QUANTITATION OF EUKARYOTIC RIBOSOMAL PROTEINS SEPARATED BY TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS

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1. Introduction

After more than a decade of research on ribosomes, the homogeneity of these sub-cellular particles is still a subject of debate. This debate, in the case of *E. coli* ribosomes, has centred around the possibility that certain proteins may be present in more or less than unitary molar amounts on average in the ribosome population [1–8]. The main technique used for quantitation of *E. coli* ribosomal proteins has been that of isotope dilution, requiring a mixture of each of the purified individual proteins, labelled with one amino acid isotope, with the total mixture of proteins, labelled with a second isotope. In the case of eukaryotic proteins, neither the appropriate labelled proteins, nor the purified individual proteins, are yet available on the scale required for such analysis. Moreover, in vivo labelling may be virtually impossible for the majority of ribosome preparations which would be of interest. To overcome these limitations, we set out to devise a densitometric procedure for quantitating eukaryotic ribosomal proteins, separated with a high degree of resolution by two-dimensional polyacrylamide gel electrophoresis [9,10]. We take advantage of the fact that the molecular weights of all the separated proteins are known from the mobilities in the second dimension of our system of electrophoresis [9,11]. Using the molecular weight values and the densities of the stained zones, measured by the new procedure, we have calculated the apparent stoichiometry of selected proteins. The results substantiate the impression gained by visual inspection of the gels: the density of most protein zones is directly proportional to molecular weight; a few proteins exhibit significantly higher or lower densities in relation to molecular weight. The method is shown to be sufficiently precise, that it may be applied without further elaboration to the measurement of the relative amounts of the same protein in different ribosome preparations. Variations in respect to particular proteins may be correlated with specific functions. Further information is required before the data obtained may be used to establish the exact stoichiometry of the proteins. The results nevertheless suggest that certain proteins may deviate significantly from the average unitary values.

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2. Experimental

2.1. *Ribosomal proteins*

Two types of preparations were examined: (1) proteins from rabbit reticulocyte polysomes washed by centrifugation through a discontinuous sucrose gradient containing 300 mM KCl, 5 mM $MgCl_2$ and 50 mM Tris–HCl, pH 8.1, and (2) proteins from ribosomal subunits prepared by zonal centrifugation through 500 mM KCl, 12.5 mM $MgCl_2$ and 50 mM Tris–HCl, pH 8.1 [10].

2.2. *Electrophoresis*

The two-dimensional polyacrylamide gel electro-

phoretic separation technique was basically that previously described [9,10]. Two modifications were employed to minimise retention of protein in the first dimensional gel rods during electrophoresis in the second dimension; (1) In work with subunit proteins, large diameter gel rods were used and rinsing of the rod surface with mercaptoethanol-free buffer before embedding in the unpolymerised slab was omitted; embedding was close to the upper edge of the slab and timed to occur shortly before the slab set. Some limitation of the resolution was accepted. (2) In work with polysomal proteins, resolution was increased, to cope with the larger numbers of proteins, by using 12 cm long gel rods in the first dimension. Protein retention at the origin of the second dimension was then minimised by setting the gel slab with riboflavin instead of persulfate catalyst. These modifications are important in measuring the relative amounts of the different proteins, since there is no guarantee against selectivity in protein retention. Thus a possible systematic error can be simply eliminated.

2.3. *Densitometry*

Two types of densitometer were used in this study. The Joyce-Loebl densitometer was used for direct measurement on gel slabs and the Optronics Autodensitometer was used for measurements on the corresponding transparent positive photographic films. In either case the densities of small adjoining rectangular areas were measured stepwise in order to map the density distribution over the entire area, the data was automatically collected and stored for computer analysis, and density maps were printed out by computer.

Measurements using the Joyce-Loebl densitometer required several hours, and meanwhile the gel had to be kept wet to prevent shrinkage. To minimise the total number of measurements, therefore, larger areas were scanned in each step than in the case of the Optronics instrument. The resolution of adjacent spots represented in the density maps was noticeably inferior as a result of the cruder measurements.

The Optronics Autodensitometer scans more quickly (about 4×10^4 measurements/min) and films may be scanned as long as necessary to obtain adequate resolution. In these studies we therefore measured relatively small areas (about 100 μm^2). The data were either stored on a magnetic tape or treated directly by the Data Corporation Nova 800 Computer with which the densitometer was interfaced. The data from the magnetic tape were analysed on an IBM 360 Computer, which is faster than Nova 800 Computer.

