The Exchange Properties of Magnesium in *Escherichia coli* Ribosomes

By A. RODGERS

Department of Cell Biology, John Innes Institute, Bayfordbury, Hertford, Herts.

(Received 19 July 1963)

Although some work has been done on the metabolism of the RNA and, to a lesser extent, of the protein in ribosomal particles (Roberts, 1960, 1961, 1962), little is known of their structure. The general physical characteristics of *Escherichia coli* ribosomes have been studied by Tissières, Watson, Schlessinger & Hollingworth (1959), of yeast ribosomes by Chao & Schachman (1956), of pea-seedling ribosomes by Ts'o, Bonner & Vinograd (1958) and of rat-liver ribosomes by Petermann, Hamilton, Balis, Samarth & Pecora (1958). The importance of magnesium in the structure of these particles is well known, and the dissociation of larger into small sub-units when magnesium is removed from them appears to be a general feature (Tissières *et al.* 1959; Bolton, Hoyer & Ritter, 1958; Chao, 1957). Protein has also been implicated in this association (Morgan, Greenspan & Cunningham, 1963). Little attention has been paid to the properties of the magnesium bound to and involved in the structure of ribosomes. The binding of magnesium to Jensen-sarcoma ribosomes has been investigated by Petermann (1960), and shown to be dependent on the pH and ionic strength of the buffer. The exchange between free Mg^{2+} ions and rabbit-reticulocyte-microsomal magnesium has been studied by Edelman, Ts'o & Vinograd (1960), who found that 100% of the microsomal magnesium exchanged in about 30 min.

The present work describes measurements of the exchangeability of the magnesium of some *E. coli* ribosomes.

The results show that probably all the magnesium in *E. coli* ribosomes exchanges fairly quickly with free Mg^{2+} ions, but that the rate of exchange depends on the site of binding. The 50s ribosomes appear to be composed of 30s sub-units. High Mg^{2+} ion concentrations are not essential for the stabilization of 70s ribosomes in crude extracts of *E. coli*.

METHODS

Chemical methods. All reagents except tris and dipotassium hydrogen orthophosphate were of analytical-reagent grade.

Tris-succinate buffer ('tris buffer') contained tris (5 mM) and succinic acid (2 mM), final pH 7.3–7.4. Magnesium acetate was dissolved in this buffer to give a final concentration of 10 mM, 5 mM, 2.5 mM or 0.1 mM.

Phosphorus was originally determined by the method of Berenblum & Chain (1938), as modified by Bartley (1953). Later, the more convenient method of Chen, Toribara & Warner (1956) was used.

Magnesium was determined by flame photometry with the Beckman model DU spectrophotometer, fitted with photomultiplier and flame attachment. Magnesium emission was measured at 285 m μ , in a H_2 (5 lb./in.²) and O_2 (10 lb./in.²) flame. Standard solutions (six or seven) covering the expected range of the samples were prepared in aq. 1% (w/v) perchloric acid.

Measurement of radioactivity. Carrier-free ^{28}Mg was obtained from The Radiochemical Centre, Amersham, Bucks. The half-life is 21.4 hr. Samples (30 μ l.) for counting were pipetted on to small disks of lens tissue (2.3 cm. diam.) or circular glass cover-slips (3.2 cm. diam.). These were dried at 37° and counted to 5000 counts in a gas-flow counter (Nuclear-Chicago Corp. model 186). Time-course experiments were corrected for the decay of the isotope over the period of the experiment and the counting period.

Analytical ultracentrifugation. This was performed in the Spinco model E analytical ultracentrifuge (Beckman Instruments Inc., Belmont, Calif., U.S.A.). Sedimentation coefficients usually quoted for purified ribosomes are $S_{20,w}^{0}$. The values obtained in crude extracts, uncorrected for viscosity and the buoyancy term, are much lower: these values were computed as a routine by the method of Cecil & Ogston (1948), and yield a sedimentation coefficient S_{20} . Typical values of S_{20} in crude extracts in tris buffer containing Mg^{2+} ions, by using schlieren optics, were ($S_{20,w}^{0}$ given in parentheses): 77–83 (100)s; 55–63 (70)s; 42–46 (50)s.

Organisms. A laboratory wild-type *E. coli* was used: it was stored at 4° on slants of the synthetic medium solidified with agar, and subcultured fortnightly. The composition of the synthetic medium was similar to C medium used by Roberts, Abelson, Cowie, Bolton & Britten (1955) but without the Na_2SO_4 . This contains: NH_4Cl , 2.0 g.; Na_2HPO_4 , 6.0 g.; KH_2PO_4 , 3.0 g.; $NaCl$, 3.0 g.; glucose, 20 g.; $MgCl_2$, 50 μ M (final concn.). Water was added to give a final volume of 1 l. Glucose and $MgCl_2$ were autoclaved separately and added to the bulk of the medium when cold. Batches (5 l.) were grown with aeration at 37° (mean generation time, 55 min.). Organisms were harvested in the late exponential phase of growth and stored at –15°. For use, they were thawed and washed twice with the buffer in which the extract was to be made.

