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The Nature of the Fluctuating Ribonucleic Acid in *Escherichia coli*

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In several species of bacteria growing cells have been shown to have much higher concentrations of ribonucleic acid (RNA) than resting cells. Apart from the work of Belosersky and his collaborators (Belosersky, 1947) there has been little investigation into the nature of the 'additional RNA' in growing cells; more attention has been paid to its possible function in the cell. The most widely accepted view, that it plays a part in the synthesis of protein, is based mainly upon the direct relationship observed between the RNA concentration and the growth rate of micro-organisms growing exponentially (Caldwell, Mackor & Hinshelwood, 1950; Price, 1952; Wade, 1952; Jeener, 1953), upon the presence of RNA in granules having the ability to synthesize proteins (Gale & Folkes, 1955a) and upon the concomitant syntheses of protein and RNA which occurs during enzyme induction (Pardee, 1954). Some experimental results, however, do not lend themselves to this theory, and there is increasing evidence that the level of RNA concentration does not reflect the rate at which a cell is synthesizing protein.

The contrasting RNA concentrations in resting cells and growing cells is more pronounced in some species than in others. In *Escherichia coli* the concentration of RNA in rapidly growing cells is approximately twice the concentration in resting cells, and in certain other species more than fivefold differences in concentration have been observed. The abundance of the 'additional RNA' in fast-growing bacteria and the conflicting evidence presented for its connexion with protein synthesis prompted the closer examination of its nature and function in *Esch. coli*.

It was important in the first instance to establish whether or not the increased RNA concentration which develops in resting cells during the early stages (division lag) of their growth in fresh medium is due to a general increase in the concentration of all RNA constituents or to only a fraction of these. To this end the chemical compositions, the solubilities, the responses to various methods of extraction and the sedimentation behaviours of ribonucleic acids in resting and in dividing cells of *Esch. coli* were compared. Concurrently with these experiments the effects of various extractions and fractionations upon the basophilia of the cells were observed in order to examine the relationship between the 'additional RNA' and the high basophilia of dividing cells. This report describes these experiments and suggests an alternative function for 'additional RNA' based upon our results and upon other published experimental data.

A preliminary note on part of this report has already been published (Wade, 1955).

MATERIALS AND METHODS

Organisms and cultural conditions. Comparisons between the RNA concentrations in resting and in dividing cells were made on *Aerobacter aerogenes* (NCTC 418), *Bacillus anthracis* (Weybridge), *Bacillus cereus* (NCTC 8035), *Chromobacterium prodigiosum* (laboratory strain), *Chromatobacterium violaceum* (NCTC 7917), *Clostridium welchii* (SR 12), *Corynebacterium hoffmannii* (NCTC 8338), *Eberthella typhosa* (Ty 479, a purine-requiring avirulent mutant kindly supplied by Dr T. W. Burrows), *Escherichia coli* (NCTC 1100), *Pasteurella pestis* (Tjiwidej), *Proteus vulgaris* (laboratory strain) and *Staphylococcus aureus* (Oxford 'H') grown on casein–casein–yeast medium (CCY) (Gladstone & Fildes, 1940) or in tryptic-meat broth (Table 1) at 37°.

The cultures were grown aerobically with the exception of *Cl. welchii* and *P. vulgaris* which were grown anaerobically in screw-capped bottles on tryptic-meat broth containing 0·5 % of glucose and 0·08 % of thioglycollic acid. Resting cells were taken from fully grown cultures and dividing cells from cultures during the phase of maximum growth and division rate. The growth rate of dividing cells was assessed from the increase in optical density, which was measured in 1 cm. cells of the Hilger Spekker absorptiometer with a neutral filter (Hilger, 508) and, within the limits of the range *E* 0·05–0·5, to which measurements were confined, was found to be proportional to the dry-weight concentration of the cells. The average cell size was assumed to be constant during this phase of growth. The cultures were treated with 4 % (w/v) formaldehyde soln., the cells were centrifuged, washed once with 20 vol. (wet cell vol.) of 0·145 M-NaCl and once with 20 vol. of water.

An investigation into the nature of *Esch. coli* RNA was carried out on resting cells and dividing cells from aerated CCY cultures grown at 37°. Each culture was cooled rapidly to 0–5°, centrifuged in a DeLaval continuous centrifuge at 2–5°, and the cells were washed twice with 20 vol. of 0·145 M-NaCl at 2–5°.

Cell suspensions. Suspensions of viable cells were prepared by suspending 1 vol. of washed cells in 2 vol. of 0·145 M-NaCl. Acid-extracted cells were prepared by suspending the cells in 10 vol. of 5 % (w/v) trichloroacetic acid (TCA) at 0–5°. After standing overnight the cells were centrifuged, washed once with 10 vol. of 5 % TCA and finally with 10 vol. of water. The cells were taken up into 1 vol. of water, and IRA-400 (OH) ion-exchange resin (British Drug Houses Ltd.) was added to remove remaining traces of TCA. Ethanol-extracted cells were prepared by delivering the saline suspension of viable cells into 10 vol. of ethanol at 0–5°. The cells were allowed to sediment from the resulting suspension and resuspended in water or 0·145 M-NaCl.

Extraction of cells. The intact cells, prepared by one of the methods described above, were extracted with at least 10 vol. (wet cell vol.) of extracting agent for 30 min. at room temperature (Table 3). Determinations of RNA carried out upon the suspension and upon the supernatant after centrifuging at 1500 *g* for 15 min. gave a measure of the RNA extracted. Changes in the physical state of the cells and basophilia were also noted.

The solubility of RNA between pH 0·3 and 8 in alkali-treated suspensions of *Esch. coli* was determined in the following way. Approximately 2 g. wet wt. of washed, viable cells was mixed with 50 ml. of water and the suspension treated with 50 ml. of 0·1 N-NaOH. After 5 min. at 20°, 10 N-HCl was added, with vigorous stirring, to pH 8. A sample of 5 ml. was withdrawn and stored at 0°. The pH was brought progressively lower by further additions of 10 N-HCl and, at suitable intervals, further samples were taken. RNA-pentose determinations (described below) were carried out on the sample taken at pH 8 and on the supernatants of all samples obtained by centrifuging at 1000 *g* for 15 min. at 20°.