2.3. *Photography*

Of several films tested, Polaroid 5S P/N gave a linear response over the widest density range and was used in all studies reported in this paper. A special light box was constructed to provide even illumination over the whole area of the slab. It was found, despite this measure, that the background stain appears to vary across the gel. Consequently, we programmed the computer to subtract the 'local' background, defined as the average of the 200 lowest values in the region of each spot (specified by its central co-ordinates) within an area of 0.5 cm^2 (2500 measurements of 100 μm^2 each). Film densities were related to those of Coomassie Blue in the orginal gel slab by photographing a series of 1 cm cuvettes containing dye solutions of accurately known concentrations, spanning the range of linear photographic response, together with the gel slabs.

2.4. *Treatment of data*

For preliminary work, we used the 'Central Density' approach, wherein we characterised each zone by the three or five highest densities. Later, we used the 'Integration' approach in which all data were analysed.

2.5. *Comparison of zones*

In order to compare the densities of zones on the same slab, the sum of all central or integrated densities (with local backgrounds subtracted) was calculated, and the contribution of each was expressed as a percentage. Reproducibility was assessed by proceeding as described for each of several slabs containing the same separation, and comparing the percentage contributions of corresponding zones. Finally, percentage values given by corresponding zones in different separations were compared.

3. Results

3.1. *Density as a function of protein load*

To establish the range of protein loads over which density is approximately a linear function, fourteen zones representing proteins of about average size

(16 000–34 000 daltons) from the small subunit were analysed by the 'Central Density' approach after submitting 25, 50, 100 and 150 μg total protein to electrophoresis as described in the Experimental Section. The results, presented in table 1 and fig.1, exhibit two notable features; (1) The percentage contributions of the various zones fall into two groups with values closer to the lower [5,4] or upper [11] limit, differing a factor of two. (2) All the curves depart from linearity in the range of 100–150 μg protein. The characteristic slopes and the saturation levels shown in fig.1, probably reflect the characteristic running properties of the individual proteins which are dependent on factors other than size and amount. For the same reason, the 'Central Densities' do not accurately reflect the relative amounts of different proteins in one separation, although they may be used to assess the relative amounts of the same protein in different

Table 1
Percentage contribution of 'Central Densities' of small subunit proteins as a function of load

Protein	25 μg	50 μg	100 μg	150 μg
S2b	6.1	5.5	5.8	5.9
S2c	5.7	5.6	5.4	5.3
S3	9.1	9.1	9.8	9.3
S4a	6.5	7.4	7.4	7.3
S4b	6.0	5.7	6.4	6.1
S5	5.9	5.7	5.6	5.9
S7b/S8	5.8	5.4	5.3	5.8
S9	6.3	6.3	5.8	6.0
S10	6.4	6.6	6.6	7.4
S11	10.2	11.1	9.8	10.0
S12a	7.8	8.5	8.5	8.6
S12b	9.4	9.1	9.2	–
S12c	6.0	5.5	6.5	6.9
S13	9.5	9.1	8.6	8.6