Organisms were disrupted by shaking with glass beads in the Mickle shaker. Approx. 2 g. wet wt. of washed cells were suspended in 4 ml. of buffer in Mickle cups which contained 6.5 g. of acid-washed no. 11 Ballotini beads and a trace of tributyl citrate anti-foam. The suspension was shaken for 5 min., when breakage was almost complete, as judged microscopically.

Grinding with alumina was carried out at 4° in a chilled mortar by using a weight of alumina (Microid polishing alumina, grade 3/50, slow-cutting; Griffin and George Ltd., London) equal to 2.5 times the wet weight of the cells. Grinding was continued for 5 min.

Cell walls and membrane fragments were removed by centrifugation at 12000g for 5 min.

Isolation of ribosomes. The 70s ribosomes were isolated in the Spinco model L preparative ultracentrifuge at 4°. The extract, prepared in tris buffer made 10 mM with respect to Mg^{2+} ions, was centrifuged at 100000g for 60 min. to sediment the ribosomes, which were purified by alternate low-speed (12000g for 10 min.) and high-speed (100000g for 60 min.; rotor 40) centrifugations in, successively, tris buffers made 10 mM, 5 mM and 2.5 mM with respect to Mg^{2+} ions. The final suspension in tris buffer made 2.5 mM with respect to Mg^{2+} ions was divided into equal portions and frozen at -7°. The purity of the preparations was determined by area measurements of the schlieren photographs of ultracentrifuge runs. Most preparations showed 65–70% of the total sedimenting material under the 70s peak. The proportions of other components were: 100s, 6–7%; 80s, 10–15%; 50s, 10–15%; 30s, 4%. As found by Huxley & Zubay (1960), the appearance of the 80s peak was variable. Mixtures of 50s and 30s ribosomes were prepared by dialysing a preparation of 70s ribosomes overnight against tris buffer made 0.1 mM with respect to Mg^{2+} ions. More than 90% of the 70s ribosomes dissociated under these conditions.

The 50s ribosomes were isolated from a mixture of 50s and 30s ribosomes by centrifugation at 125000g for 120 min. (40000 rev./min. in the SW 39 rotor of the Spinco model L ultracentrifuge) at 20°. The upper seven-tenths of the supernatant was discarded and the tube swirled momentarily to suspend the upper layer of the pellet: this material was removed. The lower part of the pellet was resuspended in tris buffer made 0.1 mM with respect to Mg^{2+} ions, and was shown to contain 90% of 50s ribosomes by analytical ultracentrifugation. Attempts to isolate the 30s ribosome gave impure preparations.

The 80s ribosomes from yeast were isolated in a similar manner to the 70s ribosomes of *E. coli* in tris buffer made 2.5 mM with respect to Mg^{2+} ions.

Precipitation of ribosomes. Separation of ribosomes from radioactive (or other) supernatant was achieved by an ice-cold-acetone-precipitation technique, similar to the alcohol precipitation used by Edelman *et al.* (1960), which was found unsatisfactory with the smaller ribosomes. The procedure adopted was as follows: to 1.0 ml. of ribosome suspension (3–5 mg.), cooled to 0°, was added 3.0 ml. of cold acetone. The mixture was stirred and then kept at 0° for 30 min. The precipitated ribosomes were removed by centrifugation (at 3000g for 5 min.). The supernatant was discarded and the residue washed twice with 1.5 ml. of ice-cold acetone each time. The acetone in the pellet was removed *in vacuo* at room temperature and the ribosomes were resuspended in 1.0 ml. of either distilled water or buffer. Magnesium was liberated from the aqueous suspension by the addition of 1.0 ml. of aq. 2% (w/v) perchloric acid, at 0°. The precipitate was removed by centrifugation at 3000g for 5 min. The supernatant was used for magnesium determination by flame photometry. No phosphate is liberated in this procedure to cause interference.

The method was checked by comparing analytical-ultracentrifuge patterns before and after the acetone precipitation. About 85–90% of the total area could be accounted for: aggregated material centrifuged out at low speeds. As a further comparison, satisfactory agreement was obtained for the magnesium content of 70s ribosomes estimated by the acetone-precipitation method and by direct precipitation with aq. 2% and aq. 10% (w/v) perchloric acid.

