The Synthesis of Ribosomes by a Mutant of *Escherichia coli*

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1. When the methionine-requiring mutant 58-161 of *Escherichia coli* was starved of methionine, ribonucleic acid was made in the absence of protein synthesis. 2. Most of this ribonucleic acid was similar to that found in ribosomes but was contained in particles differing from ribosomes both in sedimentation coefficient and in chromatographic behaviour on diethylaminoethylcellulose. 3. When methionine was added to a starved culture, the ribonucleic acid synthesized during starvation was almost completely undegraded as growth resumed. A transient loss of 5-10% could be largely attributed to breakdown of messenger ribonucleic acid accumulated during starvation. 4. After the addition of methionine, ribosomes were formed from the particles, and during this period preferential synthesis of ribosomal protein took place. 5. It is suggested that under these conditions the direct synthesis of ribosomes from the particles may occur.

This paper describes work carried out with the methionine-requiring mutant 58-161 of *Escherichia coli* K12. Normally, if a bacterial mutant that requires an amino acid is incubated in a growth medium lacking the amino acid, neither RNA nor protein is synthesized (Pardee & Prestidge, 1956). However, Borek, Ryan & Rockenbach (1955) discovered that if the mutant 58-161 is starved of methionine, considerable synthesis of RNA occurs. In this strain the normal 'stringent' control of RNA synthesis by amino acids appears to be 'relaxed' and mutants requiring amino acids other than methionine have been obtained from mutant 58-161 which continue to synthesize RNA when starved of their new requirements (Stent & Brenner, 1961). The prototrophic *E. coli* strain K10, derived from mutant 58-161 by loss of the requirement for methionine, also shows relaxed control of RNA synthesis under certain conditions (Neidhardt, 1963).

The nature of the RNA synthesized when mutant 58-161 is starved of methionine has been investigated in some detail. Transfer RNA is made which lacks the minor methylated components normally present in this form of RNA (Fleissner & Borek, 1962). Two species of RNA with the same sedimentation coefficients as the RNA in ribosomes are also synthesized (Mandel & Borek, 1963). However, during methionine starvation, ribosomes themselves are not formed. Instead, the newly synthesized ribosomal RNA is contained in particles visible in the analytical ultracentrifuge and which in crude cell-free extracts have sedimentation coefficients of about 14-16s: these particles are

more sensitive to ribonuclease and ultrasonic vibrations than are the ribosomes (Dagley, Turnock & Wild, 1963).

Apparently similar particles are found in extracts of cells of *E. coli* that have been incubated in the presence of chloramphenicol (Nomura & Watson, 1959; Dagley & Sykes, 1959), puromycin (Dagley, White, Wild & Sykes, 1962) or chlortetracycline (I. A. Holmes & D. G. Wild, unpublished work). These antibiotics inhibit protein synthesis but permit synthesis of RNA to continue. Inhibited cells thus contain RNA made in the absence of protein synthesis, as do starved cells of mutant 58-161.

It has been suggested that the 14-16s particles accumulated by the mutant or produced during inhibition by chloramphenicol are related to precursors of the ribosomes (Kurland, Nomura & Watson, 1962; Dagley *et al.* 1962). The work reported below is concerned primarily with the events following the addition of methionine to starved cultures of mutant 58-161. It provides evidence that under these conditions conversion of 14-16s particles into ribosomes takes place. In addition, some further observations on the RNA accumulated by this mutant are presented. A preliminary account of some of this work has been given (Turnock & Wild, 1964).

EXPERIMENTAL

Organism. *Escherichia coli* K12, mutant 58-161, was obtained from Dr W. Hayes, Microbial Genetics Research Unit, Hammersmith Hospital, London, W.12,

Cultural conditions. For large-scale experiments, batches (101.) of cells were grown at 30° with forced aeration in a medium adjusted to pH 7.0 with NaOH and containing (per l.): KH_2PO_4 , 9g.; $(\text{NH}_4)_2\text{SO}_4$, 1g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.6g.; DL-methionine, 0.05g.; glucose, 0.4g. After growth overnight to a population that was limited by the amount of glucose supplied, 2.3g. of glucose/l. was added and the cells were harvested 1 hr. later while in the exponential phase.

For incubation in the absence of methionine, the cells were washed by centrifuging with 800 ml. of methionine-free medium containing 2.5g. of glucose/l., resuspended in 3.8l. of the same medium and vigorously aerated at 30° for 3 hr. If necessary, the RNA synthesized during starvation was labelled by including $[2\text{-}^{14}\text{C}]\text{uracil}$ ($10\mu\text{C}/\text{l.}$) and unlabelled uracil (40mg./l.) in the medium. Growth of cultures starved of methionine was initiated by harvesting cells and resuspending them in an equal volume of fresh medium containing 100mg. of DL-methionine/l. and (if the cells contained labelled RNA) 100mg. of uracil/l.

Investigation of RNA metabolism by using $[2\text{-}^{14}\text{C}]\text{uracil}$ also involved experiments with only small volumes (30–50 ml.) of cell suspension, which were aerated by shaking at 30° in 250 ml. conical flasks. Cultures for these experiments were grown overnight with forced aeration in the same medium as described above except that the glucose concentration was 0.066g./l. Growth was restarted by the addition of 0.7g. of glucose/100 ml. of medium and allowed to proceed for at least 1 hr. before starting an experiment. The extinction of these cultures was less than in the large-scale experiments and the concentration of unlabelled uracil in the medium used for methionine starvation was decreased to 5mg./l.

