When pyruvic acid [^{14}C]phenylhydrazone is injected, it is excreted in the urine largely as such (93%) but partly as α-oxoglutaric acid phenylhydrazone (7%). When administered orally it is hydrolysed in the gut and excreted in the urine as conjugated *p*-hydroxyphenylhydrazine (33%) together with the phenylhydrazones of pyruvic acid (31%) and α-oxoglutaric acid (5%). Similar results were obtained with α-oxoglutaric acid [^{14}C]phenylhydrazone.

4. Acetyl[^{14}C]phenylhydrazine, when administered orally to rabbits, is slowly excreted as metabolites and in 2 days about 29% of the dose is found in the urine as conjugated *p*-hydroxyphenylhydrazine (14%), unchanged acetylphenylhydrazine (4·7%) and pyruvic acid phenylhydrazone (1·4%).

One of us (W.M.M.) is grateful to St Mary's Hospital Medical School and to the Leverhulme Trust for Research Fellowships.

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The Incorporation of Phosphorus into Fractions of *Escherichia coli* made by Centrifuging and by Chemical Means

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(*Received* 30 *October* 1957)

Experiments described earlier (Wade & Morgan, 1957) suggested that the increase in ribonucleic acid concentration which occurs in resting cells of *Escherichia coli*, when they are inoculated into fresh medium and start growing and dividing rapidly, does not represent a general increase in all ribonucleic acid-containing constituents but an increase in a fraction which sediments at 25 000–100 000 *g*. It was postulated that the 'additional ribonucleic acid' which develops has some role in cell division or deoxyribonucleic acid synthesis and the term 'fluctuating ribonucleic acid' was applied to it to distinguish it from other ribonucleic acid constituents ('constitutional ribonucleic acid'), the concentrations of which remain constant.

Sedimentation fractionation cannot provide unequivocal proof for the existence of biologically distinct ribonucleic acid constituents since changes in their states of aggregation may occur during the transition from the resting to the dividing state. The marked influence of magnesium upon the aggregation of ribonucleic acid (Mallette & Lamanna, 1954) may be of importance in this connexion for equivalent sedimentation fractions in resting cells and dividing cells have different ratios of magnesium concentration to ribonucleic acid concentration (Wade & Morgan, 1957). Other evidence is required to confirm the heterogeneity of the ribonucleic acid in this bacterium and the individuality of the 'fluctuating ribonucleic acid' in its dividing cells.

Much information on the metabolism of ribonucleic acid has been gained recently from studies with radioactive tracers, of which ^{32}P has proved particularly useful. In metazoan cells, for example, there is now abundant evidence that phosphorus is incorporated at different rates into the ribonucleic acid of the nucleus and cytoplasm (Marshak & Calvet, 1949; Jeener & Szafarz, 1950; Barnum & Huseby, 1950; Smellie, MacIndoe, Logan & Davidson, 1953; Elson, Trent & Chargaff, 1955), and into other sedimentation fractions of the cell (Jeener, 1949; Hultin, Slautterback & Wessel, 1951; Smellie *et al.* 1953; Tyner, Heidelberger & LePage, 1953).

Such successful demonstrations of heterogeneity in the ribonucleic acid of other cells recommended the application of similar methods to the study of *E. coli*. Experiments were therefore designed to determine whether or not the ribonucleic acid in sedimentation fractions obtained from rapidly dividing cells differed significantly among themselves in their rates of phosphorus incorporation and to examine the suggestion (Wade & Morgan, 1957) that 'fluctuating ribonucleic acid' is a precursor of deoxyribonucleic acid. The opportunity was also taken to observe the incorporation of phosphorus into other cell constituents, since the method of isolating nucleic acid phosphorus facilitated it.

This paper describes these experiments and their results.

MATERIALS AND METHODS

Cultural conditions. Escherichia coli (NCTC 1100) was cultured at 25° on the casein hydrolysates–yeast medium (CCY) of Gladstone & Fildes (1940) from which the sodium glycerophosphate had been omitted. The distribution of phosphorus between constituents of the medium is set out in Table 1; the concentration of only orthophosphate decreases during growth. A volume (18 l.) of CCY in a 20 l. aspirator was aerated with 18 l. of air/min. through a P27 Aerox ceramic cylinder (Aerox Ltd., England), 2·5 cm. diam. and 10 cm. long. Silicone Antifoam A (Hopkin and Williams Ltd., England) was added to reduce foaming.

Determination of cell growth. The medium was inoculated with bacteria from a fully grown culture and growth was followed by determining the optical densities of samples withdrawn from the culture at regular intervals. Each sample was treated with 40% (w/v) formaldehyde soln. to a final concn. of 4% (w/v) and diluted with CCY which had been similarly treated. The optical density, within the range 0·05–0·5, was measured in 1 cm. cells of the Hilger Spekker absorptiometer with filter H 508.

