The Autodegradation of Ribonucleoprotein in *Escherichia coli*

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The ribonucleic acid fractions responsible for a wide fluctuation in concentration of ribonucleic acid in *Escherichia coli* have been shown to be confined to one sedimentation fraction and called, for convenience, 'fluctuating ribonucleic acid' (Wade & Morgan, 1957). When efforts were made to separate the ribonucleoprotein from other cell constituents, difficulties arose from the lability of the ribonucleic acid. Even under very mild conditions of dialysis and storage, the ribonucleic acid quickly became degraded into acid-soluble substances apparently by the action of one or more heat-labile enzymes.

Ribonucleic acid depolymerases have been demonstrated in *Escherichia coli* but only their behaviour towards free ribonucleic acid has been fully examined (Brummond, Staehelin & Ochoa, 1957; Speigelman, 1958; Elson, 1959). The present study has been directed more towards the behaviour of endogenous ribonucleic acid. The results, it was hoped, would provide not only information which could be put to immediate practical use by suggesting methods of keeping preparations of ribonucleoprotein stable but also information on the structure of undegraded ribonucleic acid and perhaps the process of its synthesis *in vivo*.

MATERIALS AND METHODS

Cultural conditions. *Escherichia coli* (N.C.T.C. 1100) was cultured at 37° on a medium (TG3/W) prepared in the following way.

A concentrate was prepared by mixing three solutions (A, B and C) together. Soln. A: 2.8 kg. of Tryptone (Oxo Ltd., London) was mixed with 1.05 l. of 2M-K₂HPO₄ and made up to 19.6 l. with water. The solution was adjusted to pH 9.3 with NaOH, brought to 100° for 5 min. and then filtered (Green's paper no. 904). Citric acid (112 g.) was added, the pH was adjusted to 7.6 with HCl and the volume readjusted with water to 19.6 l. The solution was sterilized in volumes of 2.8 l. with steam at 10 lb./in.² for 30 min. Soln. B: 406 g. of NaCl, 140 ml. of m-ferric citrate, 14 ml. of m-CaCl₂ and 560 ml. of m-MgSO₄ were made up to 2.8 l. with water. Volumes of 750 ml. were sterilized with steam at 10 lb./in.² for 20 min. Soln. C: 2.8 kg. of glucose was made to 5.6 l. with water and the solution sterilized in volumes of 1 l. with steam at 10 lb./in.² for 10 min. The concentrate was prepared by mixing 2.8 l. of soln. A, 750 ml. of soln. B and 1 l. of soln. C together and was stored at 2–5°.

For the preparation of resting cells, the concentrate was

diluted with 4 vol. of water; for the preparation of dividing cells it was diluted with 9 vol. of 55 mM-NaCl.

The culture vessel consisted of a length (30.5 or 91.5 cm.) of Quickfit pipeline (Quickfit Visible Flow Ltd., Stoke-on-Trent, England), 15 cm. diam., supported vertically from beneath by a stainless-steel plate (1.5 cm. thick) through which services for temperature control, aeration, stirring and drainage were provided. The top of the vessel was sealed by a second plate (6 mm. thick) in which were incorporated an opening for effluent air, pH electrodes and an antifoam spray unit. The temperature control was provided by an electronic thermostat, type TcFl (Fielden Electronics Ltd., Manchester, England), operating alternately two solenoid valves, type RBG (Teddington Industrial Equipment Ltd., Sunbury-on-Thames, England), which allowed either hot water (or steam) or cold water to pass through a stainless-steel finger protruding into the vessel.

Air passed through the culture (1 vol. of air/vol. of medium/min.) was dispersed by the combined actions of a bottom-drive impeller (14 000 rev./min.) and a static system of stainless-steel vanes fixed to the bottom of a down-draught tube (10 cm. diam.), the length of which could be adjusted to suit the length of pipeline. A filtered solution of Antifoam A (Midland Silicones Ltd., London) in carbon tetrachloride (20% soln.) was sprayed on to the surface of the culture at regular intervals to combat the extensive foaming which would otherwise occur. Both influent air and, after condensation of the moisture, the effluent air were passed through cotton-wool filters. The whole system was sterilized by passing moist NH₃ through it for 18 hr. The pH of the culture was adjusted to 7.5 when necessary by passing the influent air through aq. NH₃ soln. (sp.gr. 0.88).

Resting cells were taken from a culture in which no further increase in extinction could be detected. The cell density was about 10 mg. dry wt./ml. Dividing cells were prepared continuously by displacing the culture with fresh medium, at the rate of 1 vol. of medium/vol. of culture/hr., corresponding to a doubling time of about 40 min. The cell density of the reaped culture was about 2.5–3.0 mg. dry wt./ml.

The harvested culture was immediately cooled to 0–2°. The cells were sedimented continuously in a De Laval centrifuge at 2–5° and washed twice by centrifuging in 20 vol. of a solution containing 0.145M-NaCl and 5 mM-MgCl₂. The cells were recovered as a thick paste from the final centrifuging and stored at –20°.

Disintegration and fractionation by sedimentation. The frozen cells were disrupted by the method of Hughes (1951) as described by Wade & Lovett (1958). The fractionation was carried out on a suspension of the disrupted cells (10% wet wt. suspension in 0.145M-NaCl) in a Spinco model L centrifuge (no. 30 rotor) at the average centrifugal forces indicated in Fig. 1. The fractions obtained were made up to

the original volume by the addition of a solution containing 0.145 M-NaCl and 5 mm-MgCl₂.

Dialysis. Dialysis was carried out in cellophan tubing (3 cm. flat width) against at least 100 vol. of salt solution at 2°. For each millilitre of suspension, 3 cm. length of tubing was used.

Buffers. The acetate-veronal buffers of Roth (1954) were adjusted to 7.0-7.5 and used to examine the effect of pH; on other occasions during the examination of endogenous ribonucleic acid (RNA), 2-amino-2-hydroxymethylpropane-1,3-diol (tris)-HCl buffer was used.

Degradation of ribonucleic acid. Method 1. In preliminary experiments the progress of RNA breakdown was followed by determining the conversion of RNA pentose (purine-bound pentose) into an acid-soluble state, by means of the orcinol reaction. Measurements on the acid extract were not suitable because nucleosidases, under certain conditions, liberated pyrimidine-bound ribose. It was necessary to rely instead upon measurements of acid-insoluble pentose (Wade & Morgan, 1957).

