capacity of the organism to utilize C_1 fragments, at both the formyl and hydroxymethyl levels of oxidation, for methionine synthesis. The non-utilization of formaldehyde, which normally functions as a C_1 donor through the intermediate formation of hydroxymethyltetrahydropteroylglutamate (Huennekens & Osborn, 1959) is probably due to its inhibitory effect. The comparatively lower synthesis of methionine obtained with *N. crassa* (cholineless), irrespective of the C_1 donor used (Table 2), explains the mutant's observed requirement of methionine for growth in the absence of choline (Horowitz & Beadle, 1943).

Swendseid & Nyc (1958) have shown that *N. crassa* has a relatively high concentration of folic acid derivatives, accounting for the lack of effect of added pteroylglutamate on methionine synthesis by resting cells (Table 3). Pteroylglutamate might not be converted by the cells into the metabolically active forms of folic acid, which are probably tetrahydro derivatives of pteroylglutamate. The inhibition of growth of wild-type *N. crassa* by aminopterin (Swendseid & Nyc, 1958) is reflected in a decreased methionine synthesis in the presence of this folic acid antagonist. The inability of leucovorin to reverse the effect might be due to some unidentified types of folic acid derivatives functioning as intermediates.

The essential similarity of cofactor requirements of the methionine-synthesizing systems in *O. malhamensis* (Dalal *et al.* 1961) and *N. crassa* is demonstrated by the ability of heat-inactivated extracts of *O. malhamensis* to replace the *N. crassa* extract as a cofactor source (Table 5).

SUMMARY

1. Resting cells of both wild-type and the mutant strain no. 34486 of *Neurospora crassa* form methionine from homocysteine. Formate is the most effective of the various C_1 precursors examined.

2. Added pteroylglutamate has no effect on methionine synthesis by resting cells of the wild-type organism. Leucovorin cannot restore the decrease in synthesis caused by aminopterin.

3. The methionine synthesis obtained by cell-free extracts of *Neurospora crassa* (wild) is decreased by Dowex 2 (Cl^- form) treatment. Addition of heat-inactivated cell-free extracts of either *Neurospora crassa* or *Ochromonas malhamensis*, or a cofactor mixture consisting of adenosine triphosphate, diphosphopyridine nucleotide, magnesium sulphate and leucovorin, partially restores the activity.

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Polynucleotide Phosphorylase in Ribosomes from *Escherichia coli*

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The ribonucleic acid in *Escherichia coli* has been shown to break down by two distinct routes (Wade, 1961). In the presence of ethylenediaminetetraacetic acid ribosomal ribonucleic acid breaks down into ribonucleoside 2':3'-cyclic phosphates (V-

route); in the presence of magnesium it breaks down into ribonucleoside 5'-phosphates (M-route). Although the latter process is not dependent on the presence of orthophosphate, its inclusion stimulates the process, suggesting that a polynucleotide

phosphorylase (Grunberg-Manago, Ortiz & Ochoa, 1956) may be partly responsible.

Tracer experiments with radioactive phosphorus were carried out to examine this possibility. The results are reported here.

MATERIALS AND METHODS

Cultural conditions. *Escherichia coli* (N.C.T.C. 1100) was cultured at 37° on a Tryptone-glucose medium (TG 3/W). A concentrate was prepared by mixing solutions A, B and C (Wade, 1961) in the proportions 7:1:2 and, when required, the medium was prepared by diluting the concentrate with 9 vol. of 55 mM-NaCl.

The cells, which were grown by continuous culture in a vessel described previously (Wade, 1961) and had a generation time of about 40 min., were washed in a solution of 0.145 M-NaCl and 5 mM-MgCl₂ at 2–5° and stored at –20°. They were disrupted in a Hughes (1951) press and stored at –20° for less than 4 weeks.

Isolation of ribosomes. A suspension of disrupted cells (7–10% wet wt. in a solution of 0.145 M-NaCl and 5 mM-MgCl₂) at 2–5° was mixed in an homogenizer (Townson and Mercer Ltd., Croydon, Surrey) for three periods of 10 sec., with intermittent cooling. A volume (270 ml.) was centrifuged at 25 000g in a Spinco model L centrifuge (no. 30 rotor) for 1 hr. at 0°. The preliminary homogenizing reduced the amount of nucleic acid deposited (Table 1) and produced a more compact pellet. The deposit was made up to 270 ml. with a solution of 0.145 M-NaCl and 5 mM-MgCl₂ (RI); the supernatant was made up to 270 ml. and centrifuged at 85 000g for 7.5 hr. at 0°.

The supernatant was made up to the original volume (SII); the deposit was made up to 90 ml. with the same salt solution and centrifuged at 85 000g for 7.5 hr. at 0°.

