Characterization of Rapidly Labelled Ribonucleic Acid in *Escherichia coli* by Deoxyribonucleic Acid-Ribonucleic Acid Hybridization

By G. H. PIGOTT* AND J. E. M. MIDGLEY†
Department of Biochemistry, University of Leeds

(Received 21 May 1968)

1. Rapidly labelled RNA from *Escherichia coli* K12 was characterized by hybridization to denatured *E. coli* DNA on cellulose nitrate membrane filters. The experiments were designed to show that, if sufficient denatured DNA is offered in a single challenge, practically all the rapidly labelled RNA will hybridize. With the technique employed, 75–80% hybridization efficiency could be obtained as a maximum. Even if an excess of DNA sites were offered, this value could not be improved upon in any single challenge of rapidly labelled RNA with denatured *E. coli* DNA. 2. It was confirmed that the hybridization technique can separate the rapidly labelled RNA into two fractions. One of these (30% of the total) was efficiently hybridized with the low DNA/RNA ratio (10:1, w/w) used in tests. The other fraction (70% of the total) was hybridized to DNA at low efficiencies with the DNA/RNA ratio 10:1, and was hybridized progressively more effectively as the amount of denatured DNA was increased. A practical maximum of 80% hybridization of all the rapidly labelled RNA was first achieved at a DNA/RNA ratio 210:1 ($\pm 10:1$). This fraction was fully representative of the rapidly labelled RNA with regard to kind and relative amount of materials hybridized. 3. In competition experiments, where additions were made of unlabelled RNA prepared from *E. coli* DNA, DNA-dependent RNA polymerase (EC 2.7.7.6) and nucleoside 5'-triphosphates, the rapidly labelled RNA fraction hybridized at a low (10:1) DNA/RNA ratio was shown to be competitive with a product from genes other than those responsible for ribosomal RNA synthesis and thus was presumably messenger RNA. At higher DNA/rapidly labelled RNA ratios (200:1), competition with added unlabelled *E. coli* ribosomal RNA (without messenger RNA contaminants) lowered the hybridization of the rapidly labelled RNA from its 80% maximum to 23%. This proportion of rapidly labelled RNA was not competitive with *E. coli* ribosomal RNA even when the latter was in large excess. The ribosomal RNA would also not compete with the 23% rapidly labelled RNA bound to DNA at low DNA/RNA ratios. It was thus demonstrated that the major part of *E. coli* rapidly labelled RNA (70%) is ribosomal RNA, presumably a precursor to the RNA in mature ribosomes. 4. These studies have shown that, when earlier workers used low DNA/RNA ratios (about 10:1) in the assay of messenger RNA in bacterial rapidly labelled RNA, a reasonable estimate of this fraction was achieved. Criticisms that individual messenger RNA species may be synthesized from single DNA sites in *E. coli* at rates that lead to low efficiencies of messenger RNA binding at low DNA/RNA ratios are refuted. In accordance with earlier results, estimations of the messenger RNA content of *E. coli* in both rapidly labelled and randomly labelled RNA show that this fraction is 1.8–1.9% of the total RNA. This shows that, if any messenger RNA of relatively long life exists in *E. coli*, it does not contribute a measurable weight to that of rapidly labelled messenger RNA.

The technique of hybridizing RNA to homologous denatured DNA is a powerful tool for the charac-

* Present address: Department of Clinical Biochemistry, University of Newcastle upon Tyne, NE1 7RU.

† Present address: Department of Biochemistry, University of Newcastle upon Tyne, NE1 7RU.

terization and estimation of various fractions of bacterial RNA. Of especial interest is its potentiality in estimating the messenger RNA contents of bacterial cells (Bolton & McCarthy, 1962; McCarthy & Bolton, 1964; Armstrong & Boezi, 1965; Friesen, 1966). In such experiments, rapidly labelled RNA

is first prepared, where messenger RNA, presumed to have a short lifetime in the cell (Midgley & McCarthy, 1962; Bolton & McCarthy, 1962; Levinthal, Keynan & Higa, 1962), is preferentially labelled by suitable nucleic acid precursors. As the messenger RNA is a product representative of a transcription of the bulk of the bacterial DNA (McCarthy & Bolton, 1964) it can be hybridized efficiently to denatured single strands of homologous DNA when relatively small amounts of DNA are given, DNA/RNA ratio 5:1 (w/w).

However, early assumptions that rapidly labelled bacterial RNA was exactly equivalent to messenger RNA (Gros *et al.* 1961) were shown to be incorrect when it was demonstrated that, however short the time of labelling RNA, this fraction contained a significant proportion of labelled ribosomal RNA in a precursor form (Midgley & McCarthy, 1962; Bolton & McCarthy, 1962). Indeed, only 30–33% of the rapidly labelled RNA was thought to be characteristic of messenger RNA in its apparent nucleotide base composition (Midgley, 1962; Midgley & McCarthy, 1962) or hybridization properties (Bolton & McCarthy, 1962). The remainder (67–70%) was proposed to be a precursor to ribosomal RNA, since its composition and hybridization properties were identical with those of the structural RNA in the ribosomes.

As pointed out by others (Levinthal, Fan, Higa & Zimmerman, 1963) these estimations are open to several criticisms. An assumption implicit in the use of nucleotide base compositions of rapidly labelled RNA fractions to estimate messenger RNA is that the 'apparent' nucleotide composition is identical with the true composition. If the nucleotides, labelled with [^{32}P]phosphate, are incorporated into the RNA with very different specific radioactivities, and the nucleotide sequences in the RNA chains are non-random, then distortions of true composition may become significant (Harris, Fisher, Rodgers, Spencer & Watts, 1963). It is also assumed that the rapidly labelled messenger RNA, transcribed from only one of the two strands of DNA (Geiduschek, Tochini-Valentini & Sarnat, 1964; McCarthy & Bolton, 1964; Hayashi, Hayashi & Spiegelman, 1964), has a true composition indistinguishable from that of the total DNA. Further, the differential activities of DNA sites producing messenger RNA in different amounts may also distort any relationship with total DNA composition. The hybridization experiments assume that no messenger RNA molecules are produced from single DNA sites at such a rate that they would be inefficiently hybridized at the low DNA/RNA ratios that are otherwise satisfactorily large for efficient binding of other messenger species produced at lower rates. Therefore both these methods of estimation of bacterial messenger RNA contents

could give a considerable underestimation of this fraction, with a corresponding overestimation of other species.

This paper demonstrates that the hybridization of RNA to denatured DNA can lead, with *E. coli*, to a valid estimate of messenger RNA. In accord with the results of McCarthy & Bolton (1964) it appears that the criticisms of the estimations based on nucleotide composition or hybridization are not practically significant. A preliminary communication of these results has been made (Pigott & Midgley, 1966).

MATERIALS AND METHODS

Organism. *Escherichia coli* K12, laboratory strain, was used.

Growth of bacterial cultures. The organism was grown, with forced aeration, at 37° in the glucose-salts medium of Roberts, Cowie, Abelson, Bolton & Britten (1957). This contained (per l.): 2g. of NH_4Cl , 3g. of KH_2PO_4 , 6g. of Na_2HPO_4 , 3g. of NaCl , 0.12g. of Na_2SO_4 , 0.05g. of MgCl_2 and 2.5g. of D-glucose. The cultures were grown to the late exponential phase of growth, if harvesting was required for DNA or RNA preparation. The mean generation time was 60–87 min.

Incorporation of radioactive substances into cultures. Rapidly labelled RNA was made by permitting cultures, which had been in the exponential phase of growth for at least four cell divisions, to incorporate exogenously supplied [^{14}C]uracil or [^{32}P]phosphate for a suitable short interval. After this time the culture was poured on to crushed ice to arrest cell metabolism rapidly. The cells were harvested in a refrigerating centrifuge (MSE High Speed 18) at 10000g for 10 min. at 2°. In all experiments labelling of nucleic acids was commenced when the dividing culture had reached a turbidity of 0.6 at 650 m μ , as measured on the SP.600 spectrophotometer (Unicam Instruments Ltd.) in glass cuvettes of 1 cm. light-path.

Randomly labelled cultures were prepared by allowing a continuous uptake of radioactive precursor over four cell divisions. Labelling was commenced at a culture turbidity of 0.1 at 650 m μ . The cells were collected as described for rapidly labelled cultures.

