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The Arrangement of Ribosomes in Ribosome Tetramers from Hypothermic Chick Embryos

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1. Ribosomes and the tetramer arrangement peculiar to the tissues of chick embryos exposed to low temperatures were separated by sucrose-density-gradient centrifugation, and the effects of variation of the concentrations of Mg^{2+}, Ca^{2+} and K^+ studied. 2. Lowering of the Mg^{2+} concentration from standard buffer conditions caused a reversible dissociation of tetramers into monomers and of these into subunits. 3. Ca^{2+} replaced Mg^{2+} in causing the re-formation of tetramers and monomers from subunits after dissociation in low Mg^{2+} concentrations. 4. Ca^{2+} also caused an almost complete conversion of monomers into dimers in the presence of Mg^{2+}. 5. The effect of Ca^{2+} on the formation of dimers was abolished by pre-treatment of the ribosomes with ribonuclease, but the re-formation of tetramers was unaffected. 6. Increase of the K^+ concentration from that of the standard buffer caused dissociation of monomers and dimers into subunits. 7. Raised K^+ concentration also caused a stepwise alteration of the tetramer from a particle with a sedimentation coefficient of 197S, which constitutes the bulk of the tetramer at low K^+ concentrations, first to a 184S peak and finally to material with a sedimentation coefficient of about 155S. 8. The implications of these results on hypotheses of the arrangement of the individual monomers in the tetramer are discussed and a new model for the structure is proposed.

Byers (1967) observed that exposure of chick embryos to low temperatures caused crystallization of the ribosomes *in vivo*. When cells containing the ribosome crystals are homogenized, the tetramers of which the crystals are composed can be observed in polyribosome profiles on sucrose density gradients (Humphreys & Bell, 1967). More recent techniques have greatly improved the resolution of different aggregates (Carey, 1970) and made possible the observations described below. There is now, therefore, hope that ribosomes may be crystallized *in vitro*, an achievement that would be of value in the elucidation of ribosome structure.

During studies designed to facilitate this end, observations on the effect of ionic conditions on the aggregation of chick embryo ribosomes have led to conclusions about the manner in which ribosomes associate in the tetramer, and the structure of individual ribosomes. These are presented in this paper.

It is shown that the tetramers are groups of ribosomes joined by bonds that probably involve the large subunit only and that the association is of a different nature from that of the more usual ribosome dimer. These results lead to the conclusion that the cleft commonly observed on one side of the ribosome in electron-microscope studies is unlikely to be simply the edge of the dividing point between the small and large ribosome subunits.

MATERIALS AND METHODS

Materials. Chemicals were A.R. grade wherever possible. Sodium deoxycholate, spermidine and pancreatic ribonuclease were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Triton X-100 was from British Drug Houses Ltd., Poole, Dorset, U.K.

Preparation of embryos. Fertile eggs obtained from Appleby Farm Ltd., Egerton, Ashford, Kent, U.K., were incubated at 38°C without humidification, and were turned automatically every 8h. After 7 days the eggs were placed in a refrigerator at 7–10°C for 20–30h. Preliminary experiments showed that cold treatment for periods of 48h or more caused a deterioration in the ribosome profiles obtained on sucrose density gradients, presumed to be due to cell death and autolysis.

Preparation of ribosomes. Initially ribosomes were prepared by the method of Blobel & Potter (1967), and this was varied as described below for particular purposes or as a result of experience gained. Embryos were removed into ice-cold 0.25M-sucrose–TKM buffer (50mM-tris–25mM-KCl–5mM-$MgSO_4$, pH 7.5). The heads were removed and the remaining tissue was homogenized in 2vol. of 0.25M-sucrose–TKM buffer containing 10% (v/v)

of rat liver cell sap prepared as described by Blobel & Potter (1967). The homogenate was centrifuged at 10000*g* for 10min, and in the basic method 7ml of the supernatant was layered over 5ml of 2M-sucrose–TKM buffer and was centrifuged at 100000*g* overnight. The tube was rinsed and the pellet resuspended in TKM buffer unless otherwise stated.

Treatment with 1% deoxycholate and 4% Triton X-100 as described by Blobel & Potter (1967) was sometimes done but could be omitted since most of the ribosomes of embryos at this stage are not attached to membranes; membranes present in the 10000*g* supernatant floated above the 2M-sucrose in the final 100000*g* centrifugation. In some experiments the rat liver cell sap was omitted from the homogenizing medium. Omission of detergent treatment or cell sap during preparation did not alter the sucrose-density-gradient profiles.

Rapid treatment of the ribosomes with ribonuclease was achieved by layering 7ml of the 20000*g* supernatant over two layers of sucrose, 4.0ml of 2M and 1.0ml of 1.0M, containing ribonuclease (0.5μg/ml) and centrifuging overnight at 100000*g*.

The ribosomes were stored in solid CO_2, after rapid freezing of the pellets with solid CO_2 and ethanol, for at least 9 months without alteration of the ribosome profiles on sucrose density gradients.