The supernatant was made up to 90 ml. (SIII); the ribosome deposit, about 20–25% of the original disrupted preparation, was made up to 27 ml. with a solution of 0.145 M-NaCl and 2 mM-MgCl₂ (RII) and stored at 0° for less than 7 days.

In addition to the preliminary homogenizing, this fractionation differed from that previously used (Wade & Lovett, 1958; Wade, 1961) by including a third centrifuging in which the ribosomes were washed. By reducing the volume at this point advantage was taken of the greater concentration dependence of the sedimentation rate of DNA compared with that of ribonucleoprotein (Peacocke & Schachman, 1954; Tissières, Watson, Schlessinger & Hollingworth, 1959), to reduce the contamination of the latter with the former. This contamination was of the order 2% in fraction RII (Table 2).

Ribosome autodegradation. The extent of breakdown was measured by comparing the u.v. absorption (260 mμ) of an acid extract (0.1 N-HClO₄) at 0° with a similar extract from an alkali hydrolysate prepared by exposing a sample to 0.3 N-KOH at 37° for 18 hr.

Each acid extract was prepared in duplicate by applying 0.05 ml. to a strip of Whatman filter paper no. 542 and immersing it in 0.1 N-HClO₄ (Wade, 1961).

Ion-exchange fractionation. The acid-soluble products of RNA breakdown were extracted with 0.1 N-HClO₄ and fractionated by pH-gradient elution (Wade, 1960) with columns (1 cm. diam.) containing 20 ml. or 40 ml. of

Dowex 1 X 2 (200–400 mesh; formate) resin. The fractions were eluted with ammonium formate under one of the following conditions: (I) 20 ml. of resin with an eluent of 0.13 M-ammonium formate flowing at 1 ml./min. and adjusted with HCl to pH 6 and to progressively lower pH values at a rate of 2.76 pH units/l. of eluent; (II) 40 ml. of resin with an eluent of 0.06 M-ammonium formate flowing at 1.7 ml./min. and adjusted with HCl to pH 5 and to progressively lower pH values at a rate of 2.44 pH units/l. of eluent.

The extinction of the eluate at 254 mμ was determined and recorded continuously (Lovett & Wright, 1961).

The radioactivity of the eluate was measured continuously by passing it through polyvinyl chloride tubing (60 cm. long, 2 mm. bore and 0.5 mm. wall) wound round a dipping counter tube type B12H (20th Century Electronics Ltd., Croydon, Surrey). This arrangement took advantage of the longer life, the ability to accept high count rates and the lower operating voltage of a halogen-quenched tube. The count rate was recorded on a chart recorder operated from a linear ratemeter type N 522 (Ekco Electronics Ltd., Southend-on-Sea, Essex) and an automatic range-changing device which also protected the counter tube from excessive count rates (Lovett, 1960). The radioactivities of the bulked fractions taken from a fraction collector were measured in a liquid counter type M 6H (20th Century Electronics Ltd.).

Norit adsorption. When further fractionation by paper ionophoresis was necessary, a nucleotide fraction (usually less than 2 μmoles) was passed through 100 mg. of Norit supported by an H3/H5 sintered-glass column (1 cm. diam.). A volume (1 ml.) of a stock suspension containing 10% of Norit SXII (Harrington Brothers Ltd., London, E.C. 1), 10% of Celite and 8% (v/v) of octan-2-ol in ethanol (Threlfall, 1957) was filtered on to the sinter and washed with 10 ml. of water. The nucleotide solution was passed through at about 2 ml./min. followed by 10 ml. of

Table 1. *Effect of homogenizing a suspension of disrupted cells upon the nucleic acid content of RI*

Period of homogenizing (min.)	Nucleic acid sedimented in RI	
	RNA (% of total RNA)	DNA (% of total DNA)
0	22.7	19
0.25	19.5	9.7
0.5	16.9	2.43
1	15.9	2.08

Table 2. *Distribution of nucleic acids and nitrogen between sedimentation fractions*

The concentrations are corrected for the volume changes made during fractionation (Fig. 1).

Sedimentation fraction	Nitrogen (mg./ml.)	RNA (mg. of purine-bound pentose/ml.)	DNA (mg./ml.)
RI	0.470	0.135	0.010
RII	0.630	0.400	0.040
SII	0.510	0.067	0.235
SIII	0.075	0.045	0.066

water. The nucleotide was eluted with 20 ml. of 0.05N- NH_3 solution in 50% (v/v) ethanol and recovered (usually more than 90%) as a solid, by evaporating under reduced pressure from a water pump.

Paper ionophoresis. The reservoir-free system described by Wade & Morgan (1955) was used with the apparatus described by Lovett (1960) and a buffer of pH 3.5, composed of 3.7% (v/v) propionic acid, 0.025N-NaOH and 0.1 mM-EDTA, or of pH 8.4, composed of 0.5M-glycine, 0.025N-NaOH and 0.1 mM-EDTA.