Harvesting of cultures. Large volumes (5–100 l.) were harvested in a continuous-flow centrifuge (Alfa Laval Co. Ltd.) at room temperature. Small volumes (up to 1 l.) were centrifuged as described above in the refrigerating centrifuge. Most harvested cultures were used immediately after preparation, but if stored were kept as packed cells at –10°.

Formation of bacterial cell extracts. Except for DNA purification, cell-free extracts of *E. coli* were prepared by first resuspending the harvested cells in a small volume of 0.01 M-MgCl $_2$ –0.01 M-tris-HCl buffer, pH 7.3 at 2°. The cells were centrifuged and resuspended in a suitable volume of the same buffer at 2°. They were then disintegrated in a French pressure cell (Aminco Bowman Ltd.) (French & Milner, 1955) at an extrusion pressure of 20 000 lb./in. 2 . At least 90% of the cells were disrupted by this treatment. To avoid undue heating, the pressure cell was precooled to 0° before use.

Preparation of bacterial DNA. This was isolated by a

method mainly derived from those of Marmur (1961) and Kirby (1964). Wet packed cells of *E. coli* were suspended in 0.1 M-EDTA-0.4 M-NaCl buffer, pH 8.0, at a concentration of 50–200 mg. wet wt. of cells/ml. Solid sodium 4-aminosalicylate was added to a final concentration of 4% (w/v). Enough sodium dodecyl sulphate (25%, w/v, concentrated solution) was added to make a final concentration of 2% (w/v). The mixture was immersed in a water bath at 60° and held at this temperature for 10 min. with occasional shaking.

Phenol-cresol mixture was prepared by the addition of 140 ml. of *m*-cresol (redistilled under reduced pressure until colourless), 110 ml. of water and 1 g. of 8-hydroxyquinoline to 1 kg. of phenol. After liquefaction, 1 vol. of this mixture was added to the lysed bacterial culture, and the mixture was shaken to a creamy emulsion on a rotary shaker (A. Gallenkamp Ltd.) until all lumps had dispersed, and for 5 min. thereafter. The emulsion was separated by centrifugation at 10000g in the MSE High Speed 18 centrifuge for 20 min. at 2°. The upper aqueous layer was carefully removed with a wide-tipped pipette and suction bulb. Denatured protein, which collects at the interface between the phenolic and aqueous layers, was not included in this fraction.

To the viscous solution was added an equal volume of 2-ethoxyethanol (Cellosolve). White strands of DNA were seen to collect at the interface between the aqueous and Cellosolve layers. The layers were gently mixed with a glass rod and the precipitated DNA fibres were spooled on to the rod. Excess of liquid was drained off the DNA by pressure against a glass surface. The denatured protein and phenolic layer from the first deproteinization step was re-extracted with a quantity of 0.4 M-NaCl corresponding to 40% of the original volume of the aqueous phase. After shaking for 20 min., the aqueous phase was separated by centrifuging as described above and the DNA was precipitated and collected. The yields from both processes were combined, and allowed to dissolve in the minimum practical volume of 0.1 × SSC (SSC is 0.15 M-NaCl-0.015 M-sodium citrate). Usually a volume of 0.1 × SSC corresponding to about 25% of the original aqueous phase was sufficient. The DNA was dissolved by gentle stirring of the solution at 3° overnight.

A 0.2% (w/v) solution of pancreatic ribonuclease (EC 2.7.7.16) (British Drug Houses Ltd., Poole, Dorset) in 0.15 M-NaCl was heated to 80° and held at this temperature for 10 min. to destroy any deoxyribonuclease activity present. Sufficient of the cooled solution was added to the DNA solution to bring the final ribonuclease concentration to 50 µg./ml. The solution was incubated for 1 hr. at 37°. If the solution viscosity was markedly less after this time, residual deoxyribonuclease was present and the sample was discarded. After this incubation, 1 vol. of phenol-cresol mixture was added and the mixture was shaken for 15 min. The emulsion was centrifuged as described above. The aqueous layer was collected and a further 0.5 vol. of phenol-cresol mixture was added. The cycle of shaking, centrifuging and recovery of aqueous phase was repeated. To the aqueous phase was added $\frac{1}{2}$ vol. of 3 M-sodium acetate in 1 mM-EDTA, pH 7.0, followed by the careful layering of 0.54 vol. of propan-2-ol on top of the mixture. The layers were carefully mixed and the DNA was spooled on to a glass rod as described above. The precipitate was redissolved in 0.1 × SSC and the precipitation with sodium acetate-

EDTA buffer and propan-2-ol was repeated. The DNA was then redissolved in 0.1 × SSC.

The solution was adjusted to 0.5 M-NaCl and 20% (w/v) sodium benzoate by adding the solid salts. One vol. of 2-butoxyethanol was layered on to the aqueous mixture and the DNA, now free from protein and polysaccharide, was collected on a glass rod as described above. The final product was dissolved in 0.01 × SSC and dialysed against 20–30 vol. of this buffer for 16 hr. at 3° with three buffer changes. The DNA solution was stored in solution at 5° with the addition of a few drops of chloroform as an antifungal agent. The DNA solutions cannot be frozen, as this leads to considerable shearing of molecules and subsequent poor hybridization properties.

Analysis in the analytical ultracentrifuge showed that almost all of the DNA sedimented at about 30 S. The yield of DNA was about 1–2 mg./g. wet wt. of packed cells.

Preparation of bacterial RNA. (a) Rapidly labelled RNA. Exponentially growing cultures of *E. coli* were injected with [²⁻¹⁴C]uracil or [³²P]phosphate and isotope incorporation into the cell nucleic acids was allowed to proceed for a short time. The cultures were cooled in ice, broken in the French pressure cell and cell-free extracts prepared as described above. The extracts were collected in a vessel containing 1 vol. of phenol-cresol mixture, and the materials were allowed to warm to 20° during deproteinization. The mixture was shaken at 20° for 10 min. and was then centrifuged as described for the preparation of DNA. The aqueous phase of the mixture was then collected by pipette, and was adjusted to 0.4 M-NaCl by the addition of sufficient 2 M-NaCl solution. A second cycle of deproteinization was carried out in the same way as the first. The aqueous phase was collected after centrifuging the mixture, and 2 vol. of ethanol was added. Precipitation of nucleic acids was permitted by standing the mixture at -10° for 1 hr. The precipitate was collected by centrifuging at 5000g for 10 min. at 0° in a refrigerating centrifuge and was redissolved in a minimum quantity of 0.01 M-sodium acetate buffer, pH 4.6. The precipitation by ethanol was repeated, and the nucleic acids were redissolved in sodium acetate buffer.

A two-layer chromatographic column (2.5 cm. diam.) was prepared. The bottom layer of Sephadex G-25, medium grade (Pharmacia Ltd., Uppsala, Sweden), was first equilibrated by exhaustive washing with 0.1 × SSC buffer. A 15 cm. depth of gel was placed in the column. On top of this was placed a 2.5 cm. depth of Dowex 50 (8% cross-linked; 200–400 mesh) ion-exchange resin [Sigma (London) Chemical Co. Ltd., London, S.W. 6] and equilibrated with the same buffer. The nucleic acids in sodium acetate buffer were loaded on to the column in 1 ml. and the thin band of material was carefully washed through the column with 0.1 × SSC. Fractions (3 ml.) were collected and their extinction at 260 mµ was monitored. The first absorbing peak corresponded to the nucleic acid fractions and was assayed for radioactivity. Samples (0.1 ml.) were pipetted on to lens-tissue disks, previously stuck to aluminium planchets with a drop of dilute glucose solution. The planchets were assayed for radioactivity in a gas-flow counter (Nuclear-Chicago Corp.). The labelled fractions were stored at -10° until required.

This technique ensures that the rapidly labelled RNA fractions, after column treatment, are free of ribonuclease activity and phenolic materials. In early experiments, DNA and transfer RNA were removed by methods described

below, but, since these materials were labelled to a negligible extent during the preparation of rapidly labelled RNA (Midgley & McCarthy, 1962), it was found that their presence had no effect on the hybridization of rapidly labelled RNA to DNA, or on the radioactivity bound to denatured DNA during hybridization. Extra steps of this kind were later omitted.