Sucrose density gradients. All sucrose solutions were Millipore-filtered. Two types of gradient were used: (1) 11.5ml linear gradients from 10 to 40% (w/v) sucrose prepared with the apparatus of Britten & Roberts (1960), centrifuged in the Spinco SW41 rotor at 40000rev./min for 1h at 2°C; (2) isokinetic gradients prepared as described by Noll (1967) and used in the same rotor as above. The authors are grateful to Dr J. F. Pardon for the calculations and computer program, applying the equations derived by Noll (1967) to this rotor. With careful control of the integral centrifuging times and rotor temperature, measurements of apparent sedimentation coefficients are greatly simplified with these gradients. The ribosome profiles obtained have been so reproducible that such careful control was not necessary on all occasions for the identification of peaks.

After centrifugation the distribution of extinction peaks along the gradients was analysed as described by Carey (1970). In the illustrations the top of the gradient is to the left and the bottom is marked by an apparent peak that results from a slight mismatching of the concentration of sucrose prefilling the spectrophotometer flow cell and that of the bottom of the gradient. The amount of material in the peaks was assessed by integrating on the spectrophotometer traces.

Procedures for varying ionic conditions. The effect of different ionic conditions on ribosomes was tested in two ways. In the first they were suspended in about 3ml of TKM buffer and divided into six portions, which were each diluted to 12ml with solutions of the different ionic conditions to be tested, then centrifuged at 48000rev./min for 1h in the Spinco SW41 rotor. The pellet was resuspended in 0.2ml of the salt solution and 0.1ml layered over a sucrose density gradient containing salts at the concentrations being tested. In the second method the ribosomes were suspended in a solution containing the lowest concentration of the ion under investigation, then subdivided into portions that were adjusted to the required ion concentration, and a sample of each portion was layered over an appropriate gradient as above. Where the concentration of Mg^{2+} was to be varied, the concentration of K^+ was held at the concentration of the standard TKM buffer and vice versa.

Electron microscopy. Negative staining of ribosome suspensions was done by a method similar to that of Haschemeyer & Gross (1967). We are grateful to Mr J. Hobbs for preparing the micrographs.

RESULTS

Profile of ribosomes prepared and centrifuged in TKM buffer. Fig. 1(*a*) shows a sucrose-density-gradient profile on which the characteristic features of these ribosomes in standard TKM buffer are illustrated. These features are always apparent, although the resolution achieved is sometimes not up to the standard illustrated. The profile consists of four major peaks and a number of minor ones. The sedimentation coefficients and description of the peaks have been published by Carey (1970).

Fig.1(*b*) shows the effect of treating the ribosomes with ribonuclease during preparation. Eight of the peaks are stable to this treatment. The poorly resolved material on the heavy side of the tetramer peaks and the small peak on the light side of the tetramer were lost, suggesting that these are

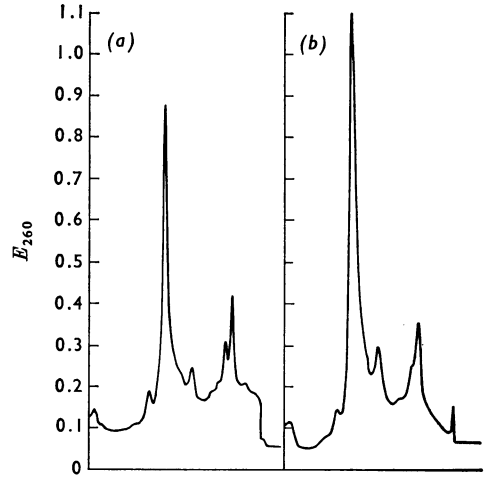


Fig. 1. Sucrose-density-gradient profile of chick embryo ribosomes. Ribosomes were prepared with or without ribonuclease treatment as described in the Materials and Methods section. A 0.1ml portion of ribosome suspension was layered over 11.5ml of 10–40% (w/v) sucrose and centrifuged for 1h at 40000rev./min in the Spinco SW41 rotor. (*a*) 2.6 E_{260} units of untreated ribosomes; (*b*) 2.3 E_{260} units of ribonuclease-treated ribosomes. The top of the gradient is on the left.

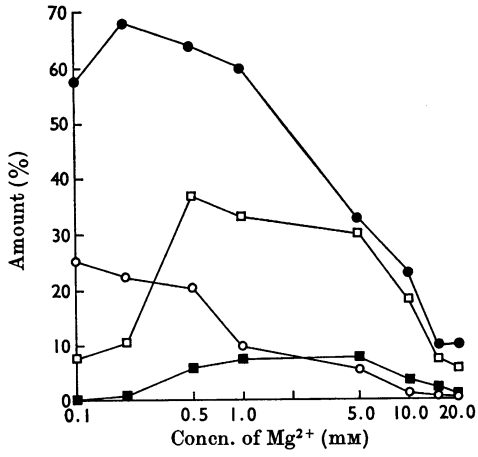


Fig. 2. Effect of Mg^{2+} concentration on the distribution of ribonuclease-treated chick embryo ribosomes on sucrose density gradients. The figure is a composite of three experiments. The ordinate represents the amount of each particle type expressed as a percentage of the total material on the gradient at 1 mM-Mg^{2+} for each experiment. The first procedure described in the Materials and Methods section was used for adjusting the Mg^{2+} concentration. ○, Subunits; ●, monomers; ■, tetramers; □, total of particles larger than monomers.

residual polyribosomes. The material in the monomer region increased in quantity.

