The Messenger Ribonucleic Acid Content of *Bacillus subtilis* 168

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(Received 16 June 1969)

Bacillus subtilis 168 messenger RNA was determined by DNA-RNA hybridization techniques, with denatured DNA immobilized upon cellulose nitrate membrane filters. The following results were obtained. (1) Cultures of *B. subtilis*, growing exponentially in enriched glucose-salts medium at 37°, incorporated [5-³H]uracil into both ribosomal and messenger RNA fractions without the kinetic delay expected from the presence of the intracellular nucleotide pools. (2) However short the time of labelling with exogenous labelled uracil (down to 7 sec.), 32–36% of the rapidly labelled RNA was messenger RNA and 68–64% was an RNA with the hybridization characteristics of ribosomal RNA. Analysis of the apparent nucleotide base composition of total ³²P-labelled rapidly labelled RNA and the two RNA fractions separated by hybridization at a DNA/RNA ratio 5:1 confirmed this finding. Of the rapidly labelled RNA, 31% readily hybridized with DNA at low DNA/RNA ratios and had an apparent base composition like that of the DNA, whereas 69% was hybridized only at low efficiency at low DNA/RNA ratios and had a composition identical with that of ribosomal RNA. (3) In cultures dividing every 48 min. at 37°, kinetic analysis of RNA labelled over a 20 min. period showed that the average life-time of messenger RNA was 2.7–3.0 min. and that its amount was 3.0% of the total RNA. (4) The hybridization of ³H-labelled randomly labelled RNA with DNA at a DNA/RNA ratio 5:1 showed that 2.9% of the randomly labelled RNA had the characteristics of messenger RNA. (5) Experiments carried out as described by Pigott & Midgley (1968) indicated that hybridization at low DNA/RNA ratios (5:1) effectively accounted for all the messenger RNA in a given specimen. The efficiency coefficient of RNA hybridization lay within the range of 90–95% input, if an excess of DNA sites was offered for RNA binding. (6) These measurements are compared with other results obtained by different methods, and reasons for any major disagreement are suggested.

Various methodological approaches have been exploited to measure the mRNA* content of bacterial RNA. A comparison of results quoted in the extensive literature on this subject shows, however, that there are considerable inconsistencies in the values obtained for a given organism. In some cases, the discrepancies are too great to be explained satisfactorily in terms, for example, of variations in the strain of the organism used, or in the conditions of growth employed.

Broadly, the techniques may be split into five main groups. In the first, mRNA was determined directly by its distinctive properties of hybridization with homologous denatured DNA (Bolton & McCarthy, 1962, 1964; McCarthy & Bolton, 1964; Nygaard & Hall, 1964; Mangiarotti & Schlessinger, 1967; Kennell, 1968; Kennell & Kotoulas, 1968; Pigott & Midgley, 1968). The kinetics of labelling of

various RNA fractions was followed after exogenous labelled nucleic acid precursors were added to growing cultures. The progress of entry of radioactivity into the RNA was then monitored. If the experiments were continued until after the mRNA pool was saturated with radioactive material, then its pool size and average life-time in the cell could be determined (Bolton & McCarthy, 1962; Mangiarotti & Schlessinger, 1967; Pigott & Midgley, 1968). When sufficient precautions were taken to ensure that all the mRNA was detected in any specimen, there is no reason to believe that this method underestimated the size of the mRNA fraction (Pigott & Midgley, 1966, 1968).

The second group of experiments made use of the fact that, if [³²P]phosphate was added to growing cells, the apparent base composition of the ³²P-labelled rapidly labelled RNA changed with time, from one typical of a mixture of unstable 'DNA-like' RNA and stable rRNA at early times to a final

* Abbreviations: mRNA, messenger RNA; rRNA, ribosomal RNA; tRNA, transfer RNA.

composition like that of the bulk cell RNA (Midgley, 1962; Midgley & McCarthy, 1962). Assuming that 'DNA-like' RNA may be equated with mRNA, and knowing the kinetics of the synthesis of rRNA in some detail (e.g. McCarthy & Britten, 1962; McCarthy, Britten & Roberts, 1962) the pool size and the rate of turnover of the mRNA fraction were determined. For *Escherichia coli* and *Proteus vulgaris*, determinations based on this method agreed well with those obtained from hybridization studies (Midgley, 1962; Midgley & McCarthy, 1962).

In the third group, these steady-state determinations were replaced by methods that measured the decay of RNA fractions labilized after RNA synthesis was halted by actinomycin D (Levinthal, Keynan & Higa, 1962; Levinthal, Fan, Higa & Zimmerman, 1963; Fan, Higa & Levinthal, 1964; Lieve, 1965; Zimmerman & Levinthal, 1967), by proflavine or by dinitrophenol (Woese, Naono, Soffer & Gros, 1963; Soffer & Gros, 1964). The assumptions implicit in these studies were (a) that the measured decay rates of pre-existing unstable RNA in the arrested state were strictly equivalent to those in conditions of steady growth and (b) that in bacterial rapidly labelled RNA the bulk of the radioactivity labilized was associated with the mRNA fraction. Assumption (b) has been shown to be incorrect, at least for *E. coli* strains BB (McCarthy & Bolton, 1964), K 12 (Pigott & Midgley, 1968) and M.R.E. 600 (Gray & Midgley, 1968) and for *P. vulgaris* (Bolton & McCarthy, 1962).