Incorporation of ^{32}P. In a preliminary experiment the incorporation was followed in a CCY culture at 37°. An amount (4·5 mc) of carrier-free $^{32}PO_4^{3-}$ (The Radiochemical Centre, Amersham, Bucks.) was added during the phase of maximum growth and division rate and the culture was harvested 15 min. later. This period of exposure was equivalent to about one-half of the generation time. The differences observed between the specific activities of ribonucleic acid (RNA) phosphorus in the sedimentation fractions of the cells were too small to yield useful information. It was necessary to reduce the period of exposure to a smaller fraction of the generation time. The culture, however, took about 15 min. to harvest completely and little improvement would have resulted from a reduction in the period of growth before harvesting. An earlier investigation (Wade, 1952) had shown that the high concentration of RNA in dividing cells is independent of the temperature of growth. In the two experiments described in this report therefore the temperature of growth was reduced to 25° and the period of exposure to about one-fifth of the generation time.

The progress of each culture was followed to a point approaching the end of the phase of maximum growth and division rate, when 20 mc of ^{32}P was added (Fig. 3). After growth for a further 20 min. (Expt. 1) or 15 min. (Expt. 2) the culture was cooled to 0° by passing it through stainless-steel coils in a refrigerated ethanol bath. The cells were sedimented from the cooled culture in a De Laval continuous centrifuge at 2–5°, and washed twice with 20 vol. of 0·145 M-NaCl at 2–5° and sedimented in a refrigerated centrifuge at 0°.

The assessment of possible damage brought about by the intensity of the radioactivity was considered unnecessary as it appears that much higher levels are necessary to bring about detectable changes in *E. coli* (Lea, Haines & Bretscher, 1941; Schmidt, 1948; Deysine & Bonet-Maury, 1951; Nishiwaki, Kawai & Furukubo, 1953).

Method of disintegration. Previously (Wade & Morgan, 1957) bacterial cells were disrupted either by shaking with glass beads (Mickle, 1948) or by exposing them to ultrasonic vibrations. The method of Hughes (1951) was more suitable because of its lower working temperature, speed and convenience. The sedimentation of 'fluctuating RNA' was not influenced by the method of disintegration.

Approximately 8 g. wet wt. of cells was cooled to about −20° in a Hughes press and pressure applied to it by means of a fly press (Norton no. 3 deep-throat press). It was found that 99·9% of the cells were disrupted.

Sedimentation fractionation. Each cell disintegrate was fractionated by two sedimentations (Fig. 1). The results of similar fractionations in a previous study (Wade & Morgan, 1957) provided no justification for separating particulate

Table 1. *Origin of phosphates in casein hydrolysates–yeast medium*

Constituent	Orthophosphate (mg. of P/ml. of medium)	Other phosphates (mg. of P/ml. of medium)
Yeast extract	0·069	0·050
Acid casein hydrolysate	0·0196	0·0144
Tryptic casein hydrolysate	0·0068	0·0082

constituents of the cell which sediment when centrifuged at 25 000 *g* for 1 hr.; this step was therefore omitted. The two fractions into which these constituents were originally divided (fractions *R*I and *R*II) are present in fraction *R*I in the present study.

The cell disintegrate (10 g. wet wt.) was made up to 100 ml. with 0·145 M-NaCl at 0° and 34 ml. of this suspension (Fig. 1*D*) retained for phosphate analysis. Of the remainder, 66 ml. was centrifuged in a Spinco model L centrifuge (no. 30 rotor) at an average centrifugal force of 25 000 *g* for 1 hr. at 0°. Since conditions of centrifuging at this stage are not critical, the same speed and time as used before were maintained, although previously a no. 40 rotor had been used (Wade & Morgan, 1957). The deposit was made up to the original volume (66 ml.) with 0·145 M-NaCl (Fig. 1, *R*I). The supernatant was also made up to this volume and then centrifuged at an average centrifugal force of 85 000 *g* for either 7·5 hr. (Expt. 1) or 4 hr. (Expt. 2). The supernatant and deposit obtained were made up to the original volume and provided fractions *S*II and *R*II respectively (Fig. 1).

In Expt. 1 the conditions for sedimentation of fraction *R*II were those calculated to sediment the 'fluctuating RNA' in the cell; conditions used successfully for this purpose in the previous study were adjusted to accommodate differences between the Performance Indices of the rotors used (*Spinco Technical Handbook*). That the adjustment was successful is shown by the similar distributions of RNA between equivalent fractions. In the earlier paper RNA was distributed between '*R*I+*R*II', '*R*III' and '*S*III' in the ratio 15:74:11; in this paper it is distributed between *R*I, *R*II and *S*II in the ratio 14:78:8.

The unfractionated disintegrate (*D*) and the three fractions (*R*I, *R*II and *S*II) were stored at −10° during the interval between sedimentation and chemical fractionations.

Chemical fractionation. The chemical fractionations of the fractions obtained by sedimentation were carried out in duplicate (Tables 2 and 3, duplicates *a* and *b*) by a scheme (Fig. 2) based upon that of Schmidt & Thannhauser (1945).

Samples (3 ml.) of *D* and 5 ml. of each sedimentation fraction were delivered separately into 10 cm. × 1·5 cm. centrifuge tubes and treated with equal volumes of 10% (w/v) trichloroacetic acid soln. at 2–5°. After 10 min. the precipitate in each tube was sedimented by a bench centrifuge at 4000 *g* for 10 min. and the supernatant poured into a 25 ml. measuring cylinder. The deposit was resuspended in 5 ml. of 5% trichloroacetic acid soln. at 2–5° and sedimented by centrifuging. It was further extracted once with 5 ml. of 5% trichloroacetic acid soln. and twice with 5 ml.