RESULTS

Magnesium:phosphorus molar ratios for ribosomes. Ribosomes were isolated by the procedures described in the Methods section. They were suspended in tris buffer containing a suitable concentration of Mg^{2+} ions. Duplicate 0.05 ml. samples were removed for the determination of total phosphorus, and duplicate 1.0 ml. samples for the determination of magnesium by the acetone-precipitation technique. Values for the magnesium:phosphorus molar ratios obtained were as follows (ribosomes suspended in the given concentration of Mg^{2+} ions): for 70s ribosomes (2.5 mM- Mg^{2+} ion), 0.551 ± 0.036 (20); for 50s ribosomes (0.1 mM- Mg^{2+} ion), 0.348 ± 0.013 (8); for yeast 80s ribosomes (2.5 mM- Mg^{2+} ion), 0.416 ± 0.014 (6); for a mixture of 50s and 30s ribosomes (0.1 mM- Mg^{2+} ion), 0.350, 0.354 and 0.363.

Exchange of ribosomal magnesium with free magnesium ions. Exchange of magnesium between the external medium and *E. coli* ribosomes was studied at 0°. A suspension of ribosomes (1.0 ml.; 5 mg.) in tris buffer containing a suitable concentration of Mg^{2+} ions was cooled to 0°. The exchange was started by the addition of 1.0 ml. of the same buffer containing $^{25}Mg^{2+}$ ions. After 60 min. the reaction was terminated by the addition of 6.0 ml. of acetone, at 0°. The mixture was stirred and then kept for 30 min. at 0°. The precipitated ribosomes were removed by centrifugation (at 3000g for 5 min.) and washed twice with 3.0 ml. of cold acetone each time. Magnesium was liberated from the precipitated ribosomes as described above (by perchloric acid) and determined by flame photometry. For counting, duplicate 0.20 ml. samples of the perchloric acid extract were taken and neutralized with 0.01 ml. of ammonia solution (sp.gr. 0.88), and 30 μ l. portions were plated out in duplicate. All exchanges were measured in duplicate. Standards were counted before and after sample counts but the difference was not significant and no correction for decay was made.

Ribosomes studied in this way were: (i) 70s ribosomes of *E. coli*, suspended in tris buffer made 2.5 mM with respect to Mg^{2+} ions; (ii) 50s ribosomes of *E. coli*, suspended in tris buffer made 0.1 mM with respect to Mg^{2+} ions; (iii) a mixture of 50s and 30s ribosomes ('derived') in tris buffer

made 0.1 mM with respect to Mg^{2+} ions; (iv) 80s ribosomes of yeast (logarithmic phase) (in tris buffer made 0.1 mM with respect to Mg^{2+} ions this system partially dissociates into the 60s and 40s forms; in tris buffer made 2.5 mM with respect to Mg^{2+} ions more than 85% of the sedimenting material is in the 80s form; tris buffer made 2.5 mM with respect to Mg^{2+} ions was used in this experiment).

Table 1 summarizes the results of an experiment with an exchange time of 60 min. Final exchange values are recorded as the percentages of the total ribosomal magnesium that had exchanged with free Mg^{2+} ions in 60 min.: this is convenient for comparison with later results. The fact that only 30% of the 70s-ribosomal magnesium exchanged in 60 min. suggested the possibility that stages in the exchange could be detected, and the time-course of the exchange of free Mg^{2+} ions with ribosomal magnesium was studied.

The experiment was conducted in a similar manner to that previously used, with three variations: (a) the time of incubation at 0° with $^{25}Mg^{2+}$ ions before the precipitation with acetone was varied, being 1.0, 2.0, 5, 15 or 30 min.; (b) the precipitation time with acetone was decreased from 30 min. to 5 min., the 70s ribosomes being precipitated completely in this time; (c) the counts were corrected for the decay of ^{25}Mg by counting standards before and after the experimental samples, and applying a correction factor; counts were corrected to the start of the counting period.

The results of this experiment are also summarized in Table 1. The exchange of free Mg^{2+} ions with 70s-ribosomal magnesium appears to

take place as rapidly as can be measured, with no further increase up to 60 min., when approx. 35% of the 70s-ribosomal magnesium has exchanged. A slightly higher proportion of the 80s-ribosomal magnesium has exchanged after this time.

The exchange of free Mg^{2+} ions with 50s-ribosomal magnesium is much slower, being about 5%/hr. The fact that the magnesium of a mixture of 50s and 30s ribosomes exchanges at a slower rate than that of 50s ribosomes alone suggests that the magnesium of 30s ribosomes alone exchanges more slowly than the 3.6%/hr. found for the mixture. Values for the extent of the exchange with the magnesium of 70s ribosomes are probably lower than the true value since the preparation contains only 65–70% of 70s ribosomes.

Magnesium involved in dissociation of 70s ribosomes. These results showed that a fraction of the 70s-ribosomal magnesium exchanged rapidly with the external Mg^{2+} ions, whereas the magnesium of the 50s and 30s ribosomes exchanged at a much slower rate. This suggested that the rapidly exchanging magnesium was associated with the formation of 70s ribosomes from 50s and 30s ribosomes.