Fourthly, mRNA was determined by its stimulatory behaviour in a system synthesizing polypeptides *in vitro* (Forchhammer, Kjeldgaard & Moldave, 1965; Maaløe & Kjeldgaard, 1966). Usually this method is restricted to give internal comparisons of mRNA concentrations in different RNA specimens from the same organism, as it is difficult to relate the stimulatory activity of mRNA to its absolute amount in any RNA regardless of origin.

Finally, a more indirect method of measuring the life-time and amount of mRNA in bacterial cells involved the study of changes in the labelling of the intracellular nucleotide pool components during the incorporation of exogenous labelled nucleic acid precursors (Nierlich, 1967; Nierlich & Vielmetter, 1968; Salser, Janin & Levinthal, 1968). Assuming a particular model for the relationships between the nucleotide pools and stable or unstable RNA fractions in *E. coli* or *Bacillus subtilis*, these authors found that the organisms contained 3 and 9% of their total RNA as mRNA.

In the present paper, I have applied the direct technique of DNA-RNA hybridization to the measurement of mRNA in *B. subtilis* 168. The method was essentially that described by Pigott & Midgley (1966, 1968), making use of all precautions

to ensure that the efficiency of RNA hybridization was taken into account and that conditions were chosen so that no mRNA was missed in the determinations. I have compared my results with those obtained by other methods and have suggested reasons to explain any serious differences.

MATERIALS AND METHODS

Organism. *Bacillus subtilis* 168 was used.

Growth of bacterial cultures. Cultures were grown, under forced aeration at 37°, in an enriched glucose-salts medium as described by Avery & Midgley (1969). They were harvested at E_{620} 0.60, as measured in the Spectrochem spectrophotometer (Hilger and Watts Ltd., London N.W.1) in glass cuvettes of 1 cm. light-path. This corresponded to the mid-to-late phase of exponential growth.

The incorporation of radioactive substances into growing cultures, harvesting of cultures and formation of cell-free extracts by the French pressure cell (Aminco Bowman Ltd., Silver Spring, Md., U.S.A.) (French & Milner, 1955) were as described by Pigott & Midgley (1968).

Preparation of RNA. This was carried out by the method of Avery & Midgley (1969).

The preparation of the various labelled and unlabelled RNA fractions, the DNA-RNA hybridization technique (Gillespie & Spiegelman, 1965) and the assay of materials immobilized upon the cellulose nitrate membrane filters were performed as described by Pigott & Midgley (1968).

Nucleotide base compositions of RNA. The method was that of Midgley (1962), using isotope dilution and ion-exchange column chromatography.

Radiochemicals. [^3H]Uracil, sp. radioactivity 1000 mc/m-mole, and [^{32}P]phosphate (as phosphoric acid), sp. radioactivity 5-200 mc/m-mole, were obtained from The Radiochemical Centre, Amersham, Bucks.

RESULTS

The initial experiments were designed to discover the kinetics of incorporation of the chosen nucleic acid precursor into growing bacterial cultures. In *E. coli* (McCarthy & Britten, 1962; Buchwald & Britten, 1963; McCarthy & Bolton, 1964; Pigott & Midgley, 1968) and in *P. vulgaris* (Bolton & McCarthy, 1962; Midgley & McCarthy, 1962), labelled nucleotide bases are taken up from the external environment into the nucleic acids without the kinetic delay expected from the intervening nucleotide pools in the cell. Thus exogenous precursors of this kind appear to by-pass the intracellular nucleotide pools, which may be an important fraction of the total nucleotide material present (the sum of the pool components plus nucleic acids). A similar phenomenon has been reported by Salser *et al.* (1968) for *B. subtilis*, where the kinetic delay of incorporation of labelled exogenous guanine was of the order of 20 sec., in spite of the existence of a considerable nucleotide pool.

Cultures of *B. subtilis* 168 were grown at 37° in enriched glucose-salts media with unlabelled uracil

for several generations. This ensured that the pathways of uracil uptake into the cells were sufficiently stabilized before labelled supplements were added. When the cultures had reached a turbidity of 0.50 at 620nm., enough [5-³H]uracil was added to give an initial specific radioactivity of 100mc/m-mole of uracil in the medium. At intervals, 1ml. samples were removed and either filtered on to membrane filters, with several washes with equal volumes of ice-cold growth medium, or otherwise were precipitated with an equal volume of ice-cold 10% (w/v) trichloroacetic acid, with four subsequent washes with ice-cold precipitant. The results are shown in Fig. 1.

These findings are essentially in agreement with

Fig. 1. Incorporation of [5-³H]uracil into cultures of *B. subtilis* 168, growing exponentially at 37°. The sample size was 1 ml. ○, Total ³H incorporation; ●, incorporation into acid-precipitable fraction.

those of Salser *et al.* (1968), in that there is a very small kinetic delay in the incorporation of exogenous nucleotide bases into the nucleic acids. For uracil incorporation, the delay seems to be not greater than 10sec. in cultures dividing every 48 min.

