Effect of Various Methods of Preparation on the Apparent Protein Composition of Eukaryotic Ribosomes

AN ESSENTIAL PRELIMINARY TO STOICHEIOMETRIC MEASUREMENTS

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1. We investigated whether there is any change in the relative amounts of ribosomal proteins during the isolation or extraction of the ribosomes by different methods, or during electrophoresis of the proteins. 2. To see whether proteins are lost (or gained) during the preparation of the ribosome we compared the two-dimensional protein pattern of three preparations: (a) ribosomes conventionally prepared by ultracentrifugation; (b) crude ribosomes obtained by pH 5 precipitation; (c) crude ribosomes prepared by gel filtration. 3. To see whether proteins were lost during protein extraction we compared the two-dimensional pattern of ribosomes by using three different extraction methods (LiCl/urea, acetic acid and guanidine hydrochloride). 4. In all experiments listed above the relative amounts of the great majority of the proteins remained unchanged. We interpret this as showing that the relative amounts of ribosomal proteins (as we observed them on a two-dimensional gel) correspond to the proportions existing in the particle *in vivo*.

The relative amounts of the different proteins in the ribosome have been the source of great interest and speculation in the case of *Escherichia coli*, the only species that has been examined in any detail so far. The original measurements by Voynow & Kurland (1971) showed that the individual proteins are not present in equimolar amounts. In the smaller subparticle some proteins seem to be present in less than one copy per ribosome, whereas in the larger subparticle the proteins can be divided into several classes: those present (1) in less than one copy, (2) in about one copy, (3) in between one and two copies and (4) in two copies per ribosome. The only possible explanation of these findings is that *E. coli* ribosomes are heterogeneous as regards their protein complement (Voynow & Kurland, 1971; Weber, 1972).

Because of the importance of the question, i.e. whether ribosomes are homogeneous or heterogeneous, several authors have reinvestigated some aspects of the original data. One of the most important questions in assessing stoicheiometric measurements is how much the composition of the ribosome, as prepared, represents the composition of the particle *in vivo*. During preparation of the ribosomes some proteins may become attached to the particles, and others (probably regulatory proteins, factors or some loosely bound structural proteins) may become detached. The 'purer' the preparation the more

proteins (involving some real ribosomal proteins) will have been washed off. The most recent investigation on the stoicheiometry of ribosomal proteins of *E. coli* (Hardy, 1975), in which particular care was taken to use ribosomes closely resembling the particles *in vivo*, has shown that probably most proteins are present in equimolar amounts.

Stoicheiometric measurements of eukaryotic ribosomal proteins have lagged behind studies of *E. coli*. The greater complexity of eukaryotic ribosomes made the purification of their proteins difficult. Also, there are difficulties with higher organisms in attaining the labelling activities obtained with *E. coli*. Thus studies of stained patterns of ribosomal proteins have considerable importance in the case of eukaryotes.

In the present paper we have concentrated on the question that is of primary importance from the point of view of ribosomal protein stoicheiometry: whether the ribosomes are of prokaryotic or eukaryotic origin, i.e. how similar ribosomes in our preparation are to the ribosomes *in vivo*. To answer this question we compared the quantities of the ribosomal proteins in a series of preparations of various degrees of purity. The preparations varied from crude cell fractions (in which minimal loss of ribosomal proteins from the particle can be expected) to highly purified ribosomes, which are commonly used for most studies. The comparisons were made on the basis of dye-binding of the proteins after separation with two-dimensional polyacrylamide-gel elec-

Abbreviation used: SDS, sodium dodecyl sulphate.

trophoresis. Since for studies of this kind one of the requirements is that ribosomal proteins have to be identified in previous studies, we have chosen to investigate a system the ribosomes of which have been characterized in detail in our laboratory; namely those of rabbit reticulocytes (Martini & Gould, 1971; Martini, 1974). Also, because loss of protein during the electrophoretic procedure cannot be followed when dye binding is used to detect the spots, it is important to use a method where such losses are minimized. The two-dimensional electrophoretic system of Martini (1974) with urea at pH 4.3 in the first dimension and SDS in the second dimension is very suitable in this respect.

In spite of problems with the evaluation of the less-purified preparations, the results show that the quantities of most of the ribosomal proteins remain fairly constant in the different preparations. This probably means that the protein composition of the purified ribosome is close to that of the particle *in vivo*.