Method 2. In later experiments, in which less precision was required, the increase in absorption at 260 m μ of an acid extract was taken as a measure of RNA breakdown. The following factors were considered about possible interference from deoxyribonucleic acid (DNA). The ratio RNA:DNA in dividing cells is about 8.5:1. At pH 7.5 10-50% of the DNA breaks down in the absence of ethylenediaminetetra-acetic acid (EDTA) in 100 min.; in the presence of EDTA no breakdown of DNA into acid-soluble substances occurred. The total concentration of RNA was deduced from the extinction of acid-soluble products from an alkali hydrolysate (0.3 N-KOH at 37° for 18 hr.) with a molecular extinction coefficient [$\epsilon(P)$] of 10 700, calculated from the mean of four determinations of base ratios (Wade & Morgan, 1957).

To overcome difficulties which arise from the turbidity of acid extracts obtained by centrifuging, the samples (0.05 or 0.1 ml.) from the reaction mixture were absorbed by Whatman no. 542 filter paper (about 10 cm. from a roll of 1 cm. width for each sample). The paper was then immersed in 8 ml. of 1% perchloric acid kept at room temperature. The test tube was inverted three times during a period of 1 hr. and, after 1-4 hr., the extinction values of clear acid extracts were measured in 1 cm. cells of the Unicam SP. 500 spectrophotometer (Cambridge Instruments Ltd., Cambridge, England). When the influence of pH upon depolymerase activity was examined the determinations were carried out on either single or duplicate samples.

Degradation of deoxyribonucleic acid. The concentration of DNA was determined by precipitation with 5% trichloro-

acetic acid, washing with 5% trichloroacetic acid and hydrolysing for 15 min. at 95° in 5% trichloroacetic acid. The hydrolysate was filtered through a sintered-glass (H 3/H 5) filter (12 cm. length, 1 cm. diam.; Baird and Tatlock Ltd., London) and then treated with diphenylamine reagent (Morse & Carter, 1949). An acid hydrolysate of thymus DNA was used as standard.

The determination of acid-soluble products of DNA breakdown (Figs. 11, 14) was carried out directly on an acid extract.

Nitrogen and phosphorus determinations. The methods described previously were used (Wade & Morgan, 1957).

Ion-exchange fractionation. The acid-soluble products of RNA degradation were prepared in the following way. Each incubated mixture was cooled to 2°, treated with 1% perchloric acid and centrifuged. The supernatant was brought to pH 6 with 5 N-KOH, saturated with chloroform and stored at 2°.

A volume (1-5 ml.), with a u.v.-absorption equivalent to 10 μ moles of RNA nucleotide, was made up to 20 ml. with 0.01 M-sodium formate, delivered on to a column (1 cm. diam.) of 20 ml. of Dowex-1 (X 2) 200-400 formate resin and eluted (0.6 ml./min.) by pH-gradient elution (Wade, 1960) with 0.04 M-ammonium formate adjusted with HCl to pH 4.5 and to progressively lower pH values at the rate of 3.5 pH units/l. of eluent. After 900 ml. had passed through, the eluent was changed to N-HCl. A mixture of ribonucleoside 5'-phosphates and ribonucleoside 2':3'-cyclic phosphates was satisfactorily resolved by this system. The same system resolved the ribonucleoside 2':3'-cyclic phosphates of uridine, adenosine and guanosine from their 3'-isomers.

Other fractionations were also used for the identification of the products (Wade, 1960).

In the analyses set out in Tables 1 and 2, the molarity of each constituent was determined by pooling the individual fractions from the fraction collector and determining the extinction at 260 m μ of a sample at pH 2. The millimolar extinction coefficients [$10^{-3}\epsilon(P)$] of cytidine, uridine, adenosine and guanosine nucleotides were taken as 6.2, 9.9, 14.6 and 11.6 respectively. Bases and nucleosides were eluted in the first 100 ml. of eluate; since they were not identified, they are described in the text as bases. An approximate measure of their concentration was obtained assuming the value of 10.7 for the average millimolar extinction coefficient.

Ionophoresis and chromatography. The neutralized acid extracts prepared for ion-exchange fractionation were concentrated 10-20-fold for examination by paper ionophoresis (Wade & Morgan, 1955) and chromatography.

Ionophoresis for part of this study was carried out with the wired sheets of filter paper as originally described (Wade & Morgan, 1954) and later with a more convenient apparatus which used the same principles and produced similar results (Lovett, 1960). The buffer (pH 3.5) consisted of 37 ml. of propionic acid and 5 ml. of 5 N-NaOH made up to 1 l. with water. A voltage of 400-600 v was applied over a length of 33 cm. for 4-7 hr. A tracing of the separation of mononucleotides is given in Fig. 2. The mobilities referred to in the text are relative to uridine 5'-phosphate.

Two solvents were used for ascending chromatography on sheets (28 cm. $\times$ 38 cm.) of Whatman filter paper no. 3 washed with N-formic acid (Wade & Morgan, 1954).

Fig. 1. Summary of fractionation procedure.

Solvent 1: 70 vol. of propan-2-ol, 5 vol. of aq. NH_3 soln. (sp.gr. 0.88) and 25 vol. of water (Brown, Magrath & Todd, 1952); solvent 2: 79 vol. of sat. $(\text{NH}_4)_2\text{SO}_4$, 2 vol. of propan-2-ol and 19 vol. of sodium phosphate soln. $[\text{NaH}_2\text{PO}_4$ (14 g./l.)– Na_2HPO_4 (1.4 g./l.)] (Markham & Smith, 1951). Since salt in the neutralized acid extract influenced the results with solvent 1, it was necessary to include about 5% of NaCl in the standards.

In both ionophoresis and chromatography each mixture was applied as a 4 cm. band which either butted adjacent bands of standard nucleotides or overlapped by 0.5 cm. Nucleotides were located under u.v. light from an Hanovia Chromatolite (Hanovia Ltd., Slough, England). Periodate-sensitive substances were detected by the method of Gordon, Thornburg & Werum (1956).

Sources of nucleotide monophosphates and ribonucleic acid. Ribonucleoside 5'-phosphates were purchases from Pabst Laboratories (Milwaukee, Wis., U.S.A.). Nucleoside 2':3'-cyclic phosphates (Smith, Moffatt & Khorana, 1958)

and yeast RNA (Holden & Pirie, 1955) were kindly prepared by Mr S. Lovett. Ribonucleoside 3'-phosphates were prepared by an ion-exchange fractionation of an alkali hydrolysate of RNA (Wade, 1960, Fig. 6A).

Abbreviations. Abbreviations applied to these mononucleotides are those used by Wade (1960).