I also found no convincing evidence that pool expansion occurred during uracil uptake into growing *B. subtilis* cultures. Experiments in which labelled uracil was given to cultures that had been given no uracil previously showed no detectable change either in the kinetic delay of uracil uptake or in the initial rate of uptake compared with those preincubated with a known concentration of unlabelled base.

Apparent nucleotide base composition of ³²P-labelled rapidly labelled RNA. The experiments initially described by Midgley (1962) and Midgley & McCarthy (1962) for *B. subtilis* A.T.C.C. 6051 were repeated for growing cultures of *B. subtilis* 168. The organism was grown in the usual enriched glucose-salts medium (Avery & Midgley, 1969), except that more than 90% of the phosphate content was replaced by an equivalent molar amount of tris-HCl buffer, pH 7.3. [³²P]Phosphate (1mc) was injected into 500ml. cultures and samples were poured on to crushed frozen growth medium at suitable intervals. The labelled RNA was isolated as described by Pigott & Midgley (1968), the DNA and tRNA being removed in the preparative procedures. The RNA was then hydrolysed with 0.4M-potassium hydroxide and analysed by isotope-dilution techniques, by using ion-exchange column chromatography on Dowex 1 (Midgley, 1962; Midgley & McCarthy, 1962). Table 1 indicates the changing apparent nucleotide base composition of the labelled RNA formed during the incorporation of [³²P]phosphate into the nucleic acids.

Table 1. *Apparent nucleotide composition of ³²P-labelled rapidly labelled RNA in B. subtilis 168*

Rapidly labelled RNA was obtained from cultures doubling every 49 min. at 37°. Determinations were to a standard error of $\pm 1.5\%$.

Labelling period (min.)	% of mean generation time	Nucleotide fraction (moles/100 moles)			
		CMP	AMP	GMP	UMP
0.25	0.51	23.0	25.2	27.6	24.2
0.50	1.02	23.7	24.9	27.5	23.9
1.0	2.04	23.3	25.6	27.4	23.7
2.0	4.08	23.1	25.6	27.7	23.6
4.0	8.16	23.3	26.0	27.2	24.5
6.0	12.24	22.9	26.1	28.0	24.0
10.0	20.40	22.5	25.0	29.8	22.7
20.0	40.80	22.6	25.3	31.4	20.7
200.0	408.0	22.5	25.0	31.1	21.4
Bulk RNA*		22.1	25.5	31.4	21.0
DNA		21.0	29.0	21.0	29.0

* Bulk RNA composition as given by Midgley (1962).

Table 2. *Hybridization of ³H-labelled rapidly labelled RNA from B. subtilis to DNA at low DNA/RNA ratios*

Rapidly labelled RNA was obtained from cultures of *B. subtilis* 168, growing exponentially with a mean generation time of 48 min. at 37°. RNA (10 µg.) was hybridized with 50 µg. of denatured DNA immobilized upon cellulose nitrate membrane filters (i.e. DNA/RNA ratio 5:1).

Time of labelling of RNA (min.)	% of labelled RNA hybridized to DNA
0.12	34.0
0.50	31.5
1.0	31.2
2.0	30.6
3.0	29.5
4.0	29.5
5.0	26.0
8.0	19.3
10.0	15.5
15.0	11.8
20.0	8.7

Table 1 shows that, over a period of 6 min. from the start of the experiment (about 10% of the cell-doubling time), the newly formed ³²P-labelled RNA had a constant apparent composition. After this initial period, the composition changed to one typical of the bulk RNA of the cell. The apparent composition at early times was like that of neither the DNA nor the rRNA of *B. subtilis*, but appeared to be like that of a mixture of materials with both compositions. This finding is in full agreement with earlier findings of Midgley (1962) and Midgley & McCarthy (1962).

Hybridization of rapidly labelled RNA to DNA. Various specimens of ³H-labelled rapidly labelled RNA were obtained by allowing [5-³H]uracil to be incorporated into steadily growing cultures of *B. subtilis*. The cultures were previously grown over several generations in the continuous presence of unlabelled uracil. The experiment was started when the cultures had reached a turbidity of 0.50 at 620 nm.

Samples of denatured DNA (50 µg.) were bound to cellulose nitrate membrane filters as described by Gillespie & Spiegelman (1965) and Pigott & Midgley (1968). A 10 µg. sample of the rapidly labelled RNA was added to the filters and DNA-RNA hybridization was carried out at 66° for 16 hr. The percentage of hybridized RNA bound to the filters was measured by scintillation counting. Table 2 shows that, over an initial period of about 4 min., there was a constant proportion of readily hybridized RNA to non-hybridized RNA at a DNA/RNA ratio 5:1. As the earliest sample for hybridization was 7 (±1) sec. after the start of

Fig. 2. Hybridization of *B. subtilis* rapidly labelled RNA with DNA immobilized upon cellulose nitrate membrane filters. ³H-labelled RNA was obtained from cultures that had incorporated the isotope over a period of 1 min. at 37°.

labelling, it is clear that the hybridization characteristics of the rapidly labelled RNA were constant from the beginning of the experiment to about 4 min. from the starting point. After this time the hybridizable RNA became a progressively smaller fraction of the labelled RNA.