Ultrasonic disintegrations. These were carried out in flat-bottomed flasks of 150–200 ml. capacity which were prepared with bases of 0·25–0·5 mm. thickness and approximately 6 cm. diam. Water suspensions, prepared by the addition of 9 vol. of water to washed cells, were used in preference to saline suspensions since disintegration was more rapid in this medium. The flask containing the suspension was supported by a retort stand 1–2 cm. above the transducer of a Mullard ultrasonic generator producing vibrations of 2 Mcyc./sec. with an energy output of 500 w. Both the transducer and the bulb of the flask were immersed in an ethanol bath refrigerated to −10°. The position of the flask was frequently adjusted to obtain the maximum turbulence. The temperature of the suspension did not rise above 7° during disintegration. Similar water suspensions were disintegrated by shaking with glass beads (Mickle, 1948) after the addition of octanol to reduce foaming. Periods of continuous disintegration at 2–5° were limited to 10 min., after which the vessels were removed and cooled in iced water. The temperature did not rise above 10° during this disintegration. Disintegrations were continued until less than 0·1 % of whole cells remained; this was normally accomplished in 1–2 hr.

Fractionation of disintegrates by centrifuging. This is summarized in Fig. 1. A volume (30 ml.) of each disintegrate was centrifuged at an average centrifugal force of 1000 *g* for 30 min. at 2–5°. Approximately 25 ml. of the supernatant was centrifuged at an average centrifugal force of 25 000 *g* for 1 hr. at 0° in a Spinco model L centrifuge (no. 40 rotor) and approx. 12·5 ml. of this supernatant was centrifuged at an average centrifugal force of 100 000 *g* for 4 hr. at 0°. Chemical determinations and the assessments of basophilia were carried out on the suspensions of the deposits in 0·145 M-NaCl and on the final supernatant (*S* III). Sedimentation analyses of the disintegrates were carried out in the Spinco model E centrifuge. The sedimentation coefficients were corrected to water at 20° and a partial specific volume of 0·75 ml./g. was assumed for all the components except deoxyribonucleic acid (DNA), for which a value of 0·55 ml./g. was used (Peacocke & Schachman, 1954).

Analysis of phosphorus, nitrogen and magnesium. The methods described by King (1951) were used for the colorimetric determinations of phosphorus and nitrogen. These determinations were carried out in triplicate and the colour intensities were measured on the Hilger Spekker absorptiometer.

Magnesium was determined spectrographically. In order to obtain samples and standards in similar solutions, the protein present in each sample was removed by precipitation with an aqueous solution of TCA and propan-2-ol. Samples (0·1 ml.) containing magnesium (20–400 p.p.m.) and 0·1 ml. of chromium soln. (250 p.p.m.) were delivered into 4·8 ml. of aqueous solution of 5 % (w/v) TCA and 10 % (v/v) propan-2-ol. The mixture was allowed to stand for 5 min. and the precipitated protein removed by centrifuging. A carbon electrode impregnated with the protein-free sample or with a standard solution containing magnesium (1–20 p.p.m.)

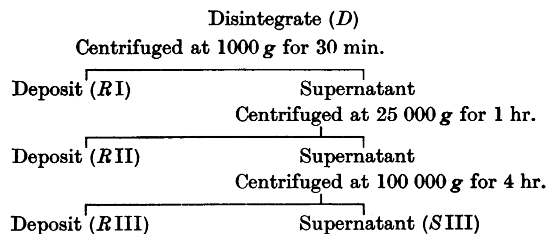

Fig. 1. Fractionation of disintegrates.

and a counter electrode were sparked for 30 sec. with a Hilger condensed-spark set. The spectrum produced by a Hilger large quartz spectrograph was recorded on an Ilford orthochromatic plate and the intensities of the 2803 Å magnesium and 2836 Å chromium lines were measured with a non-recording microphotometer. The determinations were carried out in triplicate; three pairs of electrodes were prepared for each sample and standard. Carefully standardized conditions were used throughout and an accuracy of ±5% was obtained.

Estimation of RNA. The concentration of RNA in bacteria was determined by the reaction of orcinol and $FeCl_3$ with acid hydrolysates of acid-extracted cells (Morse & Carter, 1949), yeast RNA (Light Co. Ltd.) containing 8·7% of phosphorus being used as standard. Samples of the hydrolysate were dried down before their reaction with orcinol in the manner described below.

RNA pentose (acid-insoluble purine-bound pentose) was determined in the following manner. Each sample, containing 5–40 μg. of RNA pentose, was delivered into 3 ml. of 5% (w/v) TCA at room temperature and, after 30 min., was centrifuged and drained free from supernatant. The residue was dried at 60° under negative pressure from a water pump. Samples taken from the TCA extract for the determination of acid-soluble pentose were similarly treated. Chromatographically pure ribose (Light Co. Ltd.) standards were prepared with each set of determinations. A volume (0·5 ml.) of orcinol reagent (Militzer, 1946) was delivered into each tube and the tubes were immersed in a boiling-water bath for 10 min. During this period each tube was shaken briefly to mix the pad of dried material which was hydrolysing. The tubes were then cooled rapidly in cold water and 4·5 ml. of *n*-butanol was delivered into each of them. The determinations were carried out in triplicate, and the colour intensities read on an Hilger Spekker absorptiometer with a red filter (Hilger 608). A standard curve was drawn with each set of determinations and the RNA pentose or acid-soluble pentose determined from it. RNA nitrogen was calculated from the RNA-pentose concentration and the ribonucleotide ratio of *Esch. coli* RNA (RNA nitrogen × 1·52 = RNA pentose).