Dialysis experiments were used to discover the amount of magnesium involved in the formation of 70s ribosomes from 50s and 30s ribosomes.

A sample (8.0 ml.) of a preparation of 70s ribosomes in tris buffer made 2.5 mM with respect to Mg^{2+} ions was dialysed at 4° in 8/32 Visking dialysis tubing (Hudes Merchandising Corp. Ltd., London) against a large volume of tris buffer made 0.1 mM with respect to magnesium acetate. Samples (1.6 ml.) were removed at intervals from the sac.

Table 1. *Exchange of ribosomal magnesium with free magnesium ions*

The exchange is given (last column) as the percentage of the total ribosomal magnesium that has exchanged in the given time-interval at 0°. Values for the 70s ribosomes are from two separate experiments. Experimental details are given in the text.

Ribosomes	Concn. of Mg^{2+} ions in buffer (mM)	Mg:P molar ratio	Sp. activity of supernatant (counts/min./ μ m-equiv. of Mg^{2+} ion)	Total Mg in ribosomes (μ m-equiv.)	Incubation time (min.)	Mg exchanged (μ m-equiv.)	Ribosomal Mg exchanged (%)
70s (<i>E. coli</i>)	2.5	0.665	49	4000	60	1093	27.3
		0.570	49	3420	60	1108	32.4
50s (<i>E. coli</i>)	0.1	0.389	1170	1480	60	82.6	5.58
		0.415	1170	1580	60	82.6	5.23
50s + 30s (<i>E. coli</i>)	0.1	—	1170	2180	60	72.4	2.32
			1170	2100	60	82.4	3.90
80s (yeast)	2.5	0.380	49	1520	60	608	40.0
		0.390	49	1560	60	649	41.6
		0.415	49	1660	60	710	42.8
70s (<i>E. coli</i>)	2.5	0.563	27.6	4000	1.0	1480	37.0
		0.563	27.6	4000	2.0	1460	36.5
		0.547	27.6	3880	5	1390	35.8
		0.563	27.6	4000	15	1510	37.8
		0.552	27.6	3920	30	1460	37.2

The composition of the material was determined by analytical ultracentrifugation, and duplicate phosphorus and magnesium determinations were performed on 0.05 ml. and 0.50 ml. respectively. Magnesium determinations were performed on material prepared by the acetone-precipitation method. The dialysis was considered complete when more than 90 % of the total material was in the 50s and 30s forms as measured by the areas under the schlieren peaks. Results of two experiments are presented in Table 2. It is clear that there is no loss of material from the dialysis sacs. When a preparation of 70s ribosomes dissociates to the 50s and 30s forms, Table 2 shows that, for the first preparation, 37.3 %, and for the second, 34.2 %, of the original 70s-ribosomal magnesium is lost. The similarity between these values and those obtained for the percentage of 70s-ribosomal magnesium which exchanges rapidly (mean, 34.9 %) suggests the identity of these fractions.

Dialysis of 50s ribosomes against magnesium ion-free buffer. The exchange data with $^{25}\text{Mg}^{2+}$ ions also suggested that the 50s-ribosomal magnesium was bound less firmly than magnesium in the 30s particles. Dialysis experiments were undertaken to see if the gentle removal of magnesium from the 50s particles would reveal any discrete species or whether complete dissociation into material with a relatively low sedimentation coefficient would occur.

The 50s ribosomes were isolated as described in the Methods section. Preparations were about 85–90 % pure, as measured by the area under the schlieren peaks. A sample (10.0 ml.) of 50s ribosomes (approx. 5 mg./ml.) suspended in tris buffer made 0.1 mM with respect to magnesium acetate was dialysed in 8/32 Visking dialysis tubing against 500 ml. of tris buffer without Mg^{2+} ions at 4°. The buffer was changed twice daily. Samples (1.6 ml.) were removed from the dialysis sac at various times: duplicate samples (0.5 ml.) were taken

(from the 1.6 ml.) for the magnesium determination, and 0.05 ml. for the phosphorus determination. A sample (0.5 ml.) was examined by analytical ultracentrifugation with schlieren and ultraviolet optics. It was not necessary to precipitate the ribosomes with acetone since the buffer did not contain Mg^{2+} ions. Direct precipitation of 0.5 ml. of ribosomes with 0.5 ml. of aq. 2 % (w/v) perchloric acid at 0° was satisfactory. The precipitate was removed by centrifugation (at 3000g for 5 min.) at 0°. The magnesium in the supernatant was determined by flame photometry.