(b) Randomly labelled RNA. Cell extracts of randomly labelled *E. coli* cultures were treated with phenol-cresol mixture as described for rapidly labelled RNA. After the first cycle of phenol-cresol treatment, the aqueous layer was extracted three times with an equal volume of diethyl ether. The aqueous phase was then freed from ether by bubbling N_2 through. Deoxyribonuclease (EC 3.1.4.5) (British Drug Houses Ltd.), previously purified by the method of Polatnich & Bachrach (1961), was then added to a final concentration of $20 \mu\text{g./ml.}$ A few drops of m-MgCl_2 were added to activate the enzyme. The solution was incubated at 37° for 20 min., and two cycles of deproteinization by phenol-cresol mixture were carried out. The RNA was then precipitated twice by ethanol as described for rapidly labelled RNA.

The RNA was taken up in a small volume of $0.4 \text{ M-NaCl-}0.01 \text{ M-MgCl}_2\text{-}0.01 \text{ M-tris-HCl}$ buffer, pH 7.3, and layered as a thin band on a $2.5 \text{ cm.} \times 20 \text{ cm.}$ column of Sephadex G-200, medium grade (Pharmacia Ltd.), equilibrated with the same buffer. The column was carefully washed through with the same buffer and the effluent was monitored as described for rapidly labelled RNA. This column separated the ribosomal and messenger RNA from transfer RNA, which is retarded by the column to emerge as a separate peak (Midgley, 1965). The first peak of material emerging from the column was precipitated with ethanol and taken up in $0.1 \times \text{SSC}$. The RNA was passed down a Dowex 50-Sephadex G-25 column as described for rapidly labelled RNA, to remove any ribonuclease activity. The RNA solution was then dialysed at 3° for 8 hr. against $0.1 \times \text{SSC}$ with one buffer change. The dialysed material was stored at -10° .

(c) Unlabelled ribosomal RNA. Cultures of *E. coli* were harvested as described above. The cells were resuspended in $0.01 \text{ M-MgCl}_2\text{-}0.01 \text{ M-tris-HCl}$ buffer, pH 7.3, and were disintegrated in the French pressure cell. The extract was incubated at 37° in the presence of $5 \mu\text{g.}$ of deoxyribonuclease/ml. for 15 min. The cell debris and unbroken cells were then removed by centrifugation at $100000g$ at 0° for 2 min. The supernatant fluid was carefully removed and then subjected to further centrifuging at $100000g$ at 0° for 90 min. (Spinco model L ultracentrifuge, angle-head rotor) (Beckman Instruments Ltd.). The supernatant fluid was discarded and the pellet taken up in $0.1 \text{ M-MgCl}_2\text{-}0.01 \text{ M-tris-HCl}$ buffer, pH 7.3. The suspension was dialysed against 20–30 vol. of this buffer for 12 hr. at 2° with two buffer changes. At this concentration of Mg^{2+} the ribosomes dissociate and release their messenger RNA. The ribosomes were then collected by centrifuging the solution for 4 hr. at $100000g$ at 0° , leaving messenger RNA in the supernatant fluid. The pellet was washed once with buffer and the RNA was extracted by the methods described above. Ribonuclease contamination was removed by the Dowex 50-Sephadex G-25 column and the RNA was precipitated with ethanol, dissolved in a minimal volume of $0.1 \times \text{SSC}$ and dialysed against 20 vol. of the same buffer for 4 hr. at 2° . The RNA was stored at -10° .

(d) Enzymically prepared RNA. DNA-dependent RNA polymerase (EC 2.7.7.6), prepared from *E. coli* M.R.E. 600, was a kind gift from Dr K. Abraham. The enzyme preparation was supplied in 50% (v/v) glycerol solution. The following reagents were used: 0.5 M-tris-HCl buffer, pH 7.5; $0.2 \text{ M-2-mercaptoethanol}$; 0.1 M-MnCl_2 ; 4 mM-ATP ; 4 mM-GTP ; 4 mM-CTP ; 4 mM-UTP ; DNA from *E. coli* (1 mg./ml.).

The reaction mixture (50 ml.) contained tris buffer (5 ml.) 2-mercaptoethanol solution (1 ml.), MnCl_2 solution (2 ml.) and solutions of the nucleoside 5'-triphosphates (2 ml. each). Then 4 mg. of DNA and the enzyme preparation were added and the volume was made up with water. The mixture was incubated at 37° for 1 hr. and incubation was then stopped by the addition of 1 vol. of phenol-cresol mixture. The RNA was then prepared as described for randomly labelled RNA except for the omission of the Sephadex G-200 and dialysis treatment. The first ultraviolet-absorbing peak from the Dowex 50-Sephadex G-25 column was collected and pooled, and stored at -10° .

Estimation of DNA and RNA. Concentrations of nucleic acid samples were calculated from measurements of extinction at $260 \text{ m}\mu$ in silica cuvettes of 1 cm. light-path in the Unicam SP.500 spectrophotometer. In each case the extinction coefficient was taken as $E_{1\%}^{1 \text{ cm.}}$ 200.

Nucleotide base compositions of RNA. Compositions were determined from RNA samples labelled with [^{32}P]phosphate before isolation. The method is that described by Midgley (1962). The ^{32}P -labelled rapidly labelled or randomly labelled RNA was prepared as described above. To a solution of RNA was added an equal volume of 10% (w/v) trichloroacetic acid, cooled in an ice bath. The precipitate of RNA was collected on an Oxoid membrane filter (Oxo Ltd.) and the filter was washed extensively with ice-cold 5% trichloroacetic acid.

Excess of unlabelled *E. coli* ribosomal RNA was added to the filter + ^{32}P -labelled RNA and the mixture was made up to 0.4 N-KOH . The RNA was then hydrolysed at 37° for 16 hr. Excess of KOH was then neutralized with N-HClO_4 . The precipitate of KClO_4 was removed by centrifuging at 0° . The supernatant fluid was brought to pH 8 by adding aq. 0.1 N-NH_3 .

Dowex 1 (X8; 200–400 mesh) was washed with 5 N-formic acid overnight, filtered at the pump and washed with water until acid-free. A $1 \text{ cm.} \times 10 \text{ cm.}$ column was made, and the hydrolysate was adsorbed on the column. Elution of nucleoside 2'(3')-monophosphates was effected by a convex gradient of formic acid (0.4 N). A five-chambered vessel was used containing (in order from the first) 80 ml. of water, 1 N-formic acid and, in the last three chambers, 4 N-formic acid. The order of elution of nucleotides was: cytidylic acid, adenylic acid, guanylic acid and uridylic acid, with partial resolution of the 2'- and 3'- isomers of adenylic acid and guanylic acid.

The extinction of the fractions (3 ml.) was determined at $260 \text{ m}\mu$, after addition of dilute HCl to adjust the pH to 2. The samples were assayed for radioactivity by plating out samples on lens-tissue disks stuck to aluminium planchets as described above. Samples were infinitely thin and at least 10000 counts were measured in each sample. Results are only quoted in this paper if all samples from a given nucleotide peak gave the same specific radioactivity, indicating the absence of co-chromatographed contaminants.

Since the excess of unlabelled ribosomal RNA supplies

virtually all the ultraviolet absorption of the sample, the composition of any labelled RNA can be calculated. The molar composition of unlabelled *E. coli* ribosomal RNA was taken as: CMP, 21.9%; AMP, 25.1%; GMP, 32.6%; UMP, 20.4% (Midgley, 1962; Stanley & Bock, 1965). Molar extinction coefficients used for calculation of amounts of nucleotides from their extinctions in digests were: ϵ_{260} 6.8 (CMP); 14.2 (AMP); 11.8 (GMP); 10.0 (UMP) (Beaven, Holiday & Johnson, 1955).

Denaturation of *E. coli* DNA. DNA solution was diluted to 100 μ g./ml. with $0.01 \times$ SSC and then made $0.15N$ with respect to NaOH. The mixture was kept at room temperature while denaturation was monitored by measuring the extinction of the solution at 260 $m\mu$ until readings had reached a stable maximum value. This process required not more than 10 min. The denatured DNA was cooled to 3° in an ice bath, and the solution was neutralized with $5N$ -acetic acid. The denatured material was stable for several days at 3° .