RESULTS

Factors which influence the rate of breakdown of ribonucleic acid in disrupted cells

Intact and disrupted cells. Preliminary experiments were carried out in the absence of buffer. The bacteria were washed and suspended in 0.145 M-NaCl (pH 6.0–6.5) and the breakdown was followed by determining the fall in acid-insoluble pentose. The process was more complete in dividing cells than in resting cells (Fig. 3). The difference could be attributed to the extensive lysis of the former and could be eliminated by disrupting the resting cells (Fig. 3).

Distribution of depolymerase activity among sedimentation fractions. Disrupted dividing cells were suspended in 0.145 M-NaCl (1.85 mg. of N/ml.) and fractionated (Fig. 1). The concentrations of nitrogen in *D*, *SI*, *RII* and *SII* were 1.85, 1.57,

Fig. 2. Ionophoresis of mononucleotides (0.1–0.2 μ mole) at pH 3.5 at 600 v for 4 hr. *A*, Ribonucleoside 5'-phosphates; *B*, ribonucleoside 2'-phosphates and 3'-phosphates; *C*, ribonucleoside 2':3'-cyclic phosphates. For definitions of abbreviations see Table 1 (p. 468). ---, Starting line.

Fig. 3. Breakdown of RNA in dividing cells and resting cells suspended in 0.145 M-NaCl (2 mg. of N/ml.) and incubated at 37°. ●, Dividing cells; ○, disrupted dividing cells; ▲, resting cells; △, disrupted resting cells; ■, disrupted dividing cells dialysed for 2 days against 0.145 M-NaCl.

0.926 and 0.562 mg. of N/ml. respectively and the concentrations of RNA pentose were 0.80, 0.64, 0.59 and 0.05 mg. of RNA pentose/ml. respectively. Equal volumes of a solution of 5% yeast RNA (in 0.15M-sodium phosphate, pH 7.5) and each sedimentation fraction were incubated at 37° and changes in the concentration of acid-insoluble pentose were determined.

Most of the depolymerase activity of the preparation was present with the bulk of the RNA in fraction *R*II (Fig. 4). When account was taken of the relative concentrations of these fractions in the cell, the rates of breakdown in *R*II and *S*I were found to be similar. Since *S*I is composed of *R*II and *S*II, this suggested that the constituents of *S*II do not exert much influence upon the process. Under other circumstances, however, the rates of breakdown of endogenous RNA at pH 6.0-6.5 in *R*II and *S*I were very different, thus indicating the presence of some inhibitor in *S*II (Fig. 5). This was also suggested by a marked increase in the rate of

Fig. 4. Breakdown of yeast RNA by fractions of dividing cells obtained by centrifuging (see Fig. 1). Each fraction, at a concentration similar to that in the original suspension of disrupted cells (1.85 mg. of N/ml.), was incubated with 25 mg. of yeast RNA/ml. and 0.15M-sodium orthophosphate at pH 7.5. ▲, *D*; □, *S*I; ●, *R*II; ○, *S*II.

Fig. 5. Inhibition of endogenous RNA breakdown in *R*II by constituents of *S*II (see Fig. 1). *R*II (●) and *S*I (□) were incubated at 37° in 0.145M-NaCl at pH 6.0-6.5 at concentrations similar to those in a suspension of disrupted cells containing 1.85 mg. of N/ml.

Fig. 6. Breakdown of endogenous RNA in disrupted dividing cells suspended in 0.145M-NaCl (2.5 mg. of N/ml.) dialysed against 0.145M-NaCl in the absence of $MgCl_2$, before (□) or after (●) heating at 100° for 5 min. or in the presence of 0.02 mM- $MgCl_2$ (■), 0.1 mM- $MgCl_2$ (△), 0.5 mM- $MgCl_2$ (▲) or 2 mM- $MgCl_2$ (○).

Fig. 7. Influence of pH upon the breakdown of RNA in disrupted dividing cells at 37°. (a) Suspended in 0.145 M-NaCl; (b) to (f) dialysed for 2 days before incubation; (b) against 0.145 M-NaCl and (c) to (f) against 0.145 M-NaCl containing 5 mM-MgCl₂. Each suspension (2 mg. of N/ml.) was then incubated at 37°. (a) and (b) were incubated without the addition of magnesium; (c) to (f) were incubated in the presence of mM-MgCl₂ together with (d) 10 mM-sodium orthophosphate, (e) 40 mM-sodium ethylenediaminetetra-acetate and (f) 10 mM-sodium oxalate.

breakdown of endogenous RNA in an unfractionated suspension of disrupted cells brought about by dialysis against 0.145M-NaCl (Fig. 3)

Inhibition of endogenous breakdown of ribonucleic acid by magnesium. The high concentration of magnesium in the ribonucleoprotein fraction of the cell (Wade & Morgan, 1957) and evidence that low concentrations cause disaggregation (Bowen, Dagley & Sykes, 1959; Tissières, Watson, Schlessinger & Hollingworth, 1959) suggested that magnesium may also contribute to the chemical stability of endogenous RNA.

Dividing cells were disrupted, taken up into 0.145M-NaCl (10% wet wt.) and dialysed against 0.145M-NaCl containing different concentrations of magnesium (0.2–2 mM). The dialysed suspension was then incubated (37°) at pH 6.0–6.5 in the absence of buffer. Determinations of acid-insoluble pentose showed that 2 mM-MgCl₂ protects the endogenous RNA against heat-labile enzymes (Fig. 6). From the data of Wade & Morgan (1957) it would appear that in this suspension the original concentration of endogenous magnesium was about 2.8 mM. The fact that this concentration is of the same order as the protecting concentration suggests that the natural inhibitor is magnesium.

Influence of pH. Its effect upon the breakdown of RNA in disrupted cells suspended in 0.145M-NaCl can be seen in Fig. 7 (a). Removal of Mg²⁺ ions by dialysing for 2 days against 0.145M-NaCl at 2° increased the rate of the process above pH 5 (Fig. 7b).

After dialysis in the presence of 5 mM-MgCl₂ the RNA became generally more stable at 37° in the presence of mM-MgCl₂, particularly between pH 5.5 and pH 6.5 (Fig. 7c). In fact, some acid-soluble ultraviolet-absorbing substances became acid-insoluble during the first 20 min. of incubation. Orthophosphate (0.01M) stimulated the process above pH 7 (Fig. 7d); arsenate (5 mM), in the presence or absence of the latter, did not alter the rate or the pattern of breakdown.

When sufficient EDTA was added to bind both the free Mg²⁺ ions and also magnesium contributed by non-diffusible constituents of the cell there was a prominent peak of activity at pH 7 (Fig. 7e).

These results suggested that the endogenous RNA could break down by more than one process and was generally more susceptible to these processes at pH 7.5.