No comparison of subparticles is made in the present paper. The relative amounts of proteins in ribosomal subunits may be different from those found in undissociated ribosomes in mammalian sources according to Martini's (1974) observations. Some minor differences were also found by Sundkvist & Howard (1974) between slightly different preparations of subparticles of rabbit reticulocytes, even though the activity in systems of these differently prepared particles *in vitro* was the same.

Apart from the comparison of the ribosomal protein content of different preparations, we also examined the effect of various methods of extracting the ribosomal proteins on the subsequent ribosomal protein pattern. Different conditions for incubating the first-dimensional gel rods before electrophoresis in the second dimension were also studied.

Materials and Methods

Preparation of lysate from rabbit reticulocytes

New Zealand White rabbits were injected once a day for 5 days with 0.8 ml of neutralized aq. 2.5% (w/v) phenylhydrazine to make them severely anaemic. On day 6, 0.2 ml of heparin (1000 units) and 0.7 ml of Nembutal were injected into the ear vein, and the animals were bled by cardiac puncture. The blood (about 120 ml) was collected into 250 ml centrifuge tubes on ice and all further operations were carried out at 0°C. The blood was centrifuged at 2500 rev./min (1020 g) for 10 min in a Sorvall Superspeed centrifuge (6 × 250 ml rotor). The supernatant plasma and leucocytes were aspirated, and the reticulocytes were resuspended in an equal volume of 0.15 M-NaCl/0.015 M-sodium phosphate buffer, pH 7.6. This saline/phosphate wash step was repeated twice. Then the packed cell volume was measured and the

cells were lysed by osmotic shock by 4-fold dilution with lysing medium (0.005 M-MgCl₂/0.02 M-2-mercaptoethanol). The cells were left in the lysing medium for 10 min, then 1 cell volume of 150 mM-KCl in 50% (w/v) sucrose solution was added before centrifugation at 10000 rev./min (16319 g) for 15 min to remove cell debris. The supernatant is the 'cell lysate'.

Preparation of crude cell fractions containing ribosomes

Preparation of pH 5 ribosomal precipitate. This was carried out by the method of Schweet *et al.* (1958). The reticulocyte cell lysate was poured into a beaker, and the pH was adjusted to 5 by dropwise addition of 5% (v/v) acetic acid while stirring. The precipitate was centrifuged at 3000 rev./min (1469 g) for 15 min. The precipitate was resuspended in TKM buffer (0.05 M-Tris/HCl (pH 7.6)/0.025 M-KCl/0.005 M-magnesium acetate). This preparation, which contains ribosomes, as well as proteins the isoelectric points of which are near 5, is called the 'pH 5 precipitate'.

Gel filtration of the reticulocyte cell lysate. Sephadex G-100 was chosen (mol.wt. fractionation range 4000–150000). Ribosomes were expected to emerge in the void volume. The column dimensions were 5 cm × 40 cm and the flow rate was 0.025 cm/min or 0.5 ml/min. Lysate (20 ml) was applied on top of the column. The void volume (about 40 ml) was collected and subjected to protein extraction. This void volume contains ribosomes and relatively high-molecular-weight proteins.

Preparation of ribosomes

Ribosomes were prepared by using cell lysate or the pH 5 precipitate (redissolved in TKM buffer) as starting material. The suspensions were layered on a 10 ml 1 M-sucrose cushion in TKM buffer in a 30 ml tube and centrifuged in the Ti 60 rotor at 55000 rev./min (215000 g_{av.}) for 90 min in the Beckman L265 B centrifuge at 4°C.

In some cases the sucrose layer was omitted and 30 ml tubes were filled with lysate and centrifuged in the Ti 60 rotor at 55000 rev./min for 90 min at 4°C. It should be noted here that what we call the 'ribosomal preparation' is a mixture of polyribosomes and monomers.

Extraction of proteins and their preparation for electrophoresis

Methods devised for the extraction of proteins from ribosomes were applied to crude extracts as well as to purified ribosomes. With the crude cell extracts, however, the methods had to be slightly modified, as will be pointed out below. We used three

procedures for the extraction of proteins: (a) the guanidine hydrochloride method as described by Cox (1968); (b) the acetic acid method [extracting the suspension once or twice with 0.1M-magnesium acetate and 66% acetic acid by stirring for 45 min at 0°C, essentially as described by Hardy *et al.* (1969)]; (c) the LiCl/urea method [keeping the suspension overnight in a solution of 2.5M-LiCl and 5M-urea as described by Nomura *et al.* (1968)].