The experiment was performed with three separate preparations of 50s ribosomes with substantially similar results. Determinations of total phosphorus indicated that there was no loss of material from the dialysis sac. In one experiment a small shoulder was noted on the leading edge of the '30s' boundary. Details of an experiment are summarized in Table 3, showing the time-sequence of a dialysis. The sedimentation coefficients quoted, S_{20} , are at a relatively high ribosome concentration (approx. 5 mg./ml.), when, at low ionic strength as in the present work, charge and concentration effects are magnified. The sedimentation coefficient of the 50s peak (photograph 1 in Table 3) at approx. 50 μg . of ribosomes/ml. was 50.6s; S_{20} for the major peak in photograph 4 of Table 3, at a similar concentration, was 29.5s. This experiment shows that, when 'derived' 50s ribosomes from *E. coli* are dialysed against tris buffer free of Mg^{2+} ions, the resultant particles have an S_{20} value similar to that of the 30s particles.

Occurrence of 70s ribosomes in tris buffer made 0.1 mM with respect to magnesium acetate. During the above experiments it was noticed that cells grown in the standard glucose-salts medium (final concn. of magnesium chloride, 49 μM), disrupted by grinding with alumina and extracted with tris buffer made 0.1 mM with respect to magnesium

Table 2. *Dissociation of 70s ribosomes in tris buffer made 0.1 mM with respect to magnesium acetate*

The 70s ribosomes were dialysed against tris buffer made 0.1 mM with respect to Mg^{2+} ions. Changes in the relative concentrations of 70s, 50s and 30s ribosomes (in arbitrary units of area) are given at the left-hand side of the Table; corresponding changes in the Mg:P ratio are given in the last column. Experimental details are given in the text.

Time (hr.)	Area under schlieren peaks (arbitrary units)				Total phosphate (μmoles)	Mg:P molar ratio
	70s	50s	30s	Total		
0	0.524	0.133	0.054	0.711	6.10	0.565
19	—	—	—	—	6.04	0.483
23	0.237	0.337	0.162	0.736	6.28	0.413
47	0.059	0.416	0.265	0.740	6.30	0.365
67	—	—	—	—	6.04	0.354
0	0.593	0.139	0.046	0.778	7.60	0.520
6	0.132	0.377	0.244	0.753	8.00	0.371
24	0.055	0.474	0.262	0.791	8.00	0.348
30	0.062	0.464	0.301	0.827	8.40	0.342

acetate, contained ribosomes with sedimentation coefficients, S_{20} , of 55–60s, i.e. 70s ribosomes, in substantial amounts. Extracts prepared by shaking a similar batch of cells with glass beads in the Mickle shaker, in the same buffer, contained 50s and 30s ribosomes only. It had previously been thought that the method of disruption of the cells had little effect on the ribosome pattern (Schachman, Pardee & Stanier, 1952).

The effect of three variables on the ultracentrifuge pattern of the cell-free extracts was examined: (a) the method of disrupting the cells; (b) variation in the concentration of Mg^{2+} ions in the growth medium; (c) variation in the concentration of Mg^{2+} ions in the extraction medium. The concentration of Mg^{2+} ions in the extract from which the ribosomes had been removed by centrifugation was also measured. The latter measurement required that the cells be grown in the absence of Na^+ ions, which interfere with the flame-photometric measurement of magnesium. Growth of the cells in a high concentration of Mg^{2+} ions required that the phosphate concentration of the standard medium be decreased. Replacement of sodium

salts by potassium salts, and decrease of the final phosphate concentration from 76 mM to 22 mM, did not affect either the growth rate of the culture or its final population density. The composition of the medium was otherwise as given in the Methods section. Organisms were grown on the standard medium with magnesium chloride added to give a final concentration of 10 mM, 0.5 mM or 0.05 mM. No precipitation of magnesium salts occurred during the growth at any concentration of Mg^{2+} ions. Growth and harvesting of cells were as described above.

Extracts were prepared as described above in tris buffer made 10 mM or 0.1 mM with respect to magnesium acetate, and diluted to 5.5 ml. with buffer. Then, 0.5 ml. of the extract was examined by schlieren optics in the ultracentrifuge, and the remainder was centrifuged at 124 000g for 120 min. (in the SW39 rotor of the Spinco model L ultracentrifuge). The upper 2.5 ml. of the supernatant was removed and duplicate 0.05 ml. samples were taken for the determination of inorganic phosphorus. Protein was precipitated from duplicate 1.0 ml. samples with 1.0 ml. of aq. 2% (w/v)

Table 3. *Dialysis of 50s ribosomes against 5 mM-tris buffer*

Schlieren diagrams of 50s ribosomes from *E. coli* after dialysis against tris buffer free of Mg^{2+} ions for the times stated. Values in parentheses are sedimentation coefficients (s) at a concentration of 50 μ g. of ribosomes/ml. Experimental details are given in the text.

Table 4. *S*₂₀ values for components of *Escherichia coli* extracts prepared by grinding with alumina or by shaking with glass beads in a Mickle shaker, and total magnesium concentrations of the extracts

Values for the total magnesium concn. of the extract refer to the values obtained after sedimentation of the ribosomes by centrifugation at 125000*g* for 120 min. Experimental details are given in the text.