Extinctions of cultures were determined at 540m μ with a Unicam SP.500 spectrophotometer. Estimations of RNA and protein were carried out as described by Dagley *et al.* (1963).

Preparation of cell-free extracts. Extracts were prepared from harvested cells by using a Hughes (1951) bacterial press cooled to about –15°. For analytical ultracentrifuging and sedimentation analysis in sucrose density gradients, the frozen disintegrated cells were taken up in 15mm- KH_2PO_4 , pH 7.0, containing magnesium acetate (0.1mM) ('phosphate-magnesium acetate buffer'). Insoluble debris was removed by centrifuging at 15000g for 1 hr. at 3–5° in an MSE centrifuge.

For the separation of ribosomes from 'soluble' material, extracts were made in 10mM-tris chloride, pH 7.2, containing MgCl_2 (20mM) ('tris-magnesium chloride buffer') with the addition of 5 $\mu\text{g.}$ of deoxyribonuclease/ml. After removal of debris, the clarified extract was centrifuged for 2 hr. at 102000g at 3–5° in the 50 rotor of the Spinco model L-HV ultracentrifuge. The top 4 ml. of the supernatant in each centrifuge tube was retained as a source of soluble protein. The ribosome pellet was rinsed twice with 1 ml. of tris-magnesium chloride buffer and then taken up in 3.5 ml. of the same buffer; insoluble material was then removed by centrifuging for 15 min. at 33000g at 3–5° in the SW 39 rotor.

Sucrose-density-gradient analysis. Extracts were prepared in phosphate-magnesium acetate buffer containing 5 $\mu\text{g.}$ of deoxyribonuclease/ml. on the basis of 1g. of crushed cells to 2.5 ml. of buffer. Clarified extract (0.07 ml.) was used for analysis in a sucrose gradient [5–20% (w/v) in 5 ml. of

phosphate-magnesium acetate buffer] by using the technique described by Dagley *et al.* (1963). After centrifuging at 124000g and 5° in the SW 39 rotor, 10-drop fractions were collected into 2.0 ml. of phosphate-magnesium acetate buffer for determination of the extinction at 260m μ , followed by precipitation with a final concentration of 5% (w/v) trichloroacetic acid for the estimation of radioactivity.

DEAE-cellulose chromatography. The procedure was essentially that of McCarthy, Britten & Roberts (1962). The extract was made in tris-magnesium chloride buffer containing 5 $\mu\text{g.}$ of deoxyribonuclease/ml. and, after clarification, was centrifuged for 4 hr. at 102000g and 5° in the 50 rotor of the model L-HV ultracentrifuge to sediment ribosomes and 14–16s particles. The pellet was dissolved in a small volume of tris-magnesium chloride buffer and a suitable portion, containing a maximum of 5mg. of RNA, was applied to a column of DEAE-cellulose.

Material for the column (Cellex-D, 0.91m-equiv./g.; California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.) was prepared by washing twice with N-HCl, followed by suspension in water and then in successive changes of tris-magnesium chloride buffer until the pH of the supernatant had attained that of the buffer. Columns (1 cm. $\times$ 15 cm.) were packed under 2–3 lb./in.² pressure.

Elution was carried out with a linear gradient of NaCl (0.2–1.2M) in 200 ml. of tris-magnesium chloride buffer. Fractions of volume about 4 ml. were collected. After measurement of the extinction of a fraction at 260m μ , trichloroacetic acid was added to a final concentration of 5% (w/v) and the precipitate assayed for radioactivity.

Chromatography of RNA. RNA isolated from cells as described by Dagley *et al.* (1963) was chromatographed on columns of kieselguhr coated with methylated bovine serum albumin (Mandell & Hershey, 1960). A 20g. portion of kieselguhr was added to 100 ml. of 0.1M-NaCl in 50mM- KH_2PO_4 , pH 6.7, and the suspension boiled to expel air. After cooling, 5 ml. of an aqueous 1% solution of esterified albumin was stirred in and a further 20 ml. of 0.1M-NaCl in 50mM- KH_2PO_4 , pH 6.7, was added. After washing by decantation with 0.4M-NaCl in 50mM- KH_2PO_4 , pH 6.7, a portion of the suspension was transferred to a column (1 cm. diam.) and excess of liquid removed under pressure. The final length of the column was about 20 cm.

A 1 ml. sample of RNA solution (3–5mg. of RNA/ml.) in 0.4M-NaCl in 50mM- KH_2PO_4 , pH 6.7, was applied to the column and eluted with a linear gradient of NaCl (0.4–1.1M) in 320 ml. of 50mM- KH_2PO_4 , pH 6.7. Fractions of volume 3–5 ml. were collected and assayed for extinction at 260m μ and for 5%-trichloroacetic acid-insoluble radioactivity.