Nucleotide ratios. The ribonucleotide ratios of RNA in resting cells and dividing cells of *Esch. coli* were determined by the ionophoresis of alkaline hydrolysates. Cells extracted with TCA and ethanol–ether-extracted cells (Schmidt & Thannhauser, 1945) were hydrolysed in 0·3 N-NaOH for 18 hr. at 37° (Crosbie, Smellie & Davidson, 1953) and the ribonucleotides formed were precipitated as their barium salts and prepared for ionophoresis (Wade & Morgan, 1955) by the method described previously for the analysis of adenosine triphosphate preparations (Wade & Morgan, 1954*b*). The separations were conducted for 4 hr., after which period the mixed nucleoside 2′- and 3′-phosphates of cytosine, adenine, guanine and uracil had moved approximately 2·0, 3·3, 5·2 and 7·3 cm. respectively. The nucleotides were detected and determined by a method described previously (Wade & Morgan, 1954*b*).

Dry weight determinations. These were made directly on washed cells dried at 105° for 18 hr. (Wade, 1952).

Basophilia estimates. The basophilia of sedimentation fractions was assessed with the aid of Oxoid membrane filters (Oxo Ltd., London). An area 6 cm. × 3 cm. was supported on six 9 cm. diam. Whatman no. 50 filter papers supported on a Büchner funnel. Suction from a water pump was applied and 2 ml. of 0·33 N-HCl delivered on to the surface of the membrane followed by 2 ml. of water. Volumes of the fractions were then applied to different positions on the membrane; acid-insoluble basophilic constituents of the fractions were precipitated by the residual acidity and retained on its surface. The deposits were washed successively with 2 ml. of 0·33 N-HCl and 2 ml. of water by passing them through the membrane. The latter was removed, soaked in toluidine blue soln. at pH 3·5 (Wade & Morgan, 1954*a*) for 20 min. at 20°, returned to the pile of filter papers and washed thoroughly with water to remove unbound dye.

The development of 'additional RNA' was followed in resting cells of *Esch. coli* inoculated into tryptic-meat broth at 37°. Samples taken at intervals were treated with formaldehyde to a concentration of 1% formaldehyde. The suspensions were centrifuged and the cells washed once with 1% formaldehyde soln. The cells were taken up into a little residual supernatant and applied to a slide. The smears were air-dried, extracted with 0·33 N-HCl for 10 min., washed with water, stained for 15 min. at 45° with toluidine blue soln. (Wade & Morgan, 1954*a*), rinsed with water, air-dried, stained with eosin soln. (Wade & Morgan, 1954*a*) for 50 sec., then rinsed with water and air-dried.

RESULTS

Concentrations of ribonucleic acid in the cells of a number of bacterial species. The RNA concentrations in the resting cells and dividing cells examined are set out in Table 1. Since the method adopted for this determination depends upon the colour formation of only purine-bound pentose, differences between the ribonucleotide compositions of RNA from different species influenced the values obtained and did not therefore permit interspecific comparisons of these values to be made. Evidence presented below, however, suggested that the ribonucleotide compositions of RNA from resting cells and dividing cells are similar, and justified a comparison between the ratios of RNA concentration in the resting and dividing cells of different species. These ratios (Table 1) varied markedly from species to species and did not reflect similar variations in the growth rates of the dividing cells.

Ribonucleotide ratios of RNA in resting cells and dividing cells of Esch. coli. The results of four determinations are set out in Table 2. They revealed no significant differences between the RNA in the two types of cell. This result enabled a rough determination of the ribonucleic acid nitrogen to be calculated from the RNA pentose (RNA nitrogen × 1·52 = RNA pentose) and applied to extracts and fractions of the cell.

Extraction of RNA from intact cells. The concentration of RNA in dividing cells of *Esch. coli* is approximately twice that in resting cells. If the 'additional RNA' in dividing cells is less strongly bound (Belosersky, 1947), and can be extracted from intact cells, this would result in the removal of

Table 1. *RNA concentration in the cells of a number of bacterial species*

Organism (1)	Medium* (2)	Doubling time† (min.) (3)	RNA/dry wt. (%) Resting cells (4)	RNA/dry wt. (%) Dividing cells (5)	RNA ratio (5)/(4) (6)
Aerobacter aerogenes	CCY	30·9	4·4	26·6	6·0
Bacillus anthracis	TMB	48	1·45	24	16·5
Bacillus cereus	TMB	23	3·5	31·5	9·0
Chromobacterium prodigiosum	CCY	28·5	7·8	32·1	4·1
Chromobacterium violaceum	TMB	60	7·2	30·3	4·2
Clostridium welchii	TMB	42·8	32·2	42·2	1·3
Corynebacterium hoffmannii	CCY	128	25·4	51	2·0
Eberthella typhosa	CCY	38·7	11·3	32·4	2·9
Escherichia coli	CCY	27·3	15·6	34·5	2·2
Pasteurella pestis	TMB	116	5·9	14·9	2·5
Proteus vulgaris	TMB	27·3	12·6	35	2·8
Staphylococcus aureus	CCY	30	5·2	10	1·9

* CCY, casein–casein–yeast medium; TMB, tryptic-meat broth.
† Period of twofold increase in optical density during the logarithmic growth of dividing cells.

approximately 50 % of the total RNA from dividing cells without any removal from resting cells. The results of various extractions carried out on viable, acid-extracted and ethanol-extracted cells are summarized in Table 3.

In water, viable dividing cells lysed more rapidly than resting cells. Both types of cell lysed rapidly in mild alkali but, in the presence of NaCl, mild alkali extractions could be carried out without disrupting them. However, by the use of 2 M-NaCl and different degrees of alkalinity conditions could not be found by which RNA was extracted specifically from dividing cells in the quantity expected from Belosersky's theory. Furthermore, the ratio RNA nitrogen/total nitrogen of these extracts approximated to 0·2, which suggested the presence of a large proportion of other nitrogenous substances.

Acid-extracted cells retained their physical state for several months in water at 2–5°. Suspensions in water or saline gave a weak acid reaction with indicators. Upon neutralization with sodium bicarbonate, suspensions both of resting cells and of dividing cells lost RNA to approximately the same extent; small differences in pH about neutrality had a marked influence upon the quantity removed (Table 3).