The pH 3.5 system differed from that previously used (Wade, 1961) by including a chelating agent to eliminate streaking of nucleoside polyphosphates. The chelating agent did not affect the mobilities of nucleoside monophosphates. Nucleoside 5'-mono-, 5'-di- and 5'-tri-phosphates (0.1–0.2 μ mole), when applied to the paper as 4 cm. bands and subjected to 400 v for 4 hr., had the following relative mobilities: CMP, 0.14; AMP, 0.34; GMP, 0.74; UMP, 1.00; CDP, 1.00; ADP, 1.07; GDP, 1.39; UDP, 1.77; CTP, 1.62; ATP, 1.62; GTP, 1.72; UTP, 2.16. Each band was about 0.05 unit wide after ionophoresis.

The pH 8.4 system was used to separate three adenosine nucleotides that were liberated during the autodegradation of ribosomes and behaved alike during ion-exchange fractionation and during ionophoresis at pH 3.5.

Autoradiography of the ionophoresis sheets was carried out as described by Wade & Lovett (1958).

Nucleotide analyses. The concentrations of nucleoside 5'-monophosphates (Table 3; Fig. 2a) were calculated from the extinctions at 260 μ (Wade, 1961) of bulked fractions after ion-exchange fractionation (Fig. 1). The concentrations of nucleoside 5'-diphosphates (Table 3; Figs. 2b and 3) produced by incubating ribosomes with inorganic [^{32}P]-orthophosphate, were calculated from the radioactivities of the diphosphates and the specific activity of inorganic orthophosphate. It was shown that inorganic [^{32}P]orthophosphate incorporated only into the terminal phosphate of these nucleotides.

The specific activities of nucleoside 5'-mono- and 5'-diphosphates (Fig. 4) were calculated from the ratio of radioactivity to u.v. absorption (260 μ). Each nucleotide fraction from ion-exchange fractionation was concentrated by Norit adsorption, purified by paper ionophoresis at pH 3.5 and then eluted with 0.1N-HCl.

The spectra of the nucleotide preparations were drawn by a recording spectrophotometer type CF4DE (Optica United Kingdom Ltd., Gateshead-on-Tyne, Co. Durham).

Preparation of ^{32}P -labelled ribonucleic acid. *E. coli* (American type B) was grown in a chemically defined medium containing 2% (w/v) of glucose, 0.01 M-glutamate,

Fig. 1. For legend see following page.

Fig. 1. Ion-exchange fractionation of the breakdown products from RNA (a) in disrupted cells, and (b, c and d) in ribosomes. (a) A dialysed suspension of disrupted cells (2.5 mg. of N/ml.) was incubated (37°) with mm-MgCl_2 , 0.1 M-tris buffer, pH 8.5, and 5 mm-orthophosphate containing $10 \mu\text{C}$ of ^{32}P /ml. at 1.0-15 for 2 hr. Ribosomes (1.2 mg. of N/ml.) were incubated with mm-MgCl_2 , 0.1 M-tris buffer, pH 8.5, $10 \mu\text{C}$ of inorganic [^{32}P]orthophosphate (c) with or (b) without 5 mm-orthophosphate at 1.0-15 for 6 hr. (d) The same ribosome preparation (6 mg. of N/ml.) was stored at -5° for 3 days and then incubated at a concentration of 1.2 mg. of N/ml. under the same conditions as (c).

0.05 M-ammonium sulphate, 4 mm-orthophosphate and 2 mc/l. of inorganic [^{32}P]orthophosphate in a specially designed vessel to be described in a later paper. Cells harvested during the phase of maximum exponential growth rate (45 min. doubling time) were cooled rapidly and washed with a solution of 0.145 M-NaCl and 5 mm- MgCl_2 .

The washed cells (about 10 g. wet wt.) were disrupted in a Hughes (1951) press and the RNA was extracted with phenol (Littauer & Eisenberg, 1959). Satisfactory preparations were also carried out later with a suspension of cells (in water) which had been treated with liquid N_2 (Moses, 1955; Postgate, 1960). The full purification procedure of Littauer & Eisenberg (1959) was not adopted. After the RNA (about 300 mg.) had been washed with ethanol and ether, it was dried in a desiccator over P_2O_5 and, when required, taken up into water (10 ml.). The RNA contained 11.5% of N, 19.6% of purine-bound ribose and gave a specific extinction in water ($E_{1\text{cm}}^{1\%}$) of 0.86 at 260 μ . The examination of this RNA in the analytical centrifuge

(Spinco model E) revealed the presence of two main components $S_{20,w}$ 15.5 and 19.5 and one minor component $S_{20,w}$ 4.2, showing good agreement with the results of Littauer & Eisenberg (1959).