Fractionation of RNA by the DNA-agar method. DNA prepared by the method of Marmur (1961) was used in the fractionation of RNA, which was carried out essentially as described by Bolton & McCarthy (1962). A 5 ml. sample of a solution of DNA (about 1mg./ml.) in 1.5mM-NaCl in 0.15M-sodium citrate was heated to 100°, mixed with an equal volume of 6% (w/v) Ionagar no. 2 (Oxo Ltd., London, S.E. 1) and rapidly cooled. When the gel had hardened it was forced through a 40-mesh sieve and the particles were washed at 60° with a solution of 0.3M-NaCl in 0.03M-sodium citrate. About 100 $\mu\text{g.}$ of RNA in 1 ml. of 0.3M-NaCl in 0.03M-sodium citrate was then incubated for 17 hr. at 60° with agar containing about 200 $\mu\text{g.}$ of DNA, and the slurry was transferred to a column heated by circulating

water. Non-hybridizable RNA was collected by washing at 70° with 5 ml. portions of 0.3M-NaCl in 0.03M-sodium citrate; the washings were collected separately and assayed for radioactivity. When no more radioactivity was eluted from the column (about ten washings), the temperature was raised to 75° and the RNA bound to DNA similarly eluted with 1.5M-NaCl in 0.15M-sodium citrate. For estimations of radioactivity, the samples were precipitated with a final concentration of 5% (w/v) trichloroacetic acid in the presence of a little carrier RNA.

Incorporation of [2-¹⁴C]uracil into intact cells. Incorporation of labelled uracil was followed by pipetting 1.0 ml. samples into an equal volume of ice-cold 10% (w/v) trichloroacetic acid. Samples were kept at 0° for at least 10 min. before filtering and then assaying the radioactivity.

Radioactivity measurements. Material precipitated by 5% (w/v) trichloroacetic acid at 0° was collected on membrane filters (Oxo Ltd.) in a stainless-steel filter tower. The filters were gummed to planchets, dried in air and counted for a minimum of 1000 counts with a thin-window gas-flow counter (Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.). Samples were always 'infinitely thin'.

Analytical ultracentrifugation. A Spinco model E ultracentrifuge equipped with schlieren optics and fitted with a rotor temperature and indicator control unit was used. Protein concentrations of crude cell-free extracts were adjusted to 1% before ultracentrifugation at 187000g and 20.0°.

Chemicals. Radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks. Crystalline deoxyribonuclease was from the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

RESULTS

Accumulation of RNA by the mutant. Fig. 1 shows changes in the RNA and protein content of a suspension of cells of the mutant which were grown in medium containing methionine, washed and resuspended in aerated methionine-free medium. After starvation for 3 hr. the RNA content of the culture had increased by about 100% in the absence of any detectable synthesis of protein.

Figs. 2(a) and 2(b) show the striking changes found when schlieren diagrams of extracts prepared from cells at zero time and after starvation for 3 hr. are compared; after starvation, boundaries additional to those caused by the 30s and 50s ribosomes appear and which sediment, in these crude extracts, at about 14–16s. These changes have been studied in more detail by Dagley *et al.* (1963); about 70% of the RNA synthesized during starvation is found in the 14–16s particles and the rest of the RNA has a sedimentation coefficient similar to that of transfer RNA.

Chromatography of RNA on kieselguhr coated with methylated bovine serum albumin. RNA extracted from cells of the mutant that had been starved for 3 hr. in the presence of [¹⁴C]uracil was subjected to chromatography on kieselguhr coated with methylated bovine serum albumin. This

Fig. 1. Variation in protein (●) and RNA (○) content of a culture of the mutant aerated in methionine-free medium at 30°. Experimental details are given in the text. Results are expressed as percentages of the values at zero time, which were: protein, 0.40 mg./ml.; RNA, 0.19 mg./ml. At zero time, the cell suspension had an extinction value of 1.44 at 540 mμ.

Fig. 2. Schlieren diagrams, after centrifuging for 16 min. at 187000g, of cell-free extracts prepared from the mutant. Cells were harvested (a) before starvation, (b) after starvation for 3 hr. in methionine-free medium, (c) 25 min. and (d) 50 min. after the addition of DL-methionine (100 mg./l.) to the starved culture. Sedimentation is to the left. The bar angle was 40°.

technique has been shown by its originators (Mandell & Hershey, 1960) to separate nucleic acids primarily according to size. Thus preparations of bacterial RNA are resolved into transfer RNA and the two species of ribosomal RNA (Otaka, Mitsui & Osawa, 1962). Under the present conditions transfer RNA was eluted immediately from the column. Fig. 3 shows the extinction at 260 mμ and the 5%-trichloroacetic acid-insoluble radioactivity of fractions containing components other than transfer RNA, the radioactivity locating the RNA synthesized during starvation.

Both the extinction and radioactivity profiles show the presence of one minor and two major components. The major components are the two

Fig. 3. Chromatography on kieselguhr coated with methylated bovine serum albumin of RNA isolated from cells of the mutant incubated with $[2-^{14}\text{C}]\text{uracil}$ ($10\mu\text{C}$ and 40mg./l.) during starvation for 3 hr. in methionine-free medium. The general procedure is described in the text. RNA (about 4 mg. in 1 ml.) was loaded on the column and eluted with a concentration gradient of NaCl ($0.4\text{--}1.1\text{M}$) in 320 ml. of $0.05\text{M-KH}_2\text{PO}_4$, pH 6.7. Fractions (3.5 ml.) were assayed for extinction at $260\text{m}\mu$ ($\circ$) and 5%-trichloroacetic acid-precipitable radioactivity ($\bullet$). Transfer RNA was eluted in fractions 0–10 and is not shown.