Ethanol [5–50 % (v/v)] extracted RNA to approximately the same extent from both resting and dividing cells (Table 3). Ethanol-extracted dividing cells lost a greater proportion of RNA when subsequently extracted with water or 0·145 M-NaCl than resting cells. However, when the progress of this extraction was followed over a longer period this difference was found to be only transitory.

In all instances where substantial removal of RNA from dividing cells was effected, the loss of basophilia was also observed (Table 3). In some instances it was possible to demonstrate this on a

Table 2. *Ribonucleotide ratios of RNA relative to adenylic acid from resting cells and dividing cells*

Condition of cells	Molar ratio of ribonucleotides: Cytidylic acid	Adenylic acid	Guanylic acid	Uridylic acid
Resting	1·01	1·0	1·32	0·99
Resting	0·98	1·0	1·36	0·93
Dividing	0·95	1·0	1·31	0·94
Dividing	0·87	1·0	1·36	0·93

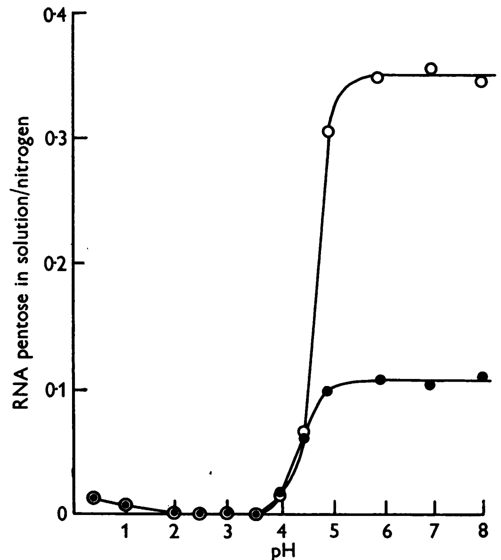

Fig. 2. Solubility of RNA of alkali-treated *Esch. coli*. For details of method used see text. ○, Resting cells (total RNA concn. = 0·165 RNA pentose/N); ●, dividing cells (total RNA concn. = 0·412 RNA pentose/N).

microscope slide. When acid-extracted cells were dried on a slide and treated with 0·145M-NaCl containing sufficient sodium bicarbonate to neutralize the suspension the dividing cells lost their high basophilia and could then be distinguished from resting cells only by their larger size. A similar effect was obtained by treating acid-extracted cells with 1 % (v/v) Tergitol 7 (British Drug Houses Ltd.) at neutral pH.

Solubility of RNA in alkali-treated Esch. coli *cells.* The influence of pH upon the solubility of RNA in alkali-treated suspensions of resting and dividing cells is illustrated in Fig. 2. There was no indication of heterogeneity in the RNA from dividing cells and the RNA in both cells was completely precipitated at pH 2·5–3·5.

Acid-soluble nucleotide content of Esch. coli *cells.* Rough assessments of the ribonucleotides present in resting and dividing cells were obtained from the pentose contents and the optical densities at 260 mμ. of 5 % TCA extracts of these cells. The results, set out in Table 4, suggest that resting cells and dividing

Table 3. *Extraction of RNA from (A) viable, (B) acid-extracted and (C) ethanol-extracted* Esch. coli *cells*

Extracting agent	% RNA extracted		RNA nitrogen*/total N		Removal of basophilia from dividing cells
	Resting cells	Dividing cells	Resting cells	Dividing cells	
(A) Viable cells					
Water	0	Lysis	.	.	.
0·01N-NaOH	Lysis	Lysis	.	.	.
0·01N-NaOH + 0·14M-NaCl	10·0	22·0	.	.	.
0·01N-NaOH + M-NaCl	4·9	21·0	.	.	.
0·01N-NaOH + 2M-NaCl	2·1	20·0	.	.	.
0·1M-$NaHCO_3$/Na_2CO_3 (pH 9·5) + 2M-NaCl	0	7·5	.	0·05	−
0·01N-NaOH + 2M-NaCl	4·5	22·2	0·01	0·19	+
0·05N-NaOH + 2M-NaCl†	5·9	27·0	0·27	0·2	+
10% Tergitol	0	0	.	.	−
1% Sodium tauroglycocholate	0	0	.	.	−
5% (v/v) ethanol	9·1	19·2	.	.	.
10% (v/v) ethanol	7·0	16	.	.	.
25% (v/v) ethanol	5·5	13·5	.	.	.
50% (v/v) ethanol	1	2·9	.	.	.
(B) Acid-extracted cells					
Water	0	0	.	.	−
Water, adjusted to pH 6·5	54·2	72·0	0·6	0·83	+
0·14M-NaCl	0	0	.	.	−
0·14M-NaCl, adjusted to pH 5·5	5·1	18·3	.	.	−
0·14M-NaCl, adjusted to pH 6·5	53·0	56·5	0·92	0·95	+
0·14M-NaCl, adjusted to pH 7·5	87·0	100·0	.	.	+
0·1M-$MgCl_2$, adjusted to pH 6·5	12·0	13·9	0·03	0·067	−
0·01N-NaOH	Lysis	Lysis	.	.	.
0·05N-NaOH + M-NaCl†	69	78	0·31	0·41	+
0·05N-NaOH + 2M-NaCl†	20	28	0·36	0·51	+
1% CTAB, adjusted to pH 6·5	Lysis	Lysis	.	.	.
1% Teepol, adjusted to pH 6·5	.	Lysis	.	.	.
1% Tergitol 7, adjusted to pH 6·5	89	99	0·95	1·05	+
(C) Ethanol-extracted cells					
Water	7·8	47·5	0·05	0·57	+
0·14M-NaCl	23·5	64·5	0·12	0·26	+

* $\frac{\text{Ribonucleic acid nitrogen}}{\text{Total acid-insoluble nitrogen}}$.

† Extracted for 30 sec. and then neutralized.