Binding of DNA to nitrocellulose membrane filters. The method is essentially that of Gillespie & Spiegelman (1965). Cellulose nitrate membrane filters (type B6, coarse, 24 mm. diam.; Schleicher and Schuell, Keene, N.H., U.S.A.) were presoaked for at least 1 min. in $6 \times$ SSC at 3° . They were transferred to a stainless-steel filter tower (2.5 cm. internal diam.) (Tracerlab). They were then washed with 10 ml. of $6 \times$ SSC and the sample of denatured DNA, diluted with 2 vol. of $10 \times$ SSC, was allowed to filter through under extremely gentle suction. A filtration rate of less than 0.25 ml./min. was essential to ensure efficient DNA binding to the filter. All manipulations were carried out at 3° to maximize DNA binding. The loaded filters were washed with 100 ml. of $6 \times$ SSC. The washed filters were allowed to dry at room temperature for at least 4 hr., followed by drying for 4 hr. at 80° over P_2O_5 in a vacuum oven.

This irreversibly attaches the denatured DNA to the membrane filter. Dry filters were stored over P_2O_5 in an evacuated desiccator at 3° .

Hybridization of RNA to DNA. Dried filters were placed in scintillation vials and the RNA samples were added. The volume was made up to 1.5 ml. and the buffer concentration to $6 \times$ SSC. If multiple filters were used, then a volume increment of 0.3 ml./filter in addition to the first was added.

The vials were stoppered and immersed in a water bath at 66° . Fig. 1 shows the course of hybridization of RNA to DNA in the concentrations of each usually used in experiments. An incubation of 16 hr. was considered sufficient for maximum hybridization. After this time, the vials were plunged into an ice bath. The filters were removed and washed on each side with 50 ml. of $6 \times$ SSC. The total nucleic acid content of the filter was then removed by the addition of 2 ml. of $0.5N$ -HClO₄, followed by incubation at 100° for 30 min. The filters were allowed to drain into the liquid for 10 min. and were then discarded. The cooled solution was then neutralized with 0.2 ml. of $5N$ -KOH, and KClO₄ was removed by centrifuging at 0° . Samples of the hydrolysate were used for measurements of radioactivity from the hybridized RNA and the assay of total nucleic acid material by phosphate analysis (see below).

All RNA samples remained completely precipitable by ice-cold 10% (w/v) trichloroacetic acid after 16 hr. at 66° .

Assay of hybridized labelled RNA. The radioactive RNA was estimated by scintillation counting in a Tritomat 6020

Fig. 1. Time-course of hybridization of ^{14}C -labelled rapidly labelled RNA from *E. coli* to denatured DNA immobilized on cellulose nitrate membrane filters. RNA was obtained from cultures that had incorporated [^{14}C]uracil for 2 min. Incubation of RNA and DNA was carried out in scintillation vials at 66° .

(Isotope Developments Ltd.). The scintillation system of Hall & Cocking (1965) was used. The following mixture was prepared: 2,5-diphenyloxazole, 4g.; 1,4-bis-(5-phenyloxazol-2-yl)benzene, 0.1g.; 2-ethoxyethanol (technical grade), 300 ml.; toluene (scintillation grade), 700 ml. A 1 ml. sample of hydrolysate was pipetted into scintillation vials containing 0.1 ml. of 90% formic acid and 2.6 ml. of 2-ethoxyethanol. Then 5 ml. of scintillation fluid, previously bubbled with O_2 -free N_2 for 10 min., was added and the vial was stoppered and shaken gently to obtain a clear solution. Samples of labelled RNA, similarly treated with membrane filters containing no DNA, were counted in the experiments to give an internal standard. The degree of hybridization of RNA was calculated by reference to this standard, after subtraction of the background rate of counting. At least 10000 counts were measured in each sample. Vials were prepared from washed filters containing no DNA, incubated with the same quantity of labelled RNA and subject to identical hybridization, washing and hydrolysis procedures. These corrected the results for non-specific RNA binding to filters.

Assay of DNA. The DNA could not be analysed readily by ultraviolet absorption of hydrolysates of filters, since appreciable (and variable) amounts of absorbing contaminants were also eluted from the membrane filters by HClO₄. The diphenylamine test of Burton (1956) could not be employed, owing to the elution of reacting contaminants in gross quantity from the filters. DNA was therefore assayed for phosphate content by analysing the hydrolysate by the method of Chen, Toribara & Warner (1956). Since the amount of RNA hybridized is negligible in amount compared with the filter-bound DNA, this method analyses the DNA content of the membrane filters satisfactorily. The following reagents were prepared: reagent A, 10% (w/v) ascorbic acid (stored at 5° for no longer than 6 weeks); reagent B, $6N$ -H₂SO₄; reagent C, 2.5% (w/v) ammonium molybdate; reagent D, 1 vol. of reagent A + 1 vol. of reagent B + 1 vol. of reagent C + 2 vol. of water (reagent D is prepared just before use). Samples (0.5 ml.) of material to be assayed were ashed by adding 1 drop of conc. H₂SO₄ and 2 drops of N -HClO₄. The tubes were heated until

white fumes appeared, and then more strongly until the drop of solution became clear (about 30 sec. after first appearance of white fumes). The tubes were cooled, and 1 ml. of water was added. The mixture was incubated in a boiling-water bath for 10 min., to ensure complete solution of the material. The volume was made up to 4 ml. with water. Then 4 ml. of reagent D was added and the tubes were stoppered, shaken and incubated for 2 hr. at 100° to develop the colour. Tubes were then removed from the bath and cooled to room temperature for 5 min. Their extinctions at 820 m μ were read on a Unicam SP.600 spectrophotometer, against a blank in which 0.5 ml. of water replaced the sample. Ashing of a nitrocellulose membrane filter with DNA samples showed no detectable change in the calibration curve.

Radiochemicals. [2-¹⁴C]Uracil, specific radioactivity 40–60 mc/m-mole, [³²P]phosphate (as phosphoric acid), specific radioactivity 5–200 mc/m-mole, and [5-³H]uracil, specific radioactivity 1000 mc/m-mole, were obtained from The Radiochemical Centre, Amersham, Bucks.

RESULTS

Satisfactory characterizations of the rapidly labelled RNA from micro-organisms depend on obtaining a specimen that can be said to be typical of this fraction. Accordingly, preliminary experiments were carried out to find the time-interval throughout which rapidly labelled RNA in *E. coli* changes least in properties such as apparent nucleotide base composition or hybridization to denatured DNA. The aim of the experiments was to find a labelling time long enough to ensure a reasonable amount of radioactivity in the rapidly labelled RNA, while maintaining the proportions of labelled materials formed in much shorter intervals.

Kinetics of incorporation of exogenously supplied precursors into cellular nucleic acids. To obtain a typical specimen of rapidly labelled bacterial RNA, it is important to know the kinetics of incorporation of precursors from the growth medium into the nucleic acids. Earlier observations have demonstrated that, whereas such precursors as ¹⁴C-labelled adenine, guanine, cytosine or uracil are incorporated without lag into the nucleic acids (McCarthy & Britten, 1962; Buchwald & Britten, 1963), [³²P]phosphate is only incorporated into the nucleic acids by using the intracellular nucleotide pool as an obligatory intermediate. This causes a considerable kinetic delay in the uptake of label into RNA and DNA (Midgley & McCarthy, 1962).

Experiments were performed in which [2-¹⁴C]-uracil or [³²P]phosphate was continuously incorporated into the nucleic acids of *E. coli* K 12. The 500 ml. cultures of *E. coli*, growing exponentially under forced aeration at 37°, were given sufficient radioactive supplements, diluted with unlabelled carrier, to allow incorporation for at least 1 hr. before exhaustion. For incorporation of [2-¹⁴C]-

uracil, unlabelled uracil was added to the growing cultures at least 10 min. before the addition of radioactive material, to allow equilibration of the pathways of uracil uptake into the cells.

At intervals, samples (0.5 ml.) of the culture were withdrawn and either filtered immediately on to membrane filters with several washes with an equal volume of ice-cold growth medium, or otherwise precipitated in an equal volume of ice-cold 10% trichloroacetic acid, filtered on to a membrane filter and washed five times with equal volumes of 10% trichloroacetic acid. The membrane filters were dried and counted as described in the Materials and Methods section. Results are shown in Fig. 2.

These results are in agreement with those reported earlier for *E. coli* and other organisms (McCarthy & Britten, 1962; Midgley & McCarthy, 1962). The trichloroacetic acid-precipitable radioactivity is, in each case, a measure of isotope incorporation into cellular nucleic acids, whereas the radioactivity in the cells simply filtered off from the supplemented growth medium is a measure of the total isotope incorporation. A comparison of Figs. 2(a) and 2(b) shows that, although incorporation of exogenous radioactive precursors into the cells is not delayed, the incorporation of [³²P]phosphate into the nucleic acids is delayed considerably, compared with the immediate incorporation of [2-¹⁴C]uracil.