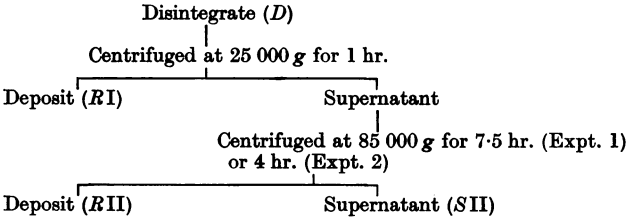

Fig. 1. Fractionation of disintegrates.

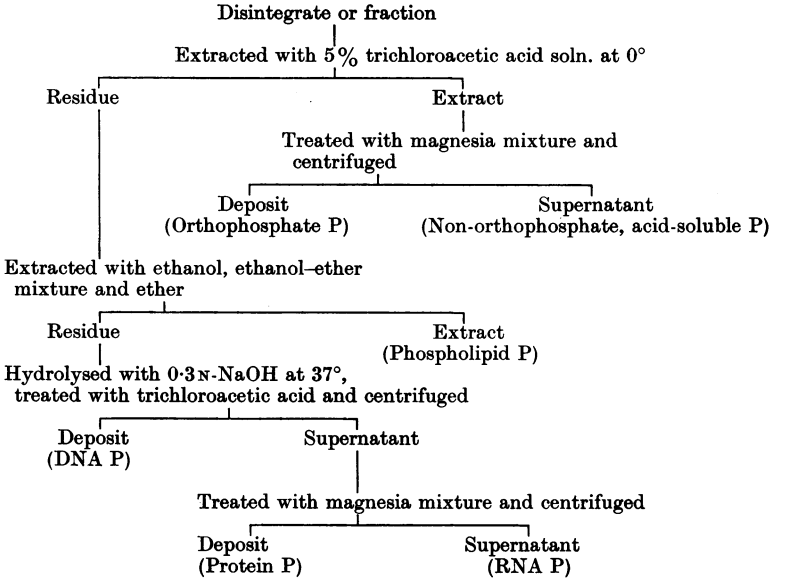

Fig. 2. Fractionation of phosphorus compounds.

of water; the extracts were added to the first supernatant. The solution of acid-soluble phosphates obtained in this way was treated with magnesia mixture (LePage, 1945) to precipitate orthophosphate. A sample (5 ml.) of the solution was treated with 1 drop of 1% (w/v) phenolphthalein soln. and sufficient aq. NH_3 soln. (sp.gr. 0·88) to turn the solution pink and then 0·5 ml. of aq. NH_3 soln. and 0·2 ml. of magnesia mixture were added and the mixture was allowed to stand for 18 hr. at room temperature. The precipitate was sedimented at 4000 *g* for 10 min. and the supernatant (Fig. 2, non-orthophosphate, acid-soluble P) drained thoroughly into a dry 10 ml. measuring cylinder. The residual liquid which remained in the tube was removed with filter paper and the deposit was taken up into 5 ml. of 0·1 N-HCl (Fig. 2, orthophosphate P).

The residue from each extraction with trichloroacetic acid was taken up into 5 ml. of ethanol and sedimented at 4000 *g* for 10 min. at room temperature. The supernatant was poured into a 25 ml. measuring cylinder and the deposit extracted twice with 5 ml. of ethanol–ethyl ether (3:1) at 60° for 5 min. and twice with 2·5 ml. of ethyl ether at room temperature. The extracts were added to the initial ethanol extract, providing a solution of phospholipids (Fig. 2, phospholipid P).

The residues were air-dried and treated with 5 ml. of 0·3 N-NaOH at 37° for 18 hr. to hydrolyse the RNA and phosphoproteins to acid-soluble phosphates. Trichloroacetic acid soln. (5 ml. of 15%) was added to each hydrolysate at 2–5° and centrifuged at 4000 *g* for 10 min. The supernatant was poured into a 25 ml. measuring cylinder and the deposit extracted twice with 5 ml. of 5% trichloroacetic acid soln. at 2–5°; the extracts obtained were added to the initial supernatant. This solution (5 ml. sample) was treated with magnesia mixture in the manner described earlier for the precipitation of orthophosphate P from the first trichloroacetic acid extract. The orthophosphate precipitated from the NaOH hydrolysate originated from protein phosphorus (Fig. 2, protein P) and the remaining phosphates from the RNA phosphorus (Fig. 2, RNA P).

The residual acid-insoluble material contained deoxyribonucleic acid (DNA) phosphorus. Each residue was suspended in 5 ml. of water, and dissolved by the addition of the minimum quantity of N-NaOH and made up to 10 ml. with water (Fig. 2, DNA P).

Chemical determinations. Phosphorus and nitrogen concentrations were determined in triplicate by the colorimetric methods of King (1951) with a Hilger Spekker absorptiometer. The distributions of phosphorus and the fiducial limits to each mean are set out in Table 2. Where triplicate readings were identical these limits were derived from the sensitivity of the instrument, which was regarded as $E \pm 0{\cdot}002$ and occupying four standard deviations.