All three methods apply extreme conditions (acidic pH, or high salt and denaturing agents) to dissociate ribosomal proteins from ribosomal RNA; under these conditions the RNA is precipitated and the proteins are left in acidic or salt solution. Before being applied to the gels the proteins have to be dialysed or precipitated and redissolved in an appropriate buffer. Precipitation was with ethanol in the guanidine hydrochloride-extracted samples (Cox, 1968), with acetone in the case of the acetic acid-extracted sample (Berger, 1974; Barritault *et al.*, 1976) and with 10% (w/v) trichloroacetic acid in the case of the LiCl/urea-extracted samples. The precipitated proteins were washed with ethanol/diethyl ether (1:1, v/v) containing 10–20mM-2-mercaptoethanol. The protein was dried and dissolved in 12mM-potassium acetate, pH 5.4, containing 6M-urea (electrophoresis buffer).

Alternatively, if the protein solutions were sufficiently concentrated, unwanted solutes (salts, guanidinium ions etc.) were removed by dialysing the samples directly against electrophoresis buffer. In most cases our samples were not concentrated enough and proteins had to be precipitated.

As mentioned, the extreme conditions used in these methods are needed for the disruption of ribosomal structure to make the extraction of ribosomal proteins possible. The non-ribosomal proteins present in the crude cell extracts do not need to be extracted like this; in fact such proteins may be irreversibly denatured during acid, or LiCl/urea, or guanidine hydrochloride treatment, and may therefore cause aggregation of ribosomal proteins as well. This in fact happened when protein precipitates were dissolved in electrophoresis buffer as described above. (The crude pH 5 fraction apparently lacked several characteristic ribosomal proteins such as proteins L₁, L₂, L_{3a} and others, when treated in the above manner, presumably because these proteins were removed from the solution through aggregation with some non-ribosomal proteins.) This apparent loss of proteins could be prevented by introducing an extra step in dissolving the protein precipitates, namely dissolving the protein precipitates first in 66% acetic acid and then dialysing the solution against electrophoresis buffer. This extra step was found to be very important in dealing with impure preparations. Purified ribosomes, however, gave very similar electrophoretic patterns after using or

omitting this 'acidifying' step in dissolving the proteins, except for proteins S₁ and L₅ and for some non-ribosomal polyribosomal proteins. The quantities of these proteins were somewhat decreased when the 'acidifying' step was omitted.

Quantitative protein determination

This was performed by the colorimetric method of Bramhall *et al.* (1969), with bovine serum albumin as standard.

Two-dimensional gel electrophoresis of ribosomal proteins. This was carried out by the method of Martini & Gould (1971) at pH 4.3 in 8M-urea in the first dimension and in SDS/phosphate buffer in the second dimension. One step of the procedure was changed occasionally (as shown below in the Results section), namely the incubation conditions of the first-dimensional gel rods before embedding them into the second-dimensional gel slab.

The pattern of ribosomal proteins of rabbit reticulocytes is essentially reproduced from Martini (1974) (see Fig. 1). Ribosomal proteins are defined as those that are present on either of the active sub-particles after dissociation of ribosomes, even though their relative amounts may deviate in some cases from the ratios found in undissociated ribosomes (Martini, 1974). The pattern we obtained is indistinguishable from this (as would be expected with the same electro-

phoretic system); minor differences in the polyribosomal proteins were observed. Fig. 1 also shows Martini's (1974) nomenclature, which we are using here. Proteins found in the smaller subparticle were designated S, those found in the larger subunit L. Non-ribosomal proteins found on polyribosomes only are labelled with small letters.

Quantitative treatment of data

Transparent photographs of gels were used for this; 2500 readings of absorbance were made in the region of each spot over an area of 0.5 cm in an Optronics Autodensitometer.