Sources of nucleotides. Nucleoside 5'-mono- and 5'-polyphosphates were purchased from Pabst Laboratories, Milwaukee, Wis., U.S.A. Adenosine 2'(3'):5'-diphosphate was a gift from Dr A. M. Michelson.

RESULTS

Breakdown of endogenous ribonucleic acid in disrupted cells in the presence of inorganic [^{32}P]orthophosphate. Disrupted cells were suspended in an equal volume of 0.145 M-NaCl containing 5 mm- MgCl_2 and dialysed at 0° for 2 days against the same salt solution. The dialysed suspension was then incubated at 37° with mm-MgCl_2 , 0.1 M-tris-HCl buffer at pH 8.5 and 5 mm- Na_2HPO_4 containing

20 μ C of 32 P/ml. The ionic strength was adjusted to 0.15 with NaCl.

After 4 hr., when about 80 % of the RNA had broken down, an acid extract was fractionated on ion-exchange resin under conditions (conditions I) which separate the 5'-mono-, 5'-di- and 5'-tri-phosphates of cytidine, uridine, adenosine and guanosine (Wade, 1960, Fig. 8). The u.v.-absorbing peaks of UMP, AMP and GMP were not associated with any radioactivity; the CMP peak was associated with the most prominent peak of activity, shown later to be entirely due to orthophosphate. The absence of radioactivity from CMP was demonstrated by passing the fraction through Norit, when the radioactivity was not retained. Of several minor u.v.-absorbing peaks, four were radioactive and were identified as CDP, ADP, GDP and UDP, from their spectra and also their behaviour during ion-exchange fractionation and paper ionophoresis at pH 3.5 and 8.4. In addition, the radioactivity of these fractions was reduced to less than 2 % when the extract was exposed to N-HCl at 100° for 10 min., conditions which hydrolyse the terminal phosphate of nucleoside 5'-diphosphates. When the individual radioactive nucleoside 5'-diphosphates were purified by paper ionophoresis and the products of acid hydrolysis subjected to ionophoresis, all the radioactivity was associated with orthophosphate, thus demonstrating that the incorporation of orthophosphate is confined to the terminal phosphate.

Under the ion-exchange conditions II, used for the analysis of nucleoside 5'-mono- and 5'-diphosphates (Tables 3 and 5; Figs. 1-4) another radioactive substance separated from the orthophosphate (Fig. 1a, substance X). It had no associated u.v. absorption of an intensity expected from a nucleotide.

Between 2 hr., when 70 % of the RNA had broken down, and 4 hr. the concentrations of bases and nucleoside 5'-phosphates increased slightly and the concentrations of nucleoside 5'-diphosphates diminished (Table 3); there was no significant change in the concentrations of substance X.

The inclusion of 5 mm-arsenate in the mixture did not affect the rate of breakdown or the extent of breakdown after 2 hr. but the concentrations of bases were slightly lower and the concentrations of nucleoside 5'-phosphates slightly higher than in its absence. The concentrations of CDP, UDP and ADP were markedly reduced; the concentration of GDP was only slightly reduced (Table 3).

Conditions which influence the rate of the M-route autodegradation of ribosomes. The breakdown of ribosomal RNA, like the breakdown of RNA in an unfractionated suspension of disrupted cells (Wade, 1961), was stimulated by orthophosphate (Table 4).

Table 3. *Products of M-route breakdown with inorganic [32 P]phosphate*

A dialysed suspension of disrupted cells (2.5 mg. of N/ml.) was incubated (37°) with mm-MgCl ₂ , 0.1 M-tris buffer, pH 8.5, and 5 mm-orthophosphate containing 20 μ C of 32 P/ml. at I 0.15. A ribosome preparation (1.38 mg. of N/ml.) was incubated with mm-MgCl ₂ , 0.1 M-tris buffer, pH 8.5, and 10 mm-orthophosphate containing 20 μ C of 32 P/ml. at I 0.15.									
	Period of incubation (hr.)	Rate of breakdown (%/hr.)	Bases and nucleoside monophosphates						Extent of orthophosphate incorporation into substance X and nucleoside 5'-diphosphates
			Bases (mm)	CMP (mm)	UMP (mm)	AMP (mm)	GMP (mm)	X (mm)	
Disrupted cells	2	35.5	2.25	1.87	1.29	1.66	2.05	0.076	CDP (mm) 0.047
	4	—	2.77	2.03	1.22	1.87	1.94	0.072	UDP (mm) 0.131
Disrupted cells with arsenate (5 mm)	2	37	1.58	1.82	1.66	1.79	2.20	—	ADP (mm) 0.041
	2	17.6	0.18	0.61	0.72	0.76	1.14	0	GDP (mm) 0.095
Ribosomes	6	—	1.40	1.32	1.57	1.52	2.33	0	0.030
									0.018
									0.057
									0.018
									0.086
									0.115
									0.221
									0.275

Table 4. *Effect of orthophosphate and freezing upon the autodegradation of ribosomes*

Two suspensions (*a* and *b*) of ribosomes (1.2 mg. of N/ml.), undialysed or dialysed at 0° for 2 days against a solution of 0.145 M-NaCl and 5 mM-MgCl₂, were incubated at 37° with mM-MgCl₂, 0.1 M-tris buffer, pH 8.5, *I* 0.15. The rate was measured over the first 2 hr.