Fig. 4. Variation in protein ($\bullet$) and RNA ($\circ$) content during recovery of starved cells of the mutant. Cells were starved of methionine for 3 hr. and then resuspended in fresh medium to which DL-methionine (100mg./l.) was added. The cell suspension then had an extinction value of 1.50 at $540\text{m}\mu$. Changes are plotted as percentages of the values at the time of the addition of methionine. These were: protein, 0.40mg./ml. ; RNA, 0.36mg./ml. During the starvation, RNA increased by 95% in the absence of detectable synthesis of protein.

species of ribosomal RNA whose sedimentation coefficients are 16 and 23s (Kurland, 1960). The correspondence between radioactivity and extinction confirms previous reports (Mandel & Borek, 1963) that the RNA synthesized during starvation and which appears in the 14–16s particles is of the same size as that found in the ribosomes. Since the molecular weight of 16s RNA is about half that of 23s RNA, roughly two 16s molecules are synthesized during starvation for each 23s molecule.

Elution diagrams for RNA from starved cells show also the presence of a minor component that appeared in fractions after the 23s RNA and can be detected in both the extinction and radioactivity profiles. This component was not present in RNA prepared from unstarved cells and thus probably represents a species of RNA synthesized during starvation and distinct from either transfer RNA or ribosomal RNA. Chromatography of pulse-labelled RNA from exponentially growing cells gives a peak of radioactivity in approximately the same position; two other peaks, not precisely coincident with 16s and 23s RNA, are then also found. These peaks are thought to represent complexes of messenger RNA with ribosomal RNA (Gros, Naono, Woese, Willson & Attardi, 1963). There is evidence, referred to below, that RNA synthesized during starvation of the mutant contains an increased proportion of messenger RNA. The appearance of the minor component may be a reflection of this.

Synthesis of RNA and protein after the addition of methionine to a starved culture. When methionine was added to a culture of the mutant that had been starved for 3 hr., growth resumed rather slowly and the extinction of the culture increased by only 50% in the first 100 min. of recovery. During this period there was a considerably greater increase in the protein content of the culture than in the RNA. This is shown in Fig. 4, where amounts of RNA and protein are plotted as percentages of the values after starvation for 3 hr. At 20 min. after the addition of methionine, the protein content of the culture had increased by 10% in the absence of detectable RNA synthesis; after 100 min., the protein had increased by 100% and the RNA by only 20%. In this particular experiment, RNA increased by 95% during starvation in the absence of protein synthesis: the considerable differential synthesis of protein during recovery therefore served to re-establish in the growing cells a ratio of RNA to protein almost the same as in the exponentially growing cells before starvation. Differential synthesis of protein does not take place when the growth of other amino acid auxotrophs resumes after the addition to a starved culture of their amino acid requirement (Schaechter, 1961).

Stability during recovery of the RNA synthesized during starvation. If ribosomes are formed directly from the 14–16s particles when methionine is

added to starved cultures of the mutant, the RNA in these particles should be incorporated into ribosomes without degradation first taking place. An investigation was therefore made of the stability of the RNA synthesized during starvation as recovery takes place. Starvation was carried out for 3 hr. in the presence of [^{14}C]uracil to label the RNA made in this period. Cells were then harvested and resuspended in medium containing excess of unlabelled uracil and methionine was then added. At intervals thereafter, samples were taken for determination of radioactivity. The results showed that the RNA synthesized during starvation was almost completely stable as growth resumed, although a 5–10% loss of radioactivity was consistently observed in the first 20–40 min. of recovery. The 5%-trichloroacetic acid-soluble products of this degradation were later reutilized. The radioactivity returned to its initial value about 60 min. after the addition of the methionine and then remained constant.

If at the end of the period of starvation methionine and a 20-fold excess of unlabelled uracil were added directly to the medium in which the cells had been starved, there was a similar fall (about 10%) in radioactivity. Synthesis of RNA from acid-soluble fragments then continued for some time, until after recovery for 3 hr. the radioactivity was 20% greater than its initial value. During starvation of this mutant, there is a considerable excretion into the medium of purines, pyrimidines and their derivatives (Borek *et al.* 1955); in these experiments, therefore, radioactive compounds derived from uracil would be present in the medium after starvation. Incorporation of such compounds into RNA thus takes place readily even in the presence of excess of non-radioactive uracil, as does also the synthesis of RNA from the degradation products produced in the early stages of recovery.

The nature and cause of the transient loss of RNA

on recovery were investigated further. At least a part of the fall might be due to the degradation of messenger RNA synthesized during starvation. In exponentially growing cells of *E. coli* about 1% of the RNA is messenger RNA (Midgley & McCarthy, 1962). Gros *et al.* (1963) and Mandel & Borek (1963) have compared the ability of RNA from starved and unstarved cells of mutant 58–161 to function as messenger RNA in cell-free systems that synthesize protein. The RNA from starved cells has about a threefold greater protein-synthesizing activity/mg. of RNA than has the RNA from exponentially growing cells. The RNA synthesized during starvation would therefore be expected to contain about 5% of messenger RNA.

Messenger RNA can also be detected by its ability to form a hybrid molecule with single strands of DNA derived from the same organism (Bolton & McCarthy, 1962). DNA was therefore isolated from the mutant and the percentage of the RNA synthesized during starvation that was able to form a hybrid with this DNA was determined. Table 1 shows that 5.7% of the RNA made during starvation for 3 hr. hybridized with DNA; when a sample of RNA was prepared from cells 30 min. after recovery had been initiated by the addition of methionine, the percentage of hybridizable material had fallen to 1.2%.