In Expt. 1 attempts were made to establish the purity of the DNA P fraction by determining the extinction coefficients of 5% trichloroacetic acid hydrolysates (Logan, Mannell & Rossiter, 1952). A volume of each DNA P fraction, containing about 8 μg. of phosphorus, was made up to 5 ml. with trichloroacetic acid soln. to a final concentration of 5% (w/v) of the acid. The suspension was sealed in a glass tube and placed in a water bath at 90° for 15 min., and cooled and then recovered. The insoluble residue which remained after this treatment and which accounted for about 10% of the total phosphorus was sedimented by centrifuging and the extinction coefficient was calculated from the phosphorus concentration and the extinction of the supernatant at 268·5 mμ measured on a Unicam spectrophotometer (Cambridge Instruments Ltd., Cambridge, England).

Paper ionophoresis. The methods described previously were used for the examination of RNA P (Wade & Morgan, 1953, 1955). Radioautographs were also prepared with Ilford Industrial B X-ray film.

Determination of radioactivity. The radioactivities of the phosphorus fractions were measured in a liquid counter (Twentieth Century Electronics Ltd., type M6H). As the phospholipid P fractions had densities considerably lower than that of water corrections were applied to their activities with an experimentally obtained graph (Barnes & Salley, 1943).

RESULTS

Experiment 1. In the first experiment cells growing at their maximum rate were exposed to 4·5 mc of $^{32}PO_4^{3-}$ and then harvested (Fig. 3). The distribution of the incorporated phosphorus between acid-insoluble phosphates of the unfractionated cells (Tables 2 and 3, *D*) suggests that similar rates of incorporation occur into phosphoproteins and nucleic acids, and that a higher rate of incorporation occurs into phospholipids. More pronounced differences were revealed, however, by an examination of sedimentation fractions (Fig. 1; Table 3, *R*I, *R*II and *S*II).

Although the duplicates for phospholipids are poor, it is clear that the rate of incorporation into

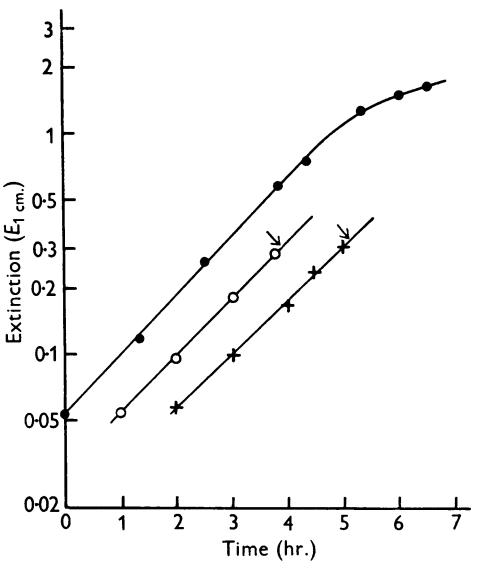

Fig. 3. Times of addition of $^{32}PO_4^{3-}$ to cultures of *Escherichia coli*. Each culture was treated with ^{32}P at the time indicated by an arrow and harvested 20 min. (Expt. 1) or 15 min. (Expt. 2) later. ●, Untreated culture; ○, Expt. 1; +, Expt. 2. The extinction is plotted on a logarithmic scale.

Table 2. *Distribution of phosphorus between chemical fractions of a disintegrate and three sedimentation fractions of* Escherichia coli

Fractionation of the whole disintegrate (*D*) into sedimentation fractions *R*I, *R*II and *S*II is described in Fig. 1. The 95% fiducial limits of each mean are given in parentheses.

Disintegrate or sedimentation fraction		Orthophosphate (μg./mg. of cell nitrogen) Expt. 2	Non-orthophosphate, acid-soluble phosphorus (μg./mg. of cell nitrogen) Expt. 2	Phospholipid phosphorus (μg./mg. of cell nitrogen) Expt. 1	Phospholipid phosphorus (μg./mg. of cell nitrogen) Expt. 2
D	*a*	25·1 (±1·24)	21·9 (±0·25)	16·2 (±0·11)*	20·8 (±0·71)
	b	26·3 (±0·14)*	18·6 (±0·12)*	16·3 (±0·11)*	18·6 (±0·08)*
*R*I	*a*	2·77 (±0·069)*	2·53 (±0·800)*	16·7 (±0·07)*	17·9 (±0·99)*
	b	2·61 (±0·066)*	2·53 (±0·800)*	16·7 (±0·07)	17·0 (±1·48)*
*R*II	*a*	0·94 (±0·063)*	1·26 (±0·088)*	1·04 (±0·025)*	2·28 (±0·202)
	b	0·92 (±0·057)*	1·42 (±0·094)	1·12 (±0·025)*	2·07 (±0·102)*
*S*II	*a*	24·9 (±0·44)	16·4 (±0·11)	2·3 (±0·042)	1·25 (±0·057)*
	b	24·9 (±0·09)*	16·0 (±0·11)*	2·4 (±0·027)*	1·25 (±0·054)*