Apparent nucleotide base composition of ³²P-labelled rapidly labelled RNA. As a check on the nature of rapidly labelled RNA in *E. coli* measurements were made of the change in apparent base composition of this fraction during the incorporation of [³²P]phosphate into exponentially growing cultures. The organism was grown in a medium containing the usual salts (Roberts *et al.* 1957) except that 90% of the phosphate content of the medium was replaced by an equivalent molar amount of tris buffer, pH 7.3. At a suitable time, [³²P]phosphate (0.1 mc) was injected into the growth medium (500 ml.) Samples were poured on to crushed ice at intervals, and the RNA was prepared as described in the Materials and Methods section. The RNA was then hydrolysed with potassium hydroxide and analysed by isotope-dilution techniques, by using ion-exchange column chromatography with Dowex 1 (Midgley, 1962). Table 1 shows the changing apparent nucleotide base composition of the labelled RNA during the incorporation of [³²P]phosphate into the cells.

Table 1 shows that, over the first 4 min. (approx. 8% of the dividing time of the cells), the newly formed ³²P-labelled RNA has an almost constant apparent composition, after which time a gradual transition occurs to a composition typical of the bulk RNA of the cells. This result is in agreement with that of Midgley (1962) and Midgley & McCarthy (1962).

Fig. 2. (a) Incorporation of $[2-^{14}\text{C}]$ uracil into the cells and into the nucleic acids of exponentially growing *E. coli* cultures. ●, Incorporation into whole cells; ○, incorporation into nucleic acids (measured by precipitation in ice-cold 10% trichloroacetic acid). (b) Incorporation of $[^{32}\text{P}]$ phosphate into the cells and into the trichloroacetic acid-precipitable fraction of exponentially growing *E. coli* cultures. ●, Incorporation into whole cells; ○, incorporation into the fraction precipitated by ice-cold 10% trichloroacetic acid.

Table 1. Apparent nucleotide base composition of ^{32}P -labelled rapidly labelled RNA in *E. coli* K12

Specimens of 'rapidly labelled' RNA were obtained from cultures dividing once every 50 min. at 37°. Determinations were to a standard error of $\pm 1\%$.

Labelling period (min.)	% of mean generation time	Nucleotide fraction (moles/100 moles)			
		CMP	AMP	GMP	UMP
1	2	23.5	24.5	28.4	23.6
2	4	24.2	24.8	28.2	22.8
4	8	23.7	25.6	28.8	21.9
8	16	22.5	24.7	31.5	21.3
16	32	22.1	24.8	32.5	20.7
200	400	21.6	25.3	33.0	20.2
<i>E. coli</i> ribosomal RNA from 70S ribosomes*		21.9	25.1	32.6	20.4

*Data from Midgley (1962).

Hybridization of rapidly labelled RNA to denatured DNA. The analytical method first described by Bolton & McCarthy (1962) was used. A 50 μg . sample of denatured *E. coli* DNA was bound to nitrocellulose membrane filters as described in the Materials and Methods section. Various specimens of rapidly labelled RNA were obtained by allowing the incorporation of $[5-^3\text{H}]$ uracil (50mc/m-mole) into growing *E. coli* cultures. A 10 μg . sample of rapidly labelled RNA was added to the filters and hybridization was carried out at 66° for 16hr. (Materials and Methods section). The percentage of hybridized RNA bound to the filters was measured by scintillation counting. Table 2 indicates that, over the first 5–6 min. of $[5-^3\text{H}]$ uracil incorporation

Table 2. Hybridization of ^3H -labelled rapidly labelled RNA from *E. coli* to DNA at low DNA/RNA ratios

Rapidly labelled RNA was obtained from cultures of *E. coli* K12 dividing exponentially in glucose-minimal medium (Roberts *et al.* 1957) at 37°. The mean generation time was 87 min. Samples of rapidly labelled RNA (10 μg .) were hybridized to denatured DNA (50 μg .) immobilized on cellulose nitrate membrane filters.

Time of labelling RNA (min.)	% of labelled RNA hybridized to DNA (DNA/RNA ratio 5:1)
0.33	29
0.67	25
1	26
2	25
4	27
7	23
10	18
20	14

into the cellular nucleic acids, there was a constant proportion of hybridized to non-hybridized RNA in the rapidly labelled fraction. After this time the hybridized RNA became a progressively smaller fraction of the total labelled RNA.

From these preliminary data it would seem that, for a measurable time after commencement of labelling of RNA in *E. coli*, the newly formed RNA has a constant apparent base composition, and uniform hybridization properties with homologous denatured DNA. Therefore, as a typical sample of the rapidly labelled RNA from the bacterium, in experiments described below a specimen was used from cells that had incorporated radioactive precursor for 2 min. Further experiments, discussed in the text, also demonstrated that this

Fig. 3. Hybridization of ^{14}C -labelled rapidly labelled RNA with increasing amounts of immobilized denatured *E. coli* DNA, bound on cellulose nitrate membrane filters. RNA was obtained from cultures that had incorporated $[2\text{-}^{14}\text{C}]\text{-uracil}$ for 2 min. Incubation of RNA and DNA was carried out in conical flasks at 66° .

choice gave a typical sample of rapidly labelled RNA.

Hybridization of rapidly labelled RNA with various amounts of DNA. Given quantities of rapidly labelled RNA, prepared from cultures allowed to incorporate $[2\text{-}^{14}\text{C}]\text{-uracil}$ for 2 min. at 37° , were hybridized to increasing amounts of denatured *E. coli* DNA on membrane filters. The hybridization curve obtained is shown in Fig. 3. This shows that, as the DNA/RNA ratio (w/w) is increased, there is a parallel increase in the hybridization of the rapidly labelled RNA from an initial 23% at DNA/RNA ratios 5:1 to a maximum of 80% at ratios 200:1 or higher. Thus we may suppose that some considerable fraction of rapidly labelled RNA can find few sites in the DNA for hybridization at 5:1 ratios, but that all (to a practical maximum) of the rapidly labelled RNA is bound to the sites provided at 200:1 ratios.

Efficiency of hybrid formation. Since a maximum of 80% hybridization to DNA was found for rapidly labelled RNA, it was obviously important to determine whether this value represented the efficiency of the process of hybridization, or a fraction of the RNA with extremely few complementary sites on the DNA. Accordingly, the efficiency of hybridization of RNA to DNA was tested (a) by subjecting the RNA not hybridized by an initial incubation with DNA to a second incubation, and (b) by removal of bound RNA from the filters, followed by incubation of the RNA with a further DNA-containing filter.

Filters were prepared containing approx. $250\text{ }\mu\text{g}$. of denatured DNA, and incubation vessels were set up in duplicate at DNA/RNA ratios 250:1 and 100:1. After hybridization of rapidly labelled RNA had proceeded for 16 hr., the filters were removed and washed as in the normal procedure.

Table 3. *Efficiency of DNA/RNA hybrid formation between E. coli DNA and rapidly labelled RNA*

Source of RNA	DNA/RNA ratio in test	% of RNA hybridized
(1) ^{14}C -labelled rapidly labelled RNA (<i>E. coli</i> K 12)	250:1	79
(2) Non-hybridized fraction from (1)	800:1	81
(3) Hybridized fraction from (1) rehybridized to DNA	700:1	78
(4) ^{14}C -labelled rapidly labelled RNA (<i>E. coli</i> K 12)	100:1	49
(5) Non-hybridized fraction from (4)	400:1	77
(6) Hybridized fraction from (4) rehybridized to DNA	700:1	80

A sample of the liquid remaining in the vessels was counted to determine the percentage hybridization of the RNA in the first challenge. A second sample was diluted to 1.5 ml. with $6\times\text{SSC}$ (Materials and Methods section) and incubated with a further filter containing approx. $250\text{ }\mu\text{g}$. of DNA for a further 16 hr. This filter was also assayed for hybridized RNA. The washed filters from the first hybridization were dipped into a beaker containing 100 ml. of ice-cold $0.01\times\text{SSC}$ to ensure removal of traces of stronger salt solution. The filters were then covered with 2 ml. of $0.01\times\text{SSC}$ and heated at 90° for 10 min., after which time the filters were removed and allowed to drain into the liquid. This procedure removed at least 95% of the hybridized RNA, and much of the DNA bound to the filters. A $100\text{ }\mu\text{g}$. of sample of purified deoxyribonuclease in 0.5 ml. of $0.01\times\text{SSC}$ was added to the cooled RNA-containing fraction and the mixture was incubated at 37° for 30 min. to destroy DNA. The mixture was then heated at 80° for 10 min. to destroy enzyme activity. After cooling, the salt concentration was adjusted to $6\times\text{SSC}$ and 1.5 ml. of the solution was incubated with another DNA-containing filter. A further sample was taken for counting to confirm the initial percentage of RNA hybridized and to provide an estimate of the RNA available for the second hybridization. After incubation at 66° for 16 hr. the second filter was washed and assayed for bound RNA as before. Results are shown in Table 3.