A convenient procedure for detecting differences between the two-dimensional gel pattern of ribosomes prepared by different methods is to compare the central absorbances (i.e. the average of the five highest values) of corresponding Coomassie Blue-stained spots, i.e. protein L_1 in one pattern with protein L_1 in a second pattern, protein S_1 with protein S_1 and so on. This is based on the observations of Martini *et al.* (1975) that, below a certain total protein loading, the central absorbance of a spot is proportional to the amount of protein (and this was demonstrated for the spots of several small subunit proteins). This method has the advantage that the difficulties encountered in determining the area of the spot are avoided. Also, differences in staining between spots corresponding to different proteins (e.g. L_2 and S_{13}) due to different degrees of dye binding caused by the different nature of the proteins are avoided. By comparing the central densities of corresponding spots of two samples run on the same two-dimensional gel, variations in staining due to experimental factors are minimized. Clearly, if two preparations are identical, then the ratio between the central absorbances of corresponding spots (e.g. protein L_1 in one pattern and protein L_1 in the second pattern) will simply represent the ratio of the total protein in the samples of the preparations applied to the gel, and will be the same for each pair of corresponding spots. Thus, if two preparations contain relatively different quantities of a particular protein, its ratio will differ from that of the bulk of the proteins. In practice the median value (as distinct from the arithmetic mean) of a set of ratios from a pair of samples run on a two-dimensional gel was taken as the ratio between the two protein loadings, and deviations from this of greater than 25% were taken to indicate a significant difference between the pair of spots, i.e. between the relative contents of a particular protein in two preparations.

Photography of gel, scanning on a densitometer, and treatment of data on a Nova 800 computer, were performed as described by Martini *et al.* (1975).

Results

Protein pattern of crude cell fractions containing ribosomes

The electrophoretic patterns of the pH 5 precipitate and of the void volume obtained by gel filtration on a Sephadex column can be seen in Figs. 2 and 3. (In Fig. 2, as a reference, we also show the proteins of purified ribosomes, not subjected to salt treatment.) Both samples contain extra proteins in the high-molecular-weight region of the gel, and in the region of proteins L_5 and S_1 , i.e. the most acidic ribosomal proteins, but seemingly none co-migrating with the bulk of ribosomal proteins, except for one protein species that can be seen closest to protein L_{7C-d} in the sample obtained by column chromatography. Ribosomes obtained by column chromatography seem to contain the greatest number of proteins, although contamination with haemoglobin is greater in the pH 5 precipitated sample.

Most of the ribosomal proteins seem to be present in very similar relative amounts in these two preparations as in isolated ribosomes, judging by the relative densities of the spots. Some of them, however, seem to be present in decreased amounts

Fig. 2. Protein pattern of rabbit reticulocyte ribosomes and pH 5 precipitates

Ribosomes (a) were prepared by layering the lysate on 1 M-sucrose solution in TKM buffer and centrifuging in the Ti 60 rotor. The pH 5 precipitate (b) was prepared as described in the Materials and Methods section. Proteins from both samples were extracted by acetic acid and precipitated with acetone (as described in the Materials and Methods section). The precipitates were taken up in 66% acetic acid and dialysed against electrophoresis buffer. Arrows point to some of the proteins mentioned in the text in this and other Figures.

Fig. 3. Protein pattern of the void-volume fraction of the rabbit reticulocyte lysate passed through a Sephadex G-100 column

Gel filtration of the lysate was performed as described in the Materials and Methods section. Proteins were extracted from the void-volume fraction by the guanidine hydrochloride method. The ethanol-precipitated proteins were dissolved in 66% acetic acid and dialysed against electrophoresis buffer.

when compared with isolated ribosomes. These are mainly acidic proteins (S_1 , L_5 and L_{10}) and one or two others (L_{3a} , L_{18a}/S_{11}).

Comparison of the protein patterns of differently prepared ribosomes

Ribosomal preparations were obtained by using several slightly different methods, all involving ultracentrifugation. These preparations can be expected to resemble each other more closely and to contain less non-ribosomal contaminants than the crude fractions analysed in the previous section. The purest preparations (those with fewest protein components) are those obtained by treating the ribosomes with high concentrations of univalent cations ('salt wash').

Total lysate was either centrifuged through a 1 M-sucrose layer (in TKM solution) or sedimented directly without using this sucrose layer, and the ribosomes thus obtained were analysed for protein content. As expected, the 1 M-sucrose layer helped in decreasing the amount of haemoglobin present in

the ribosomal preparation. The relative amounts of ribosomal proteins (and of the non-ribosomal polyribosomal proteins) remained, however, unchanged. Similarly, no difference in the relative amounts of ribosomal or polyribosomal proteins of ribosome preparations were found when either the lysate or the pH5 precipitate was used as starting material for the sedimentation of ribosomes (results not shown).