Concn. of Mg ²⁺ ions in extraction medium ...				0.1 mM		10 mM	
Concn. of Mg ²⁺ ions in growth medium (mM)				Method of extraction		Method of extraction	
				Alumina	Mickle	Alumina	Mickle
10.0	<i>S</i> ₂₀ (s)	...	...	27, 42, 55	21, 31	50, 61, 85	49, 59, 77
	Total Mg concn. (mM)	...	...	0.69	0.92	12.0	9.8
0.5	<i>S</i> ₂₀ (s)	...	...	27, 41, 53	18, 36	49, 69, 95	35, 68, 93
	Total Mg concn. (mM)	...	...	0.48	0.25	9.0	11.0
0.05	<i>S</i> ₂₀ (s)	...	...	25, 40, 54	20, 30	—, 66, —	50, 70, 90
	Total Mg concn. (mM)	...	...	0.46	0.62	10.6	11.0

Fig. 1. Occurrence of 70s ribosomes in extracts of *E. coli* prepared by grinding with alumina in tris buffer made 0.1 mM with respect to magnesium acetate. The photograph was taken after 20 min. at 42000 rev./min. (phase-plate angle, 45°; temp., 4.5°). Sedimentation is from left to right. *S*₂₀ values of ribosomes: 53s, 41s and 27s. The cells were grown in medium containing MgCl₂ at a concentration of 0.5 mM.

perchloric acid. The precipitate was removed by centrifugation at 3000*g* for 10 min. and the supernatant was retained for the determination of magnesium. Magnesium standards were prepared in 1% (w/v) perchloric acid containing an amount of inorganic phosphate equal to that of the samples.

The results are summarized in Table 4. The relative amounts of components are shown in Fig. 1, from which it is clear that a considerable proportion of the ribosomal material is in the 70s form. Growth of cells on a higher concentra-

tion (10 mM) of Mg²⁺ ions caused a slight increase in the amount of the 70s component. Growth in a medium containing yeast extract and tryptic digest of casein, in which the cells grow with a mean generation time of 20 min., resulted in extracts showing only small amounts of 30s and 50s ribosomes; in tris buffer made 0.1 mM with respect to Mg²⁺ ions, the majority of the ribosomal material occurred in the 70s form.

DISCUSSION

The results presented show that about 35% of the magnesium in the 70s ribosome of *E. coli* is exchanged very rapidly with free Mg²⁺ ions. The magnesium involved in this rapid exchange is probably that associated with the formation of 70s ribosomes from 50s and 30s ribosomes, since the magnesium in the sub-units exchanges slowly and the dialysis experiments show that the magnesium involved in the association reaction amounts to about 35% of the total 70s-ribosomal magnesium. The observation that the magnesium in a mixture of 50s and 30s ribosomes exchanges more slowly than that in isolated 50s ribosomes suggests that the 30s-ribosomal magnesium is more tightly bound than some or all of the 50s-ribosomal magnesium. Magnesium in the 50s particles is probably not homogeneous with respect to its exchange properties since dialysis against buffer free of Mg²⁺ ions results in the formation of a particle with a sedimentation coefficient (at low concentrations) of 29.5s. Although it is not certain that this represents a fall in molecular weight and not a change in shape or hydration or both, both the magnitude of the effect and the fact that the sedimentation coefficient is close to that of a known ribosome make this less likely. No other large particles were observed during the dialysis, although Elson (1961) has described species with

sedimentation coefficients of 47s, 43s and 41s produced by the action of sodium chloride on 70s ribosomes: it is possible that some of these latter effects may be due to changes in shape, hydration or charge. If the fall in sedimentation coefficient may be taken as representing a fall in molecular weight, it is likely that two 30s particles give rise to one 50s particle, since only a small proportion of the total sedimenting material appears as 6s particles and no phosphorus-containing components are lost from the dialysis sac. This would be feasible if the molecular-weight data of Tissières *et al.* (1959) (mol.wt. of 30s particles, 0.7×10^6 ; mol.wt. of 50s particles, 1.8×10^6) are accepted. Bowen, Dagley & Sykes (1959) have also found that the 50s ribosome may be dissociated into smaller components, although it is difficult to identify them since the sedimentation data are given at a relatively high ribosome concentration and also the starting material, 40s particles, may differ from the 'derived' 50s particles. Differences between the 'native' and 'derived' 50s ribosomes have been observed by Green & Hall (1961). Although the nucleotide compositions of 30s and 50s ribosomes are very similar (Spahr & Tissières, 1959), the results of Aronson (1962) do not support the idea of the formation of a 50s ribosome from two 30s ribosomes alone, since some base sequences are different in the two ribosomes. As suggested by Aronson, there may be other RNA components in 50s ribosomes apart from the 30s sub-units to account for the sequence difference, and this explanation would not be inconsistent with the finding reported above. The published molecular-weight data are not sufficiently accurate to confirm this.