It may be concluded that the maximum value of approx. 80% hybridization of the rapidly labelled RNA at high DNA/RNA ratios is indicative of the efficiency of the hybridization process, and does not represent a situation where 20% of the RNA is incapable of hybridization by virtue of decomposition, lack of complementary sites on the DNA etc. Nor can it be explained as a loss of DNA, with hybridized RNA attached, from the filters during

Table 4. *Apparent nucleotide base compositions of ³²P-labelled rapidly labelled RNA fractions after DNA-RNA hybridization*

³²P-labelled rapidly labelled RNA was obtained from an exponentially growing culture of *E. coli* K12. The mean generation time was 50 min. at 37°. [³²P]Phosphate was incorporated into cells for 2 min. Determinations were to a standard error of $\pm 1.5\%$.

RNA fraction	Nucleotides (moles/100 moles)				% of RNA hybridized
	CMP	AMP	GMP	UMP	
Total ³² P-labelled rapidly labelled RNA	23.5	25.2	29.9	21.8	—
Hybridized RNA (DNA/RNA ratio 10:1)	24.0	25.0	27.1	23.9	24
Non-hybridized RNA (DNA/RNA ratio 10:1)	21.5	25.2	33.2	20.1	—
Hybridized RNA (DNA/RNA ratio 200:1)	23.0	25.0	30.8	21.2	80
Non-hybridized RNA (DNA/RNA ratio 200:1)	22.8	25.1	31.2	21.5	—
<i>E. coli</i> ribosomal RNA*	21.9	25.1	32.6	20.4	—
<i>E. coli</i> DNA	25	25	25	25†	—

* Nucleotide base composition of *E. coli* ribosomal RNA taken from Midgley (1962).

† As thymidylic acid.

the annealing process, as assays of DNA before and after hybridization of RNA show that virtually none is lost.

Apparent nucleotide base composition of hybridized and non-hybridized rapidly labelled RNA at low and high DNA/RNA ratios. Bolton & McCarthy (1962) reported that, if low DNA/RNA ratios were used to hybridize the rapidly labelled RNA of *Proteus vulgaris* to its DNA, the hybridized RNA had a nucleotide base composition resembling that of the bacterial DNA, and the non-hybridized RNA resembled the ribosomal RNA. These experiments were repeated with *E. coli* K12, with the membrane filter-DNA system instead of agar gels (Bolton & McCarthy, 1962).

Filters with approx. 200 µg. of denatured DNA/filter were separately incubated with 20 µg. or with 1 µg. of rapidly labelled RNA prepared from *E. coli* cultures exposed to [³²P]phosphate for 2 min. The filters containing hybrids and the supernatant fluids were analysed for the base composition of the RNA. The apparent compositions obtained are shown in Table 4.

The results show that hybridization at the 200:1 DNA/RNA ratios gives no differentiation of apparent composition between the 80% hybridized and the 20% non-hybridized fractions. However, hybridizations at DNA/RNA ratios 10:1 divide the rapidly labelled RNA into two fractions with different apparent base compositions. The hybridized fraction (24%) is very like the DNA in its composition, whereas the non-hybridized fraction resembles the ribosomal RNA. As the hybridization process has an overall 80% efficiency, then 30% of the rapidly labelled RNA can be said to be capable of hybridization to DNA at low DNA/RNA ratios, and has the apparent composition of the

Table 5. *Hybridization of ³H-labelled rapidly labelled RNA to E. coli DNA at high DNA/labelled RNA ratios*

Rapidly labelled RNA was obtained from cultures of *E. coli* K12 dividing exponentially in glucose-minimal medium (Roberts *et al.* 1957) at 37°. The mean generation time was 87 min. Samples of rapidly labelled RNA (1 µg.) were hybridized to denatured DNA (200 µg.) immobilized on cellulose nitrate membrane filters.

Time of labelling RNA (min.)	% of labelled RNA hybridized to DNA (DNA/labelled RNA ratio 200:1)	
	Without added ribosomal RNA	With 50 µg. of ribosomal RNA
0.33	78	24
0.67	80	24
1	81	24
2	77	23
4	81	25
7	77	23
10	81	16
20	76	11

DNA. This exactly agrees with earlier work on *E. coli* and other organisms (Midgley, 1962; Midgley & McCarthy, 1962; Bolton & McCarthy, 1962, 1964).

Competition between rapidly labelled RNA and unlabelled RNA in hybridization. To obtain further information about the nature of the various fractions of rapidly labelled RNA that may be hybridized to denatured DNA, purified fractions of unlabelled RNA were added to hybridization mixtures to assess the degree of competition for binding sites on the DNA.

(i) Competition with ribosomal RNA. A series of filters containing DNA was challenged with a

fixed quantity of rapidly labelled RNA from cultures labelled with [^3H]uracil for various short intervals, at DNA/RNA ratios 200:1. Approx. 80% of the rapidly labelled RNA was hybridized in each case. A parallel series of filters was set up, in which a 50-fold excess of unlabelled *E. coli* ribosomal RNA was added to DNA and rapidly labelled RNA in a 200:1 ratio (Table 5).

These results show that, if excess of unlabelled ribosomal RNA is added to experiments where 80% rapidly labelled RNA will otherwise hybridize to DNA, the hybridized label is decreased to 20–25% of the total. This conclusion was enlarged upon for a sample of rapidly labelled RNA from a culture of *E. coli* labelled for 2 min. with [^{14}C]uracil. A series of filters containing DNA was challenged with a fixed amount of rapidly labelled RNA and increasing quantities of unlabelled *E. coli* ribosomal RNA, free from messenger RNA (Materials and Methods section). The DNA/rapidly labelled RNA ratio was 200:1, i.e. the minimum ratio at which 80% rapidly labelled RNA will hybridize. A parallel series of filters was set up in which the competing unlabelled ribosomal RNA was from *Pseudomonas aeruginosa*. The ribosomal RNA from this organism was chosen because, although *E. coli* and *Ps. aeruginosa* are genetically unrelated and their DNA species have very different nucleotide compositions, their ribosomal RNA species have the same overall composition (Midgley, 1962). The resulting competition curves are shown in Fig. 4.

When *E. coli* ribosomal RNA was used as the competing fraction there was a steady lowering of the rapidly labelled RNA binding to the DNA until, with excess of ribosomal RNA added, a level of 23% hybridization of rapidly labelled RNA was reached. Further additions of *E. coli* ribosomal RNA did not reduce this value. On the other hand, *Ps. aeruginosa* ribosomal RNA was not an effective competitor with hybridized *E. coli* rapidly labelled fractions.

(ii) Competition with 'synthetic' RNA. Since the fraction of rapidly labelled RNA from *E. coli* with a base composition like that of the DNA was efficiently hybridized at low DNA/RNA ratios (e.g. 10:1), low ratios must be used in experiments designed to show competition of unlabelled RNA species with this fraction. Otherwise, the number of free DNA sites available for RNA hybridization becomes very large, and excessive amounts of unlabelled RNA may have to be added before competition with the labelled RNA can begin. A specimen of RNA was synthesized, by using DNA-dependent RNA polymerase and nucleoside 5'-triphosphates as described in the Materials and Methods section. The enzyme was used with either *E. coli* or *Ps. aeruginosa* DNA as primer.

Three sets of filters containing 10 $\mu\text{g.}$ of *E. coli*

Fig. 4. Competition of *E. coli* or *Ps. aeruginosa* unlabelled ribosomal RNA with *E. coli* ^{14}C -labelled rapidly labelled RNA for hybridization sites on denatured *E. coli* DNA. The labelled RNA sample was obtained from cultures allowed to incorporate [^{14}C]uracil for 2 min. A DNA/rapidly labelled RNA ratio 200:1 was used throughout the experiment. ●, Competition with *E. coli* ribosomal RNA; ○, competition with *Ps. aeruginosa* ribosomal RNA. Unlabelled RNA samples were specimens free from messenger RNA contamination.