Disintegrate or sedimentation fraction		Protein phosphorus (μg./mg. of cell nitrogen) Expt. 1	Protein phosphorus (μg./mg. of cell nitrogen) Expt. 2	RNA phosphorus (μg./mg. of cell nitrogen) Expt. 1	RNA phosphorus (μg./mg. of cell nitrogen) Expt. 2	DNA phosphorus (μg./mg. of cell nitrogen) Expt. 1	DNA phosphorus (μg./mg. of cell nitrogen) Expt. 2
D	*a*	1·34 (±0·077)*	9·05 (±0·618)	176 (±0·5)*	189 (±8·3)	25·2 (±0·11)*	30·1 (±0·20)
	b	1·34 (±0·074)*	8·85 (±0·075)*	179 (±0·5)*	202 (±4·7)	23·1 (±0·09)*	30·4 (±0·06)*
*R*I	*a*	0·71 (±0·049)*	2·71 (±0·073)*	21·9 (±0·13)*	32·4 (±1·97)	13·9 (±0·33)	7·4 (±0·032)
	b	0·71 (±0·049)*	3·17 (±0·069)*	21·9 (±0·13)*	30·9 (±2·52)	14·0 (±0·06)	7·2 (±0·018)*
*R*II	*a*	2·14 (±0·048)*	1·8 (±0·072)*	125 (±1·7)	95·6 (±2·26)	4·9 (±0·400)	0·925 (±0·021)*
	b	2·24 (±0·228)	2·03 (±0·072)*	128 (±0·6)*	94·6 (±2·25)	4·9 (±0·168)	0·925 (±0·0174)*
*S*II	*a*	0·41 (±0·052)*	2·49 (±0·87)	14 (±0·06)*	85·3 (±5·08)	9·45 (±0·189)	19·4 (±0·13)
	b	0·60 (±0·052)*	1·92 (±0·072)	13·8 (±0·06)*	71·0 (±1·60)*	9·25 (±0·061)*	17·3 (±0·07)*

* Fiducial limits were derived from sensitivity (see text for full details).

Table 3. *Specific activities of phosphorus fractions in a disintegrate and three sedimentation fractions of* Escherichia coli

Fractionation of the whole disintegrate (*D*) into sedimentation fractions *R*I, *R*II and *S*II is described in Fig. 1. The 95% fiducial limits of each mean are given in parentheses.

Disintegrate or sedimentation fraction		Orthophosphate (counts/min./μg. of phosphorus) Expt. 2	Non-orthophosphate, acid-soluble phosphorus (counts/min./μg. of phosphorus) Expt. 2	Phospholipid phosphorus (counts/min./μg. of phosphorus) Expt. 1	Phospholipid phosphorus (counts/min./μg. of phosphorus) Expt. 2
D	*a*	1115 (±30)	560 (±13)	351 (±6)	134 (±4)
	b	1153 (±15)	555 (±13)	278 (±9)	177 (±5)
*R*I	*a*	507 (±28)	270 (±17)	420 (±5)	152 (±3)
	b	554 (±30)	262 (±21)	315 (±4)	177 (±3)
*R*II	*a*	504 (±43)	147 (±36)	263 (±16)	123 (±11)
	b	499 (±45)	155 (±36)	256 (±15)	136 (±11)
*S*II	*a*	1081 (±16)	570 (±20)	21·3 (±22)	78·3 (±13·3)
	b	1041 (±12)	521 (±13)	49·7 (±22)	95·7 (±13·3)

Disintegrate or sedimentation fraction		Protein phosphorus (counts/min./μg. of phosphorus) Expt. 1	Protein phosphorus (counts/min./μg. of phosphorus) Expt. 2	RNA phosphorus (counts/min./μg. of phosphorus) Expt. 1	RNA phosphorus (counts/min./μg. of phosphorus) Expt. 2	DNA phosphorus (counts/min./μg. of phosphorus) Expt. 1	DNA phosphorus (counts/min./μg. of phosphorus) Expt. 2
D	*a*	238 (±17)	86·7 (±8·1)	238 (±2)	46·1 (±1·2)	243 (±4)	55·6 (±1·6)
	b	252 (±18)	107 (±8)	238 (±4)	44·6 (±0·9)	233 (±3)	62·2 (±1·7)
*R*I	*a*	291 (±36)	74 (±10)	296 (±6)	80·6 (±3·1)	367 (±7)	97·3 (±3·1)
	b	216 (±31)	109 (±11)	315 (±7)	68·2 (±3·3)	328 (±5)	95·7 (±3·2)
*R*II	*a*	219 (±13)	25·6 (±11)	224 (±2)	29·1 (±0·8)	176 (±9)	93·8 (±12·5)
	b	219 (±15)	16·9 (±11)	220 (±3)	31·9 (±0·8)	167 (±7)	110 (±12·5)
*S*II	*a*	138 (±40)	39·1 (±12)	240 (±7)	49·8 (±1·7)	167 (±4)	36·5 (±1·2)
	b	91 (±33)	18·9 (±11)	238 (±7)	49·5 (±1·0)	164 (±3)	35·9 (±1·2)

this fraction decreases as the particle size decreases. A similar pattern of incorporation into phosphoprotein occurs but not into the nucleic acids (Table 3). In RNA the rate of incorporation decreases in the order *RI*, *SII*, *RII*; in DNA the highest rate of incorporation occurs in *RI* and low similar rates of incorporation into *RII* and *SII*. The ionophoresis of alkaline hydrolysates of RNA in different sedimentation fractions did not reveal the presence of any phosphates other than the ribonucleotides expected, suggesting that the differences observed between the specific activities of RNA fractions were not the result of contamination. A closer examination of the DNA fractions, however, did suggest this as a likely explanation for the differences between DNA in *RI* on the one hand and in *RII* and *SII* on the other. The extinction values $\epsilon(P)_{268\cdot5\,m\mu}$ of acid hydrolysates of the DNA P fractions (Fig. 2) were 2490, 8530 and 9380 in *RI*, RII and *SII* respectively compared with 9850 for thymus DNA (Logan *et al.* 1952), suggesting that the DNA P fraction in *RI* is heavily contaminated with a phosphate which exhibits a faster rate of incorporation than the authentic DNA in *RII* and *SII*.