Concn. of orthophosphate (mM)	Rate of breakdown of RNA (%/hr.)		
	Undialysed preparations		Dialysed preparation
	(<i>a</i>)	(<i>b</i>)	(<i>a</i>)
0	16.5	12.5	8.5
1	17	12.5	8.5
2	18.5	14.3	8.5
5	21	16.7	10.5
5	—	37*	—
10	26.5	21.0	14.5

* The rate of breakdown after the ribosome preparation had been stored at -5° for 3 days before incubation.

The variation in the rate of the process observed from one preparation to another is indicated by the results of two experiments (*a* and *b*) in Table 4. The rate of breakdown with mM-MgCl₂ and 5 mM-orthophosphate usually falls between 15 and 22 %/hr.

The rate was not greatly influenced by the concentration of magnesium (0.4–2 mM) in the presence of 5–10 mM-orthophosphate, by the period of storage (1–11 days) of the preparation at 0° or by the concentration of ribosomes (0.3–1.2 mg. of N/ml.) during incubation at 37°. There was, however, a tendency for the extent of breakdown to increase from 85 to 95 % as the concentration of ribosomes during incubation was reduced.

Dialysis against a solution of 0.145 M-NaCl and 5 mM-MgCl₂ reduced the rate to about one-half over the first hour's incubation with mM-MgCl₂ and 5 mM-orthophosphate (Table 4).

Arsenate (5 mM) did not affect the rate of the process in the presence of mM-MgCl₂ and 5 mM-orthophosphate. The rate was substantially increased when the ribosome preparation was frozen at -5° for 3 days before being incubated (Table 4). This treatment also caused a marked change in the acid-soluble products (Fig. 1*d*) in a way to be described below.

Autodegradation of ribosomes in the presence of inorganic [³²P]orthophosphate. A similar experiment to the one described above with an unfractionated preparation of disrupted cells, was carried out with ribosomes. The latter were incubated with mM-MgCl₂ and 10 mM-orthophosphate containing 20 μC of ³²P/ml. After incubating for 2 hr. about 45 % of the RNA had broken down; after 6 hr. about 90 % had broken down. As before, the radioactivity in the products was associated with nucleoside 5'-diphosphates; the

extent of incorporation into nucleoside 5'-diphosphates, however, did not diminish towards the end of the process (Table 3).

The specific activities of CDP, UDP, ADP and GDP, produced by incubating ribosomes (1.2 mg. of N/ml.) with mM-MgCl₂ and 5 mM-orthophosphate containing 10 μC of ³²P/ml. for 6 hr., were 380, 367, 352 and 322 counts/min./μm-mole respectively. The close agreement with the specific activity of the orthophosphate present (360 counts/min./μm-mole) suggested the absence of any significant incorporation of other phosphates into this site and enabled the concentration of each nucleoside 5'-diphosphate to be calculated from its radioactivity and the specific activity of orthophosphate.

During incubation of ribosomes with mM-MgCl₂ and 5 mM-orthophosphate, nucleoside monophosphates were formed at a constant rate until about 80 % of the RNA had broken down (Fig. 2*a*); the rate of formation of diphosphate, however, fell off when about 40 % of the RNA had broken down (Fig. 2*b*). The influence of orthophosphate concentration upon the prominence of nucleoside 5'-diphosphates in the products was also examined.

Ribosomes were incubated with mM-MgCl₂ and inorganic [³²P]orthophosphate without added unlabelled orthophosphate and with concentrations of up to 20 mM-orthophosphate. The period of incubation was adjusted (3–9 hr.) to ensure the breakdown of between 80 and 90 % of the RNA. The concentration of each base fraction (Fig. 1) or nucleoside 5'-phosphate was determined from the u.v. absorption of the ion-exchange fraction. In addition to the nucleoside 5'-diphosphates, a radioactive substance Y (Fig. 1*c*) was produced, which was very prominent in the presence of low concentrations of orthophosphate.

Some incorporation of ³²P occurred into nucleoside 5'-diphosphates and substance Y in the absence of added unlabelled orthophosphate (Fig. 1*c*). After incubation for 9 hr., about 0.3–0.6 mM-orthophosphate is normally liberated from the RNA; however, initially, the concentration of orthophosphate is less than 0.1 mM.