Influence of some naturally occurring acids upon the process. Since the dialysis in the presence of 5 mM-MgCl₂ endowed the RNA with stability between pH 5.5 and 6.6 (Fig. 7c) which it did not previously possess, certain naturally occurring substances were added for the incubation after dialysis. Amino acids, applied individually (0.05M) or as a complete mixture, or fumaric acid, malic acid,

malonic acid, succinic acid or tartaric acid (0.01M) did not stimulate the process in this region. Oxalic acid, however, increased the rate over the whole range examined. The pattern (Fig. 7f) resembled the combined patterns in Figs. 7(c) and (e) and may be the result of an affinity of oxalic acid for magnesium which is intermediate between those of the other dibasic acids examined, on the one hand, and of EDTA on the other.

Influence of magnesium during incubation at 37°. In one experiment which had already demonstrated the influence of magnesium upon the rate of RNA breakdown (Fig. 6), both dialysis and incubation were carried out at similar concentrations of magnesium. Later experiments suggested, however, that, after dialysis in the absence of magnesium, the presence of magnesium during incubation did not afford the same degree of protection to the RNA. The effect of different concentrations of magnesium (0.5–10 mM) during incubation was

Fig. 8. Influence of dialysis (2 days) against 0.145M-NaCl, (a) in the presence or (b) in the absence of 2.5 mM-MgCl₂, upon the breakdown of endogenous RNA in disrupted dividing cells. The dialysed suspensions (1.5 mg. of N/ml.) were incubated at 37° at I 0.15 in the presence of 0.5–10 mM-MgCl₂ and 0.1M-tris buffer, pH 7.5.

therefore examined with two dialysed preparations of disrupted cells: one (a) dialysed in the presence of magnesium and the other (b) in its absence.

The result (Fig. 8) showed that in both (a) and (b) the rate of breakdown decreased as the concentration of magnesium increased; however, the character of the progress curves differed. In (a),

with 0.5 mM-Mg²⁺ and mM-Mg²⁺ ion, the rate increased with time (Fig. 8a); in (b), a much faster rate of breakdown occurred at these concentrations and, for a short time, at the higher concentrations also (Fig. 8b).

These differences suggested that more than one process was involved and the permanent changes

Fig. 9. Influence of pH upon the breakdown of RNA during the incubation (37°) of disrupted dividing cells (i) dialysed for (a) 2 days or (f) 9 days against 0.145 M-NaCl containing 5 mM-MgCl₂, or (ii) dialysed for (b) 1 day, (c) 2 days, (d) 4 days or (e) 7 days against 0.145 M-NaCl followed by 2 days' dialysis in the presence of 5 mM-MgCl₂. The dialysed suspensions were incubated at 1.0-15 in the presence of mM-MgCl₂ and 10 mM-sodium orthophosphate. Concentrations of cell nitrogen in the incubated mixtures (a)-(f) were 1.54, 1.26, 0.96, 0.85, 0.62 and 1.32 mg. of N/ml. respectively. Ratios of RNA pentose/cell nitrogen (w/w) were 0.46, 0.58, 0.62, 0.70, 0.60 and 0.43 respectively.

caused by dialysis against 0.145M-NaCl were therefore examined more thoroughly.

Omission of magnesium during dialysis. Disrupted dividing cells were taken up into a solution containing 0.145M-NaCl and 5 mM-MgCl₂ (50% wet wt.) and dialysed against 0.145M-NaCl for different periods of time (1-7 days) and then for 2 days against a solution of 0.145M-NaCl and 5 mM-MgCl₂. Each dialysed preparation was incubated at *I* 0.15 with 3 vol. of buffer (pH 4.0-8.5) and 1 vol. of 0.05M-Na₂HPO₄ adjusted with HCl.

Dialysis against 0.145M-NaCl for only 1 day resulted in a significant loss in stability which previously existed at pH 6 (Figs. 9*a*, *b*) and which was otherwise maintained for at least 9 days (Fig. 9*f*). More prolonged dialysis against 0.145M-NaCl (Figs. 9*c-e*) resulted in a progressive increase in acid-soluble non-diffusible products, a greater loss in stability and a change in pattern of breakdown towards that produced by EDTA (Fig. 7*e*).

A similar pattern of breakdown to that in Fig. 9(*e*) was produced by dialysing for 2 days against 0.145M-NaCl and storing for 7 days at 2°, suggesting that the changes occurring after more than 2 days' dialysis were independent of the further loss of diffusible substances.

Ionic strength. The experiments described above (Figs. 6, 7*b-d*) suggested that, by maintaining the concentration of magnesium above 2 mM during dialysis, the stability of the endogenous RNA at pH 6 (mM-Mg²⁺ ion) was ensured. A concentration of magnesium above 2 mM also avoided an acceleration in the rate of the process at pH 7.5 (Figs. 8*a* and 12). A similar concentration was also critical to the end products in a way to be described later.

All these experiments were carried out at *I* 0.15. When, after dialysis at *I* 0.15 in the presence of 2-5 mM-Mg²⁺ ion, the suspension of disrupted cells was incubated with mM-Mg²⁺ ion at *I* 0.2, the stability at pH 6 was decreased (Fig. 10*a*) and was

Fig. 10. Influence of pH upon the breakdown of RNA in disrupted dividing cells dialysed for 2 days against 0.145M-NaCl with 5 mM-MgCl₂ and incubated at 37° (2 mg. of N/ml.) in the presence of mM-MgCl₂ and 10 mM-sodium orthophosphate with NaCl added to produce ionic strengths of (a) 0.2, (b) 0.3 or (c) 0.5. (d) Progressive inhibition at *I* 0.5 (after 40 min.) by increasing concentrations of MgCl₂.

further decreased at I 0.3 and 0.5 (Figs. 10*b*, *c*). At I 0.5, stability could be restored by increasing the concentration of magnesium to 5 mM (Fig. 10*d*). The acceleration in the rate of the process, which was previously avoided at I 0.15 by increasing the concentration of magnesium from 1 to 2 mM (Figs. 8*a* and 12), was also avoided by decreasing I to 0.1 (Fig. 13).

The critical concentration of magnesium (2 mM) which governs the stability of endogenous RNA at pH 6 and the character of the process at pH 7.5 is thus dependent upon ionic strength. At a given ionic strength the concentration of Mg^{2+} ions, relative to others, is more important than the absolute concentration.

*Products of breakdown of ribonucleic acid
in disrupted cells*

A suspension of disrupted dividing cells was taken up into 0.145M-NaCl (10% wet wt. suspension) and incubated at 37° with 4 vol. of 0.02M-tris buffer, pH 7.5 (I 0.15), for 2 hr. Four anionic substances could be detected after ionophoresis, having the relative mobilities 0.17, 0.37, 0.72 and 1. They were identified from their mobilities, spectra and positive periodate reactions as cytidine 5'-phosphate, adenosine 5'-phosphate, guanosine 5'-phosphate and uridine 5'-phosphate respectively (Fig. 2). Later, their identities were confirmed by ion-exchange fractionation (Wade, 1960).