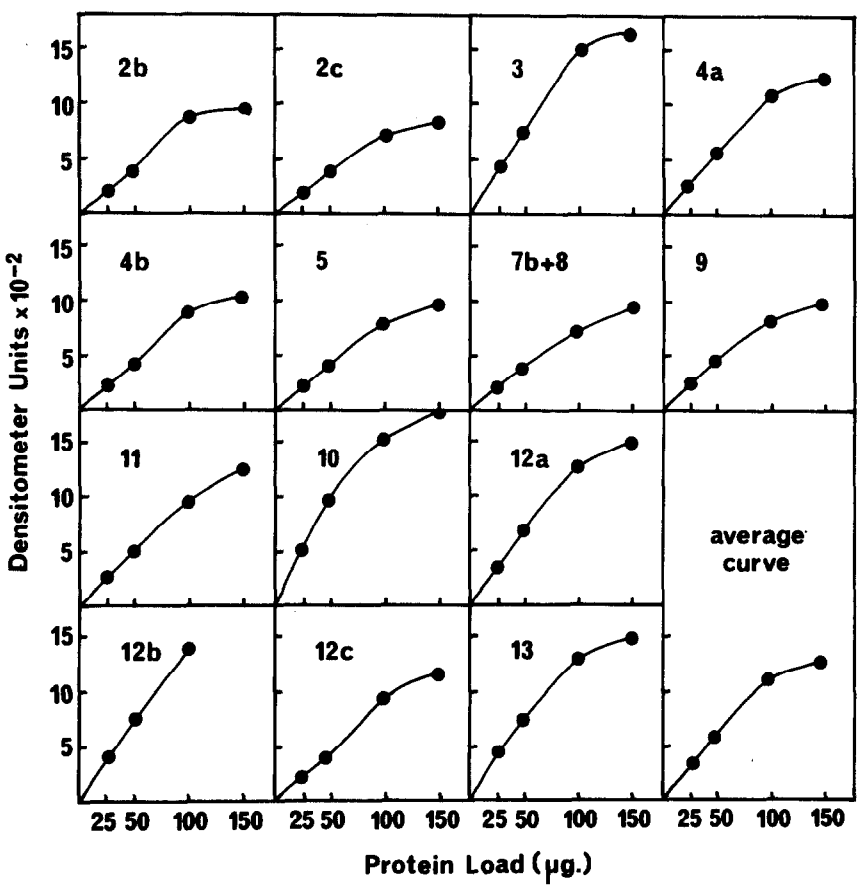

Fig.1. Relationship between total protein load and 'Central Densities' for 14 small subunit proteins.