Effect of Mg^{2+} *concentration.* It is well known that Mg^{2+} is required to maintain the structure of ribosomes *in vitro*, and it was decided to investigate the effect of this ion on the association of ribosomes into tetramers. In the early stages of this study it was not known whether the effect of changes in ion concentration would be reversible. Changes from the standard TKM buffer concentrations were therefore made by diluting the ribosome suspension with a large volume of buffer at the new concentration and resedimenting the ribosomes (first procedure, Materials and Methods section). The effects of altering the Mg^{2+} concentration on ribosomes prepared with ribonuclease treatment are shown in Fig. 2. This gives the results of three experiments that have been normalized by expressing the quantity of material in each peak as a percentage of the total material in the gradient at 1 mM-Mg^{2+} and then averaging between experiments. The results show that, in common with other ribosomes, low Mg^{2+} concentration causes disaggregation into subunits such that at 0.1 mM there was a high proportion of subunits. At high Mg^{2+} concentration the subunit concentration was lower and the amount of monomers and higher aggregates increased up to about 1 mM, the increase of monomers preceding that of the higher aggregates. At the higher Mg^{2+} concentrations the amount of material on the gradients decreased almost to nothing, presumably as a result of non-specific aggregation.

The reversibility of the effect of Mg^{2+} was indicated by a preliminary experiment in which the tissues were homogenized and the ribosomes prepared in 0.5 mM-Mg^{2+} in tris–potassium chloride buffer. A part of the product was resuspended in this concentration and part in 5 mM-Mg^{2+} in tris–potassium chloride buffer. In the former case no clear tetramer peak was present and the subunit peaks were increased. In the latter a normal tetramer peak was observed.

This result was confirmed in the experiment shown in Fig. 3. Ribosomes prepared in TKM buffer were resuspended in 0.1 mM-Mg^{2+} in tris–potassium chloride buffer and the concentration was adjusted before centrifuging. It was clear that the effect of low Mg^{2+} concentration in causing disaggregation was easily reversed by raising the concentration. It is also apparent that the decreased amounts of material on the gradient shown in Fig. 2 are mainly due to the inability to reverse the aggregating effects of centrifuging, since the decrease in Fig. 3 is much less marked.

The experiments shown in Figs. 2 and 3 were carried out with ribonuclease-treated ribosomes. If ribosomes were not so treated the result shown in Fig. 4 was obtained. At all concentrations of Mg^{2+} there was material on the gradient of much higher sedimentation coefficient than the monomer. The peak was broad and altered in position by changing the Mg^{2+} concentration. There appeared to be sufficient material in the peak to account for all the tetramer, which was restored to its normal positions by raising the Mg^{2+} concentration above 5 mM.

Effect of Ca^{2+} *concentration.* There are two major effects of the addition of Ca^{2+} to the ribosomes in buffer. First, it can replace Mg^{2+} in causing aggregation, and, secondly, if the ribosomes are not pretreated with ribonuclease they result in an unusually high dimerization.

When Ca^{2+} was added to TKM buffer the most noticeable effect, as shown in Fig. 5, was a decrease in the total material on the gradient as the bivalent ion concentration increases. The ribosomes in this experiment were treated with ribonuclease during preparation. If ribosomes were resuspended in a buffer of low Mg^{2+} concentration the addition of Ca^{2+} restored the normal pattern of distribution on the gradient that allowed the formation of dimers, trimers and tetramers at the expense of monomers and subunits. Fig. 6 shows the effect with 0.5 mM-Mg^{2+}, and a similar result was obtained with 0.1 mM-Mg^{2+}. Here again the ribosomes had been treated with ribonuclease.