Thus the loss of RNA as cells recover could be at least partially due to degradation of the increased percentage of messenger RNA present after starvation. However, the following experiment (Fig. 5) indicated that the RNA which is degraded as cells recover is not necessarily more unstable than the rest of the RNA under other conditions. Cells were starved of methionine for 45 min. in medium containing [^{14}C]uracil, harvested and divided into two portions. One was resuspended in medium containing excess of unlabelled uracil and methionine. As recovery took place there was

Table 1. *RNA complementary to DNA in Escherichia coli mutant 58–161*

Details of the experiment are given in the text. Cells were starved for 3 hr. in the presence of [^{14}C]uracil (10 μC and 40 mg./l.) and then resuspended in fresh medium containing non-radioactive uracil (100 mg./l.) and DL-methionine (100 mg./l.). RNA was prepared from samples taken as indicated and portions (about 100 $\mu\text{g.}$) were incubated with DNA-agar (about 200 $\mu\text{g.}$ of DNA) for 17 hr. at 60°. Non-hybridized RNA was eluted by washing at 70° with portions (5 ml.) of 0.3 M-NaCl in 0.03 M-sodium citrate; the temperature was then raised to 75° and hybridized RNA similarly eluted with 1.5 M-NaCl in 0.15 M-sodium citrate. Measurements were on 5%-trichloroacetic acid-insoluble radioactive material.

Conditions	Total radioactivity (counts/min.)		Percentage of hybridized RNA
	Fractions containing hybridized RNA	Fractions containing non-hybridized RNA	
(1) Starvation (3 hr.)	1470	23215	5.7
(2) Starvation (3 hr.) plus recovery (30 min.)	330	26700	1.2

Fig. 5. Stability of the RNA accumulated by the mutant. Cells were starved of methionine for 45 min. in medium containing $[2-^{14}\text{C}]\text{uracil}$ ($10\mu\text{C}$ and 5mg./l.), harvested and divided into two portions. (a) One was resuspended in fresh medium containing unlabelled uracil (100mg./l.), and DL-methionine (50mg./l.) added. (b) The other was suspended in medium containing non-radioactive uracil (100mg./l.) but lacking methionine and starved for 150 min. (c) The culture in (b) was harvested after 150 min. of starvation and cells were resuspended in medium containing non-radioactive uracil (100mg./l.) and DL-methionine (50mg./l.) was added. Measurements were of 5%-trichloroacetic acid-insoluble radioactive material and are plotted as percentages of the values at the beginning of each part of the experiment, which were (a) 1390, (b) 1260 and (c) 1010 counts/min./ml.

the usual transient fall (about 10%) in the 5%-trichloroacetic acid-insoluble radioactivity. The other portion was starved for a further 150 min. in medium lacking methionine but containing excess of unlabelled uracil; samples taken for radioactivity estimations showed that during this time there was a loss (about 4%/hr.) of the RNA synthesized during the first period of starvation. When these cells were harvested and allowed to recover in medium containing methionine and excess of uracil a further transient loss of 10% of the radioactivity occurred. Thus if the temporary loss of RNA on recovery is caused by degradation of a small fraction of 'unstable' RNA, this fraction is not appreciably more unstable than the total RNA when cells are starved in methionine-free medium. Neidhardt & Gros (1957) showed that recovery of *E. coli* from inhibition by chloramphenicol is similarly accompanied by a transient loss of about 20% of the RNA accumulated during inhibition. Only about 5% of the accumulated RNA is messenger RNA (Midgley, 1963). Thus, in the mutant, species of RNA other than messenger RNA could be subject to nuclease action during both starvation and recovery.

Synthesis of ribosomes from the RNA accumulated during starvation. To try to decide the fate of the RNA synthesized during starvation when growth resumes after the addition of methionine, experi-

ments were carried out to observe possible differences in schlieren diagrams of cell-free extracts and to follow changes taking place by means of analysis on sucrose density gradients and by chromatography on DEAE-cellulose. The typical results obtained by these three methods, and which are described below, are from the same experiment and thus directly comparable. Cells were starved for 3 hr. in the presence of $[^{14}\text{C}]\text{uracil}$, harvested and resuspended in fresh medium containing excess of non-radioactive uracil and methionine.

(a) Changes in schlieren diagrams. Fig. 2 shows diagrams for extracts (in phosphate-magnesium acetate buffer) of cells harvested at the beginning and end of starvation and after recovery for 25 and 50 min. The extract prepared at the end of starvation had boundaries caused by the 30s and 50s ribosomes and by the particles accumulating during starvation (Fig. 2b). At 25 min. after the methionine was added (a period during which no net synthesis of RNA took place) the amount of 14–16s material was greatly diminished (Fig. 2c). At the same time there was an increase in area of the boundary associated with the 30s ribosomes and an additional small boundary appeared, sedimenting between the 30s and 50s ribosomes. After recovery for 50 min., few 14–16s particles were detectable (Fig. 2d); the area under the 50s-ribosome boundary had increased relative to the 30s boundary, and the small component sedimenting between the 30s and 50s ribosomes was still present.

The diagrams show that, during recovery, the 14–16s particles disappear. Since little degradation of the RNA accumulated during starvation takes place during recovery and because there is evidence (below) that extensive breakdown of 14–16s particles to RNA with a smaller sedimentation coefficient does not take place, the increase in area of the 30s-ribosome boundary observed after 25 min. and the other changes may represent the conversion of 14–16s material into ribosomes or ribosome-like particles.