The use of tris buffer made 1–10 mM with respect to Mg^{2+} ions for the isolation of 70s ribosomes has seemed arbitrary to some authors (Bowen *et al.* 1959), who have suggested that ribosomes larger than 50s ribosomes may not be indispensable for normal cell metabolism. As Table 4 shows, high concentrations of Mg^{2+} ions are not essential for the observation of 70s ribosomes in normal cell extracts. Considerable amounts of 70s ribosomes are present in extracts made with tris buffer made 0.1 mM with respect to Mg^{2+} ions, provided that the cells are disrupted by grinding with alumina. Values for the total magnesium concentration in the extracts given in Table 4 are maximum values and include magnesium bound to small molecules and proteins. Values for free Mg^{2+} ions are probably slightly in excess of 0.1 mM.

Tissières, Schlessinger & Gros (1960) have reported that a partially synthesized protein provides a stabilizing influence on 70s ribosomes in buffers containing low concentrations of Mg^{2+} ions. If this explanation for the relative stability of 70s

ribosomes in crude extracts reported above is accepted, it is interesting that such a high proportion of the ribosomal material in rapidly dividing cells can be observed in this form.

SUMMARY

1. The structure of *Escherichia coli* ribosomes has been investigated by ultracentrifugation, dialysis and ^{25}Mg exchange.

2. About 35% of the magnesium in the 70s ribosomes exchanges in less than 2 min. at 0°. Magnesium in the 50s particles exchanges at 5–6%/hr., and that in a mixture of 50s and 30s ribosomes at 3–4%/hr.

3. Dialysis experiments show that 35% of the 70s-ribosomal magnesium is involved in association of the sub-units. It is concluded that the rapidly exchanging magnesium in 70s ribosomes is that associated with the formation of 70s ribosomes from the sub-units.

4. Dialysis of 50s ribosomes against tris buffer free of Mg^{2+} ions suggests that two 30s ribosomes are involved in the structure of the 50s ribosome.

5. Tris buffers made 1–10 mM with respect to Mg^{2+} ions have been shown not to be essential for the observation of 70s ribosomes in crude extracts of *E. coli*. Substantial quantities of 70s ribosomes are observed in extracts in tris buffer made 0.1 mM with respect to Mg^{2+} ions if these extracts are made by grinding with alumina but not by shaking with glass beads.

REFERENCES

- Aronson, A. I. (1962). *J. molec. Biol.* **5**, 453.
- Bartley, W. (1953). *Biochem. J.* **54**, 677.
- Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
- Bolton, E. T., Hoyer, B. H. & Ritter, D. B. (1958). In *Microsomal Particles and Protein Synthesis*, p. 18. Ed. by Roberts, R. B. Washington: Washington Academy of Sciences.
- Bowen, T. J., Dagley, S. & Sykes, J. (1959). *Biochem. J.* **72**, 419.
- Cecil, R. & Ogston, A. G. (1948). *Biochem. J.* **43**, 592.
- Chao, F.-C. (1957). *Arch. Biochem. Biophys.* **70**, 426.
- Chao, F.-C. & Schachman, H. K. (1956). *Arch. Biochem. Biophys.* **61**, 220.
- Chen, P. S., Toribara, T. Y. & Warner, H. (1956). *Analyt. Chem.* **28**, 1756.
- Edelman, I. S., Ts'o, P. O. P. & Vinograd, J. (1960). *Biochim. biophys. Acta*, **43**, 393.
- Elson, D. (1961). *Biochim. biophys. Acta*, **53**, 232.
- Green, M. H. & Hall, B. D. (1961). *Biophys. J.* **1**, 517.
- Huxley, H. E. & Zubay, G. (1960). *J. molec. Biol.* **2**, 10.
- Morgan, R. S., Greenspan, C. & Cunningham, B. (1963). *Biochim. biophys. Acta*, **68**, 642.
- Petermann, M. L. (1960). *J. biol. Chem.* **235**, 1998.
- Petermann, M. L., Hamilton, M. G., Balis, M. E., Samarth, K. & Pecora, P. (1958). In *Microsomal Particles and Protein Synthesis*, p. 70. Ed. by Roberts, R. B. Washington: Washington Academy of Sciences.