The effect of exposure to different KCl concentrations on the electrophoretic patterns of ribosomal proteins was investigated by dividing the lysate into several portions and centrifuging them on 1 M-sucrose cushions containing various concentrations of KCl. The electrophoretic patterns subsequently obtained are shown in Fig. 4, and the experimental details are contained in the legend to that Figure. As expected, salt washing decreased the amount of non-ribosomal polyribosomal proteins. The relative amounts of ribosomal proteins remained essentially unchanged throughout this treatment. Proteins L_5 and S_1 content seemed to increase slightly in the salt-washed samples. This may indicate that these proteins tend to aggregate with some of the non-ribosomal proteins during extraction from the ribosomes.

We confirm the previous observation of Martini (1974) that the polyribosomal protein 'c' withstands salt washing. We also found proteins 'i', 'h' and 'k' rather resistant to salt washing (traces of these proteins could be found after washing with 0.7 M-KCl).

Preparation of ribosomal proteins by different methods: effect on the relative amounts of ribosomal proteins

We extracted ribosomal proteins by three different methods: (1) with acetic acid, (2) with LiCl/urea and (3) with guanidine hydrochloride (see the Materials and Methods section).

The protein pattern of the same ribosomal preparation subjected to LiCl/urea and guanidine hydrochloride respectively can be seen in Figs. 5(a) and 5(b). An example of the protein pattern obtained with acetic acid-extracted ribosomal proteins can be seen in Fig. 2(a).

The following general trends in the variation of the relative amounts of proteins with differences in the extraction method can be noted: proteins L_5 , S_1 and L_4 seemed to be relatively easily lost during all extractions, or later in the procedure. Guanidine hydrochloride sometimes underextracted proteins L_{3a} , L_{7a} and S_{7b} and more often protein L_{10} , whereas guanidine hydrochloride-extracted preparations showed slightly higher amounts of protein L_{14} when compared with preparations obtained by the other two extraction methods. Differences were detected between samples in which the protein was extracted by the same method, but was treated subsequently in a different manner (i.e. whether proteins were

(b)

(a)

(d)

(c)

Fig. 4. Protein pattern of salt-washed reticulocyte ribosomes

Ribosomes were prepared by layering the lysate on 1 M-sucrose solution containing: (a) TKM buffer; (b) 0.21 M-KCl/magnesium acetate and Tris buffer as in TKM buffer; (c) 0.35 M-KCl (other constituents as in TKM buffer); (d) 0.71 M-KCl (other constituents as in TKM buffer). Proteins were extracted by the LiCl/urea method, as described in the Materials and Methods section, and the proteins were precipitated by trichloroacetic acid and redissolved in electrophoresis buffer.

precipitated etc.; see the Materials and Methods section). Protein L_{3b} seemed to be underextracted

(a)

(b)

Fig. 5. Pattern of proteins extracted from ribosomes by the LiCl/urea or by the guanidine hydrochloride method

Rabbit reticulocyte ribosomes were prepared by layering the lysate on 1 M-sucrose solution containing TKM buffer (as in Fig. 2a). Proteins were extracted by the LiCl/urea (a) or by the guanidine hydrochloride method (b) as described in the Materials and Methods section. The LiCl/urea-containing protein sample was dialysed directly against electrophoresis buffer. Proteins extracted by guanidine hydrochloride (after precipitation with ethanol) were dissolved in 66% acetic acid and dialysed against electrophoresis buffer.

with LiCl/urea when the proteins were precipitated with trichloroacetic acid after extraction (Fig. 4). This was not found, however, when the proteins were directly dialysed into electrophoresis buffer (Fig. 5). Thus this apparent extraction effect is due to the subsequent manipulation (precipitation).

Apart from these variations, the relative quantities of the individual proteins seemed to be fairly constant with different methods used for the extraction of proteins.