Expt. 1 provided the evidence of RNA heterogeneity in *E. coli* that was sought and also yielded the information that, of all the RNA fractions, the RNA in the sedimentation fraction which contains the 'fluctuating RNA' (Expt. 1, *RII*) exhibits the lowest rate of incorporation. As to the possibility that 'fluctuating RNA' is a direct precursor of DNA (Wade & Morgan, 1957), the specific activity of the authentic DNA in *RII* and *SII* is the lowest of all the nucleic acid fractions in this experiment, but it is too similar to that of RNA in *RII* to make this relationship likely.

Experiment 2. In the second experiment the chemical analysis was widened to include acid-soluble phosphates and the period of exposure to $^{32}PO_4^{3-}$ and the sedimentation fractionation were changed slightly in order to obtain further information on the heterogeneity of the nucleic acids.

Expt. 2 demonstrated the presence in *RI* and *RII* of acid-soluble phosphates with lower specific activities than the bulk of these phosphates in *SII* (Tables 2 and 3). The orthophosphate in *RI* could have arisen from labile, highly charged phosphates such as polyphosphates adsorbed on to or chemically bound to these sedimentation fractions. This possibility is of particular interest in view of the metachromatism exhibited by a similar sedimentation fraction reported previously (Wade & Morgan, 1957). The low specific activity of non-orthophosphate, acid-soluble P (Fig. 2) in *RI* and *RII* suggests that these fractions may contain degradation products of less-active acid-insoluble phosphates.

The distributions of phospholipid and phosphoprotein differed from those observed in the first experiment (Table 2) in ways which could not be explained by differences in the sedimentation fractionation (Fig. 1). These discrepancies probably arise from the very low concentrations in some of the sedimentation fractions. The pattern of phosphorus incorporation into phospholipid on the other hand is similar in the two experiments (Table 3).

The alteration made to the sedimentation fractionation in this experiment (Fig. 1) resulted in the lighter constituents sedimenting in fraction *RII* in Expt. 1 passing instead into *SII*. The influence of this change upon the relative specific activities of nucleic acids provides interesting information on the heterogeneity of RNA in the sedimentation fraction which contains the 'fluctuating RNA' (Expt. 1, fraction *RII*). Whereas in Expt. 1 the specific activity of RNA in *RII* is greater than the mean specific activity of the authentic DNA in *RII* and *SII*, in Expt. 2 it is significantly lower. This suggests that, of all the nucleic acid fractions, the heavier RNA particles in fraction *RII* of Expt. 1 exhibit the lowest rate of phosphorus incorporation, not the authentic DNA as deduced previously from this experiment.

In common with the previous experiment, the specific activity of phosphoprotein is higher in *RI* than in *RII* or *SII* (Table 3); the reliability of these data, however, is seriously undermined by the dissimilar estimates of phosphoprotein concentration in *D* and the unsatisfactory accounts of its distribution between sedimentation fractions (Table 2).

DISCUSSION

Chemical fractionation employed in the analysis of biological materials is sometimes unsatisfactory when applied to the determination of specific activity in tracer studies since a small amount of contamination can lead to erroneous conclusions. The nucleic acid fractions of the Schmidt & Thannhauser fractionation (Fig. 2) are particularly susceptible to such contamination and this possibility must be considered.

There is good evidence that two established contaminants of the RNA phosphorus fraction (Fig. 2), metaphosphate (Juni, Kamen, Reiner & Spiegelman, 1948) and polyglycerophosphate (Mitchell & Moyle, 1951*a*), are absent from *E. coli* (Duguid, Smith & Wilkinson, 1954; Mitchell & Moyle, 1954). The absence of these and other contaminants is confirmed by paper ionophoresis, and also by the low specific activity of protein phosphorus, which testifies to the satisfactory removal of acid-soluble phosphates (Fig. 2). The examination of the DNA phosphorus fraction, on the other hand, did reveal contamination in *RI* (Fig. 1).

Another possible source of error is the presence of unlabelled organic phosphates in the medium (Table 1). The interpretation of the results is not complicated by this, however, since the total concentration remains constant during growth. It is probable therefore that the estimates of nucleic acid specific activities, with the exception of DNA in *R*I, are reliable.

Much has been published on the incorporation of ^{32}P into living cells which have not been subjected to sedimentation fractionation, and interesting comparisons can be made with the results obtained from the unfractionated disintegrate of *E. coli* (Fig. 1, *D*). As in most living cells, the phospholipid phosphorus fraction (Fig. 2) has a higher specific activity than the nucleic acids (Table 3, *D*). In most tissues (Brown & Roll, 1955), and in *Micrococcus pyogenes* (Mitchell & Moyle, 1953), the rate of incorporation into RNA is greater than into DNA. The reverse relationship in *E. coli* (Table 3, *D*) is the result of the contamination of DNA P in *R*I with a more highly active phosphate. The authentic DNA in the lighter fractions of the cell has a lower specific activity than the bulk of the RNA.