Table 4. *Concentration of pentose compounds soluble in 5 % trichloroacetic acid and substances absorbing at 260 mμ. in resting and dividing cells of* Esch. coli

Extracted material	Pentose extracted (mg.)/Cell N (mg.)		Optical density at 260 mμ. ($E_{1\,\text{cm.}}^{1\,\text{mg. cell N/ml.}}$)	
	Resting cells	Dividing cells	Resting cells	Dividing cells
Whole cells	0·029	0·022	2·35	2·06
Disintegrates	0·040	0·042	2·56	2·95

Table 5. *Distribution of nitrogen and RNA pentose in resting and dividing cells of* Esch. coli

M, cells disintegrated by the Mickle method; US, cells disintegrated by exposure to ultrasonic vibrations.

Fraction	Nitrogen distribution		RNA-pentose distribution		RNA nitrogen/ total nitrogen (%)	
	M	US	M	US	M	US
Resting cells						
D	100	100	17·0	17·1	11·2	11·2
R I	4·5	1·3	1·0	0·2	14·7	10·1
R II	33·0	18·7	3·8	3·4	7·6	12·0
R III	25·4	28·1	8·3	7·7	21·7	18·7
S III	35·8	46·9	2·9	5·0	5·3	7·0
Dividing cells						
D	100	100	36·2	37·3	23·8	24·6
R I	7·3	1·9	2·0	0·2	18·1	6·9
R II	16·2	11·6	3·5	2·7	14·2	15·4
R III	49·0	42·7	26·6	26·5	35·7	41·2
S III	23·1	36·9	4·0	6·7	11·4	11·9

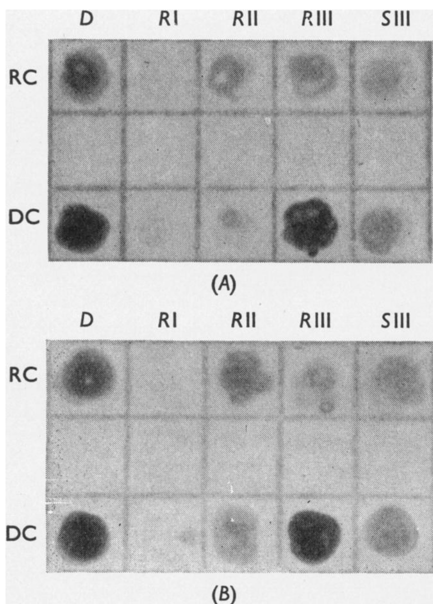

Fig. 3. Qualitative assessment of basophilia in sedimentation fractions of cell disintegrates obtained by (*A*) the Mickle method and (*B*) exposure to ultrasonic vibrations. RC, Resting cells; DC, dividing cells; *D*, unfractionated disintegrate; *R* I, deposit from 1000 *g*; *R* II, deposit from 1000–25 000 *g*; *R* III, deposit from 25 000–100 000 *g*; *S* III, supernatant from 100 000 *g*. The quantities applied were equivalent to approx. 25 μg. of N unfractionated disintegrate. Magnification ×2.

cells do not have widely different concentrations of these constituents.

Fractionation of Esch. coli *disintegrates by centrifuging.* The course of this fractionation is given in Fig. 1. The results of nitrogen and RNA-pentose determinations carried out on samples of *D* and *S* III and also on *R* I, *R* II and *R* III after resuspension in 0·145 M-NaCl are presented in Table 5.

The results showed that the increase in RNA concentration which occurs during the division lag of *Esch. coli* is due to an increase in RNA-containing constituents which sediment at 25 000–100 000 *g* and not to an increase in all RNA-containing constituents. In both disintegrates, one prepared by the Mickle method (M-disintegrate) and the other by exposure of the cells to ultrasonic vibrations (US-disintegrate), the difference between fractions *R* III of resting cells and dividing cells accounted for the difference in the total RNA of the two types of cell.

The analyses (Table 5) also showed that the distribution of cell constituents between sedimentation fractions is influenced by the method used to disrupt the cells. The significantly smaller values for nitrogen and RNA-pentose in fractions *R* I and *R* II and the greater values in fractions *S* III from disintegrates of either resting or dividing cells disrupted by ultrasonic vibrations suggests that a greater fragmentation of the larger particles is brought about by this method.

A qualitative assessment of basophilia showed that the contrasting basophilia of resting cells and dividing cells (Fig. 3 *D*) was due to a highly basophilic constituent of *R* III in dividing cells (Fig. 3, *R* III). In the course of this assessment it was observed that fractions *R* I and *R* II of both resting and dividing cells were metachromatic in reaction, unlike other fractions. This metachromatism, first observed during the drying of the stained fractions on the membrane, was readily demonstrated by the addition of small quantities of the suspended fractions to a weak aqueous solution of toluidine blue.

The magnesium contents of sedimentation fractions from M-disintegrates of resting cells and

dividing cells are set out in Table 6. The total concentration of magnesium in dividing cells was approximately 50 % greater than in resting cells; fraction *R* III of dividing cells was mainly responsible for this difference.

Sedimentation characteristics of resting-cell and dividing-cell disintegrates. An examination of the sedimentation patterns of disintegrates and fractions *S* III revealed marked differences between resting and dividing cells and also between the two methods of disintegration. The sedimentation pattern obtained from resting cell/M-disintegrate [Fig. 4 (*A*) (*a*)] was composed of two main peaks, 3·8 *S* and 10·8 *S*; the sharp nature and the sedimentation rate of the latter suggested it to be deoxyribonucleic acid (DNA). The pattern obtained from dividing cell/M-disintegrate [Fig. 4 (*A*) (*b*)] had three additional peaks, 19·8 *S*, 27·1 *S* and 34·7 *S*, only traces of which could be detected in the disintegrate of resting cells. The pattern from resting cell/US-disintegrate was composed of one major peak, 3·8 *S* [Fig. 4 (*B*) (*a*)]; that from dividing cell/US-disintegrate had two additional peaks, 20·6 *S* and 29·4 *S* [Fig. 4 (*B*) (*b*)] the sedimentation constants of which approximated to two of the peaks of the dividing cell/M-disintegrate. The constituents responsible for the additional peaks in the patterns of dividing-cell disintegrates were not present in the final supernatants *S* III [Fig. 4 (*A*) (*c*); (*B*) (*c*)].