When a similar suspension was stored for more than 1 day at 2° before incubation under these conditions, traces of four other ultraviolet-absorbing substances with mobilities 0.33, 0.47, 0.85 and 1.15 contaminated the products. The prominence of the last-named two, and also the bases, increased as the period of storage (1–30 days) increased. This change in behaviour of the RNA could be avoided by having 5 mM- Mg^{2+} ion present during storage.

The influence of magnesium upon the products of breakdown was even more marked after dialysis. After dialysis against 0.145M-NaCl for more than 1 day, the products of incubation produced a streak on the ionophoresis paper; when, after 2 days' dialysis, mM- $MgCl_2$ and 10 mM-orthophosphate were included in the incubation mixture, the main products were nucleoside 5'-phosphates with traces of the contaminating nucleotides referred to above. The contamination could be decreased by including 5 mM- $MgCl_2$ during dialysis.

Although extensive dialysis against 0.145M-NaCl removed the Mg^{2+} ions, it was considered probable that part of the magnesium originally bound to the ribonucleoprotein would remain undialysed and be liberated in the course of RNA breakdown during incubation. The effect of including EDTA during incubation was therefore studied. The amount was

sufficient to bind all the magnesium calculated to be present (Wade & Morgan, 1957). Under these conditions, whether an undialysed or a dialysed preparation of disrupted cells was used, four anionic ultraviolet-absorbing substances could be distinguished in the products, with mobilities similar to those of substances which had earlier contaminated the nucleoside 5'-phosphates. They had the spectra of the four nucleoside 5'-phosphates but a weaker periodate reaction. Their behaviour during ionophoresis resembled those of the nucleoside 2'(3')-phosphates (Fig. 2), but ion-exchange fractionation (Fig. 11*b*) and chromatography with solvent 1 showed them to be ribonucleoside 2':3'-cyclic phosphates together with small quantities of the 3'-phosphates.

Two routes of breakdown could now be distinguished: the *M* route and the *V* route.

M route. When a suspension of disrupted cells was dialysed in the presence of 2–5 mM- Mg^{2+} ion at I 0.15 for less than 2 days and incubated at pH 7.5–8.5 (I 0.15) in the presence of 1–2 mM- Mg^{2+} ion the RNA was degraded mainly into nucleoside 5'-phosphates (Fig. 11*a*). The process was stimulated by orthophosphate (10 mM) (Figs. 7*c*, *d*). In the presence of 10 mM-orthophosphate at 37°, 50% became acid-soluble in 120 min. (mM- Mg^{2+} ion) or 200 min. (2 mM- Mg^{2+} ion). The process was retarded by higher concentrations of magnesium (Fig. 8*a*).

V route. When an undialysed or dialysed suspension of disrupted cells was incubated at pH 6.5–7.5 in the presence of sufficient EDTA to bind the magnesium present, the RNA was broken down rapidly into nucleoside 2':3'-cyclic phosphates (Fig. 11*d*). Fifty per cent was degraded in about 20 min. As the period of incubation increased, the prominence of ribonucleoside 3'-phosphates increased, suggesting that the cyclic phosphates are subsequently hydrolysed at the 2-carbon ester link.

The nucleoside 3'-phosphates were distinguished from their respective 2':3'-cyclic phosphates by the fractionation system in Fig. 11 and a system described earlier (Wade, 1960; Fig. 9).

Nucleoside cyclic phosphates also appeared in the products of the *M* route under certain circumstances. At concentrations of magnesium intermediate between those required for the two routes, either during dialysis or during incubation, guanosine 2':3'-cyclic phosphate and uridine 2':3'-cyclic phosphate appeared in the products; after prolonged dialysis (9 days) in the presence of magnesium all four cyclic phosphates appeared in the products.

Dialysis in the absence of magnesium. The dialysed suspensions of disrupted cells, upon which the pattern of breakdown between pH 4.0 and 8.5 was examined (Fig. 9), were also incubated under

conditions of the *M* route at pH 7.5 with 10 mM-orthophosphate and 5 mM-arsenate included to reduce dephosphorylation (Fig. 12). The products after 4 hr. were examined by an ion-exchange system which distinguished the two series of

nucleotides (Fig. 11). As the period of dialysis without magnesium increased, the rate of the process increased (Fig. 12), and the concentrations of guanosine 2':3'-cyclic phosphate, uridine 2':3'-cyclic phosphate and bases increased rela-

Fig. 11. Ion-exchange fractionation of products from the breakdown of endogenous RNA in disrupted dividing cells by (a) the *M* route and (b) the *V* route. (a) The preparation (50% wet wt.) was dialysed for 2 days against 0.145 M-NaCl with 5 mM-MgCl₂ and incubated at 37° at 1.0-15 (1.54 mg. of N/ml.) with mM-MgCl₂, 10 mM-sodium orthophosphate, 5 mM-sodium arsenate and 0.075 M-tris buffer, pH 7.5, for 4 hr.; 83% of the RNA was degraded (96% maximum at 5 hr.). Contamination with deoxyribonucleotides (calculated from the diphenylamine reaction) was < 13%. (b) A similar preparation was incubated with 40 mM-EDTA and 0.075 M-tris buffer, pH 7, for 1 hr.; 82% of the RNA was degraded (90% maximum at 2 hr.). The numbers associated with the peaks are concentrations in $\mu\text{moles}/100\text{ }\mu\text{moles}$ fractionated.

Fig. 12. Influence of dialysis in the absence or presence of Mg^{2+} ion upon the *M* route. A suspension of disrupted dividing cells (50% wet wt.) was dialysed under the conditions indicated in Table 1 (in which the symbols are identified).

Fig. 13. Influence of ionic strength upon the *M* route. A suspension of disrupted dividing cells (50% wet wt.) was dialysed for 2 days against 0.145M-NaCl with 5mM- $MgCl_2$ and incubated (2 mg. of N/ml.) at 37° with mM- $MgCl_2$, 10 mM-sodium orthophosphate, 5 mM-sodium arsenate, 0.03M-tris buffer, pH 7.5, and sufficient NaCl to bring the ionic strength to 0.1 (○), 0.15 (●), 0.2 (△), 0.3 (▲), 0.5 (□) or 0.7 (■).

tive to the nucleoside 5'-phosphates (Table 1, lines 1-7).