Fig. 5. Competition of *E. coli* or *Ps. aeruginosa* unlabelled RNA with *E. coli* ^{14}C -labelled rapidly labelled RNA for hybridization sites on denatured *E. coli* DNA. Two specimens of competing RNA were obtained by synthesis of RNA from nucleoside 5'-triphosphates, by using DNA and DNA-RNA polymerase. The third specimen was unlabelled *E. coli* ribosomal RNA, free from messenger RNA. A DNA/rapidly labelled RNA ratio 10:1 was used throughout the experiment. The rapidly labelled sample of RNA used was the same as in Fig. 4. ■, Competition with *E. coli* ribosomal RNA; ●, competition with RNA synthesized *in vitro* on *E. coli* DNA; ○, competition with RNA synthesized *in vitro* on *Ps. aeruginosa* DNA.

DNA were incubated with 10 $\mu\text{g.}$ of ^{14}C -labelled rapidly labelled RNA. Increasing amounts of 'synthetic' RNA, prepared by using *E. coli* DNA as primer, were added to the first set, 'synthetic' RNA prepared by using *Ps. aeruginosa* DNA as primer was added to the second set, and *E. coli* ribosomal RNA (free from messenger RNA) to the third set. Results are shown in Fig. 5. Whereas neither *Ps. aeruginosa* 'synthetic' RNA nor *E. coli* ribosomal RNA affected the hybridization of that

Table 6. *Hybridization of ^{14}C -labelled randomly labelled RNA from *E. coli* to DNA at high and low DNA/RNA ratios*

^{14}C -labelled randomly labelled RNA was obtained from cultures of *E. coli* dividing exponentially in glucose-minimal medium (Roberts *et al.* 1957) at 37°. The mean generation time was 90 min. Samples of randomly labelled RNA, specific radioactivity 880 counts/min./ μg ., were hybridized to DNA. In the experiments with low DNA/RNA ratios (5:1), 10 μg . of labelled RNA was hybridized with 50 μg . of DNA. Where high DNA/RNA ratios (250:1) were used, 3 μg . of labelled RNA was hybridized with 750 μg . of DNA.

Specimen of RNA	% of labelled RNA hybridized to DNA (uncorrected for hybridization efficiency)		
	(DNA/RNA ratio 5:1)	(DNA/RNA ratio 250:1)	
		Without added ribosomal RNA	With 1 mg. of ribosomal RNA
1	3.5 (± 0.2)	81.8 (± 0.5)	1.4 (± 0.2)
2	3.8 (± 0.2)	76.6 (± 0.5)	1.5 (± 0.2)
3	3.5 (± 0.2)	79.1 (± 0.5)	1.5 (± 0.2)

fraction of rapidly labelled *E. coli* RNA bound at DNA/rapidly labelled RNA ratios 10:1, *E. coli* 'synthetic' RNA was completely competitive with this fraction.

Hybridization of ^{14}C -labelled randomly labelled RNA to DNA. Cultures of *E. coli*, dividing at 37° every 90 min., were allowed to incorporate [^{14}C]uracil for at least six generations. After this time, the ribosomal and messenger RNA contents of the cells may be presumed to have the same specific radioactivity, even though the former is a stable end product in the cell and the latter is unstable. Randomly labelled ribosomal and messenger RNA fractions were obtained together and hybridized to *E. coli* DNA. Three sets of filters were set up. To the first set, the randomly labelled RNA was added, at DNA/RNA ratios 5:1. To the second, the RNA was added in a 250:1 DNA/RNA ratio. To the third, the RNA was again added in a 250:1 DNA/labelled RNA ratio, in the presence of a large excess of unlabelled ribosomal RNA, free from messenger RNA. Results, obtained in triplicate, are shown in Table 6.

DISCUSSION

The object of this paper is to show that the analysis and estimation of the content of messenger RNA in *E. coli* cells, by hybridization to DNA or by nucleotide base composition analyses (Midgley, 1962; Midgley & McCarthy, 1962; Bolton & McCarthy, 1962, 1964; McCarthy & Bolton, 1964; Armstrong & Boezi, 1965; Mangiarotti &

Schlessinger, 1967), give accurate values. In the literature, a considerable divergence exists between the estimations of messenger RNA contents in bacteria by 'direct' methods, as described above, and 'indirect' methods involving, for example, an examination of the kinetics of decay of rapidly labelled RNA after inhibition of cellular RNA synthesis by actinomycin (Levinthal *et al.* 1962, 1963; Lieve, 1965; Salser, Janin & Levinthal, 1968) or studies on the kinetics of labelling of the components of the bacterial nucleotide pools (Nierlich, 1967; Nierlich & Vielmetter, 1968).

Criticisms have been made (Levinthal *et al.* 1963) that the 'direct' methods of estimation of messenger RNA contents of bacterial RNA may be subject to considerable errors, leading to underestimations of this fraction in rapidly labelled fractions. However, our experiments have established that, at least for *E. coli*, no significant underestimations of messenger RNA are made by DNA-RNA hybridization analysis.

First, we have again demonstrated that the kinetics of labelling of nucleic acids in *E. coli* by [^{14}C]uracil and by [^{32}P]phosphate give different curves of isotope incorporation. Whereas the incorporation of [^{14}C]uracil into RNA occurs without a detectable delay (McCarthy & Britten, 1962; Buchwald & Britten, 1963), the kinetics of [^{32}P]phosphate incorporation indicate that the entry of the isotope into nucleic acids is delayed by the bacterial nucleotide and phosphate pools (Midgley & McCarthy, 1962). Secondly, the hybridization experiments, with either DNA/RNA ratios 5:1 or DNA/RNA ratios 200:1 in the presence of excess of added ribosomal RNA, have shown that, however short the period of incorporation of [^{14}C]uracil into *E. coli* rapidly labelled RNA, the proportion of readily hybridizable and non-hybridizable RNA remains approximately constant over the first 5-6 min. of ^{14}C -labelling (in organisms dividing every 80-90 min.). During this time, the analysis of the apparent nucleotide composition of the ^{32}P -labelled RNA also showed constancy of composition (Midgley & McCarthy, 1962). The apparent composition is never exactly like that of the DNA of *E. coli*, but appears to be typical of a mixture of 67% (by wt.) ribosomal RNA precursor and 33% an RNA like the DNA in base composition (Midgley & McCarthy, 1962). These results are fortified by composition estimations from ^{32}P -labelled hybridized RNA at low DNA/RNA ratios. By using such ratios, the rapidly labelled RNA may be divided into a readily hybridizable fraction, with a composition like that of the DNA (messenger RNA), and a non-hybridized fraction like ribosomal RNA in composition. The ratios of materials obtained in this way are exactly those predicted from the intermediate composition of total rapidly

labelled RNA (Midgley & McCarthy, 1962; Bolton & McCarthy, 1962, 1964).

If increasing DNA/rapidly labelled RNA ratios are used in hybridization experiments, more and more labelled RNA is bound until, at a DNA/RNA ratio 200:1, 80% of the rapidly labelled fraction will hybridize. This is true for any sample of rapidly labelled RNA obtained from cells given less than 6 min. incorporation of radioactive supplements. Higher DNA/RNA ratios give no further increase in hybridization. If, at DNA/rapidly labelled RNA ratios 200:1, competition experiments are carried out with unlabelled *E. coli* ribosomal RNA, then about two-thirds of the hybridized rapidly labelled RNA is found to compete for the appropriate DNA sites. The remaining one-third does not compete even after a large excess of ribosomal RNA has been added. Further, the unlabelled ribosomal RNA will not compete with the 30% fraction of rapidly labelled RNA bound at DNA/RNA ratios 5:1, which has the apparent characteristics of messenger RNA. The behaviour of *E. coli* ribosomal RNA may be contrasted with that of *Ps. aeruginosa* ribosomal RNA, which has no competitive effect on the binding of *E. coli* rapidly labelled RNA at low or at high DNA/RNA ratios. The competition phenomenon is thus shown to be specific for ribosomal RNA derived from homologous sources, and is not due to non-specific effects. We have thus demonstrated that, however short the time of labelling of growing *E. coli* K 12 cultures, the rapidly labelled RNA always consists of about 30% messenger RNA and 70% ribosomal RNA, presumably in a precursor form.