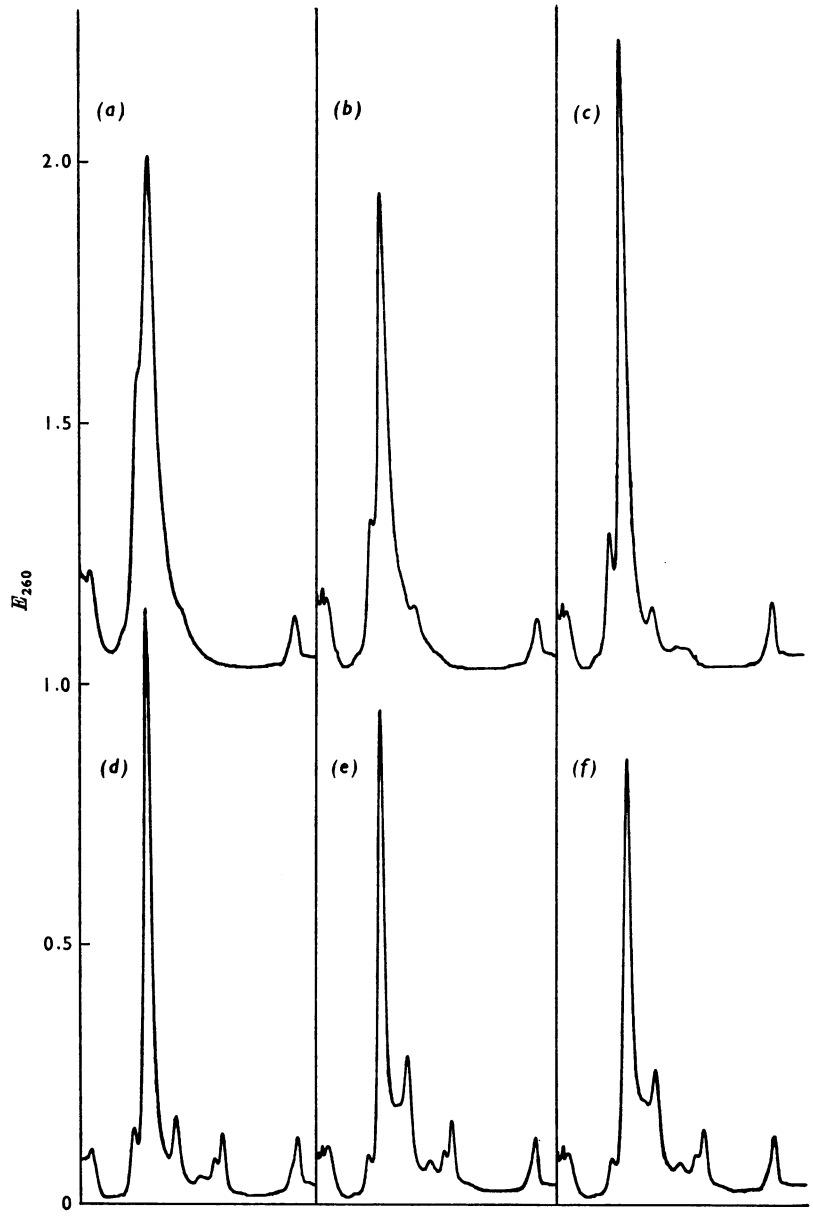


Fig. 3. Effect of Mg^{2+} concentration on the sucrose-density-gradient profiles of ribonuclease-treated chick embryo ribosomes. The second procedure described in the Materials and Methods section was used for adjusting the Mg^{2+} concentration. The ribosomes (1.7 E_{260} units) were layered over 11.5ml isokinetic gradients and centrifuged for 65min at 40000rev./min in the Spinco SW41 rotor. Concentration of Mg^{2+}: (*a*) 0.1mM; (*b*) 0.5mM; (*c*) 1mM; (*d*) 5mM; (*e*) 10mM; (*f*) 20mM.

If both these experiments are performed on ribosomes that had not been treated with ribonuclease a different result was obtained. In both cases at certain Ca^{2+} concentrations a preponderance of dimers was obtained, which is illustrated in Fig. 7 with ribosomes resuspended in TKM buffer. The formation of tetramers was unaffected, but the monomers were largely converted into dimers. The

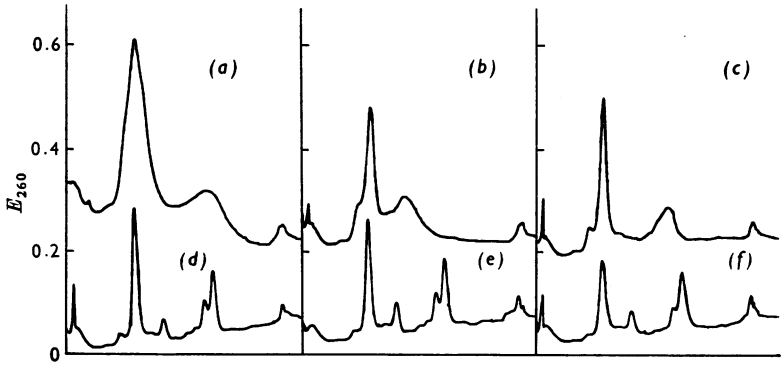


Fig. 4. Effect of Mg^{2+} concentration on the sucrose-density-gradient profiles of chicken embryo ribosomes. The experiment was the same as that in Fig. 3 except that the ribosomes were not treated with ribonuclease. The ribosomes (2.3 E_{260} units) were added to the gradients and centrifuged for 65 min at 40000 rev./min in the Spinco SW41 rotor.