(b) Analyses in sucrose density gradients. Fig. 6 shows analyses in sucrose density gradients of extracts prepared from starved cells and from cells after recovery for 25 and 50 min. In the sample from the starved cells (Fig. 6a), about 25% of the RNA synthesized during starvation sedimented with transfer RNA. There was no significant change in the proportion of this sort of RNA in the later samples. Most (75%) of the RNA made during starvation appears in the 14–16s particles, which are not well resolved by this method from the 30s ribosomes. It has been shown by Dagley *et al.* (1963), and is confirmed below, that few ribosomes are synthesized during starvation.

After recovery for 25 min. (Fig. 6b), when schlieren diagrams show that a considerable

Fig. 6. Sucrose-density-gradient analyses of cell-free extracts containing accumulated RNA prepared from mutant cells incubated with $[2\text{-}^{14}\text{C}]\text{uracil}$ ($10\text{ }\mu\text{C}$ and 40 mg./l.) during starvation for 3 hr. in methionine-free medium. The general procedure is described in the text. After starvation the cells were harvested and resuspended in fresh medium containing unlabelled uracil (100 mg./l.) to which DL-methionine (100 mg./l.) was added. Samples were taken for analysis (a) after starvation, (b) 25 min. and (c) 50 min. after the addition of methionine. Sedimentation in the gradient was at $124000g$ for 190 min. in (a) and (b) and for 170 min. in (c). Measurements were of extinction at $260\text{ m}\mu$ (○) and 5%-trichloroacetic acid-insoluble radioactive material (●).

decrease in the amount of 14–16s material had taken place, the density-gradient profile had a shoulder of radioactivity associated with the 50s ribosomes: by 50 min. (Fig. 6c) this shoulder had become a definite peak. At 50 min., by which time few 14–16s particles were present, there was a close correspondence between radioactivity and extinction in fractions containing both 50s and 30s ribosomes. However, there was also a considerable amount of radioactivity associated with material sedimenting between the main ribosome components. This material corresponded to that visible in schlieren diagrams and was apparent also in the extinction profiles of the density gradient, thus making the 30s and 50s ribosomes appear less well resolved from each other. It had a high specific activity and thus probably corresponds to a (radioactive) component involved in the synthesis of ribosomes rather than a (non-radioactive) component concerned in a degradative process.

Fig. 7. Chromatography on DEAE-cellulose of ribosome preparations from mutant cells incubated with $[2\text{-}^{14}\text{C}]\text{uracil}$ ($10\text{ }\mu\text{C}$ and 40 mg./l.) during starvation for 3 hr. in methionine-free medium. After starvation, the cells were harvested and resuspended in fresh medium containing non-radioactive uracil (100 mg./l.), and DL-methionine (100 mg./l.) was then added. Samples were taken for analysis (a) after starvation and (b) 50 min. after the addition of methionine. The preparation of ribosomes from the samples is described in the text. A portion of each preparation (containing about 4 mg. of RNA) was loaded on the column and eluted with a concentration gradient of NaCl ($0.2\text{--}1.2\text{ M}$) in 200 ml. of tris-magnesium chloride buffer. Measurements of extinction at $260\text{ m}\mu$ (○) and 5%-trichloroacetic acid-insoluble radioactive material (●) were made on fractions of volume about 4 ml. In (a), fraction 10 contained 0.40 M -NaCl and fraction 21 0.55 M -NaCl.

(c) Chromatography on DEAE-cellulose. Chromatography on DEAE-cellulose is a technique that has been used with considerable success to distinguish the precursor stages in ribosome synthesis from each other and from the ribosomes (Britten, 1963). In our experiments, extracts were made in tris-magnesium chloride buffer, the concentration of magnesium chloride being 20 mm. At this concentration of Mg^{2+} ions, 70s ribosomes were formed from the 30s and 50s species (Tissières, Watson, Schlessinger & Hollingworth, 1959). If 14–16s particles were present, they underwent aggregation and were sedimented down with the ribosomes in the preparative procedures.

Ribosomes prepared from exponentially growing cells were eluted from the column at a salt concentration of about 0.4 M . Fig. 7 shows the radioactivity and extinction of fractions derived from cells after starvation and after recovery for 50 min. The

sample from starved cells showed (Fig. 7a) that the RNA in the 14–16s particles was eluted at about 0.55M-sodium chloride and was thus well resolved from fractions containing the ribosomes. The latter had only a small amount of radioactivity associated with them; this confirms previous observations (Dagley *et al.* 1963) that few ribosomes are synthesized during starvation. After recovery for 50min. the particles accumulated during starvation were lost and radioactivity appeared in the ribosomes (Fig. 7b). The elution diagram shows the presence of a third radioactive component that was eluted between the fractions containing the ribosomes and accumulated RNA and at about 0.48M-sodium chloride. A considerable proportion of the radioactivity was located in this region. A component that was eluted in this position has also been found to comprise a stage in the synthesis of ribosomes by exponentially growing cells (Britten, 1963). The appearance of this particular component when starved cells of the mutant recover is discussed further below.

Nature of the protein synthesized during recovery. Since RNA from the 14–16s particles is incorporated into ribosomes when methionine is added to starved cultures, the differential synthesis of protein relative to RNA which takes place during this period might be to provide these particles with the protein needed to complete the synthesis of ribosomes. Accordingly, the early stages of recovery were investigated to determine whether they involved selective synthesis of ribosomal protein.