Criticisms that the hybridization techniques do not take account of all the messenger RNA in rapidly labelled RNA may be discounted by the following argument. First, in all samples of rapidly labelled RNA formed in cells given radioactive supplement for periods of less than 6 min., the percentage of rapidly labelled RNA that hybridizes to DNA at low DNA/RNA ratios (5:1) is not different from that proportion hybridizing at much higher ratios (200:1), after the addition of unlabelled ribosomal RNA in excess. As, in each case, a DNA/RNA ratio 200:1 binds (to a practical maximum) all the rapidly labelled RNA, in the absence of competitors, we must conclude that all the messenger RNA bound at high DNA/RNA ratios (200:1) is also bound at low ratios (5:1). Further, the results also indicate that probably no messenger RNA species are produced at rates approaching the rate of transcription of ribosomal RNA from its DNA sites.

A similar argument can be made for ^{14}C -labelled randomly labelled RNA in hybridization experiments. Of the randomly labelled RNA 1.8% is attributable to messenger RNA bound at low

Fig. 6. Kinetics of labelling hybridization of *E. coli* RNA in exponentially dividing cultures. Samples of ${}^3\text{H}$ -labelled rapidly labelled *E. coli* RNA were hybridized to denatured DNA at DNA/RNA ratios 5:1. The hybridization curve was corrected for (i) the 80% efficiency of hybridization processes, (ii) the exponential expansion of the messenger RNA pool during cell growth in the experiment and (iii) the contribution of ribosomal RNA to the hybridization curve (determined as 2.5% of the non-hybridized labelled RNA at any time point). This is the ratio of the DNA/RNA ratio 5:1, used in the experiment, over the DNA/RNA ratios 200:1 at which rapidly labelled RNA will just bind to DNA at 80% efficiency. O, Incorporation of $[5\text{-}{}^3\text{H}]\text{uracil}$ into total RNA; ●, incorporation of $[5\text{-}{}^3\text{H}]\text{uracil}$ into hybridized RNA.

DNA/RNA ratios (5:1). A similar percentage is also bound with DNA/RNA ratios 200:1, in the presence of excess of unlabelled ribosomal RNA, whereas 80% randomly labelled RNA is bound in the absence of competitor. Once again it appears that messenger RNA bound at high DNA/RNA ratios (200:1) is bound with equal efficiency at low ratios (5:1). This technique therefore gives an accurate measurement of the messenger RNA content of randomly labelled RNA in *E. coli*, in agreement with the results of others (Bolton & McCarthy, 1964; Friesen, 1966, 1968).

Finally, a graphical analysis of the results in Table 2, after the manner of Bolton & McCarthy (1962), should permit an estimate of the percentage of rapidly labelled messenger RNA in total bacterial RNA. For such a graphical analysis, the hybridization percentages in Table 2 have to be multiplied by 1.25, to take account of the 80% overall efficiency of hybridization of RNA, even when enough binding sites on the DNA are offered. Fig. 6 shows the results obtained. Two points are immediately apparent from the graph. The first is that, however short the time of labelling of the cells, the proportion of radioactivity in messenger and ribosomal RNA precursor has a 1:2 relationship. Secondly, after 5–6 min. labelling, the pool of messenger RNA

becomes saturated with labelled precursors. Thus, if we extrapolate this saturation plateau to the curve of total radioactivity incorporated into the nucleic acids and drop a perpendicular to the abscissa (time axis), we obtain a point corresponding to the time (in min.) it would take to make an amount of RNA equivalent to the messenger RNA pool. This value is 1.7 min. Thus, as the division time of the organism is 87 min., the percentage messenger RNA in the total RNA must be $(1.7/87) \times 100 = 1.9\%$. The close agreement between the estimations by the use of rapidly labelled RNA and randomly labelled RNA shows that unstable messenger RNA, with a lifetime of about $5\frac{1}{2}$ min. in these cultures, constitutes the overwhelming bulk of the total messenger RNA in the cell. Stable messenger RNA, or messenger RNA with a lifetime in the cell much longer than $5\frac{1}{2}$ min., must be a negligible fraction of the total messenger RNA.

G. H. P. thanks the Science Research Council for provision of a grant for a postgraduate studentship. J. E. M. M. thanks the Medical Research Council for a Research Fellowship, in which some of this work was undertaken. We thank Mrs M. Liddle for her excellent technical help in some of these experiments.

REFERENCES

- Armstrong, R. L. & Boezi, J. A. (1965). *Biochim. biophys. Acta*, **103**, 60.
- Beaven, G. H., Holiday, E. R. & Johnson, E. A. (1955). In *The Nucleic Acids*, vol. 1, p. 135. Ed. by Davidson, J. N. & Chargaff, E. New York: Academic Press Inc.
- Bolton, E. T. & McCarthy, B. J. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 1390.
- Bolton, E. T. & McCarthy, B. J. (1964). *J. molec. Biol.* **8**, 201.
- Buchwald, M. & Britten, R. J. (1963). *Biophys. J.* **3**, 155.
- Burton, K. (1956). *Biochem. J.* **62**, 315.
- Chen, P. S., jun., Toribara, T. Y. & Warner, H. (1956). *Analyt. Chem.* **28**, 1756.
- French, C. S. & Milner, H. W. (1955). In *Methods in Enzymology*, vol. 1, p. 64. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Friesen, J. D. (1966). *J. molec. Biol.* **20**, 559.
- Friesen, J. D. (1968). *J. molec. Biol.* **32**, 183.
- Geiduschek, E. P., Tochini-Valentini, G. P. & Sarnat, M. T. (1964). *Proc. nat. Acad. Sci., Wash.*, **52**, 486.
- Gillespie, D. & Spiegelman, S. (1965). *J. molec. Biol.* **12**, 829.
- Gros, F., Hiatt, H., Gilbert, W., Kurland, C. G., Risebrough, R. W. & Watson, J. D. (1961). *Nature, Lond.*, **190**, 581.
- Hall, T. C. & Cocking, E. C. (1965). *Biochem. J.* **96**, 626.
- Harris, H., Fisher, H. W., Rodgers, A., Spencer, T. & Watts, J. W. (1963). *Proc. Roy. Soc. B*, **157**, 177.
- Hayashi, M., Hayashi, M. N. & Spiegelman, S. (1964). *Proc. nat. Acad. Sci., Wash.*, **51**, 351.
- Kirby, K. S. (1964). *Progr. Nucleic Acid Res. molec. Biol.* **3**, 1.
- Levinthal, C., Fan, D. P., Higa, A. & Zimmerman, R. A. (1963). *Cold Spr. Harb. Symp. quant. Biol.* **28**, 183.
- Levinthal, C., Keynan, A. & Higa, A. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 1631.
- Lieve, L. (1965). *J. molec. Biol.* **13**, 862.
- McCarthy, B. J. & Bolton, E. T. (1964). *J. molec. Biol.* **8**, 184.
- McCarthy, B. J. & Britten, R. J. (1962). *Biophys. J.* **2**, 35.
- Mangiarotti, G. & Schlessinger, D. (1967). *J. molec. Biol.* **29**, 365.
- Marmur, J. (1961). *J. molec. Biol.* **3**, 208.
- Midgley, J. E. M. (1962). *Biochim. biophys. Acta*, **61**, 513.
- Midgley, J. E. M. (1965). *Biochim. biophys. Acta*, **108**, 340.
- Midgley, J. E. M. & McCarthy, B. J. (1962). *Biochim. biophys. Acta*, **61**, 696.
- Nierlich, D. P. (1967). *Science*, **153**, 1186.
- Nierlich, D. P. & Vielmetter, W. (1968). *J. molec. Biol.* **32**, 135.
- Pigott, G. H. & Midgley, J. E. M. (1966). *Biochem. J.* **101**, 9P.
- Polatnich, J. & Bachrach, H. L. (1961). *Analyt. Biochem.* **2**, 161.
- Roberts, R. B., Cowie, D. B., Abelson, P. H., Bolton, E. T. & Britten, R. J. (1957). *Publ. Carnegie Instn no. 607: Studies of Biosynthesis in Escherichia coli*, p. 5.
- Salser, W., Janin, J. & Levinthal, C. (1968). *J. molec. Biol.* **31**, 237.
- Stanley, W. M. & Bock, R. M. (1965). *Biochemistry*, **4**, 1302.