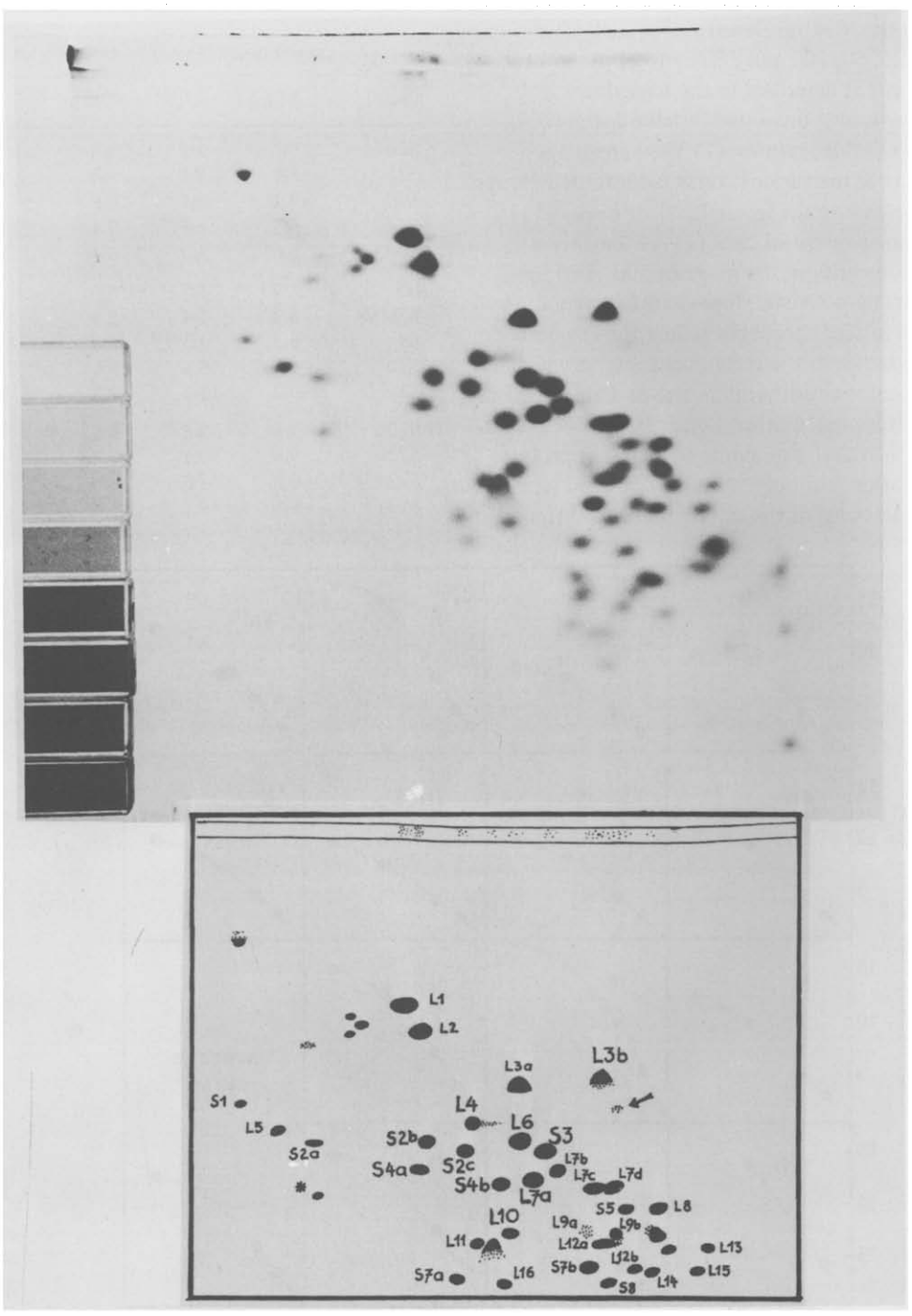

Fig. 2. Electrophoretic pattern of polysomes. The gel was photographed together with cuvettes containing known amounts of Coomassie Blue to provide a density calibration curve. Note that the amount of protein retained in the first dimensional gel rod at the top of the slab is small. Below is a guide to the zones present in the gel. Zones S2b, S2c, S3, S4a, L3b, L4, L6, L7a and L10 were subjected to stoichiometric analysis by the 'Integrated Density' approach.

Table 2
Relative amounts of ten ribosomal proteins using the 'Central Density' approach

Protein	Percentage contribution Triplicate analyses 1	2	3	Average	Molecular weight × 10^{-3}	Apparent relative amount*
S2b	6.7	6.6	6.3	6.5	34.2	6.4
S2c	8.0	7.8	8.4	8.1	33.2	8.2
S3	11.0	14.0	13.3	12.8	32.9	13.0
S4a	4.2	4.3	4.3	4.2	30.9	4.6
S4b	6.5	8.6	8.6	7.9	29.8	8.6
L3b	20.4	12.7	13.5	15.5	39.9	13.0
L4	5.5	8.1	8.8	7.5	36.0	7.0
L6	10.6	14.1	13.6	12.8	33.6	12.8
L7a	22.3	16.4	17.8	18.8	29.9	21.2
L10	4.8	7.4	5.4	5.9	25.3	7.8

* The average percentage contribution to the Central Density has been divided by the molecular weight and the percentage contribution recalculated.

separations, in relation to some standard. Vertical comparison of the numbers in table 1, however, is suggestive. The relatively high density of S12a, S12b and S13 may be explained by the comigration of two proteins (S12a/d, S12b/e and S13a/b) which may be resolved by changing the electrophoretic conditions [9]. The 'Central Density' should reflect overloading at an earlier stage than the 'Integrated Density'. Therefore, in restricting protein loads to the equivalent of less that 150 μg small subunit proteins (approx. 6–12 μg of the individual proteins) in all studies, we ensured the accuracy of the measured relative intensities.

3.2. *Reproducibilities of 'Central' and 'Integrated' densities*

Polysomal proteins were analysed by both methods. A typical result with 150 μg protein is shown in fig.2, and the results of triplicate analyses of 10 zones in different separations by the 'Central Density' and the 'Integrated Density' approaches are shown in tables 2 and 3, respectively. Horizontal comparisons show triplicate determination to be in close agreement in both methods. Reproducibility was somewhat better in the case of the 'Integrated Densities' than in the case of the 'Central Densities'. Thus, although the 'Central Density' method is quicker and simpler, and may have limited applications, the 'Integrated Density' method is preferable for two reasons: firstly, it gives more reproducible results, and, secondly, the resulting values are directly proportional to the absolute protein mass and thus may be used to estimate the stoichiometry.