Thus there was agreement between the results from analyses of apparent nucleotide base composition and from hybridization studies at a DNA/RNA ratio 5:1, in that rapidly labelled RNA from *B. subtilis* had constant apparent composition and hybridization characteristics in the early stages of pulse labelling. However, at no time would rapidly labelled RNA hybridize more effectively than 34% of input at a DNA/RNA ratio 5:1.

Hybridization of rapidly labelled RNA with various amounts of DNA. It is important to know the efficiency coefficient of hybridization of RNA in the conditions employed, as corrections have to be made if the coefficient is consistently less than unity (Pigott & Midgley, 1968). Accordingly, ³H-labelled RNA from cultures that had incorporated [5-³H]uracil for 1 min. at 37° was used as a typical sample for testing. The hybridization curve is shown in Fig. 2. As in the case of *E. coli* RNA (Pigott & Midgley, 1968), when the DNA/RNA input ratio was increased, there was a parallel increase in the hybridization of the rapidly labelled RNA from an initial value of 33% to a maximum value of 95% of input. This latter value was first reached at a DNA/RNA ratio 210:1. Thus all, to a practical maximum, of the rapidly labelled RNA hybridized with appropriate DNA sites at ratios greater than 210:1. The shape of the hybridization curve over the range of DNA/RNA ratios 10:1–200:1 is very reminiscent of the curve for *B. subtilis* rRNA (Avery & Midgley, 1969).

Hybridization of ribosomal RNA with denatured DNA. To compare the hybridization character-

istics of rapidly labelled and randomly labelled RNA species, ^3H -labelled rRNA, containing mRNA, was prepared as described by Pigott & Midgley (1968). In the case of randomly labelled RNA, the mRNA was labelled to the same specific radioactivity as the major component of stable rRNA.

The RNA was hybridized to denatured DNA over the range of DNA/RNA ratios 2:1–250:1 (Fig. 3). In all respects the curve was like that observed by Avery & Midgley (1969). Maximum hybridization of the RNA (90% of input) was first achieved at a DNA/RNA ratio 215:1. At low DNA/RNA ratios, however, not more than 4–5% of the randomly labelled RNA input would hybridize. This puts an upper limit of 4–5% on the amount of mRNA bound at low DNA/RNA ratios.

To determine more accurately the absolute percentage of RNA binding to DNA at low DNA/RNA ratios as mRNA, three sets of filters were set up, each set in quadruplicate. Each DNA-bearing filter contained 200 μg . of DNA. To the first set ^3H -labelled rRNA + mRNA was added at a DNA/

RNA ratio 5:1. To the second set the ^3H -labelled RNA was added to the filters at a DNA/RNA ratio 250:1, and to the third set 0.8 μg . of ^3H -labelled RNA and 1 mg. of unlabelled rRNA (free from mRNA) were added. The results are shown in Table 3.

Whereas 4.7% of input RNA would hybridize at a DNA/RNA ratio 5:1 and 92% of input RNA would hybridize at a ratio 250:1, when excess of unlabelled rRNA was added at a DNA/labelled RNA ratio 250:1, only 2.6% of labelled RNA was hybridized. Therefore, of the 4.7% of input RNA hybridized at low DNA/RNA ratios (5:1), 2.1% must be ascribed to the binding of rRNA (we would expect $5/210 \times 92/100 = 2.2\%$ of rRNA to be hybridized; see Fig. 3). This is no longer bound in the presence of excess of unlabelled competitors, even at high DNA/RNA ratios, leaving 2.6% of the labelled RNA input hybridized as mRNA. Thus low DNA/RNA ratios are as effective as high ratios in efficiently binding the mRNA fraction. We can therefore put an upper limit on the amount of mRNA in the labelled specimen, as, with an efficiency coefficient of 0.92 in the experiment, the corrected hybridization of the randomly labelled RNA should be $2.6 \times 100/92 = 2.9\%$ input. Further experiments described in the text support this value.

Apparent nucleotide base composition of hybridized and non-hybridized rapidly labelled RNA at low and high DNA/RNA ratios. The experiments described by Pigott & Midgley (1968) using the rapidly labelled RNA from *E. coli* were repeated for the equivalent ^{32}P -labelled material from *B. subtilis*. The labelled RNA was obtained from cultures that had incorporated [^{32}P]phosphate into the cells for 90 sec. at 37°. Filters containing 200 μg . of DNA were incubated with either 40 or 1 μg . of RNA, as described by Gillespie & Spiegelman (1965). The hybridized RNA and the non-hybridized RNA in

Fig. 3. Hybridization of randomly labelled rRNA and mRNA from *B. subtilis* with DNA. The RNA was obtained from cultures that had incorporated the labelled [^3H]-uracil over five cell divisions.

Table 3. Hybridization of ^3H -labelled randomly labelled mRNA + rRNA from *B. subtilis* to DNA at high and low DNA/RNA ratios

Randomly labelled RNA was obtained from cultures of *B. subtilis* 168 dividing exponentially at 37°. The doubling time was 48 min. In experiments with low DNA/RNA ratios (5:1), 10 μg . of RNA was hybridized with 50 μg . of DNA. Where high DNA/RNA ratios (250:1) were used, 0.8 μg . of labelled RNA was hybridized with 200 μg . of DNA. Results are uncorrected for efficiency of hybridization.