Influence of ionic strength upon the products of the M route. A similar progressive change was observed when the ionic strength was increased during incubation by additions of NaCl, thereby reducing the concentration of magnesium below the critical level referred to earlier (Fig. 13). As *I* was increased from 0.1 to 0.5, guanosine 2':3'-cyclic phosphate, uridine 2':3'-cyclic phosphate and bases became more prominent (Table 2).

Dialysis in the presence of magnesium. Up to this point the appearance of cyclic nucleotides in the end products of the *M* route (Table 1, lines 1-7; Table 2) had been associated with the loss in stability of the endogenous RNA at pH 6 (Figs. 9, 10). Prolonged dialysis (3-9 days) in the presence of 5 mM- $MgCl_2$, however, resulted in an increasing prominence of all four nucleoside cyclic phosphates (Table 1) unaccompanied by a decrease in stability (Fig. 9f). When the concentration of magnesium used for the incubation was increased from 1 to 2 mM the rate of breakdown was slower (Figs. 12, 8a) and, in addition, less of the uridine and guanosine cyclic phosphates were formed (Table 1), suggesting that the loss in stability is specifically associated with the production of these nucleotides.

Resting cells. The RNA in disrupted resting cells also breaks down by the two routes referred to above.

Breakdown of ribonucleic acid in a ribonucleoprotein fraction RII

In an earlier experiment most of the depolymerase activity in the cell had been found in the ribonucleoprotein fraction RII (Fig. 4). It was impossible to deduce from the results, however, that all the enzymes responsible for the *M* route and the *V* route were present in this fraction. The behaviour of RNA in RII was therefore re-examined.

Disrupted dividing cells were suspended in a solution of 0.145M-NaCl with 2 mM- $MgCl_2$ and fractionated according to the scheme in Fig. 1. The deposit RII was resuspended in the same salt solution (20 vol.) and centrifuged at 78 000*g* for 7.5 hr. The deposit was finally resuspended in a solution containing 0.145M-NaCl and 5 mM- $MgCl_2$ to a concentration ten times that in the unfractionated suspension. The additional washing of RII removed about 4% of the nitrogen.

Effect of pH. The pattern of depolymerase activity between pH 4.0 and 8.5 in the presence of mM- $MgCl_2$, after dialysing for 2 days against 0.145M-NaCl with 5 mM- $MgCl_2$, was similar to that in Fig. 7(c). In the presence of EDTA it was similar to that in Fig. 7(e).

M route. When conditions for the *M* route were applied at 37° (1.5 mM- Mg^{2+} ion, 10 mM-orthophosphate, 0.075M-tris buffer, pH 7.5, *I* 0.15) the rate

Table 1. *Influence of magnesium upon the products of the M route at I 0.15*

Each dialysed suspension of disrupted dividing cells (0.5–2 mg. of N/ml.) was incubated (37°) with 1 or 2 mm-MgCl₂, 10 mm-sodium orthophosphate, 5 mm-sodium arsenate and 0.075 M-tris buffer, pH 7.5, for 4 hr. The progress of RNA breakdown is illustrated in Fig. 12. C5P, Cytidine 5'-phosphate; U5P, uridine 5'-phosphate; A5P, adenosine 5'-phosphate; G5P, guanosine 5'-phosphate; C23P, cytidine 2':3'-cyclic phosphate; U23P, uridine 2':3'-cyclic phosphate; A23P, adenosine 2':3'-cyclic phosphate; G23P, guanosine 2':3'-cyclic phosphate.

Dialysis			Incubation										Symbols used in Fig. 12	
0.145 M-NaCl (days)	0.145 M-NaCl + 5 mM-MgCl ₂ (days)	Mg ²⁺ ion (mM)	RNA degraded (%)	Bases	C5P	U5P	A5P	G5P	C23P	U23P	A23P	G23P		Total recovered
0	2	1	95	21	14.3	14.0	13.5	14.5	<0.5	<0.5	<0.5	2.5	80	○
0	2	1	93	31	14.8	9.3	11.2	11.4	<0.5	<0.5	<0.5	2.4	80	○
1	2	1	85	43.7	9.15	7.6	8.6	7.1	<0.5	0.8	<0.5	3.3	80	●
2	2	1	89	50	7.7	3.7	5.0	4.7	<0.5	1.8	<0.5	4.6	78	△
2	2	1	85	65.5	9.8	4.4	4.6	3.7	<0.5	1.8	<0.5	4.3	94	△
4	2	1	81.5	54.5	10.1	3.0	5.9	8.6	<0.5	3.0	<0.5	3.7	89	▲
7	2	1	91	64.2	11.4	0.9	0.5	1.8	<0.5	5.0	<0.5	6.0	90	□
0	9	1	95	40	6.5	2.8	3.0	5.3	3.1	2.8	3.6	11.7	79	■
0	3	2	90	25.9	13.5	13.1	16.0	15.1	1.3	1.7	1.4	2.6	91	—
0	9	2	83	38.9	10.6	4.4	3.3	6.9	3.1	1.8	3.1	8.8	81	▽

Table 2. *Influence of ionic strength (0.1–0.5) upon the products of the M route*

A dialysed suspension of disrupted dividing cells (2 mg. of N/ml.) was incubated (37°) at I 0.1–0.5 with mm-MgCl₂, 10 mm-sodium orthophosphate, 5 mm-sodium arsenate and 0.03 M-tris buffer, pH 7.5, for 3–6 hr. The progress of RNA breakdown is illustrated in Fig. 13. For definitions of abbreviations see Table 1.

I	Duration (hr.)	RNA degraded (%)	Products (moles/100 moles of RNA nucleotide)										Symbols used in Fig. 13
			Bases	C5P	U5P	A5P	G5P	C23P	U23P	A23P	G23P	Total recovered	
0.1	6	60	19	18.3	16.0	20.0	15.0	<0.5	<0.5	<0.5	<0.5	88	○
0.2	4	87	42.2	11.7	8.5	6.8	6.6	0.7	1.2	0.6	2.8	81	△
0.3	3	87	56.7	8.1	3.0	1.8	4.4	<0.5	1.3	0.8	10.7	87	▲
0.5	3	85	78.3	8.9	2.0	0.4	3.8	<0.5	<0.5	<0.5	5.1	98	□

of breakdown was uniform and complete when about 80 % of the RNA had become acid-soluble. The products were mainly nucleoside 5'-phosphates (Fig. 14a). Bases were less prominent than in a similar digest of an unfractionated suspension of disrupted cells.

Traces of other nucleotides were also present. Four of them corresponded in the ratio $E_{280\text{ m}\mu}/E_{260\text{ m}\mu}$, and position, to the nucleoside 2':3'-cyclic phosphates (Fig. 14a).