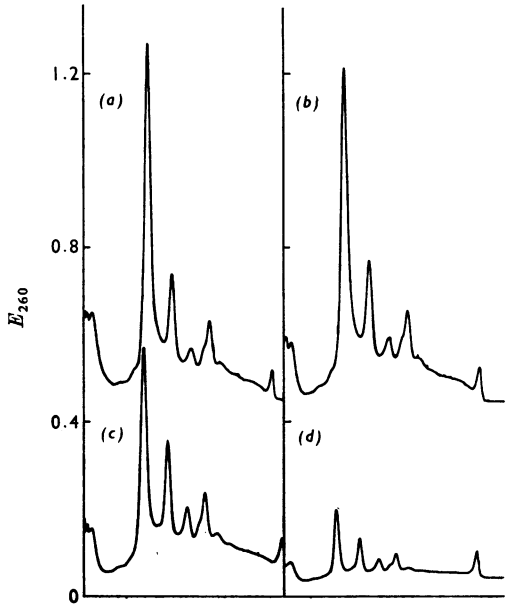


Fig. 5. Effect of Ca^{2+} concentration on the sucrose-density-gradient profiles of ribonuclease-treated chick embryo ribosomes. Samples in TKM buffer containing 2.14 E_{260} units were centrifuged for 90 min at 40000 rev./min in the Spinco SW41 rotor after adjustment of Ca^{2+} concentration by the second procedure described in the Materials and Methods section. Ca^{2+} concentration: (*a*) 0; (*b*) 0.1 mM; (*c*) 0.5 mM; (*d*) 1.0 mM.

effect was most noticeable in TKM buffer at about 5 mM-Ca^{2+}.

Finally, in the absence of Mg^{2+}, Ca^{2+} at 1.0 mM will maintain the presence of tetramers, but these are obviously modified in some way since the peak in the tetramer region, although double as with Mg^{2+}, was much broader. The addition of a low concentration of Mg^{2+} (0.2 mM) restored the normal pattern.

Effects of other bivalent cations. A few other bivalent cations were added to the ribosomes in addition to those described above. These studies were not very extensive, but the results can be summarized as follows: Mn^{2+} has a similar effect to Ca^{2+} in that it restores the normal distribution on a gradient with low Mg^{2+} concentrations and at the appropriate concentration causes increased formation of dimers. There was no effect of Ni^{2+} or Zn^{2+} up to 1 mM when added to TKM buffer. Spermidine also had no effect up to 1 mM, the highest concentration tested.

Effect of K^+ concentration. The ribosomes of other tissues are known to dissociate under the influence of high K^+ concentration. As with Mg^{2+}, it was decided to observe the effect of K^+ concentration on the ribosome structure. The effect of increased K^+ concentration from that in the normal TKM buffer is shown in Fig. 8, and this was achieved by the second procedure described in the Materials and Methods section, which does not include centrifuging. The results are averages of two experiments and the quantities are expressed as percentages of the total material added to the gradient.

Several effects can be noted. First, the amount of material in the monomer peak was fairly constant up to 0.2 M-K^+, then decreased sharply. The dimer was also lost at high K^+ concentrations. In parallel with the decreases in monomer and dimer the proportion of subunits increased greatly at concentrations higher than 0.20 M.

Material was present in the tetramer region up to a concentration of 0.15 M-K^+, above which it was no

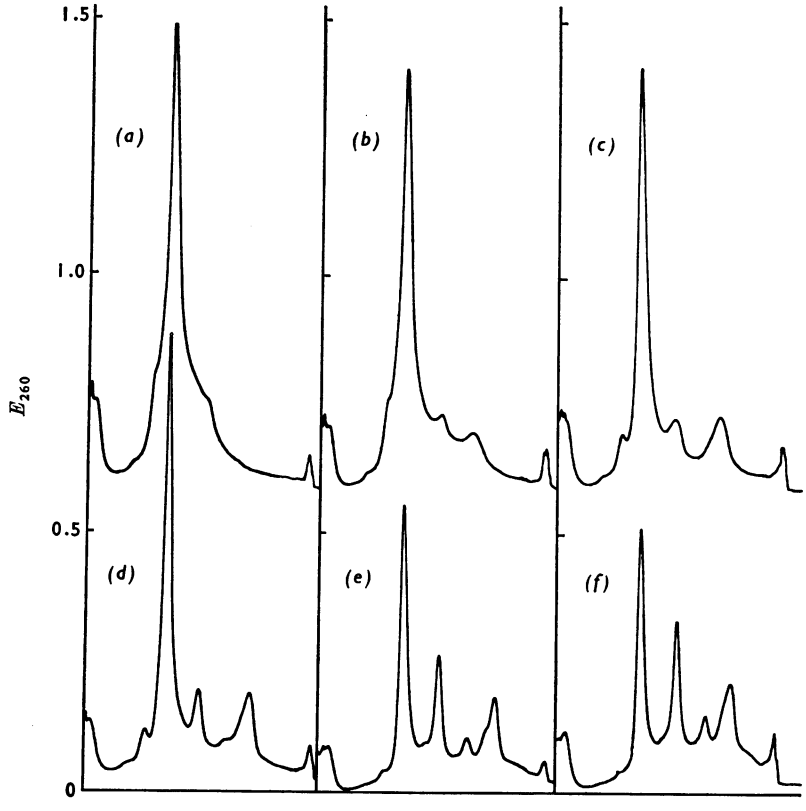


Fig. 6. Effect of Ca^{2+} concentration on the sucrose-density-gradient profiles of ribonuclease-treated chick embryo ribosomes. Conditions were as for Fig. 5 except that the samples contained 1.75 E_{260} units and the ribosomes were suspended in 0.5 mM-Mg^{2+} in tris–KCl buffer instead of TKM buffer. Ca^{2+} concentration: (*a*) 0; (*b*) 0.1 mM; (*c*) 0.5 mM; (*d*) 1.0 mM; (*e*) 5.0 mM; (*f*) 10.0 mM.