3.3. *Relative molar amounts of ten ribosomal proteins*

The average relative integrated densities recorded in Column 3 of table 3 have been used, together with the mol wts (Column 4), to calculate the relative molar amounts of 10 proteins, 5 from the small and 5 from the large subunit of reticulocyte polysomes, falling in a narrow molecular weight range (25 300–39 900 daltons). Four of these (S3, L3b, L6 and L7a) are seen to be present in approximately twice the stoichiometric amounts of the remaining 6 (S2b, S2c, S4a, S4b, L4 and L10). The average contribution of the members of the first group is in fact 2.1 times that of the second. In the former group, L3b yielded somewhat lower values than the other three (Column 2), and the decreased yields in some preparations may be attributed to a tendency for this proteins to be degraded [9]. In the latter group, S4a also yielded noticeably lower values than the remaining 5 proteins. Since yields were reproducible, the values may be significant, but interpretation is difficult.

4. Discussion

We previously observed that the majority of eukaryotic ribosomal proteins, judging from visual

inspection of staining intensities in the mol. wt based two-dimensional separations, are present in roughly equimolar amounts. Some apparent exceptions, yielding conspicuously dark zones, were brought into line by changing the technique to improve resolution, when single zones broke into two or more. Several conspicuously dark zones still remained after all attempts to resolve additional proteins failed [9]. The present quantitative analysis confirms that four of these these zones, S3, L3b, L6 and L7a, definitely contain significantly more dye than six neighbouring zones, and the apparent stoichiometric ratio of approx. 2:1 strongly suggests duplicate copies in the ribosome. We cannot, of course, exclude the possibility that two different proteins in these zones remain unresolved, despite our efforts to improve resolution. Previous authors [13–15], using a somewhat different method of two-dimensional polyacrylamide gel electrophoresis, without SDS, resolved about the same number of (though not necessarily the same) proteins. Furthermore, when Lin and Wool [16] subjected the discs of protein from the two-dimensional slabs to electrophotoresis in third-dimensional polyacrylamide gel rods containing SDS, no additional proteins were detected. It therefore seems likely that most of the ribosomal proteins are resolved in both of the two-dimensional systems, or else that the number of unresolved proteins is the same in both cases. In the absence of further resolution, it may be necessary to resort to chemical analysis to determine whether zones represent more than one protein. A further complication in interpreting the data in terms of stoichiometric ratios is that proteins may absorb different amounts of dye per unit mass. However, two separate studies [5,17] have revealed that proteins of widely varying net charge exhibit minimal (less than ± 20%, e.g. for lysosyme and albumin) dye binding capacities with Coomassie Blue, the dye used here. Other dyes proved less uniform in this respect. Though the ribosomal proteins are relatively basic, as compared with most proteins, we deliberately chose a group of proteins of roughly the same net charge. Therefore, it is reasonable to assume that the relative densities reflect, at least to a close approximation, the stoichiometric ratios.

Bielka et al. [18] claim to have purified 31 (about half) of the proteins from rat liver ribosomes. Such purified proteins will greatly facilitate stoichiometric analyses. In the first place, the isotope dilution procedure may then be applied to ribosomal proteins which may be labelled in tissue culture. In the second place, the dye-binding approach, whether by densitometry or by dye elution, could be placed on an absolute basis by determining the exact uptake/unit mass of all the purified proteins and applying a correction factor to results such as those shown in table 3. Labelling may be the method of choice when only limited amounts of protein of high specific activity are available. On the other hand, the dye binding approach may be more generally applicable to eukaryotic ribosomal proteins, which cannot always be prepared in a suitably labelled form.

Table 3
Relative amounts of ten ribosomal proteins using the 'Integrated Density' approach

Protein	Percentage contribution Triplicate analyses 1	2	3	Average	Molecular weight × 10^{-3}	Apparent relative amount
S2b	6.8	6.6	6.4	6.6	34.2	6.3
S2c	8.9	7.1	7.6	7.8	33.2	7.7
S3	14.0	16.0	15.2	15.1	32.9	15.0
S4a	4.7	4.8	4.5	4.7	30.9	4.9
S4b	6.6	7.9	7.3	7.3	29.8	8.0
L3b	15.1	12.2	14.2	13.8	39.9	11.3
L4	7.0	7.6	7.4	7.3	36.0	6.6
L6	14.7	15.3	15.2	15.1	33.6	14.6
L7a	16.4	16.6	15.7	16.2	29.9	17.7
L10	5.8	5.8	6.5	6.0	25.3	7.8

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