As the concentration of unlabelled orthophosphate was increased to 5 mM, the concentrations of CDP, UDP and ADP increased proportionately (Fig. 3). The concentration of substance Y, on the other hand, remained constant at about 1.5 μM (with respect to the incorporated orthophosphate). The concentration of GDP did not change proportionately with the other nucleoside 5'-diphosphates: in the presence of mM-orthophosphate it was the least prominent diphosphate, in the presence of more than 2 mM-orthophosphate it was the most prominent.

The results in Fig. 2 suggested that the formation

Fig. 2. Formation of (a) nucleoside 5'-monophosphates and (b) nucleoside 5'-diphosphates during the auto-degradation of ribosomes with orthophosphate. Ribosomes (1.2 mg. of N/ml.) were incubated (37°) with mM-MgCl₂, 0.1 M-tris buffer, pH 8.5, and 5 mM-orthophosphate containing 10 μ C of ³²P/ml. at *I* 0.15.

of nucleoside 5'-monophosphates was independent of the formation of nucleoside 5'-diphosphates. With 20 mM-orthophosphate, the competition between the two systems for the available RNA became more evenly balanced (Table 5).

Dialysis of the ribosomes against 0.145 M-NaCl and 5 mM-MgCl₂ had no significant influence upon the prominence of nucleoside 5'-diphosphates produced during incubation with 1–2 mM-MgCl₂ in the presence or the absence of 5 mM-orthophosphate.

Arsenate (5 mM) reduced the prominence of nucleoside 5'-diphosphates without altering the rate of the process. When the ribosome preparation was allowed to freeze (–5°) for 3 days before being incubated with mM-MgCl₂ and 5 mM-orthophosphate the rate was greatly increased (Table 4) and a more complicated process of degradation took place (Fig. 1d). Nucleoside 5'-diphosphates were produced but in much smaller quantities.

The relationship between orthophosphate concentration, the rate of breakdown and the prominence of nucleoside 5'-diphosphates in the products

revealed by these experiments could be explained in two ways. The diphosphates could be produced by the phosphorylation of the monophosphates by using energy derived from RNA degradation or directly by the phosphorolysis of the RNA. Evidence of the absence of any equilibrium between the monophosphates and diphosphates of each nucleoside (Table 3, Fig. 2) suggested that the formations of nucleoside 5'-monophosphates and nucleoside 5'-diphosphates were independent, favouring the latter hypothesis. More conclusive evidence was sought by following the fate of ³²P-labelled RNA added to a suspension of ribosomes which had been exhaustively degraded by the *M*-route.

Breakdown of exogenous ³²P-labelled ribonucleic acid. A suspension of ribosomes (1.2 mg. of N/ml.) was incubated for 6 hr. at 37° when the breakdown of endogenous RNA was complete. Sodium ribonucleate, labelled with ³²P, was then added and the changes in specific activity of the nucleoside 5'-mono- and 5'-di-phosphates formed were followed

over a period of 45 min. When this experiment was first carried out, extensive degradation of the sodium ribonucleate into other nucleotides occurred, probably through the chelation of Mg^{2+} ions by the ribonucleate resulting in the stimulation of the *V*-route of breakdown (Wade, 1961). When a concentration of $MgCl_2$ equivalent to the molar concentration of RNA-nucleotide was included, the breakdown was more closely confined to the *M*-route.

After a preliminary fractionation on ion-exchange resin of each acid extract under conditions II, the nucleotides were concentrated on Norit and

further purified by paper ionophoresis at pH 3.5 before the specific activities were determined. As previous experiments had shown that the orthophosphate is incorporated specifically into the terminal phosphate of nucleoside 5'-diphosphates, the specific activities derived (Fig. 4) were those of the phosphate group adjacent to the ribose, the terminal phosphate being unlabelled.

The low specific activity of the orthophosphate during this period (Fig. 4) demonstrated the low phosphatase activity of the ribosome preparation and also suggested that, had any substantial exchange between the orthophosphate and the

Fig. 3. Influence of orthophosphate concentration upon the concentrations of nucleoside 5'-diphosphates in the products of ribosome autodegradation. Ribosomes (1.2 mg. of N/ml.) were incubated (37°) with mM- $MgCl_2$, 0.1M-tris buffer, pH 8.5, with 10 μ C of inorganic $[^{32}P]$ orthophosphate and different concentrations of unlabelled orthophosphate at 1 0.15 for 9 hr. (mM-orthophosphate), 6 hr. (2 mM- and 5 mM-orthophosphate), 4 hr. (10 mM-orthophosphate) or 3 hr. (20 mM-orthophosphate). ●, Rate of RNA breakdown (%/hr.).