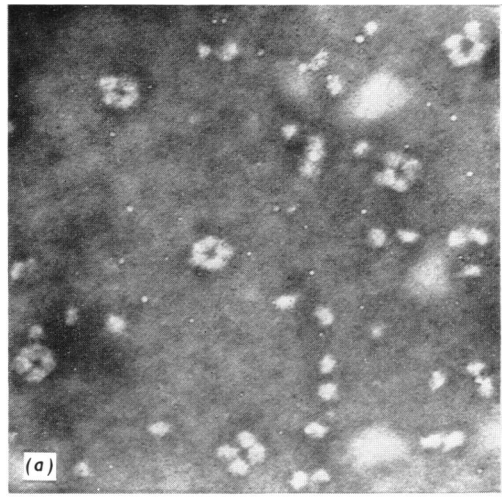
longer found, but a similar quantity of material was recovered in the trimer region. This feature is better illustrated in Fig. 9. It is clear that material originally in the tetramer region can occupy any of three positions. At low K^+ concentrations the tetramer peak was double, with most of the material in the heavier (197 S) peak. At 0.15 M-K^+ most of the material was in the lighter (184 S) peak. Above 0.20 M-K^+ the material was found in the region normally ascribed to the trimer at about 155 S. The fact that this peak contained tetramers was shown by electron-microscope observation of negatively stained preparations (Plate 1*a*). A sample of tetramers in TKM buffer is included for comparison (Plate 1*b*). Although the structure of the aggregate was less distinct in high K^+ concentration than in TKM buffer, in that the individual ribosomes seem to have expanded to the point that their edges were less clearly separated, there can be no doubt about the presence of tetramers. It is also clear that the aggregates formed in the presence of high K^+ concentrations are of similar dimensions to the original tetramer, certainly not smaller.

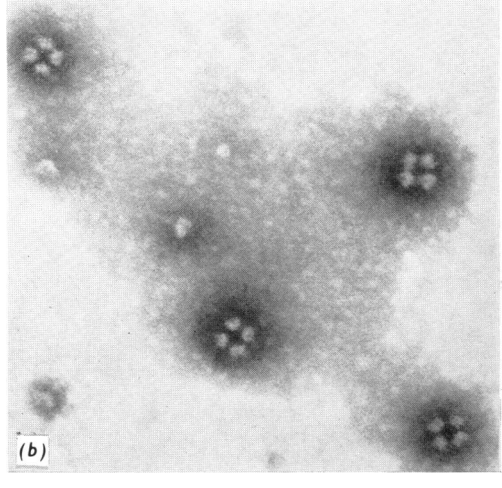
DISCUSSION

Carey (1970) gave details of the sucrose-density-gradient profiles of ribosomes from cold-treated chick embryos with estimates of the sedimentation coefficients of the peaks. Material in the tetramer region was resolved into two sharp peaks at 184 S and 197 S.

The present paper gives information about the association and dissociation of the tetramers under various ionic conditions and leads to conclusions about the structure of the individual ribosomes in the tetramer.

The effects of bivalent cations on chick embryo ribosomes are similar to those on ribosomes from other tissues. For example they dissociate into subunits when the Mg^{2+} concentration is lowered, and this occurs at a similar concentration to that





EXPLANATION OF PLATE 1

(*a*) Electron micrograph of ribosome tetramers in 0.25 M-K^+ in tris–$MgSO_4$ buffer from a sucrose-density-gradient separation. (*b*) Electron micrograph of ribosome tetramers in TKM buffer from a sucrose-density-gradient separation. Magnification ×100 000.

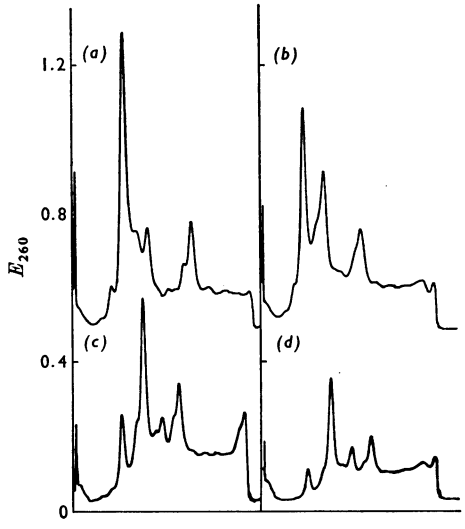


Fig. 7. Effect of Ca^{2+} concentration on the sucrose-density-gradient profiles of chick embryo ribosomes. Conditions were as for Fig. 5 except that the sample contained 3.75 E_{260} units and the ribosomes were not treated with ribonuclease during preparation. Ca^{2+} concentration: (*a*) 0.1mM; (*b*) 1.0mM; (*c*) 5.0mM; (*d*) 10.0mM.