Specimen of RNA	% of labelled RNA hybridized to DNA		
	DNA/RNA ratio 5:1	DNA/RNA ratio 250:1	
		Without added rRNA	With 1 mg. of rRNA
1	4.6 $\pm$ 0.2	93.0 $\pm$ 0.5	2.7 $\pm$ 0.1
2	4.7 $\pm$ 0.2	92.5 $\pm$ 0.5	2.4 $\pm$ 0.1
3	5.1 $\pm$ 0.2	89.8 $\pm$ 0.5	2.8 $\pm$ 0.1
4	4.6 $\pm$ 0.2	91.0 $\pm$ 0.5	2.6 $\pm$ 0.1

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

Rapidly labelled RNA was obtained from *B. subtilis* cultures growing exponentially at 37° with a doubling time of 48 min. [³²P]Phosphate was incorporated for 90 sec. Determinations were to a standard error of $\pm 1.5\%$.

RNA fraction	Nucleotides (moles/100 moles)				% of RNA
	CMP	AMP	GMP	UMP	
Total ³² P-labelled rapidly labelled RNA	23.1	25.5	27.5	23.9	—
Hybridized RNA (DNA/RNA ratio 5:1)	21.9	28.1	22.5	27.5	31
Non-hybridized RNA (DNA/RNA ratio 5:1)	23.0	25.0	31.8	20.2	69
Hybridized RNA (DNA/RNA ratio 200:1)	22.5	26.0	27.0	24.5	95
<i>B. subtilis</i> rRNA*	22.3	25.9	31.0	20.8	
<i>B. subtilis</i> DNA	21.0	29.0	21.0	29.0†	

* Composition from Midgley (1962).

† As thymidylic acid.

the supernatant were analysed for their apparent base compositions. These results are shown in Table 4.

As with *E. coli* ³²P-labelled rapidly labelled RNA, hybridization at DNA/RNA ratio 200:1 bound a representative major fraction of the labelled RNA. With a DNA/RNA ratio 5:1, however, the filters divided the labelled RNA into a fraction comprising 31% of the total and having an apparent composition of an RNA very like the DNA and 69% having an apparent composition like that of rRNA. Thus, taking the efficiency coefficient as 0.95 in this experiment, about 32–33% of the rapidly labelled RNA was capable of hybridizing with DNA at a DNA/RNA ratio 5:1 and had a composition like that of *B. subtilis* DNA, whereas the remainder bound to the DNA with low efficiency at low DNA/RNA ratios and had a hybridization curve and an apparent base composition typical of rRNA.

Competition between rapidly labelled RNA and unlabelled RNA in hybridization. Membrane filters containing 200 µg. of DNA were challenged with 1 µg. of ³H-labelled rapidly labelled RNA obtained from a culture of *B. subtilis* allowed to incorporate [^{5-³H}]uracil over a short period. In each case 90–95% of input RNA would hybridize with the DNA. To another set of filters a 50-fold unlabelled excess of *B. subtilis* rRNA (free from mRNA) was added (Table 5).

The results demonstrated that the addition of excess of unlabelled RNA led, in each case, to a lowering of the hybridization of the rapidly labelled RNA from 90% of input to 32% or less. *B. subtilis* rRNA thus competed effectively with a fraction of the rapidly labelled RNA for the available DNA sites.

This finding was investigated in more detail by using a sample of rapidly labelled RNA from *B. subtilis* cultures that had been labelled for

Table 5. *Hybridization of ³H-labelled rapidly labelled RNA with DNA at a DNA/RNA ratio 200:1, in the presence or absence of excess of unlabelled rRNA*

Rapidly labelled RNA specimens were as described in Table 2.

Time of labelling RNA (min.)	% of labelled RNA hybridized	
	With DNA	With DNA + 50-fold excess of rRNA
0.12	88	30
0.50	86	33
1.0	91	35
2.0	94	32
3.0	87	30
4.0	91	29
5.0	90	24
8.0	83	16
10.0	92	13
15.0	96	10
20.0	90	7

30 sec. at 37° by [^{5-³H}]uracil. Table 5 shows that this was one of the early RNA specimens, at which time competition with excess of unlabelled ribosomal RNA at high DNA/RNA ratios lowers the efficiency of rapidly labelled RNA hybridization from 90% of input to 32% of input.

A series of DNA-bearing filters was challenged with a fixed amount of ³H-labelled rapidly labelled RNA (DNA/rapidly labelled RNA ratio 200:1) and increasing amounts of unlabelled *B. subtilis* rRNA. Fig. 4 shows that the additions of rRNA steadily decreased the hybridization of the labelled RNA to a constant value of 30% of input, whereafter larger additions of rRNA had no further effect.

Kinetic analysis of labelling of mRNA and rRNA. The data from Fig. 1 and Table 2 were analysed

Fig. 4. Competition of ^3H -labelled rapidly labelled RNA from *B. subtilis* with unlabelled homologous rRNA for hybridization sites on the DNA. RNA was obtained from cultures that had incorporated $[5\text{-}^3\text{H}]\text{uracil}$ for 30 sec. at 37° . The DNA/rapidly labelled RNA ratio was 200:1 (w/w) throughout. The percentage of rapidly labelled RNA hybridized is normalized to a maximum of 100%.