Fig. 4. Products of ^{32}P -labelled RNA, degraded by an unlabelled ribosome preparation. Ribosomes (1.2 mg. of N/ml.) were incubated (37°) with mM- $MgCl_2$, 0.1M-tris buffer, pH 8.5, and 10 mM-orthophosphate at 1 0.15 for 6 hr. $MgCl_2$ (9 mM) and ^{32}P -labelled RNA (about 1 mg./ml.) were then added and the products liberated during the following 45 min. examined. ●, Orthophosphate; ▨, nucleoside 5'-phosphates.

Table 5. Influence of orthophosphate upon the rate of formation of nucleoside 5'-monophosphates and nucleoside 5'-diphosphates

Ribosomes (1.3 mg. of N/ml.) were incubated (37°) with mM- $MgCl_2$, 0.1M-tris buffer, pH 8.5, 1 0.15, with 20 μ C of ^{32}P /ml. The rates of formation of nucleotides were measured during the breakdown of 30% of the total RNA.

Orthophosphate concn. (mm)	Rate of formation of nucleotides during the autodegradation (mm/hr.)							
	CMP	UMP	AMP	GMP	CDP	UDP	ADP	GDP
5	0.18	0.22	0.27	0.27	0.016	0.029	0.009	0.04
20	0.35	0.32	0.52	0.52	0.22	0.26	0.12	0.36

terminal phosphate occurred, it would not have affected the specific activities of the nucleoside 5'-diphosphates.

The marked differences between the mono- and di-phosphates (Fig. 4) in fact suggested the absence of any rapid equilibrium between the two and the higher specific activities of the diphosphates strongly suggested that these could not have arisen from the monophosphates by phosphorylation but were more likely derived from the RNA by phosphorylase.

The exceptionally high specific activity of ADP (Fig. 4) was shown later to be due to its contamination with two other adenosine nucleotides during ion-exchange fractionation and ionophoresis at pH 3.5. The three nucleotides were separated by ionophoresis at pH 8.4, when the contaminating nucleotides had mobilities of 0.78 and 1.25 with respect to ADP. The fastest nucleotide behaved like adenosine 2'(3'):5'-diphosphate during ion-exchange fractionation and during paper ionophoresis at pH 3.5 and 8.4. After the autodegradation of ribosomes with inorganic [^{32}P]orthophosphate only ADP, of the three, became radioactive.

Insignificance of contamination from RI

The above experiments demonstrated the presence of two, apparently independent, processes of ribosome autodegradation in the presence of magnesium: one occurs in the absence of orthophosphate and produces nucleoside 5'-phosphates (Fig. 1b); the other occurs together with the first in the presence of orthophosphate and produces nucleoside 5'-diphosphates (Fig. 1c). The question arose whether or not the enzymes responsible are present in the ribosomes or are contaminants from other sedimentation fractions. Since the ribosomes first sedimented are washed by a second centrifuging the most likely contamination was from fraction RI. This was all the more plausible since occasionally a small quantity of turbid material drains from the deposit RI when the supernatant is poured off, and polynucleotide-phosphorylase activity has already been reported in particles from *Ascaris lumbricoides*, which sediment like RI (Entner & Gonzalez, 1959). A previous experiment (Wade, 1961, Fig. 4) had shown that the removal of RI from a suspension of disintegrated cells does not influence the rate at which exogenous RNA is broken down in the presence of 0.15M-orthophosphate, suggesting that a polynucleotide phosphorylase is not present in this fraction. The activity of RI towards the breakdown of ribosomal RNA, however, has not been examined. A sedimentation fractionation was therefore carried out in which the period of the first centrifuging was increased to 3.5 hr., resulting in about one-quarter of the ribosomes sedimenting in fraction RI. The fraction RII

produced in this way was then subjected to auto-degradation with mM-MgCl₂ both in the presence and in the absence of 5 mM-orthophosphate.

The results were similar to those illustrated in Figs. 1b and c, suggesting that the enzymes responsible for the formation of nucleoside 5'-monophosphates and nucleoside 5'-diphosphates are intimately associated with, or a part of, the ribosomes.

DISCUSSION

Earlier experiments (Wade, 1961) showed that ribosomes from *E. coli* are capable of breaking the RNA moiety into ribonucleoside 2':3'-cyclic phosphates in the presence of a chelating agent (*V*-route) and into nucleoside 5'-monophosphates in the presence of Mg²⁺ ions (*M*-route). At that time, the rate of breakdown into nucleoside 5'-monophosphates was observed to be stimulated by orthophosphate but the formation of nucleoside 5'-diphosphates was obscured by the presence of other nucleotides.