Changing parameters of the incubation of the gel rods before embedding them into the second-dimensional gel

Before embedding the first-dimensional gel rods into the second-dimensional gel, the gel rods are incubated three times for 25 min at 50°C in a series of SDS / phosphate buffer / mercaptoethanol-containing solutions. To investigate whether proteins were specifically lost during this procedure, the conditions of the incubations were varied. Gel rods were incu-

bated: (1) in the usual way (three times for 25 min at 50°C); (2) changing the medium three times for 10 min at 50°C; (3) changing the medium three times for 25 min at room temperature; (4) not incubated at all. Then the gels were embedded and subjected to electrophoresis in the second dimension in the usual way. The patterns obtained after incubation for three times 10 min at 50°C and after no incubations are shown in Fig. 6. The electrophoretic patterns obtained after incubations under conditions (1), (2) (see Fig. 6a) and (3) were the same. Changing the conditions of incubation within some limits made no difference in the relative quantities of proteins. Thus we do not think that proteins are specifically lost during incubation, although a very easily degradable species could have been lost in all samples.

However, when incubation was left out completely, the embedded gel rod retained a substantial amount of protein (Fig. 6b). Also the numerous 'extra spots'

found, with simultaneous loss of some ribosomal ones, shows that some equilibration of the gel with SDS/phosphate buffer is important. The large dark spot above the position of protein S₆ coincides with haemoglobin in its first-dimensional mobility, so it is probably haemoglobin released from the first-dimensional gel after the bulk of the protein has moved into the second-dimensional gel.

Discussion

In studies on the stoichiometry of ribosomal proteins it is essential to know whether the ratios of individual proteins to one another are altered in any way during the many steps of their preparation. Investigations of the stoichiometry of ribosomal proteins of prokaryotic species have dealt with the problems of whether proteins are lost during isolation of the particles or extraction of protein. Substantial differences between prokaryotic and eukaryotic cell ribosomes as well as between methods for isolation of the particles warranted a detailed study of this kind on a eukaryotic species.

In the present paper we tried to follow the fate of rabbit reticulocyte ribosomal proteins during isolation of ribosomes, extraction of proteins and subsequent treatment during electrophoresis in two dimensions.

One step where major losses of protein may occur is the electrophoretic procedure itself. The widely used Kaltschmidt-Wittmann (1970) electrophoretic system was found to cause serious loss of material (unequal for different proteins) throughout the first-dimensional run, owing to the very high pH. When the stoichiometry of bacterial ribosomal proteins was studied (Voynow & Kurland, 1971; Hardy, 1975) this electrophoresis system was used, but, since proteins were radioactively labelled, the loss of any individual protein could be followed by using the isotope-dilution method (i.e. co-purification of purified labelled proteins with the sample being investigated).

Since quantification of proteins by dye binding does not allow checking of potential losses through electrophoresis, we had to use an electrophoretic system where losses of proteins were minimal. The electrophoretic procedure we were using was specifically developed to minimize losses by avoiding electrophoresis at high pH and by using SDS in the second dimension. The system was characterized and described at length by Martini (Martini & Gould, 1971; Martini, 1974; Martini & Gould, 1976), so we checked only one detail of the procedure, namely whether alteration of the incubation conditions of the first-dimensional gel rods would have an effect on the electrophoretic pattern. This emerged as an important technical point, for we found that omitting incubation of the gel rods with SDS-

Fig. 6. Comparison of the protein pattern of the same ribosomal preparation after subjecting the first-dimensional gel rods to different incubation before embedding

Preparation of the ribosomes was by the usual ultracentrifugation, but the sucrose layer was omitted in this case. Proteins were extracted by the acetic acid method. After electrophoresis in the first dimension the gel rods were incubated for 10 min in 1% SDS/0.1 M-phosphate buffer (pH 7.1)/6 M-urea, for 10 min in 1% SDS/0.01 M-phosphate buffer/6 M-urea and for another 10 min in 0.1% SDS/0.01 M-phosphate buffer/6 M-urea at 50°C (a), or the gel rods were not incubated at all (b). The gels were embedded into the second-dimensional slab and electrophoresis was carried out under standard conditions.

containing buffer leads to a distorted final pattern. We did not search for a minimum length of incubation of the gel rods that has to be used for preventing distortion of the pattern and retention of proteins at the start line; we found, however, that the time or temperature of the original conditions (3×25 min at 50°C) could be decreased without distorting the final protein pattern.

Extraction of proteins and purification of the particle is another step where proteins may be lost (or gained from the cell sap). The most direct way of checking if ribosomal proteins are lost during isolation of the particles is to compare the relative amounts of ribosomal proteins in whole cells with the corresponding values in ribosomes. This comparison has been performed with *E. coli*, where the ribosomal proteins represent about 60% of total soluble protein (Tissières *et al.*, 1959), and it has been shown that for most proteins the two sets of values were very similar (Hardy, 1975).