Fig. 5. Kinetics of labelling and hybridization of *B. subtilis* RNA in exponentially dividing cultures. Samples of ^3H -labelled rapidly labelled RNA were hybridized with DNA at a DNA/RNA ratio 5:1 (w/w). Hybridization curves were corrected for (1) the expansion of the mRNA pool during exponential growth of the cultures, (2) the 90% efficiency of hybridization processes and (3) the contribution of rRNA to the hybridization curve (determined as 2.5% of the non-hybridized labelled RNA at any time-point). ●, Incorporation of $[5\text{-}^3\text{H}]\text{uracil}$ into total RNA; ○, incorporation of $[5\text{-}^3\text{H}]\text{uracil}$ into hybridized RNA.

graphically as described originally by Bolton & McCarthy (1962). In Fig. 5 a plot was made of the progress of entry of $[5\text{-}^3\text{H}]\text{uracil}$ into the mRNA and the stable RNA fractions. The mRNA curve was corrected for (1) the exponential expansion of its pool during bacterial growth, (2) the efficiency coefficient of RNA hybridization with DNA and (3) the contribution of labelled rRNA to the hybrids at the DNA/RNA ratio 5:1 used. As with *E. coli* RNA (Pigott & Midgley, 1968), this was determined as 2.5% of the non-hybridized labelled RNA at any time. Fig. 5 shows that (a) the proportions of ^3H label in mRNA and total RNA at early times (0–4 min.) had a constant 1:3 relationship and (b) the percentage of messenger RNA was 3.0% of the total. This estimate was obtained by extrapolation of the saturation plateau of mRNA labelling to the curve for total RNA and dropping a perpendicular at this point to the time axis. Then this time value $\times 100\%$ /cell doubling time = percentage of total RNA as mRNA (Bolton & McCarthy, 1962). The average life-time of the mRNA was obtained by using the equation:

$$M = La / (La + Db)$$

where M = fraction of mRNA in total RNA, L = its average life-time in min., D = cell division time in min., a = fraction of labelled precursors entering mRNA in unit time and b = fraction of labelled precursors entering stable RNA in unit time. In the experiments, $M = 0.030$, $D = 48$ min., $a = 0.33$ and $b = 0.67$. Thus the average life-time of mRNA is 3.0 min.

The values obtained agreed well with the corresponding value obtained from the hybridization of

^3H -labelled randomly labelled rRNA + mRNA. The latter value could be represented in Fig. 5 by an mRNA hybridization point placed at infinite time. This means that the mRNA pool was truly saturated with ^3H label after incorporation of $[5\text{-}^3\text{H}]\text{uracil}$ for 4 min. It is therefore unlikely that mRNA with a much longer life-time exists as an important part of the total messenger pool.

DISCUSSION

In any model designed to throw light on the interrelationships between the intracellular nucleotide pools, unstable mRNA and stable tRNA and rRNA in bacterial cells, the observed kinetics of nucleotide flow into the RNA fractions must fit a coherent pattern. My results have shown that the essential features of RNA labelling in *B. subtilis* are explicable only in terms of a limited number of formal schemes.

We may list the findings which any adequate model must explain as follows. (1) If we assume that pre-existing material mixes completely with incoming exogenously supplied nucleotide bases, no matter what pool the precursors enter initially, the flow of bases enters the mRNA and rRNA pools in a steady 1:2 weight/time relationship. (2) There is no kinetic delay greater than 10 sec. in the entry of labelled uracil into either rRNA or mRNA, in spite of the presence of the nucleotide pools (Nierlich & Vielmetter, 1968). (3) The addition of exogenous uracil to the growth medium causes little or no

Fig. 6. (a) Model of the chemical flows and the kinetic inter-relationships between exogenous nucleic acid bases, the pools of nucleotides in the cell and the stable and unstable RNA fractions (Levinthal *et al.* 1963). (b) Model of the same relationships, according to Britten (1963).

expansion of the internal nucleotide pools (Nierlich & Vielmetter, 1968). (4) The mRNA pool is saturated with labelled precursors after incorporation for 4 min. from the external environment. In comparing these results with those with 3H -labelled randomly labelled RNA, there does not appear to be a detectable 'slow' component in the labelling pattern of mRNA that might otherwise give a misleadingly low estimate of mRNA at early times.

The two alternative models that may be invoked to explain these results are shown in Fig. 6. In Fig. 6(a) the model depicted is that proposed by Levinthal and his associates (Levinthal *et al.* 1962, 1963; Fan *et al.* 1964; Nierlich, 1967; Zimmermann & Levinthal, 1967; Nierlich & Vielmetter, 1968; Salser *et al.* 1968). In this model exogenous and endogenous nucleic acid precursors are presumed to enter the intracellular nucleotide pools (B). There it is proposed that they mix adequately with pre-existing pool components. Nucleotides for unstable mRNA (A) are then withdrawn from the pool and are returned later, after RNA breakdown. Nucleotides for stable RNA (C) (tRNA + rRNA) are also withdrawn from the pool (B).