Location of 'additional RNA' in dividing Esch. coli *cells.* The strongly basophilic character of the 'additional RNA' permitted its development in freshly inoculated resting cells to be followed under the microscope. It was observed that this RNA developed evenly throughout the cell during the division lag phase, a period of approximately 40 min., and that subsequently, during the period of maximum growth and division rates when RNA concentration was maximum, there was no marked difference between the concentrations in fully grown and in newly divided cells.

Table 6. *Distribution of magnesium in Mickle disintegrates of* Esch. coli

Fraction	Magnesium (g.)/100 g. of cell N	Mg (μg.)/ mg. of N	Mg (μg.)/ mg. of RNA pentose
Resting cells			
R I	0·14	31·7	151
R II	0·29	9·2	80
R III	0·35	14·2	44
S III	1·1	30·7	380
Dividing cells			
R I	0·38	52	189
R II	0·29	18·8	87
R III	0·82	17·5	31·6
S III	1·34	58·0	335

DISCUSSION

The RNA concentration in micro-organisms is known to vary with the physiological state of the cell, and a number of independent observations on several species of bacteria have suggested a

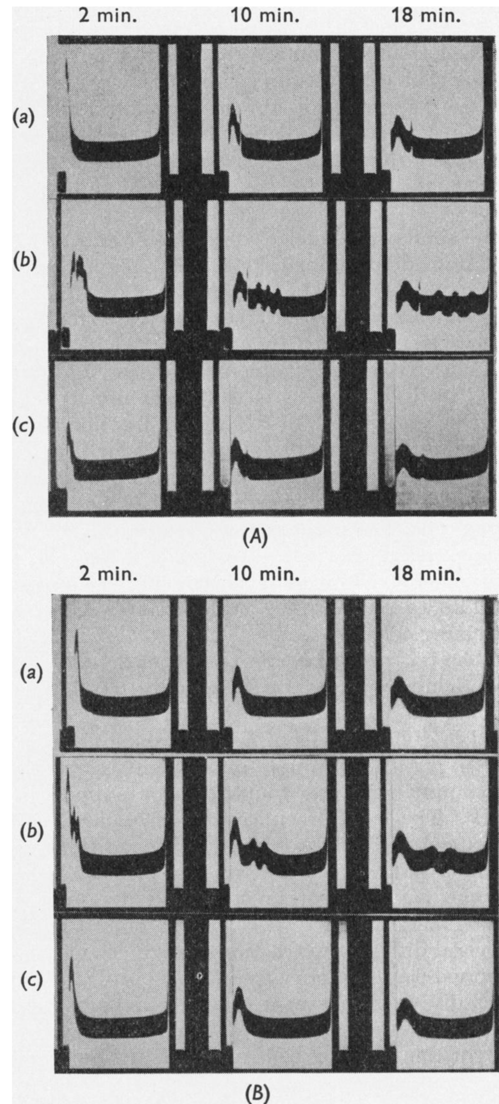

Fig. 4. Sedimentation patterns of disintegrates obtained by (*A*) the Mickle method and (*B*) exposure to ultrasonic vibrations. RC, Resting cells; DC, dividing cells. (*a*) RC disintegrate, (*b*) DC disintegrate, and (*c*) DC supernatant, after centrifuging at 100 000 *g* for 4 hr. (*A*) RC disintegrate, 1·5 mg. of N/ml.; DC disintegrate, 1·41 mg. of N/ml. (*B*) RC disintegrate, 1·31 mg. of N/ml.; DC disintegrate, 1·27 mg. of N/ml. Photographs were taken at 2, 10 and 18 min. after a maximum speed of 59 780 rev./min. had been reached.

correlation between RNA concentration and growth rate. The results set out in Table 1 provide qualitative evidence for this; the fast-growing cells have a higher concentration of RNA than the non-growing cells of the same species. For reasons already given an interspecific comparison of these values cannot be made; however, the evidence obtained from *Esch. coli* that the ribonucleotide compositions of ribonucleic acids from resting and dividing cells are similar (Table 2) justifies a comparison of the ratios of RNA concentration in resting and dividing cells. Such a comparison (Table 1) reveals the absence of a direct quantitative relationship between the changes in the RNA concentration and growth rate.

The similar ribonucleotide compositions of RNA in resting and dividing cells of *Esch. coli* provides no information on the heterogeneity of the RNA in dividing cells. However, the analyses of four sedimentation fractions (Fig. 1, *R*I, *R*II, *R*III and *S*III) have revealed (Table 5) that the 'additional RNA' in dividing cells is the result not of a proportional enrichment of RNA in all fractions but of an enrichment in only one of these, which suggests that the 'additional RNA' does not have a composition widely different from that of RNA present in resting cells. The similar compositions of RNA from sedimentation fractions of resting cells of *Esch. coli* observed by Elson & Chargaff (1955) appears, therefore, to extend to the 'additional RNA' present in dividing cells.

It has not been possible to obtain details of the experiments reported by Belosersky (1947) which are claimed to demonstrate that the 'additional RNA' in dividing cells of *Esch. coli* is not bound to protein, but the results of experiments carried out to examine this possibility do not support this theory. The acid-precipitation of 0·05 N-NaOH-treated cells of this bacterium (the conditions used for an initial 'extraction' by Belosersky) showed that the RNA of both resting and dividing cells behaved alike in this respect. Furthermore, not one of several different extraction techniques (Table 3) examined would remove free RNA specifically from intact dividing cells in a quantity expected from this theory. The possibility that preliminary treatment of the dividing cells analysed by Belosersky resulted in a partial degradation of the RNA is suggested by the analyses of TCA extracts (Table 4), the results of which do not confirm his observation that young cells (dividing cells) have a much higher proportion of mononucleotides than resting cells. However, the possibility was further entertained that free RNA, originally present, combined with protein upon extraction or disintegration of dividing cells. In one experiment the concentration of RNA in a disintegrate of resting cells was doubled by the addition of yeast sodium ribonucleate and the mixture centrifuged at 100 000 *g*. Although 50% of the added RNA was sedimented with the deposit, none of the additional peaks present in the sedimentation patterns of dividing-cell disintegrates (Fig. 4) was formed, suggesting that the RNA-rich macromolecular particles of dividing cells occurred naturally and were not artifacts of naturally free RNA.