For these experiments, [^{35}S]methionine was added to cultures of the mutant; after a time the cells were harvested and extracts prepared in tris-magnesium chloride buffer. The ribosomes were then separated from 'soluble' (i.e. non-ribosomal) protein by ultracentrifugation and the specific

activities of ribosomal and soluble proteins compared. Table 2 shows that, after cells had been grown overnight in the presence of radioactive methionine, the specific activities of soluble and ribosomal protein were almost the same. The methionine content of the two sorts of protein is thus closely similar. Again, when radioactive methionine was added to an exponentially growing culture for 25min. and the newly synthesized soluble protein bound to the ribosomes removed by growth for a further 20min. in the presence of excess of non-radioactive methionine, the specific activities of ribosomal and soluble protein were identical.

However, 25min. after the initiation of recovery in a starved culture, the specific activity of ribosomal protein was more than twice that of soluble protein. This value was scarcely altered when nascent protein was removed from the ribosomes by a further period of growth in the presence of excess of non-radioactive methionine. Thus, during the early stages of recovery, ribosomal protein is made at about twice its normal rate relative to soluble protein. It is tempting to think that this preferential synthesis of the structural protein of the ribosomes is due to completion of the synthesis of ribosomes from the precursors the cells contain.

DISCUSSION

When growth is initiated by the addition of methionine to starved cells of *E. coli* mutant 58–161, ribosomes are formed from the RNA accumulated during starvation. Two pieces of evidence suggest that the RNA made during starvation and which has the sedimentation characteristics of transfer RNA does not play a role in this transformation, and that ribosomes are

Table 2. *Specific radioactivities of soluble and ribosomal protein fractions labelled with [^{35}S]methionine*

Cells of the mutant were grown with [^{35}S]methionine as detailed below and cell-free extracts prepared. These extracts were centrifuged, as described in the text, to give a preparation of ribosomes and a 'soluble' fraction containing non-ribosomal protein. Radioactivity measurements were on 5%-trichloroacetic acid-insoluble precipitates. Concentrations of DL-methionine were (per l.): (1) 12.5 μC and 50 mg.; (2) overnight growth on 40 mg., then 125 μC added at zero time and 200 mg. after 20 min.; (3) (a) 125 μC and 10 mg., then (b) 200 mg.

Conditions	Specific activity (counts/min./mg. of protein)		R/S ratio
	Soluble fraction (S)	Ribosomal fraction (R)	
(1) Overnight growth with [^{35}S]methionine	570	610	1.1
(2) Cells labelled for 25 min. during exponential growth; further growth (20 min.) with excess of unlabelled methionine	18840	18820	1.0
(3) (a) Cells starved for 3 hr. labelled for 25 min. after addition of methionine; and	5050	10600	2.1
(b) then grown for 35 min. with excess of unlabelled methionine	4060	9380	2.3

therefore synthesized only from the RNA contained in the 14–16s particles. First, the sucrose-density-gradient analyses show that the percentage of RNA synthesized during starvation and which sediments with transfer RNA remains constant as recovery takes place. Secondly, the transfer RNA made in the absence of methionine lacks the methylated bases ordinarily present. Fleissner & Borek (1962) showed that, when methionine is added to starved cultures, methylation of this RNA takes place, thus rendering unlikely the use of this species of RNA for the synthesis of ribosomes.

The RNA that accumulated during starvation is almost completely stable when recovery is initiated by the addition of methionine. The 14–16s particles, from which the ribosomes are formed, contain RNA with the same sedimentation coefficients as the RNA in ribosomes and which behaves in the same way as ribosomal RNA on a column of kieselguhr coated with methylated bovine serum albumin. It is therefore likely that the 14–16s particles are converted directly into ribosomes without intervening degradation. This is rendered even more probable by the discovery that ribosome-like particles can be formed *in vitro* by the addition of protein to the particles that accumulate in cells of *E. coli* inhibited with chloramphenicol (Spirin, 1963).

The small transient loss of about 5–10% of the RNA synthesized during starvation when recovery occurs was not detected in the chemical analyses of RNA made by the orcinol method. The fall would be only 2–5% of the total RNA and could be masked by slight synthesis of new RNA during the early stages of recovery. A large part of the loss can be attributed to the degradation of messenger RNA present in increased amount after starvation and which falls to a more nearly normal percentage of the total RNA after recovery for 30 min. Incubation of cells of *E. coli* with puromycin causes the accumulation of RNA and the appearance in cell-free extracts of particles sedimenting at about 16s (Dagley *et al.* 1962). When cells are washed free from the antibiotic, ribosomes are formed from the (mostly ribosomal) RNA accumulated during the inhibition. Although the RNA formed during the inhibition is said to be retained during recovery, the published values (Sells, 1964) show a transient loss of about 10% in a way similar to that found for the mutant.

RNA accumulated during starvation of the mutant for 45 min. is only slowly degraded during continued starvation. However, the possibility that accumulated RNA is extensively broken down during recovery and that this degradation is masked by simultaneous resynthesis from the fragments has not been eliminated by the experiments described above, although it is rendered unlikely

by the other considerations discussed. Levinthal, Fan, Higa & Zimmermann (1963) have claimed that a substantial fraction of the RNA which accumulates during inhibition of *Bacillus subtilis* by chloramphenicol is degraded before being incorporated into ribosomes when the antibiotic is removed, even when cells are allowed to recover in a medium enriched with amino acids so that no significant loss of accumulated RNA occurs. Much of the RNA synthesized during inhibition for 20 min. was heterogeneous, with sedimentation coefficients of between 4 and 16s; it was assumed that ribosomal RNA is synthesized directly as the 16s and 23s species and that this heterogeneous RNA is therefore degraded ribosomal RNA. This assumption is, however, not supported by extensive kinetic evidence (see below) that at least two stages are involved in the synthesis of ribosomal RNA, the first stage involving RNA which is polydisperse and with an average sedimentation coefficient of about 8s. RNA prepared after starvation of the mutant for 3 hr. is, however, almost completely of the 4s, 16s and 23s species; so is RNA accumulated by *E. coli* inhibited by chloramphenicol for comparable periods (Kurland & Maaløe, 1962).