We made some attempts to draw similar comparisons between eukaryotic cells and ribosomes. However, as the reticulocyte lysate contains a great excess of a special protein (haemoglobin) over the ribosomal proteins (300-fold excess), layering the total cell lysate on gels for following the intensity of ribosomal protein spots was, not surprisingly, unsuccessful. Thus to obtain data on ribosomal proteins of whole cells we tried to use a less specialized cell than the reticulocyte, where soluble proteins comprise many different rather than one main species. We have chosen rat liver cells, where ribosomal proteins comprise 5–10% of the postmitochondrial protein content (P. Huvos, unpublished work) and used the postmitochondrial supernatant as representative of whole cells (i.e. ribosome and cell sap) (see Fig. 7). Ribosomal proteins, being more basic than the bulk of the proteins, migrate faster in the first dimension. [The rat liver ribosomal pattern and the relative densities of the spots are very similar to that found in rabbit reticulocytes, the most conspicuous difference being the absence of protein L_{3b} in the rat (see Martini, 1974).] The ribosomal protein spots are very faint, showing that ribosomal proteins represent a small proportion of total cell protein. As can be seen in Fig. 7, no valid comparison can be drawn about the relative densities of the majority of the spots (the two or three possible exceptions are proteins L_1 , L_2 and L_{3a} , i.e. the proteins having the highest absorbances and largest molecular weights); loading more protein on the gel increased blurring. Thus the postmitochondrial supernatants of mammalian cells seemed to contain too much non-ribosomal protein to allow reasonable comparisons to be drawn between the ribosomal proteins in the unfractionated lysate.

Therefore to check whether proteins are lost during the isolation of the ribosomal particles we com-

Fig. 7. Protein pattern of the postmitochondrial supernatant of rat liver

Rat liver was chopped and homogenized in 5 vol. of TKM buffer in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at $10000g$ for 10 min in the Sorvall centrifuge at 0°C to remove nuclei and mitochondria. The resulting supernatant was subjected to protein extraction by acetic acid and dialysed against electrophoresis buffer. Electrophoresis was carried out as described in the Materials and Methods section. The broken line shows where the ribosomal protein spots are located.

pared the pattern of the rabbit reticulocyte ribosomal proteins from particles prepared by ultracentrifugation with the pattern of two other (less pure) preparations. As was shown in the Results section (Figs. 2 and 3), the relative amounts of ribosomal proteins were very similar (as judged by visual inspection of the spots) in the lysate after subjection to (a) ultracentrifugation, (b) pH5 precipitation or (c) gel filtration. Since the methods of preparation in the three cases are quite different, it seems unlikely that the similar result obtained in the three cases is an artifact due to similar losses in amounts of the same proteins. We also found that the relative amounts of proteins were very similar after different steps of purification in the ultracentrifuge.

A quantitative demonstration of the data obtained in Figs. 2, 4 and 5 is shown in Figs. 8(a), 8(b) and 8(c). For each ribosomal protein the ratio of protein

Fig. 8. Ratios of protein weight in ribosomal proteins in the pairs of preparations shown in Figs. 2, 4 and 5. Ratios of protein weight in corresponding pairs of spots were calculated as described in the Materials and Methods section (by comparing central densities). The thick vertical line in the middle shows the median. The two horizontal lines indicate $\pm 25\%$ limit of the median value.

material was estimated in corresponding spots in pairs of preparations and the values are listed in decreasing order (see the Materials and Methods section). In the case of the pH5 preparation it was difficult to estimate ribosomal protein content because of excessive contamination of the sample, especially with haemoglobin (Figs. 2 and 8a). In the other two cases (Figs. 4 and 5) the amount of ribosomal protein layered on the two sides of the slab gel is nearly the same. This is reflected in that the median values are very near unity (0.93 and 1.2). Values for the majority of the proteins fall between $\pm 25\%$ limit of the median value, confirming conclusions gained by visual inspection of the gels, i.e. that the relative amounts of most proteins did not change during the preparation or extraction procedures, as compared with one another.