Although it was not possible to demonstrate free RNA in cell disintegrates, it was possible to remove RNA from both acid-extracted resting cells and dividing cells practically free from other nitrogenous substances (Table 3). The ability to carry out these extractions on a microscope slide and observe the removal of basophilia under the microscope confirms the identity of the basophilic constituent of these cells (Belosersky, 1947; Tulsane & Vendrely, 1947). Unlike Tulsane & Vendrely (1947) and Onisi & Kato (1955), however, who revealed central basophilic bodies after they had removed the RNA by ribonuclease treatment or 0·145 M-NaCl extraction, no central bodies were revealed by this treatment.

There have been few reports describing the sedimentation patterns of bacterial disintegrates. Schachman, Pardee & Stanier (1952) and Billen & Volkin (1954) have described patterns which resemble those reported here. The dissimilar conditions employed prevent a direct comparison of sedimentation coefficients, but the identity of the particulate fraction (deposited at 160 000 *g* for 1 hr., after centrifuging at 7000 *g* for 10 min.) reported by Schachman *et al.* (1952), with fraction *R*III (Fig. 1) from dividing cells, is suggested by the similar RNA concentrations in these fractions (Table 5).

Considered together, the sedimentation patterns and chemical analyses of dividing cells disintegrated by the two methods described yielded some interesting information on the chemical nature of the fastest-moving component. Disintegration by exposure to ultrasonic vibrations resulted not only in the absence of the DNA peak from the sedimentation patterns, an effect expected from the work of Laland, Overend & Stacey (1952), but also in the gross reduction of the fastest-moving component of the Mickle-disintegrated dividing cells. [Compare Fig. 4 (*A*) (*b*) with Fig. 4 (*B*) (*b*).] The chemical analyses (Table 5), however, revealed no great difference between the RNA in fractions *R*III of the dividing-cell disintegrates obtained by the two methods, and suggested that the fastest-moving component of the Mickle-disintegrated dividing cells contained little if any RNA.

Although chemical analyses suggested a composition of approximately 41% of RNA (Table 5) for the two constituents which sediment at about 20*S* and 28*S*, it is possible that the 'additional RNA' in dividing cells is composed of new constituents, which, in dividing-cell fraction *R*III (US) (Table 5),

are present in addition to those of resting-cell fraction *R*III (US) and that fractions 20*S* and 28*S* have, in fact, an average composition of approximately 85 % of RNA.

The biological significance of the 'additional RNA' in growing micro-organisms is not understood. The low rate of RNA-phosphorus (Cohen, 1948) and RNA-purine (Koch, Putnam & Evans, 1952; Manson, 1953) turnovers reported in phage-infected *Esch. coli* and in normal cultures (Hershey, 1954), has led to the belief that RNA is an inert by-product (Manson, 1953; Pardee, 1954), but such demonstrations of inactivity do not influence the general opinion that RNA is concerned in protein synthesis. The template theory of protein synthesis (Haurowitz, 1949; Dounce, 1952) does not depend upon a turnover of RNA and, as experiments by Hershey (1954) have shown, in growing cells of *Esch. coli* the phosphorus turnover of RNA, although small, is significantly greater than that of DNA.

The view that quantitative differences in RNA concentration are associated with differences in rate of protein synthesis stems chiefly from studies of metazoan cells which have shown that cells actively engaged in protein synthesis are rich in RNA (see reviews by Davidson, 1947; Caspersson, 1947; and Brachet, 1947). In micro-organisms the only positive evidence for this has been obtained from cells growing and dividing exponentially (Caldwell *et al.* 1950; Price, 1952; Wade, 1952; Jeener, 1953), a correlation which had been noted earlier between total nucleotide concentrations and rates of protein synthesis (Malmgren & Heden, 1947). This correlation has not been observed in other phases of growth. During the first generation of growth no correlation was observed between the growth rates and nucleotide concentrations (Malmgren & Heden, 1947) or RNA concentration (Wade, 1952), and the coincidence of the maximum concentration of RNA during this period with the middle of the first generation, has suggested that the varying concentration of RNA is more closely associated with the division process (Wade, 1952), an opinion also expressed by Davidson (1953) and Brachet (1955). The absence of a correlation between RNA concentration and growth rate was also observed during growth subsequent to exponential growth (Jeener, 1952*a*), during the growth stimulation of cells in nutritionally limiting medium (Abrams, Hammarsten, Reichard & Sperber, 1949; Jeener, 1952*b*), and during growth inhibition by cobalt ions (Levy, Skutch & Schade, 1949). Evidence from micro-organisms growing exponentially, however, is unsound, for under these conditions of growth the RNA concentration can be correlated with the rate of any process which, like the rate of protein synthesis, is proportional to the growth rate. Less equivocal evidence is needed before fluctuations in RNA concentration can be attributed to changes in protein synthesis.

It is possible to resolve some of the conflicting views on RNA function by assuming the presence of at least two RNA constituents in living cells, each with a distinct role. It is possible that whereas the concentration of one form ('constitutional RNA') remains constant and is directly concerned with protein synthesis, the concentration of another ('fluctuating RNA') varies directly with the rate of some other process which, during exponential growth, is directly related to the growth rate.