The presence of acid-soluble phosphates with low specific activity in the particulate fractions (Tables 2 and 3) has been reported also in liver cells (Johnson & Albert, 1953) and may be of general occurrence.

The distribution of phospholipids (Table 2) reveals their close association with the large particles in the disintegrated cell. The absence of evidence that particles of this size exist in the whole cell of *E. coli* (Bradfield, 1956) suggests that this fraction may originate entirely from the cell wall and plasma membrane of the living cell. The different patterns of distribution reported in other studies of micro-organisms do not conflict with this interpretation. The relatively low content of phospholipid in particulate fractions of *Micrococcus pyogenes* (Mitchell & Moyle, 1951*b*) can be explained by the low lipid concentration in the cell walls of Gram-positive species (Salton, 1956), of which this species is one. The low phospholipid content of a large particulate fraction in *E. coli* reported by Billen & Volkin (1954) can be explained by the removal of the cell wall and plasma membranes as cell debris before sedimentation fractionation. The high specific activity of phospholipids in large particles in the disintegrates of the cell (Table 3), the high rate of turnover of its phosphorus in resting bacteria (Mitchell & Moyle, 1953) and the reported impermeability of the plasma membrane to orthophosphate ions (Mitchell & Moyle, 1956) suggest that part of this phosphate may be in process of transport through the cell wall and plasma membrane.

Although both experiments suggest that incorporation into protein phosphorus takes place more rapidly into *R*I than into *R*II, the data on this phosphate are not very reliable (Tables 2 and 3), a conclusion reached also by Barnum & Huseby (1950), and do not merit further attention.

The incorporation of ^{32}P into the RNA in sedimentation fractions has been studied mainly in cells with established intracellular organizations. A detailed comparison of these results with those from *E. coli* would be of doubtful value. It is clear, however, that in common with more highly organized cells *E. coli* contains RNA constituents of widely different metabolic activities (Table 3) and that, of all the RNA fractions obtained by sedimentation, a particulate fraction (Table 3, Expt. 2, *R*II) exhibits the lowest rate of phosphorus incorporation. A recent study of phage infection in this bacterium affords a closer comparison with these results. Volkin & Astrachan (1956), studying the incorporation of ^{32}P into the nucleic acids of two particulate fractions P_1 and P_2 (similar to *R*I and *R*II in Expt. 2) and a soluble fraction *S* (similar to *S*II) in T2r+ infected *E. coli*, observed that the specific activities decreased in the order P_1, *S*, P_2. The similar relationship found in uninfected cells (Table 3) suggests that the pattern of incorporation they observed is not a feature of the infection process but the result of normal metabolism continuing for a short period after the infection has taken place.

The contamination of the DNA in the DNA phosphorus fraction with other phosphates has been reported previously (Sherratt & Thomas, 1953; Juni *et al.* 1948), but their distribution among sedimentation fractions has not been pursued. The location of the bulk of the authentic DNA in *S*II is in agreement with observations made by Schachman, Pardee & Stanier (1952) and Alexander & Wilson (1955), and also with the sedimentation coefficients published by Peacocke & Schachman (1954).

The different rates of phosphorus incorporation exhibited by RNA in different sedimentation fractions of growing and dividing *E. coli* provides good evidence that these RNA fractions are composed of cell constituents which have individuality *in vivo* and are not artifacts produced by the random segregation of otherwise homogeneous RNA. This supports the broad differentiation of RNA in dividing cells into 'constitutional RNA' and 'fluctuating RNA' based previously upon the different sedimentation behaviour and basophilia of RNA in resting cells on the one hand and in dividing cells on the other (Wade & Morgan, 1957). The results, however, provide no evidence that the 'fluctuating RNA' plays some part in the division

process (Wade & Morgan, 1957). The suggestion that it is a precursor of DNA is unlikely in view of the similar specific activities of the nucleic acid fractions concerned. The inability to demonstrate this relationship cannot be attributed to wide differences in rates of phosphorus turnover for there is ample evidence that these are very low in the nucleic acids of growing micro-organisms. Whatever other relationship may exist between the 'fluctuating RNA' and the division process it is unlikely that the bulk of the phosphorus becomes incorporated into DNA.

SUMMARY

1. Growing cells of *Escherichia coli* were exposed to $-^{32}PO_4^{3-}$ for about one-fifth of their generation time, and disintegrated and fractionated into a heavy particulate fraction (*R*I), a light particulate fraction (*R*II) and a soluble fraction (*S*II). The unfractionated disintegrate and its three fractions were then chemically fractionated by the Schmidt & Thannhauser (1945) method and the specific activities of these fractions determined.

2. Of the acid-insoluble phosphates in the unfractionated disintegrate the highest rate of incorporation is into the phospholipids and the lowest into ribonucleic acid. When account is taken of the presence in the deoxyribonucleic acid fraction of a heavy, more highly active contaminant, however, deoxyribonucleic acid is observed to exhibit the lowest rate of incorporation.