V route. When the preparation of RII was incubated with 10 mM-EDTA and 0.038M-tris buffer,

Fig. 14. Ion-exchange fractionation of products from the breakdown of endogenous RNA in RII (see Fig. 1) by (a) the *M* route and (b) the *V* route. (a) A suspension of RII (1.4 mg. of N/ml.) was incubated (37°) at I 0.15 with 1.5 mM-MgCl₂, 10 mM-sodium orthophosphate and 0.075M-tris buffer, pH 7.5, for 6 hr.; 77% (maximum) of the RNA was degraded. Contamination with deoxyribonucleotides (calculated from the diphenylamine reaction) was < 6%. (b) A similar suspension was incubated with 10 mM-EDTA and 0.038M-tris buffer, pH 7.5, for 2 hr.; 94% (maximum) of the RNA was degraded. The numbers associated with the peaks (in parentheses) are concentrations in μ moles/100 μ moles fractionated.

pH 7.5, about 90% of the RNA became acid-soluble (Fig. 14b) and the products after 2 hr. were mainly ribonucleoside 2':3'-cyclic phosphates with smaller quantities of nucleoside 3'-phosphates. The latter were identified by their spectra, their behaviour during ion-exchange fractionation (Wade 1960, Fig. 9) and chromatography in solvent 2. Their greater prominence compared with that in Fig. 11b can be attributed to the longer period of incubation. The *V* route, unlike the *M* route, resulted in a marked increase in turbidity.

The RNA in the ribonucleoprotein fraction *R*II is thus independent of other sedimentation fractions both with regard to its optimum stability at pH 6 and to its ability to breakdown by two distinct routes.

DISCUSSION

The two routes of depolymerization resemble those catalysed by enzymes which have already been purified.

M route. Certain phosphodiesterases hydrolyse RNA into nucleoside 5'-phosphates (Cohn & Volkin, 1953) but the stimulation of the process by orthophosphate, particularly striking in *R*II, strongly suggests that a polynucleotide phosphorylase (Grunberg-Manago, Ortiz & Ochoa, 1956) or pyrophosphorylase (Speigleman, 1958) is responsible.

The association of a polynucleotide phosphorylase with this fraction of the cell presents an interesting anomaly. Polynucleotide phosphorylase and pyrophosphorylase synthesize RNA from nucleoside polyphosphates; the presence of either enzyme in the ribonucleoprotein fraction of the cell (Fig. 1, *R*II) would suggest that this is the primary site of RNA synthesis. Tracer experiments, however, suggest the contrary. When orthophosphate (^{32}P) is added to an unlabelled culture of *Escherichia coli* the RNA in fraction *R*II develops the lowest specific activity (Wade & Lovett, 1958). Similar conclusions are reached from pulse experiments (Countryman & Volkin, 1958).

The progressive inhibition of the *M* route as the concentration of magnesium is increased (Fig. 9a) can be attributed to the multi-stranded configuration magnesium imposes upon RNA (Felsenfeld & Rich, 1957), which inhibits phosphorylase (Grunberg-Manago, 1959).

V route. Some of the known ribonucleases resemble the one or more enzymes responsible for the *V* route in that they produce nucleoside 2':3'-cyclic phosphates. Pancreatic ribonuclease breaks RNA at the pyrimidine residues (Markham & Smith, 1952) and leaf ribonuclease at every residue (Markham & Strominger, 1956; Reddi, 1958).

In this respect, the *V*-route ribonuclease re-

sembles more closely the leaf ribonuclease. But whereas the latter hydrolyses the purine 2':3'-cyclic phosphates to the 3'-phosphates, the *V*-route ribonuclease appears to hydrolyse all the cyclic phosphates in this way. It also differs in having optimum activity at pH 7 instead of pH 5 (Holden & Pirie, 1955; Frisch-Niggemeyer & Reddi, 1957), resembling more closely pancreatic ribonuclease in this respect (Kunitz, 1940; Holden & Pirie, 1955).

The influence of magnesium upon ribonuclease is difficult to assess. It has been shown to activate pancreatic ribonuclease (Ceriotti, 1949) and leaf ribonuclease (Holden & Pirie, 1955) and also partially to inhibit these enzymes under other circumstances (Mallette & Lamanna, 1954; Holden & Pirie, 1955; Frisch-Niggemeyer & Reddi, 1957). Although the inclusion of a chelating agent is necessary for the exclusive formation of cyclic nucleotides by the *V* route it does not necessarily follow that the *V*-route ribonuclease is inhibited by magnesium. Indeed there is evidence to the contrary. After prolonged dialysis in the presence of 5 mM- Mg^{2+} ion, there is an increase in the concentrations of all four nucleotide cyclic phosphates in the products of incubation with 2 mM- Mg^{2+} ion (Table 1). Since these do not appear after a similar period of storage the result suggests that a slowly diffusing substance, in addition to Mg^{2+} ion, inhibits *V*-route ribonuclease. The results of Wagman & Trawick (1958) suggest that this substance may be a peptide. A protein inhibitor of pancreatic ribonuclease is also known (Roth, 1956, 1958a), and Pirie (1957) has suggested that the inhibition of ribonuclease by magnesium may depend upon the presence of protein. The RNA-depolymerase activity found associated with ribonucleoprotein particles isolated chemically from *E. coli* by Elson (1959) is probably more closely associated with the *V* route than with the *M* route since it resembles ribonuclease and, in common with the *V*-route enzymes, results in an increase in the turbidity of the preparation.

Ribonucleoprotein stability. The association of decreasing stability at pH 6 (Figs. 9, 10) with an increasing prominence of guanosine and uridine cyclic phosphates (but not of cytidine or adenosine cyclic phosphates) in the products of incubation at pH 7.5 (Tables 1 and 2) might be accounted for in the following way.

Under conditions of magnesium deficiency, even during dialysis at 2°, initial stages of the *V* route occur with RNA breaking at the uridine and guanosine residues to form oligonucleotides each with terminal uridine 2':3'-cyclic phosphate or guanosine 2':3'-cyclic phosphate. Subsequent incubation under conditions of the *M* route breaks each oligonucleotide into one equivalent of nucleoside cyclic phosphate, orthophosphate and nucleoside,

and one or more equivalents of nucleoside 5'-phosphate. The activity of a nucleosidase in the unfractionated disintegrate prevents quantitative proof of these changes being obtained from Tables 1 and 2. There is no doubt, however, that oligonucleotides are produced during dialysis against 0.145 M-sodium chloride since there is a progressive increase in non-diffusible, acid-soluble nucleotide as the period of dialysis or storage increases (Fig. 9). The acid-soluble polynucleotides which have been reported in preparations of microsomal ribonucleoprotein by Roth (1958b) may have originated in a similar way.