- Roberts, R. B. (1960). *Yearb. Carneg. Instn*, **59**, 229.
 Roberts, R. B. (1961). *Yearb. Carneg. Instn*, **60**, 263.
 Roberts, R. B. (1962). *Yearb. Carneg. Instn*, **61**, 244.
 Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T. & Britten, R. J. (1955). *Publ. Carneg. Instn: Studies of Biosynthesis in Escherichia coli*, no. 607, p. 5.
 Schachman, H. K., Pardee, A. B. & Stanier, R. Y. (1952). *Arch. Biochem. Biophys.* **38**, 245.
 Spahr, P. F. & Tissières, A. (1959). *J. molec. Biol.* **1**, 237.
 Tissières, A., Schlessinger, D. & Gros, F. (1960). *Proc. nat. Acad. Sci., Wash.*, **46**, 1450.
 Tissières, A., Watson, J. D., Schlessinger, D. & Hollingworth, B. R. (1959). *J. molec. Biol.* **1**, 221.
 Ts'o, P. O. P., Bonner, J. & Vinograd, J. (1958). *Biochim. biophys. Acta*, **30**, 570.

Biochem. J. (1964) **90**, 555

Studies on a Herring-Egg Phosphoprotein

By T. E. BARMAN,* NGUYEN-KIM BAI AND NGUYEN-VAN THOAI
Laboratoire de Biochimie générale et comparée, Collège de France, Paris, France

(Received 26 August 1963)

Phosvitin is the major phosphoprotein of hen's-egg yolk. All its phosphorus (10 %) is monoesterified to serine (Mecham & Olcott, 1949) and blocks of phosphorylserine sequences make up an important part of its structure (Williams & Sanger, 1959). Schmidt & Laskowski (1961) suggest that in these blocks alkali-labilization of the phosphate groups occurs by enolization of the carbonyl groups. This may explain the susceptibility of the phosvitin phosphorus to alkali, a property common to other classical phosphoproteins (Plimmer & Scott, 1908), and its possible role as a source of energy (Rabinowitz & Lipmann, 1960).

Poorly characterized phosphoproteins have been obtained from the eggs of herring (Levene & Mandel, 1907), cod (Levene, 1901) and tunnyfish (Dezani, 1908) by methods relying on alkali extraction. Much dephosphorylation and degradation could have occurred under these conditions. Though there is evidence that rainbow-trout eggs contain a phosphoprotein (Ito, Fujii & Yoshioka, 1963), it is not known whether the phosphorus of this material (4 %) is alkali-labile and whether it is esterified to serine.

The present paper deals with the isolation and characterization of a phosphoprotein (herring-egg phosvitin) from the mature unfertilized eggs of the herring (*Clupea harengus*). This protein is of particular interest as its phosphorus content (12 %) is greater than that of any other phosphoprotein yet isolated.

MATERIALS AND METHODS

Preparation of herring-egg phosvitin. Herring was obtained at a local fish market during the winter months of 1963 and the eggs, which represented as much as one-sixth

of the weight of the fish, were cleaned of adhering material and blood and treated as shown in the following typical experiment.

Eggs (400 g.) were homogenized in a Waring Blendor, and the homogenate was diluted with 1.2M-MgSO₄ (1 vol.). The mixture was stirred for 30 min., separated by centrifugation and the residue re-extracted with 1.2M-MgSO₄ (0.5 vol.). The combined extracts were dialysed (at 4–5°) against several changes of distilled water in Visking tubing and the precipitate that formed was removed by centrifugation. The supernatant was made 80 % with respect to saturated (NH₄)₂SO₄ and the precipitate was collected by filtration on fluted filter paper overnight at 4–5°. The filtrate contained less than 5 % of the organic phosphorus of the egg. The precipitate was dissolved in 1.5 vol. of a 4:1 (v/v) mixture of 1.7M-NaCl and M-acetate buffer pH 4, and 0.75 vol. of butan-1-ol was added slowly, with stirring. The mixture was stirred at room temperature for a further 4 hr. After centrifugation, the organic top layer (containing herring-egg phospholipids) was poured off and the aqueous bottom layer was carefully collected from underneath the intermediate solid material by siphoning. The insoluble material was re-extracted with buffer and butan-1-ol but with half the volumes used above. The combined aqueous layers were extracted with ether (3 × 0.5 vol.) and clarified by filtration on fluted filter paper. Solid (NH₄)₂SO₄ was added until a permanent crystalline deposit was present; this entrained with it much anthrone-positive material, but little phosphorus, and was removed by centrifugation. The supernatant was dialysed against several changes of distilled water and the phosvitin was precipitated as its calcium salt by the addition of 2 ml. of 5M-CaCl₂. The material was washed several times with water and ethanol and dried under vacuum over CaCl₂ to provide a light white powder. The total yield was 210 mg., containing 12.0 % of phosphorus.

Hen's-egg phosvitin. This was prepared according to the method of Mecham & Olcott (1949).

Phosphorylserine and phosphorylthreonine. These were prepared according to the method of Neuhaus & Korkes (1958).

Nucleic acids. Yeast RNA (10.9 % phosphorus) and herring-sperm DNA (9.1 % phosphorus) were obtained from L. Light and Co. Ltd.

* Present address: National Institute for Research in Dairying, Shinfield, near Reading, Berks.