60	45	+ 50	++ 96	+ 87
53	++ 101	50	53	53	87	45	39	++ 103	+++ 158	65
51	51	56	55	++ 91	+ 57	++ 106	++ 107	++ 106	++ 106	69
54	++ 101	53	56	++ 94	++ 104	+ 57	57	+++ 108	51	68
52	++ 167	55	++ 105	++ 77	++ 107	++ 112	++ 88	++ 114	++ 108	53
58	+++ 119	++ 96	++ 93	+ 59	++ 109	++ 107	42	++ 121	++ 99	+ 56
52	+ 51	52	55	56	++ 115	++ 121	54	47	++ 114	++ 118
55 (a)	50	54	53	57	51	56	55	48	50	52
55	61	52	53	55	54	51	54	44	53	56

were then counted for radioactivity. The counts were recorded on a grid and the presence or absence of ninhydrin-positive material lying in the square in question was recorded. The results of such an analysis are summarized in Table 3. It is clear that there is no radioactive material away from the ninhydrin peptides.

DISCUSSION

The results of Khairul Bashar *et al.* (1971) and those in the present paper demonstrate that it is possible to isolate from induced cultures of *E. coli* M.R.E. 600 either polyribosomes capable of completing tryptophanase synthesis *in vitro* or RNA capable of directing the initiation of tryptophanase synthesis *in vitro*. The conclusions from our results are summarized in Fig. 6. This evidence suggests that after prolonged incubation, the 'chain completion reactions' of the polyribosomes achieve the synthesis of the bulk of the chains *de novo*. We have established (Khairul Bashar *et al.* 1971) that after short periods of incubation, the synthesis *de novo* that resulted in the labelling of identification subunit chains commenced at a point nearer to the C-terminus. Our present analysis suggests that after longer incubations, the section of unlabelled chain (N-1 in Fig. 6) is relatively short. The results of Khairul Bashar *et al.* (1971) also demonstrated that after long incubations, there

is a concomitant increase in the amount of tryptophanase activity in the polyribosome preparation.

Our results imply that there is no re-initiation in the polyribosome system. It is well established that re-initiation is extremely inefficient in such systems (otherwise the preincubation of S-30 preparations for subsequent use in RNA-dependent protein synthesis would not decrease the endogenous incorporation in the way it does). We cannot state whether the difference between the RNA-directed and the polyribosome-directed synthesis is that initiation is genuinely more efficient in the S-30 system or whether there is an extremely inefficient initiation in both cases (which is undetectable in the polyribosome system, because it is completely masked by the products of chain completion). We cannot make such a differentiation because there is no means of knowing the concentration of tryptophanase mRNA in the RNA preparation that is used for the RNA-directed synthesis. Certainly the total RNA concentration in this system (see the Methods section, and Nathans, 1968) is greatly in excess of the ribosome concentration.

The RNA-directed system results in the synthesis of incomplete tryptophanase chains (N-2 in Fig. 6). This is in agreement with the observation that the protein labelled in this system is incapable of forming the quaternary structure characteristic of tryptophanase. We cannot examine the accuracy

N		2
Met		Val
1,7?,	2?,3,4,5,6,8?,9,10*,11*,12*,	19*,20*,21*,22*,
30.	13?,14*,15,16*,17,18?,	23*,24*.
	26?,27,28,29,31,32.	

Fig. 6. Representation of the tryptophanase subunit chain with a tentative grouping of the tryptic peptides into three groups. We have omitted peptide 25 as no significant labelling of this material was obtained. Other peptides the labelling of which is extremely low or ambiguous (some of which may not contain leucine) are identified by a question mark. A superscript x indicates that the peptide in question is definitely not labelled in the methionine-labelled RNA system. In several other peptides the labelling with methionine is very low and probably not significant (see Table 2).

of the RNA-directed system in more detail with our present techniques. We have only characterized the protein that co-chromatographs with tryptophanase on DEAE-cellulose columns. It is possible that there are other classes of fragmentary tryptophanase subunits formed in the RNA-directed system that are discarded during the purification procedure for tryptophanase.

Two points emerge from our studies of the biosynthesis of tryptophanase. One is that the techniques for protein synthesis and characterization of the products (in the presence of added tryptophanase) described in the present paper provide an assay system for tryptophanase mRNA. Even though the subunits so produced are clearly incomplete, the controls (summarized in Fig. 2) establish clearly that the DEAE-cellulose chromatography system is adequate for this purpose.

The second point relates to the translation of mRNA for tryptophanase *in vivo*. These results (and those of Khairul Bashar *et al.* 1971) imply that polyribosomes with the mRNA corresponding to the tryptophanase structural gene occur intact in the cell. It is known that for certain *E. coli* operons, such as the one containing the structural gene for tryptophan synthetase (EC 4.2.1.20) transcription and translation are uniquely coupled. There are two interpretations of our results on tryptophanase. One is that at least a proportion of the tryptophanase polyribosomes are free in the cell and unattached to DNA. The alternative is that if a coupled transcriptional and translational system of the type operating in tryptophan synthetase (Morse, Mosteller & Yanofsky, 1969) is operative, the section of mRNA containing the transcribed copy of the tryptophanase structural gene can be isolated intact, either as a fragment of a polyribosome (attached to DNA *in vivo* via RNA polymerase) or as a deproteinized RNA molecule.

We are unable to decide between these two possibilities as the length of the operon containing the *tna* (tryptophanase) gene is unknown (Gartner & Riley, 1964). If the mRNA is as large as its apparent sedimentation constant (23S) implies, it is clearly polycistronic. However we believe that the former alternative (that separate tryptophanase polyribosomes exist in *E. coli*) is more likely. Khairul Bashar *et al.* (1971) were unable to demonstrate any effect of deoxyribonuclease on the sedimentation behaviour of the polyribosome preparation, or on the sedimentation of the small amount of tryptophanase that we tentatively identified as 'polyribosome-bound', or on the protein synthetic capacity of the polyribosome. Thus, tryptophanase may be an example of an inducible enzyme of *E. coli* that, unlike tryptophan synthetase or the products of the *lac* operon, is translated in a system uncoupled to transcription. There is indirect evidence of another type in support of this view. Pastan & Perlman (1969) demonstrated that 3':5'-cyclic AMP enhances the rate of tryptophanase synthesis in *E. coli* that had been pre-treated with EDTA to make the cells permeable to the nucleotide. This effect was apparently due to translational control as it was observed both in cells in which transcription was proceeding and in cells in which transcription had been inhibited. Interestingly, there was no enhancement by 3':5'-cyclic AMP of the synthesis of tryptophan synthetase, nor of total protein synthesis. Pastan & Perlman (1969) showed that the effect is not due to activation of tryptophanase subunits and they also estimated the half-life of tryptophanase mRNA to be 3 min. Clearly the existence of such systems should facilitate the isolation and characterization of undegraded *E. coli* mRNA. If tryptophanase is an example of a gene product the translation of which is uncoupled to transcription, are there already any clues to what might distinguish tryptophanase from other enzymes in this way? The only obviously distinguishing feature of tryptophanase is that its induction is maximal in the very late exponential phase (Happold & Scott, 1963; Khairul Bashar *et al.* 1971). Perhaps more attention should be paid to enzymes induced in late exponential or early stationary phase in future experiments on *E. coli* mRNA.

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