The cytidine moiety in RNA is relatively insensitive to the consequences of magnesium deficiency; it does not undergo a marked drop in concentration as the period or extent of magnesium deficiency increases (Tables 1 and 2). If the mechanism for the origin of guanosine 2':3'-cyclic phosphate and uridine 2':3'-cyclic phosphate is correct, it suggests that the other end of the oligonucleotide is predominantly an adenine residue.

The maintenance of stability after prolonged dialysis in the presence of magnesium (Fig. 10f), in spite of the appearance of all four nucleoside cyclic phosphates after incubation at pH 7.5, may be attributed to the loss of the slowly diffusing inhibitor referred to above, leaving a proportion of the RNA molecules susceptible to the *V*-route ribonuclease. Since the rate of breakdown under these circumstances is not increased, as might be expected from the fast rate of the *V* route in the presence of EDTA (Fig. 8e), the presence of magnesium may be influencing the process by maintaining the RNA in a multi-stranded condition (Felsenfeld & Rich, 1957) and allowing the enzymes to act only upon the terminal residues. The higher incidence of end groups resulting from the breaking of the RNA at the guanosine and uridine residues is probably the primary factor responsible for the loss of stability at pH 6 (Fig. 10).

SUMMARY

1. Most of the ribonucleic acid-depolymerase activity of *Escherichia coli* is present in a fraction (*R*II) which sediments between 25 000 and 78 000g and contains the bulk of the ribonucleic acid.

2. In an undialysed suspension of disrupted dividing cells or a suspension that has been dialysed in the presence of more than 2 mM-magnesium chloride, the breakdown of ribonucleic acid is inhibited by mM-Mg²⁺ ion at pH 5.5–6.5 and *I* 0.15. At *I* 0.5, 5 mM-Mg²⁺ ion is required for stability.

3. Two routes of breakdown at pH 7.5 and 37°

can be distinguished: the *M* route in the presence of magnesium and the *V* route in its absence.

4. The *M* route occurs at pH 7.5–8.5 in the presence of 1–2 mM-Mg²⁺ ion at *I* 0.15 and is stimulated by orthophosphate. The nucleotide end products are mainly nucleoside 5'-phosphates.

5. The *V* route occurs at pH 6.5–7.5 when ethylenediaminetetra-acetic acid is present. Nucleoside 2':3'-cyclic phosphates are produced and these are further hydrolysed to nucleoside 3'-phosphates.

6. The ribonucleic acid becomes unstable when the concentration of magnesium is allowed to fall below 2 mM at *I* 0.15. The evidence presented suggests that the instability might be the result of the ribonucleic acid breaking at the uridine and guanine residues to form 2':3'-cyclic phosphate end groups.

7. The ribonucleic acid in the ribonucleoprotein fraction (*R*II) behaves in a similar way to ribonucleic acid in an unfractionated preparation of disrupted cells both with respect to conditions for optimum stability at pH 6 and in its ability to break down by more than one route at pH 7.5.

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The Metabolism of *Plasmodium berghei*, the Malaria Parasite of Rodents

2. AN EFFECT OF MEPACRINE ON THE METABOLISM OF GLUCOSE BY THE PARASITE SEPARATED FROM ITS HOST CELL*

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Glucose is essential for the growth of the erythrocytic forms of several malaria parasites *in vitro*, and certain antimalarial drugs such as mepacrine inhibit development and multiplication and cause a rapid decrease in the utilization of added glucose and lactate (McKee, 1951). Although the relation of these latter effects to the primary action of the drug is obscure, mepacrine is known to have an inhibitory effect on several enzymes of the Embden-Meyerhof glycolytic pathway present in the parasites of avian malaria (Speck & Evans, 1945a; Marshall, 1948) and on the oxidation by the same parasite of intermediates of the tricarboxylic acid cycle (Speck, Moulder & Evans, 1946; Moulder, 1948, 1949; Bovarnick, Lindsay & Hellerman, 1946; Marshall, 1948). It is not, however, known whether glucose is metabolized exclusively by these pathways in the intact parasite.

In the present work, *Plasmodium berghei* has been used as a readily available mammalian parasite. The metabolism of specifically and uniformly labelled [¹⁴C]glucose by the parasite separated from its host cell, the reticulocyte, has been studied and it has been concluded that an enzyme concerned with the further catabolism of hexose 6-phosphate is the most sensitive to approximately therapeutic concentrations of mepacrine. This appears to be a selective inhibitory effect since an appreciable increase in drug concentration was without effect on the uptake of glucose by the reticulocyte.

* Part 1: Bowman, Grant & Kermack (1960).

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Part of this work has already been briefly reported (Bowman, Grant, Kermack & Ogston, 1959).

EXPERIMENTAL

Suspension of infected cells. The strain of *P. berghei* used was obtained from Dr J. D. Fulton (see Fulton & Spooner, 1956). Infected blood was obtained by cardiac puncture from white mice (about 20–25 g.) which had been inoculated intraperitoneally 5–7 days previously with 0.2 ml. of blood containing about 10⁷ infected cells.

White cells were removed by percolation of freshly shed blood through small columns of filter-paper powder (1 g./10 ml. of blood; Fulton & Grant, 1956). The blood was then centrifuged at 1000 g for 10 min., the supernatant was discarded and the cells were resuspended in an equal volume of a phosphate-buffered iso-osmotic salt solution, pH 7.2 (Krebs & Eggleston, 1940). This washing procedure was repeated twice. The suspension was then centrifuged at 1000 g for exactly 10 min. in 10 cm. × 1 cm. graduated centrifuge tubes and the sedimented cells were resuspended in 19 times their volume of phosphate-buffered salt solution which contained 2 μmoles of NaHCO₃/ml. Suspensions prepared in this way contained about 10⁶ cells/ml., of which not less than 70% were infected.

Suspensions of free parasites. Free parasites were obtained by haemolysis of infected red cells with the serum of rabbits which had been immunized against mouse red cells. Fresh guinea-pig serum was used as a source of complement. The washed parasites were centrifuged at 1000 g for exactly 10 min. and resuspended in 19 times their volume of a buffered iso-osmotic salt solution, pH 7.2, which contained K⁺ and Na⁺ ions in the ratio 7:1. Details of these procedures have been given previously (Bowman, Grant & Kermack, 1960). The final suspension contained in 1 ml. the free parasites originally present in about 10⁶ infected red cells, and such preparations will subsequently be referred to as 'standard suspensions' of free parasites.