In Fig. 6(b) the model proposed by Britten (1963) is depicted. Here, the essential features of the model are that exogenous nucleotide bases enter a small non-expandable compartment (D), which

equilibrates with the nucleotide pool (B) relatively slowly (the 'by-pass' phenomenon). From compartment (D) material is drawn either from exogenous or endogenous sources directly into the RNA fractions (A) and (C). On the other hand, the phosphate moieties of the nucleotides incorporated into the RNA are derived primarily from the nucleotide pool (B). Pool (B) thus acts essentially as a 'storage' pool to one side of the main flow of RNA base precursors. mRNA degradation products are then fed into a further pool (E) that does not readily equilibrate with either component (D) or the nucleotide pool (B), but supplies material for the synthesis of both tRNA and DNA (Midgley & McCarthy, 1962; Midgley, 1963).

In both models the primary problem is the need to explain the kinetics of uptake of exogenous nucleotide bases from the external environment into the cellular nucleic acids. The kinetics of [^{32}P]phosphate incorporation must also be fitted into the general scheme.

In Fig. 6(a) there are certain consequences for the expected rates of labelling of the unstable mRNA and stable RNA species, if we assume complete mixing of exogenous precursors in the nucleotide pools and find an undelayed entry of precursors into the nucleic acids. Briefly, if the flows 1 and 2 in Fig. 6(a) are not greater than flow 3 into stable RNA, and if the nucleotide pool contains as much as 5–10% of the total amount of bases in the cell (as pool + nucleic acid) (Nierlich & Vielmetter, 1968; J. E. M. Midgley, unpublished work), the entry of precursors into RNA from the external environment must show a detectable kinetic delay. If, as has been argued by Salser *et al.* (1968), the flows 1 and 2 are as much as seven times flow 3, and if the pool of mRNA is as large as 9% of the total RNA, the kinetic delay imposed by the nucleotide pools will be virtually abolished, as the unstable RNA pool then acts as a major trichloroacetic acid-precipitable compartment of the nucleotide pools, owing to rapid equilibration. Thus the kinetics of labelling mRNA and rRNA must have the form shown in Fig. 7(a).

In Fig. 6(b) the lack of kinetic delay in RNA labelling is explained by postulating compartmentalization of the flow of material into the nucleotide pools and into nucleic acids. If flows 1 and 2 are both greater than flows 3 and 4 in this model, then exogenous material will pass directly into the nucleic acids while the pool (B) is incorporating labelled precursors more slowly. In this model, discussed in much greater detail by McCarthy & Britten (1962), Britten (1963) and Buchwald & Britten (1963), there is no need to postulate a 'filling in' of the expected kinetic delay of RNA labelling by a relatively large pool of mRNA that is turning over rapidly. The kinetics of labelling of

Fig. 7. (a) Representation of the expected kinetics of labelling of stable and unstable RNA, on the basis of the assumptions in the model by Levinthal *et al.* (1963) in Fig. 6(a). (b) Representation of the early part of the kinetics of labelling of stable rRNA and unstable mRNA according to the model by Britten (1963) in Fig. 6(b). In both Figs. 7(a) and 7(b), the additional complications of labelling of DNA and tRNA have been ignored, as have the effects of nucleotide-pool labelling on the later period of labelling of the cellular nucleic acids in Fig. 7(b). This can be done by considering only the initial short period of labelling after addition of the exogenous nucleic acid precursor, when distortions from these other factors are minimal (Britten, 1963).

mRNA and rRNA have the form shown in Fig. 7(b), if we postulate that the degradation products of RNA breakdown do not re-enter the nucleotide pools (B) but are used instead to supply material for the synthesis of other nucleic acids (Britten, 1963; Midgley, 1963).

An inspection of the kinetic plots in Figs. 7(a) and 7(b) allows us to make a choice between these two models. At early times after the start of labelling by [$5\text{-}^3\text{H}$]uracil, the model of Levinthal *et al.* (1962) demands that the bulk of the radioactive RNA is mRNA; that is, it must be readily hybridized to homologous denatured DNA at low DNA/RNA

ratios (Bolton & McCarthy, 1962, 1964; Pigott & Midgley, 1966, 1968). There can be no period of time over which the proportions of labelled mRNA and stable RNA remain constant until after both pools are saturated with labelled precursors. At times close to the start of labelling, the proportion of labelled mRNA is at its maximum, and falls off steadily as the labelled precursors begin to enter the stable RNA pools to a greater extent at later times. In short, because of its size and rapid equilibration with the nucleotide pools, the mRNA pool should apparently substitute for the nucleotide pool as a precursor of stable RNA.

According to the model proposed by Britten (1963), this feature is not necessary to explain the results. When the nucleotide pools are no longer obligatory intermediates in the incorporation of exogenous precursors into the nucleic acids, there is no need to postulate a high rate of turnover of a relatively large pool of mRNA to explain the kinetics. Further, the kinetics of labelling the nucleotide pools have now no rigid precursor relationship with the rates of RNA labelling.

The evidence I have presented for *B. subtilis* 168 strongly favours the model suggested by Britten (1963), which was devised originally for *E. coli* and other organisms. Conversely, certain findings do not favour the model proposed by Levinthal *et al.* (1963). The proportion of radioactive precursors in rRNA and mRNA is constant over a period of 0–4 min. from the start of labelling. This indicates that there is a constant ratio of 1:2 between the flows into the mRNA and rRNA pools (Midgley, 1962; Midgley & McCarthy, 1962). Therefore, as the kinetics of entry of radioactivity into total RNA show little initial delay, it follows that there is little delay of entry into either the mRNA or the rRNA pools. This is not in agreement with the results expected from the model proposed by Levinthal *et al.* (1963) (Figs. 6a and 7a). If the mRNA + nucleotide pool is as much as 10–15% of the total (pool + unstable RNA + stable RNA), we would expect a kinetic delay of about 3 min. of the entry of labelled uracil into stable RNA pools (in an organism doubling every 48 min.).