The specific labelling of nucleoside 5'-diphosphate during the *M*-route process in the presence of inorganic [^{32}P]orthophosphate suggests that one of the three following schemes operated:

- (1) RNA + orthophosphate → nucleoside 5'-diphosphates → nucleoside 5'-monophosphates + orthophosphate
- (2) RNA → nucleoside 5'-monophosphates. Nucleoside 5'-monophosphates + orthophosphate → nucleoside 5'-diphosphates
- (3) RNA → nucleoside 5'-monophosphates. RNA + orthophosphate → nucleoside 5'-diphosphates.

The fact that breakdown into nucleoside 5'-monophosphates occurs at an appreciable rate even in the absence of orthophosphate (Table 4, Fig. 3) strongly suggests that the formation of monophosphates is independent of the formation of diphosphates. It is unlikely therefore that scheme (1) is correct since the formation of monophosphates would be dependent upon the presence of orthophosphate. It may be argued that only traces of orthophosphate would be required; in this event it is surprising that changes in the concentration of orthophosphate at much higher levels (5–20 mM) should influence the rate of the process.

Scheme (2) is not supported by the experiment with ^{32}P -labelled RNA in which the diphosphates had higher specific activities than the monophosphates at all times (Fig. 4).

The interpretation which best fits the results is summarized in (3). The *M*-route is largely the result of production of nucleoside 5'-phosphates by a phosphodiesterase. In the presence of ortho-

phosphate, however, a polynucleotide phosphorylase contributes to the degradation. One interesting similarity between the latter phosphorylase and the one described by Grunberg-Manago *et al.* (1956) is the marked influence the concentration of orthophosphate has upon the activity of the enzyme towards GDP (Singer, Hilmoie & Grunberg-Manago, 1960). As the concentration of orthophosphate is increased, the prominence of GDP in the product increases relative to the other diphosphates (Fig. 3).

The constant rate of formation of nucleoside 5'-monophosphates during autodegradation in the presence of orthophosphate (Fig. 2a) is consistent with the reactions being confined to the terminal residues and, in this respect, it behaves like the phosphodiesterase from snake venom (Razzell & Khorana, 1959). The earlier deterioration in the rate of formation of nucleoside 5'-diphosphates (Fig. 2b) can be attributed to the equilibrium constant of the phosphorylase being less completely towards the formation of nucleotides (Grunberg-Manago *et al.* 1956).

The identity of substance X (Fig. 1a) has not been pursued. Its absence from the products of ribosomes (Fig. 1b and c) suggests that it is produced by an enzyme that is not present in this sedimentation fraction of the cell.

For reasons stated previously (Wade, 1961) the function of polynucleotide phosphorylase in the ribosome fraction is not clear. One possibility, however, is that its substrates *in vivo* are oligonucleotides with terminal pyrophosphate groups.

SUMMARY

1. The stimulation of ribosome autodegradation into ribonucleoside 5'-phosphates by orthophosphate, has been examined by using inorganic [³²P]phosphate and ³²P-labelled ribonucleic acid.

2. Dialysed preparations of disrupted *Escherichia coli* incorporated inorganic [³²P]orthophosphate into the terminal phosphate of ribonucleoside 5'-diphosphates and also into an unidentified substance X.

3. Arsenate did not alter the rate of degradation of ribonucleic acid but reduced the extent of incorporation into nucleoside 5'-diphosphates and inhibited the formation of substance X.

4. A ribosome preparation, incubated under similar conditions, also produced ribonucleoside 5'-diphosphates with ³²P confined to the terminal phosphates of nucleoside 5'-diphosphates but not substance X. Dialysis reduced the rate of the process by about 50%. Arsenate did not affect the rate but reduced the concentrations of nucleoside 5'-diphosphates produced.

5. An increase in the rate of the process, as the concentration of orthophosphate was increased, was accompanied by a progressive increase in the concentrations of nucleoside 5'-diphosphates in the products. The relative concentrations of cytidine 5'-diphosphate, adenosine 5'-diphosphate and uridine 5'-diphosphate produced in the presence of mM–20 mM-orthophosphate remained about constant; guanosine 5'-diphosphate, however, changed from the least prominent to the most prominent diphosphates as the concentration of orthophosphate was increased from mM to 2 mM.

6. After the exhaustive autodegradation of ribosomes and the addition of ³²P-labelled ribonucleic acid, the specific activities of the nucleoside 5'-diphosphates exceeded those of the nucleoside 5'-monophosphates, suggesting that the nucleoside 5'-diphosphates are produced directly from ribonucleic acid by phosphorolysis.

7. The results suggest that both a polynucleotide phosphodiesterase and a polynucleotide phosphorylase are present in ribosomes from *Escherichia coli* and that the phosphorylase, during the early stages of the process, accounts for 14 or 36% of the total activity when 5 mM- or 20 mM-orthophosphate respectively is present.

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