A number of demonstrations that the microsome fraction of the cell has protein-synthesizing ability (Hultin, 1950; Keller, 1951; Siekevitz, 1952; Allfrey, Daly & Mirsky, 1953; Keller, Zamecnik & Loftfield, 1954; Oota & Osawa, 1954; Gale & Folkes, 1955*a*, *b*) suggest that the RNA associated with this fraction is the 'constitutional RNA'. The distribution of RNA pentose in sedimentation fractions of *Esch. coli* (Table 5) is consistent with this theory; the aggregate fraction '*R*I plus *R*II', which contains particles similar in size to microsomes, is present in similar concentrations in both resting and dividing cells. The constancy of a particulate RNA fraction in yeast cells has been reported before by Brachet & Jeener (1943). The dependence of the maximum potential rate of protein synthesis upon this concentration is suggested by the results of Allfrey *et al.* (1953), who compared tissues of widely different metabolic activities and associated differences in microsome concentrations with differences in rates of protein synthesis.

Sedimentation data from *Esch. coli* suggest that, unlike the larger microsome particles of 'constitutional RNA' which are of the order 60–160 mμ. diameter (Davidson, 1953), the 'fluctuating RNA' exists in particles of the order 10 mμ. diameter. This RNA probably corresponds to the volutin-RNA of Belosersky (1947), the '20–40*S*' fraction of Schachman *et al.* (1952) and most of the 'soluble ribonucleoprotein' of Brachet & Jeener (Brachet & Jeener, 1943, 1944; Brachet, 1947). The function of such an abundant cell constituent merits a little speculation. RNA has been found in chromosomes (Frolova, 1944; Kaufmann, McDonald & Gay, 1948; Turchini, 1949), and its role in nuclear division (Brachet, 1947; Kaufmann & Das, 1954; Jacobson & Webb, 1952; Ledoux & Baltus, 1954) and cellular division (Thomas, Rostand & Gregoire, 1946; Wade, 1952) has been postulated. The association of RNA-rich particles with division in *Esch. coli* (Table 5) and in regenerating liver cells (Petermann, Mizen & Hamilton, 1953; Porter, 1954) has also suggested a function in the division process. It is tempting, therefore, to correlate the fact that a high concentration of magnesium is

associated with the 'fluctuating RNA' in fraction *R*III of dividing cells (Table 6) with the fact that a controlled deficiency of this element inhibits division in certain bacteria (Webb, 1949), and to postulate that this RNA is directly concerned with the division process, and that under conditions of magnesium deficiency its normal formation is prevented and the process it governs is inhibited. The dependence of the division process upon the strongly basophilic 'fluctuating RNA' would also account for the influence of several basic compounds which, under certain conditions, inhibit division but not growth, e.g. proflavine (Davies, Hinshelwood & Pryce, 1944), penicillin (Gardner, 1940), streptomycin (Stenderup, 1953), sulphonamide (Tunnicliff, 1939), methyl violet (Ainley-Walker & Murray, 1904), triethylenemelamine and ethyleneimine (Loveless, Speorl & Weissman, 1954).

The cytological observation that 'fluctuating RNA' is as abundant in fully grown cells as it is in newly divided cells suggests that it is not catabolized during the division process. The possibility of it being a source of energy, however, cannot be discredited by this observation since it may function by transferring energy in the manner postulated by Dounce (1952). Another possibility is that it plays a part in DNA synthesis. The failure of most tracer studies (Villee, Lowens, Gordon, Leonard & Rich, 1949; Abrams, 1951; Mitchell & Moyle, 1953; Hershey, 1954) to demonstrate a conversion from RNA into DNA (Brachet, 1947; Mitchell, 1942, 1943; Caldwell & Hinshelwood, 1950; Argell, 1952) could be attributed to the low concentrations of 'fluctuating RNA' present in the systems examined. The little information available at present leaves both possibilities equally acceptable and justifies their further entertainment.

SUMMARY

1. The analyses of resting and dividing bacterial cells have shown that in each of twelve species examined a higher concentration of ribonucleic acid (RNA) is present in dividing cells than in resting cells. A comparison of different species has further shown that the growth rate and the ratio of these concentrations are not related in a simple manner. The nature of the 'additional RNA' in dividing cells of *Escherichia coli* has been studied in greater detail.

2. The ribonucleotide compositions of RNA in resting cells and dividing cells are similar.

3. Attempts to extract the 'additional RNA' specifically from intact dividing cells were unsuccessful.

4. The analyses of sedimentation fractions from cell disintegrates have shown that the 'additional RNA' is present in a fraction (*R*III) which is sedimented at 25 000–100 000 g. The difference between the RNA content of this fraction from dividing cells and the same fraction from resting cells accounts for the difference between the total RNA concentration in these cells.

5. Depending upon the method used for disrupting the cells, fraction *R*III from dividing cells contains two or three prominent constituents with different sedimentation characteristics; only low concentrations of these constituents are present in resting cells.

6. Constituents of fraction *R*III are responsible for the difference in basophilia between resting cells and dividing cells and partly responsible for the higher concentration of magnesium in dividing cells.

7. The possibility of a connexion between the division process and the fluctuations in 'additional RNA' normally observed in batch cultures is discussed in the light of these results and other experimental data.

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The Interconversion of Serine and Glycine: Preparation and Properties of Catalytic Derivatives of Pteroylglutamic Acid

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The catalytic activity of hydrogenated forms of pteroylglutamic acid (PGA; I) in the enzymic synthesis of serine is now well established (Blakley, 1954; Kisliuk & Sakami, 1955; Alexander & Greenberg, 1955). The mechanisms involved are obscure, but a possible mechanism is the enzyme-catalysed combination of hydrogenated PGA with formaldehyde to form a relatively stable intermediate. This intermediate may then react with glycine under the influence of a second enzyme to produce serine and regenerate hydrogenated PGA.

Many attempts have been made in this laboratory to isolate such a pteridine intermediate, since its structure would be the key to the mechanism of enzyme reactions involving PGA derivatives. Furthermore, if sufficiently stable, it might conceivably have pharmaceutical applications. Among difficulties encountered has been the heterogeneity and instability of the pteridines isolated, and this has led to a reinvestigation of the nature and stability of hydrogenated derivatives of PGA. Evidence is presented that most preparations of