We must now attempt to reconcile the results of Levinthal *et al.* (1962, 1963), Fan *et al.* (1964), Nierlich (1967), Zimmermann & Levinthal (1967), Nierlich & Vielmetter (1968) and Salser *et al.* (1968) with Britten's (1963) model. The above authors have estimated the size of the mRNA pool in *B. subtilis* as some 8–9% of the total RNA. In some experiments (Levinthal *et al.* 1962, 1963; Zimmermann & Levinthal, 1967) actinomycin D was used to arrest RNA synthesis. The rate of decay of rapidly labelled RNA was followed after inhibition of RNA synthesis by measuring the fall in trichloroacetic acid-precipitability of the labelled

material with time. These authors found that the bulk of the rapidly labelled RNA decayed into non-precipitable products. However, I have shown that, at all early times in pulse labelling, only 33% of the rapidly labelled RNA has the base composition and the hybridization characteristics of mRNA.

If the time required for the synthesis of complete RNA chains is about 30 sec.–1 min. at 37° (Kepes, 1963; Goldstein, Kirschbaum & Roman, 1965; Lieve, 1965; Kaempfer & Magasanik, 1967; Mangiarotti & Schlessinger, 1967; Bremer & Yuan, 1968; Mueller & Bremer, 1968; Manor, Goodman & Stent, 1969), then with much shorter periods of incorporation of labelled precursors a high proportion of the labelled rRNA chains will be still unfinished when synthesis is stopped by actinomycin D. We must therefore assume that these are also degraded to trichloroacetic acid-soluble material after actinomycin treatment. There is also no evidence as to the fate of newly finished rRNA chains that have not received their stabilizing protein before the addition of actinomycin. As I have shown that the mRNA detectable by DNA–RNA hybridization is no more than 3.0% of the total RNA, then, as the ratio of flow of radioactive precursors into mRNA and rRNA is 1:2 at early times, the estimation of mRNA by decay after actinomycin treatment could give a value as much as three times too large. Thus, from my results, use of the actinomycin treatment of *B. subtilis* could give an estimate of the mRNA pool as being as much as 9% of the total RNA, in agreement with the determinations of Levinthal *et al.* (1962, 1963), Nierlich & Vielmetter (1968) and Salser *et al.* (1968). Therefore I would agree with the conclusions of Acs, Reich & Valenju (1963) and Kennell (1964) that arrest of RNA synthesis by actinomycin D must lead to the decay of non-messenger RNA molecules in amounts comparable with, or indeed greater than, that of true mRNA itself. I agree with the results of Kennell (1964) for *B. megaterium* that mRNA is less than 5% of the total RNA.

In examining the estimation of mRNA pools by the kinetics of labelling of the nucleotide pools in *B. subtilis* (Nierlich & Vielmetter, 1968; Salser *et al.* 1968), I must emphasize that in Britten's (1963) model the labelling of these two pools pursues different paths. It may be that the flows of material into and out of the nucleotide pools from the external environment are considerably slower than, or at most comparable with, the rate of incorporation of material into stable nucleic acids through the 'by-pass' compartment (Buchwald & Britten, 1963) (Figs. 6*b* and 7*b*). Thus a calculation based on the slow flow of nucleotides through the intracellular pool and the faster flow into stable and unstable RNA would lead to a large overestimate of the contribution of any unstable mRNA that may

equilibrate with the nucleotide pool in the model by Levinthal *et al.* (1963). In other words, the reason for the slow equilibration of the nucleotide pools may be ascribed to the presence of a large proportion of mRNA, when other reasons for this phenomenon are operative. I cannot evaluate the results of inhibition by actinomycin and nucleotide-pool labelling as determinants for mRNA content as being in other than fortuitous agreement.

If the mRNA pool of *B. subtilis* is only 3% of the total RNA, this indicates that there is no excess of mRNA over available ribosomal particles. There should be one 70s ribosome per 50 000-mol.wt. unit of mRNA, a value in reasonable agreement with results from other studies (Maaløe & Kjeldgaard, 1966; Mangiarotti & Schlessinger, 1967; Pigott & Midgley, 1968). I therefore conclude that *B. subtilis* is not essentially different from other micro-organisms so far studied in either the labelling patterns of its stable and unstable RNA pools, or in its mRNA content. Indeed, the average mRNA life-time/cell doubling time ratio in this organism is 0.063 at 37°, which may be compared with the ratio of 0.054 for *E. coli* in glucose–salts medium and 0.095 for *E. coli* in enriched broth medium (W. J. H. Gray & J. E. M. Midgley, unpublished work). The evidence suggests that the model by Britten (1963) to explain the flows of nucleotide bases into RNA of *E. coli* applies with equal rigour to *B. subtilis*.

I am indebted to Mrs M. Liddle for her excellent technical help. This work was carried out in the Medical Research Council Research Group in the Structure and Biosynthesis of Macromolecules.

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