3. Interesting differences exist between the sedimentation fractions. Acid-soluble phosphates with high specific activities are located mainly in *S*II. The concentration and specific activity of phospholipid decreases as the particle size decreases. The rate of incorporation into ribonucleic acid is highest in *R*I and lowest in *R*II. Authentic deoxyribonucleic acid with low specific activity is located mainly in *S*II. Of all the nucleic acid fractions examined, the lowest rate of phosphorus incorporation is into a ribonucleic acid constituent of *R*II.

4. The results confirm the heterogeneity of ribonucleic acid suggested previously by its staining properties and its sedimentation behaviour but do not furnish evidence that 'fluctuating ribonucleic acid' is a precursor of deoxyribonucleic acid.

We are pleased to acknowledge the technical assistance of Mr D. M. Morgan and Mr S. W. F. Restall and record our thanks to Dr D. Herbert for his advice and interest. The statistical treatment of results was carried out under the guidance of Mr S. Peto. Acknowledgement is made to the Chief Scientist, Ministry of Supply, for permission to publish this paper.

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The Synthesis of Bilirubin Glucuronide in Animal and Human Liver

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(*Received* 30 *September* 1957)

A system conjugating various alcohols and acids with glucuronic acid has been described by Storey & Dutton (1955) and Dutton (1956). It is located in liver microsomes and requires uridine diphosphate glucuronic acid. Isselbacher (1956) has shown that steroid glucuronides are formed in a similar manner. The finding that bilirubin is excreted as a glucuronide (Billing, Cole & Lathe, 1957; Schmid, 1957) raises the question of whether conjugation is effected by the same system. This problem was examined in order to throw light on the reduced capacity of new-born infants (Billing, Cole & Lathe, 1954) and of Gunn's strain of jaundiced rats (Malloy & Lowenstein, 1940) to excrete bilirubin. A preliminary report of this work has already been given (Lathe & Walker, 1957).

METHODS AND MATERIALS

Preliminary examination of rat bile. A 300 g. rat was anaesthetized with Nembutal and the bile duct was cannulated as described by Billing & Weinbren (1956). Bile was collected into a 0·1 ml. graduated pipette. After collection of control samples of bile, bilirubin was injected into the femoral vein. Over a period of 10 min. 3·8 ml. of a 0·2% solution of bilirubin in 0·5% Na_2CO_3 and 0·52% NaCl soln. was given. Bile was collected continuously in 0·1 ml. samples which accumulated in 3–10 min., while the secretion of pigment rose and fell again. Total and conjugated bilirubin and glucuronic acid were estimated in each sample.

Materials. *o*-Aminophenol was resublimed before use. The β-glucuronidase was an ox-liver preparation (Ketodase) from Warner-Chilcott Laboratories, New York. A solution of bilirubin (British Drug Houses Ltd. and Hoffman la Roche) was prepared by dissolving 10 mg. in a minimum amount of 0·25 N-NaOH soln. (about 0·15 ml.) with stirring. A phosphate–bicarbonate solution (see below) (0·85 ml.) was added and mixed, and the solution was centrifuged to remove any material not in solution; calcium was not included in this solution, as it precipitated bilirubin.

The uridine diphosphate glucuronic acid (UDPglucuronic acid) concentrate was prepared by a modification of the method of Storey & Dutton (1955) from a boiled extract of rabbit liver, by precipitation with acetone in acid solution. The precipitate was dried *in vacuo* over P_2O_5, and was stored at −12° in small ampoules sealed *in vacuo*.

Analytical techniques. The conjugated bilirubin in incubation media was estimated by the direct diazo reaction. Test and control (without diazo solution) values were obtained, and the difference between them gave the amount of conjugated pigment. This was determined before and after incubation.

To 1 ml. of medium in a 3 in. × ½ in. test tube was added 0·5 ml. of freshly prepared diazo reagent (10 ml. of 0·1% sulphanilic acid in 0·25 N-HCl, plus 0·3 ml. of 0·5% $NaNO_2$ soln.). A control tube was set up with 1 ml. of medium and 0·5 ml. of 0·25 N-HCl. After 30 min. 0·1 ml. of 5% ascorbic acid soln. was added to the test to neutralize the diazonium chloride, the tubes being inverted to ensure mixing; 0·1 ml. of water was added to the control. After 5 min. 0·1 ml. of saturated $(NH_4)_2SO_4$ soln. and 3 ml. of ethanol were added and the tubes were mixed by inversion. The tubes were placed at −12° for 15 min. and then centrifuged for 5 min. at 2000 *g*. The supernatant was read in a spectrophotometer (Unicam SP. 500) at 525 mμ. A solution of methyl red in sodium acetate buffer, pH 4·63, was used as a standard (Haslewood & King, 1937).

In experiments with suspensions samples were pipetted directly into the diazo reagent used for the estimation of conjugated bilirubin, and allowed to react for only 5 min. before the addition of ascorbic acid. It was established by Lathe & Ruthven (1958) that the reaction of the direct pigment was almost complete within this period.

Total and conjugated bilirubin in bile were estimated by the method of Lathe & Ruthven (1958). Glucuronic acid was estimated in bile according to Fishman & Green (1955).

o-Aminophenyl glucuronide was estimated by the method of Levvy & Storey (1949).