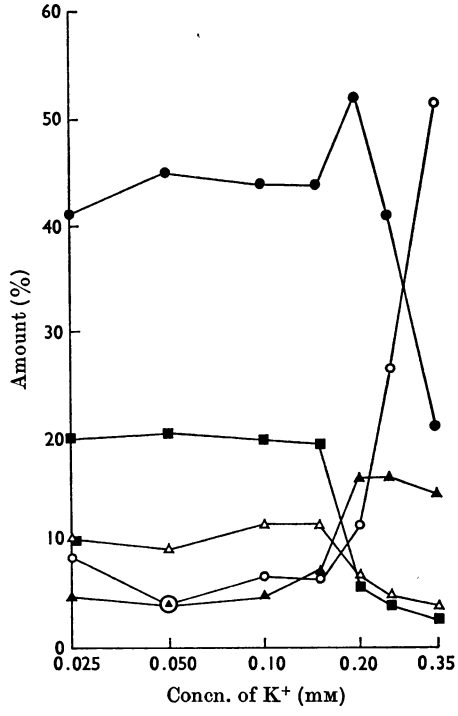


Fig. 8. Effect of K^+ concentration on the distribution of chick embryo ribosomes on a sucrose density gradient. The figure is a composite of two experiments. The ordinate represents the amount of each particle type expressed as a percentage of the amounts of material on the gradient at each concentration. The second procedure described in the Materials and Methods section was used to adjust the K^+ concentration. ○, Subunits; ●, monomers; △, dimers; ▲, trimer region; ■, tetramers.

shown by Peterman & Pavlovec (1967) for rat liver ribosomes. Tetramers from ribonuclease-treated preparations also dissociate into monomers and subunits when the bivalent ion concentrations are lowered. This effect was easily reversed by raising the Mg^{2+} concentration.

Ca^{2+} or Mn^{2+} were effective in re-forming tetramers in preparations that had been dissociated by exposure to low Mg^{2+}. In addition, both these ions caused the association of monomers into dimers, the proportion of tetramers being unchanged. The conversion into dimers was abolished if the ribosomes were pretreated with ribonuclease, but the enzyme had no effect on the ability to re-form tetramers. These results strongly suggest that tetramers are not simply two of the more usual ribosome dimers associated side by side, since in that case the effects of ions on their association and dissociation would be linked. This conclusion is reinforced by the observation that the dimer is not stable, as is the tetramer, to high K^+ concentrations. It is therefore considered that the dimer and tetramer have a different mechanism of association.

There are three types of tetramer found on sucrose gradients of the embryo ribosomes. Two of these, at 197 S and 184 S, are apparent under the conditions of TKM buffer and the proportions of these are changed by altering the K^+ concentration. At 0.025M-K^+ the material of highest sedimentation coefficient predominates. At 0.15M this peak is greatly decreased in amount and material is found predominantly in the 184 S peak. Above 0.20M-K^+ material was found in a position that would normally be ascribed to a trimer (about 155 S), but this was found, by electron-microscope observation, to be composed of tetramers.

There are three possible explanations for this change in sedimentation coefficient of the tetramers on raising the K^+ concentration: (1) loss of ribosome subunits, (2) loss of protein and (3) conformation changes, such as expansion of the particles. Since K^+ concentrations above 0.25M caused extensive dissociation of monomers into subunits it seems likely that the tetramer ribosomes at these concentrations also dissociate into subunits, and since the residual aggregate is quite large it is probable that the small subunit is lost. The tetramer (155 S)

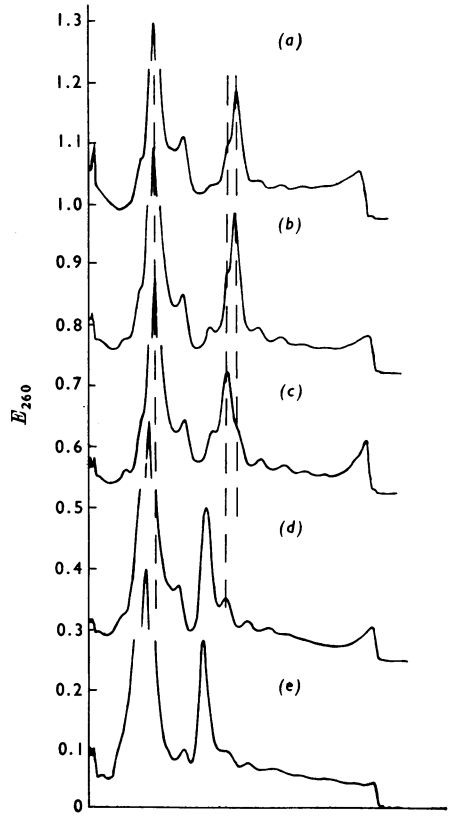


Fig. 9. Effect of K^+ concentration on the sucrose-density-gradient profiles of chick embryo ribosomes. Samples containing 2.10 E_{260} units were layered at 11.5ml isokinetic gradients and centrifuged for 50min at 40000rev./min in the Spinco SW41 rotor. K^+ concentration: (*a*) 0.025M; (*b*) 0.10M; (*c*) 0.15M; (*d*) 0.20M; (*e*) 0.25M.