Two or three proteins, the amount of which seemed to be the most variable with different methods of purification of the particle or protein extraction, belong to the relatively acidic ribosomal proteins. We cannot decide at present why these particular proteins are the ones that are easily lost. In many cases we found that acidifying the protein solution in 66% acetic acid before dialysis against electrophoresis buffer seemed to increase the quantities of these proteins (L_5 , S_1 , L_{10} and L_{2a}) in the gel. Probably it helped to dissolve some aggregates containing the proteins. This effect of acidifying the solution may mean that aggregation is due to electrostatic forces. It is noteworthy that some of the proteins (S_1 , L_9 , L_7 and L_{12}) easily lost during isolation or extraction of ribosomal particles in *E. coli* also belonged to the acidic proteins (Hardy, 1975).

Proteins with the lowest values in Fig. 8(a) are those that are present in relatively low amounts in ribosomes as compared with the less pure pH 5 preparation. Proteins with the lowest values in Fig. 8(b) are those which may have been partially washed off by salts. There is no close correspondence between the two groups, suggesting that the low values do not reflect specific washing-off of these proteins, rather that these values simply fall into the lower part of the dispersion.

For some of the ribosomal proteins listed in Fig. 8 the percentage contribution of central absorbances to the sum of all central absorbances was calculated previously by Martini *et al.* (1975), whose preparation was comparable with our LiCl-extracted dialysed sample. For the sake of comparison we computed our results for the corresponding proteins in a similar manner. As can be seen in Table 1, the agreement between the two sets of values is reasonable, with the exception of protein S_{4a}.

Comparison of the extraction of eukaryotic (rat liver) ribosomal proteins by the LiCl/urea and acetic acid methods (as well as some other less-satisfactory methods) has been carefully examined by Sherton & Wool (1974), who found that the acetic acid extraction was the most reliable. We found the differences between the extraction methods less pronounced than they did, and also included the convenient guanidine hydrochloride method.

We find only some minor quantitative differences between samples prepared with acetic acid, LiCl/urea or the guanidine hydrochloride method, if proteins are taken up in acetic acid before dialysis into electrophoresis buffer. This latter step was most important with the less purified preparations. We did not find derivative spots with either the LiCl/urea or guanidine hydrochloride method, as Sherton & Wool (1974) have with the LiCl/urea extraction; however, the Kaltschmidt-Wittmann (1970) electrophoretic system used by these authors gives better separation than ours, so more subtle changes can be detected.

These experiments on different ribosomal preparations of variable purity seem to point to the conclusion that the protein composition of ribosomes prepared by the conventional methods is very similar to that of the particle *in vivo*.

Experiments of this kind can only be conducted and evaluated when the soluble ribosomal protein pool of the cell is small. Although there have been contradictory reports on this subject (Dice & Schimke, 1972), more recent evidence seems to indicate that the postmicrosomal supernatant of cells does not contain (Delaunay & Schapira, 1974; Wool & Stoffler, 1976) ribosomal proteins.

On the basis of general considerations applied to ribosomal proteins, we may try to identify the real non-ribosomal polyribosomal proteins as those that are present in the same amount whatever the purity of the preparations. There are two proteins in the preparations that can be relatively unambiguously identified as contaminants, namely c' and c". Both of these seem to be present in higher amounts in the pH 5 fraction than in ribosomes. One of them (c') is present in very high amount in the sample prepared by gel filtration. On the other hand the very weak x and y spots did not seem to be present in greater quantities in the crude preparations than in ribosomes, so these would qualify as genuine polyribosomal proteins or bound factors. The same is true for protein c, which was identified by Martini (1974) as a messenger-bound protein.

Polyribosomal proteins are in many cases overlapped by other protein spots, making evaluation rather difficult. In fact it is possible that some of the 'polyribosomal' proteins comprise two or three molecular species in the crude preparations. Identifications of the 'true' polyribosomal proteins are more conveniently achieved by salt-wash studies. In accordance with Martini (1974), we find protein c the most resistant to salt washing (see Fig. 4). Protein c is possible a messenger-binding protein (Blobel, 1972).

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Table 1. Comparison of the percentage contribution of the central absorbance of some spots obtained in different studies

Protein	Percentage contribution given by Martini <i>et al.</i> (1975)	Percentage contribution in present study
S _{2b}	7.7	9.9
S _{2c}	9.6	8.8
S _{4a}	4.8	8.4
S _{4b}	9.02	11.2
L _{3b}	17.7	16.2
L ₄	8.5	7.2
L ₆	14.6	14.4
L _{7a}	21.4	16.8
L ₁₀	6.7	6.5

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