The synthesis of ribosomes by exponentially growing cells of *E. coli* has been investigated in some detail by labelling cells with [^{14}C]uracil for periods of a few seconds upwards and analysing the flow of radioactivity through the various fractions: a review of this work is given by Britten (1963). Two precursor stages in the synthesis of ribosomes have been recognized and the particles comprising these stages called 'eosomes' and 'neosomes' respectively, in the order of their synthesis. On a column of DEAE-cellulose, elution with a linear gradient of sodium chloride takes place in the sequence: ribosomes (0.4M-sodium chloride); neosomes (0.5M-sodium chloride); eosomes (0.6M-sodium chloride). These differences in behaviour are primarily due to a difference in the ratio of RNA to protein in the three sorts of material. The 14–16s particles are eluted at about the same salt concentration as eosomes; the formation of ribosomes from these particles, with the simultaneous appearance of material eluted at an intermediate salt concentration and corresponding to the position occupied by neosomes, thus bears a striking resemblance to the process taking place in exponentially growing cells.

Differences in sedimentation coefficient can also be used to distinguish the precursor stages in ribosome synthesis from each other and from the ribosomes. The neosomes, which are the immediate precursors of the 50s ribosomes, have a sedimentation coefficient of about 43s, and thus are similar in this respect to the component visible in schlieren diagrams during recovery and which the sucrose-

density-gradient analyses show to have a high specific activity. A similar component has been observed in cells of the mutant that have accumulated RNA as a consequence of inhibition by 5-methyltryptophan and are recovering from this inhibition (Turnock & Wild, 1963). The neosomes from which 30s ribosomes are formed have a sedimentation coefficient of not much less than 30s, as also has a second neosomal component destined for 50s ribosomes. The increase in area of the peak sedimenting with 30s ribosomes as starved cells of the mutant recover and the appearance of radioactivity in this region is thus consistent with the synthesis of 30s ribosomes and/or precursors of both ribosomal species from 14-16s particles.

Although similar in their behaviour on columns of DEAE-cellulose, there are differences between the particles that accumulate on starvation of the mutant and the eosomes from exponentially growing cells. When corrected to infinite dilution, the sedimentation coefficients of accumulated material are about 20 and 23s, greater than the average value of 14s quoted (by Britten, 1963) for eosomes. Eosomal material seems also to be more heterogeneous in sedimentation properties than the 14-16s particles; moreover, the RNA derived from eosomes is polydisperse with an average sedimentation coefficient of about 8s (McCarthy *et al.* 1962). This is in sharp contrast with the two species of RNA with the sedimentation coefficients of ribosomal RNA that are contained in the 14-16s particles. More gentle methods of preparation of RNA from cells labelled for short periods with [¹⁴C]uracil have yielded preparations with a greater range of size, although it is clear that 16s and 23s RNA, as such, are not present (Otaka *et al.* 1962; Gros *et al.* 1963). It seems likely that, although the formation of ribosomes from 14-16s particles is in many respects similar to the synthesis of ribosomes from eosomes, these two components are not the same. Further work is still necessary to decide whether the particles are present in exponentially growing cells but are undetected in the kinetic analyses, or whether they are peculiar to cells that have accumulated RNA in the virtual absence of protein synthesis.

Previous work (Dagley *et al.* 1963) in which 14-16s particles were isolated and analysed indicated a composition similar to that of the ribosomes, namely 60% of RNA and 40% of protein. The origin of this protein presented difficulties because it appeared on the particles in the absence of net synthesis of protein. A possibility that was considered and observations have now shown to be correct is that contamination of isolated particles by material derived from ribosomes takes place during the isolation procedures.

Preliminary observations suggest that the particles from the mutant contain less protein than do ribosomes. This is in accord with their behaviour on DEAE-cellulose, the approximate composition of eosomes (about 90% of RNA and 10% of protein) and the composition of the particles produced by chloramphenicol inhibition (Nomura & Watson, 1959). Since the particles appear to contain complete chains of RNA it is likely that synthesis of ribosomes from them corresponds primarily to the addition of protein, and that this leads to the differential synthesis of protein and in particular of ribosomal protein found to occur during recovery. Recovery from inhibition by puromycin similarly is accompanied by an apparent oversynthesis of ribosomal protein (Sells, 1964). Ennis & Lubin (1963) have shown a similar situation to occur when growth of a mutant deficient in a mechanism for transporting K⁺ ions and which accumulates RNA in K⁺ ion-deficient media is restarted by the addition of potassium salts. The events taking place during recovery of the mutant from methionine starvation may therefore be of general occurrence when growth resumes in cells that have accumulated RNA in the absence of protein synthesis.

After this paper was submitted for publication, Nakada, Anderson & Magasanik (1964) published experiments on the recovery from starvation of a relaxed methionine-requiring mutant of *E. coli* similar to mutant 58-161. They found that during recovery exogenous uracil can enter the cells, and RNA with some of the properties of messenger RNA is synthesized. However, the ribosomes made in the early stages of recovery appear to contain RNA derived intact from the particles accumulated during starvation.

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