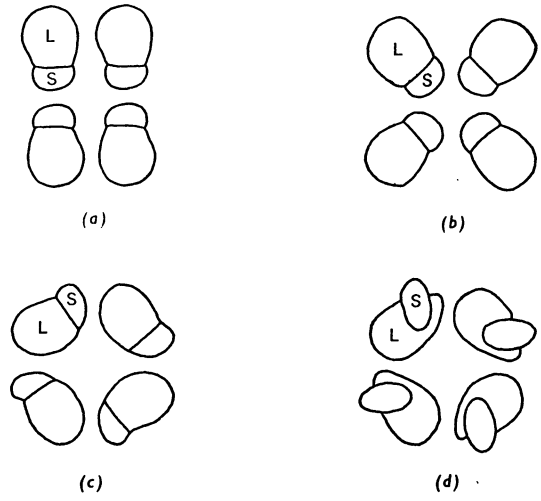


Fig. 10. Four models of the arrangement of monomer ribosomes in tetramers induced in chick embryos by hypothermia. L, large subunit; S, small subunit. For further description see the text. The diagram is not to scale.

formed in the presence of high K^+ concentrations is therefore considered to consist of large subunits only.

This explanation is unlikely to account for the difference between the other two tetramer peaks (197S and 184S) formed in the presence of low K^+ concentrations, since the transition between them occurs at a K^+ concentration that does not cause significant dissociation of monomers (<0.15M). Dissociation into subunits at low K^+ concentration would be incomplete, so that multiple peaks would be expected from the presence of tetramers with one, two or even three small subunits. This was not observed, so it eliminates alternative (1) as an explanation for the transition between the 184S and 197S tetramers, but it is not yet possible to suggest the true basis of this transition.

The conclusions that the tetramer is not composed of two conventional dimers and that the tetramer formed in the presence of high K^+ concentrations is composed of large subunits only have bearing on the structure of individual monomers. Byers (1967) observed a cleft in the outer edge of the ribosomes in the tetramers in the crystals obtained *in vivo*, and we have also seen this in negative-stained preparations of free tetramers (Carey, 1970; and Plate 1*a*). Byers (1967) suggested either that the tetramers were composed of monomers in a similar association to that of the dimer proposed by Huxley & Zubay (1960) or that the cleft represented the edge of a join between the large and small subunits. These considerations lead to three possible models for the tetramer, which are illustrated in Figs. 10(*a*), 10(*b*) and 10(*c*). Fig. 10(*a*) shows a tetramer composed of two dimers of the type described by Huxley & Zubay (1960). Fig. 10(*b*) shows a model in which the four small subunits are towards the centre of the group. Fig. 10(*c*) shows a model in which the cleft referred to above is taken as the position of the dividing line between the subunits, leading to a head-to-tail arrangement in the tetramer.

That the tetramer is composed of a side-to-side arrangement of two conventional dimers (Fig. 10*a*) was eliminated for reasons given above, and, further, the suggestion by Byers (1967) that the tetramer is similar to the dimer in that it is joined by juxtaposition of all four small subunits (Fig. 10*b*) can be eliminated because such a grouping would not give rise to the large residual tetramer observed at high K^+ concentrations. The group of four small subunits that would be obtained would have a much

lower sedimentation coefficient than is observed for the tetramer formed in the presence of high K^+ concentrations.

The model in Fig. 10(*c*) is also considered to be unlikely in view of the results given here, since a particle so arranged would be expected to dissociate completely when the small and large subunits of each ribosome are separated by raised K^+ concentration. Since the tetramer formed in the presence of high K^+ concentrations is probably composed of large subunits only, it is suggested that the tetramers formed in the presence of low K^+ concentration are joined by bonds between the large subunits and that the small subunits are not involved in tetramer formation. From this it follows that the cleft in the prolate shape of the individual monomers in the tetramer does not mark the dividing line between its two ends, one composed of the small and the other the large subunit, as shown in Fig. 10(*c*). If the cleft has anything to do with the disposition of the subunits it suggests a degree of asymmetry in their arrangement, not a simple division of the monomer into two unequal ends.

The results presented in this paper support the model illustrated in Fig. 10(*d*). In this model the bonds between the monomers are formed by the large subunits, and the small subunit is arranged above the large in such a way that it enhances the elongated outline and produces the kidney shape, or cleft, observed in electron micrographs.

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